MDS Pathways and DNMT1 Control

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MDS, Myelodysplastic Syndrome, is a complex hematopoietic disorder that appears to be drivin by epigenetic methylation of transcription factor regions in various cell lines. A standard procedure has been to use high doses of DNMT1 suppressors and these have demonstrated efficacy in almost all cases for a period of time. However there is the question of what targets are methylated, how and what the proper dosages should be. Recent research has argued for a substantially lowered dose, which may have substantial efficacy in view of the fact that system demethylation could be problematic. We examine this process herein.

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1 INTRODUCTION

MDS is a proto-malignant state where the causative factor putatively is methylation of hematopoietic control genes often along the myelo line from stem cells. The result is a proliferation of blasts and immature cells and often with an aberrant growth in one or more cell lines including erythrocytes and platelets.

The typical initial approach is to use one of two possible suppressors on DMNT1 which can facilitate methylation in new cells. The two most common DMNT1 inhibitors are azacitidine and decitabine. However the doses used are often quite large and have some morbidity and sequellae. In a recent paper by Sauntharajah et al the authors note:

MDS is genetically heterogeneous. Thus, in the effort to identify pretreatment characteristics that predict response to therapy, there has been a focus on potential mutational predictors, but without a strong mechanistic rationale. From a mechanistic perspective, however, a minimum requirement for response is achievement of the intended molecular pharmacodynamic effect (DNMT1 depletion), whatever the disease mutations.

DNMT1 depletion is S-phase dependent and hence drug exposure time dependent: effectiveness meriting FDA approval was achieved when decitabine doses were reduced to less than 10% of those initially evaluated but administered more often (lower doses caused less toxicity that enabled more frequent administration).

A further decrease in this initial FDA-approved dose from 45 mg/m2/day to 20 mg/m2/ day and a further increase in frequency of administration (5 days every 4 weeks instead of 3 days every 6 weeks) doubled the overall response rate from 30% to 63%. Underscoring the importance of frequency of administration, giving this latter lower dose less often (3 days/28-day cycle) decreased overall responses to 23%.

Thus a reduction actually improved response.

However the authors of this paper demonstrate a collection of genes which we will discuss herein as being of significance in this disorder. They state:

Hence, especially for some subtypes of myeloid malignancy, there is a need for treatments that are not mediated through p53 and apoptosis (noncytotoxic treatments). Several groups have observed that terminal differentiation is induced in vitro when treating myeloid and other cancer cells with drugs or conditions that inhibit gene-silencing, chromatin-modifying enzymes (chromatin relaxation).

These differentiation- mediated cell-cycle exits, like those that occur during normal tissue differentiation, do not require p53 and are readily induced in p53/p16-null cancer cells. The same chromatin-relaxing conditions increase the differentiation of normal progenitors as well but, in contrast, increase self-renewal of NHSCs. The reasons for this cell context–dependent

response have been evaluated: differentiation is driven by relatively few master transcription factors.

Myeloid cancer cells express master myeloid differentiation– driven transcription factors (e.g., CEBPA and PU.1) at high levels, yet the target genes of these transcription factors are epigenetically silenced because of aberrant recruitment of silencing — instead of activating — chromatin- modifying enzymes to the transcription factors. Inhibition of silencing enzymes with drugs such as decitabine restores expression of numerous target genes of the transcription factors, including MYC antagonists (e.g., CEBPE and p27/ CDKN1B), that terminate proliferation.

Normal stem cells, on the other hand, express master stem cell transcription factors (e.g., HLF and HOXB4) and activate stem cell genes and stem cell fate in response to the same treatments (good therapeutic index).

The above focuses on the importance of CEBPA, HLF and PU.1. The authors also focus on GATA1 as well. These all are control elements in hematopoiesis. Now it is known that there may be methylation on CpG islands ahead of these genes and such methylation would result in suppression of the gene. Suppression would then result in loss of control and putatively a resulting unstable set of growth.

We will use this paper to examine several issues. Specifically:

1. The effect of methylation.

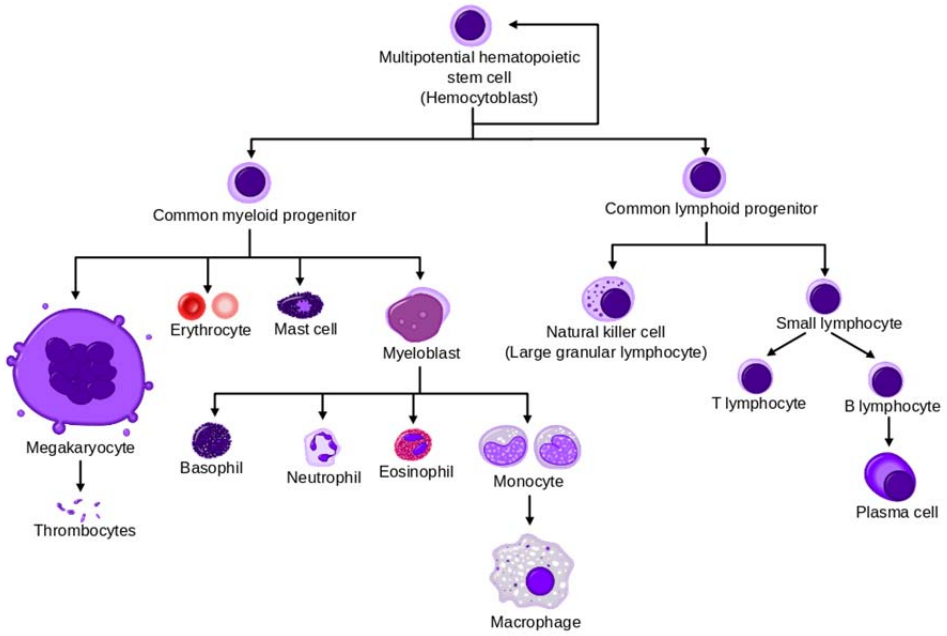
2. The location of methylation.

3. The cause of methylation.

4. The impact of suppressing DMNT1 and thus re-methylation of stem cell proliferates.

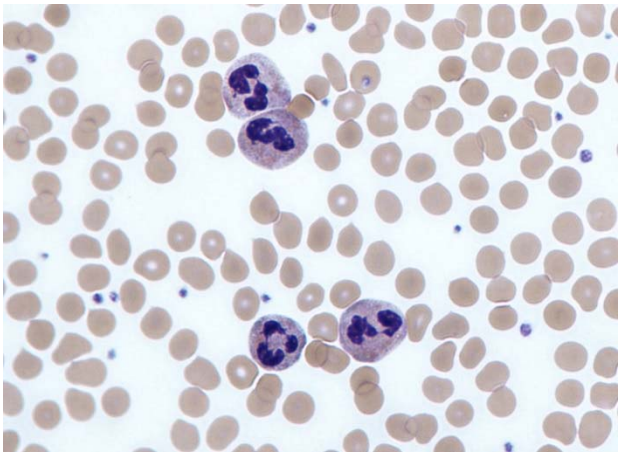
5. A re-analysis of the results of recent work using DNMT1 suppression followed by BMT and then the use of CIK cells. We had examined this in 2013 and results clinically have been favorable in several Phase 2 Trials.

Now when we examine MDS we often ask; when and where does the methylation occur. The following is the classic breakout of cells from the stem cell:



Now we often see low erythrocytes or thrombocytes (platelets) as well as low neutrophils. Low red cells result in an anemia and this is often one of the major presentations of MDS. Low platelets are the cause of poor clotting and excessively low platelets lead to catastrophic DIC. Low neutrophils result in possible uncontrolled infections including sepsis. Oftentimes the diagnosis of MDS is incidental to these and other findings.

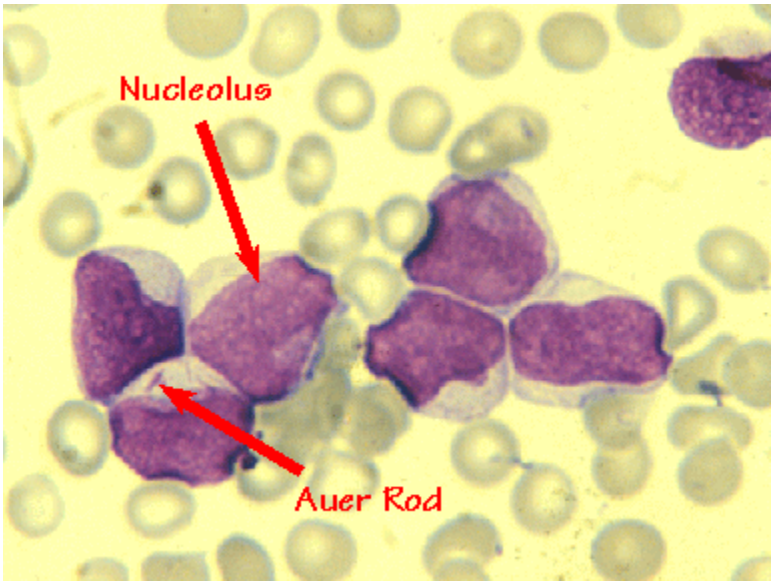
If we examine neutrophils we typically see the following:



The neutrophils are multilobed and mature is what one would expect in the circulatory system. Immature neutrophils, called bands, are often indicative of infection and blasts are very immature cells often indicative of an advanced MDS process.

One question is that in MDS we see just one line, say the myeloid line, showing the effects of methylation. However the natural sequella to MDS is AML and this is a broad loss of control across the myeloid line.

Blast cells are immature precursors of either lymphocytes (lymphoblasts), or granulocytes (myeloblasts). They do not normally appear in peripheral blood. When they do, they can be recognized by their large size, and primitive nuclei (ie the nuclei contain nucleoli), as in the picture. When present in the blood, they often signify acute myelocytic leukemia, AML. This particular case below demonstrates the presence of an Auer Rod, which is pathognomonic for Acute Myeloid Leukemia. Otherwise, special stains and surface marker techniques are needed to identify the lineage of the cells.



As Pang et al note:

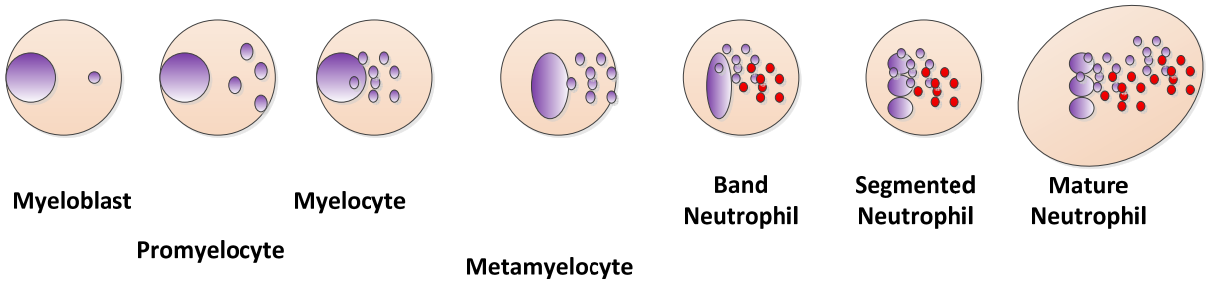
Myelodysplastic syndromes (MDS) are a group of disorders characterized by variable cytopenias and ineffective hematopoiesis. Hematopoietic stem cells (HSCs) and myeloid progenitors in MDS have not been extensively characterized. We transplanted purified human HSCs from MDS samples into immunodeficient mice and show that HSCs are the disease-initiating cells in MDS.

We identify a recurrent loss of granulocyte-macrophage progenitors (GMPs) in the bone marrow of low risk MDS patients that can distinguish low risk MDS from clinical mimics, thus providing a simple diagnostic tool. The loss of GMPs is likely due to increased apoptosis and increased phagocytosis, the latter due to the up-regulation of cell surface calreticulin, a prophagocytic marker.

Blocking calreticulin on low risk MDS myeloid progenitors rescues them from phagocytosis in vitro. However, in the high-risk refractory anemia with excess blasts (RAEB) stages of MDS, the GMP population is increased in frequency compared with normal, and myeloid progenitors evade phagocytosis due to up-regulation of CD47, an antiphagocyticmarker. Blocking CD47 leads to the selective phagocytosis of this population.

We propose that MDS HSCs compete with normal HSCs in the patients by increasing their frequency at the expense of normal hematopoiesis, that the loss of MDS myeloid progenitors by programmed cell death and programmed cell removal are, in part, responsible for the cytopenias, and that up-regulation of the "don't eat me" signal CD47 on MDS myeloid progenitors is an important transition step leading from low risk MDS to high risk MDS and, possibly, to acute myeloid leukemia.

Now if one looks at the neutrophil line one sees the progression from the myeloblast, blast, in the bone marrow, to the mature neutrophil in the blood stream. We demonstrate that below.



Now it is the presence of adequate neutrophils that result in a strong initial immune response. The failure of the marrow to allow maturation results in excess blasts and fewer mature neutrophils. That is one of the problems of having methylated areas around the genes forcing that maturation process.

There are three transcription factors that play a prominent role here. They are:

1. CEPBA
2. PU.1
3. GATA-1

Also discussed in Fiedler and Brunner are GATA-2. The authors state:

Several other studies dealing with certain aspects of the molecular interaction of PU.1 and GATA-1 as well as their gene regulatory capacity revealed the cross-antagonism between these proteins involving direct physical interaction of both factors that results in an inhibition of the transactivation trans-activation potential of the counterpart. Based on these findings, GATA-1 is prospected as the erythroid/megakaryocyte lineage determinant, whereas PU.1 is regarded as the myeloid/ lymphoid lineage determinant…..

Moreover, lineage choice between monocytes and granulocytes depends on the expression level of PU.1 and C/EBPα, which has been shown by studies using different mouse as well as in vitro models for diminished PU.1 expression in the hematopoietic system.

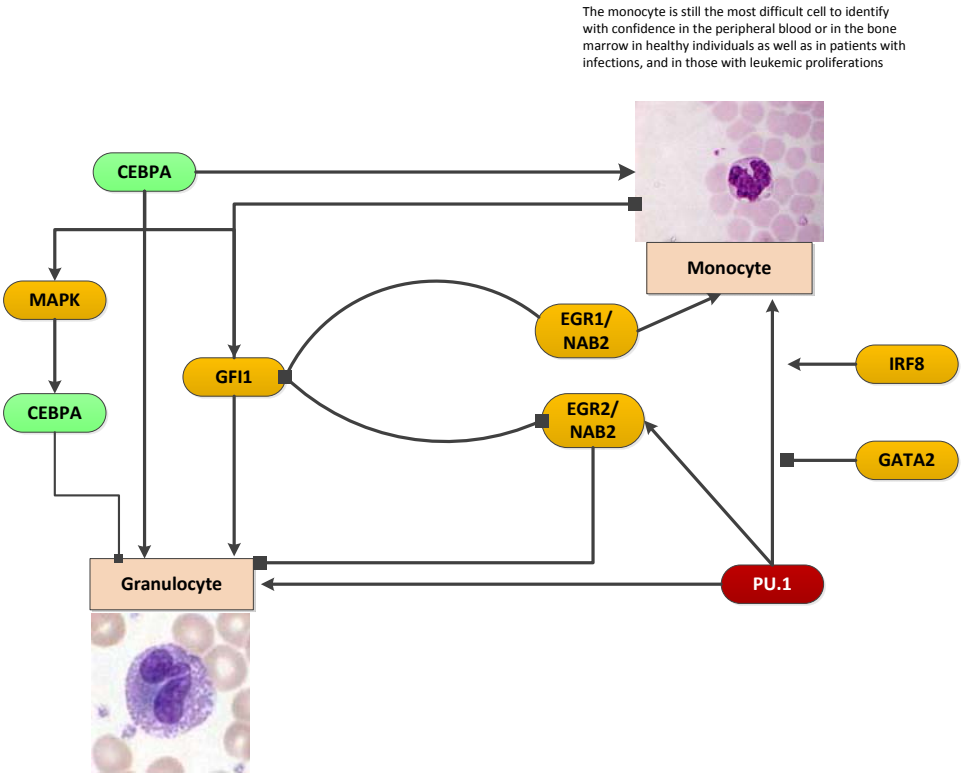
In all experimental setups, reduced expression of PU.1 is followed by an augmented granulopoiesis to the disadvantage of monocyte development. In line with these findings, we

have demonstrated that loss-of-function mutations of Btk in myeloid cells diminished the C/EBPα as well as PU.1 expression resulting consequently also in an increased granulopoiesis at the expanse of monopoiesis.

Additionally, gene expression analyses of PU.1-deficient progenitors revealed a decreased or even absent expression of several monocyte-specific genes, like the macrophage scavenger receptor or the M-CSF receptor….

Thus for neutrophils there needs to be increased PU.1 and CEBPA to allow full maturation. It is thus expected that for lack to mature one must have an altered level. We will demonstrate that from the results in the focus paper.

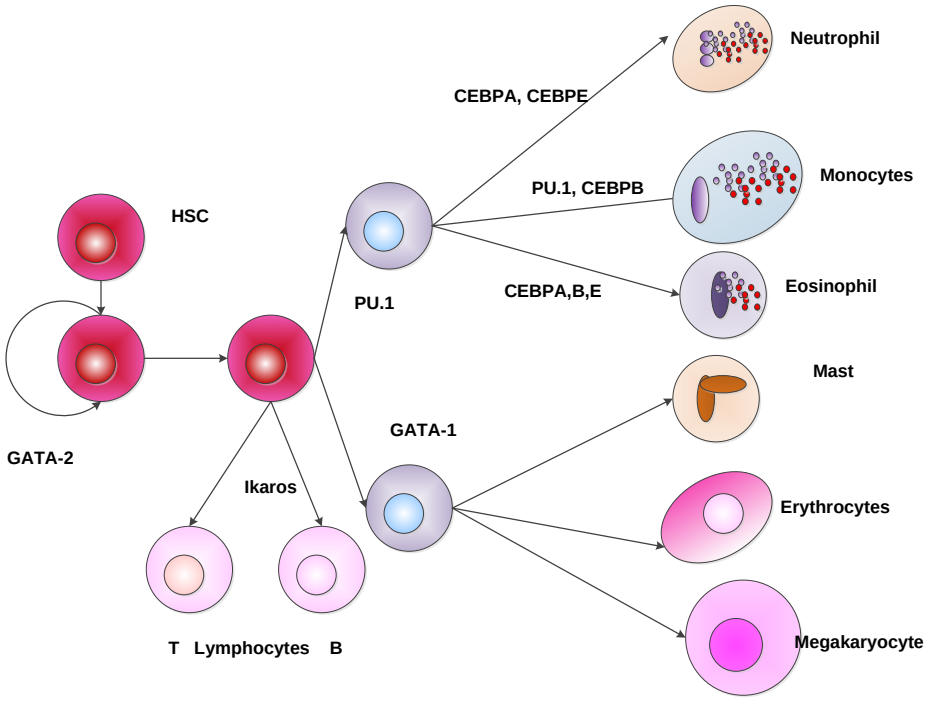
The management of monocyte to granulocyte is shown below with two of the genes we discuss herein. We return to this later.



2 GENE EXPRESSIONS

We now will examine the genes referenced in the focus paper. Specifically we examine HLF is a transcription factor and has been associated with leukemias. It also prolongs cell life and can be a facilitator for other transcription factors. CEBPA is another transcription factor gene and is a leucine zipper domain entity. It is well know that any mutations of this gene, or perhaps suppression by methylation, results in AML. CEBPA provides a control element is normal hematopoietic control. STI1 or PU.1 is another transcription factor which controls myeloid maturation as well as B cell maturation. We can often see the two names used interchangeably. GATA1 is also a transcription factor of the GATA family and plays a significant role in hematopoietic stasis.

The following is a descriptive from Hoffman et al (Modified Fig 25.3) regarding the active genes which are noted:



It should also be noted that the genes identified are transcription factors which activate genes which themselves are the operative genes. The above from Hoffman et al is indicative of the importance of these transcription factors.

Hoffman et al also there note:

Lineage-specific maturation of committed hematopoietic progenitor cells is ultimately driven by transcription factors, which have been hypothesized to be the final common pathway leading to commitment and differentiation of the pluripotent stem cell.

The role of transcription factors in cellular proliferation, differentiation, and survival of stem cells during hematopoiesis in the mammalian BM has been well established. Studies of the regulation of individual genes that show tissue- and stage-specific myeloid expression have implicated a small number of transcription factors that are responsible for directing both phenotypical myeloid maturation and the expression of functionally important myeloid genes. As described in detail subsequently, this role is underscored by the observations in AML, in which disruption of differentiation and defective myeloid-specific gene expression are linked to pathognomonic chromosomal translocations that result in the dysregulation of transcription factor expression.

Maturation of multipotent progenitor stem cells into specialized blood cells (lymphocytes, erythrocytes, neutrophils, monocytes, and eosinophils, among others) is regulated by a well-orchestrated interplay of transcription factors that are capable of instructing the expression of a specific set of genes within a specific lineage.

Here Hoffman et al emphasize the importance of the transcription factors. The question is however in the discussion on MDS; if we see an excess of these factors in the cells then there must be some other factor occurring and that factor is the methylation and effective blocking of the products of these genes, the transcription factor proteins, from doing what they were intended to do. Thus a secondary protein or sets of proteins are not being produced and the resulting maturation is blocked.

Gene knock-out technology and overexpression studies, in conjunction with newer techniques that involve the use of multicolor fluorescence-activated cell sorting (FACS), have aided in delineating several transcription factors critical to the development of specific hematopoietic lineages. On the basis of these studies, critical transcription factors have been classified into two major categories. The first category includes factors such as stem cell leukemia transcription factor (SCL), GATA2, and AML factor-1 (AML-1) now known as Runx1, that influence differentiation to all of the hematopoietic lineages; the second category comprises the master regulators of lineage development, including GATA1, PU.1, and CCAAT enhancer–binding protein-α (C/EBPα).

These factors not only promote lineage-specific gene expression but also suppress alternative lineage pathways. (The Figure above) summarizes the postulated role of several key transcription factors during hematopoietic development. Myeloid progenitors exhibit multilineage patterns of gene expression. Studies by Laslo et al elegantly demonstrated that cell fate determination is dependent on subtle changes in expression levels of transcription factors, which regulate differential lineage maturation. For example, levels of PU.1 expression are increased by Egr-1/Nab-2 in developing macrophages; at the same time, Egr-1 represses the expression of the neutrophil specific Gfi-1 transcription factor, thereby simultaneously repressing the neutrophil development program

The following is a summary table of each of these genes. These are the genes examined as markers for the efficacy of reduce DMNT1 inhibitors in the MDS work by Sauntharajah et al. One of the key questions is; are these correct markers? A second question is: if they are then what caused their change in expression and how?

Gene	*Location*	*Function*
HLF[1]	17q22	This gene encodes a member of the proline and acidic-rich (PAR) protein family, a subset of the bZIP transcription factors. The encoded protein forms homodimers or heterodimers with other PAR family members and binds sequence-specific promoter elements to activate transcription. Chromosomal translocations fusing portions of this gene with the E2A gene cause a subset of childhood B-lineage acute lymphoid leukemias. Alternatively spliced transcript variants have been described, but their biological validity has not been determined. It drives the hematopoietic stem cell fate.
CEBPA[2]	19q13.1	This intronless gene encodes a transcription factor that contains a basic leucine zipper (bZIP) domain and recognizes the CCAAT motif in the promoters of target genes. The encoded protein functions in homodimers and also heterodimers with CCAAT/enhancer-binding proteins beta and gamma. Activity of this protein can modulate the expression of genes involved in cell cycle regulation as well as in body weight homeostasis. Mutation of this gene is associated with acute myeloid leukemia. The use of alternative in-frame non-AUG (GUG) and AUG start codons results in protein isoforms with different lengths. Differential translation initiation is mediated by an out-of-frame, upstream open reading frame which is located between the GUG and the first AUG start codons.
STI1[3] also PU.1	11p.11.2	This gene encodes an ETS-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development. The nuclear protein binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. The protein can also regulate alternative splicing of target genes. Multiple transcript variants encoding different isoforms have been found for this gene.
GATA1[4]	X.p.11.23	This gene encodes a protein which belongs to the GATA family of transcription factors. The protein plays an important role in erythroid development by regulating the switch of fetal hemoglobin to adult hemoglobin. Mutations in this gene have been associated with X-linked dyserythropoietic anemia and thrombocytopenia.

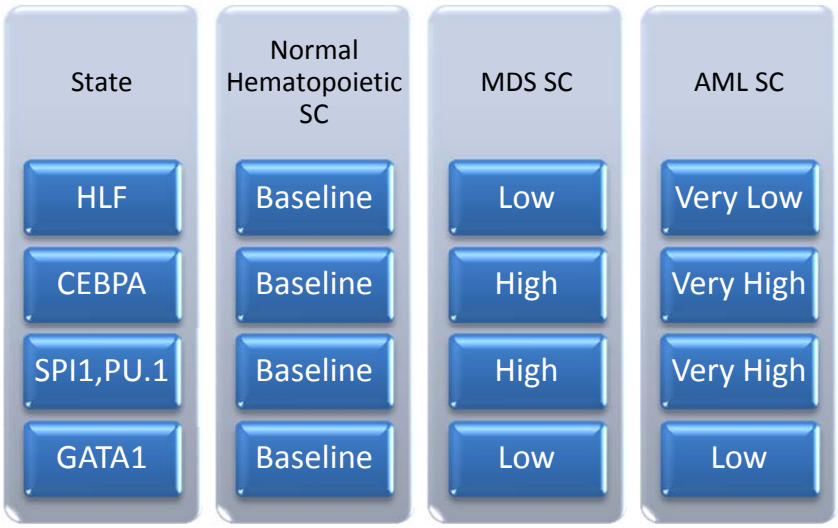
[1] http://www.ncbi.nlm.nih.gov/gene/3131

[2] http://www.ncbi.nlm.nih.gov/gene/1050

[3] http://www.ncbi.nlm.nih.gov/gene/6688

[4] http://www.ncbi.nlm.nih.gov/gene/2623

We can summarize the results of Sauntharajah et al in the following graphic as regards to these four genes. We show the normal baseline and then for MDS stem cells and then for Acute Myelogenous stem cell the expression of each of these genes relative to the normal state. Note the significant increases in CEBPA and PU.1 and the decrease in HLF. We will examine these in some detail. Our intent here is not to examine the work of Sauntharajah et al but to use it as a stepping off point for examining the issue of methylation. Namely what, where, and when are these sites methylated, if indeed that is the case.



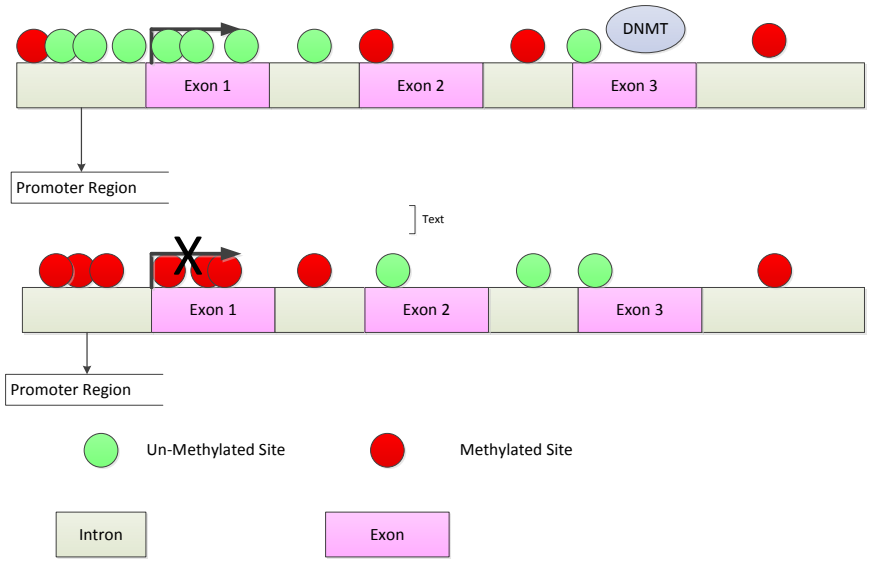
As stated in the reference article by Sauntharajah:

Myeloid cancer cells express master myeloid differentiation– driven transcription factors (e.g., CEBPA and PU.1) at high levels, yet the target genes of these transcription factors are epigenetically silenced because of aberrant recruitment of silencing — instead of activating — chromatin- modifying enzymes to the transcription factors.

Inhibition of silencing enzymes with drugs such as decitabine restores expression of numerous target genes of the transcription factors, including MYC antagonists (e.g., CEBPE and p27/ CDKN1B), that terminate proliferation.

Normal stem cells, on the other hand, express master stem cell transcription factors (e.g., HLF and HOXB4) and activate stem cell genes and stem cell fate in response to the same treatments (good therapeutic index)

Namely although the CEBPA and PU.1 are high, they as transcription factors cannot function because the gene which they control has been methylated and thus is blocked. We demonstrate that below:



Namely with the methylation of say a promoter region even though we have a strong transcription factor it cannot function and we obtain aberrant cell growth.

2.1 HLF

We start with HLF, the hepatic leukemia factor gene and its product.

From Waters et al:

Physiological variation related to circadian rhythms and aberrant gene expression patterns are believed to modulate therapeutic efficacy, but the precise molecular determinants remain unclear. Here we examine the regulation of cell death by hepatic leukemia factor (HLF), which is an output regulator of circadian rhythms and is aberrantly expressed in human cancers, using an ectopic expression strategy in JB6 mouse epidermal cells and human keratinocytes.

Ectopic HLF expression inhibited cell death in both JB6 cells and human keratinocytes, as induced by serum-starvation, tumor necrosis factor alpha and ionizing radiation. Microarray analysis indicates that HLF regulates a complex multi-gene transcriptional program encompassing upregulation of anti-apoptotic genes, downregulation of pro-apoptotic genes, and many additional changes that are consistent with an anti-death program.

Collectively, our results demonstrate that ectopic expression of HLF, an established transcription factor that cycles with circadian rhythms, can recapitulate many features associated with circadian-dependent physiological variation.

Thus HLF has a strong capability to allow a cell to avoid apoptosis and survive long periods. HLF is amongst the many transcription factors stabilizing cell life.

From earlier work by Honda et al, the authors had noted:

Transcription factors are frequent target of chromosomal translocations observed in acute lymphoblastic leukemia (ALL). Among them, E2A gene, encoding a basic helix-loop-helix (bHLH) transcription factor on chromosome 19, has been known to be involved in two chromosomal translocations, t(1;19)(q23;p13)3,4 and t(17;19)(p22;q13)5,6 that are observed in human B-lineage leukemias.

As a result of t(1;19)(q23;p13), the C-terminal region of E2A gene, including the bHLH DNA binding and dimerization domains, is replaced with the DNA binding domain of PBX1 homeobox gene on chromosome 1.

On the other hand, following the t(17;19)(p22;q13), the same region of the E2A gene is fused to the DNA-binding and dimerization domains of hepatic leukemic factor (HLF) gene belonging to the basic region/leucine zipper (bZIP) family on chromosome 17. These events create novel fusion gene products, E2A-PBX1 and E2A-HLF, respectively, and the expression of these chimeric transcription factors with altered structural and functional features would play a substantial role in the leukemogenic process(es).

2.2 SPI1 (PU.1)

PU.1 is another transcription factor involved in hematopoeisis. We have seen its function generally before and we have discussed its overall function. We here examine it in a bit more detail. Again it should be noted that it is not PU.1 but the genes it transcribes that are at fault.

As Hoffman et al state (Chapter 25):

PU.1 is a member of the Ets family of transcription factors and is expressed abundantly in B cells and macrophages. Expression of PU.1 has also been reported in granulocytes and eosinophils as well as in CD34+ hematopoietic progenitor cells. Whereas high levels of PU.1 expression in fetal livers of mice preferentially direct macrophage development, low levels of PU.1 result in B-cell development. C-Jun, another member of the b-Zip family of transcription factors, serves as a coactivator of PU.1 during macrophage development.

The ETS family transcription factors form or have loops (loop-helix-loop) and are activated by kinases. (See Marks et al pp 404-406). The above continues:

It has been demonstrated that overexpression of c-Jun in myeloid progenitor cells results in macrophage development. Recent studies have revealed that downregulation of c-Jun by C/EBPα is necessary for granulocytic maturation and appears to be the mechanism through which C/EBPα blocks macrophage development. C/EBPα not only binds to the promoter of the c-jun gene and decreases its expression but also binds PU.1, thereby inhibiting its activity.

PU.1-binding sites have been reported in almost all myeloid-specific promoters reported to date, including those for M-CSF, GM-CSF, and G-CSF receptors, all of which play critical roles in myeloid cell development. PU.1 activity is modulated both by covalent modifications and by

protein–protein interactions. For example, phosphorylation of PU.1 by casein kinase II or by JNK kinase leads to increased transcriptional activity.

Abrogation of PU.1 expression in PU.1−/− mice results in perinatal lethality accompanied by the absence of mature monocytes/macrophages and B cells and delayed and reduced granulopoiesis. After in vitro differentiation, embryonic stem cells derived from PU.1−/− blastocysts fail to express mature myeloid cell markers, suggesting that PU.1 is not essential for the initial events associated with myeloid lineage commitment but is necessary for the later stages of development.

From Young et al we have a more detailed discussion on monocyte development:

Numerous studies have established that a core set of genes act as 'master regulators' of myeloid differentiation. Monocytic differentiation is promoted by the PU.1-induced transcription factors EGR2 and NAB2, whereas granulocytic differentiation is promoted by GFI1 and CEBPA .

Note the differentiation with PU.1 and CEBPA. Myeloid differentiation is complex and controlled via these transcription factors. Granulocytes result from the CEBPA lineage.

Our data show that loss of Hnrnpa0 leads to the suppression of pro-monocytic genes (Egr2, Nab2, Irf8, Emr1) and induction of pro-granulocytic genes (Cebpa, Gfi1, Csfr3), within this network. EGR1, like HNRNPA, is located within the CDS of 5q31.2, and is expressed at reduced levels in CD34+ cells from patients with a del(5q). Similar to HNRNPA0, loss of EGR1 expression favors granulocytic over monocytic differentiation.

Thus, loss of a single allele of EGR1 and HNRNPA0, as a result of a del(5q), may lead to a synergistic disruption of EGR1, and the functionally redundant family member, EGR2, leading to aberrant myeloid differentiation, a hallmark of t-MN. Any disruption to the gene program that regulates the transition from the CMP to the GMP and beyond can uncouple lineage commitment and proliferation control during myeloid differentiation and lead to malignant transformation….

(The following Figure is as…) Model of the impact of 5q deletion upon myeloid cell fate. A schematic diagram showing some of the factors that regulate myeloid cell fate.

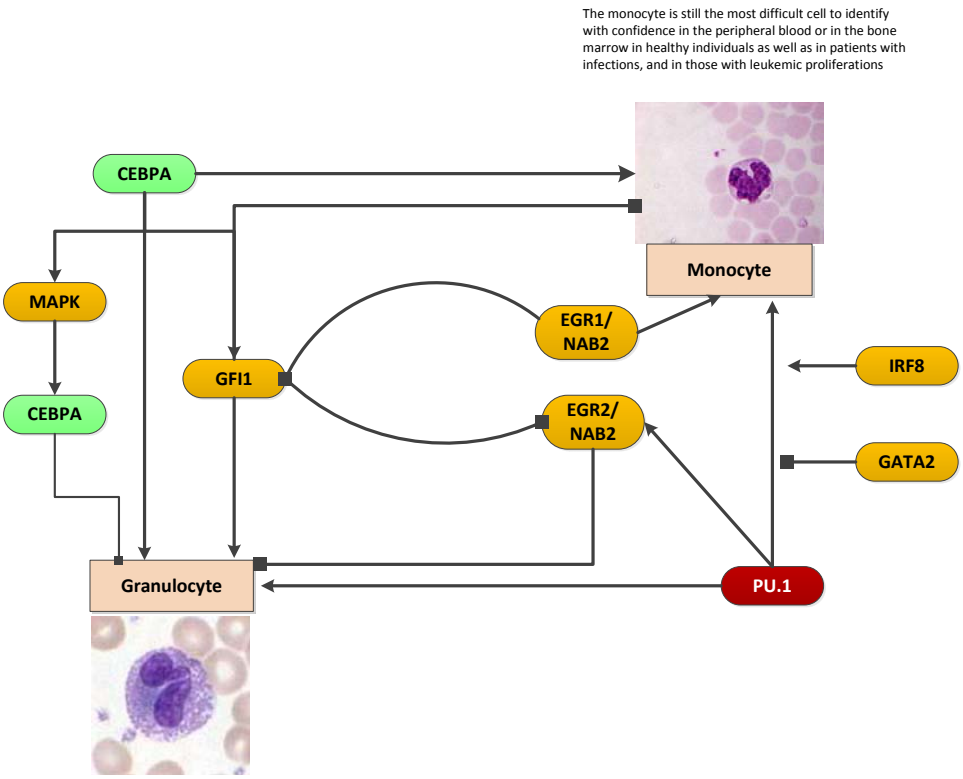
PU.1 normally induces EGR2 and NAB2 to induce monocytic differentiation. CEBPA and GFI1 promote granulocytic differentiation, partially through suppression of EGR1, EGR2 and NAB2.

Again note the focus on PU.1 is monocyte and not granulocyte. Thus when we examine a neutropenic MDS patient we are looking at a blockage of CEBPA transcription action and if the monocyte line is normal we can see a benign state there.

Knockdown of Hnrnpa0 in murine cells leads to the suppression of pro-monocytic transcripts (Egr2, Nab2, Irf8, and Emr1 (F4/80)), many of which contain AREs (light gray boxes), and

induction of pro-granulocytic genes (Cebpa, Gfi1, Csf3r (G-CSFR)). MAPK-induced phosphorylation of CEBPA has been shown to inhibit granulopoeisis. KD of Hnrnpa0 leads to a decreased expression of many genes within the MAPK signaling pathway, consistent with the observed shift towards granulocytic differentiation. Loss of EGR1 (also mapped to human 5q) also favors granulocytic over monocytic differentiation36. Egr1 and Egr2 function in a redundant manner. In del(5q) patients, haploinsufficiency for both EGR1 and HNRNPA0 may synergistically disrupt myeloid differentiation.

We now demonstrate the above discussion with the Figure below. Note that we have a monocyte and a granulocyte at both ends of this process and we have included the respective transcription factors associated with each.



2.3 CEBPA

CEPBA is another key transcription factor. It is a major one especially for neutrophils and other granulocytes. As Hoffman et al state (Chapter 25):

C/EBPα (Namely CEPBA) has been postulated to be a master regulator of the granulocytic developmental program. It is expressed at high levels throughout myeloid differentiation and has been shown to bind to the promoters of multiple myeloid-specific gene promoters regulating gene expression at many different stages of myeloid maturation. Although C/EBPα−/− mice die perinatally because of defects in gluconeogenesis that result in fatal hypoglycemia, they also have a selective early block in the differentiation of granulocytes without affecting either monocyte/macrophage maturation or the differentiation of other hematopoietic lineages.

Myeloid cells from C/EBPα−/− mice lack the G-CSFR, and it has been postulated that lack of mature neutrophils in these mice may be caused by the lack of G-CSFR. However, the myeloid defect in C/EBPα−/− mice is more severe than that seen in G-CSFR−/− mice, suggesting that C/EBPα has additional functions vital to granulocytic maturation.

C/EBPα is a single exon gene, but it is expressed as two isoforms that arise from alternate translation start sites that give rise to a full-length C/EBPαp42 and a truncated dominant negative C/EBPαp30 isoform.13 Translational control of C/EBPα isoform expression is orchestrated by a conserved upstream open reading frame (uORF) in the 5' untranslated region (5'UTR). This region is thought to be responsive to the activities of the translation initiation factors eIF4E and eIF214 such that an increase of eIF2 or eIF4E activity results in an increase in expression of the shorter p30 isoform.13

Again, it appears that CEBPA is expressed but that when it is so the transcription site is blocked due putatively to methylation From Pabst and Mueller:

This review intends to highlight recent reports on dysregulation of the differentiation factor CEBPA at various levels in human AML. The CCAAT enhancer binding protein alpha (CEBPA) is a member of the basic region leucine zipper family of transcription factors. It is composed of two transactivation domains in the N-terminal part, and a leucine zipper region mediating dimerization with other CEBP family members and a DNA binding domain in the C-terminal part.

As a condition for DNA binding, dimerization depends on the basic amino acid residues, and genomic alterations in the exact distance between basic region and leucine zipper impair DNA binding. Two inframe start codons give rise to two CEBPA isoforms: The p30 protein is initiated at an AUG codon further downstream and thus lacks the amino terminal sequences, whereas the C terminus is identical to the full-length p42 protein .

As a consequence, the p30 isoform lacks domains mediating the contact with the transcriptional apparatus, whereas other functions such as dimerization or regions involved in protein-protein interactions are preserved in both p30 and p42 proteins. CEBPA is an intronless gene located at chromosome 19q13.1. It was originally isolated as a rat liver transcription factor regulating hepatic and adipocyte genes. Studies in adipocyte lines have founded the role of CEBPA as an inhibitor of cell proliferation and as a tumor suppressor. In hematopoiesis, the interest in CEBPA is based on its crucial role during the development of granulocytes and on its deregulation associated with myeloid transformation

Isoforms are presentations which may alter the effectiveness of CEBPA.

As Sonnet et al state:

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy associated with severe morbidity and poor prognosis. It comprises a highly heterogeneous group of blastic myeloid malignancies and constitutes the most frequent type of acute leukemia in adults [1]. AML can arise de novo but also secondarily from preceding myelodysplastic syndrome (MDS),

or after cytotoxic treatment or radiotherapy. It is characterized by an aggressive clonal proliferation of immature hematopoietic progenitor cells (myeloblasts) and impaired differentiation.

Recurrent chromosomal aberrations and rearrangements occur in more than 50% of cases and represent important predictive factors for response to therapy and outcome of the disease.

Altered gene function in AML is often a consequence of distinct cytogenetic aberrations, but also results from mutations in genes like CEBPA (CCAAT/enhancer-binding protein, alpha), FLT3 (fms-like tyrosine kinase receptor-3), or NPM1 (nucleophosmin 1. Although novel high resolution genome-wide technologies have enabled the detection of numerous gene mutations, the multistep process of leukemogenesis is still poorly understood.

It is not the mutation of CEBPA in MDS that is the issue but its failure to transcribe. However it appears that in progression to AML that CEBPA itself is mutated.

2.4 GATA1

We now consider GATA1 and its impact. We summarize some details but the concern as we shall show is that GATA1 control an alternative line. Let us begin with a summary of GATA1 operations.

From Morceau et al:

The zinc finger protein GATA-1 is considered as one of the most critical transcription factors in erythropoiesis as well as megakaryopoiesis. Besides GATA-1 that belongs to the GATA-family of transcription factors, GATA-2 is also involved in erythropoiesis and megakaryopoiesis regulation .

Both GATA-1 and GATA-2 transactivation activities require interaction with friend of GATA (FOG)-1 cofactor. In addition, both transcription factors have GATA binding sites in their cis-acting elements allowing a cross-regulatory mechanism in which GATA-1 can control the expression of GATA-2 and vice versa. GATA-2 is overexpressed in early immature hematopoietic progenitors to ensure their maintenance and proliferation whereas GATA-1 is essential for the survival of erythroid progenitors as well as the terminal differentiation of erythroid cells. In fact, increased expression of GATA-2 determines megakaryocytic differentiation whereas its down-regulation is required for erythroid differentiation.

Recently, a role for GATA-2 in the regulation of quiescence in human hematopoietic stem and progenitor cells has been reported. GATA-1 activation has been correlated to its phophorylation. Epo-induced phosphorylation of GATA-1 is important for maturation of fetal liver erythroid progenitor cells, specifically on serine 310 by PI3K/AKT that enhances GATA-1 transcriptional activity in vitro and in erythroid cells .

However, GATA-1 acetylation by CBP/p300 is also described as crucial for the binding to its DNA target GATA sequence possibly involving phosphorylation. Moreover, phosphorylation of

GATA-1 could be mediated by MAPK pathway, as an ubiquitination signal for its proteasomal degradation .

On the other hand, besides FOG1, GATA-1 activity is highly dependent on interaction with many cofactors including EKLF, SP1, CBP/p300, Lmo2, Ldb1, RUNX1, Fli1 and PU.1, which represent a part of the best-described interacting proteins. These cofactors can constitute a very complex network regulating erythropoiesis and megakaryopoiesis, by promoting or repressing GATA-1 activity.

Particularly, PU.1 is a strong inhibitor of GATA-1 DNA-binding activity and erythroid differentiation.

Before examining this observation it is worth a brief review of the NF-kB gene complex. We demonstrate this below (taken from Marks et al pp 408-416). The Figure below is a modification from Marks et al of the NF-kB complex. It functions as follows:

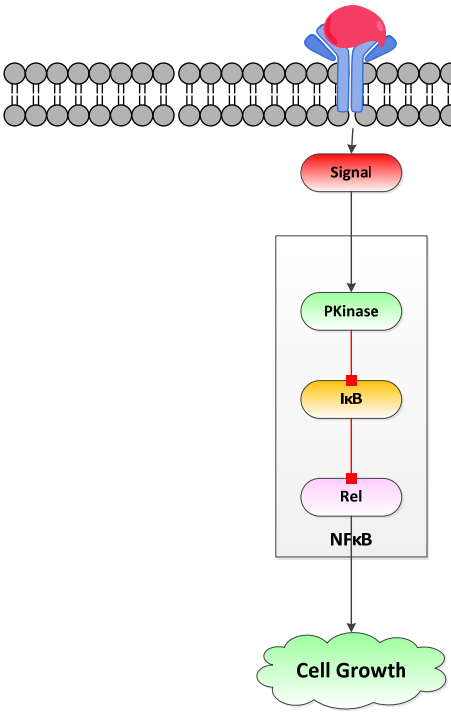
1. A receptor is activated via a ligand starting a path down a signalling chain to the NF-kB complex.

2. This signal then activates the PK kinase which can block the IkB portion of the transcription factor.

3. The transcription factor is the combined IkB and Rel proteins.

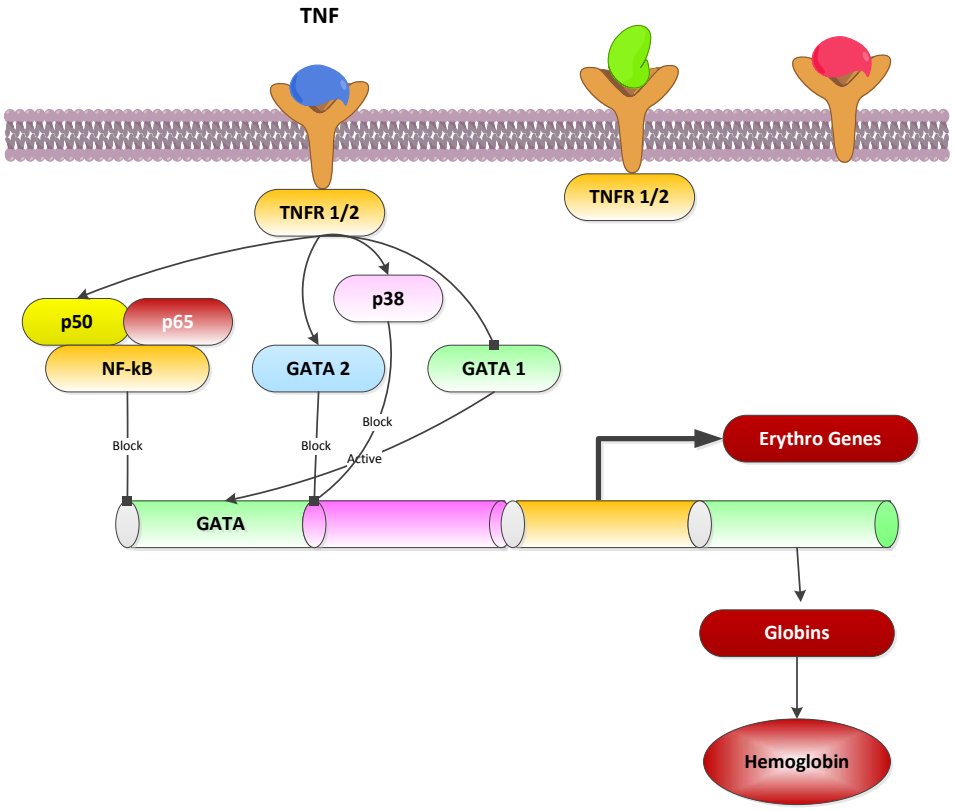
4. The Rel protein, if allowed to be activated, can facilitate survival and development.

5. Note that there is a switch mechanism in the NF-kB complex. If it is activated then PK blocks IkB and no IkB means Rel is active and thus we have the result. If on the other hand there is no input activation then PK is not active and IkB is not blocked but active, and thus Rel is blocked, and there is no survival or development resulting from Rel transcription.



Gene	State	State
Receptor	On	Off
Activator	On	Off
P Kinase	On	Off
IkB	Off	On
Rel	On	Off
State	On	Off

Now using the above from Morceau et al we have the following Figure demonstrating GATA1 and its activation.

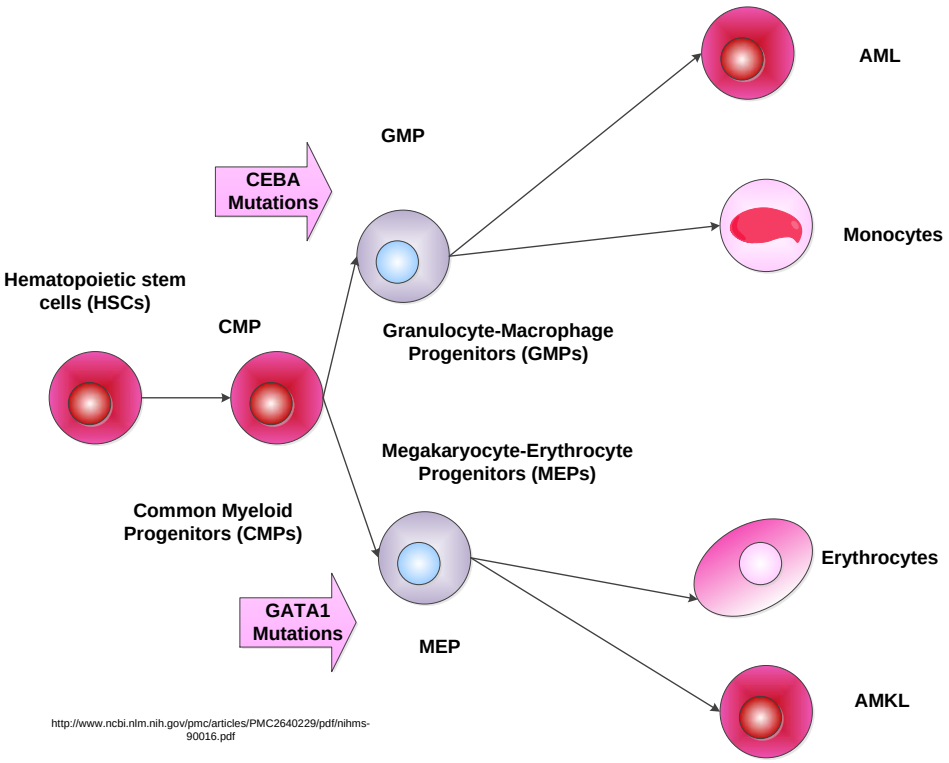


However with GATA1 we really have control over an alternative line and thus there is a concern as to what GATA1 deviation truly measures. From Look:

Mutations in the gene encoding the transcription factor GATA1 contribute to a specific type of leukemia in people with Down syndrome. This finding suggests a multistep pathway of leukemia development that involves GATA1 and one or more genes on chromosome 21.

Human myeloid leukemias are clonal disorders arising from the accumulation of mutations in hematopoietic stem cells (HSCs) that retain the capacity for self-renewal1. Most leukemogenic mutations are acquired somatically and affect a growing cadre of proto-oncogenes and tumor suppressor genes.

From Look we have the following Figure discussing this issue as stated above. Note that we have two arms of progression. The CEBA line leads to an AML path whereas the GATA1 line leads to an AMKL path with erythrocytes. Both are myeloid products but they grow into two separate lines; erythroid and monocytes. For example, neither is neutrophil linked. Thus one may ask if such a study on MDS with such a disparate set of expressions when agglomerated makes any sense in separating factors. Specifically we may reasonably ask; is MDS so different from patient to patient that any generalization can be problematic?



We now move to several observations which can be made.

Also from Hoffman et al there is a detailed discussion regarding GATA1 and AMKL[5]:

The molecular pathogenesis of TMD and AMKL in children with Down syndrome is now providing valuable insights into myeloid leukemogenesis.20 Recent studies have shown that virtually all patients with TMD and most patients with AMKL harbor mutations in the hematopoietic transcription factor GATA1.

GATA1 is a double zinc finger DNA-binding transcription factor expressed primarily in hematopoietic cells. It is required for the development of red blood cells, megakaryocytes, mast cells, and eosinophils. A number of different mutations in GATA1 have been identified, including insertions, deletions, missense mutations, nonsense mutations, and slice site mutations. All of these mutations lead to a block in the expression of the full-length 50-kd isoform of GATA1 but allow for the expression of a smaller, 40-kd isoform (GATA1s).

The zinc finger transcription proteins have loops of nucleic acids bound by two zinc molecules and then the bottom loop portions can bind as a transcription factor to the DNA and assist in

[5] Novel Somatic Mutations in JMML, Dr. Carl Allen, MD, PhD; Baylor College of Medicine, Date Published: 10Mar2014, On Line in Hoffman et al. Chpt 62.

effecting transcription. Mutations are possible but it is essential to still have the exposed loops between each of the zinc fingers.

This smaller isoform lacks the N-terminal transactivation domain but retains both zinc fingers involved in DNA binding as well as interactions with its cofactor, friend of GATA1 (FOG1). Recent studies have shown that mutations that alter GATA1–FOG1 binding in the N-terminal zinc finger or result in the expression of the GATA1s isoform uncouple megakaryocyte growth and differentiation.

Similar studies in cell lines derived from children with Down syndrome and AMKL have demonstrated that expression of GATA1 led to erythroid differentiation whereas expression of GATA1s did not alter the characteristics of the cell line. Taken together, current data suggest that the loss of GATA1 and expression of GATA1s directly contribute to leukemogenesis.

It thus appears that GATA1 is a crucial gene. However in the article in question the GATA1 seem to still be present but that their ability to effect a transcription factor is blocked by methylation.

Although mutations in GATA1 may be sufficient to cause TMD, these mutations are not sufficient for the development of AMKL, as evidenced by the latency period between resolution of TMD and the development of AMKL as well as the observation that not all children with TMD and GATA1 mutations will ultimately develop AMKL. Therefore, a multistep pathogenesis model is proposed in patients with Down syndrome in whom AMKL develops in clones with GATA1 mutations and additional cooperating mutations.

One potential "second-hit" mutation may occur in the JAK3 (Janus kinase 3) gene, a member of the JAK family of nonreceptor tyrosine kinases. Several gain-of-function mutations and loss-of-function mutations in JAK3160160 have been identified in both TMD and AMKL patient samples. Although mutations in JAK3 might indeed represent a "second hit," the finding of mutations in TMD patients who have not progressed to AMKL may argue against JAK3 as a second cooperating mutation.

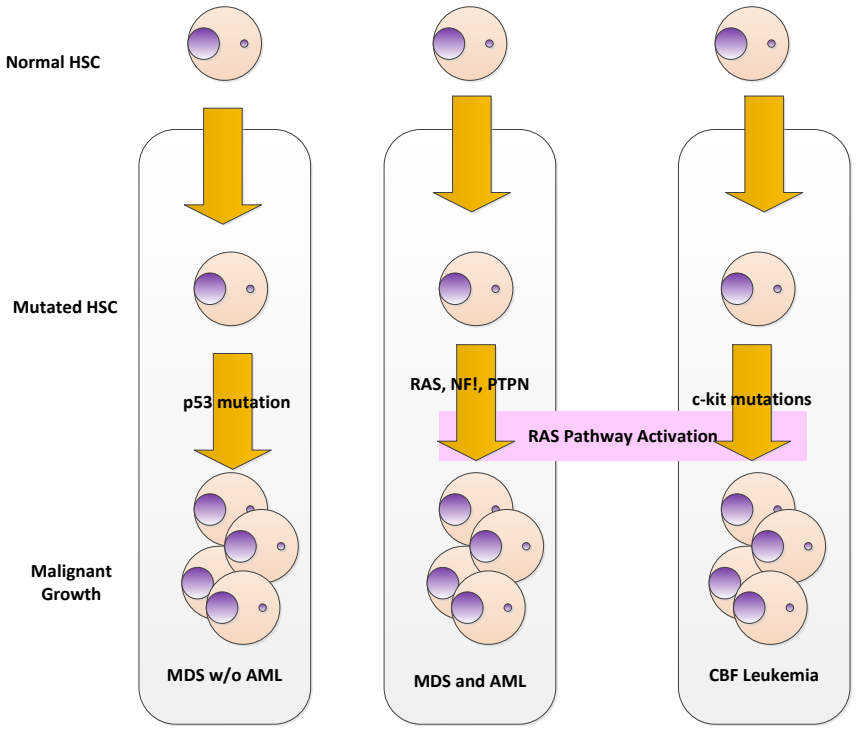
Thus examining the effect of loss of function in genetically changed patients is helpful in understanding its function overall.

3 OBSERVATIONS

We end this section with some general observations regarding MDS, methylation and the use of transcription factor markers and their expression.

1. Is AML a definitive sequella to MDS: MDS is not a single disorder? There are multiple representations of the disease and there is no single clear representation. Many MDS patients end with AML and that is often the end state. AML can be severe and there are few treatments for it once it has commenced. In younger patients a BMT is sometimes effective but the problem with those who start with MDS is that they are too old for BMTs.

From Niimi et al we have the following graphic which demonstrates the progression:



The normal progression of untreated MDS to AML is a common pathway and it does involve RAS activation. In addition there are also p53 mutations in some MDS but not all.

2. What are the Causes of Methylation: We continue to struggle to understand what is the cause of the methylation that blocks the transcription factors and understanding that could be the most beneficial. Also why the specific methylation targeting?

From Sonnet et al we have:

Hypermethylation and subsequent inactivation of genes are hallmarks of AML pathogenesis. Prominent examples include the epigenetically silenced tumor suppressor genes CDH1 or p15/CDKN2B. In addition, gene hypomethylation is frequently found in myeloid malignancies. The mechanistic link, however, between promoter hypomethylation and tumorigenesis is incompletely understood.

Global hypomethylation is common in many cancers, including AML, and is suspected to destabilize genome integrity by re-activating retrotransposons. Alterations in DNA methylation contribute to initiation, expansion, and evolution of the leukemic clone and promoter hypermethylation is a frequent observation in specimens of patients with MDS and AML. The mechanisms underlying the establishment of aberrant DNA methylation patterns are still largely unknown. Aberrant DNA methylation might be explained by the aberrant binding of transcription factors to their genomic target sequences.

3. What role do miRNAs play, if any, in the expression of these transcription factors?

As stated in Mendelsohn et al (see p 429):

The miRNAs miR-143, miR-145, and miR-146a mapping at 5q33 are significantly reduced in bone marrow cells isolated from 5q− syndrome patients. miR-145 and miR-146a regulate the toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) and tumor necrosis factor receptor–associated factor 6 (TRAF6). TIRAP is a regulator of TRAF6, an E3 ubiquitin ligase, required for nuclear factor κB (NFκB) activation. Collectively, these proteins regulate innate immunity. Haploinsufficiency of miR-145 and miR-146a resulted in increased TIRAP and TRAF6 expression and activity in hematopoietic stem/progenitor cells. Transplantation of haploinsufficient miRNA145/146a cells or TRAF6-overexpressing cells into recipient mice resulted in several hallmark MDS/5q− syndrome phenotypes, such as thrombocytosis, neutropenia, dysplastic megakaryopoiesis, and propensity to transform to AML. These findings link innate immunity to MDS pathogenesis.

4. General use of a DMNT1 blocker and have putative systemic effects. It may demethylate many other genes and even demethylate histones thus uncovering genes which have been closed off by histone methylation. Is it thus better long term to have lower doses for that reason as well?

5. Cytogenic Abnormalities are more classic means for identifying MDS and AML as well as assessing prognostic values.

From Hoffman et al (Table 59.8) we have a complex array of cytogenic abnormalities amongst MDS patients. The Table shows the frequency of each and the mean survival when the abnormality remains. The observation can be made that they are so disparate and complex that any attempt to examine MDS in a broad sense is difficult. This is compounded by the low incidence as well.

Anomaly	Frequency (%)	Median Survival (Months)
Good-Prognosis Cytogenetics		
del(9q), NC	0.4	NR
del(15q), NC	0.4	NR
t(15q), NC	0.4	NR
del(12p), NC	0.8	108.0
+21, NC	1.1	100.8
–Y, +1	0.4	84.6
del(5q), isolated	8.2	80.0
+21, or +1	0.8	80.0
del(5q), NC	10.7	77.2
del(20q), isolated	1.9	71.0
del(20q), NC	2.2	71.0
–X, NC	0.5	56.4
No (normal karyotype)	49.5	53.4
del(5q), +1	2.5	47.0
+8, or +1	1.2	44.0
–Y, NC	2.7	39.0
–Y, sole	3.5	36.0
+1/+1q, NC	0.4	34.7
t(1q), NC	0.6	34.7
t(7q), NC	0.6	34.7
t(11q), NC	0.5	32.1
−21, NC	0.5	32.0
Intermediate-Prognosis Cytogenetics		
del(11q), NC	0.9	26.1
+8, NC	5.0	23.0
+8, isolated	3.8	22.0
t(11q23), NC	0.5	20.0
Rea 3q, NC	0.5	19.9
+19, NC	0.4	19.8
del(7q), isolated and NC	0.6	19.0
Any 3 abnormalities	2.8	17.1
del(11q), isolated	0.6	15.9
−7, +1	0.9	14.4
−5, NC	0.4	14.6
−7, sole	2.3	14.0
−7, NC	3.2	14.0
Poor-Prognosis Cytogenetics		
Complex, all	13.4	8.7
t(5q), NC	0.4	4/4
4-6 abnormalities	5.3	9.0
>6 abnormalities	3.9	5.0

6. Therapeutic Targeting: The approach taken in dealing with MDS is using the DNMT1 suppression drugs. These drugs have a broad spectrum use and frankly may have less than long term beneficial effects. However when we see specific links being broken, then for example can we replace the genes not being expressed in the methylated cells? That would mean a targeting of specific proteins and more importantly an understanding of the proteins which have failed in terms of expression.

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