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The stem cell fitness landscape and pathways of molecular leukemogenesis

Grover C. Bagby[1,2,3], Angela G. Fleischman[1]

[1]Department of Medicine, Division of Hematology and Medical Oncology, Oregon Health and Science University, Portland, Oregon 97239, [2] Knight Cancer Institute at Oregon Health and Science University, Portland, Oregon 97239, [3] NW VA Cancer Research Center, Portland, Oregon, 97239

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1. ABSTRACT

The relative risk of clonal evolution to either myelodysplasia (MDS) or acute myelogenous leukemia (AML) is high in patients with chronic bone marrow failure. From 10 to 20% of acquired aplastic anemia survivors will develop clonal evolution within the decade following their diagnosis as will 40% of patients with some of the inherited bone marrow failure syndromes. Studies on bone marrow failure states have provided some perspective on molecular pathogenesis of marrow failure and have also provided insights on the adaptive nature of clonal evolution. We review the scientific evidence validating this model, emphasize the importance of the fitness landscape in the stem cell pool, outline the clinical and investigative implications of the model, suggest that the lack of fitness in the starting pool accounts for the phenomenon of oncogene addiction, promote the value of the model for the evaluation of prevention strategies, and argue that experiments focusing attention on the relative phenotypes of neoplastic stem cell clones and pools of unfit stem cells from which they evolved will provide a useful paradigm of carcinogenesis in general.

2. INTRODUCTION

Acute myeloid leukemic clones evolve from somatically mutated hematopoietic stem cells (HSCs) or progenitors (HPCs) that have acquired (or aberrantly retained) the capacity self- replicate but have lost the capacity to give rise to myeloid precursor cells capable of undergoing orderly terminal differentiation. Recent evidence from high-density SNP arrays and massively parallel DNA sequencing analyses of AML samples have identified multiple point mutations(1) and significant alterations in gene copy number(2) even in samples that appear normal by standard metaphase cytogenetic analysis. Recurrent mutations have been identified in AML and the identification of some of them can help with assessments of prognosis.(3-7) Unfortunately, less is known about the function of the mutant proteins or the consequence of gene inactivation and, in light of the enormous case-to-case genomic heterogeneity and multiplicity of genomic changes, nothing is known about the temporal order of appearance of genomic alterations during the course of clonal evolution; that is, from the starting point of the very first genetic change.

Many published studies have revealed factors that likely influence disease progression in AML but until recently, few reliable *in vivo* models of myeloid leukemogenesis existed that might shed light on early genetic events in this process. Only a clear picture of the first molecular events that ignite clonal evolution in HSCs will pave the way to the development of rational approaches to leukemia prevention, particularly in patients at high risk. Fortunately, there are now new genetic paradigms of spontaneous myeloid leukemogenesis, (8, 9) that can be used to illuminate ordered events in leukemic clonal evolution in bone marrow failure states. These models have also confirmed earlier predictions that when clones evolve in the context of bone marrow failure states they do so by adapting to the environmental stresses to which all other cells in the stem cell pool were vulnerable.(10-12)

3. CANONICAL LEUKEMOGENESIS

Dominant acting oncogenes are clearly involved in the molecular pathogenesis of a variety of hematologic neoplasms and for cases in which promiscuous kinase activation is the cause, knowledge of the key pathway in individual cases has enormous therapeutic significance.(13-15). The capacity of aberrantly regulated or mutated genes to transform hematopoietic cells and to induce stem cell expansion *in vitro* and *in vivo* has been demonstrated in many cases, leading to the presumption that the function of the oncogene is sufficient to account for the process of leukemogenesis in nature. This presumption is overdrawn because the experimental model usually involves the ectopic expression of the gene in question. In addition, it cedes to the activated oncogene the capacity to function as a complete leukemogen in a pool of stem cells that are completely normal and in all environmental contexts. In fact, few studies have focused on the possibility that, in nature, the stem cells in the non-neoplastic pool may be dysfunctional and only because they are unfit can the evolutionary process proceed.

Moreover, it is increasingly clear that genes (HOXA9 for example) whose activation regularly occurs when AML clones evolve(16) and are therefore considered to be leukemogenic(17, 18) are often insufficient, when ectopically over-expressed on their own, to induce leukemic transformation.(19, 20) Instead, such genes maintain key cellular functions or encode critical co-factors upon which other leukemogenic factors depend.(21) They may also protect a cell in which the gene is highly expressed from the vicissitudes of the environment, and may, only under certain conditions of stress, allow that cell to acquire a competitive survival advantage.(22)

Highly informative studies on human cells have shown that some genomic alterations considered to be classically involved in leukemogenesis are insufficient, on their own, to account for the outgrowth of leukemia clones. For example, in studies by Greaves and his colleagues, the translocation t8;21 found in the leukemic cells of children with AML was also found to have existed in prenatal life indicating that this classical leukemic marker may have provided a key "parental phenotype" in the stem cell pool but was insufficient to induce rapid clonal evolution.(23) This notion is confirmed by a related study from the same group reporting that the "leukemogenic" translocations TEL-AML1 and AML1-ETO can be found in cord blood samples of normal neonates at a frequency at least two orders of magnitude greater than the prevalence of the leukemia that sorts with the translocation.(24)

Based on their analyses of TEL-AML1-transduced cord blood cells, the group argues that TEL-AML1 functions as a first-hit mutation by endowing a cell with altered self-renewal and survival properties(25) and that the early TEL-AML1 clone is truly preleukemic but requires some additional environmental events to occur before the mutation leads to clonal evolution. Based on these and like studies and on results of recent high-throughput genomic analyses of AML cells(1, 2), it is obvious that complex molecular interdependencies exist during the course of clonal evolution to AML and that the earliest changes are necessary but not sufficient for the full development of the neoplastic clone. What then is the relevant function of the early molecular aberrations and how does clonal evolution begin to occur? If they are weak mutations in the classic sense(26) what does the environment contribute to their selectability? From studies we have conducted on the rare bone marrow failure syndrome Fanconi anemia,(9, 12) using both human and murine hematopoietic cells, we believe that clonal evolution, at least in that particular context, meets every standard of a Darwinian selection process(27) and that this disease provides a robust opportunity to identify early genetic events in leukemogenesis.

4. ALTERING SELECTION COEFFICIENTS IN STEM CELL POOLS

4.1. Selection coefficients defined

Applied legitimately to both sexual (e.g. mammalian) and asexual (e.g. HSC) populations, the coefficient of selection ("s") represents the proportionate reduction in the pool contribution made by a specific genotype, relative to the contribution made by another genotype. That is, "s" expresses the relative advantage or disadvantage of specific traits with respect to survival and expansion. A selection coefficient may have a value from zero to one. If s=0 a selected genotype is neutral compared to the favored phenotype. Increasing "s" from 0 to 1 describes decreasing fitness of the genotype in question. Therefore, if we apply this standard to the work on t8;21 in normal prenatal cells,(24) and grant that at least one HSC carries the translocation, that cell can be assigned s=0. The neutrality of this cell in its environment raises important questions. Was the translocation the first molecular event that occurred in an untransformed stem cell? If not, what event initially sent a mutant stem cell down the path of clonal evolution? What role if any does the macro- or microenvironment play in the process?

There are two ways that selection coefficients can be altered to favor clonal evolution. The first, and most commonly promoted in the literature is the circumstance in

which the new clone arises in the context of a *normal* pool of stem cells and at some point acquires a significant growth advantage by acquiring new mutations that alter its response to environmental cues in favor of growth or differentiation blockade. Inflammation is commonly invoked as a driver.

4.2 . Enhanced fitness of the new clone relative to cells in a normal stem cell pool

There is abundant clinical and experimental evidence to support the idea that inflammation plays a role in the molecular pathogenesis of neoplastic processes. Patients with ulcerative colitis are at high risk for the development of colon cancer(28) and rodent models of colon cancer(29) have been developed which are tightly associated with diffuse colitis resembling ulcerative colitis in humans.(30) Colitis is inducible by exposing mice to dextran sulfate sodium and in this context, tumors develop.(31) Interestingly, genetic or pharmacological inhibition of TNFα signaling reduces both inflammation and tumorigenesis.(31) There are a number of other cancers that arise in the context of inflamed tissues including hepatitis C virus induced hepatocellular carcinoma(32, 33), adenocarcinoma of the esophagus associated with Barrett's esophagus,(34) gastric cancer associated with Helicobacter pylori infection,(35) and others. Some have even argued that inflammation should become a hallmark of cancer as important as uncontrolled growth and metastasis.(36)

Notwithstanding the evidence that the inflammatory process is capable of promoting cancer progression,(37-39) and the theoretical potential for components of the inflammatory response (e.g. reactive oxygen species [ROS]) to induce DNA damage,(40-42) there is no evidence that inflammation per se is sufficient to account for the earliest steps of clonal evolution. This is in large part because the intense focus on the inflammatory response has strangely ignored the fundamental principle that inflammation is, more often than not, a consequence, not a primary cause of tissue damage. Hepatitis C virus, for example, increases apoptotic responses in infected hepatocytes,(43) and the apoptotic fraction of colonocytes is increased in ulcerative colitis.(44) The landscape in those organ systems includes unfit pools of pressured non-transformed cells from which a mutant clone arises but the condition of the cells in these pools is commonly ignored as a determinant in clonal evolution. Environmental factors are capable of altering clonal selectability from moment to moment changing any given mutation from weak and physiologically insignificant to strongly selectable and unambiguously "beneficial" at least in the short term and at the level of a single cell. Therefore, the fitness landscape simply can't be ignored.

4.3. Altering the coefficient of selection by reducing fitness of stem cells in the untransformed pool

A second way of altering selection coefficients is by a reduction in fitness in the general stem cell pool. The evolutionary impact of genetic change is determined by comparing fitness of one genotype to that of another. For studies on carcinogenesis normal cells are most often used as a reference point. This isn't always appropriate. Normal cells vs. a mutant clone might have an "s" of zero, but when a less fit pool of cells (a pool of unfit hepatitis C infected hepatocytes for example) is compared to the same mutant, their score might be closer to 0.5 or more. Stated another way, when a mutant clone is compared to a normal population a given genotype might be insignificant, but when compared to cells in an unfit pool under attack from factors in the microenvironment, it might be significantly more fit. Naturally the increase in fitness would depend upon a match between the acquired genetic change (stochastic) and the stressful challenge the mutation relieves. The mutation must precisely match and relieve the stress that makes the pool unfit in the first place. Under these environmental conditions, the mutation would meet the standard of a "beneficial" mutation(45) at the cellular level although clearly for the whole organism it would be precisely the opposite. Evolution of mutants from a globally unfit pool is a general mechanism of clonal evolution that we see in Fanconi anemia and will be described in more detail below. An interaction between mutant stem cells and their microenvironment plays a seminal role in defining the fitness landscape in this disease and it is this aberrant relationship that defines the molecular pathogenesis of both bone marrow failure and leukemic clonal evolution.

5. THE FITNESS LANDSCAPE

5.1. Lessons from lava dwellers

The molecular genetics of adaptive coat color variation in rock pocket mice is an instructive example. These animals are light-colored and live in light colored environments. Dark colored mice have habitats on darker colored lava beds. Mutations in the melanocortin-1-receptor gene, Mc1r, are responsible for melanism in the lava-dwellers.(46) In a laboratory, the dark colored mice have no inherent advantage over the lighter ones. They are equally fit congenic strains but in the wild the coefficient of selection is massively altered such that on lava beds s=1 (a lethal score) for the light colored mice because they can be more easily seen by predatory birds and mammals.(46, 47) In the absence of predators, scores for both absolute and relative fitness, concepts that can be defined mathematically (reviewed in (48)), of these two strains are identical but both scores differ in the real world. In effect, the environment does its work to select the fit population not by influencing directly that population but by purging its unfit competitors. There is evidence that this is true not only for bacteria in which adaptation to antibiotic challenge results in resistance(49, 50) but for mammalian cells as well.(51) This Darwinian model fits perfectly with results of studies on stressed HSCs, namely those found in the bone mice and humans with bone marrow failure.

5..2. Molecular Pathogenesis of Aplastic Anemia

Acquired aplastic anemia is most often an autoimmune disease. The evidence from a number of laboratories, recently summarized by Young *et al*, (52) has revealed that; (1) aberrantly activated oligoclonal T-cell populations(53) suppress hematopoiesis by releasing cytokines (importantly IFNγ and TNFα) (2) these cytokines

and other factors induced by them cause apoptotic responses in hematopoietic stem and progenitor cells, (3) clinical responses to immunosuppressive therapy vary directly with the power of the treatment to suppress aberrant T-cell function,(54) and (4) immune mediated bone marrow failure can be modeled in mice and in that model monoclonal antibodies to TNFα and IFNγ prevent fatal aplasia.(55) Recent gene expression microarray analysis confirms on a genome wide scale that T-cell populations(56) are on the attack and that the progenitor cell pool is suffering as a consequence.(57)

5.3. Clonal evolution in acquired aplastic anemia

Stem cells assaulted by cytotoxic lymphoid populations(54) represent perfect models of a disadvantaged population. Unless the offending T-cell population is eradicated or inactivated, the selective pressure they exert on the stem cell pool would favor the evolution of any somatically mutated stem cell that had acquired, by virtue of the mutation, the capacity to resist the attack of T-cells. An identical somatic mutation might occur randomly in a single cell in an otherwise normal stem cell pool. In this case, because the selection coefficient is zero, clonal evolution would not be favored. It is precisely this situation that might explain how potentially leukemic clones can be found during normal fetal development yet not raise their heads even later in life.(24) However, if some environmental stressor were to disadvantage the cells in the normal pool, the situation could then favor clonal evolution. The environmental challenge therefore reduces fitness in the stem cell pool and creates an opportunity for selection of somatic mutants or epigenetically altered stem cells to emerge – mutant cells that may have existed for years in advance of the environmental insult. The most compelling experimental evidence in support of this paradigm derives from studies on humans and mice with an inherited form of bone marrow failure, Fanconi anemia.

5.4. Molecular pathogenesis of fanconi anemia

Fanconi anemia is a rare autosomal recessive (and in the case of FANCB, X-linked) disease characterized by congenital skin and skeletal anomalies, bone marrow failure, short stature, and high relative risks of acute myelogenous leukemia and epithelial malignancies.(58-60) Biallelic inactivation of any one of at least 13 genes are known to be responsible for this disease.(58, 61) Excessive chromosomal breakage in response to DNA crosslinking agents was one of the first reliable *in vitro* disease markers(62) and, indeed, forms the basis of the current diagnostic test. Consequently, most of the scientific work on this rare disorder has focused upon chromosomal instability phenotype. However, Fanconi himself observed that this disease has a strong phenotype of hematopoietic failure(63) and hematologic defects are the most consistent abnormalities seen in practice. Virtually all patients will exhibit some degree of bone marrow hypocellularity, albeit of varying severity, and up to 40% of children and young adults with FA will exhibit signs of clonal evolution in the bone marrow. This is a process in which aberrant HSCs with acquired genetic alterations self-replicate with more success than their competitors and give rise to progeny that overtake the bone marrow leading to myelodysplasia (MDS) and acute myelogenous leukemia (AML). (64, 65)

The historical presumption of the FA research community has been that the DNA damage phenotype is the direct cause of stem cell failure, but recently biochemical pathways of hematopoietic dysfunction have been discovered that can be, in some cases, formally uncoupled from DNA damage.(66, 67) More than 15 years ago in seeking to unravel the molecular pathogenesis of marrow failure in FA, we took notice of reports of parents and referring pediatricians that viral infections in children with this disease were often followed by periods of prolonged pancytopenia. For that reason, we considered that this aberrant response reflected an unusual sensitivity of hematopoietic progenitor cells to inflammatory cytokines that evolve in response to the infection. We also supposed that recurrent inflammatory events in patients with FA might sweep through the stem cell pool and result in its contraction. As will be described below, this turned out to be the case. It is also now very clear that such inflammatory events can serve as classical "selective sweeps" permitting the evolution of neoplastic clones.(9)

Hematopoietic cells from children and adults with Fanconi anemia and in mice nullizygous for *Fancc* not only release more TNFα in response to TLR ligation and in the ground state(68-70) but hematopoietic progenitor cells are also hypersensitive to TNFα and other well known growth suppressive cues. Specifically, the growth of FANCC- and Fancc-deficient progenitor cells is suppressed by very low doses of IFNγ, TNFα, mip1α and TRAIL.(71-75) HSCs are also hypersensitive to IFNγ because continuous infusions of that cytokine enhance engraftment of gene-corrected HSCs in competitive repopulation experiments not only in Fancc-deficient mice but in Fancg and Fanca deficient mice as well.(76, 77) Similar hypersensitive responses occur in FA progenitors and stem cells exposed to TNFα through direct exposure to that cytokine or by exposure to TLR-activating agents that induce its overproduction.(78, 79) While apoptotic responses seem to be one pathway of suppression by cytokines in these cells,(71, 80, 81) more recently we have also noted that TNF suppresses the replicative activity of Fancc-deficient multipotential progenitor cells (unpublished). Either way, FA stem cell pools are unambiguously unfit.

Some of the biochemical mechanisms of this hypersensitivity are now being clarified. For example, two serine/threonine kinases are involved in TNFα hypersensitivity because their activity is directly influenced by FA proteins. One is the protein kinase PKR a key molecular effector of the anti-viral response. The other is the apoptosis signal regulating kinase 1 (Ask1).(80, 82-84) Suppression of either one of these kinases relieves the cytokine hypersensitivity phenotype *in vitro*.

5..5. Overproduction of cytokines by FA cells

To add fuel to the fire, FA mononuclear phagocytes over-produce TNFα. Schultz and Shahidi(85) were the first to describe elevations of TNFα levels in

Table 1. Two genetic pathways of clonal adaptation and maladaptation in Fanconi anemia

Phenotype	FA Gene Correction (mosaicism) (Normal Hematopoiesis)	Stem cell sparing somatic mutation (MDS/AML)
Mitomycin C Hypersensitivity	Absent	Present
Cytokine Hypersensitivity	Absent	Absent*
TNF overproduction	Absent	Present

*in some cases TNF paradoxically augments myeloid colony growth (marrow CFU-GM)

serum of children with Fanconi anemia but the significance of and mechanisms that explain those findings have only recently emerged. Pang's group reported that Fancc$^{-/-}$ mice treated with endotoxin (lps) had higher mortality rates than wild type mice, had higher serum levels of TNFα and IL-6, and developed profound bone marrow suppression in response to lps that was formally attributed to an effect of TNFα (anti-TNFα antibodies protected FA hematopoietic cells).(78) They also showed that transplantation of HSCs from wild type mice protected Fancc$^{-/-}$ mice from endotoxin-induced TNF-overproduction and mortality, clarifying that both the TNF-hypersensitive cell population and the TNF-overproducing population were of hematopoietic origin.(78) What biochemical mechanisms underlie the overproduction phenotype have not been fully clarified but one pathway of significance seems to be that of Toll-like receptor 8 (TLR8) which is hyper-activated by the ligand R848 in FANCC- and Fancc-deficient mononuclear phagocytes. (70)

5.6. Bad stem cell pools in marrow failure states

In summary, abnormalities of stem cell self-renewal(86) and survival(9, 71, 82, 84) are found in patients with both acquired and inherited aplastic states. Both patient populations have high relative risks of clonal evolution to MDS and AML and there is strong evidence (indirect in humans and direct in mice) that the clones appear in response to aberrant production of or responses to suppressive cytokines or both. In acquired aplastic anemia the stem cell stress depends upon clones of T-cells that overproduce factors that result in stem cell death and loss of self-replicative potential.(52, 87) In Fanconi anemia the production of some of the same factors is increased because the normal FA proteins set response thresholds for TLR ligands including R848(70) and endotoxin(78)). In addition, the FA stem cell pool is inherently hypersensitive to those factors because the normal FA proteins somehow set response thresholds for exactly the cytokines over-expressed in auxiliary cells.(59) Experimental evidence supporting stem cell stress in other inherited marrow failure syndromes is not as robust, but preliminary studies support the idea that highly disadvantaged stem and progenitor cells exist in the hematopoietic microenvironment in these disorders as well.(88-90) Because of their lack of fitness, the selection coefficient is such that mutated stem cell clones have acquired the capacity to rectify the abnormal relationship of the defective stem cell with its environment. These clones will have a huge competitive advantage when they are in competition with the unfit cells but might not have an advantage at all if they are tested against normal ones.

6. CLONAL EVOLUTION IN FANCONI ANEMIA

6.1. Adaptive responses

Fanconi anemia stem cells, with time, will either adapt or perish. There are two pathways for adaptation. The perfect adaptive response in a pressured FA stem cell is, in fact, seen in nature and accounts for a phenomenon known as mosaicism. In this condition, a single HSC undergoes a corrective genetic rearrangement or mutation that corrects one of the FA alleles. This can happen through compensatory missense mutations, gene conversion, or intergenic crossovers.(91-93) In these cases, it isn't uncommon for the bone marrow to be dominated by the new clone and for hematopoiesis to be either normal or nearly normal.(94) Unlike the uncorrected mutant cells, the progeny of the clone are: (a) resistant to mitomycin C (MMC), (b) respond to TLR ligation normally vis-à-vis TNFα production, and (c) show perfectly normal responses to suppressive cytokines (Table 1). To emphasize some important points to be deduced from these experiments of nature, we summarize here experiments conducted using hematopoietic cells and fibroblasts from twins with biallelic mutations of FANCA.(94)

Monozygotic twin sisters were born with skeletal and cutaneous stigmata of FA but without hematologic defects. Mitomycin C tests of peripheral blood lymphocytes showed no evidence of excessive chromosomal breakage diagnostic of FA. Because fibroblasts did show MMC-induced breakage, they were clearly somatic mosaics for mutations in the FANCA gene. In skin fibroblasts, molecular analysis revealed a frameshift causing deletion in exon 27 (2555DeltaT) and an exon 28 missense mutation (2670G >A / R880Q). The latter resulted in reduced function of the mutant FANCA (R880Q) protein. An acquired exon 30 missense change (2927G >A / E966K) was detected in the hematopoietic cells of both sisters, but was not found in fibroblasts of the patients or of the parents. This compensatory mutation existed in cis with the maternal exon 28 mutation, and restored the function of the resulting protein in hematopoietic cells.(94) Neither sister has had abnormal blood counts for more than 3 decades.

There are some profound implications of such experiments of nature. First, because the compensatory mutation was identical in both twins, this de novo "corrective" mutation occurred prenatally in a single HSC in one twin and the progeny of this HSC repopulated the blood lineages of both sisters through a shared intrauterine circulation. Secondly, because the corrected clone replaced the entire marrow of both twins indicates that FA stem cells are unfit early in development (a conclusion supported experimentally by recent studies on embryonic stem cells(86)) and that the selection coefficient in the pool was high. Third, because one stem cell was capable of taking over the entire marrow of both twins suggests that effective gene therapy in patients with FA might require successful transduction of only a small number of stem cells.(94)

6.2. Myelodysplasia and AML

The second "adaptive" pathway for FA stem cells results in clonal evolution to either myelodysplasia or acute myelogenous leukemia. While the "adaptive" steps might assure the survival of the new stem cell and its progeny, the clonal progeny are not normal and reveal multiple abnormalities of growth and differentiation control. The syndromes that ultimately result from expansion of the neoplastic clones are often untreatable and more often fatal than not. It therefore seems reasonable, for purposes of this discussion, to consider this type of response as "maladaptive" and the category of mutations "pseudo-beneficial."

To emerge as a new clone, the unfit stem cell must overcome the aberrant pathways that reduced its fitness in the first place. This does occur in humans and has been modeled in mice.(9, 95, 96) For example, as mentioned above, TNFα hypersensitivity exists in progenitor cells of patients and mice with Fanconi anemia in the hypoplastic phase.(9, 12, 78, 79) However, in longitudinal studies on FA patients who enter the phase of clonal evolution to myelodysplasia, we find that the progenitor cells exhibit either resistance to TNFα or a paradoxical proliferative response *in vitro*. Likewise, in FA siblings discordant for clonal evolution, cytokine hypersensitivity exists in the hematopoietic progenitor cell population from the sibling without clonal evolution but resistance or a paradoxical (replicative) response is found the progenitors of the sibling with MDS.(12)

Unlike the example of mosaicism, these are not perfectly adaptive responses because: (a) the progenitor cells are more resistant to TNFα than even normal progenitor cells, (b) TNF-α production by the neoplastic mononuclear phagocytes remains very high in response to TLR ligation (unpublished), and (c) the clonal progeny show diagnostic chromosomal breakage responses to MMC (Table 1). In effect, the selection of neoplastic progenitors has resulted in exploitation of the macrophage defect by adapting in a way that leads not to their demise but to their outgrowth and survival. The neoplastic clone has a genotype that is "beneficial" to the survival of the stem cell, but is catastrophic for the host because it is fundamentally a new population that has learned how to grow in response to suppressive cues, overproduces the cue, and retains its genetic instability.

This process has been modeled in mice. A number of groups have observed acquired cytokine resistance and clonal evolution in murine models of FA.(8, 9, 96, 97). In one of these models, murine *Fancc*$^{-/-}$ stem cells and their progeny were cultured *ex vivo* for up to 90 days. During this culture period in some instances TNFα – resistant cytogenetically abnormal clones appeared. These clones had the capacity to induce ongoing clonal evolution to AML in transplanted mice.(9) That the appearance of these clones depends upon the continued presence of TNFα(9) emphasizes the importance of selective pressure in accelerating this process. That we have universally seen TNF-resistance in clonally evolved FA progenitor cells suggests that the cytokine hypersensitive phenotype is required for clonal evolution. It is therefore reasonable to expect that the development of cytokine resistance would be one of the earliest steps in myeloid leukemogenesis.

We do not argue that the change is sufficient to initiate the entire leukemogenic process. In addition, over time the new clone may also become resistant to other factors as well,(98) a phenomenon that would only make the neoplastic clone even more capable of competing against the disadvantaged stem cells. Nor would we argue that clonal evolution is always a formally genetic process because epigenetic events have been described as factors in the evolution of hematologic neoplasms as well.(99-101) In fact, genetic loss and epigenetic silencing may cooperate in some instances.(99, 100, 102) In myeloid leukemic clones with allelic losses due to chromosomal deletions, for example, the retained allele can be suppressed epigenetically(103) resulting in a functional loss of heterozygosity.

7. GENERALIZATION OF THE MODEL

Unfit stem cell pools plays an important role as a breeding ground for maladaptive mutations in the context of bone marrow failure. Because the development of malignant neoplasms is often an outcome of chronically damaged tissues, we argue that the fitness landscape in other stressed stem cell pools might play a role in carcinogenesis in general. We suspect that oncogenic mutations that lead to other hematologic malignancies might evolve in a like context; namely in pools of disadvantaged stem cells.

7.1. Jak2 mutations and myeloproliferative diseases

An activating mutation in the tyrosine kinase JAK2 (V617F) is identified in the majority of patients with Philadelphia chromosome-negative myeloproliferative neoplasms (MPN).(104-108) This mutation arises at the level of the hematopoietic stem cell(109) and results in expansion of one or more of the myeloid lineages each of which retains the ability to execute a complete differentiation program. Animal models of this disease abound. A polycythemia vera (PV)-like disease with secondary myelofibrosis arises in lethally irradiated mice transplanted with bone marrow transduced with JAK2 V617F retroviral vectors.(110-112) Transgenic(113) and knock-in mice expressing JAK2 V617F or Jak2 V617F under the influence of the endogenous Jak2 promoter, (114-116) develop an ET or PV-like MPN demonstrating that in mice the JAK2 V617F mutation is sufficient to induce a myeloproliferative phenotype. It should be emphasized, however, that the design of these experiments does not address the *earliest* steps in leukemic evolution in which a single cell and its progeny gain a competitive advantage. Admitting to the speculative nature of the idea, we suggest that the MPNs might evolve in ways analogous to the evolution of clones in patients with marrow failure syndromes, that is, specifically from unfit pools of HSCs.

Clinically relevant cytopenias have not been recognized as a prodromal feature of MPN so how could the leukemogenesis model we have described for the

marrow failure syndromes be conceivably applied to these diseases? First, substantial stem cell defects can occur in nature without attendant changes in blood counts in the ground state.(117) Only by providing environmental challenges to those pools can the vulnerability of stem cells and their progeny be revealed.(9, 76-79) Secondly, in every murine model of MPNs described, some sort of environmental stress exists precisely at the point clones might take hold. Thus, JAK2 V617F might have provided an advantage only in the context of transiently disadvantaged stem cells. For example, in both of the recent descriptions of conditional knock-in models, a pool of normal stem cells was transiently exposed to high levels of IFNγ (induced by the polyI-polyC required to activate Mx dependent Cre) so the initial outgrowth of JAK2 V617F expressing cells cannot be said to have occurred in an unstressed landscape. Indeed, exposure to pI:pC would likely have been sufficient to suppress expansion of Fanconi stem cells.(76, 77) In secondary transplants however, the landscape would have improved (or at least normalized) which might explain why there was no competitive advantage seen in the studies by Li *et al.*(115) However, even in secondary transplants, the cytokine storm induced by lethal radiation(118) could be a sufficient early stressor to induce the selective emergence of stem cells that express the mutant JAK2 (provided the mutant protected stem cells from one or more of the suppressive cytokines). Therefore, because none of the models tested begin with a single mutant cell in an unperturbed ground state environment, they do not directly address the molecular pathogenesis of the earliest steps in clonal evolution.

No one can sanely argue that in MPN patients the JAK2 V617F mutant cells have no advantage compared to the JAK2 wild-type cells in that patient. However, it is equally true that no one can yet argue, at least until the definitive experiments are done, that JAK2 wild type cells in MPN patients are perfectly fit. We believe that in humans the unmutated stem cells might well be unfit and that the evolution of the JAK2 mutant clone might represent an adaptive response. In fact, some genetic observations provide opportunities to test this notion directly. First, there exists a familial predisposition to acquire MPN. Nearly 8 percent of patients will have a strong family history of MPN.(119) Second, a specific JAK2 haplotype (termed 46/1) is strongly associated with JAK2V617F+ MPN, with a relative risk of 2.6.(120-122) and, interestingly, healthy controls with this haplotype have decreased granulocyte macrophage colony growth as compared to controls,(122) compatible with the notion that this haplotype (and the predisposition to acquire MPN) is associated with subclinically unfit progenitor and stem cell pools.

8. IMPLICATIONS OF CLONAL ADAPTATION

8.1. Oncogene addiction

The effectiveness of molecularly targeted agents (some of which can induce regression of a mutant clone but not suppress normal stem cells) has posed some challenging questions. Why do cancers that depend upon one wayward gene to maintain its neoplastic state simply die when that one gene is inactivated when it ought to simply suppress the proliferative advantage that the mutation confers? This phenomenon of "oncogene addiction"(123-125) can be modeled in conditional "oncomice" in which oncogene deprivation can induce sustained regression via induced differentiation or programmed cell death.(124) Whether this model represents the situation in nature is unclear. One alternative explanation is that suppression of oncogene function in evolved neoplastic clones can be lethal because the stem cells that were transformed by that oncogene were unfit to start with and required the activation of the oncogene to survive in the first place.

8.2. Oncogenes vs. non-oncogenes: distinguishing "passengers" from "drivers"

To credential mutations of tyrosine kinase alleles as "driver" mutations, a common and fairly reliable approach is to determine whether ectopic expression of the mutant in cytokine dependent murine hematopoietic cells results in; (a) a phenotype of cytokine independence and (b) enhancement of or constitutive activation of tyrosine kinase activity.(126-128) A mutation that fails to meet these standards is considered to be more likely a "passenger" mutation (one plays no role in disease pathogenesis).(128) There are exceptions to this rule. For example, some BRAF mutants that have *lost* catalytic activity are known to promiscuously activate CRAF so the downstream consequences are identical to those ignited by BRAF mutants with elevated kinase activity.(129) In addition, and more to our point, mutations might not be sufficient to induce factor independent growth but might be sufficient to protect exposed cells from perishing under stressful conditions. The experimental approach would be difficult because discovering the right mix of inhibitory factors and environmental conditions (that a potential passenger mutation might resist) would require a "fishing expedition" of sorts but with high throughput screening strategies, the task would not be unachievable. Adding such experiments to the process of credentialing mutant enzymes might provide evidence of growth and survival advantages in cells expressing mutant enzymes that have no catalytic activity.

8.3. Leukemia Prevention

Systems biology experiments can now be simply done using stem cell pools at high risk of AML and MDS and as a result a clearer picture of the temporal events that precede clonal evolution will be soon apparent. Even if the nature of the key stem cell pool stressor is not known, by identifying early molecular alterations in the march toward clonal evolution, one can backtrack to narrow the list of what those stressors might be. For example, if an early alteration in a stem cell pool suppressed expression of a receptor for inhibitory factor X, one could then directly test the notion that factor X was somehow initially contributing to the suppression of fitness in the parental stem cell pool. So unique signatures of adaptation ought to lead to the identification of factors that contribute to the reduction of fitness in stem cell pools. Once these factors are credentialed, they naturally would become molecular targets for prospective studies on leukemia prevention. It is this prospect that excites us the most.

9. ACKNOWLEDGEMENTS

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Abbreviations: LPS- Bacterial Endotoxin, TNFα- Tumor necrosis factor alpha, IFNγ - Interferon gamma, SCF - Stem cell factor (Steel factor), JAK - Janus kinase, STAT - signal transducer and activator of transcription, CFU-GM - Colony forming unit- granulocyte/macrophage, BFU-E - Burst forming unit, erythroid, IL-11 - Interleukin eleven, Flt3 - fms-related tyrosine kinase 3, Flt3L - Flt3 ligand, HOXA9 - Homeobox A9, HSC - Hematopoietic stem cell, SNP - Single nucleotide polymorphism, TLR - Toll-like receptor

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Send correspondence to: Grover C. Bagby, NW VA Cancer Research Center; R&D, B103, E221B; Portland VA Medical Center, 3710 SW VA Hospital Road, Portland, Oregon 97239, Tel: 503-273-5008, Fax 503-721-7946, E-mail: grover@ohsu.edu

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