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EPIGENETIC MODIFICATION AS AN ENABLING MECHANISM FOR LEUKEMIC TRANSFORMATION

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TABLE OF CONTENTS

1. ABSTRACT

Cancer is now thought of as a fundamentally genetic disease, in that changes in the genome result in aberrant gene expression of oncogenes and tumor suppressor genes to promote oncogenesis. However, with our increasing knowledge of gene regulation, it is becoming obvious that changes in nucleotide sequence are not the sole mechanism for eliciting changes in transcription. An additional layer of regulation of gene expression, called epigenetics, is now being realized as increasingly important in oncogenesis. Epigenetics is defined as non-sequence based changes in chromatin that elicit changes in gene expression that are propagated through mitosis and/or meiosis. The alleles of the genes containing these epigenetic marks are termed epialleles. Epigenetics has been linked to cancer since 1983 by the work of Andy Feinberg and Bert Vogelstein (1-2), but has largely remained in the shadows. These changes in chromatin are now at the forefront of research in the field of oncogenesis, both as mechanisms of oncogenesis and as prognostic indicators of cancer risk.

Leukemia, due to the defects in cellular differentiation associated with the disease, has important connections to epigenetic gene regulation. Cellular differentiation has been studied as a model system for epigenetic gene control in *Drosophila*. Homeobox genes in the antennapedia (3) and bithorax (4, 5) gene clusters have long been known to be regulated by trithorax group (trxG) and Polycomb group (PcG) of genes, which regulate transcription through chromatin remodeling mechanisms. The ectopic expression of the mammalian homologs of the homeobox genes has been linked to leukemic transformation since 1988 (6), and has continued to show extensive connections (7-14). These connections that leukemia has with cellular differentiation make this group of diseases amenable to exploring the mechanisms of epigenetic gene regulation as they pertain to oncogenesis.

This review will examine leukemia, with an emphasis on myelogenous leukemia, as a defect in cellular differentiation and examine possibilities of epigenetic gene regulation of oncogenes and tumor suppressor genes.

2. THE PRESENT THEORY OF ONCOGENESIS IN EVOLUTIONARY TERMS

My collaborators and I have referred to components of epigenetic gene regulation as Waddington's widget (15), because they both fulfill the requirements for Conrad H. Waddington's model of evolutionary canalization proposed in 1942 (16) and are still inadequately understood. Unfortunately, few scientists accepted his ideas at the time because his scientific program was suspect, presumably because it appeared Lamarckian (17). Since Waddington invented the term "epigenetics" which has its roots in evolutionary biology, it is instructive to view epigenetic's relationship to oncogenesis in these terms.

A complicated network of cooperation exists among the cells of multicellular organisms, which is entrenched in the genomes of the cells making up the organism. Oncogenesis is a process where cells of a multicellular organism sever these bonds of mutual dependence and cooperation characteristic of these organisms, becoming for all intents and purposes a separate organism. The loss of cooperation typically begins with the over-proliferation of the premalignant cell, which is followed by the organism's attempt to restrain the cell. In evolutionary terms, oncogenesis can be viewed as a Darwinian survival of the fittest, as the premalignant cells attempt to survive in a hostile new environment, to which they must adapt or be destroyed. In this process of premalignant cells becoming fully transformed, the proliferation urge drives the cell to a cancerous state, while the mutual dependence and cooperation of the multicellular organism that is entrenched in the genome of the cell work against it.

To survive in the host, the precancerous cell struggles to acquire four characteristics: 1) proliferative autocrine signaling, 2) insensitivity to antigrowth signals,

3) protection from apoptosis, and 4) unlimited self-renewal. These characteristics are reviewed elsewhere including two other characteristics of solid tumors not pertinent to leukemia, specifically angiogenesis and metastasis (18). The acquisition of these four characteristics can be viewed as a survival of the fittest in the truest Darwinian fashion, where the order of acquiring these characteristics may vary, but the endpoint is the same in all forms of cancer. The first three characteristics involve communication and cooperation with the organism as a whole. These characteristics are detrimental to the precancerous cell, as the needs of the organism are at odds with the proliferation urge of the cell. Unlimited self-renewal is a function of cell senescence, which is a limitation that must be overcome. The precancerous cell acquires these characteristics by altering the expression pattern of genes controlling these functions. The unique nature of epigenetic gene regulation (in comparison to other forms of gene regulation) gives it tremendous importance in oncogenesis, just as it has in evolution (19). This importance is due to epigenetics being a unique enabling mechanism for oncogenesis.

Often, the first step in oncogenesis is sustained proliferation of the cell (18). Examples of mechanisms that can lead to sustained proliferation include exposure to inflammatory cytokines in chronic conditions, production of growth factors by the malignant cell to stimulate itself (autocrine signaling), mutations resulting in constitutive activation of cell proliferation pathways at the signal transduction or transcriptional activation levels, or malfunctions at cell cycle checkpoints (tumor suppressor genes). Sustained proliferation of the cell has multiple advantages for the premalignant cell. First, the number of premalignant cells increases, thus increasing the likelihood that one of the cells will acquire all of the remaining three characteristics to become fully transformed. Second, as the mitotic index of a cell increases, the cell comes closer to cellular senescence, a time when replicative capacity is exhausted. If the cell continues to proliferate due to sustained stimulation by the oncogene, the cell enters cellular crisis characterized by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of immortalized cells (20). Thus, cellular crisis is an enabling mechanism for oncogenesis, in that it allows for more efficient acquisition of the four necessary characteristics of oncogenesis by altering gene expression through gross chromosomal alterations.

Genes that are important in the progression of cancer can be grouped into two categories, those that are involved in the molecular pathways of the four characteristics mentioned above and those that give the cell adaptability, or in evolutionary terms “increased fitness.” Genes in the former category are necessary for oncogenesis. Genes falling into the latter category are enabling mechanisms that allow the cell to acquire the necessary characteristics with more success. Indeed, it has been argued that without these enabling mechanisms, the probability of cancer formation is highly unlikely within a human life span (21). Genes in this category are known as “genomic caretakers” as they ensure the integrity of the genome through monitoring and repair. These genes include, but are not limited to, those that are involved in DNA damage repair, chromosomal separation in mitosis, or cell-cycle checkpoint regulation such as *p53*, *Ataxia telangiectasia mutated* (*ATM*), *human homolog of E. coli mutY* (*MYH*), *Breast cancer 1* (*BRCA1*), and *Breast cancer 2* (*BRCA2*) (22, 23). Damage to the genomic caretaker pathways allows a cell to make changes in the pathways involved in the four conserved characteristics of cancerous cells. Genomic damage is random but, just as in Darwinian evolution, those changes that are advantageous to oncogenesis allow survival and are selected. Thus, genes in the enabling category increase the likelihood that one of the cells of a premalignant group will shed their genomically entrenched mutual dependence and cooperation to evolve into a fully transformed cell.

3. EPIGENETICS AS AN ENABLING MECHANISM FOR ONCOGENESIS

DNA methylation is the epigenetic gene regulatory mechanism that has seen the most research in oncogenesis, perhaps due to its long standing functional role in human diseases such as Prader-Willi and Angelman syndromes (24). DNA methylation occurs at CpG islands that are defined as regions greater than 200 bp with a CG content greater than 50% and an observed to predicted ratio of CG greater than or equal to 0.6 (25). Imprinted genes are often organized in clusters, sometimes in megabase-range chromosomal regions containing key control elements – the differentially methylated regions (DMRs) (26-28). Two well-characterized clusters are present on chromosome 7 in the mouse (29). DMRs are CpG rich and subject to epigenetic modifications. DMRs can function as boundary elements (30, 31) that indirectly affect expression of neighboring genes by repression when methylated or unmethylated depending upon the particular element.

In addition to DNA methylation, epigenetic modifications take the form of posttranslational modifications to the amino-terminal tail and internal sites of histones. These modifications include phosphorylation, acetylation, methylation, ADP-ribosylation, glycosylation, and ubiquination (32-36). DNA methylation acts by inhibiting the binding of trans-acting factors, typically repressing transcription if at promoter sequences (37-40), but sometimes methylation inhibits the binding of repressive factors and thus activates transcription (41-44). Histone modifications affect the ability of nucleosomes to interact and form repressive complexes. The histone modifications are complex with evidence that interaction between modifications is occurring (33, 34). This has led to the recently formulated "histone code" hypothesis by Allis, suggesting the type, timing, placement, and sequence of histone posttranslational modifications comprise a code for controlling chromatin conformation (45, 46).

Genes involved in epigenetic gene regulation, e.g. genes that are responsible for DNA methylation or histone modification pathways, fall into the enabling category because they give the premalignant cell increased fitness, and are in that way important in oncogenesis. The

genes that these epigenetic regulators target are those that are involved in the four characteristics of malignant cells and are not themselves enablers, but the targets of the enabling machinery. The adaptability epigenetics allows premalignant cells is augmented tremendously by the unique nature of epigenetics. The unusual aspect of epigenetic gene regulation compared to other forms of gene regulation is the stability these modifications can possess through meiosis and mitosis. With few exceptions, such as maternal effect and asymmetric cell division, changes in gene expression resulting from non-coding factors are not propagated through cellular division with stability. It is the stability of epigenetic modifications, such as X-inactivation, that results in cellular memory and heritable epialleles. Currently, there are more than 65 genes involved in oncogenesis that are known to be regulated by DNA methylation (47). Also, nearly 50% of the genes that cause familial cancer when mutated in the germline are epigenetically inactivated by hypermethylation in sporadic cancers of the same type (48). The increasing awareness of the connection between oncogenesis and epigenetics has contributed to the emergence of a new branch of study – epigenetic epidemiology (49). In these studies, researchers are investigating the effects of epigenetics on the occurrence and distribution of diseases.

Even with the propagation of epigenetic modifications through meiosis and mitosis, many epialleles are metastable, in that the fidelity of transmission does not appear as robust as genetic information (50-53). This is seen with some cases of epigenetic modifications known as metastable epialleles, in which the epigenetic state can switch from on to off or vise versa and is established in a probabilistic event (54). Metastable epialleles are seen in mice with the *agouti viable yellow* (55), *agouti hypervariable yellow* (56) and *axin* fused (57) alleles, as well as in the *Drosophila* ectopic outgrowth from the eye phenotype (19) and color variegation in plants (58). In these cases, transmission of the epiallele, or the gene containing epigenetic modifications, is not 100% as is the case with classically imprinted genes, but has a probability of being erased during transmission through cell division. This metastability offers increased adaptability to the premalignant cell, not possible with genetic mutation, by offering a mechanism of gene regulation that is reversible.

Adaptability is also increased by epigenetics' regulatory roles at the chromosomal and regional levels of gene expression, as well as involvement in chromosome, centromere, and kinetochore stability (36). Multiple genes may be targeted by epigenetic changes because of epigenetic control mechanisms for large regions of chromatin. Thus, epigenetic gene regulation can affect many genes simultaneously, leading to increased likelihood that a gene important to the four characteristics of oncogenesis will be targeted. This regional chromatin control is in addition to the chromosome architectural role of epigenetics important to chromosomal stability that is alone sufficient to place it in a potent class of oncogenic enabling mechanisms (59-61).

4. THE MOLECULAR CHARACTERIZATION OF MYELOGENOUS LEUKEMIAS

The current paradigm of leukemia states that the leukemias start from the indolent myelodysplastic syndromes (MDS) or myeloproliferative disorders (MPD) and eventually result in the more aggressive leukemias. In terms of our evolutionary model, the premalignant cells that have increased proliferation and are struggling to survive are represented by the indolent stage. The malignant cells of leukemias exhibit all the characteristics of cancers including increased proliferation, increased self-renewal, and inhibited apoptosis. Genes that are associated with these traits are misexpressed in leukemias as they are in other cancers. Leukemias and MDS are also characterized by impaired differentiation resulting from expression of BCR-ABL or the AML-associated fusion proteins (62-65). Two experimental approaches that have been instrumental in identifying the genes affecting the various stages of development of the myelogenous leukemias are the study of cytogenetics in human disease and murine models displaying a predisposition for developing leukemia.

Eventually oncogenesis results in drastic changes in the karyotype of a cell resulting in aneuploidy, deletions, duplications, and translocations. Analysis of these chromosomal aberrations has become an established method in the discovery of genes important to the etiology of cancer. Various approaches are taken, ranging from the classical analysis of frequent sites of translocations to the relatively new comparative genomic hybridization with microarrays (66). Many different translocations and other chromosomal aberrations have been found within the various forms of myeloid leukemia with specific translocations being associated with disease subtypes that manifest themselves through the accumulation of immature myeloid cells at varying stages of differentiation. For instance, 95% of chronic myeloid leukemia (CML) patients have the Philadelphia chromosome, a shortened chromosome 22 arising from the reciprocal translocation t(9q34;22q11). The remaining 5% have translocations resulting in the net same result of the production of the *BCR-ABL* gene (67). Genes important to hematopoietic differentiation have also been identified by karyotypic analysis of myeloid leukemias. These AML-associated fusion proteins usually contain a transcription factor involved in differentiation (such as the retinoic acid receptor α) fused to a factor associated with one of the four characteristics of malignant cells (68). Recently, the analysis of chromosomal aberrations has resulted in the discovery of a connection between leukemia and microRNAs (69), small transcripts of 19 to 25 nucleotides that interfere with the expression of genes. Evidence now exists connecting these microRNAs to translocation breakpoints in other forms of cancer (70), suggesting that chromosomal instability acts on these small RNA species as an enabling mechanism similar to the effects seen in tumor suppressor and oncogenes. It will be interesting to see if epigenetic mechanisms are employed directly in the silencing of microRNAs or indirectly in chromosomal instability that targets these microRNAs.

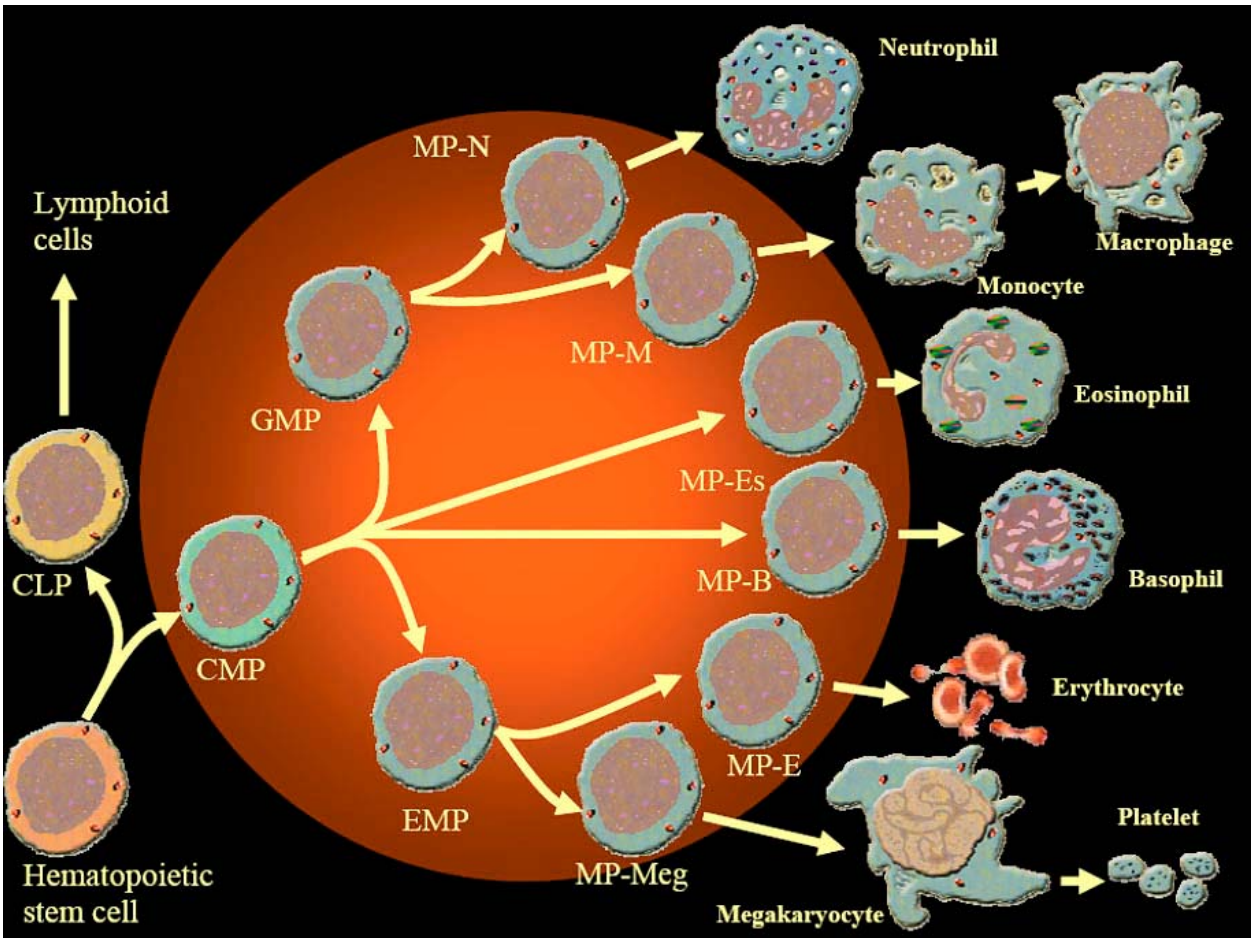

Figure 1. Myeloid cell differentiaion. The differentiation of the six major myeloid cell types from the HSC, CMP, and commited myeloid progenitors types is shown with the MPC compartment depicted as the shaded area. Abreviations: hematopoietic stem cell (HSC), common myeloid progenitor cell (CMP), common lymphoid progenitor cell (CLP), erythroid and megakaryocyte progenitor cell (EMP), and granulocyte and monocyte progenitor cell (GMP). The committed progenitor cells are abbreviated as follows: neutrophil (MP-N), monocyte (MP-M), eosinophil (MP-Es), basophil (MP-B), erythrocyte (MP-E), and megakaryocyte (MP-Meg).

Identification of genes important in leukemias, as well as the elucidation of the mechanisms involved in oncogenesis, has been aided by use of murine model systems. In particular, mouse strains containing ecotropic murine leukemia viruses (MuLVs) have been useful in identifying factors important in leukemias. The SL/Kh, AKXD, and BXH-2 inbred mouse strains contain ecotropic MuLVs that act as mutagenic agents predisposing these strains to the development of leukemia (71-74). Analysis of proviral integration sites in these mice has identified factors such as *Evi2*, *Meis1*, *Hoxa7*, *Hoxa9*, and *Nf1*. Furthermore, these strains exhibit increased hematopoietic proliferation long before they show symptoms of leukemia (72, 75) (Sollars and Buchberg, unpublished observations). This proliferative stage occurs as early as four weeks after birth in BXH-2 and SL/Kh mice. In BXH-2 mice, progression of the disease is reminiscent of human AML with the indolent period for the MPD existing for up to a year before the mice succumb to AML.

5. HEMATOPOIETIC DIFFERENTIATION

About five hundred billion blood cells need to be replaced in the human body each day (76). This feat is accomplished by the exponential amplification of cells from a pluripotent precursor cell known as the hematopoietic stem cell (HSC). The HSC does not generate the required 5 x 10^{11} cells directly on a daily basis, but instead generates highly prolific progenitor cells that produce the nine major hematopoietic cell types. These progenitor cells serve the function of cellular amplification between the largely quiescent HSC and the non-dividing mature cell. The progenitor cells form a gradient of cell types that begins at a level of pluripotence capable of generating all the cells of the hematopoietic system and ends up in a state of commitment where they are only capable of generating a specific cell type (Figure 1). Understanding the mechanisms controlling differentiation in this milieu of progenitor cells, known as the myeloid progenitor cell compartment (MPC), is important to our comprehension of myelogenous leukemias.

Differentiation events in the adult myeloid lineages of the immune system start from the common myeloid progenitor (CMP) (76), the most pluripotent

progenitor cell of the myeloid lineages that is derived from the HSC. This cell eventually produces committed myeloid progenitor cells (committed MPs) that are only capable of generating cells of one of the six major myeloid cell types. The committed MPs will proliferate, mobilize from the marrow, and differentiate to mount the cellular immune response of terminally differentiated myeloid cells in the periphery (77).

6. EPIGENETICS AS A CONTROL MECHANISM FOR DIFFERENTIATION

Waddington was a developmental biologist, and it is in this field that the study of epigenetics has been fruitful in determining biological significance. His studies in *Drosophila*, along with other researchers, led to the discovery of the importance of the chromatin remodeling trxG and PcG genes in cellular differentiation events. Since the control of differentiation is increasingly understood to be important in leukemia, it is critical that the role of epigenetics in differentiation be understood.

Differentiation of the various cell types of an organism is the result of activating or repressing the expression of sets of genes by epigenetic changes. Such epigenetic events reprogram the genome in normal development into different types of differentiated somatic cells (29, 78). Epigenetic modifications influence the flexibility of differentiating cells, and these modifications may eventually become sufficient to serve as the basis for stable gene activation or silencing in subsequent cell generations. The importance of differentiation in oncogenesis is due to its effects on the characteristics a premalignant cell must acquire in order to become fully transformed. Differentiating cells take on a quiescent cell state, where they remove themselves from the cell cycle, entering the G_0 stage and thus becoming less proliferative. Differentiated cells are less protected from apoptosis, in that they lose the expression of several anti-apoptotic factors that stem cells express. Differentiated cells are more prone to senescence because they have very low level or no expression of telomerase that stem cells possess (79), thus they have limited ability to propagate. The loss of self-renewal capacity is the first characteristic of differentiation of the hematopoietic stem cell, being the difference between the long-term subset and short-term subset of HSCs (80). Consequently, differentiation takes a cell further and further away from the attributes of malignant cells.

The relationship between differentiation and leukemia is best understood through the study of the homeobox (Hox) class of genes. These are transcription factors expressed during differentiation that elicit changes in gene expression necessary for morphological and physiological transformations of the cell. The mammalian counterparts to the 39 human homeobox class I genes are best known for their roles in axial patterning during development (81). However, in 1988 Hox genes were linked to murine leukemic transformation when the WEHI-3B leukemic cell line was found to contain proviral integrations resulting in transcriptional activation of *Hoxb8* and *Interleukin 3* (*Il3*) (6). Later, direct evidence for Hox involvement in leukemic transformation was reported when mice transplanted with bone marrow cells engineered to overexpress *Hoxb8* and *Il3* were shown to suffer from acute, aggressive, polyclonal leukemia (11). Other Hox genes have also been implicated in leukemia, including *Tlx1*, *Hoxa9*, *Hoxa10*, and *Hoxb3* (8, 9, 12, 13). Conservation from mice to humans of Hox-induced leukemic transformation is indicated by a recurrent reciprocal translocation t(7;11) predominantly associated with acute myelogenous leukemia (AML), which results in a fusion of a subdomain of *NUP38*, a member of the GLFG nucleoporin family, with *HOXA9* (7). Further evidence for the validity of the murine models was found when human AMLs with favorable cytogenetic features associated with low overall HOX gene expression while poor prognostic cases had high levels (14).

Further evidence illustrating the involvement of *Hoxa9* in hematopoiesis was found when *Hoxa9* knockout mice were found to have abnormal B lymphopoiesis and hypoproliferative granulocyte-macrophage progenitors (10). The importance of *HOXA9* in human leukemias was also demonstrated in an analysis of 6817 genes in leukemias with treatment failure, which found the most highly correlating factor to be the expression of *HOXA9* (82). Studies *in vitro* have shown that *Hoxa9* is capable of immortalizing a promyelocyte that is dependent upon granulocyte-macrophage colony stimulating factor (GM-CSF) and capable of differentiating into at least two myeloid cell types upon removal of GM-CSF and addition of other cytokines (83). Later *in vivo* studies were able to elicit AML in mice by overexpression of *Hoxa9* in hematopoietic cells used to reconstitute the immune system of immunodepleted mice (12, 84). The role of *Hoxa9* in murine AML appears to be the arrest of hematopoietic cells in a primitive myeloid progenitor state characteristic of this disease, i.e. cellular differentiation has been prevented in these cells (85).

Evidence indicates that the prevention of differentiation in this system requires the expression of *myeloid ecotropic integration site 1* (*Meis1*) (9, 74), another gene encoding a homeobox containing protein. The *Drosophila* homolog of *Meis1*, *homothorax*, has been implicated as a target in epigenetic gene regulation by Hsp90 (encoded by *Hsp83* in *Drosophila*) and the trx-G proteins (Sollars, unpublished observations). Therefore, not only are homeobox genes important to human leukemias, there is evidence that the specific genes involved are subject to epigenetic regulation. Since enabling mechanisms are so important in oncogenesis, determining the role of epigenetic gene regulation in the control of Hox genes necessary for eliciting AML is crucial for our treatment of the disease.

The connection between mammalian Hox genes and epigenetics can be explained by examining the role of their homologs in *Drosophila* during embryogenesis. In this developmental period, the anteroposterior (head to tail) axis of the body plan for the organism is laid out in a segmental pattern by the actions of a group of transcription factors encoded by the segmentation genes. By acting in a combinatorial manner, they provide the positional information

necessary for determination of segment identities. However, expression of the segmentation genes ceases long before this determination has resulted in differentiation. The role of trxG and PcG genes is to preserve the positional information of the segmentation genes in the form of chromatin conformation after their expression has ceased. In the case of PcG genes, this occurs by repressing gene expression while the trxG genes preserve the active state of genes already being expressed. Both PcG and trxG genes are thought to alter chromatin structure to carry out their affects, likely involving histone H4 acetylation (86). Thus, it is possible for segments to preserve their determination after the segmentation gene protein products are degraded so that differentiation can occur later.

The positional information takes the form of expression patterns of the Hox genes contained in the Antennapedia (3) and bithorax complexes (4, 5). These Hox genes are responsible for gene regulation necessary for proper differentiation of the segments of the developing embryo. In this manner, cells are locked into a specific state of determination resulting in differentiation via the Hox genes. A mutation in the homeobox genes causes the transformation of one body part into another as positional informational is wrongly represented. These homeotic transformations are also seen when mutations occur in trxG or PcG of genes (19), indicating that changes in positional information can occur as a result of changes in epigenetic gene regulation. These transformations induced by perturbations in epigenetic regulation are heritable, even in the absence of the original mutation in the chromatin remodeling factor (19). Thus, epigenetic gene regulation affects the expression pattern of the Hox genes and thereby influences differentiation.

The ability of epigenetic changes to override transcription factor regulation has tremendous implications for chemotherapy, as it implies that epigenetics is epistatic to genetics in some cases, e.g. Hoxa9 overexpression leads to AML by preventing differentiation, but it may be possible to induce even these cells to enter a quiescent differentiated state less refractory to traditional chemotherapeutic approaches. Differentiation therapy for leukemia has been centered on the instigators of the signal transduction pathways leading to Hox expression, namely cytokines. Examinations of whether malignancy in myeloid leukemic cells can be suppressed by inducing differentiation with normal cytokines have been conducted. It was found that these cells can be induced to differentiate to non-dividing mature granulocytes and/or macrophages by adding different cytokines including IL-6, IL-1, GM-CSF, G-CSF and IL-3 (87-91). It was then shown that differentiation can be induced in some myeloid leukemic cells by LIF, OSM, IL-11 (92) and TNF (93). These results suggest that leukemic cells can be reprogrammed by normal cytokines to behave again like non-malignant cells. This approach has been used in the form of retinoic acid and cytokine therapies following high dose cytotoxic agents (90, 94, 95), but has had limited success. Epigenetics may offer a solution to the difficulties of this therapeutic approach, because as differentiation progresses, non-expressed cytokine loci move from euchromatin to regions of pericentromeric heterochromatin, a subnuclear microenvironment correlated with gene silencing and DNA replication late in S phase (96). Since epigenetic suppression of malignancy by inducing differentiation bypasses the genetic abnormalities, such as chromosomal abnormalities in malignant cells, epigenetic-related chemotherapy may be a more successful differentiation therapy (97, 98). Several clinical trials are now ongoing using histone deacetylase inhibitors and DNA methylase inhibitors.

7. EPIGENETIC MODIFICATIONS AND THEIR RELATIONSHIP WITH LEUKEMIA

The role of epigenetic gene regulation in hematopoietic differentiation and the importance of differentiation in both the MDS and leukemic stages of disease progression make epigenetics vitally important in both the prevention and treatment of leukemia. Scientists have recognized the importance of epigenetics in oncogenesis. This recognition has resulted in an exciting era of research in the field with an increase from 45 genes shown to exhibit DNA methylation in 2001 (26-28) to 66 genes shown to exhibit DNA methylation (specifically in oncogenesis) in 2004 (47). Thus, epigenetic modifications are becoming increasingly important in the etiology of cancer, but should be viewed as one of many layers of control on gene expression, albeit one with unique characteristics.

Discoveries of epigenetic gene regulation in differentiation of myeloid cells, T-helper (T_H) cells, and T lymphocytes show that this form of regulation is robust in the hematopoietic system. Differentiation of T_H cells has proven a particularly tractable system for studying how epigenetics regulates cellular identity (99-102). The existing data reveal that T_H1 and T_H2 cell identity is established and maintained by a dynamic interplay between epigenetic modifications and self-reinforcing transcription factors that act through *cis*-regulatory DNA elements to manage the threshold for subsequent factor binding, chromatin remodeling and transcription (103). Studies of T lymphocytes point out that the murine homologues of the well characterized SWI-SNF complexes are an integral part of hematopoietic differentiation (104, 105), indicating conservation of basic mechanisms from yeast to mice. The myeloid differentiation specific cytokine IL-6 (87, 106) can induce expression of *DNA methyltransferase 1* (*DNMT-1*) (107), which was found to occur via the transcriptional activation of *FLI-1*, a transcription factor that up-regulates *DNMT*-1 (108). Additionally, cDNA microarray analysis of the KAS-6/1 multiple myeloma cell line treated with the demethylating drug, zebularine, revealed the methylation of several genes contributing to the growth and survival of these cells. Cells treated with zebularine recovered their viability and methylation status of these apoptosis and proliferation related genes after treatment with IL-6 (109).

The involvement of epigenetics in leukemia is seen in several notable examples. In CML patients, the genes making up the major breakpoint cluster region display epigenetic regulation with disease advancement. These epigenetic effects includes increased methylation of the Pa promoter of *Abl* seen in advanced phases (110, 111),

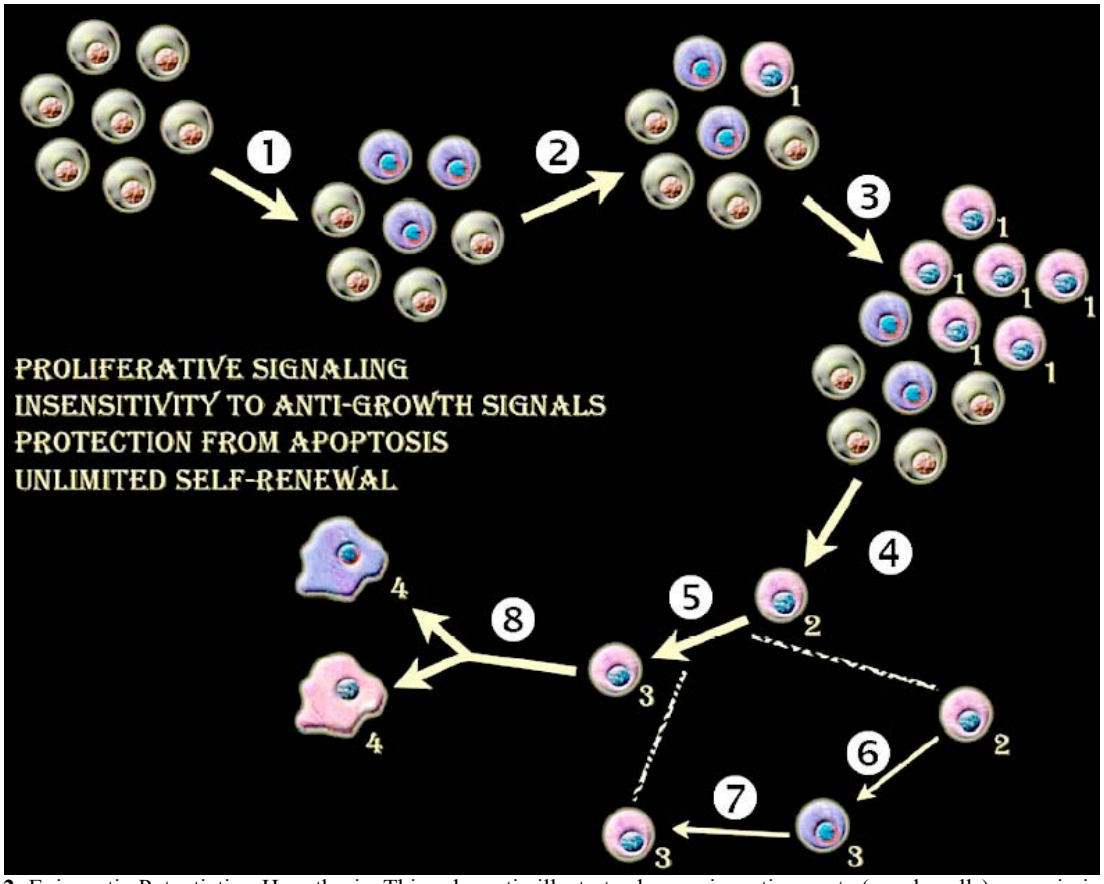

Figure 2. Epigenetic Potentiation Hypothesis. This schematic illustrates how epigenetic events (purple cells) can mimic genetic alterations (pink cells) or prime a cell for a genetic mutation. In this hypothesis the epigenetic events do not have to occur at each stage (similar to the hypothesis proposed by Dr. Ilyas *et al*. for oncogenesis[1]), the hypothesis simply states the possibility of where these events can occur to aid in the process of cellular transformation. Normal cells (yellow cells) may be exposed to environmental factors that prime these cells by producing a pool of cells that have epigenetic modifications making the cells more susceptible to genetic mutation (step 1). The epigenetic modifications may act as an enabling mechanism which results in the cell acquiring one of the four characteristics of malignant cells listed in the figure (step 2). The numbers in yellow indicate the total number of characteristics the premalignant cells have acquired. The cells that have acquired the mutation begin to proliferate and struggle with the host to survive (step 3). As this process continues the cells must acquire more of the characteristics of malignant cells to survive. During the acquisition of the remaining characteristics epigenetics may act repeatedly as an enabling mechanism (step 5) by producing an epigenetic mimic of a genetic mutation (step 6). Epigenetic changes can alter the expression patterns of genes important in cellular pathways that are critical in the four characteristics of malignant cells, thus creating an epigenetic mimic of a genetic change. This epigenetic mimic may be solidified by an eventual genetic mutation (step 7). Eventually, a combination of epigenetic and genetic alterations accumulate in a cell resulting in a fully transformed state (step 8).

as well as methylation of *Bcr* in lymphoid blast crisis (112). Blast phase CML is also associated with increased expression levels of DNA methyltransferase genes *DNMT1*, *DNMT3A*, and DNMT3B (113). Hypermethylation of *p15*, an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, whose expression is induced by transforming growth factor β, is also associated with CML transformation (114). The importance of *p15* is seen in disease progression from MDS to AML, where *p15* is targeted for hypermethylation in 78% of samples at the time of leukemic transformation (115). Epigenetics may be a significantly more important enabling mechanisms in AML than in other cancers, because chromosomal instability does not seem to be predominant, with 57.6% *de novo* AML patients having normal cytogenetics (116).

Epigenetic regulation of key factors involved in oncogenesis can precede actual genetic changes, temporarily altering the phenotype of a particular cell or group of cells. This regulation can alter cells in a way that makes them more susceptible to genetic alteration or can mimic a genetic mutation with an epigenetic change. Evidence suggests that this mechanism is an adaptation of the standard apparatus of gene regulation in the hematopoietic system, where before the onset of stable transcription factor binding, specific chromatin alterations are manifested (117). Thus, epigenetic alterations can act as an enabling mechanism for acquisition of the characteristics of oncogenesis by providing a mimic of an oncogenic mutation preceding the genetic changes that allow the oncogenic phenotype to become more stable (Figure 2). This idea is an extrapolation of Waddington's canalization model and a model for evolution involving

chromatin remodeling that others and I proposed earlier (19), which has also been postulated in a similar form applied to oncogenesis by Andrew Feinberg (118). According to Waddington, "By such a series of steps, then, it is possible that an adaptive response can be fixed without waiting for the occurrence of a mutation which, in the original genetic background, mimics the response well enough to enjoy a selective advantage."

The study of cancer has jumped forward with our deepening knowledge of how the environment and the history of the organism can affect genomes. It is clear that cells retain a "cellular memory" in the form of epigenetic modifications of the genome. This cellular memory can be influenced by past and present conditions, as well as conditions of the founder cells and environmental effects on ancestral genomes of the organism. Thus, cellular memory can result in polymorphisms which are not genetic, but may exist among a population both at the organism and cellular levels. It is critical to our understanding of oncogenesis that we elucidate the system whereby epigenetics acts as an enabling mechanism for oncogenesis.

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