Stem Cell Reports
Perspective
ISSCR
OPEN ACCESS

A Prospective Analysis of Human Leukemogenesis

Connie J. Eaves[1,*]
[1]Terry Fox Laboratory, British Columbia Cancer Agency and Department of Medical Genetics, University of British Columbia, British Columbia Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada
*Correspondence: ceaves@bccrc.ca
https://doi.org/10.1016/j.stemcr.2018.10.016

Decades of lack of progress in treating many fatal malignancies of the blood-forming system is commanding interest in new approaches. Targeting early events in the leukemogenic process has long been recognized as likely to offer the information required for these diseases. Analysis of the representation of different mutations in the leukemic cells from individual patients offers a retrospective method to infer their sequence of acquisition and associated subclonal dynamics. An alternative, prospective approach is to exploit strategies for recreating human leukemia *de novo* using defined genetic methods. This concept is not new, but has been historically difficult to realize. A brief review of our experience in generating insights into the properties and regulation of primitive normal human hematopoietic cells serves as a reminder of how this has enabled our recent advances in developing this approach using both primary sources of chronic myeloid leukemic cells and normal cord blood cells as targets.

Prologue

This perspective traces some highlights from our laboratory research efforts spanning several decades that have worked toward the long-term goal of improving outcomes of patients with acute myeloid leukemia (AML). It is also written with the purpose of formally recognizing the extraordinary insight and generosity of the Stiftelsen family and the Tobias Foundation they created. Their vision to promote hematopoietic stem cell research as a primary avenue to obtain better treatments of life-threatening blood diseases is unprecedented. Also to be recognized at the outset is the combined effort of an outstanding and dedicated team of trainees and colleagues who played a major role in the work described below. Many of the methods developed to enable primitive normal and malignant human cells to be characterized, as well as key findings thereby enabled, are due to their creativity and efforts. It is an honor and a privilege to link this jointly inspired and executed work with the Tobias Award, necessarily summarized here in very brief form.

Introduction

Evidence that human diseases classified as acute myeloid leukemia (AML) represent clonal disorders arising in a single hematopoietic cell dates back many years. Early studies indicated that the generation of AML is commonly, albeit not always associated with the acquisition by the malignant cells of a variety of chromosome abnormalities. These are now known to reflect different mutations that deregulate proliferation and block the differentiation of primitive normal blood cell precursors, features considered diagnostic of AML (Kayser and Levis, 2018). Additional features of most AMLs include the suppression of residual normal hematopoiesis and a continuing genetic and biological evolution of the leukemic cells. Recent large-scale collections of genomic, transcriptomic, and epigenomic data from AML patients' cells have now identified the genes most commonly altered, including mutations affecting epigenetic control systems rather than those directly altering signaling, receptor, or transport mechanisms (Bullinger et al., 2017; Cancer Genome Atlas Research Network et al., 2013; Welch et al., 2012). The large diversity of subclones thereby evident in many AML patients already at diagnosis is likewise now well established. Nevertheless, the field is now facing many additional challenges to the use of patients' samples for developing more effective treatments. This is due to the sheer numbers of AML blasts often needing to be eliminated ($>10^{12}$ cells/patient), the limited numbers of cells that can be accessed for analysis (typically <1/10,000th of the entire clone), their distribution over many sites, and the heterogeneity of their biological properties (Dick, 2005; McCulloch, 1993; Ng et al., 2016). Taken together, these issues greatly undermine the likelihood that continued direct examination of patients' samples will be sufficient to identify treatments capable of significantly advancing the management of human AML.

A related hurdle has been the long-standing paucity of detailed information about cellular and molecular mechanisms that control primitive stages of normal human hematopoiesis. Standard microscopic examination of the bone marrow and blood remains limited to defining the last few steps involved in the terminal differentiation of granulocytes, monocytes, erythroid cells, and megakaryocytes. The development of clonal assays able to detect and quantify subsets of cells with these different output capabilities in the 1960s and 1970s was thus a major breakthrough. Eventually such assays enabled the detection of cells that produced pure colonies of mature neutrophils and/or macrophages, or erythroid cells, or megakaryocytes, or mixtures of these in semi-solid cultures containing appropriate growth factors (Figure 1). Analogous to the terminally differentiating cells they generate, these clonogenic cells could be classified based on the number, time of generation, and ultimate spectrum of mature cells each

Dr. Eaves holds a BA in Biology and Chemistry and a MSc in Genetics from Queen's University, Kingston, Ontario, and a PhD in Immunology from the University of Manchester, UK. Following post-doctoral training in experimental hematology at the Ontario Cancer Institute under Dr. James Till in collaboration with Dr. Ernest McCulloch, she joined the faculty of the BC Cancer Agency and the University of British Columbia in 1973. In 1981, she and her husband co-founded the Terry Fox Laboratory at the BC Cancer Agency. Over the last four decades, Dr. Eaves has developed an internationally recognized research program that continues to address fundamental questions in normal and cancer stem cell biology. Her contributions have included the development and use of quantitative methods to molecularly and biologically characterize rare, functionally defined cells of the blood-forming system, the mammary gland, leukemia, and breast cancer that have become gold standards in these disciplines. More recently, her group has pioneered the creation of several models of *de novo* leukemia and breast cancer starting from primary sources of human cells. She has published more than 500 papers and has a long track record as a scientific leader and devoted mentor of more than 100 post-graduate trainees at all levels in multiple disciplines. She has also been an energetic lifelong contributor to the development of science policy and management in Canada and abroad, and maintains an active role in editing and reviewing scientific publications. She has received many prestigious national and international awards for her numerous and diverse accomplishments, including election as a Fellow of the Royal Societies of Canada and Edinburgh, the Noble Prize for Cancer Research, the Metcalf Lecture and Prize from the International Society of Hematology, and the Stratton Lifetime Achievement Award and Donall Thomas Lecture and Award from the American Society of Hematology.

produced. Optimization of conditions for their detection also enabled the development of methods for their prospective isolation as separately enriched compartments of cells. Taken together, these findings were interpreted as indicative of an early lineage restriction process that involved sequential stepwise events spanning a finite number of cell divisions, thus creating a readily visualized hierarchy incorporating these coordinated changes (Eaves, 2015).

Later studies identified an even more primitive subset of human (as well as mouse) hematopoietic cells. Their detection was based on their ability to produce directly clonogenic cells and their short-lived mature progeny for much longer periods (many weeks) when co-cultured with certain types of stromal cells. In addition, these more primitive cells could be phenotypically distinguished from the short-term clonogenic progenitors they produced (Eaves et al., 1992). The discovery that highly immunodeficient mice support all early stages of human blood cell production (Doulatov et al., 2012) led to a further but analogous stratification of primitive human hematopoietic cell types, again according to the durability of their mature cell outputs (Figure 1) (Miller et al., 2013).

Chronic-Phase Chronic Myeloid Leukemia Cells as Targets for Genetically Engineering Disease Progression to AML

The mutational diversity of human AML on top of the genetic dissimilarities in the individuals that develop these diseases have always raised questions about the likely utility of using forward genetic approaches to recapitulate the genesis of AML experimentally in primary sources of human cells. From a practical viewpoint, the very defining biological features of AML—a deregulated proliferation activity and suppressed terminal differentiation—also preclude the use of most differentiation landmarks to relate the mechanisms perturbed to the hierarchically ordered normal processes.

This challenge is not unique to AML. It is shared by many types of human cancer. Indeed its early recognition prompted a strong effort to create and investigate the malignant cells obtained in genetically engineered mouse models. Moreover, early efforts to extend this approach to primary sources of normal human hematopoietic cells were generally unsuccessful, despite progress in primary human cell isolation and continuously improved methods for their efficient genetic modification using lentiviral vectors.

As a way of potentially side-stepping the failures encountered using normal human hematopoietic cells as targets of genetic alteration, we opted to start with chronic-phase chronic myeloid leukemia (CP-CML) cells. In the CP of CML, expansion of the leukemic clone has generally already advanced to the point of achieving dominance throughout the entire system, but without perturbing

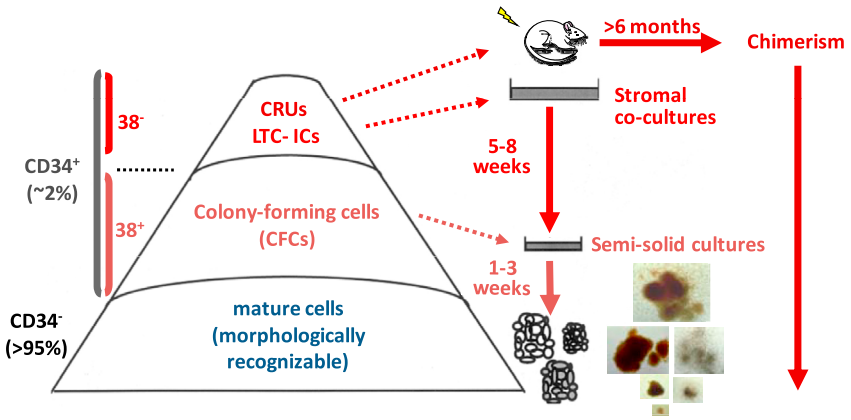

Figure 1. Schematic Visualization of the Hierarchical Organization of Normal Human Hematopoietic Progenitor Cell Types
This schema is based on the number, type, and duration of time over which different progenitor cell types produce mature blood cells when optimally stimulated. Cell surface expression of CD34 generally separates all of these cells (2% of all nucleated bone marrow cells) from their CD34-negative, morphologically recognizable maturing progeny (98% of all nucleated bone marrow cells). CD38 then further subdivides the $CD34^+$ subset as shown. CRUs refers to competitive repopulating units (in immunodeficient mice), LTC-ICs to long-term culture initiating cells (in stromal cell-containing cultures), and CFCs to colony-forming cells or short-term clonogenic progenitors.

normal differentiation. Moreover, studies of CP-CML cells suggested that these, in fact, mirrored the envisaged normal hierarchy (Figure 2). In addition, in the era preceding the introduction of tyrosine kinase inhibitor therapy for CML, CP-CML cells were known to ultimately transform into very aggressive leukemias, typically within a few years, even if the CP-CML clone was kept in check with drugs such as hydroxyurea. Thus it seemed likely that primitive CP-CML progenitors might be already primed to progress to an AML state and hence display an increased susceptibility to an oncogenic perturbation (Sloma et al., 2010).

Models Based on Genetically Engineering CP-CML Cells

To rationalize how best to approach testing this idea, we first considered available clues from analyses of CP-CML progenitors. One of these was the unexpected identification of a "normal" representation of different lineages of early progenitor types, all of which were inappropriately enlarged (Figure 2). Thus the characteristic excess output of neutrophils and monocytes of CP-CML, and the usual associated suppression of erythropoiesis, could not be explained as an early programmed switch or commitment bias toward the neutrophil/monocyte lineage. In addition, accompanying this observation was the finding that all CP-CML clonogenic progenitors, regardless of their lineage types, display an increased cycling activity. Importantly, we also found that these primitive CP-CML cells produce and respond to an autocrine interleukin-3 and granulocyte colony-stimulating factor mechanism that is activated within them, but is then lost when they terminally differentiate. This unusual stage-specific autocrine mechanism thus provides a potential explanation for the characteristically marked lineage-independent expansion of CP-CML clonogenic progenitors (Holyoake et al., 2002).

However, examination of the more primitive CP-CML cells from which short-term clonogenic cells are derived in co-cultures containing stromal cells revealed a different picture. The numbers of the upstream CP-CML cells detected using this culture system appeared not to be grossly expanded. In fact, they are usually outnumbered by a residual population of their normal counterparts that remain functional but whose progeny are outcompeted or suppressed by the later types of CP-CML cells whose proliferative activity becomes deregulated. Moreover, when the cell-cycle status of this very early subset of normal and CP-CML cells was compared, both normal (Ph-negative) and CP-CML ($Ph/BCR\text{-}ABL^+$) subsets were found to be quiescent when isolated directly from patients, without any evidence of an autocrine mechanism in the CP-CML cells (Holyoake et al., 2002). Not only was this discovery of a primarily quiescent subset of primitive CP-CML progenitors unexpected, to my knowledge, it was the first report of a reversibly dormant subset of malignant cells. On the other hand, despite their apparently "normal" proliferative state, the self-maintenance behavior of these primitive CP-CML cells *in vitro*, as shown by their reduced outputs of daughter long-term culture initiating cells, (LTC-ICs) proved to be highly deficient by comparison with their normal counterparts both in the presence and absence of stromal cells (Figure 2) (Eaves et al., 1998).

Accordingly, our initial thoughts about how to engineer their progression to AML were focused on strategies to overcome this "self-renewal defect." For this we turned to the *NUP98HOXA10* homeodomain (*NA10HD*) fusion gene construct based on its previously demonstrated ability to enhance the expansion *in vitro* of primitive mouse hematopoietic cells (>1,000×) (Ohta et al., 2007), and a significant, albeit more modest effect, on their similarly treated human counterparts (Sloma et al., 2013). As illustrated in Figure 3,

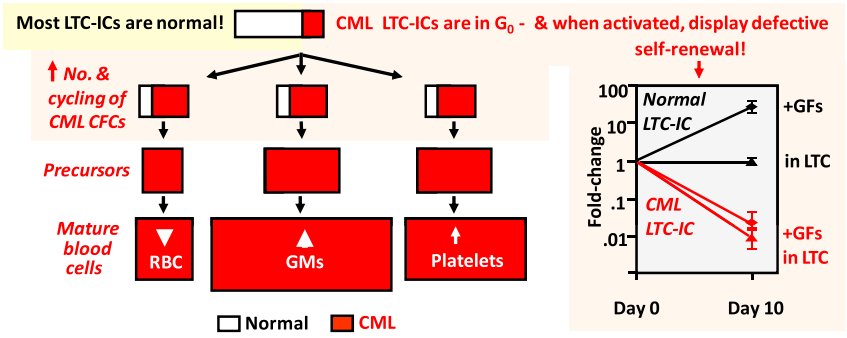

Figure 2. Schematic Visualization of the Different Types of Hematopoietic Cells Present in Patients with Chronic-Phase Chronic Myeloid Leukemia

This diagram shows the increasing representation of CP-CML elements (shown in red) compared with their normal (Ph/BCR-ABL-negative) counterparts (shown in white) in the same samples, together with the generally observed stage-dependent, increased cycling activity of CP-CML short-term clonogenic progenitors. Also shown in the right panel is the much poorer self-renewal behavior of the CP-CML LTC-ICs under different conditions (Eaves et al., 1998). GFs, growth factors; RBC, red blood cells; GMs, granulocytes and monocytes. Other abbreviations are as in Figure 1.

when the same *NA10HD* vector was lentivirally introduced into CD34$^+$ CP-CML cells that were then cultured on supportive human growth factor-producing stromal cells, the typical failure of primitive Ph/BCR-ABL$^+$ cells to be sustained (Figure 2) was reversed. Instead, the output of Ph/BCR-ABL$^+$ clonogenic progenitors was markedly and consistently increased for up to 6 weeks. Moreover, this effect stood in sharp contrast to the concomitantly declining progenitor outputs from primitive normal cells initially also present in these same samples. However, despite this ability of NA10HD expression to expand the progenitor output of CP-CML cells, the latter showed no deficiency in their ability to further differentiate into granulopoietic and erythroid progeny and there was also no significant effect on cell outputs in transplanted immunodeficient mice (Sloma et al., 2013).

Based on these findings, we next designed an experiment using the stromal co-culture assay system to screen a series of 16 candidate mutant genes that we anticipated might have even greater effects when expressed in CD34$^+$ CP-CML cells. To facilitate this, we initiated each culture with equal numbers of these cells transduced either with a vector encoding the test gene plus GFP or just YFP, so that their progeny generated over time in the same cultures could be compared. This experiment identified three genes that markedly enhanced both clonogenic and mature cell outputs from CP-CML cells over a 12-week period *in vitro*. These were *NA9*, *IK6*, and *c-MYC* (Beer et al., 2015). *NA9* (*NUP98HOXA9*) was of immediate interest because *HOXA9* was known to be associated with the progression of CP-CML to blast phase (BP-CML), and it had been found that a fatal AML is rapidly produced by primitive BCR-ABL$^+$ hematopoietic mouse cells transduced with an NA9-encoding vector (Mayotte et al., 2002). Interestingly, when we then examined the effect of introducing *NA9* into human CD34$^+$ CP-CML cells, we found that this elicited features of a more advanced disease phenotype than had been achieved with the *NA10HD* construct. NA9 produced a rapid activation of a "stem cell signature," some perturbation of granulopoietic differentiation, and even delayed mortality in some transplanted immunodeficient mice. However, the more complete block of terminal myeloid differentiation diagnostic of CML BP was not obtained with NA9 (our unpublished data).

Transduction of CD34$^+$ CP-CML cells with *IK6* (a dominant-negative form of *IKAROS*) also produced features of disease progression. However, in this case the alterations seen were more closely associated with, and limited to, characteristics of accelerated-phase disease (AP-CML); i.e., an enhanced self-renewal of CML cells with *in vivo* repopulating activity (in transplanted mice) and their increased output of clonogenic cells that produce eosinophils (Beer et al., 2015). Interestingly, this finding prompted the exciting discovery of its relevance to disease progression in patients developing AP-CML. This came from a survey of bone marrow biopsies taken from CML patients with different stages of disease. An immunohistochemical analysis of these biopsies revealed that expression of IKAROS, although prevalent in CP-CML cells (and in their normal counterparts and most AML cells), generally disappears in both AP- and BP-CML cells (Beer et al., 2015), albeit through as yet unknown mechanisms. Since effects of suppressing IKAROS in primitive normal human hematopoietic cells were not then known, we also undertook similar experiments using these as targets. These latter studies showed that the IK6-mediated inhibition of IKAROS activity also enhanced primitive normal human hematopoietic cell self-renewal and modestly enhanced eosinophil outputs, thus uncovering a previously unknown role of this transcription factor as a negative regulator of normal stem cells and eosinophil production (Beer et al., 2014).

A New Model Based on MYC Transformation of Normal Human Hematopoietic Cells

The last of the three positive candidates identified in the co-culture survey was *c-MYC*. As for IKAROS, there existed

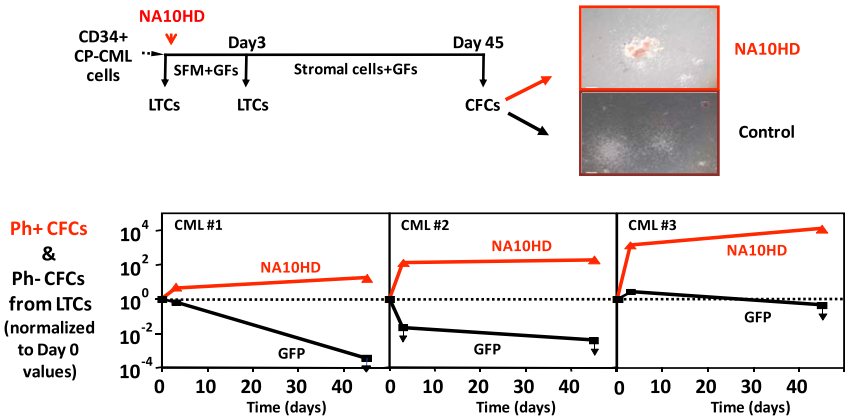

Figure 3. Effect of NUP98HOXA10 (NA10HD) Expression on Primitive CP-CML (LTC-ICs) *In Vitro*

The top left panel shows the design of the experiments. The lower panel shows results for three different CML cell samples. The top right panel shows examples of the normal neutrophil/macrophage and erythroid differentiation of clonogenic progenitors obtained from NA10HD-transduced CP-CML LTC-ICs at the end of 6 weeks *in vitro*. Data are redrawn from Sloma et al. (2013). SFM, serum-free medium, Ph-, Ph-negative (i.e., normal). Other abbreviations are as in Figures 1 and 2.

a wealth of data on the effects of altering MYC expression in various cell lines, as well in many mouse tissues including primitive hematopoietic cells (Bahr et al., 2018). However, there remains little information as to the effects that altered MYC expression may have on primitive normal human hematopoietic cells. Given the novel insights obtained from analyzing the effect of IK6 in normal hematopoietic cells (Beer et al., 2014), we decided to first determine whether and how MYC overexpression in normal human CD34$^+$ cord blood cells might alter their subsequent growth and differentiation. The results of these experiments were again unexpected. We soon found that this single maneuver reproducibly enables primary normal human CD34$^+$ hematopoietic cells at high frequencies to generate a rapidly fatal (within 3–6 weeks) human growth factor-dependent AML in transplanted immunodeficient mice, and is subsequently serially transplantable (C.J.E., unpublished data). These preliminary findings now offer a highly tractable new model for determining how a single genetic alteration can trigger the biological and molecular events in naive human cells that are sufficient to cause their rapid production of a lethal leukemia *in vivo*.

Conclusions

This brief historical review highlights the importance of understanding the process of normal tissue development in making advances in disease management. It also underscores the importance of clonal tracking to quantify and analyze critical properties, responses, and events in primitive cell types that cannot be readily visualized directly *in situ* and may also be quite rare. Furthermore, they illustrate the power of genetic manipulation to recreate disease features with ease and within time frames that are readily amenable to further investigation. Finally, it is clear that there is new excitement and a team spirit among the scientific community globally to share new ideas, methods, and information as rapidly as possible to accelerate progress in meeting the challenges still posed by disease.

ACKNOWLEDGMENTS

The work presented in this article was made possible by a long history of peer-reviewed competitive grant funds awarded to the author and trainees from the Terry Fox Foundation, the Canadian Cancer Society Research Institute and its predecessor, the National Cancer Institute of Canada, the Canadian Institutes of Health Research, the Canadian Stem Cell Network, the Cancer Research Society, the British Columbia Cancer Foundation and the Leukemia and Lymphoma Society of Canada to whom enormous thanks is owed. The added input of technical and administrative support, as well as the samples consented by patients and normal donors and used according to procedures approved by the University of British Columbia Research Ethics Board, is also gratefully acknowledged.

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