Epigenetics in Medicine: The Rise and Fall and Rise of Lamarckian Thought

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Summary
A number of common and serious adult diseases such as obesity, dementia, and cancer, have long been known to result from some combination of genetic propensity and environmental exposure. However, the general mechanisms by which these two forces interact to shape disease phenotypes has been unclear until relatively recently. Somewhat surprisingly, principles of human development often turn out to be critical for understanding the basis for such adult diseases. Development, in turn, requires exquisite, broad, and coordinated control of gene expression through epigenetic pathways. To the extent that environmental stimuli directly alter stable chromatin marks, epigenetic signatures link external forces with the phenotypic expression of our genome even after long latency periods. The result of such epigenetic modifications, stamped either early or late in life, can form the basis for chronic disease in adulthood.

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[1] Dr. Terada does not have any financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Terada will be discussing off-label uses in his presentation. He is a Professor of Internal Medicine in the Pulmonary and Critical Care Division and holds the Dr. Carey G. King Jr. and Dr. Henry W. Winans Sr. Chair in Internal Medicine. His research interests include vascular cell signal transduction, mechanosensation of cellular anchorage, and epigenetic control of metastasis. The purpose and overview of this talk is summarized above. The educational objective is to appreciate the impact that epigenetic pathways have on the development of common adult diseases.

1. Epigenetics Arises

The French botanist and zoologist Jean-Baptiste Lamarck (1744-1829) is said to have synthesized the first comprehensive theory of organic evolution, drawing on long-existing but poorly articulated principles of development and evolution. Lamarck identified an individual's interaction with its environment as the critical component driving diversification of life forms and thus the creation of divergent species. The two cardinal forces driving evolution, he stated, were *le pouvoir de vie*, a tendency for increasing complexity, and *l'influence des circonstances*, the tendency for organisms to adapt to their environment. These forces led to two natural laws: first, that through use and disuse, individuals gain or lose characteristics during their own lifetime; second, that characteristics so acquired are inherited by the next generation. These laws were incorporated into a general theory which became known as the *inheritance of acquired traits*, although Lamarck apparently did not use that specific term. Notably, Lamarck did not originally conceive of this notion nor claim it as his own, and it was not central to Lamarck's main theory of Transformationism, for which he was best known in his day. It is, however, the phenomenon of inheritance of acquired traits that has come to be most closely linked to his name, hence the repopularization of his

moniker and its association with the modern field of epigenetics.

Lamarck's theories were extensively detailed in his *Philosophie Zoologique*, which was published in 1809, the year Charles Darwin was born. Darwin thus formulated his own theories on the origin of species in the context of Lamarck's prevailing theories, which emphasized the real-time transformation of individual's bodies during their lifetime in response to their environment, and their ultimate inheritance. Darwin's main point of departure was the theory of natural selection, which proposed stochastic variation and propagation of individuals surviving their environmental and biological challenges. Less well known is the fact that he clearly allowed for the inheritance of acquired traits in his later work *Variation in Plants and Animals under Domestication*. In this third of his four major publications, Darwin postulated that "pangenesis" involved the transmission of acquired developmental information to germ cells through hypothetical "pangenes" or "gemmules." This theory was based largely on his observations of plants, which were known to transmit resistance to environmental insults to the seeds of the next crop. Ironically, pangenes have found modern correlates in the delivery of noncoding RNAs through exosomes and other microparticles, a well-described mode of epigenetic transmission.

Evolutionary thought over the next century, buffeted between the polar philosophies of Lamarck and Darwin by both scientific and sociopolitical forces, were reformulated in the 1920's and 1930's as neo-Lamarckism and neo-Darwinism. The former, though largely grounded in honest and careful observation, reached notorious proportions in the guise of the botanist Trofim Lysenko. Lysenko was ideologically driven along with his patron Joseph Stalin, who, like many, favored a theory in which the individual could alter evolutionary history. Unfortunately, Lysenko routinely fabricated data and used the repressive machinery of the Soviet Union to dispose of his scientific opponents. The politics of the time did nothing to enhance Lamarck's reputation, which progressively sank early in the twentieth century. In contrast, neo-Darwinism, formulated on the heels of Gregor Mendel's laws of inheritance and championed by rising geneticists such as Theodosius Dobzhansky and Thomas Morgan, rejected any possibility that acquired characteristics could be inherited.

By the early 1940's, a large gap between the relatively new field of genetics and the stagnant discipline of embryology had formed, owing to the lack of any apparent mechanism to explain the phenotypic diversity arising from genetically identical cells in any given individual. In response to this growing schism, Conrad Waddington, an embryologist working chiefly with *D. melanogaster*, coined the term "epigenetics" as a fusion of Aristotle's epigenesis and contemporary genetics of the day, to explain the process by which varied phenotypes are assigned from any single genotype. His intent was to reformulate the study of development in genetic terms. Epigenesis was an old concept which recognized the close association of ontogeny with evolution, thus epigenetics from the outset arose in the context of both evolution and development. Waddington also introduced the concept of the epigenetic landscape to describe the differentiation of totipotent cells as a ball whose path is determined by valleys and hills (Figure 1). The emphasis was on the former, with each single valley being carved deeper by multiple genetic and environmental factors through a process he termed canalization [1]. What was not emphasized was the formation of

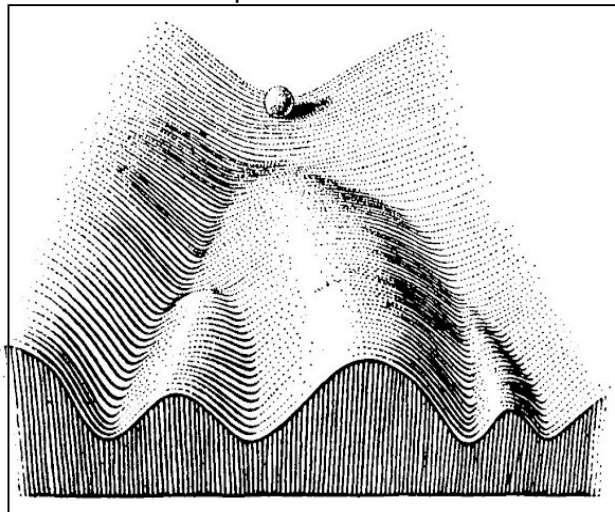

Figure 1. Line drawing by C.H. Waddington depicting the epigenetic landscape that progressively restricts cell differentiation fates. From Waddington, C.H. *The Strategy of the Genes*, 1957.

the hills which restrict passage and are now recognized as important vis-à-vis gene repression. The evolutionary equivalent of canalization occurred through "genetic assimilation," exemplified by the disappearance of the crossvein pattern on wings of flies exposed to heatshock, which reappears after 14 generations in the absence of heatshock. Thus the theory of genetic assimilation as a consequence of semipermanent epigenetic stamping was clearly a nod towards Lamarck's inheritance of acquired characteristics.

Waddington's emphasis on epigenetics as an environmentally responsive process which translates genotype into phenotype, especially in the context of development, is a key concept which has reemerged in a number of far-ranging fields of medicine and biology. Currently, the most widely used redefinition of epigenetics is the study of heritable changes that occur without a change in DNA sequence. At the present time, "heritable" is taken to mean inheritance across multiple mitotic generations, though inheritance through meiosis and thus across familial generations is included in this definition. A principal characteristic of both the original and modified definitions of epigenetic processes is the implied durability of the phenotypic change, which survives for a significant portion of an individual's lifetime.

With this in mind, we consider in broad outline the impact of epigenetic processes in three widely divergent, clinically important fields: metabolism, memory, and cancer.

2. Metabolism

Studies on the development and inheritance of obesity and metabolic syndrome have yielded a large number of potential genetic loci that, in combination, account for a significant proportion of inherited obesity [2]. However, it is increasingly clear that environmental factors, particularly variations in diet at certain critical times in human development, also greatly influence the risk of obesity and related metabolic disorders. This latter observation fits well with the long latency period between exposure to an environmental stimulus and the onset of subsequent disease, typified by the development of cancers but not applied to obesity until relatively recently.

One of the first large scale studies of environment on obesity rates arose from a study of 94,800 individuals conceived during the Dutch hunger winter which resulted from the German occupation of western Netherlands in World War II. In October of 1944, the exiled Dutch government directed a national railway strike in an effort to cripple the mobility of the German army. In retaliation, Germany cut off all food deliveries into the occupied western region, leading to severe food rationing. While rations initially were partially supplemented for pregnant women, by the spring of 1945 overall rations had declined to roughly 500 kcal/person/day, with no additional allotment for pregnant, ill, or debilitated citizens. Food influx was promptly restored after liberation in May of 1945. In the initial study, obesity rates were assessed in 19 yr old men inducted into the Dutch military from 1964 to 1967, placing their conception before, during, and immediately after the Dutch hunger winter [3]. In theory, one critical period of human development for adipose tissue was thought to extend from the last trimester through the first year of life, a period during which caloric restriction was thought to decrease the number of adipocytes. In this study, young men whose mothers survived the hunger winter during their last trimester indeed had significantly lower obesity rates than contemporaneous controls born in the unoccupied territories of the Netherlands. A further theory held that the development of the hypothalamus, an organ which controls appetite and satiety, occurred during midgestation; thus, a prevailing thought was that the ultimate size of an organism was determined by nutrient availability during this time. However, young men whose periconceptual period (first and into the second trimester) fell within the hunger winter instead had highly significant increased rates of obesity. In a later study following both men and women conceived during the hunger winter to an average age of 50, results of oral glucose tolerance tests indicated that in utero exposure to caloric restriction increased both glucose and insulin levels as adults, consistent with insulin resistance [4]. Accordingly, basal proinsulin levels were higher in this group as well. Consistent with the first study, BMI's of 50

year-old adults exposed as early fetuses to nutritional deprivation were also significantly increased. A third follow-up study with subjects now ~58 yrs of age demonstrated increased rates of hypertension, increased BMI, and increased waist circumference of subjects exposed to famine in utero compared with either sibling or hospital controls [5]. Interestingly, elevations in total cholesterol, triglycerides, and LDL cholesterol were also found but only in women. Of uncertain significance, birthweight of the grandchildren of the original mothers exposed to nutritional deprivation while pregnant were not different, suggesting perhaps a limit to the transgenerational effects of starvation [6].

Limits on nutritional delivery during embryogenesis are also thought to mediate differences in metabolic parameters in twins. Monozygotic twins share a single placenta, thus discrepancy in birth size is thought to reflect a disproportionation in nutrient delivery. A study of twins of average age 32 found that of 13 monozygotic and 8 dizygotic twin pairs found to be discordant for glucose intolerance, the glucose intolerant twin consistently had a lower birth rate than his/her twin [7]. While BMI's were not different, monozygotic twins born smaller had a higher rate of metabolic syndrome (54 vs 0%). A different study of older monozygotic twins (age 67±2) found twins born smaller to have increased BMI's (30.2±1.4 vs 26.8±1.5), glucose intolerance, and dyslipidemia [8].

Overall, such human studies have caused rethinking about the inheritance of obesity to include a prominent role for nongenomic mechanisms, albeit not to the exclusion of genetic factors. The "thrifty genotype" hypothesis, proposed in 1962 by J.V. Neel, held that thrifty alleles were selected for in Neolithic environments marked by poor or uncertain nutrition [9]. However, no plausible thrifty alleles have been identified [10], and recent studies such as those above indicate that exposure to nutritional extremes acts within a generation to cause metabolic disorders decades later and into the next one to two generations. Neel's hypothesis has thus been revised as a parallel "thrifty phenotype" to explain more rapid inheritance patterns. The thrifty phenotype is postulated to arise during embryonic life in response to nutritional availability. Key to this phenomenon is a requirement for significant developmental plasticity, in turn controlled by environmentally-influenced epigenetic pathways (Figure 2) [11]. This hypothesis fits well with a role for epigenetic changes in controlling developmental phenotypes. In brief, the theory holds that exposure to inadequate nutrition early in development reprograms the hypothalamus and other organs to prepare the individual for a similar poorly nourished existence as an adult, thus increasing their risk

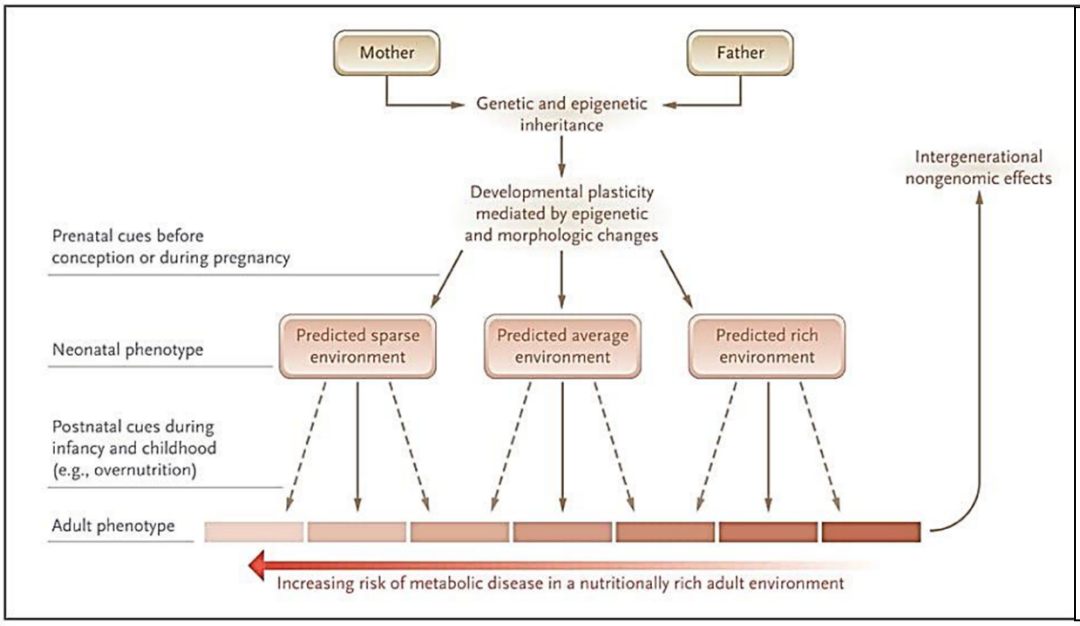

Figure 2. The Thrifty Phenotype as an explanation for the effect of nutritional vari-ation in utero on adult obesity. Develop-mental plasticity at key stages is pro-posed to leave epigenetic marks on relevant genes to anticipate various nutritional environ-ments as an adult. From Gluckman et al, *NEJM* 2008;359: 61.

for obesity, type 2 diabetes, and metabolic syndrome if food availability becomes unlimited during adulthood.

While much remains to be understood regarding the specific chromatin marks placed during this process, DNA methylation appears to play an important role. The vast majority of genomic methylation occurs on cytosine residues which precede guanosine, or CpG sites (the p stands for the phosphodiester bridge and seems superfluous). CpG sites are canonical (they read CG on the opposite strand), thus methylation occurs on both strands. During mitosis, the DNA replication fork is equipped with both CpG readers and DNA methyl transferases (DNMT), thus methylation of the nascent strand is replicated and ensures inheritance of this particular epigenetic mark. The majority of the CpG sites in the human genome are methylated, and many correspond to intergenic regions, particularly redundant repeat sequences contained within transposon elements, or within gene bodies. Methylation of retrotransposons, or "jumping genes," prevents their expression and thus excessive genomic recombination, while methylation within gene bodies presumably minimizes illegitimate transcriptional starts. A minority of CpG sites remain unmethylated and are densely clustered in structures called CpG islands (CGI) that frequently overlie promoter sites; roughly half of such CGI-containing genes are ubiquitously expressed (housekeeping) genes. In contrast, most if not all genes with non-CGI promoters are variably methylated and accordingly are expressed in a tissue and organ-specific fashion. These latter genes are therefore critical for cell lineage specification and consequently control organ development.

Recently, the importance of DNA methylation in human development was confirmed through genome-wide analysis of the human starting with preconceptual male and female gametes, through the single cell zygote, the inner cell mass containing pluripotent stem cells, and the post implantation early embryo [12, 13]. In human embryos, global though incomplete genome-wide demethylation is complete by the 2-cell stage. Such global demethylation is required for erasure of tissue-specific epigenetic marks and is therefore required for pluripotency. Consequently, genome methylation nadirs at ~30% of available CpG sites at the pluripotent stem cell stage then increases immediately upon implantation [12]. The latter increase in DNA methylation initiates the formation of new epigenetic memories which define cellular identity and restrict lineage [14]. In essence, differential methylation at this stage is the biochemical equivalent of the canalization of Waddington's epigenetic landscape. Notably, sites that escape demethylation include retrotransposons, although in a diverse and class-specific fashion [13], and known imprinted genes [12]. The latter includes the *IGF2* gene cluster, which coordinately controls both pro- and anti-proliferative genes. In brief, imprinted genes are expressed by only maternal or paternal alleles, reflecting a need for reduced gene dosage. Frequently, as in the case of *IGF2*, the products of imprinted genes control cellular proliferation or cytostasis. This latter observation in particular makes a case for the importance of intergenerational epigenetic modifications in the origins of obesity.

Indeed, methylation of at least two of several *IGF2* differentially methylated regions (DMR) is critical for regulated body growth during development. Abnormal loss of imprinting of two DMRs leads to an increase in expression of the pro-proliferative *IGF2* or silencing of the antiproliferative *CDKN1C*, leading to an epigenetically-mediated form of Beckwith-Widemann syndrome, characterized by gigantism, macroglossia, abdominal wall defects, and embryonal cell carcinomas [15]. Loss of imprinting control in the opposite direction leads to silencing of *IGF2* and the Silver-Russell syndrome, marked by growth failure and dysmorphic features [16]. Notably, methylation of one of the DMRs of the *IGF2* locus was quantified in individuals prenatally exposed to famine during the Dutch hunger winter, revealing a ~5% decrease in methylation even ~60 yrs after the famine [17]. While quantitatively small, the samples were taken from peripheral blood and not from more relevant organs, and only a 50% change in methylation results in gross developmental disorders described above. Of note, a subsequent study examined differential

methylation of a wider range of relevant genes and found both imprinted and non-imprinted gene promoters to be differentially methylated in subjects exposed to famine in utero [18], suggesting that epigenetic marks are placed during early development through mechanisms which do not necessarily require imprinting processes.

Mouse studies support human findings in that maternal caloric restriction causes obesity, impaired glucose tolerance, and insulin resistance even out to the F2 (grandpup) generation [19]. A recent study, again using caloric restriction of mice during pregnancy as a model, used a genome-wide expression analysis to identify differential expression of a panel of genes involved in regulating triacylglycerol formation [20]. The upstream regulator found to be epigenetically altered was *Lxra*, the gene expressing the liver X-receptor alpha. Of note, lipogenic genes were differentially expressed in the grandsons of nutritionally deprived grandmothers, and differential methylation of *Lxra* in the sperm of F1 fathers was noted, providing a possible explanation for intergenerational transmission of phenotypic effects [20].

While caloric restriction of humans and mice at an embryonic stage provides proof-of-principle evidence for the importance of epigenetic control of development in causing adult obesity, caloric excess at later developmental stages has also been demonstrated to be associated with subsequent obesity. The slow growth period (8-10 yrs for girls and 9-12 yrs for boys), before the prepubertal peak in growth, is considered to be a sensitive period where differences in nutritional intake may translate into divergent transgenerational responses to metabolic handling of calories. Cardiovascular mortality rates, for example, worsen for subjects whose father had excess compared with insufficient food availability [21]. Human studies looking across three generations are rare but data from the isolated northern Swedish farming community of Overkalix were obtained from church and community records of family trees and harvest yields, in years ranging from 1800 to 1920. Because of its isolated nature, members of this community relied on their own crop yields to tide them through the winter, leading to a “feast or famine” existence. When transgenerational effects of having a consistent surfeit of food versus poor food availability during the slow growth period were analyzed, a strong sex-specific influence of the grandparent’s nutrition was found. Specifically, an excess of food supplies during the slow growth period worsened mortality rates for a man’s paternal grandson or a woman’s maternal granddaughter, but not the other way around [22]. The effect was linked with food availability specifically during this prepubertal period, again suggesting the existence of critical events during this developmental period [23].

Animal data support human epidemiologic studies. In mice, males whose mothers consumed a high-fat diet become diabetic, insulin resistant, and obese. Conversely, female rats of fathers fed a high-fat diet have increased body weight and adiposity and impaired glucose tolerance and insulin sensitivity, despite being fed normal diets and having no social contact with their fathers [24]. Transcriptomic analysis of the pancreatic beta cells of these female rats demonstrated differential expression of a large number of genes which control insulin and glucose metabolism, with specific differential methylation in *Il13ra2* [24]. Interestingly, a similar analysis of retroperitoneal white adipose tissue in obese daughters showed a broad downregulation of 387 olfactory receptor genes, suggesting epigenetic control of peripheral nutrient sensing [25]. In a similar model of low protein (high sucrose)-fed fathers, livers of the offspring show differential expression of a large number of genes controlling lipid, steroid, and cholesterol biosynthesis [26]. Differential methylation of genes at a coverage of ~1% of the genome was also identified, including a putative enhancer of *Ppara*, a key lipid biosynthesis transcription factor. Notably, no differences were found in methylation patterns in the sperm of high sucrose-fed fathers, suggesting alternate mechanisms for intergenerational transmission of epigenetic information.

3. Memory

The linked processes of learning and memory would seem to be unlikely targets for epigenetic regulation, since they occur entirely in terminally differentiated, postmitotic neurons. However, the encoding, consolidation, and retrieval of long term memories requires gene expression and protein synthesis, and inhibition of these processes completely blocks long term but not short term memory formation. Furthermore, long-term memories become semi-permanent records of life events, many surviving for the lifetime of the individual. In this sense, memory consolidation can be seen as a form of neuronal development; thus, memory itself represents the long-term modification of gene expression in response to environmental stimuli, appropriately mediated by epigenetic modifications [27, 28]. Indeed, disorders characterized by gross deficiencies in memory and cognition such as Angelman syndrome, Rubinstein-Taybi syndrome, fragile X mental retardation, and Rett syndrome are thought to arise from epigenetic derangements [29].

Synaptic plasticity refers to the ability of synapses to dynamically increase or decrease neurotransmission, and is required for long-term memories to occur. Synaptic plasticity is measured electrophysiologically as long-term potentiation and morphologically as dendritic spine remodeling. Notably, a memory can be perpetuated for years, while LTP and enzymes required for memory encoding are transient, consistent with the existence of more durable cellular alterations such as epigenetic chromatin modifications in the maintenance of memory. In this sense, synaptic plasticity can be seen as a neuronal correlate of developmental plasticity.

Alzheimer's disease is a common disorder marked by loss of both long and short term memory. Studies of monozygotic twins show a relatively low concordance rate of dementia (19.2% at the high end), independent of age, which suggests both genetic and nongenetic effects [30, 31]. Indeed, postmortem studies of neocortex samples show dramatic differences in global DNA methylation and hydroxymethylation between normal and Alzheimer's brains (Figure 3) [32].

Long term memory requires extensive post-translational modification of histone proteins, a process which does not require DNA replication and thus becomes actively managed in memory-storing hippocampal neurons. In mice, extensive studies suggest that de novo histone acetylation, in general required for gene expression, fails in various models of dementia. Thus inhibitors of histone deacetylases (HDAC) which would be expected to nonspecifically increase histone acetylation, lead to improvement long term potentiation, synaptic plasticity, and fear-conditioned memory [33, 34]. Likewise, genetic overexpression of specific HDACs (e.g. *Hdac2*) worsens synaptic plasticity and memory formation, while deletion or RNAi-mediated knockdown of *Hdac2* restores neuronal gene expression, synaptic plasticity, and long term memory in mice [35, 36]. These findings support studies of human brains, which demonstrate increased levels of HDAC2 protein in the CA1 hippocampal nucleus in Alzheimer's brains.

To the extent that the epigenetic state of many neuronal genes is set by environmental context, it is noteworthy that environmental enrichment of caged mice (access to running wheels, climbing devices, toys, and food hidden within bedding) improves synaptic function.

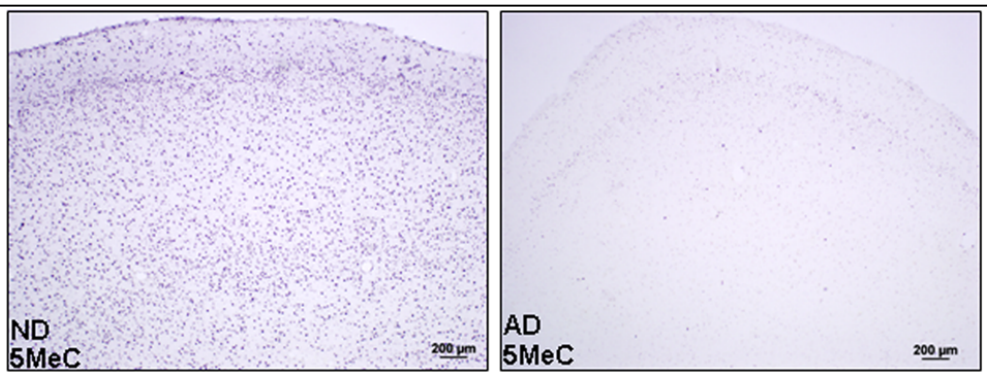

Figure 3. Immuno-histochemical stain for 5-methylcytosine in the anterior temporal neo-cortex of control and Alzheimer's disease patients, showing mar-ked loss of global DNA methylation. From Mastroeni et al, *PLoS One*, 2009;4:e6617.

Environmental enrichment was studied in a mouse model of dementia which involves doxycycline-induced expression of p25, a protein implicated in various neurodegenerative disorders [37]. Remarkably, despite a reduction in brain size comparable to control-caged mice, synaptic density, learning-associated gene expression, and memory improved in p25-expressing mice exposed to environmental enrichment. The beneficial effect was seen whether the training period preceded or succeeded p25 induction, suggesting that environmental effects are able to improve retrieval of memories thought to be "lost." Environmental enrichment was accompanied by histone acetylation in hippocampal cortical neurons, particularly acetylation of lysines 8 and 12 of histone protein H4 (H4K8ac/H4K12ac) [37]. Indeed, substitution of environmental enrichment with a nonspecific HDAC inhibitor, sodium butyrate, restored hippocampal synaptic density and both associative and spatial memory of p25-induced mice back to that of normal controls.

Similar studies of age-related memory loss in mice again implicate epigenetic control of gene expression as being required for long term memory [38]. Remarkably, fear conditioning of young (3 mo) mice induces 2229 genes, 1539 of which are associated with associative learning; in contrast, 16 mo old mice, who do more poorly on memory tests, display only 6 differentially regulated genes. Again, loss of H4K12 acetylation correlates with memory loss, and treatment with an HDAC inhibitor (in this case intraventricular SAHA) restores H4K12 acetylation, its association with specific learning-induced genes, and associative memory performance [38].

Opposite from memory loss, other memory disorders include conditions caused by unregulated retrieval of stressful memories. Memories imprinted during early childhood are thought to mediate behavioral disorders in adulthood, including depression, post-traumatic stress disorder, and schizophrenia. A common biochemical readout for abnormal stress response is an exaggeration of HPA axis activation following a stressful stimulus. Notably, reduced expression of the neuron-specific glucocorticoid receptor (*NR3C1*), a negative regulator of the HPA response, was found in hippocampi of suicide victims with a history of child abuse, compared with other suicide victims or normal dying suddenly of unrelated causes [39]. In these brains, overall and CpG-specific increases were seen in a regulatory domain of *NR3C1*, with corresponding loss of binding of the transcription factor NGFI-A. A broader analysis of the 6.5 Mb region surrounding the *NR3C1* promoter revealed hundreds of differentially methylated sites, many over promoters of genes implicated in dendrite remodeling [40]. A mouse correlate of early life memory is seen in natural variations in maternal licking and grooming during the nursing period. A low level of licking/grooming attention correlates strongly with excessive HPA responses to stress as an adult, an effect that is transmitted across generations [41]. As with humans, large differences are seen in the methylation status of the mouse ortholog of *NR3C1* depending on early life exposure [41].

Autism spectrum disorders are also thought in many cases to result from epigenetic errors, as fewer than 10% of subjects harbor known DNA mutations. A minority of individuals with autism display savant-like long term memory capacity, providing a clue to the molecular pathogenesis of the disorder. Indeed, deep sequencing of genomic regions associated with trimethylated H3K4, a mark associated with actively-expressed genes, shows excess spreading of this mark downstream of transcriptional start sites well into gene bodies, suggesting unregulated increases in neuronal gene expression [42]. Recent morphometric studies of autistic brains have shown a corresponding loss of developmental pruning of dendritic spines, also suggestive that epigenetic and neurophysiological processes opposite in direction from dementias occur in this disorder, though potentially in different brain regions [43].

Perhaps most surprisingly, a recent study suggests that certain memories, at least in mice, can be passed transgenerationally through inheritance of epigenetic marks. Fear conditioning in this model entails a mild foot shock associated with exposure to acetophenone, known to trigger a specific odorant receptor encoded by *Olfr151*. Sons of

fear-conditioned males, with no previous exposure to acetophenone, display a heightened fear response to a loud noise when sounded following acetophenone exposure [44]. Further, grandsons of fear-conditioned males, two generations removed from acetophenone exposure, also display a heightened acetophenone-associated fear response. Olfactory bulbs of both sons and grandsons were found to have markedly increased expression of *Oflr151*, confirming relevant and specific developmental changes induced by an associative memory and transmitted to subsequent generations. The developmental and behavioral differences survived in vitro fertilization and cross-fostering, ruling out an effect of parental behavior. Of importance, CpG-specific hypomethylation of the *Olfr151* promoter was demonstrated in sperm of both conditioned mice and their sons, suggesting a potential mechanism for transgenerational passage of epigenetic effects down three generations [44].

4. Cancer

Cancer is widely (and correctly) regarded as a genetic disease. Recent large-scale sequencing efforts, particularly The Cancer Genome Atlas (TCGA), have revealed a large number of mutations in many tumors. Interestingly, the median number of mutations per tumor ranges from only 8-12 for leukemias to ~200 for melanomas and lung cancer [45]. Overall, however, the vast majority of these mutations are considered passenger mutations that confer no selective advantage, and these mutations are frequently found in adjacent normal tissue as a consequence of aging. On average, tumors will contain two to six functionally important "driver" mutations [46], each of which provides only a ~0.4% growth advantage over normal cells [45]. Importantly, as tumors progress, a large degree of phenotypic heterogeneity develops, as rare cells gain, for instance, invasive or metastatic capability. Indeed, recent whole exome sequencing studies of different sites within resected lung cancer tumors support the progression of branched evolution within the primary tumor [47, 48]. However, most known driver mutations appear to occur early in tumor development, with few appearing in later branches [47]. When genomic features of metastatic sites are compared with corresponding primary tumors, most and oftentimes all of the driver mutations of metastatic sites can be found in the majority of ancestor cells from their primary tumors, indicating that cumulative stochastic mutations do not explain tumor cell evolution [45, 49, 50]. In contrast, widespread and stereotypical changes in gene expression can be found in association with such phenotypic shifts, strongly suggesting epigenetic dysregulation as an important driver of phenotypic diversity within any given tumor.

Perhaps the earliest indication that tumors are epigenetically unstable was the demonstration of widespread DNA demethylation in colon cancer tumors [51]. This loss of methylation is extensive, covering over half of the genome, and is seen in every cancer type examined. In some ways, such widespread demethylation resembles the initial wave of genomic demethylation which occurs during normal embryonic development, and both are required for erasure of tissue-specific memories and the loss of differentiation characteristics. As with early embryogenesis, the mechanisms responsible for this wave of demethylation are poorly understood, but it occurs early and can be seen in premalignant adenomas. However, beyond causing a loss of differentiation, tumor-associated demethylation diverges from that seen in development in most other aspects. In cancer cells, demethylation of transposable elements and other regions of heterochromatin cause extensive chromosomal rearrangements, leading to deletions, translocations, and aneuploidy. Further, imprinting control regions are frequently disrupted, causing loss of imprinting and expression of tumor promoters, as is seen with the *IGF2* gene in colon cancer. In contrast, both of these demethylation patterns are tightly controlled and restricted in early human embryos [12, 13].

More recent whole-genome studies of various cancer samples have revealed distinct spatial patterns of hypomethylation. Notably, demethylation occurs in large, discrete blocks covering roughly half of the human genome, with each block occupying a median size of 28

kb but ranging in length up to 10 Mb and containing dozens of genes [52, 53]. Hypomethylation of these blocks causes a shift in the frequency distribution of genomic methylation, in essence destroying tissue-specific methylation patterns (Figure 4). In this sense, tumor-associated demethylation can be seen as a flattening of Waddington's epigenetic landscape with consequent deprogramming of epithelial expression patterns.

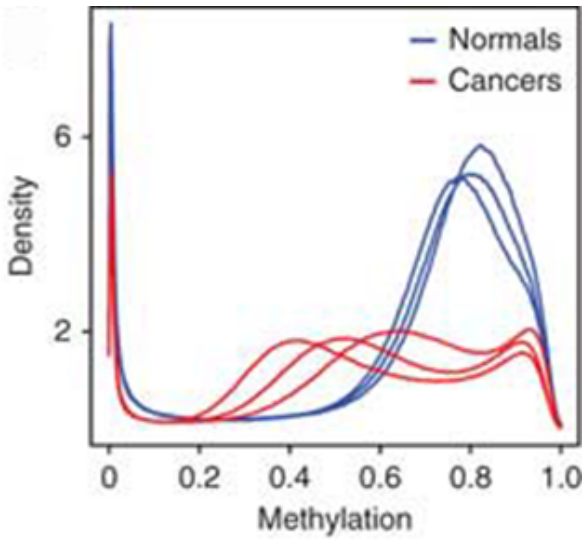

Figure 4. Distribution of smoothed methylation values for three colon cancers (red) and corresponding normal tissues (blue) showing overall hypomethylation of the genome and loss of hypermethylated regions. From Hansen et al, *Nature Genet* 2011;43:768.

Many blocks correspond closely with nuclear lamina-associated domains (LADs), normally hypermethylated regions of repressed heterochromatin physically associated with the nuclear matrix [52, 53]. Following malignant transformation, LADs are variably demethylated and released for accession by transcriptional factories within the nucleus. Of importance, genes within these large hypomethylated blocks display hypervariable expression across tumor samples when compared with adjacent normal tissues or with each other [53], providing an explanation for wide phenotypic variations within cells of a single tumor that arise through epigenetic deregulation, driving tumor evolution.

At the same time, much smaller regions primarily centered over CpG island promoters become hypermethylated [52]. These events are associated with gene silencing and again explain phenotypic variation within and across tumors. Of note, many of the larger hypomethylated blocks appear to border CpG islands, and differential methylation on either end of these islands appears as erosion of normal methylation boundaries [53, 54]. This latter observation corresponds to the earlier identification of critical control regions within 2 kb outside of CpG island borders which are differentially methylated between cancer and normal cells, regions termed CpG island "shores." Differential methylation of these same shores discriminates between normal endodermal (liver), mesodermal (spleen), and ectodermal (brain) tissues [55], as well as induced pluripotent stem cells reprogrammed to differentiate along these lines [56]. These studies strongly suggest that the same epigenetic modifications which specify lineage during normal development are erased during malignant transformation. A companion set of histone modifications has long been noted, mediated by the complementary actions of different polycomb repressor and trithorax complexes [57, 58]. Thus, genome-wide chromatin alterations reverse developmentally committed cell fate pathways in cancer cells, unlocking pathways that lead to previously restricted phenotypes.

Aside from the process of epigenetic deprogramming of differentiation pathways, one might ask whether the opposite process, epigenetic reprogramming along specific lineages, in a forward sense, also occurs. The rationale for asking this question stems from the observation that subpopulations of cancer cells seem to mimic identity-dissonant cell types. The clearest example of this process is vasculogenic mimicry. Here, glioblastomas, lymphomas, melanomas, lung carcinomas, and myelomas have been shown to incorporate malignant cells into the neovasculature [59-62]. These cancer cells morphologically and functionally behave like their surrounding, normal endothelial cells, and contribute up to 60% of the tumor's vascular endothelial surface [61].

A more common and lethal phenotype switch may account for the acquisition of metastatic behavior. In this regard, a small subpopulation

of cells derived from the primary tumor gains the ability to traffic to other organs, mimicking normal blood cell behavior. Leukocytes, and in particular lymphocytes, acquire several distinctive phenotypic characteristics during their development: the loss of permanent matrix and cell-cell adhesion, a gain in anchorage-independent survival, the expression of specific homing receptors, and the ability to shape the local inflammatory milieu. Importantly, cancer cells mirror each such behavioral trait upon acquisition of metastatic competence. Do specific epigenetic changes account for such lymphocyte mimicry?

Examination of the *SHC1* gene provides a convenient starting point for answering this question. *SHC1* expresses three proteins with divergent actions. The smaller 52 and 46 kD isoforms are constitutively expressed in all cell types, and serve as scaffolds linking mitogenic receptors with Ras and Rac1 [63-65]. $p52^{Shc}$ binds normal and oncogenic forms of receptor tyrosine kinases, and when overexpressed can mediate anchorage independence [66]. In contrast, the longer $p66^{Shc}$ isoform promotes differentiation, cell death, and senescence. Importantly, $p66^{Shc}$ also mediates epithelial-like dependence of tissue cell adhesion to firm matrix, an obligate requirement for epithelial cell survival [67]. Accordingly, $p66^{Shc}$ is strongly expressed in epithelial cells, variably expressed in mesenchymal cells, and repressed in hematopoietic cells. Not surprisingly, silencing of $p66^{Shc}$ is also found in metastatic lung carcinomas and deregulates Ras and Rho proteins, uncoupling Ras from anchorage context and acting as a strong metastasis-suppressor [68].

Separate promoters drive $p66^{Shc}$ and $p52^{Shc}$ expression, consistent with differential expression of their transcripts. As one might expect, the $p52^{Shc}$ promoter lies within a CpG island as is usual for housekeeping genes. In addition, a differentially methylated region containing five CpG sits within a downstream CpG island shore and marks a critical enhancer which interacts directly with the $p66^{Shc}$ promoter. This enhancer becomes methylated and associates with the repressive H3K9me2 histone mark in SCLC cells, and is consequently delocalized from its cognate promoter. The key protein which initiates epigenetic repression of $p66^{Shc}$ was found to be the lymphocyte-specific chromatin regulator, Aiolos. Aiolos binds three sites within the enhancer and reconfigures higher order chromatin structure, deacetylates $p66^{Shc}$ promoter histones, and selectively silences $p66^{Shc}$ expression [69]. Importantly, Aiolos is commonly expressed in both NSCLC and SCLC, as assessed by immunohistochemistry, and high expression levels predict strikingly worse survival rates in both early and late stage NSCLC [69].

The clinical significance of Aiolos deregulation becomes clear upon considering its known function during hematopoiesis. Aiolos and its close paralog Ikaros both regulate chromatin structure and gene expression during lymphocyte development, and together they are required for lymphopoiesis [70]. Aiolos and Ikaros are induced in normal lymphocytes as adhesion-related genes are downregulated and immature lymphocytes prepare to leave matrix-rich bone marrow and thymus niches. Accordingly, deletion of the gene encoding Ikaros causes mouse lymphoid progenitors to flatten, assume an epithelioid morphology, and become anchorage dependent [71]. Oppositely, human lung cancer cells which aberrantly express the lymphocyte protein Aiolos assume a rounded morphology, downregulate multiple genes in cell-cell and cell-matrix adhesion functional groups, gain

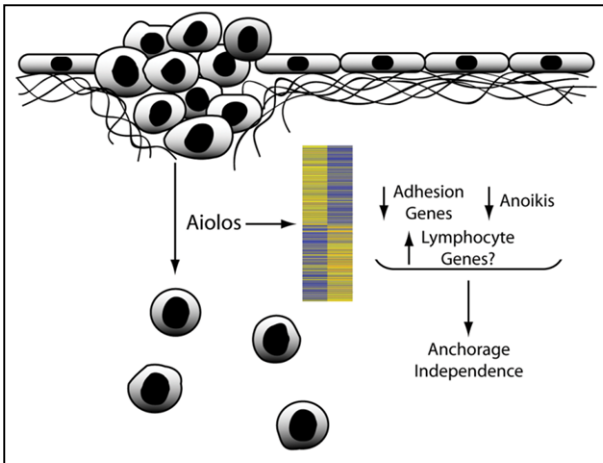

Figure 5. Schematic indicating the role of Aiolos in reprogramming transformed cells to gain lymphocyte-like characteristics. From Terada and Liu, *Mol Cell. Oncology*. In press.

anchorage independence in vitro, and metastasize avidly in mice in vivo [69]. Aiolos thus appears to coopt normal lymphocyte developmental pathways and epigenetically reprogram epithelial carcinomas to gain lymphocyte characteristics (Figure 5). The overall effect of this phenotypic shift can be considered to represent an epithelial-to-immune cell transition. As opposed to the well-described epithelial-to-mesenchymal transition, which describes the initial deprogramming and erasure of epithelial lineage marks, such an epithelial-to-immune cell transition in theory reflects abnormal progression towards lymphoid lineage restriction, enabled by the cancer cell's epigenetic instability and extreme developmental plasticity.

5. Conclusions

When seen in the context of various mechanisms cells and tissues use to respond to their environment, it is clear that nature has endowed us with a set of tools which operate over a very broad dynamic range along a time axis. These mechanisms include signal transduction, with effects lasting seconds to hours, and genetics, whose effects are stable over years to tens of thousands of years. At time scales in between, epigenetic mechanisms hold an important middle ground that allows the individual to respond to persistent environmental challenges with relatively long-lasting modifications in developmental pathways. That such epigenetic marks are dynamic and to some extent reversible may in the future allow therapeutic intervention for epigenetically-mediated diseases.

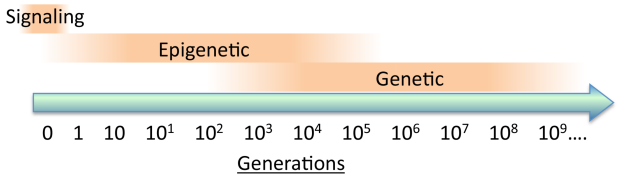

Figure 6. General schematic indicating the relative temporal impact of three major classes of phenotypic responses to the environment, ranging from signal transduction to epigenetic chromatin modifications to genetic mutations.

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