Gene Expression Changes in Normal Hematopoietic Cells

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ABSTRACT

The complexity of the healthy hematopoietic system is immense, and as such, one must understand the biology driving normal hematopoietic expression profiles when designing experiments and interpreting expression data that involves normal cells. This chapter seeks to present an organized approach to the use and interpretation of gene profiling in normal hematopoiesis and broadly illustrates the challenges of selecting appropriate controls for high-throughput expression studies.

List of keywords: Hematopoiesis, microarrays, cDNA, transcriptome, proteome, cytokine, interleukin, malignancy, genome, epigenome, differentiation, polymorphism, aging, somatic mutation, pharmacogenetics, post-translational modification, stem cell.

INTRODUCTION

As discussed in other chapters, malignancies demonstrate significant “disease-specific” expression changes compared to normal hematopoiesis. However, these findings must be interpreted with caution and in full view of critical differences between normal and diseased cellular biology.[1-3] Several fundamental issues (outlined below) must be addressed to effectively design experiments and to appropriately interpret results.

What are the characteristics of “normal” hematopoiesis? Hematopoietic cells must constantly recognize a wide variety of internal and external signals in order to maintain a balanced yet flexible blood system. Consequentially, normal hematopoietic cells will often display widely divergent transcription profiles depending upon their environmental cues and immediate surroundings. For example, a hematopoietic system undergoing the stress of an acute hemorrhage will present a markedly different molecular expression signature than one in homeostasis.[4] Likewise, hematopoietic stem cells (HSCs) from a 65 year-old smoking male on multiple medications are likely very different from healthy newborn HSCs, despite a shared immunophenotype.[5] Therefore, unrecognized biological variables within the subject may be responsible for expression changes that can be inappropriately ascribed to pathologic conditions. For this chapter, normal hematopoiesis will be defined as any non-malignant state. Although too broad for many comparisons, this definition highlights the absolute necessity to acknowledge and control for “normal” biological factors that may cause inter-individual variation in expression data.

A similar but distinct issue relates to the heterogeneity of cells within the hematopoietic system. What are the appropriate populations of cells for comparisons between normal and diseased states? The answer to this question will profoundly impact the biological and technical aspects of an experiment. To illustrate this point, consider that “normal” CD34+/CD38- and CD34+/38+ cells have overlapping yet unique expression signatures,[6,7] and similar differentiation specific expression signatures have been found in leukemic blasts.[8] Therefore, if one compares the expression profiles between relatively homogenous CD34-positive AML blasts and heterogeneous normal bone marrow samples, there will certainly be a large number of expression differences that initially appear to be associated

with the disease phenotype. However, upon further investigation, it becomes clear that many of the "AML-specific" expression changes may be secondary to lineage-specific variations in the trascriptome.[3] To further complicate matters, many hematopoietic malignancies inappropriately express a variety of differentiation markers, making precise determination of the optimal comparison population a challenge.[9] Due to the lack of an absolutely correct choice, the appropriate control will depend on the intellectual bias, available resources, and specific goals of the investigator.

How do investigators inadvertently change the biology of cells? Cells require extensive handling prior to most expression profiling studies, and many times, the normal and diseased populations undergo different processing. For example, leukemic samples obtained from large cooperative groups are frequently shipped from outside institutions, resulting in a delay of processing, while normal samples are often obtained and processed locally. In many cases, studies use frozen samples, introducing another potential variable that can impact expression.[3] Processing, thawing, and other typical laboratory manipulations impact the biology and expression profiles of normal and abnormal cells.[10] Together, these factors may introduce uncontrolled or unknown methodologic biases that can lead to erroneous findings and decrease the experimental power to detect true biological differences.

A firm grasp of the biology governing normal hematopoiesis is essential to the study of the molecular aspects of hematopoietic diseases. This chapter focuses on expression profiles of normal cells and factors that alter expression in these cells. It is intended to provide a framework to facilitate the reader's evaluation of studies that focus on gene profiles of normal hematopoietic cells, or use them as a point of comparison. In general, the term "expression" will refer to either mRNA or protein expression in order to illustrate the concept in its broadest sense. When appropriate, the authors will clarify the type of expression by specifically referring to either mRNA or protein expression. Obviously, a comprehensive review of any specific topic regarding the transcriptome, its regulation, or translated products is beyond the scope this chapter. Additional concepts and details are available in the cited references and reviews.

<u>Chapter Goals:</u>

- Outline the current understanding of gene expression in normal hematopoietic cells.
- Provide a basic understanding of the factors that govern the normal hematopoietic transcriptome.
- Explore the impact of intra-individual, inter-individual and environmental factors on gene expression.
- Discuss the pitfalls of current approaches examining normal hematopoietic gene expression.

FIGURE 1 TO BE INSERTED HERE.

FACTORS INFLUENCING EXPRESSION IN NORMAL HEMATOPOIETIC CELLS

Intrinsic and extrinsic factors that control and alter expression profiles are broadly considered in this section, starting with the genome and working outward to the environment (Figure 2). It is incorrect to derive from this structured description that the regulators of expression function independently of one another, and in fact, considerable cross-talk and coordination occurs between them.

FIGURE 2 TO BE INSERTED HERE

Impact of Genomic Changes on Expression

For years, investigators have been aware of genetic divergence across populations and among family members, which is demonstrated by inter-individual differences in behavioral and physical attributes. Historically, the genome was modeled as rigidly static after conception, and monozygotic twins were expected to share an exact genetic unity. This assumption has recently been shattered by studies using high resolution genomic arrays.[11,12] Unexpected levels of copy number variation (CNV) between monozygotic twins provide support for a model of sub-chromosomal genetic divergence *after* the splitting of the embryo, indicating that the normal genome may be more dynamic than once appreciated.[13] An even more dramatic example of post-conception genomic divergence has recently been described in mammals. Microsatellite mutations in mice occur with sufficient frequency during early organogenesis that the genetic variability can be used to model the developmental relationship of different tissues within the animal.[14]

To further complicate matters, an individual's genome continues to change *ex utero,* probably as a result of the intrinsic aging processes and environmental factors.[15] For example, non-allelic homologous recombination naturally increases as a function of normal aging in humans, and studies reveal increased DNA damage in the hematopoietic cells from older animals in the absence of malignant disease.[16,17] If these genetic changes affect critical regulatory sequences within the gene, then transcription, translation, and post-translational modifications could be altered and result in a modified phenotype. The effect or significance of incidental age-related acquired genomic variation on gene expression is unclear; however, there is an effort to better describe the degree of genetic variation in humans and identify the factors that cause genetic changes within normal cells.[18] Until we have a better understanding of the mechanisms governing genetic variation across individuals and acquired genetic alterations within individuals, studies comparing normal and diseased states must be interpreted cautiously. A real possibility exists to misinterpret genetic "lesions" or expression changes in the diseased population that actually represent normal divergence.[13]

Epigenetic Regulation of Expression

Epigenetic factors regulate gene expression.[19,20] For example, methylation of cytosines modulates transcription factor binding at critical promoter sequences of some genes, thereby altering expression.[5,20] As with the genomic variability discussed above, methylation states are dynamic.[21] Genes that govern methylation indirectly control the expression of an even larger number of genes.[22] Regulators of methylation vary with the cell cycle, overall health of the cell, and differentiation stage.[5,21] In addition, external exposures (e.g., stroma), molecules (e.g., folate), environmental factors (e.g.,

radiation and tobacco smoke), and drugs (e.g., 5-azacytidine) impact the methylation states in hematopoietic cells, providing another level of transcriptome modification and regulation.[5,20]

Chromatin scaffolding houses the genome and is an epigenetic regulator of transcription.[20,23] Chromatin is composed of proteins, primarily histones, which actively organize the genome, engaging and regulating transcriptional machinery.[24,25] A variety of genes, conditions, and mechanisms dictate the dynamic and plastic nature of chromatin structure.[26] For example, histone acetylation/deacetylation plays a critical role in modifying chromatin configuration and impacts transcription.[27]

Role of Transcription Factors in Gene Expression

Nuclear proteins bind to transcription factor binding sites (TFBS) that lie within promoter regions and initiate synthesis of RNA.[28] There are probably 100s – 1000s of transcription factors expressed at any one time in normal and malignant cells, and transcription factor may have thousands of binding sites and control hundreds of genes.[28] For example, TP53 (a tumor suppressor gene and transcription factor) binds to over 500 loci,[29] while MYC may have 10,000 – 20,000 unique TFBS within the genome.[30] Furthermore, transcription factors can have multiple isoforms that have different affinities to the TFBS and produce opposing biological effects.[31]

Numerous context-dependent genetic and epigenetic components influence the function of a transcription factors. The complexity of the system is challenging to model because transcription factors can be directly involved in expression of genes at multiple levels. For example, IKAROS family zinc finger 1 (IKZF1) promotes transcription, recruitment and modification of other transcription factors, and influences chromatin remodeling.[32] The interactions between the genome, primary sequence methylation, chromatin structure, and thousands of transcription factors set the stage for a versatile and adaptable transcriptome, which is capable of simultaneously maintaining homeostasis yet has the capacity to rapidly respond in times of stress.

The Changing Transcriptome

There are numerous components to the transcriptome: messenger (m)RNA, ribosomal (r)RNA, transfer (t)RNA, and the recently described non-coding (nc)RNA,[33,34] and each of these components play a critical role in regulating protein expression. mRNA transcripts are perhaps the most studied of all the different transcriptional components. After transcription, pre-mRNA products undergo post-transcriptional modifications and are eventually processed into mature mRNA transcripts. These mature mRNA transcripts form the template for translation and thus, directly regulate protein expression. Due to their pivotal role in regulating this expression, the levels of mRNAs are tightly regulated *via* timely expression, maintenance, and degradation. The inherent instability of mRNA can work against accurate assessment of transcript levels, introducing a potential experimental pitfall that may be difficult to control for on a gene by gene basis.

For many years the biological activity of ncRNAs was unrecognized, and they were thought to arise from non-specific transcriptional activity or as the by-product of normal RNA processing. Interest in ncRNAs increased in the mid-late 1990s when it became clearly apparent that ncRNAs are highly

conserved across species and that some ncRNAs have functional activity.[33] Functional experiments demonstrated that these simple but eloquent gene products (i.e. microRNAs, or miRNA) provide a versatile method for controlling expression by binding and modulating the stability of mRNA and/or its translation.[35-38] The expression of many miRNAs (e.g., miR-181) fluxuate during hematopoiesis, consistent with recent studies demonstrating a critical role for these transcripts in normal differentiation programs.[35,36,39] Other ncRNAs may serve as a physical linkers between RNA-binding proteins (e.g., translocation in liposarcoma, TLS) and DNA promoter sequences. In this model, the ncRNAs cause an allosteric change within the RNA-binding protein, which, in turn, promotes the association of other transcriptional proteins and represses promoter activity.[40] Although ncRNAs are sometimes expressed at very low levels, they may still have profound biological effects. Additionally, recent data suggest that ncRNA species have greater variety than the mRNA transcripts in the cell, although further characterization is required.[34] Even if only half of the projected ncRNAs are functional, they add yet another level of complexity to the biological equation that has not been encountered since the discovery of the genome.

The Vast and Varied Proteome

Analogous to the transcriptome, the proteome is the sum of all translation products, and is the point at which genetic changes become relevant to the biology of the cell. Proteins are responsible for adaptation, metabolism, and survival. The modern biologist must model real-time non-linear behavior of subcellular signal transduction pathways, microcompartments, and organelles that have critical spaciotemporal associations with diverse cellular apparatuses, ion gradients, cytoskeletons, carbohydrates, etc. Compared to the leviathan effort needed to sequence the human genome, the work required to comprehensively model the proteome is vastly greater because not all protein characteristics are encoded by genomic elements. Post-translational modifications (PTMs) can activate, modulate, and/or terminate the function of a protein, while the intracellular context of a protein can profoundly alter the cell phenotype.[41,42] The four-dimensional model of the biology mediated by proteins is described by the field of proteomics with subclassifications of data referred to by novel terms including the "interactome"[43] or the "metabalome,"[44,45] and recently extended to include the important concept of carbohydrate modification of proteins called the "glycome."[46] Experimental approaches to these diverse fields require specialized techniques and software that are beyond the scope of this chapter, and the reader is referred to the many excellent reviews for additional information.

A common application of proteomics is the screening of candidate pharmacologic molecules prior to animal studies.[47] Thousands of potential agents can be screened for specific interactions with key biologically active proteins. Candidate molecules that pass the first *in vitro* tests move forward to the next round, while molecules with presumed deleterious effects, demonstrated by specific affinities, are eliminated. If the underlying interaction is misunderstood, potentially beneficial may be overlooked and potentially dangerous compounds may be inappropriately advanced. There is optimism about using a similar *in vitro* approach to predict a patient's response to therapy in a burgeoning discipline of pharmacogenomics.[48]

The field of proteomics is in its infancy, but has already demonstrated utility. Nevertheless, current challenges include isolation of specific cell sources and managing the vast amount of information generated. An additional critical component is the need to define appropriate thresholds to delineate the biological relevance of a protein-protein interaction. This challenging issue may vary for each target because protein interactions are not binary, and both non-specific and low-affinity binding can be biologically significant.

Regulation of Expression *via* Cell Membrane Proteins

Communication between hematopoietic cells and the microenvironment occurs through receptors embedded in the plasma membrane. These receptors may function to anchor the cell to a niche[49-52] and often provide critical points of contact for internal cytoplasmic stuctures.[53,54] Receptors are pivotal to the biology of the cell and often define a cell's identity, differentiation stage, and disease state (i.e., "normal" vs. "abnormal").[9,55] Membrane-associated receptors and integrins are numerous, and their role in hematopoiesis is a field of investigation under constant flux that has regularly been reviewed in detail.[51,56-70] We will briefly highlight major points of interest for several of the most studied receptors below and in Table 1.

Receptor tyrosine kinases (RTKs) and cytokine receptors (CKRs) are classes of proteins that play critical roles in hematopoietic biology and are expressed on normal hematopoietic cells (Table 1). RTKs contain intrinsic kinases that drive intracellular signaling activity and are divided into more than 20 subclasses.[71] The RTK subclass III (the PDGFR family) is the best-studied in normal and abnormal hematopoiesis, and is composed of 5 homologous members (CSF1R, FLT3, KIT, PDGFRA, and PDGFRB).[71-73] The shared structure includes: five extracellular immunoglobulin-like domains (EMD), a transmembrane domain (TMD), juxtamembrane domain (JMD), two intracellular tyrosine kinase domains (TKD), and a hydrophilic insertion/linker domain between the TKDs (KID).[72,73] Compared to RTKs, CKRs do not have intrinsic tyrosine kinase activity and must recruit kinases to initiate intracellular signaling. Two classical examples of hematopoietic CKRs include CSF2R (GM-CSF receptor) and CSF3R (G-CSF receptor).[59] At the plasma membrane, the extracellular domains of RTKs and CKRs bind specific cytokines or growth factors (Table 1).[72] Ligand binding induces conformational changes that in turn either activate intrinsic kinase activity (RTK only) or recruit cytoplasmic kinases to initiate diverse signal transduction pathways. Therefore, the membrane receptor expression pattern of a specific cell dictates ligand binding and, ultimately, defines the cell responses to the repertoire of ligands in its environment.

The Notch and WNT families of receptors initiate intracellular signals that have been increasingly implicated in normal and abnormal hematopoiesis. Notch signaling plays an active role in tissue homeostasis, with initially studies first describing its role in embryonic development.[65,74,75] In humans, Delta-like and/or Jagged ligands bind to four Notch receptors, and studies suggest that Notch activation may to play some role in HSCs maintenance and/or expansion.[51,65] In fact, recent work has found that Notch activation can be utilized to expand primitive HSCs for transplantation.[76] However, it remains unclear if Notch is required for perpetual maintenance of these cells.[65,74,75] WNT signaling may also play some role in promoting the expansion of primitive HSCs and has been implicated in HSCs self-renewal.[75] Although Notch and WNT pathways represent the "state-of-the–art" in our understanding of

how membrane receptors influence hematopoiesis, many aspects of these receptors' biology remains unknown, highlighting the need for additional studies.

TABLE 1 TO BE INSERTED HERE

METHODS TO EXAMINE THE REGULATORS OF EXPRESSION AND EXPRESSION

Since the completion of the human genome project, there has been an explosion of novel platforms to examine genomic, epigenetic, and expression changes in hematopoietic cells (Table 2). Current genomic technologies have the capacity to examine millions of single nucleotide polymorphisms (SNPs) and acquired mutations using relatively little starting material,[77,78] and these assays have already been found to be extremely useful for identifying nucleotide changes, uniparental disomy (UDP), and loss of heterogeneity (LOH) in both normal and diseased states.[79] As this technology evolves, array-based SNP and comparative genomic hybridization platforms will enable researcher to study the genetic variability in hematopoietic cells with increasing precision and genomic coverage. Recently, high-throughput full genome sequencing (FGS), or whole genome sequencing (WGS), has become a reality, with novel pyrosequencing technologies able to sequence > 400 – 600 Megabases in 10 hours.[80] Using this technology, investigators have sequenced the entire genome for selected subsets of human cells, including malignant blasts from an AML patient with normal cytogenetics.[81,82] These cutting edge technologies have rapidly advanced our understanding of how genomic changes impact expression, identifying thousands of new polymorphisms and promoter loci.[78,83,84]

Currently, there are a number of different techniques to examine DNA methylation and histone marks – two major epigenetic mechanisms for controlling transcription. DNA methylation changes were first examined using restriction enzyme digest/PCR assays. The key to this approach is the use of "methylation specific" restriction enzymes that cleave unmethylated alleles but fail to digest methylated alleles.[85] Gene-specific PCR primers can be developed that flank the digestion site and amplify only the protected, methylated DNA sequences. Using this method, investigators first demonstrated that the G-SCF promoter displays different methylation states in granulocytes (hypomethylated, expressed) and lymphocytes (hypermethylated, not expressed).[86] One major limitation of this technique is that only sequences with a methylation-specific restriction enzyme sites can be assessed. The discovery that bisulfite treatment of DNA converts unmethylated cytosines to uracil provided a major advancement in the development of more comprehensive, high-throughput techniques to examine global DNA methylation patterns.[87] By changing the DNA sequence, investigators were able to design primers or hybridization probes that differentially target non-modified (methylated) and modified (unmethylated) sequences, leading to the development of global methylation arrays.[87-89] Currently, there are a number of techniques that can be used to examine the relationship of chromatin structure and genes, but the workhorse of these investigations is the chromatin immunoprecipitation (CHiP) assay.[90] With CHiP assays, chromatin is precipitated with an antibody, and the pellet is interrogated by a variety of methods, including PCR of specific genomic fragments of interest, and as with methylation arrays, high

throughput CHiP arrays are becoming increasingly more comprehensive and popular in the research community.[91] Together, these novel techniques provide a powerful set of tools to characterize the multitude of epigenetic changes in hematopoietic cells.

High-throughput technology for examining mRNA, or total RNA, expression has been in use for over a decade.[92,93] The first arrays examined expression of a limited number of genes, using relatively simple platforms, but the technology quickly proved to be a powerful tool of characterizing diseases and interrogating the molecular biology of cells.[94] With refinements in RNA array technology, there has been a dramatic increase in the number of targets that can be evaluated – with the current arrays displaying the capacity to examine more than 45,000 transcripts and 35,000 unique genes.[95] As the importance of non-coding RNAs (discussed elsewhere in this and other chapters) became known,[36,38] arrays were developed to specifically examine transcription of miRNAs.[96] Investigators have also adapted the high-throughput "deep sequencing" techniques used in FGS to the transcriptome, identifying novel isoforms and alternative splice variants, suggesting that 92% of genes may undergo alternative splicing.[82] Capitalizing on these data, more sophisticated microarrays are being developed that will allow researchers to discriminate between isoforms and quantify alternative splicing.[97,98]

At the level of the proteome, the most basic question is whether or not a protein is present within a cell or in a comparison of cell lysates. Two-dimensional protein electrophoresis (2DPE), based on separation of proteins first by isoelectic point and then by denaturation electrophoresis, provided the first insight into differential protein expression in a holistic fashion.[99] Antibodies remain the most typical approach for specific identification of proteins and are used in a variety of applications, including ELISA assays, western blotting, affinity chromatography, and immunoprecipitation. Some antibodies have been raised to specific activation or phosphorylation states of proteins, allowing a modicum of functional data.[100] However, mass spectrometry (MS) does not necessarily require specific antibodies to identify proteins or peptides; rather MS relies upon ionization of peptides into charged particles that can be characterized after passing them through electric or magnetic fields.[101,102] Another major strength of MS is the ability to detect PTMs on peptide fragments, and the technique is versatile enough to use many sources of materials.[103,104] Thus, MS has been increasingly adapted to provide specific protein identification and high throughput analyses of the proteome.[104] However, maintaining a system to simultaneously investigate the biological context of a range of protein interactions during manipulation will require creative approaches and sophisticated experimental design.[102,103] Examples of the tools used to evaluate the proteome are listed in Table 2, but experimental approaches to interrogating the proteome are diverse and manifold.

Although these techniques may hold the key to unlocking the biological mysteries of normal and abnormal hematopoietic cells, high-throughput platforms have significant limitations. Importantly, they can only provide "snapshots" of the genetic, epigenetic, and expression changes that occur over time. Therefore, momentary events (cell processing, target isolation and enzyme efficiencies) directly impact the cells and the data.[3,10] Consequentially, quality controls are essential for proper analyses and interpretation of the results. In addition, most of these platforms have a finite number of potential targets that can be examined, potentially missing critical genetic, epigenetic, and/or expression changes. Finally, the amount of data generated can be daunting, pushing the limits of current computing

technology and creating a need for special statistical methods of normalizing, analyzing, and interpreting the data.[105,106]

TABLE 2 TO BE INSERTED HERE

DIFFERENTIATION PROGRAMS AND THE TRANSCRIPTOME

The hematopoietic system is amazing in its complexity, flexibility, precision, and capacity. Aging alone does not exhaust the normal hematopoietic system, nor does recurring hemorrhage or chronic and repetitive infections. In addition, under normal circumstances all terminal cellular lineages are precisely maintained without excess. This is particularly impressive because the disparate internal and external regulation within each compartment independently act on a shared population of primordial cells. The activities of the hematopoietic system can be artificially separated into different functions: i) maintenance of the stem cell pool; ii) cell fate/lineage decision making; iii) expansion of daughter cells; and iv) feedback regulation from the terminal functional cells. Collectively, these and other tasks are highly regulated by expression changes within the cells to secure maintenance of the hematopoietic system and dictate differentiation of hematopoietic cells. Examples of differentiation regulation that broadly illustrate these complex mechanisms follow below, starting at global transcriptional assessment of HSCs and proceeding to specific examples of expression changes that control differentiation.

One of the greatest challenges in the study of the normal hematopoietic system is characterization of the pluripotent stem cell. This cell compartment has two mutually exclusive roles: either to commit to proliferation and generate functional circulating cells, or to maintain “stem-ness” as an undifferentiated normal stem cell.[107,108] The cells that make this decision are exceedingly rare, currently do not have an absolute marker of identity, and therefore, can only truly be identified by transplantation experiments demonstrating successful engraftment of enriched cell populations.[109] Clonal stem cell experiments are possible in mice, but even in this system approaches are limited to evaluation of mature stem cell progeny and retrospective extrapolation. An ideal experiment to define the expression changes associated with critical HSC decisions would involve sequential array assessment of a pure population of HSCs over time and as the cells respond to specific ligand challenges.

By comparing neuronal, hematopoietic and embryonic stem cell transcriptomes, investigators have identified a cadre of transcripts common to maintaining “stem-ness”, as well as expression signatures of individual stem cell groups.[110,111] The Ivanova group further subdivided populations into Long Term HSC (LT-HSC), Short Term HSC (ST-HCS), Lineage Committed Progenitors (LCP), and mature blood cells. Each of these hematopoietic subpopulations had an overrepresentation of specific signaling ligands, receptors, and adhesion molecules. The most primitive LT-HSC notably expressed several critical genes Wnt10A, Erg, Hegfl, Bmp8a, and Agpt, many of which had previously been recognized as playing a critical role in maintenance or proliferation of the primitive HSC compartment.[110] In addition, the studies found that primitive HSCs may simultaneously express ligand/receptor pairs (e.g., Wnt10A/Frizzled and Agpt/Tek), suggesting autonomous regulation of some signaling pathways may be occurring in HSCs.[110] The same studies found that the HOX family displayed differential expression patterns among the subpopulations. For example, Hoxa5 and Hoxa10, both of which disrupt

differentiation, were expressed primarily in cells retaining stem-ness (LT-HSC), while Hoxb4 and Hoxa9, both of which are associated with the cell expansion, were expressed in more differentiated, proliferative populations.[110] However, when data from the two above-mentioned publications were compared by a third party, the degree of concordance between the studies regarding the transcripts common to stem cells was low.[112,113] The published exchange of opinions on this data (the primary papers, the counter analysis, and the rebuttal) are well worth reading as a primer regarding the complex issues involved in expression profiling and the application of this technology to rare cells.[112-114]

Other investigators have returned to similar target populations as isolation techniques, definitions of cell populations, and microarrays have improved. Using improved arrays, Fosberg et al. confirmed some of the LT-HSC transcriptome elements of previous authors (e.g.,ROBO4 and Slit-like2 ligand receptor pair) and the general trend of the preservation of "stem-ness" genes in earlier cells (Hoxa5 and Hoxb5), with proliferation genes arising in more mature forms (Hoxa9).[115] However, intriguing new elements were also described (e.g., previously undescribed adherens junctional complex proteins, like Esam1), suggesting novel elements of HSC biology.[115]

Array RNA data and the transcriptome only tell the beginning of the story. Translated proteins are responsible for much of the functional biology governing differentiation, and these regulatory proteins may have unique and overlapping functions. For example, CRE binding protein (CBP) and p300 have a significant amount of structural homology, and both proteins play very active roles in chromatin remodeling, suggesting a potential functional redundancy.[116,117] In fact, CBP and p300 proteins both harbor the "KIX" domain, which is a binding site for the transcriptional factors CREB and c-myb. However, site-directed mutagenesis studies reveal that mutations in the KIX domain of the two genes produced distinct functional responses. Mice with a CBP-KIX mutation displayed no significant alterations in hematopoiesis, while the mice harboring the same KIX mutation in p300 had multilineage abnormalities.[118] This study stands in contrast to the effect seen by a frank single knockout of each gene, in which a more dramatic disruption of hematopoiesis was seen in the CBP knockout.[119] Certainly, experimental technique variation, specificity of the mutants, and/or the mouse strains in each experiment may have a role in the discordant results; alternatively, each protein may have a distinct function in HSC biology that will be elucidated with further investigation. Consistent with this assertion is the data provided by a third study, which found distinct roles for the two proteins, with CBP influencing HSC renewal and p300 influencing differentiation.[120] Interestingly, when the p300 double-knockout HSCs were transplanted into WT animals, the differentiation defect was overcome, suggesting the intriguing possibility that the p300-deficient phenotype is influenced by the stroma rather than a HSC autonomous mechanism.[120] Given the wide range of roles that CBP and p300 play in the cell, it is perhaps not surprising that distinct facets of their biological roles are evident in different experimental systems. This example underscores the complexity of the functional response of even relatively homologous proteins and how subtle changes in the environment may dramatically change impact their effect.

As a cell moves through differentiation, innate programs are activated within cells at immature stages maintain lineage commitment *via* positive and negative feedback loops. Transcriptional factor upregulation can serve as the key to making cell fate decisions, and may act *via* repressor mechanisms

to maintain the decision. For example, GATA-1 and PU.1 are expressed and promote differentiation in the erythroid and megakaryocytic lineages, respectively. Furthermore, each protein drives a positive feedback loop for its own expression, and a negative feedback loop for the expression of the other transcription factor.[121,122] These types of complex and tightly regulated programs help to prevent inappropriate expression of genes at the wrong stage of differentiation.

As previously described ligands and their receptors orchestrate a complex symphony of self-renewal, proliferation, and differentiation during hematopoiesis (Table 1). Cells express a unique set of receptors and ligands at specific points of in the differentiation process, at times forming auto/paracrine feedback loops. A recent study carefully evaluated the FLT3/FLT3LG system in CD34+ cells, an important pathway in undifferentiated hematopoietic cells, and showed that FLT3 receptor and ligand were both produced. Additionally, the a FLT3 receptor inhibitor (lesaurtinib) promoted upregulation of both the ligand and receptor, suggesting autonomous regulation.[123] In another microarray based study, human CD34+ cells expressed multiple different cytokines (e.g., VEGF, HGF, IGF1, IL16, TGFB1, and TPO).[124] This study correlated mRNA expression with protein production and biological function, and addressed some of the potential difficulties in modeling gene profiling assessment in the form of microarrays.[125]

EFFECTS OF AGING ON HEMATOPOIETIC EXPRESSION

The hematopoietic system undergoes a number of significant functional and anatomic changes with age. Overall, there is a diminution of the immune and growth factor responsiveness.[126-130] Furthermore, the incidence of most hematopoietic diseases (e.g., anemia, malignancies) dramatically increases with age.[131] Currently, it is unclear exactly what biological factors are responsible for the age-related changes in the hematopoietic system. Genetic and epigenetic changes within hematopoietic progenitor/stem cells (HPCs/HSCs) and their progeny definitely increase over the life of an organism.[108] For example, HSCs from older mice display a marked reduction in their capacity to repair DNA damage, and this deficiency leads to decreased self-renewal, increased apoptosis, diminished capacity to maintain normal hematopoiesis, and "functional exhaustion".[16,132] Despite these findings (or perhaps due to this dysfunction) older mice have an *increased* number of HSCs, although these cells demonstrate reduced repopulating potential and a propensity towards myeloid skewing.[108,133-135] Together, these findings strongly support the conclusion that aging expands one or more pools of dysfunctional HPCs/HSCs that limit the hematopoietic system response, especially in times of stress. Since DNA repair processes are disrupted with aging, DNA damage accumulates in the cells of older adults. Like many other cancers, multiple genetic, epigenetic, and/or transcriptional events are required to transform normal hematopoietic progenitor/stem cells.[136-139] Therefore, dysfunctional HPCs/HSCs may acquire the necessary and sufficient transforming events over time, leading to the development of hematopoietic malignancies and explaining the increased incidence of hematopoietic malignancies in older adults.[16] If so, the characterization of the nature and identity of ARECs may provide insight into the biology of normal aging and the development of age-associated diseases such as cancer.

Both genetic and epigenetic mechanisms are involved in generating specific age-related expression changes (ARECs) in hematopoietic cells. Werner syndrome is an extensively studied premature aging disorder that is caused by a specific mutation in the WRN gene.[140,141] The WRN protein

functions as a DNA helicase and exonuclease, aiding normal recombination and repair.[142] The common WRN mutations result in a truncated protein that lacks appropriate and necessary nuclear localization,[143] and the subsequent decrease of WRN activity leads to the accumulation of genetic damage, genetic instability, premature aging, and cancer.[142] It is unclear whether the decrease in WRN expression and/or its activity plays a role in "normal" hematopoietic aging; however, expression profiles in fibroblasts of Werner syndrome patients and older adults reveal a set of common ARECs as compared to young healthy controls.[144] In addition, WRN activity is lost in a number of age-related diseases, including several malignancies.[145,146] Taken together, these data suggest that Werner syndrome may be a reasonable model for "normal" aging and the development of age-related disease.[144]

Some ARECs may directly facilitate the transformation process (T-ARECs). We recently examined the ARECs in human and murine HPCs/HSCs and found that interferon regulatory factor 8 (IRF8) displayed robust ARECs with in human CD34+ cells, murine long-term repopulating HSCs (LTR-HSCs), and human T-cells.[147] In all three hematopoietic cell types, IRF8 expression decreased with aging. Previously, IRF8 insufficiency has been associated with the development of hematopoietic malignancies in murine models,[148,149] and many human hematopoietic malignancies (AML, CML, MDS) display decreased IRF8 expression.[150-152] These results suggest that IRF8 insufficiency with aging *may* facilitate transformation, but additional studies will be required to confirm these results and to identify potential other T-ARECs, if they exist.

In an interesting twist, some ARECs appear to play a role preventing the development of age-related diseases such as cancer. For example, cyclin-dependent kinase inhibitor 2A (CDKN2A, a.k.a., $p16^{INK4a}$) is a tumor suppressor gene that regulates the cell cycle,[153] and loss of CDKN2A function is associated with hematopoietic malignancies, most notably acute lymphoblastic leukemia.[154,155] However, numerous studies have found that CDKN2A expression increases in an age-dependent manner in most tissues, including the hematopoietic system.[133,156,157] TP53, another tumor suppressor gene, also displays a similar paradoxical expression pattern in aging and malignant transformation. Like CDKN2A, loss of TP53 activity promotes the development of hematopoietic malignancies,[158-161] and in mice, a "hyperactive" TP53 phenotype has been found to protect against cancer development. However, these same mice displayed a 20% reduction in life-span as compared to their TP53 wild-type counterparts, and in fact, the TP53 hyperactive mice prematurely develop classic signs of aging (e.g., osteoporosis and tissue atrophy).[162] Furthermore, genetic polymorphisms in TP53 that modulate its expression have been found to be associated with age-related conditions and altered human survival curves.[163,164] For example, subjects with the $TP53^{72\text{-}Pro/Pro}$ genotype (associated with a reduced TP53 apoptotic potential and activity) have an increased risk of cancer (2.54 fold, $P = 0.007$), but they also have a 41% increased overall survival ($P = 0.032$) when compared to $TP53^{72\text{-}Arg/Arg}$ carriers.[164] These controversial results suggest that increased TP53 activity may protect against cancer at a cost of longevity. A more recent study found the apparent $TP53^{72\text{-}Pro/Pro}$ benefit may be more related to improvements in survival after the diagnosis of life-threatening diseases than an actual decrease in the "aging" process *per se*.[108,163,165,166] Taken together, these and other studies suggest a delicate, evolving balance between the need for cell survival and an increased susceptibility for transformation as we age. In this model, DNA damage promotes "protective" ARECs (P-ARECs) that induce DNA repair and/or apoptosis, preventing

transformation. Unfortunately, this protection comes at a price, namely, an age-related decline in the ability to replenish the tissues and cellular exhaustion of organ systems. If P-ARECs are lost due to genetic or epigenetic events, aging cells may more readily acquire necessary and sufficient transforming hits that lead to development of malignant clones (Figure 3).

New advancements in microarray design and labeling techniques have reduced the quantity of starting material necessary to detect ARECs. Most investigations have examined either non-hematopoietic tissue (e.g., fibroblasts, muscle, brain) or murine-derived hematopoietic cells. Chambers et al. identified approximately 3100 ARECs in murine HSCs. These investigators found an age-dependent down-regulation of genes involved in chromatin remodeling and maintenance of genomic integrity, while there was a concomitant increase in the expression of genes associated with stress responses, inflammation, and protein aggregation.[135] Rossi et al. examined a slightly less differentiated population of murine LTR-HSCs, and this study found marked ARECs for genes associated with myeloid development, which may provide some insight into the age-associated myeloid skewing.[167] Furthermore, older LTR-HSCs also displayed increased expression of some genes that have previously been recognized as being involved in leukemic transformation.[167] Due to the limited capacity to obtain highly purified populations of human HSCs, there have been relatively few studies examining ARECs in human hematopoietic progenitor cells. We recently found a number of genes with ARECs in human CD34+ and CD34+/38- cells, some of which also displayed similar ARECs in murine HSCs from other studies.[147] Prall et al. has also examined ARECs in a limited number of human CD34+ cells.[168] These studies identified three genes with major ARECs: KU-antigen 70 kD (KU70), microsomal glutathione S-transferase 1 (MGST1), and BCL2-interacting killer (BIK).[168] The relatively small numbers of samples used in each of these studies limit the power to identify significant ARECs and assess potential inter-individual variations in expression. Additionally, even the most highly purified populations of cells are probably composed of relatively heterogeneous subpopulations. Thus, many apparent ARECs may merely reflect the inability to select and examine truly identical cell populations in young and older adults.

FACTORS IMPACTING HEMATOPOIETIC EXPRESSION

Hematopoietic cells are constantly receiving, sending, and managing signals that are integral to the control of normal hematopoiesis. Key issues assigned to the hematopoietic microenvironment (i.e. “niche”) are the maintenance and protection of the HSC pool, regulation of mitogenic signals, and oversight of appropriate differentiation. The importance of the microenvironment is illustrated in the case of an ablative transplantation. Immediately after transplantation, HSCs rapidly “home” to the appropriate hematopoietic niches within the bone marrow microenvironment.[169] There are numerous factors that contribute to the homing nature of HSCs, some of which are cell-dependent, while others are microenvironment-dependent. For example, primitive hematopoietic progenitors express calcium-sensing receptor (CaR) molecules. These CaRs detect and act upon subtle differences in calcium gradients and control the homing of HSCs to the appropriate stem cell niches.[170]

At a macroscopic level, the hematopoietic system must respond to environmental stressors such as radiation, toxins (tobacco), medications, and industrial exposures, and these environmental factors

directly impact the expression profile of normal hematopoietic cells.[5] Radiation exposure is one of the most extensively studied environmental exposures. Numerous studies indicate that even low doses of radiation promote expression changes in normal hematopoietic cells.[171-175] Many radiation-induced expression changes involve genes that control cell cycle and DNA-repair pathways, with p53 signaling being the classical example,[175,176] and even low doses of radiation have been found to induce expression of CDKN1A (a.k.a., CIP1, p21), DDB2, and XPC, all of which play direct or indirect roles in the p53 signaling pathway.[177] The fact that such low doses of radiation exposure can induce significant expression changes may be directly relevant to expression studies of normal and abnormal hematopoietic cells. For example, at least one study has found that airline pilots have a relative increase frequency of number of chromosomal translocations in their peripheral blood cells, and retrospective analyses suggest that flight attendants may have a slightly higher risk of certain cancers.[178,179]

Investigators have also examined the effect of toxins, such as tobacco smoke, metal fumes, and bacterial endotoxins, on gene expression in human hematopoietic cells.[180-182] Tobacco smoke inhalation clearly induces the expression of numerous genes in peripheral blood cells.[183-185] Specifically, smoking promotes expression changes associated with the inflammatory response (increased CCR2 and IL1β), carcinogen metabolism (increased CYP1B1), oxidative stress responses (increased SOD2), and apoptosis/cell cycle (increased CDKN2B).[183,184] Physical displacement of populations of cells (for instance, due to local toxic damage/death or inflammatory cell recruitment) may be responsible for some of these expression changes; however, even in studies that carefully controlled for “cell population shifts,” there continues to be smoking-related expression changes.[185,186] These molecular changes certainly seem appropriate in light of data that indicate increased inflammation and DNA-damage within peripheral blood cells from tobacco smokers.[187,188]

A critical environmental factor, often overlooked in expression studies, is medications. Approximately 80% of people over the age of 18 take at least one medication each week, and the number of medications taken per individual increases with age, with 23% of women over the age of 65 years taking at least 5 medications.[189] It is unknown how most medications impact the transcriptome in hematopoietic cells from healthy volunteers or patients. To complicate the matter further, recent studies reveal that some disorders may be associated with reproducible expression changes in the hematopoietic system, despite the absence of a known abnormal hematopoietic phenotype. For example, characteristic “abnormal” expression signatures in hematopoietic cells have now been identified for depression, bipolar disease, and psychosis.[190] In the case of depression, drugs such as selective serotonin uptake inhibitors (SSRIs) decrease expression of serotonin transportors,[191] and in fact, may reverse the underlying “abnormal” expression profile in the peripheral blood.[190] Likewise, patients with bipolar disease or schizophrenia display increased transcription of dopamine D3 receptor (DRD3) in peripheral lymphocytes that also reverts back to near normal levels after treatment with anti-psychotic medications.[190]

LIMITATIONS AND FUTURE DIRECTIONS

Expression in normal hematopoietic cells is a tightly controlled and poorly understood process, which is impacted by numerous factors including intrinsic changes within the cell (e.g., age) and environmental exposures (toxins, ligands, etc.). In addition, these expression responses must be overlaid upon the inherent genetic variation in the population. In order to better understand the interactions of genetics and environment on the hematopoietic organ, there is a crucial need for a more precise and detailed characterization of ***normal*** hematopoietic subpopulations. This includes the development of techniques to identify and isolate the most primitive hematopoietic stem cells. Advancements in the field of single-cell capture methods, as well as methods to perform wholesale genetic analyses on ever-lower amounts of DNA, RNA, and protein, hold considerable promise. In addition, there is a profound need to better understand how the micro- and macro-environment are controlling hematopoiesis. It is difficult to imagine that an isolated hematopoietic cell in culture has a similar or identical biological response as that same cell in the blood or bone marrow niche. In the future one can imagine that the study of peripheral blood gene expression will tell us much about disease propensity, potential treatment response and toxicity, and perhaps be a “canary” of environmental insult.

The authors have no conflict of interest to declare.

FIGURES

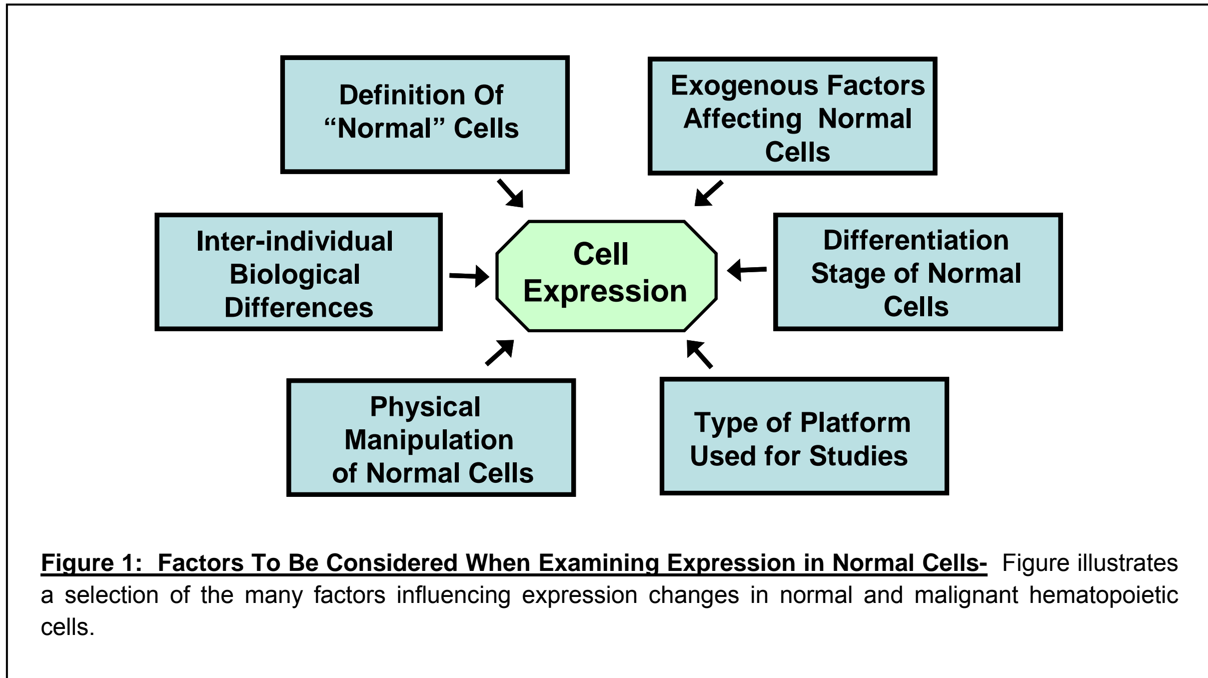


Figure 1: Factors To Be Considered When Examining Expression in Normal Cells- Figure illustrates a selection of the many factors influencing expression changes in normal and malignant hematopoietic cells.

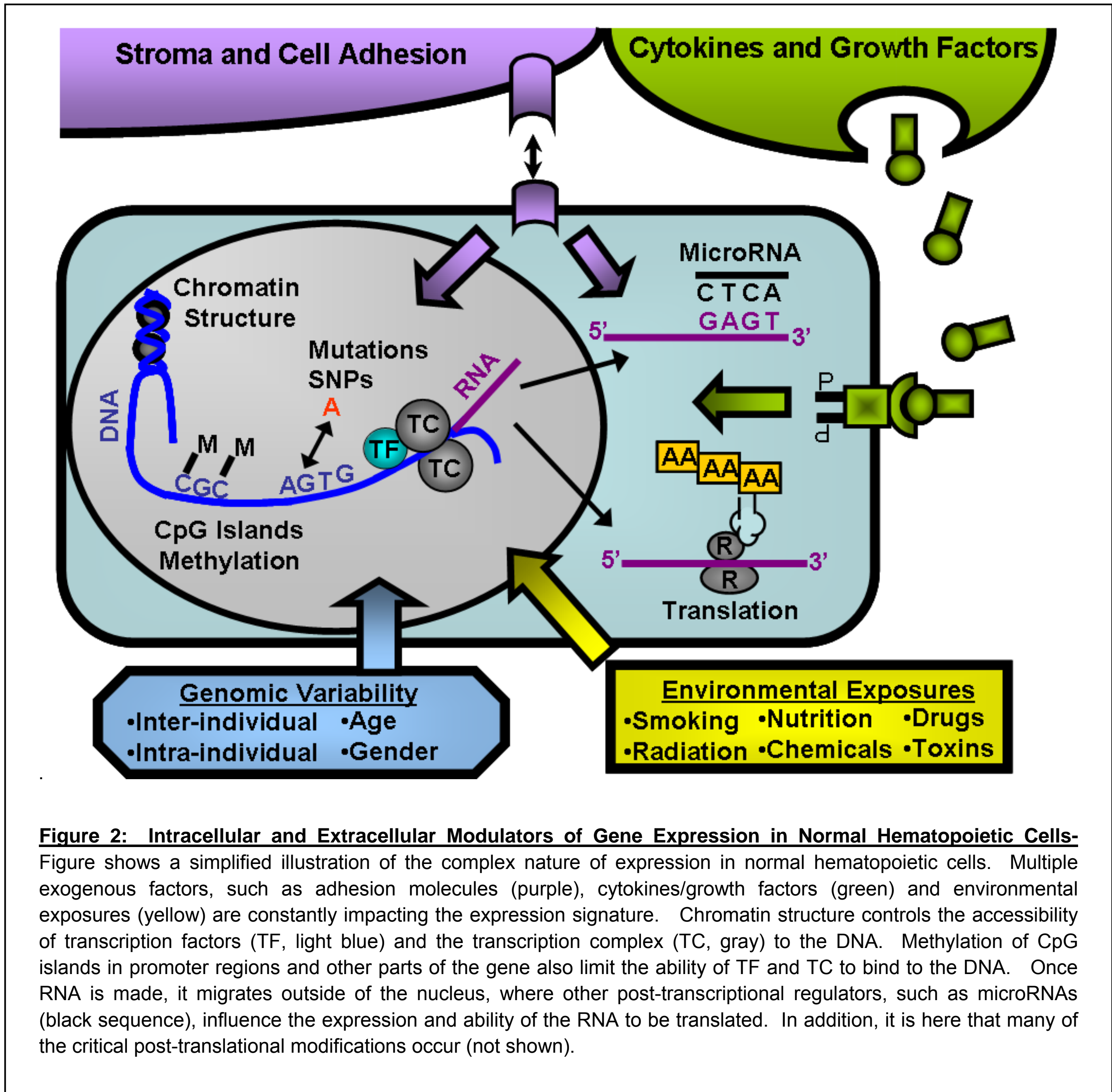
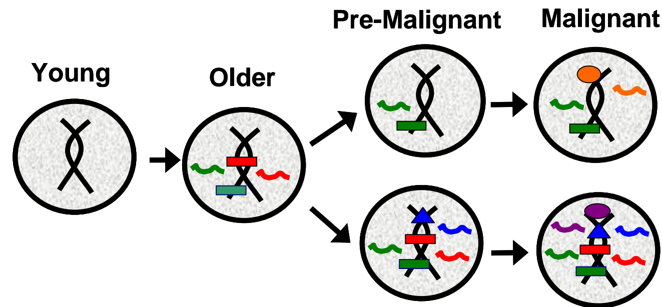


Figure 2: Intracellular and Extracellular Modulators of Gene Expression in Normal Hematopoietic Cells- Figure shows a simplified illustration of the complex nature of expression in normal hematopoietic cells. Multiple exogenous factors, such as adhesion molecules (purple), cytokines/growth factors (green) and environmental exposures (yellow) are constantly impacting the expression signature. Chromatin structure controls the accessibility of transcription factors (TF, light blue) and the transcription complex (TC, gray) to the DNA. Methylation of CpG islands in promoter regions and other parts of the gene also limit the ability of TF and TC to bind to the DNA. Once RNA is made, it migrates outside of the nucleus, where other post-transcriptional regulators, such as microRNAs (black sequence), influence the expression and ability of the RNA to be translated. In addition, it is here that many of the critical post-translational modifications occur (not shown).



<u>Figure 3: Model of Malignant Transformation in Older Patients-</u> As humans age, HSCs acquire age-related genetic and epigenetic changes (red and green rectangle), which produce ARECs that either protect cells from transformation (red line, P-ARECs) or promote transformation (green line, T-ARECs). In some cases, the P-ARECs are lost either through genetic or epigenetic changes, resulting in a pre-malignant phenotype that transforms with additional changes (orange). Even if the P-ARECs are not lost, T-ARECs may promote the acquisition of additional genetic and epigenetic changes (blue triangle and purple circle), which facilitate the development of pre-malignant and eventually, malignant clones.

Table 1. Type of Membrane Bound Receptors on Hematopoietic Cells.

Receptor				Ligand		
Name	Symbol	Alias	Family	Name	Symbol	Alias
Receptor Tyrosine Kinase (RTK). All 5 members of RTK family.[57,71-73]				Ligand		
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	CD117, C-KIT	PDGFR, RTK III	KIT ligand	KITLG	SCF, KL-1
Platelet-derived growth factor receptor, alpha[192]	PDGFRA	CD140A	PDGFR, RTK III	Platelet-derived growth factor, alpha	PDGFA	
Platelet-derived growth factor receptor, beta[192]	PDGFRB	CD140B	PDGFR, RTK III	Platelet-derived growth factor, beta	PDGFB	SIS, SSV
FMS-related tyrosine kinase 3[192]	FLT3	CD135, FLK2	PDGFR, RTK III	FLT3 ligand	FLT3LG	FL
Colony stimulating factor 1 receptor	CSF1R	CD115, FMS	PDGFR, RTK III	Colony stimulating factor (macrophage)	CSF1	MCSF
Cytokine Receptors (CKRs). Selected set of CKRs involved in hematopoiesis.[58,59,193]				Ligand		
Colony stimulating factor 2 receptor, composed of two different subunits[194,195]	CSF2RA, Unique to CSFR	CD116, GMCSFR	Cytokine Receptor Superfamily, Type 1, CKR I	Colony stimulating factor (granulocyte - macrophage)	CSF2	GMCSF
	CSF2RB, Common also to IL3 and IL5 receptors	CD131, IL3RB, IL5RB				
Colony stimulating factor 3 receptor	CSF3R	CD114, GCSFR	CKR I	Colony stimulating factor (granulocyte)	CSF3	GCSF
Erythropoietin receptor	EPOR	------------	CKR I	Erythropoietin	EPO	----------
Myeloproliferative leukemia virus oncogene	MPL	CD110, TPOR	CKR I	Thrombopoietin	THPO	TPO
Interleukin receptors (Most interleukin receptors but not IL10 or IL 20)	IL2RA, IL2RB, IL2RG, IL3RA, IL6R respectively	CD25, CD122, CD132, CD123, CD126 respectively	CKR I	Interleukin 2, Interleukin 3, Interleukin 6	IL2, IL3, and IL6 bind to their appropriate receptors.	
Interferon receptors, IL10, and IL20	IFNAR1,IFNAR2, IFNGR1,IFNGR2	IFNGR1 (CD119)	CKR 2	Interferon alpha, interferon beta, interferon gamma	IFNA and IFNB bind IFNAR1/2; IFNG binds IFNGR1/2	
Protein Tyrosine Phosphatatases Receptors (PTPRs). Selected members from 38 classical PTPRs.[60,61]				Ligand		
Protein tyrosine phosphatase, receptor type, C	PTPRC	CD45, LCA	Receptor Type I	Specific ligands are not well characterized for PTPRs. Many PTPRs interact and regulate other molecules/receptors in the cell membrane or are involved in cell-cell and cell-matrix interactions. There is also a separate class of non-receptor protein tyrosine phosphatases (PTPNs).		
Protein tyrosine phosphatase, receptor type, F	PTPRF	LAR	Receptor Type II			
TGF-β Superfamily of Receptors[62,196]				Ligand		
Transforming growth factor Beta Receptor I	TGFBR1	ALK5	Type 1 Receptors	Numerous ligands that bind to the various combinations of receptors. In most cases, a type 1 receptor will pair with a type 2 receptor to form the functioning receptor complex. Some ligands include TGFβ1, TGFβ2, TGFβ3, activin β_A, activin β_B, activin β_C, nodal, BMP2 - 7, GDFs, and AMH.		
Specific activin receptor-like kinases (ACVRL) and activin receptors (ACVR)	ACVRL1, ACVR1, ACVR1B, ACVR1C	ALK1, ALK2, ALK4, ALK7 respectively	Type 1 Receptors			
Bone morphogenetic protein receptors (BMPR)	BMPR1A, BMPR1B	ALK3, ALK6 respectively	Type 1 Receptors			
Transforming growth factor Beta Receptor I	TGFBR2	TGFβRII	Type 2 Receptors			
Activin receptors (ACVR)	ACVR2, ACVR2B	ACTRII, ACTRIIB	Type 2 Receptors			

Bone morphogenetic protein receptors 2	BMPR2	BMPRII	Type 2 Receptors	
Anti-Mullerian hormone receptor 2	AMHR2	MISRII	Type 2 Receptors	
TNF Receptors (Selected members from more than 29 different TNF receptors)[63,64]				Ligand
Tumor necrosis factor receptor 1	TNFR1	CD120a	------------	Both TNF (a.k.a. TNFA, TNF-α) and TNFB can bind to either receptor.
Tumor necrosis factor receptor 2	TNFR2	CD120b	------------	
Tumor necrosis factor receptor, superfamily 10A	TNFRSF10A	CD261, DR4, TRAILR1	------------	Tumor necrosis factor ligand superfamily, member 10 (aka TNF-related apoptosis- inducing ligand, TRAIL) binds to both receptors.
Tumor necrosis factor receptor, superfamily 10B	TNFRSF10B	CD262, DR5, TRAILR2	------------	
Notch Receptors[51,65,197]				Ligand
Notch homolog, 1 - 4	NOTCH1-NOTCH4	------------	Type 1 Transmembrane Receptors	Delta-like family of ligands (DLL1, DLL3, DLL4) and Jagged family (JAG1, JAG2) bind Notch Receptors.[65]
Wingless-type MMTV integration site family members (WNT) Receptors[66,198]				Ligand
Frizzled homolog, 1 - 10[199,200]	FZD1 - FZD10,	------------	G-Protein-coupled Receptors, Class 6	FZD and LRP combine to form a receptor complex that binds to Wingless-type MMTV integration site family members (WNTs)
Low density lipoprotein receptor-related protein, 5 and 6[201]	LRP5 and LRP6	LRP5 (a.k.a., BMND1, EVR1); LRP6 (a.k.a., ADCAD2)	------------	
CXC Chemokine Receptors (Selected members from the 6 known CXCRs)[67]				Ligand
CXC chemokine receptor 4	CXCR4	CD184, Fusin	G-Protein-coupled Receptors, Class 1, Subfamily A2	Binds C-X-C motif ligands, 12; CXCL12
CXC chemokine receptor 5	CXCR5	CD185, Burkitts lymphoma receptor 1		Binds C-X-C motif ligands, 15; CXCL15
Chemokine Receptors (Selected members from the 10 known CCRs)[68]				Ligand
Chemokine receptor 1	CCR1	CD191	G-Protein-coupled Receptors, Class 1, Subfamily A1	Binds C-C motif ligands, 3 or 5; CCL3, CCL5
Chemokine receptor 2	CCR2	CD192		Binds C-C motif ligands, 2; CCL2
Other Types of Receptors: Toll-like Receptors, Integrins, and multichain immune recognition receptors (MIRRS).				

Table 2: Tools for Gene Profiling					
Assessing Genomic Changes:					
Genomic	Throughput	Resolution	Material Required	Limitations	Data Provided
Pyrosequencing	High	Primary Sequence	DNA, nanograms	Expense	Complete Sequence
SNP Arrays	High	Up to 1 million SNPs per Genome	DNA, nanograms	Expense	SNP variation, CNV, LOH
PCR/Sequencing	Moderate-low	Primary Sequence	DNA, picograms	Limited number of genes	Sequence between primers
Epigenetics	Throughput	Max Resolution	Material Required	Limitations	Data Provided
Methylation Arrays	High	Increasing	DNA, nanograms	Requires sequence modifications	Methylation at CpG islands
Methylation-specific PCR	Low	Specific target sequence	DNA, nanograms	Requires sequence modifications	Methylation at CpG islands
Methylation-specific Restriction Enzyme Protection PCR Assays	Low	Specific target sequence	DNA, nanograms	Requires methylation be at restriction site	Methylation at restriction enzyme cutting sites
CHiP-on-chip Assay	High	> 200,000 specific DNA and/or promoter sequences	Cells, ≥ 10,000	Antibody quality and technically challenging	DNA sites of histone binding and type of histone
Assessing Transcriptome Changes:					
Transcription	Throughput	Max Resolution	Material Required	Limitations	Data Provided
Whole transcriptome sequencing	High	Primary sequence	RNA, nanograms	Cost	All transcript isoforms
RNA and microRNA arrays	High	> 20,000 transcripts and/or genes	RNA, micro - nanograms	Cost, miss genes or isoforms not included on array	Quantitative expression of transcripts included on the array
RT/PCR amplification	Moderate	Selected gene	RNA, picograms	Not useful for discovery studies	Quantitative expression of gene
Assessing Proteomic Changes:					
Mass Spectrometry based	Throughput	Max Resolution	Material Required	Limitations	Data Provided
Peptide fingerprinting	High	Digested peptides	Protein, low amounts	Limited to known peptides in database	Potentially identifiable fragments of protein
MALDI-TOF or SELDI-TOF	High	Digested peptides	Protein, low amounts	Limited to known peptides in database	Protein interaction, dissociation constants, manifold applications
Microarray	High	Any protein recognized by available antibodies	Protein, low amounts	Limited by antibodies	Protein expression
Electrophoresis and Antibody based	Throughput	Max Resolution	Material Required	Limitations	Data Provided
Two-dimensional electrophoresis	Moderate-low	Separation of potentially 1000s of proteins	Protein, micrograms	Potential protein co-migration	Separation of protein by isoelectic point and molecular weight
Western Blotting	Low	Specific proteins	Protein, micrograms	Antibody specificity and sensitivity	Semi-quantification of protein expression
Immunoprecipitation	Moderate-low	Specific proteins	Protein, micrograms	Antibody specificity and sensitivity	Protein expression and binding partners
ELISA	High-moderate	Specific proteins	Protein, nanogram	Antibody specificity and sensitivity	presence of a specific protein

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