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14. ABSTRACT
The purpose of this proposal is to understand the molecular pathways regulating Bcr-Abl positive CML cells. We demonstrated that the transcription factor, CREB, is highly expressed in K562 cells and cells from patients with chronic phase CML. This led us to hypothesize that CREB may play a critical role in regulating proliferation of CML cells. To determine whether CREB and CREB-dependent pathways may be bonafide targets for CML therapy, we chose to downregulate CREB using RNA interference. There are two specific aims. In Aim 1, we will test the hypothesis that downregulation of CREB inhibits proliferation and survival of CML cells. In Aim 2, will test the hypothesis that downregulation of CREB inhibits leukemia progression in vivo and in primary CML cells. We have generated CREB shRNA lentivirus and infected primary mouse and human bone marrow stem cells. We have also infected Ba/F3 cells expressing the T315I mutation of Bcr-Abl with and without CREB shRNA and followed leukemia progression in vivo. Our results suggest that CREB is necessary for both normal stem cell proliferation and differentiation, and leukemic progression.

15. SUBJECT TERMS
CREB, RNA interference, CML, signaling

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INTRODUCTION

CREB is a transcription factor that regulates proliferation and survival in both hematopoietic and neuronal cells. We found that overexpressed in the blood and bone marrow from patients with chronic phase CML. We hypothesize that CREB and CREB-dependent signaling molecules may be effective targets for CML therapy. To test this hypothesis we have chosen to downregulate CREB using RNA interference.

BODY

Statement of Work

Task 1. To test the hypothesis that downregulation of CREB will inhibit the proliferation and survival of CML cells

These tasks have been accomplished and are following the timeline outlined in the proposal.

a. We have generated CREB shRNA constructs and generated lentivirus (Months 1 to 3).

b. We have infected various CML cell lines with lentivirus and characterize expression of CREB by Western Blot analysis and RT-PCR (Months 3 to 4).

c. We have tested the effects of CREB shRNA on cell proliferation, survival, and apoptosis (Months 4 to 10) and showed that CREB is necessary for proliferation and survival of K562 cells (CML cell line).

d. We have examined CREB downstream gene expression, using microarray analysis (Months 10 to 12) and have identified 200 genes that are significantly upregulated and downregulated that also have CREB binding sites according to “chip on chip” data published by Marc Montminy.

Task 2. To test the hypothesis that downregulation of CREB inhibits leukemia progression *in vivo* and in primary CML cells

a. We have generated CREB shRNA, bcr-abl, and control retrovirus (Months 1 to 3).

b. We have examined the effects of CREB shRNA retroviral infection on normal stem cells and follow mice for engraftment in bone marrow transplantation assays (Months 3 to 12). We have also shown that primary hematopoietic stem cells require CREB for proliferation and differentiation *in vitro*.

c. We infected mouse bone marrow with CREB and bcr-abl, or control shRNA retrovirus and perform bone marrow transplantation assays. Latency and type of leukemia will be characterized (Months 12 to 24).

We have also infected Ba/F3 cells containing the T315I mutation that also express the luciferase gene and injected these cells into SCID mice. Ba/F3 T315I mutation cells infected with CREB shRNA have delayed progression of leukemia compared to scrambled shRNA controls.

So far, our results are novel and have not been previously described. In addition, we have performed microarray analysis on shRNA transduced K562 cells to study possible mechanisms downstream of CREB. Our results showed that beclin1 and UBE2B were downregulated significantly in CREB knockdown cells (Fig. 1). We also showed using Ingenuity software, signaling networks (Fig. 2). We also showed using heatmaps that histones were significantly downregulated (Fig. 3). Finally, results from real-time PCR experiments demonstrated that expression of specific histones, 1H2BJ, 1H3B, and 2H2AA, were significantly decreased (Fig. 4).

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration that CREB is required for normal hematopoietic stem cell proliferation and differentiation.
2. Demonstration that CREB is necessary for CML cell proliferation and survival.
3. Demonstration that CREB inhibits leukemia progression of resistant Bcr-Abl cells *in vivo*.

REPORTABLE OUTCOMES

Papers:

1. Cheng JC and KM Sakamoto. Novel Technologies in Stem Cells: RNA interference and Stem Cells. Stem Cells, 25 :1070-88, 2007.

2. Cheng JC, Kinjo K, Wu WS, Schmid I, Shankar DB, Stripecke R, Kasahara N, Bhatia R, Landaw EM, and KM Sakamoto. CREB is a critical regulator of normal hematopoiesis and leukemogenesis, Blood, 111: 1182-1192, 2008.

3. Pellegrini M, Cheng JC, Voutila J, Judelson D, Taylor J, Nelson SF, and KM Sakamoto. Expression profile of CREB knockdown in myeloid leukemia cells. BMC Cancer, *in press*.

Abstracts:

1. KM Sakamoto. Requirement of CREB in normal myelopoiesis and leukemogenesis. Presentation at Myeloid Workshop, American Society for Hematology, Orlando FL, December 2006.

2. Cheng JC, Shankar D, and KM Sakamoto. Requirement of CREB in Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

3. Cheng JC, Judelson D, Kinjo K, Chang J, Landaw EM, and KM Sakamoto. CREB Plays a Critical Role in the Regulation of Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society of Hematology, Atlanta, GA, December 2007.

PERSONNEL
Kathleen Sakamoto, M.D., Ph.D.
Jerry Cheng, M.D.
Kentaro Kinjo, M.D., Ph.D.
Jenny Chang
Dejah Judelson

CONCLUSIONS

Our results suggest that CREB plays a critical role in normal cells and CML cells. We are continuing to validate the requirement for CREB in primary CML cells *in vitro* and *in vivo*. These are novel findings and will advance our understanding of normal and malignant hematopoiesis.

Work funded by the DOD showed the following:

1. CREB plays a critical role in leukemogenesis and prognosis (see reprints of Cheng and Sakamoto in Stem Cells and Cheng et al in Blood.

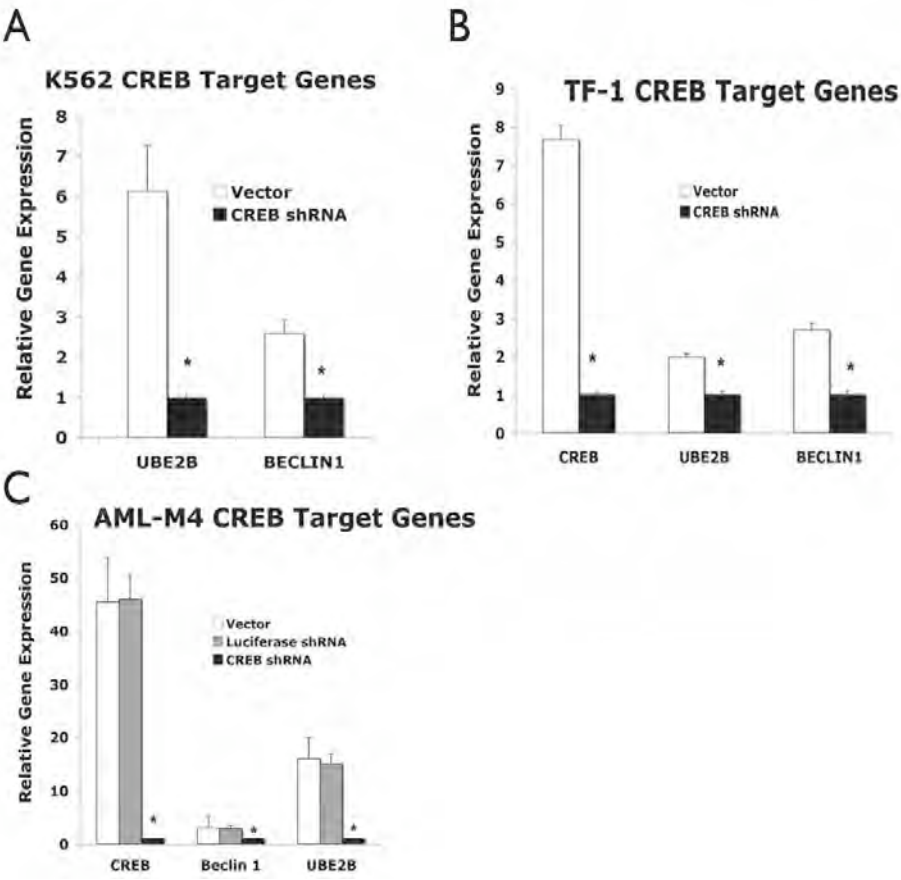
2. CREB appears to regulate histone expression in addition to UBE2B and beclin 1 (see manuscript *in press* in BMC Cancer).

REFERENCES: Not applicable.

APPENDICES: Curriculum Vitae.

SUPPORTING DATA: See figures and attached papers.

Figure 1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K562 CREB Target Genes
Relative Gene Expression
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CREB shRNA
UBE2B
BECLIN1
B
TF-1 CREB Target Genes
Relative Gene Expression
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CREB
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BECLIN1
C
AML-M4 CREB Target Genes
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UBE2B

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Figure 2

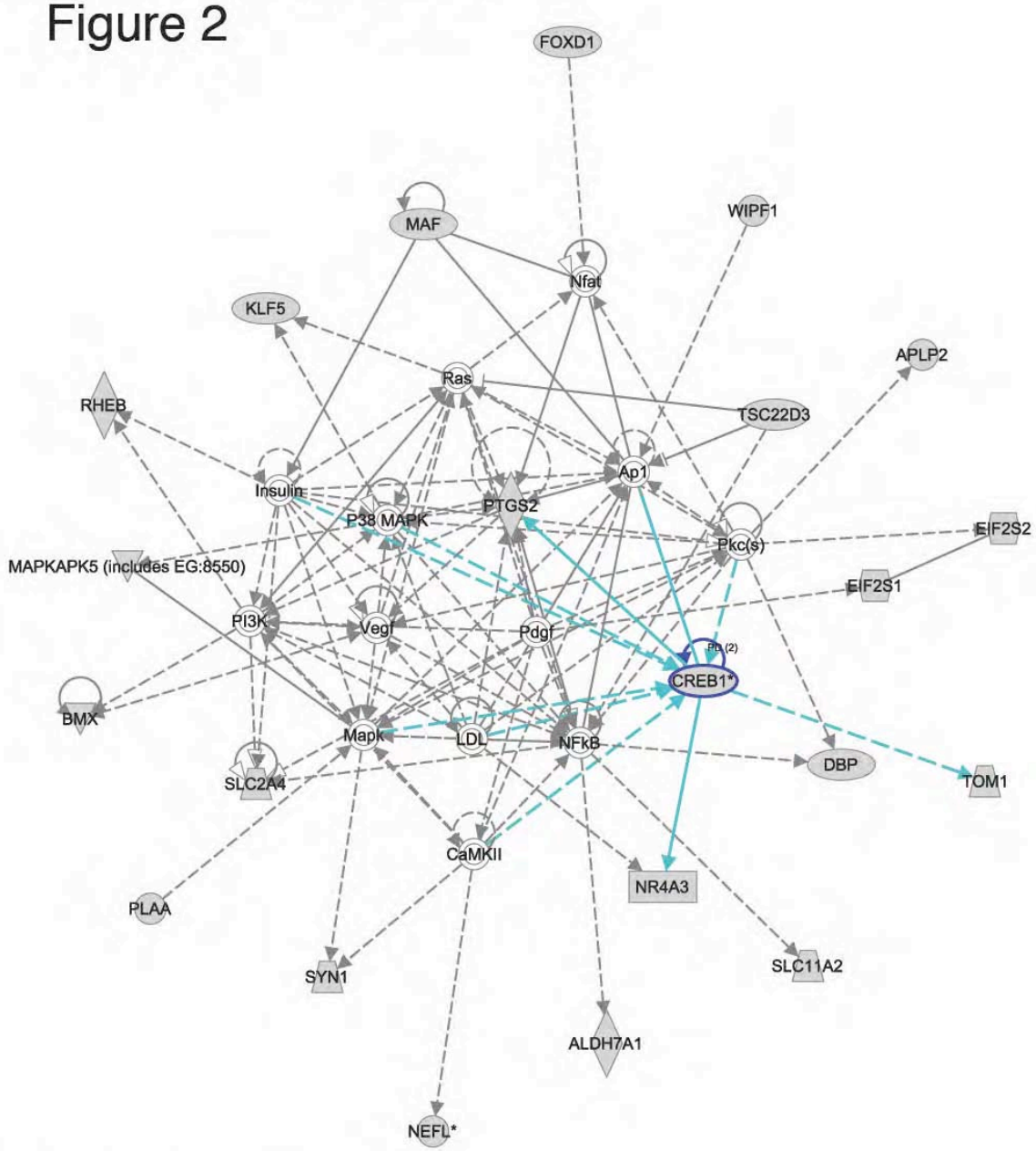


Figure 3

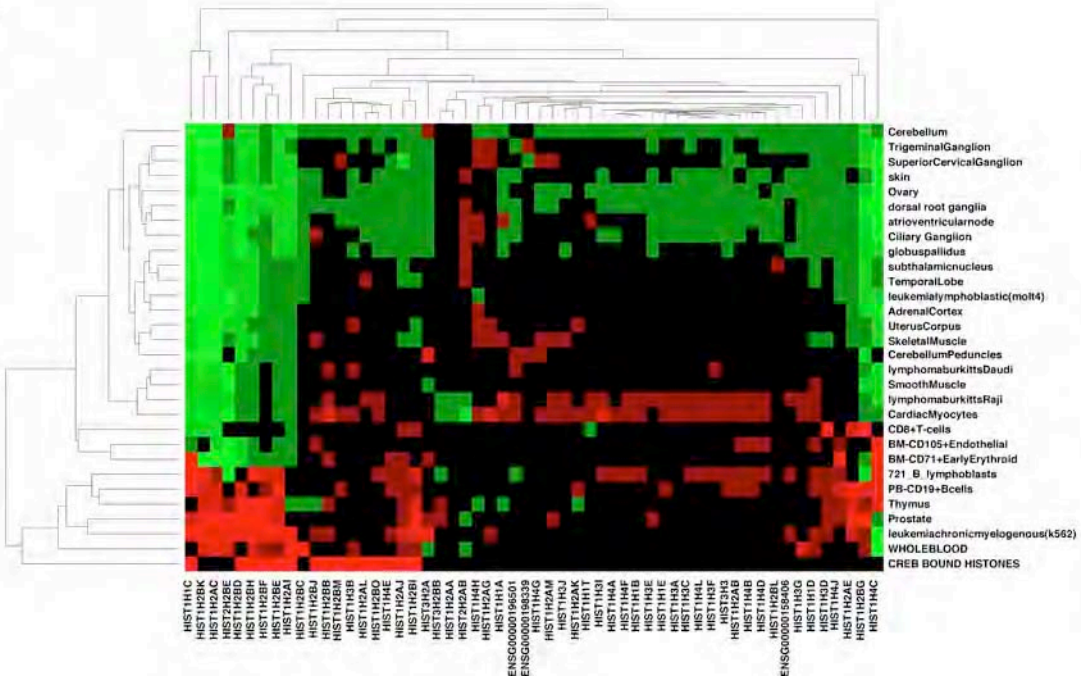
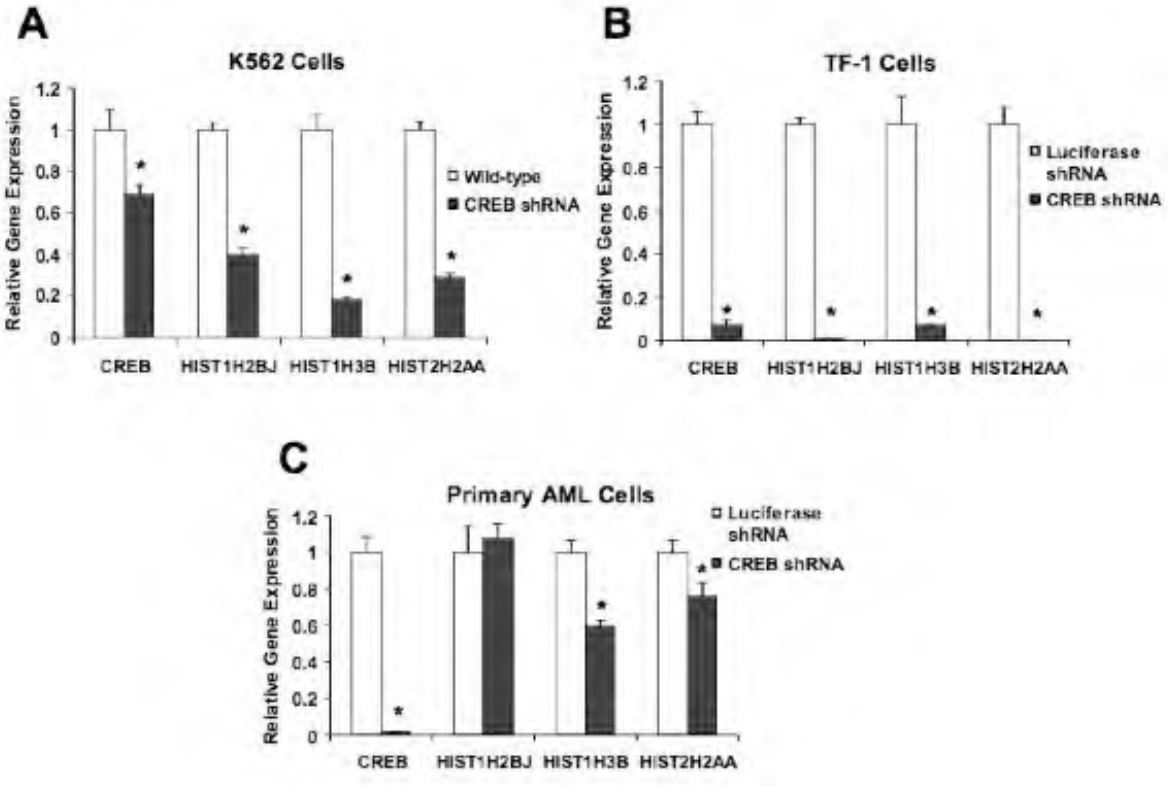


Figure 4



STEM CELLS®

Report on the Workshop "New Technologies in Stem Cell Research," Society for Pediatric Research, San Francisco, California, April 29, 2006

Jerry C. Cheng, Kathleen M. Sakamoto, Edwin M. Horwitz, Stanislav L. Karsten, Lorelei Shoemaker, Harley I. Kornblumc and Punam Malik

Stem Cells 2007;25;1070-1088; originally published online Jan 25, 2007;
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STEM CELLS®

MEETING REPORT

Report on the Workshop "New Technologies in Stem Cell Research," Society for Pediatric Research, San Francisco, California, April 29, 2006

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Key Words. Stem cells • RNA interference • Expression profiles • Neural stem cells • Mesenchymal stem cells • Purification Gene therapy • Hematopoietic stem cells

INTRODUCTION

This is a meeting report on the workshop "New Technologies in Stem Cell Research," which was presented to pediatric residents, fellows, and faculty at the Society for Pediatric Research meeting in San Francisco, California, on April 29, 2006. Four speakers presented an overview of selected topics related to the current status of methods used to study stem cells. The topics presented at the workshop focused on RNA interference, mesenchymal stem cells, expression analysis, and gene therapy. In the first report, Drs. Jerry Cheng and Kathleen Sakamoto summarize the application of RNA interference in stem cells. Second, Dr. Edwin Horwitz describes basic approaches to the isolation and purification of mesenchymal stem cells. Third, Drs. Stanislav Karsten, Lorelei Shoemaker, and Harley I. Kornblum discuss methods in expression analysis of stem cells. Fourth, Dr. Punam Malik reports on the use of gene therapy for hemoglobinopathies using autologous stem cells. STEM CELLS *2007;25:1070–1088*

Disclosure of potential conflicts of interest is found at the end of this article.

RNA Interference and Stem Cells

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Key Words. RNA interference • Stem cells • Lentivirus

ABSTRACT

RNA interference (RNAi) is a powerful tool with which to study gene function, especially in stem cells. Small interfering RNAs (siRNAs) can effectively be introduced either with a vehicle or through viral vectors to transiently or stably inhibit the expression of a particular gene target. Much is known about the optimization of siRNAs and method of delivery in mammalian cells. In this review, we discuss design considerations for siRNAs, methods of delivery, optimization of siRNAs, applications to study genes in stem cells, therapeutic applications, and remaining hurdles. With recent advances in RNAi, it is likely that application of this technology will increase in the future.

Correspondence: Kathleen M. Sakamoto, M.D., Ph.D., Division of Hematology-Oncology, Mattel Children's Hospital at UCLA, 10833 Le Conte Avenue, Los Angeles, California, 90095-1752. Telephone: 310-794-7007; Fax: 310-206-8089; e-mail: kms@ucla.edu Received June 28, 2006; accepted for publication January 5, 2007; first published online in STEM CELLS *EXPRESS* January 25, 2007. 1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2006-0397

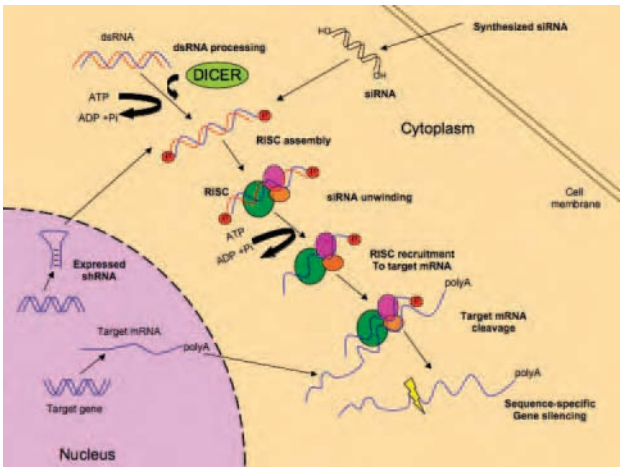

Figure 1. siRNA pathways that target mRNA for degradation. Abbreviations: dsRNA, double-stranded RNA; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA.

INTRODUCTION

RNA interference (RNAi) describes the inhibition of gene expression by double-stranded RNAs (dsRNAs) developed in the mid-1990s [1]. Guo and Kemphues discovered that sense RNA was as effective as antisense RNA for suppressing gene expression in nematode worms (*Caenorhabditis elegans*) [2]. This was followed by the introduction of dsRNA into worms. When single-stranded antisense RNA and double-stranded RNA were introduced into worms, it was found that dsRNA was more effective than either strand individually in downregulating genes [1].

RNAi is a multistep process that involves the generation of small interfering RNAs (siRNAs) in vivo through the activity of the RNase III endonuclease Dicer. The resulting 21- to 23-nucleotide (nt) siRNAs mediate degradation of their complementary RNA [3]. It is now thought that RNAi induces gene silencing through various mechanisms. One is by sequence-specific targeted gene silencing. The second is through translational repression (microRNAs). Finally, it has been reported that RNAi maintains silenced regions of chromosomes [3].

BASIC MECHANISMS OF RNAI

Long dsRNAs are the precursors of the siRNAs that trigger the RNAi effect. When dsRNAs enter cells, they are cleaved by an RNase III-like enzyme known as Dicer into siRNAs (Fig. 1). These 21–23-nt siRNAs form part of a siRNA$^-$ protein complex known as RNA-induced silencing complex (RISC), which contains helicase activity that unwinds the two strands of RNA molecules, allowing the antisense strand to bind the targeted RNA [4–7]. RISC also has endonuclease activity that hydrolyzes the target RNA at the site where it binds the antisense strands. Formation of RISC is critical for mRNA degradation. Therefore, the RISC complex mediates the sequence-specific degradation of the target RNAs that contain homologous sequences to the siRNA.

WHAT IS A DESIRABLE TARGET FOR RNAI?

Desirable targets of RNAi include genes that are amplified or overexpressed in cells leading to a specific phenotype. Additional targets include aberrant proteins that are encoded by dominant mutant alleles. An example is oncogenes that produce transformation in mammalian cells. However, genes that are abundantly expressed or have a prolonged half-life may not be efficiently inhibited. Similarly, genes that are redundant may not be effectively downregulated.

The advantages of RNAi are that the targeted degradation is very specific and can result in variable levels of downregulation such that gene dosage effects can be studied. This technology is much easier, quicker, and less expensive than generating knockout mice. RNAi can also be used to inhibit expression of multiple genes at the same time [8–10].

DESIGN OF SIRNA

The use of siRNAs has become a common method of downregulating gene expression to screen gene function in many cell types, including stem cells. Although long dsRNAs (>30 nt) are effective in suppressing gene expression in plants, *Drosophila*, and *C. elegans*, long dsRNAs are cleaved by Dicer to form siRNAs when introduced into mammalian cells, and these siRNAs lead to mRNA degradation. However, in mammalian cells, long dsRNAs activate the interferon response pathway, leading to nonspecific mRNA degradation. The dsRNA-dependent protein kinase (PKR) is activated, resulting in nonspecific translational inhibition [11, 12]. Therefore, the usefulness of dsRNA in mammalian cells is limited.

In general, 21–23-nt siRNAs are too short to activate the nonspecific dsRNA response pathway, but they are effective in inhibiting the expression of specific targets. There are several limitations of using this technology in mammalian cells. In fungi, plants, and worms, siRNAs can be replicated in vivo. In mammalian cells, siRNAs do not prime the synthesis of dsRNA to form additional siRNAs, which may explain why this technology is less effective [9]. Nevertheless, there are several examples in which siRNAs are effective in a variety of mammalian cell types, including stem and progenitor cells [1, 13].

Optimization of siRNAs in mammalian cells is dependent on several factors. One is the accessibility of the target sequence to the desired mRNA substrate. Previous reports have suggested that selecting a target sequence 100–200 nts away from the translational initiation sequence AUG of the gene is desirable [1]. However, successful inhibition of gene expression has also been reported for siRNAs targeting various sequences, including the 3′ untranslated region [14]. Targeting of the 3′ untranslated region is also useful if rescue experiments are to be performed. There is no reliable way to predict or identify the ideal sequence for siRNA. Several reports have suggested that sequences that form the stems of the hairpin siRNAs, the loop size, and the sequences at the base of the loop might also affect siRNA-induced gene inhibition. Other determinants include thermodynamic stability; siRNA with lower thermodynamic stability for base pairing at the 5′ end of antisense (guide) strand and in the middle of the siRNA were more effective at RNAi than those that had stronger base pairings in these regions due to affects on uptake of guide strand into RISC and enhancing RISC binding to target mRNA.

The sequence of siRNAs should be carefully designed. The number of nucleotides should be between 19 and 23. The GC

content should be between 30% and 50%. The preferred format is $AAN_{19}TT$. Sequence specificity to at least two nucleotides should be confirmed by Blast comparison of the National Center for Biotechnology Information GenBank database. Finally, one should query against the single nucleotide polymorphism database [10].

Optimization of siRNA

To ensure that the gene of interest is effectively downregulated by the siRNA, it is now recommended that at least three different siRNA sequences per target be designed [15, 16]. More robust knockdown of genes has been reported using this approach of creating "multiplicity" controls. Inhibition of expression has been reported for up to 5–10 days when using "pools" of siRNAs in transfected cells.

siRNA concentrations must also be optimized. In general, concentrations of siRNAs greater than 100 nM are considered to be toxic. Various amounts of siRNAs should be tested for each specific cell type. This should be considered when one is using multiple siRNA sequences. Multiple cell lines should also be tested to validate response and downregulation. Finally, a nucleotide Blast search should be performed to determine whether the siRNA sequence would target another gene. In terms of controls, scrambled or mutated sequences (http://www.sirnawizard.com) and unrelated genes (e.g., luciferase) are commonly used. To validate successful downregulation of the target gene, it is recommended that a Western blot analysis be performed to assess protein levels and Northern blot analysis or reverse transcription-polymerase chain reaction (RT-PCR) to measure RNA levels. Demonstration of lower mRNA levels is critical to rule out a microRNA effect and translational inhibition of gene expression. To control for off target effects, one can measure interferon response genes, including *OAS1*, *OAS2*, and *INFB1*, by RT-PCR [1].

Delivery of siRNA to Cells

In mammalian cells, efficiency of siRNA to cells transiently depends on the vehicle or mode of delivery and the cell types. Approaches to introduce siRNAs into cells include a lipid-based vehicle (e.g., Lipofectamine) or a non-lipid-based approach (e.g., calcium phosphate or electroporation). The disadvantages of this approach are that the siRNAs are nonrenewable and are only effective as long as they are bath-applied to cells. An alternative strategy has been to deliver siRNAs through a DNA vector-mediated RNAi approach.

Because of the transient nature of gene silencing produced by oligonucleotide siRNAs and their high costs of chemical synthesis, alternative approaches to introduce siRNAs in plasmid vectors have been developed. A variety of expression vectors are now available. Expression is driven by either the U6 (small nuclear RNA) or H1 RNA polymerase III promoters to drive expression of sequence-specific short hairpin RNAs (shRNAs) in mammalian cells [2]. These systems are based on the expression of siRNAs either as two separate strands or as a single shRNA. It is thought that the shRNAs are processed by Dicer to active siRNAs in vivo [17–19].

For stable expression in stem cells, successful delivery has been demonstrated with viral vectors. Various recombinant viral vectors have been developed to deliver shRNAs in mammalian cells [10, 20]. Lentiviral vectors are especially effective. The reasons for this are that lentiviruses have broader tropism and receptor-independent delivery, that they have the ability to integrate into the genome for stable gene silencing, and that lentiviral transduction and expression of shRNAs do not require cell division for integration into the genome [21]. Lentiviral transduction has been successfully performed in cell lines, mouse hematopoietic stem cells (HSCs), and embryonic stem (ES) cells [22–24].

Adenoviral vectors have also been reported to be useful for delivering siRNAs to target cells. This vector system has been used to downregulate genes in liver. However, this vector system has limited utility in stem cells, since low transduction rates have been found in ES cells and HSCs. This is most likely due to the fact that the receptor for adenovirus is not highly expressed in stem cells [25]. Similarly, adenoviral-associated vectors have been successfully used to deliver RNAi to nonstem cells [1].

If the stable transfection or transduction of siRNAs results in toxic effects to cells, an alternative approach is to use the inducible expression of shRNAs. The tetracycline/doxycycline regulated form of U6 or H1 promoter has been successfully used. If there is leakiness, other inducible systems, such as an ecdysone-inducible system, are more tightly regulated with less background. A newer approach has been a CRE-lox-inducible system [26]. Most recently, a doxycycline-inducible vector that contains a KRAB domain from one-third of zinc finger domains was used in cell lines, mouse ES cells, epithelial breast cancer cells, rat brains, $CD34^+$ cells, and transgenic mice [27].

Application of RNAi in Stem Cells

There is now emerging evidence that RNAi can be used to study gene function and for therapeutic application. ES cells are pluripotent stem cells that are derived from the inner cell mass of the 3.5-day-old mouse blastocyst [1, 28]. These cells are desirable models to study the regulation of development and cell lineage commitment and differentiation, since ES cells can give rise to all three germ layers. This system is a powerful tool with which to study development.

Interestingly, long dsRNA has been used in ES cells, but only when undifferentiated. The reason for this is unknown. In differentiated ES cells, siRNAs have been found to be effective in inhibiting genes, such as PU1 and c-EBPa [1]. A variety of other genes have been downregulated in ES cells, such as Shp-2 and Oct-4. Synthetic shRNAs recently have been shown to be efficiently transfected transiently with Lipofectamine [29]. More commonly, viral vector systems have been used to transduce genes of interest for stable expression of shRNAs.

HSCs are a self-renewing population of cells in the bone marrow that gives rise to all differentiated hematopoietic cells [1]. A number of genes have been targeted using RNAi in HSCs. Growth factor receptor genes, clusters of differentiation, chemokines, oncogenes (*bcr-abl*), tumor suppressors, human immunodeficiency virus genes, globin genes, and RPS19 expression have all been successfully targeted. In most cases, retroviral or lentiviral vector systems were used. Electroporation has been used successfully to introduce dsRNA in HSCs [13]. Lipofectamine has also been reported to effectively transfect oligonucleotide siRNAs into hematopoietic progenitor cells [30]. HSCs that are transduced with shRNAs can then be studied in vitro using methylcellulose colony assays or in vivo in bone marrow transplantation experiments.

Neural Stem Cells and MSCs

Neural stem cells (NSCs) have also been transduced with shRNAs to downregulate genes. Examples of genes inhibited in

NSCs by RNAi are MELK, PPARγ and B27.a genes [31–33]. MSCs have been studied using both viral and nonviral methods. Genes inhibited using viral vectors were β-catenin, Msx2, and mecdin [2, 34]. Nonviral liposomal methods to introduce siRNAs into MSCs have been used to inhibit epidermal growth factor receptor and connective tissue growth factor [35, 36]. Recently, a transfection microarray approach was generated in which siRNAs were applied onto slides that are coated with poly-L-lysine and fibronectin. MSCs were then placed on top of the poly-L-lysine and siRNA sandwich. Fluorescent microscopy was used to then visualize and quantify the degree of downregulation [37, 38]. A similar approach was used with HeLa cells placed on slides treated with siRNAs, in which cells were then followed in real time using time-lapse fluorescent microscopy as a high-throughput method to screen for genes involved in chromosomal segregation [39].

shRNA Libraries

One of the technological advances in the RNAi field has been the development of shRNA libraries to screen for genes that regulate a specific pathway or biological function. Many of the libraries rely on lentiviral vector-based expression. Libraries have been used to identify deubiquitinating enzymes [40], sensitivity to small molecule inhibitors, novel cancer genes, and previously unidentified components of signaling pathways. A recent report from the Broad/Massachusetts Institute of Technology group (The RNAi Consortium) used an shRNA library with 72,600 clones targeting 10,500 human and 5,300 mouse genes [41]. It is anticipated that the numbers of genes targeted could be as high as 15,000 human or mouse genes. Viruses expressing shRNAs can be transiently or stably transduced into mammalian cells [41]. Genes that are involved in a particular cellular process will be identified through identification of the shRNA clones that block the function of the gene. An inducible shRNA library has also been used recently to identify genes that regulate proliferation or survival of diffuse large B cell lymphoma cells to seek novel targets for therapy [42].

Therapeutic Applications of RNAi

The field of RNAi is advancing at a rapid pace. The application of RNAi as gene therapy is now being realized. In mice, delivery of siRNA to downregulate Fas by hydrodynamic tail injection resulted in protection from fulminant hepatitis [43]. A recent report by Samakoglu et al. has demonstrated that sickle globin gene can be downregulated in $CD34^+$ cells using a lentiviral shRNA, with a concomitant increase in γ-globin expression in erythroid-specific manner [44]. Another advance has been the successful RNAi-mediated gene silencing in nonhuman primates. The first report of systemic delivery of *APOB* siRNA in nonrodent species was recently reported [45]. APOB is a component of low-density lipoprotein (LDL) and regulates the storage and metabolism of cholesterol. A liposomal formulation of *APO-B* siRNAs was intravenously administered into cynomolgus monkeys with effective inhibition of APOB levels after 48 hours and 11 days. Plasma levels demonstrated that not only LDL and cholesterol levels were lower than controls, but high-density lipoprotein levels were not affected. Although previous success was shown with hydrodynamic tail injection of oligonucleotide siRNAs in rodents, this was the first report of siRNAs successfully targeting a gene in nonrodent models.

Remaining Challenges

Although the field of RNAi has progressed rapidly, there are several hurdles that remain before this technology can be fully applied in humans. The specificity and toxicity of siRNAs must be more rigorously examined. The use of lentiviral vectors in gene therapy has led to insertional mutagenesis and malignancies, which must be overcome. Newer generations of lentiviral vectors are currently being studied. Stability of siRNAs is also problematic for long-term use. However, recent advances in nanotechnology have demonstrated that delivery of siRNAs using nanoparticles has potential in the clinics [46]. Given the advances in the field, it is highly likely that within the next few years, RNAi will become a viable approach to treat human disease.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Fundamentals of MSC Isolation and Purification

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Key Words. Mesenchymal stem cells • Purification • Isolation

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INTRODUCTION

Mesenchymal stromal cells are the spindle-shaped adherent cells isolated from bone marrow and other tissues [1, 2]. Designated mesenchymal stem cells by some investigators, these cells are increasingly being investigated as cell therapy to rebuild diseased or damaged tissues [3–6] and as immunomodulatory therapy for the treatment of graft-versus-host disease [7] and autoimmune disorders [8, 9]. It is quite important, then, to understand the various approaches of isolation purification and fundamental characterization of these potentially powerful therapeutic cells.

The notion of a stromal stem cell thought to repopulate the marrow microenvironment in analogy to the hematopoietic stem cell that can repopulate hematopoiesis was proposed by Owen and Friedenstein [10], largely based on the work of Friedenstein et al. [11, 12]. This stem cell concept was extended to all mesenchymal tissues, and the term "mesenchymal stem cell" was popularized by Caplan, who pioneered much of our early understanding of this cells [13]. Indeed, mesenchymal stromal cells seem to function as stem cells in vitro.

Our general concept of a stem cell evolved from our understanding of hematopoietic stem cell. Till and McCulloch suggested that the stem cell could be defined as a cell with extensive self-renewal capacity and the potential to terminally differentiate to two or more lineages [14]. Based on this definition, the mesenchymal stromal cells do, in fact, meet these criteria in vitro; however, true "stemness" is likely is much more complex and is most often operationally defined. As this idea has become increasingly recognized, many investigators suggested that convincing data supporting mesenchymal stromal cells as stem cells was lacking [15]. Hence, the Mesenchymal and Tissue Stem Cell Committee of International Society for Cellular Therapy has proposed that the term "mesenchymal stromal cell" is a more appropriate designation for this heterogeneous population of cells, maintaining the abbreviation

"MSC" for "mesenchymal stromal cell" [16], while reserving the term "mesenchymal stem cell" for a subset of these (or other) cells that demonstrate stem cell activity in vivo by clearly stated criteria.

Overview of the Isolation of Mesenchymal Stromal Cells

All strategies to isolate mesenchymal stromal cells must take into account that the cells are quite rare within their tissue source. For example, mesenchymal stromal cells are estimated to comprise 0.01% of bone marrow mononuclear cells [17]. With this in mind, there are currently four principal strategies for the isolation of mesenchymal stromal cells. First, the cells can be isolated by "adherence selection," in which the mesenchymal stromal cells are selected by their capacity for adherence to plastic in vitro. Second, the mesenchymal stromal cells can be selected by surface antigen expression using fluorescence-activated cell sorting (FACS). Third, surface antigen expression can be exploited to isolate mesenchymal stromal cells by magnetic label-activated cell sorting using antibodies conjugated to magnetic beads. Finally, populations of cells can be enriched for mesenchymal stromal cells by depleting the bone marrow cells of all other cells. Antibodies to non-mesenchymal stromal cell antigens can be conjugated to beads and then separated from the fraction of cells containing the mesenchymal stromal cells by centrifugation. This is not truly an isolation approach; rather, it is an enrichment of mesenchymal stromal cells within a still crude cell preparation. The mesenchymal stromal cell-enriched populations of cells must undergo a second isolation step, most often by adherence selection, to obtain mesenchymal stromal cells.

Isolation of the Mononuclear Cells

For the following discussion of the isolation of mesenchymal stromal cells, we will use bone marrow as the prototypic tissue since it is currently the most common source of mesenchymal stromal cells. The principles are equally applicable to other cell sources. In general, the first step to isolate mesenchymal stromal cells is to obtain mononuclear cells (MNCs) and rid the preparation of debris, typically by density centrifugation. Isolation of MNCs is important regardless of the subsequent approaches to obtaining a population of mesenchymal stromal cells. The two most common media for density centrifugation are Ficoll (1.077 g/cm^3) and Percoll (1.073 g/cm^3). Ficoll is frequently used to isolate bone marrow mononuclear cells and has also been extensively used in the isolation of mononuclear cells in anticipation of isolating mesenchymal stromal cells by adherent selection. Percoll may also be used to isolate mesenchymal stromal cells by two different approaches. First, a discontinuous gradient can be used, where the bone marrow mononuclear cells will band at the interface in a similar fashion as when using Ficoll. Alternatively, investigators can generate a continuous gradient with Percoll. In this case, the mesenchymal stromal cells will band at approximately 1.07 g/cm^3. In practice, a large layer of Percoll is harvested from the centrifuge tubes to maximize the recovery of mesenchymal stromal cells [13]. Whether one medium in particular offers an advantage is unclear; thus, investigators should use the medium with which they have the most experience. Regardless of which medium is used, the resulting mononuclear cells must now undergo a further procedure, as stated above, to actually isolate the mesenchymal stromal cells.

Adherence Selection

The most common and best-characterized method to isolate mesenchymal stromal cells is by adherence selection. The mononuclear cells resulting from the density centrifugation are transferred to a tissue culture vessel and maintained at 37°C in a standard incubator for 3 days. Then, the medium is replaced, which removes the nonadherent cells. The typical MNC density is 1.65×10^5 cells per cm^2 [18]; however, lower densities also generate an acceptable yield. Any type of plastic culture vessel (dish, flask, or multilayer "cell factory") may be used with an equivalent recovery of mesenchymal stromal cells.

Media

Several media have been used for this initial mesenchymal stromal cell isolation and subsequent cell expansion. In our laboratory, we typically use Dulbecco's modified Eagle's medium, but α-modified Eagle's medium and McCoy's 5A also support the growth of mesenchymal stromal cells. McCoy's 5A culture medium may be preferable because it contains ascorbic acid (E. Clarke, personal communication); however, in our laboratory, we have not been able to demonstrate a significant difference among the various media.

All synthetic media will require growth factor supplementation; most often, investigators simply add fetal bovine serum (FBS), as this has proved an effective medium supplement to support the growth and in vitro differentiation of mesenchymal stromal cells [19]. The standard practice is to supplement with 10% FBS; however, in the laboratory, 20% FBS often results in more prolific cell growth. Importantly, FBS preparations can differ in their potential to support mesenchymal stromal cells; therefore, investigators generally screen several lots of FBS to identify the product that best supports bone marrow stromal stem cells (CFU-F) formation and mesenchymal stromal cell propagation and purchase a large stock of that particular lot.

Adult human serum, specifically autologous serum, has been reported to support the growth of human mesenchymal stromal cells [20, 21], although many laboratories are not able to reproduce these published results and consequently there are few scientific or clinical reports using autologous human serum. However, supplementing adult human serum with cytokines such as basic fibroblast growth factor, epidermal growth factor, and/or platelet-derived growth factor will support mesenchymal stromal cell growth [22, 23] (unpublished observation) and may be used if animal serum must be avoided. Serum-free medium has also been reported [22] but has not been extensively used by independent laboratories, and development of new serum-free media to isolate and expand mesenchymal stromal cells is an area of investigation within the biotechnology industry. Recently, human serum with a platelet lysate was shown to support mesenchymal stromal cells in culture [24]. Human serum containing a suspension of platelets can be frozen at −80°C and thawed just before the preparation of tissue culture media. The precipitate and other particulate matter must be removed by centrifugation. Then this serum/platelet lysate can be used to supplement (final volume, 5%) any of the synthetic media discussed above. There are currently few reports documenting the efficacy of serum/platelet lysate supplementation of media for mesenchymal stromal cell expansion, but the scientific community is currently showing great enthusiasm for this approach, especially when translating mesenchymal stromal cell-based therapy to the clinical setting, where the elimination of animal

products may prove advantageous. Hence, it is likely that many studies will be forthcoming over the next few years.

At this juncture, the MNCs have been placed into tissue culture and maintained in the medium of choice for 3 days, after which the medium should be replaced, which removes the nonadherent cells. The cells remaining adherent to plastic are the mesenchymal stromal cells. Other time intervals prior to the initial medium change have been used. Some investigators replace the medium in a few hours to 1 day, whereas others choose to wait up to 5 days. The longer intervals often result in a greater recovery of mesenchymal stromal cells but lesser initial purity. Conversely, shorter intervals result in a lesser mesenchymal stromal cell recovery but greater initial purity. Regardless of the time interval used, there remains significant non-mesenchymal stromal cell contamination of the cultures. Macrophages will also directly adhere to the plastic surface. Some cells, such as hematopoietic progenitors and mature B-cells, will adhere to the mesenchymal stromal cells. Thus, the mesenchymal stromal cell preparation will require a greater level of purity than that afforded by the initial isolation protocol. Further enrichment occurs as the mesenchymal stromal cells are cultured and passaged, as the other cell types do not expand to any appreciable extent. This can be demonstrated by flow cytometry.

Other Isolation Methods

Mesenchymal stromal cells may also be isolated by multiparameter FACS technology with select antibodies that can define mesenchymal stromal cells, which is a subject of some debate. Initially, the two antibodies SH2 and SH3 were used to identify the heterogeneous population of cells designated mesenchymal stromal cells [2, 13]. Although often used by many laboratories through the generosity of the original investigators, these antibodies were not commercially available. More recently, SH2 and SH3 were found to recognize epitopes on CD105 and CD73, respectively [25, 26]. In theory, then, $CD45^-$ $CD105^+$ $CD73^+$ marrow cells could be used to isolate mesenchymal stromal cells by FACS; however, since this approach is tedious and does not offer a proven advantage, it has not been used.

The monoclonal IgM antibody STRO-1, developed by Simmons and Torok-Storb in 1991 [27], identifies a subset of human marrow cells that is composed of erythroid precursors and CFU-F cells [27]. In fact, most, or all stromal precursors seem to reside in the STRO-1 fraction of marrow cells; however, this population remains heterogeneous. STRO-1 has been extensively studied by Gronthos et al. [28] and Shi and Gronthos [29], who showed that two color isolation strategies using $STRO\text{-}1^+$/$CD106^+$ or $STRO\text{-}1^+$/$CD146^+$ yield a cell product highly enriched for high proliferative adherent cells. Whether these phenotypes represent a bona fide mesenchymal stem cell or a more highly enriched population of progenitors, as well as the biologic significance/therapeutic value of this phenotypically defined subset of cells, awaits broad independent scientific confirmation. Interested investigators can obtain STRO-1 through the Developmental Studies Hybridoma Bank (Iowa City, IA, http://www.uiowa.edu/~dshbwww), which is under the auspices of the National Institute of Child Health and Human Development.

Other antibodies have been used to isolate mesenchymal stromal cells. The antibody D7FIB recognizes mesenchymal stromal cells, and $CD45^-$ $D7FIB^+$ marrow cells have been show to represent mesenchymal stromal cells [17, 30]. The low-affinity nerve growth factor receptor CD271 also recognizes mesenchymal stromal cells and can be used to prospectively isolate cells [31]. At recent scientific meetings focused on mesenchymal stromal cells in North America and Europe, CD271 seems to be gaining the interest of many clinician scientists. However, the value of CD271 selection is unproven. Currently, most investigators studying the potential applications of the heterogeneous population of mesenchymal stromal cells isolate cells by adherence selection. Investigators focused on the biologic properties of mesenchymal stromal cells, especially those investigators seeking a more homogenous population of cells or trying to define a purified population of stem cells, use more specific phenotypic criteria. There is still considerable debate on how best to define the heterogeneous population of mesenchymal stromal cells, as well as a putative mesenchymal stem cell. Hence, the importance of various phenotypic markers engenders extensive discussion and surface antigen expression as a means of isolating the cells is subject to some uncertainty.

Similar to FACS isolation of mesenchymal stromal cells, antibodies conjugated to magnetic beads can be used to isolate mesenchymal stromal cells [31]. The cells, bound with magnetically labeled antibody, can be sorted by passing through a magnetic field. This so-called magnetic label-activated cell sorting is a highly effective method to isolate a wide variety of cells. However, the caveat of using specific antigens for FACS is equally applicable to magnetic label-activating cell sorting.

Mesenchymal Stromal Cell Expansion

The initial isolation of mesenchymal stromal cells, by any method, generally yields a relatively small population of cells. This finding is not surprising considering that mesenchymal stromal cells are minor constituents of most tissue sources (e.g., 0.01% of bone marrow MNCs). Hence, the mesenchymal stromal cells will require substantial culture expansion prior to most experimental applications. The medium used for the isolation of mesenchymal stromal cells is most often used throughout the culture expansion. The cells are maintained in tissue culture under standard conditions with medium replacement every 3–4 days. The cultures should be monitored often by visual examination with an invested microscope (daily if possible), and the cells should be passaged when the population attains approximately 80% confluence on the bottom of the tissue culture vessel [2]. The cells should not be allowed to contact each other, as this may alter the phenotype [2]. Mesenchymal stromal cells can be released from the vessel by trypsinization and then collected by pipette, washed, and replaced into a new culture vessel. Although most expansion protocols suggest a replating cell density of 2,000–4,000 cells per cm^2 for general experimental applications, the optimal cell density depends, in part, on the desired outcome. A study of cell plating density showed that very low densities, as low as 2.5 cells per cm^2, yield a significantly greater number of population doublings (expansion) than higher cell densities over a given time interval [32]. However, the total number of cells obtained at the end of the expansion is less. Thus, if clonal expansion is desired, very low *plating* cell densities are best, but if the goal is to obtain a large number of mesenchymal stromal cells, higher cell densities (e.g., 1,000–4,000 cells per cm^2) may be preferable.

The mesenchymal stromal cells may be expanded until the desired number of cells is attained; however, the expansion potential is not infinite (i.e., mesenchymal stromal cells will senesce in culture). Most studies use cells between passage 1 and passage 8.

PURIFICATION

As noted above, the isolation of mesenchymal stromal cells yields a preparation still "contaminated" with non-mesenchymal stromal cells. This seems to be true whether mesenchymal stromal cells are isolated by adherence selection or magnetic bead-based cell sorting. However, as the cells propagate and the cultures undergo further medium changes, the nonadherent cells will be removed. Thus, expansion *is* purification. Further purification of the mesenchymal stromal cells is generally not required; however, proving the lack of non-mesenchymal stromal cells (e.g., hematopoietic cells) is required. Such analyses are most readily accomplished with flow cytometry, demonstrating that cells expressing hematopoietic antigens are not present in the cell preparation.

CHARACTERIZATION

The final product of mesenchymal stromal cells should be characterized to prove the identity of the cells. Although mesenchymal stromal cells are clearly a heterogeneous population, the International Society for Cellular Therapy has suggested a working definition for mesenchymal stromal cells as (a) plastic adherent cells that (b) express CD105, CD73, and CD90 on the cell surface determined by flow cytometry and lack expression of CD45, CD34, CD11B or CD14, CD19, or CD79α and human leukocyte antigen-DR. The latter is most important to exclude hematopoietic contamination as a means of confirming purity rather than identity. Finally, the population of cells should (c) have the capacity for in vitro differentiation to osteoblast, adipocytes, and chondroblasts. Thus, adherence, surface antigen expression, and in vitro differentiation collectively define the heterogeneous population of mesenchymal stromal cells, and experimental data demonstrating these properties may be presented as evidence of mesenchymal stromal cells [33].

This definition is quite cumbersome, and a simpler defining phenotype is clearly needed; however, a single antigenic determinant (e.g., STRO-1 or CD271 [LNGRF]) to define the heterogeneous population has yet to gain universal acceptance in analogy to the biomedical scientific community's acceptance of CD34 expression as a marker of an enriched population of hematopoietic stem cells. Moreover, a combination of antigens to define subsets of mesenchymal stromal cells, or perhaps a mesenchymal stem cell, although reported [17, 28, 29, 31], is not yet widely accepted. Indeed, phenotypic analysis and correlation of the antigenic phenotype with biologic activity, especially in vivo activity, is an area of considerable effort within the field of mesenchymal stromal cell biology.

PARTING THOUGHTS

This short primer has highlighted the fundamental features of the isolation and purification of mesenchymal stromal cells. Certainly, as technology advances, new methods will evolve and, hopefully, improve our efforts. Current methods of magnetic bead isolation will likely gain prominence as we better define the phenotype of mesenchymal stromal cells and cell subsets with unique biologic properties. New investigators can best gauge the state of the art by observing the methodology used by the preponderance of recent reports. Currently, there are many feasible approaches; the most important aspect of mesenchymal stromal cell isolation is to develop the protocols that work best in your laboratory.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Methods in Expression Analysis of Stem Cells

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ABSTRACT

Recent advances in stem cell technology have opened the door to the study of stem cell biology, including mechanisms underlying the fundamental properties of stem cells: self-renewal and cell fate. These analyses can be greatly enhanced by large-scale studies of gene and protein expression. Such studies can be used to categorize stem cells and their progeny, as well as to determine specific genes, proteins, and molecular pathways involved in functional processes. This review provides examples of how expression analysis can be used by the stem cell biologist, as well as methodological guidance in determining what questions can be asked. Furthermore, we provide descriptions of currently available microarray platforms and analysis tools.

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Stem cell biology is at an important stage in its development. Recent years have seen an explosion in the amount of information available regarding both adult and embryonic stem cells. Initial studies have documented both the existence and isolation of numerous cell types and have provided promising evidence that therapeutic strategies using stem cells may be possible. Subsequently, a number of investigators have begun to develop studies to unravel the molecular mechanisms of stem cell function, including studies of cell fate and self-renewal. In conducting these studies, many investigators are now using modern methods of large-scale, high-throughput characterization of stem cells and their progeny. Methods used can interrogate large percentages of the transcriptome or proteome and allow for a more detailed description of the cells and tissues being studied, as well as insight into fundamental mechanisms of stem cell biology. This review is meant to give the stem cell scientist an introduction to and an update on some of these methods, including what specific questions can be resolved through their use. Since gene expression microarray technology is the most commonly used approach, we will devote the greatest attention to this topic.

WHAT TYPES OF QUESTIONS CAN BE ANSWERED WITH TRANSCRIPTOME/PROTEOME-WIDE PROFILING?

The advent of microarray and proteomic technologies opened the door for numerous types of studies. Early studies using microarrays made comparisons of gene expression of cells in two (or more) different states to compare what sets of genes are regulated during normal cellular processes, such as the cell cycle, or following a particular perturbation, such as a drug treatment. These studies generally compare large numbers of genes (or proteins) and determine which ones are significantly different between the two populations. Such design, although using heterogeneous cellular populations, can provide important clues about gene function, especially when dramatically different cell culture conditions are tested (e.g., growth factor withdrawal [1]). Subsequent functional studies can then determine whether individual genes are then active in determining the state change being examined. For example, in a few recent studies, we found a number of genes enriched in proliferating, as compared to differentiating, neural stem/progenitor cell populations [2, 3]. Among the gene candidates identified was *MELK*, a poorly characterized kinase. We then performed functional studies of *MELK* in neural progenitors and determined that it is a critical regulator of their self-renewal [4].

Similar to determining the differences between two populations of cells following perturbations, microarrays have been successfully used to understand differences in stem cell populations created by a germline mutation, as in a knockout or transgenic mouse. Molofsky et al. [5], in an elegant manner, used microarrays to discover the molecular mechanisms underlying the effects of the polycomb transcription factor Bmi-1 by analyzing the differences in gene expression between mutant and wild-type neural progenitor cells. Recently, we have analyzed genes upregulated by the elimination of the tumor suppressor gene *PTEN* in neural progenitors in the hope that we would discover candidates for mediating tumorigenesis [6].

Another way in which array studies can be used to derive information on genes relevant for the function of particular stem cells or universal "stem cell signature" is to analyze those genes that are shared by two or more populations. For example, we and others [7–9] have uncovered sets of genes that are shared by multiple stem cell populations. These genes may then be considered candidates for mediating stem cell-specific processes, such as self-renewal, rather than simply being indicators of the cell or tissue of origin of the cells.

Genomic and proteomic studies can also be used to characterize disease states and to delineate markers or sets of markers [10]. For example, proteomic analysis of blood or tissue samples from cancer patients can yield diagnostic and prognostic biomarkers. Microarray analysis of brain tumors, in some cases, provides better prognostic categorization than traditional histologic/pathological categorization. The delineation of cancer- or disease-specific genes or proteins can also yield valuable targets for therapeutic intervention [11].

Large-scale analyses of protein or gene expression may also be the optimal way to define a particular cell type and to compare cells obtained by different investigators under different conditions [12]. Often, the sets of markers used for immunocytochemistry or fluorescence-activated cell sorting (FACS) are insufficient to define a particular cell type. Neural stem cells, for example can be cultured from different central nervous system (CNS) regions under a variety of conditions. These cells all appear to express the intermediate filament nestin and produce neurons, astrocytes, and oligodendrocytes. However, depending on CNS region, stage of development, and culture conditions, neural stem cells may vary greatly in many properties, including self-renewal and differentiation potential [13]. Through the use of genomics or proteomics methods, one can identify fingerprints of the "specific" neural stem cell types, creating unique molecular identifiers facilitating integration of the stem cell data from different laboratories.

An emerging area of research within the "-omics" field is an analysis of massive data sets with the goal of the delineation of pathways that regulate specific regulatory processes. By looking at coordinated gene expression and making use of the ever-increasing annotated databases, one can define sets of genes or proteins that are coregulated by a particular manipulation or disease state. In such a way, we have found that the pRB pathway is important for regulating the proliferation of postnatal neural progenitors [1] Properly designed postarray functional studies can reveal the degree to which these sets of genes or proteins interact, determining the key functional regulators of the particular regulatory pathway.

The examples delineated above represent only a few of the potential uses for large-scale expression; numerous other uses exist, and still more will be created as methodology becomes readily accessible to investigators without specialized training.

Choosing the Appropriate Cells/Tissues To Study

The key to success of any profiling study rests in the choice of starting material. Studies range from use of whole tissue to highly purified, sorted cells. Although whole tissue has the advantage of being plentiful, it is generally true that the more pure the starting material, the more specific the profiling data will be. Several approaches can be taken to minimize cellular heterogeneity and maximize purity. The most homogeneous starting material is usually based on clonally expanded cell lines. For those who study neural stem cells, for example, there are several transformed lines available that have many of the properties of neural stem cells. However, these lines do not represent stem cells in their normal state, and caution must be taken in any profiling experiment using them. For some stem cell types, such as hematopoietic stem cells, sufficient numbers of positive and negative extracellular markers exist to allow for FACS-based purification and subsequent study [14]. Neural stem cells, on the other hand, can be enriched by cell sorting using cell surface markers or other methods (such as size or dye exclusion) [15–17], but not to purity. One approach to get at least partway around this heterogeneity is to use promoter-driven green fluorescent protein expression followed by FACS analysis [18]. For example, the nestin promoter, when transduced into freshly dissociated human fetal brain, appears to specifically drive enhanced green fluorescent protein expression in neurosphere-forming multipotent neural progenitors, allowing subsequent purification by FACS and array analysis [18]. Similar methods can be used when green fluorescent protein (GFP) (or another fluorescent protein) expression is driven by a specific promoter in transgenic animals. D'Amour and Gage used the *SOX2* promoter to create a transgenic mouse and then performed array analysis on sorted brain-derived neural progenitors, which express *SOX2* [19]. One caveat to using this approach is that most promoter fragments that are used to drive reporter gene expression are not 100% faithful to the endogenous gene. That is, expression of the transgene may not entirely mimic expression of the native mRNA or protein. The advent of bacterial artificial chromosome transgenic technology [20], however, should alleviate many of these concerns, since the full gene sequence, or a large part of it, will be used to drive reporter expression. Another problem with using any single gene-based approach is that no marker is absolute. For the more homogeneous population of the progenitors, several rounds of sorting using a combination of a cell surface and GFP markers may be used.

Another way to get around the issue of heterogeneity is to perform genetic subtraction between two different populations of cells that differ largely in the number of cells of interest. Several mRNA/cDNA subtraction methods exist, such as representational difference analysis (RDA), polymerase chain reaction (PCR)-based differential display, and comparison of cDNA profiles obtained by serial analysis of gene expression [21–24]. In our previous study, we performed an RDA subtraction on two neurosphere (NS) populations [3]. Neurospheres are derived from neural stem cells, containing a variety of their progeny at different stages of differentiation. Using RDA, we compared mRNA populations of proliferating to differentiating NSs. Proliferating neurospheres contained 10-fold greater numbers of NS-forming neural stem cells. Currently, many microarray platforms are sensitive and broad enough (whole genome size) such that the comparisons between heterogeneous populations can be reasonably made, provided that the two populations mainly differ in the cell types of interest.

Except for the expected problems of high-throughput methodologies present in gene expression microarray profiling, the field of proteomics faces additional challenges. One of them is the absolute range of protein expression within one cell or one biological fluid. The protein field has yet to benefit from the protein equivalent to PCR, and thus the success of any proteomics study relies on reducing the complexity of the sample matrix through subcellular fractionation or through depletion of abundant proteins that would otherwise mask low-abundance, yet potentially important, proteins. And although transcript variants can be readily predicted and identified, proteins will often have multiple post-translational modifications, which increases greatly the complexity and diversity of the proteome [25]. The question in proteomics is quickly turning from what proteins are present to what posttranslational modifications are present.

Types of Profiling Tools

In any type of profiling approach, investigators are faced with a choice of exactly what should be measured. Several approaches exist to measure mRNA expression, whereas others measure protein expression. Recent studies have also begun to perform profiling of small, regulatory RNAs, termed microRNAs [26].

Proteomics

Technological and informatics advances have paved the way for the advent of proteomics: the study of large sets of proteins expressed by a particular cell type, tissue, or biological fluid [27]. The use of proteomics complements genomic methods and allows investigators to overcome some drawbacks of genomic approaches [28]. This is an important consideration as more proteins are discovered that are not under classic transcriptional regulation. Recent estimates in hematopoietic stem cells [29] suggest less than 50% agreement between protein and transcript expression levels.

Contemporary proteomics has three components: analytical separation to reduce the complexity of the protein matrix, mass spectrometry (MS) analysis of the proteins, and bioinformatic analysis. The most common approach to separation of complex protein mixtures is two-dimensional (2D) gel electrophoresis. Although there are still some drawbacks, advances in the field have greatly improved resolution, reliability, and MS compatibility [30]. Significant improvements specific to the analysis of membrane proteins include immobilized pH gradient gels, which offer extended pH range and steady-state focusing. 2D gel electrophoresis has advanced to the point where protein isoforms can be reliably detected [31] and intact protein mass can be measured [32, 33].

Additional separation techniques, such as two-dimensional liquid chromatography, are also available and offer their own sets of benefits and drawbacks. Recent advances have improved the quantitative nature of protein profiling and include, but are not limited to, isobaric tags [34] and the incorporation of stable isotopes into either living tissue or cell cultures [35].

These separation methodologies are then coupled to various MS platforms [36]. MS is precise, rapid, independent of antibodies, requires nano- to femtomole amounts of protein, generally does not demand 100% protein purity, and is capable of identification of unknown proteins. Using MS, it is possible to analyze the total protein complement, the intact protein mass [37], the amino acid sequence of small peptides (enabling the identification of gene sequence errors), and the nature and location of post-translational modifications [38, 39]. Acquisition of MS data is typically automated, and the interpretation of these data is facilitated by publicly available software and databases, such as Mascot, National Center for Biotechnology Information (NCBI), and SwissProt. Generally, MS analysis programs compare the experimentally determined MS/MS scan of the peptide against all existing peptide sequences from a selected database (such as NCBI or SwissProt), calculate match probabilities, and predict protein identity. The presence of post-translational modifications, such as phosphorylation, can be accommodated and detected within these software programs. De novo sequencing is also possible but can be experimentally challenging.

Genomics

Simultaneous measurements of messenger RNAs encoding a large number of genes can be accomplished in a number of ways, starting with simple subtraction techniques such as RDA. The most common method used is microarray analysis, which is discussed in detail below. Alternatives that are more or less comprehensive to microarrays also exist. Multiplex quantitative reverse transcription (RT)-PCR can be used to assay expression levels of tens of genes. This method allows for a more precise level of quantitation and does not require the user to have access to an array analysis facility. Thus, multiplex quantitative PCR could be of significant use when one wants to study a limited number of genes [40, 41]. Recent advances in multiplexing, based on specific oligonucleotides tagged with beads or signature molecules or particular mass, will make the screening of a limited number of genes in a large number of samples highly efficient [42–44]. On the other hand, methods exist that give an even broader picture of gene expression. The massively parallel signature sequencing method uses a proprietary technology to determine the number of each transcript and is purported to have a much higher sensitivity than microarray for low copy number transcripts. This method has been successfully used to delineate global gene expression in embryonic stem cells and compare them to differentiating cells [45–50].

With the introduction of cDNA and oligonucleotide microarrays in the mid-1990s [51–53], high-throughput, simultaneous monitoring of gene expression became possible. DNA microarrays consist of a group of methods that allow the instantaneous study of the expression patterns of thousands of genes in the same tissue or cell in parallel [54]. cDNA and oligonucleotide arrays have been used successfully in studying the nervous system, in health (e.g., [1, 3, 55, 56]) and disease [57–62].

Microarray Experimental Flow

The typical procedures involved in a microarray experiment include isolation of a messenger or total RNA from the tissue or cell culture sample; labeling of the sample with fluorophores (e.g., Cy3-dCTP or Cy5-dCTP), often in conjunction with amplification; hybridization of samples onto array (slide); raw data acquisition; and subsequent analysis. Postarray steps include data interpretation that typically results in "hypothesis generation" and its independent confirmation or "hypothesis testing." Here, we will briefly discuss the use of microarray technology in the stem cell research, reliable commercial microarray platforms, available resources for microarray data analysis and interpretation, and importance of "postarray" studies as a standard of microarray-based research.

The most common microarray experiment has traditionally used the *two-channel design.* In this scenario, two samples are labeled with different fluorophores that emit different wavelengths and can be independently quantified. Both samples are hybridized onto the same slide, and the signals from the two samples can be directly compared. Differences in gene expression are then given as a ratio rather than as an absolute value.

Such a system was routinely used with all custom and commercial cDNA microarrays, as well as with some commercial oligonucleotide platforms (e.g., Agilent Technologies, Palo Alto, CA, http://www.agilent.com).

Over the last 5 years and with the introduction of novel methods of array printing, oligonucleotide-based platforms became more popular because of their advantages in reproducibility and sensitivity over cDNA-based platforms. As a rule, commercial oligonucleotide platforms tend to use a single-channel design, allowing more flexibility in experimental comparisons. In a single-channel experiment, all samples are labeled with one dye, and only one sample is hybridized onto the slide. The signal detected for each probe upon laser excitation is directly proportional to the amount of labeled target bound to it, thus allowing for semiquantitative analysis of the transcript abundance in a given sample. The samples (slides) can be compared between each other, and the differences in the expression are identified. Examples of such platforms are Affymetrix GeneChips (Santa Clara, CA, http://www.affymetrix.com), CodeLink Expression Bioarrays (GE Healthcare, Little Chalfont, Buckinghamshire, U.K., http://www.gehealthcare.com/) and Illumina BeadChips (Illumina, San Diego, http://www.illumina.com). Agilent arrays can also be used for one-channel experiments because of their high level of reproducibility from slide to slide [55].

Depending on the labeling technique and source of experimental RNA, an entire microarray experiment from RNA isolation to raw data acquisition might take up to 3 days, especially when working with finite amounts of starting materials, such as laser-captured or FACS-collected cells [55].

Due to the large scale of a microarray experiment, there are a number of procedural considerations in almost every step of the experimental flow. One of the restricting factors in applying microarray technology in a laboratory is the cost of replicating experiments. To produce reliable results, replication of experiments is prerequisite for reduction of biological and experimental noise [63, 64]. The number of replicates for an experiment will vary depending on the amount of experimental noise; however, replicates introduce greater reliability to the expression data and should not be neglected. When using T7-based labeling technique and high-quality arrays, it is standard to run from three to five independent replicates, each duplicated with switched dyes (in case of two-channel design), to obtain a low enough number of false-positive signals [65]. Noise measures can be empirically derived and screening thresholds set appropriately based upon the technical and biologic noise in a particular system [56, 63, 64]. Increasing the number of independent replicates will permit the detection of smaller changes in expression (e.g., 1.5-fold) with higher confidence (e.g., [55]). Statistical methods that estimate variance to increase statistical power are very useful when small numbers of replicates are available compared with the number of measurements being made [66, 67]; several excellent reviews of statistical methods are available [67–70], and tools are available on line (as described in Online Resources).

Whether or not to pool samples is another question that is often raised. Pooling is an effective way to diminish the effects of individual variability within biological samples. But the power of this approach depends upon the integrity of the samples being pooled, and one sample with significant deviation from the rest of the pool may spoil an experiment consisting of a comparison of two pools.

Sample Amplification and Labeling

The purity and quality of the starting RNA has a major effect on the results of microarray experiments; therefore, it is essential that all steps of RNA isolation be carried out with maximum care and speed. The major limiting factor in cell-specific gene expression experiments is an ability to reliably amplify and label finite amounts of starting RNA, avoiding introduction of amplification bias [54]. The signal intensity from hybridization depends on the target concentration, the amount of immobilized probe molecules, and the method of labeling. Today, the most common method used with commercial microarray platforms is a T7-directed in vitro transcription and amplification [65]. It was shown to be reliable in generating labeled products from small quantities of RNA on a consistent basis, in some cases from a single cell or a few laser-captured or FACS-collected cells [55, 71].

Commercial Microarray Platforms

A typical DNA microarray consists of tens of thousands of elements, called probes, densely deposited onto a solid surface, such as glass, beads, or a membrane. The probes comprise either cDNA sequences [53] or short synthetic oligonucleotides of up to 70 nucleotides (Affymetrix) [72–74]. Microarray platforms can therefore be divided into two major formats, oligonucleotide arrays and cDNA arrays [51, 75, 76]. Due to the increasing popularity of commercial (typically oligonucleotide-based) microarray slides, we will discuss several of the most commonly used commercial platforms.

Today, practically all available commercial platforms cover nearly the entire genome, containing up to 50,000 genes on one slide [77], while also offering specific arrays with customer-selected probe content for more detailed and focused gene expression studies. It should be noted that availability of so-called "whole genome" arrays is a bit misleading, as these arrays operate with the number of genes on the array, not the number of actual transcripts detected. Most of the genes in the genome generate multiple transcripts, often with different functions, and are expressed in a specific tissue at a particular time of development. Currently, there is no good estimate of how many transcripts human transcriptome possesses, but it is most likely at least 5–10 times more then the number of genes in the genome, bringing us to nearly 400,000 different transcripts. Therefore, when working with whole-genome microarrays, one should be aware that at best one has in one's possession 40,000 different probes, where a specific gene probe can often recognize either one or multiple transcripts of the same gene. Therefore, it is difficult to estimate what part of transcriptome a particular whole-genome microarray represents.

Oligonucleotide microarrays can be manufactured either using in situ synthesis by photolithography (e.g., Affymetrix) or deposition of already synthesized oligonucleotides (e.g., ink jet technology, Agilent; Illumina). Some of the strategies for probe selection are common to all oligonucleotide arrays. Melting temperature of an oligonucleotide probe is calculated based on experimentally derived computer models calculating hybridization behavior of target sequences in complex mixtures under particular conditions. Commercial platforms are summarized in Table 1.

Affymetrix. The GeneChip (Affymetrix) arrays are the most widely used of the commercial platforms. They are manufactured using a combination of photolithography and combinatorial chemistry [74]. This allows the synthesis of hundreds of thousands of different oligonucleotides on the same surface at an extremely high density. Because the resulting surface area is very small, it enables researchers to use small sample volumes, therefore reducing the amounts of starting RNA. Affymetrix offers a range of preprinted arrays covering up to 54,000 genes. Each transcript is represented by 11–16 short 25-mer oligonucleotides selected according to their specificity to the desired transcript and low cross-hybridization with similar but unrelated

Table 1. Commercial microarray platforms

Vendor	Affymetrix	Agilent	CodeLink	Illumina
Platform	Oligonucleotide	Oligonucleotide/cDNA	Oligonucleotide	Oligonucleotide
Labeling	T7	T7	T7	T7
Reliability	High	High	High	High
Cost	High	Medium	High	Medium/low
Genome	Whole genome	Whole genome	Whole genome	Whole genome
Custom	Yes	Yes	Yes	Yes
Organism	Human, mouse	Human, mouse, rat	Human, mouse, rat	Human, mouse

This information was obtained from the vendor web sites in June 2006.

sequences. Because probes are designed for significantly unique regions of genes even among gene family members, GeneChip arrays can distinguish transcripts that are up to 90% identical. In addition, some probes are designed to distinguish multiple splice or polyadenylation variants (Table 1).

Agilent. An industrial noncontact inkjet printing process is used for the manufacturing of Agilent microarrays. Both oligonucleotide and cDNA can be deposited. The reproducible deposition of oligonucleotide or cDNA molecules onto specially treated glass slides is achieved without actual contact with a surface, thereby reducing the risk of potential anomalies due to the physical contact of slide and printer surfaces (http://www.chem.agilent.com). The technology requires only picoliters of DNA per spot. The 60-mer oligonucleotides are synthesized using standard phosphoramidite chemistry. Microarrays covering up to 50,000 genes per slide are available. This platform has proved to be very sensitive and reliable, and all types of experiments, including ones using FACS cells, have been performed successfully [55].

GE CodeLink. CodeLink Activated Slides (General Electric Healthcare) are specially treated to covalently immobilize amine-modified DNA. The combination of cross-linked polymer and endpoint attachment allows the oligonucleotides to be more accessible to the labeled targets hybridized onto the slides (http://www1.amershambiosciences.com). Whole Genome Bioarrays are available containing functionally validated, specific, prescreened 30-mer probes. As is the case for the other cDNA and oligonucleotide arrays described here, publications support the sensitivity, reproducibility, and validity of the data obtained with this platform [79–81].

Illumina BeadChips. Illumina BeadChips are another oligonucleotide-based platform that uses 50-mer probes. The unique feature of this platform is the ability to process multiple samples (currently up to six for the whole genome screen) on a single slide, greatly increasing specificity and reducing cross-array variability. Illumina BeadArray technology uses gene-specific probe sequences concatenated with "address" sequences, which are immobilized to a bead along with hundreds of thousands of probes of the same sequence [82]. BeadChip arrays provide extensive genomic coverage for well-annotated genomes such as human or mouse. The labeling protocol uses a T7 amplification technique that has been optimized for single-round amplification of as little as 50 ng of total RNA. As with other vendors, Illumina offers custom probe content for more focused multiple experiments.

Data Analysis and Interpretation

Currently, there is no standard or consensus on the best way to represent or analyze microarray data. This is a rapidly changing field, and methods are continuously evolving. However, there are several general data analysis and interpretation steps that are requisite for most microarray experiments. In addition, consensus has been reached about how microarray data should be presented, shared, and annotated in the minimal information about a microarray experiment (MIAME; http://www.mged.org).

In general, microarray data are normalized, and the relative expression of each gene within a sample is determined. Following initial assignment of expression values, the data can be subject to a wide variety of analyses. As stated in the section above, statistical analyses are used to determine which genes are enriched or reduced in one experimental condition or another. In this way, individual genes of interest can be identified. Before individual signal intensity values are compared, normalization is necessary. This critical step compensates for technical variability that includes inconsistency between slides, different rates of fluorescent dye incorporation (e.g., Cy3 is generally incorporated more efficiently them Cy5), and other systematic sources of error. Normalization adjusts measured signal intensities appropriately. Raw data filtering is also performed by removing poor or questionable spots (signals). Several types of data normalization are used, and depending on a particular experiment and the microarray platform used, a particular type (often suggested and developed by a vendor) should be used. In this regards, we have had success with the Microarray Data Analysis system of The Institute for Genomic Research (TIGR) that combines several analytical and data conversion software in one suite (http://www.tm4.org/). It provides users with an intuitive interface to design data analysis flow, array normalization, and gene identification tools.

After normalization and filtering, expression values can be analyzed and compared between experimental samples. Many sophisticated algorithms for microarray data clustering, visualization, classification, statistical analysis, and biological theme discovery have been developed (e.g., http://www.tm4.org/). At this final stage of data analysis, the use of a variety of analytic techniques is critical, as every algorithm or statistical method has strengths and weaknesses. Therefore, several analytical strategies should be exploited to generate a reliable set of candidate genes affected in the experiment. Frequently used analytical tools include direct statistical interrogation for significantly deregulated genes (e.g., *t* tests or analysis of variance) and various types of data clustering, where groups of genes with similar behavior across experimental conditions can be identified. The latter include such clustering algorithms as hierarchical clustering [83], self-organizing maps, K-means clustering [84], and principal components analysis [85]. Using the TM4 software suite allows researchers to label and track identified gene clusters through other analyses, giving the ability to compare expression behavior from experiment to experiment.

A global sense of the similarities or differences between sample sets can be obtained using clustering algorithms. For example, using hierarchical clustering, we performed an analysis of the expression profile specific for PTEN-deficient neurospheres and confirmed its specificity [6]. This cluster analysis can be extended to include hundreds of genes in hundreds of

individual samples and can become a powerful tool in the grouping of samples. For example, Freije et al. have used cluster analyses to group glioblastoma multiforme samples into novel categories with prognostic significance [86]. In addition, as described above, gene expression data can be mined to examine functional groupings of differentially expressed genes. Analysis tools, such as DAVID/Ease (http://david.niaid.nih.gov/david/ease.htm), allow one to determine whether a tissue or cell type differentially expresses particular kinds of genes or genes involved in specific processes. As more functional data are amassed about individual genes, the annotation becomes more and more detailed and, hence, more sophisticated. Numerous other methods and resources exist for the analysis of array data. Some of these resources are listed at the end of this review.

Microarray Data Confirmation: "Postarray" Studies

Even with good statistical methods, confirmation of some small cross-section of the results using an alternative method, such as quantitative RT-PCR, in situ hybridization, or Northern blot, is necessary. Quantitative and real-time RT-PCR are both especially suitable in validating a large number of gene expressions (e.g., [1, 55]); however, in situ hybridization experiments can also be performed to confirm the expression of dozens of genes within a relatively short period of time and provide in vivo validation of gene expression data [2, 3]. Northern blots also offer a way to confirm the expression of transcripts and have demonstrated the consistency and validity of cDNA microarray data [80] .

Microarrays—Conclusions

The field is changing rapidly, and new techniques and platforms are introduced every year. Commercial oligonucleotide platforms are becoming standards in the field. Whole genome chip sets are now available, and the general cost of microarray experiments is decreasing. Simple and reliable amplification and labeling techniques are still needed as more and more researchers are looking at the cell-specific expression levels. There are a variety of analytic tools, and certain standards are starting to appear. Data mining and interpretation is still a challenge, as complete information on gene function is not always available. Functional confirmation becomes a standard and a part of microarray experimental design; this is one of the most exciting areas of progress. Internet resources are rapidly changing, so we refer readers to some general array websites that should keep them up-to-date.

Online Resources

General Microarray Sites

Microarray Gene Expression Database group: http://www.mged.org
TIGR: http://www.tigr.org/tdb/microarray
Stanford University: http://cmgm.stanford.edu/pbrown/protocols/index.html
DeRisi Laboratory, University of California San Francisco: http://derisilab.ucsf.edu/
Y.F. Leung's Functional Genomics: http://ihome.cuhk.edu.hk/%7Eb400559/array.html

Data Collection, Annotation, and Interpretation Tools

PubMed: http://www.ncbi.nlm.nih.gov/PubMed
Database for Annotation, Visualization and Integrated Discovery (DAVID): http://david.abcc.ncifcrf.gov
PubGene, University of Oslo: http://www.pubgene.org/
SOURCE, Stanford University: http://source.stanford.edu
GenMapp, Gene Microarray Pathway Profiler: http://www.genmapp.org/
2HAPI, High-density Array Pattern Interpreter, version 2, University of California San Diego: http://david.abcc.ncifcrf.gov
ChiliBot: http://www.chilibot.net/
Genomatix microarray data interpretation tools: http://www.genomatix.de/
MeSHer biological literature mining: http://biocomp.dfci.harvard.edu/mesher.html
MatchMiner translates between different gene identifications: http://discover.nci.nih.gov/matchminer/index.jsp

Data Analysis Tools

TM4 microarray analysis software, TIGR: http://www.tigr.org/softlab
Cyber-T, Institute for Genomics and Bioinformatics, University of California: http://visitor.ics.uci.edu/genex/cybert/
EMBL, European Bioinformatics Institute: http://ep.ebi.ac.uk
Patterns from Gene Expression (PaGE), University of Pennsylvania: http://www.cbil.upenn.edu/PaGE
SCAN-ALYZE, Lawrence Berkeley National Laboratory: http://www.microarrays.org/software.html
Rosetta Resolver System: http://www.rosettabio.com/products/resolver/default.htm
ImaGene and GeneSight, BioDiscovery, Inc.: http://www.biodiscovery.com
GeneSpring, Silicon Genetics, Inc.: http://www.sigenetics.com
Spotfire DecisionSite for Functional Genomics, Spotfire, Inc.: http://www.spotfire.com

Metabolic and Regulatory Pathway Databases

Kyoto Encyclopedia of Genes and Genomes (KEGG): http://www.genome.ad.jp/kegg/
TRANSPATH database describes the signal transduction from the ligand at the surface of a cell up to the transcription factor: http://www.gene-regulation.com/cgi-bin/pub/databases/transpath
The Signaling PAthway Database (SPAD): http://www.grt.kyushu-u.ac.jp/spad/
Wnt signaling pathway database: http://www.stanford.edu/~rnusse/wntwindow.html
Boehringer Mannheim Biochemical Pathways: http://www.expasy.org/cgi-bin/search-biochem-index
Enzymes and Metabolic Pathways (EMP) database: http://www.empproject.com
BioCarta: http://www.biocarta.com

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Gene Therapy for Hemoglobinopathies Using Autologous Hematopoietic Stem Cells

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Key Words. Thalassemia • Hemoglobin • Retroviral vectors • Lentiviral vectors

ABSTRACT

Hemoglobin disorders constitute the most common single-gene disorders that are potentially amenable to gene therapy. However, retroviral vectors carrying the human β-globin cassette are notoriously unstable, express the transgene at low levels, or are unable to hold large erythroid regulatory elements. In the past 5 years, tremendous progress has been made in this field with the use of lentiviral vectors. Our laboratory investigated lentiviral vectors for erythroid lineage-specific expression, long-term expression, and silencing following transduction of hematopoietic stem cells. In addition, we have been able to overcome the chromatin position effects with insulated self-inactivating lentiviral vectors that have increased probability of expression from individual integrants and reduced clonal variegation in expression in long-term transplanted mice. We have shown complete correction of the human thalassemia phenotype in vitro and in xenografts in the red blood cell progeny of CD34$^+$ cells from patients with β-thalassemia major. This article provides a concise review of the current status of gene therapy for hemoglobin disorders and the steps needed for safe human clinical trials.

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Expressing a normal β-globin gene or an antisickling globin in red blood cells following permanent gene transfer into hematopoietic stem cells (HSCs) can result in permanent cure for hemoglobinopathies, such as β-thalassemia and sickle cell anemia. Therefore, retroviral vectors, which integrate permanently into host genome, have been explored for gene transfer into HSCs. Gene therapy for hemoglobinopathies, however, suffered from several obstacles: the commonly used onco-retroviral vectors carrying the β-globin gene and its regulatory elements have notoriously suffered from problems of vector instability, low titers, and variable expression of the human β-globin gene

Table 2. Summary of studies on lentiviral vectors for gene therapy for hemoglobin diseases

Investigators	LTRs in LV[a]	Transgene	LCR	Hb/vector copy (g)[b]	Model/phenotype	Summary of results
Thalassemia						
May et al. [24]	HIV-1 LTR	β	3.2 kb	3.8	Murine thal. intermedia	Correction
Imren et al. [27]	HIV-1 LTR	β-87	2.7 kb	1.5	Murine thal. intermedia	Correction
Rivella et al. [25]	HIV-1 LTR	β	3.2 kb	2.3	Murine thal. major	Rescue of lethality
Persons et al. [26]	SIN[c] HIV-1 LTR	γ	1.7 kb	1.1	Murine thal. intermedia	Correction
Hanawa et al. [51]	SIN HIV-1 LTR	γ	3.4 kb	2.0	Murine thal. intermedia	Correction
Imren et al. [40]	SIN HIV-1 LTR	β-87	2.7 kb		Human normal xenografts	High β^{87} expression Inserts near oncogenes
Puthenveetil et al. [28]	SIN (Ins[d]) HIV LTR	β	3.2 kb		Human thal. major/xenografts	Correction
SCA						
Pawliuk et al. [32]	HIV-1 LTR	β-87	2.7 kb	1.9	Murine SCA (Berk, SAD mice)	Correction
Levasseur et al. [35]	HIV-1 SIN LTR	β-AS3	3.4 kb	2.1	Murine SCA (Townes mice)	Correction

[a] The configuration of the HIV-1 LTR in the lentivirus.
[b] Gram rise in Hb per deciliter, in mice for an average vector copy of 1.
[c] Enhancer-deleted 3′ LTR that results in a SIN deletion in the 5′ LTR upon integration into host genome.
[d] The chicken hypersensitive site 4 chromatin insulator element flanks the integrated SIN lentiviral vector.
Abbreviations: Hb, hemoglobin; kb, kilobase(s); LCR, locus control region; LTR, long terminal repeat; SCA, sickle cell anemia; SIN, self-inactivating deletion; thal., thalassemia.

[1–6]. AAV vectors, which initially held promise, did not integrate efficiently into HSCs [7] and were restricted to carrying only the core elements of the large β-globin locus control region (LCR) due to their size limitation [8–10]. RNA-based approaches, such as use of hammerhead and trans-splicing ribozymes [11, 12], antisense RNA against β^S globin [13, 14], or DNA short-fragment homologous recombination [15] to convert β^S to β-globin have also been tried and show promise, but they require improvements in methods of gene delivery and demonstration of efficacy in animal models. For detailed recent reviews on gene therapy for hemoglobinopathies, the reader is referred to [16–20].

Gene Therapy for β-Thalassemia

Lentiviral vectors have the distinct advantage of integrating into nondividing cells, such as the hematopoietic stem cells. We designed several erythroid human immunodeficiency virus 1 (HIV-1)-based lineage-specific lentiviral vectors [21] in a self-inactivating (SIN) vector backbone, whereby the 3′ long terminal repeat (LTR) enhancer deletion gets copied over into the 5′ LTR, removing viral enhancer elements in the integrated virus. These SIN-lentiviral vectors expressed in a highly lineage-specific manner [21] and resulted in correction of murine protoporphyria [22]. However, SIN lenti-provirus was subject to chromosomal position effects [23].

In a pioneering study performed by May et al., HIV-1-based lentiviral vectors were shown to stably transmit the human β-globin gene and large elements of the LCR, resulting in the correction of β-thalassemia intermedia phenotype in mice [24]. Several other groups subsequently showed correction of β-thalassemia intermedia phenotype in mice, using either β- or γ-globin lentiviral vectors. These results are summarized in Table 2.

Homozygous β-thalassemia (thalassemia major) is embryonic-lethal in mice, since the globin switch occurs in utero. Rivella et al. [25] developed a mouse model of β^0-thalassemia major by transplanting fetal liver cells from thalassemia major fetuses, prior to fatality, into lethally irradiated normal adult mice. Fully engrafted mice died of severe anemia (hemoglobin ~3 g/dl) within 6–8 weeks following the transplantation, whereas genetic correction of the fetal liver cells with a lentiviral vector, previously shown to correct thalassemia intermedia phenotype in mice [24], rescued their lethality. However, it is to be noted that most mice were still severely anemic, with hemoglobin ranging from 4.7–7.5 g/dl (a severe thalassemia intermedia phenotype), whereas one mouse showed complete correction (hemoglobin [Hb] 12 g/dl).

Similar data on variable expression of β/γ globin have emerged from other laboratories, where despite therapeutic levels of globin gene expression, there was marked variability in transgene expression among different mice [25–27]. Persons et al. showed that the γ-globin/LCR vector expression did not correlate with vector copy number, and this was due to chromosomal position effects [26]. The same laboratory has shown that the same vector, TNS9, resulted in an average of 3.8 g/dl increase in hemoglobin/vector copy number in thalassemia intermedia mice in one study [24] versus a 2.3 g/dl increase in hemoglobin/vector copy number in the thalassemia major mouse model in another study [26]. Imren et al. showed pan-erythroid correction of murine β-thalassemia intermedia only with 3–5 copies per cell, whereas minimal correction occurred at single vector copy per cell in the mice [27]. They attributed this to chromosomal position effects [27].

Our laboratory designed a β-globin gene/LCR containing lentiviral vector carrying the chicken hypersensitive site 4 (cHS4) chromatin insulator element, such that it flanks the provirus upon integration, to address chromatin position effects and, in addition, improve vector biosafety. This vector was used to transduce $CD34^+$ cells from bone marrow of four patients with transfusion-dependent thalassemia major [28]. There was high-level transduction with complete correction of the in vitro model of human thalassemia erythropoiesis, and this correction was sustained long-term in vivo in immune-deficient mice.

Gene Therapy for Sickle Cell Anemia

Lentiviral vectors have also paved the way for gene therapy for sickle cell anemia (SCA). At a molecular level, sickling occurs when sickle hemoglobin (Hb S) pairs between the mutant valine-6 in the β_2 chain of one hemoglobin molecule and a hydrophobic pocket, formed by phenylalanine-85 and leucine-88 in the β_1 chain of another hemoglobin molecule

($\alpha_2\beta^S{}_2$). Gamma globin is a natural antisickling hemoglobin, because glutamine-87 of γ-globin aligns with threonine-87 of β^S-globin, resulting in mixed tetramers ($\alpha_2\gamma\beta^S$) that do not participate in polymer formation. Several synthetic antisickling β-globins have been designed based on similar principles [29–31] and are an attractive approach to gene therapy for SCA.

Pawliuk et al. [32] were the first to express an antisickling β-globin (β-T87Q-globin) from a lentivirus, with correction of phenotype in two murine models of SCA: (a) the SAD [33] mice that express a "super-sickling" β^{SAD} globin (with Sickle, Antilles, and hemoglobin D-Punjab mutations in human β-globin), and (b) the BERK mice [33] that express human α and human β^S globins and, in addition, are knockouts for murine α and β globins. Ryan et al. had also generated transgenic sickle mice at the same time the BERK mice were generated [34] that exclusively produced human α and human β^S globins. The same group used a self-inactivating lentiviral vector carrying antisickling globin (β-AS3) to correct the disease in these transgenic sickle mice [35]. Notable features of this study were very high gene transfer efficiency into hematopoietic stem cells and a very short cytokine-free exposure in primary and secondary recipients. Recently, Samakoglu et al. have used short hairpin RNA in lentiviral vectors to destroy the β^S globin and introduce the antisickling γ-globin gene [36]. All of these studies are tremendous strides toward gene therapy for SCA.

Safety of Integrating Vectors

The development of leukemia in three children treated with a γ-oncoretroviral vector carrying the IL2R-γ chain and oncogene activation by the integrated proviral LTR [37] underscore the need for generating safer vectors. Lentiviral vectors, like oncoretrovirus vectors, preferentially integrate near or around cellular genes [38]. Although γ-oncoretroviral vectors have now been shown to prefer integration start sites of actively transcribed genes, lentiviral vectors integrate within active genes, with no predilection for promoters and integration start sites [38, 39]. Imren et al. showed preferred intragenic integration of the β-T87Q-globin/LCR lentiviral vector in human cord blood progenitor cells that were transplanted into immune-deficient mice, with several integrations occurring near oncogenes [40]. These studies emphasize the need for safe vector design. The latter is especially important, given that three children cured of X-linked severe combined immunodeficiency in the French trial went on to develop a T-cell leukemia, which was found to be due to activation of surrounding LMO2 oncogene by the provirus.

Chromatin insulator elements, such as those from cHS4, have been shown to have chromatin barrier activity and an enhancer-blocking effect, two separable activities [41, 42]. Although the barrier function of the chromatin insulator elements has been tested in γ-oncoretroviral vectors [43–45] and in SIN-lentiviral vectors [46, 47] by several groups, the enhancer-blocking activity of the insulators in the context of viral vectors has not been studied. Chromatin insulator elements should be tested in the context of β- or γ-globin lentiviral vectors, since these vectors carry the LCR, a strong erythroid enhancer, and the LCR's propensity to activate erythroid genes surrounding the integrated provirus needs to be determined. In its native configuration, the β-globin LCR can activate erythroid genes over large distances. We have shown improved barrier activity by incorporating cHS4 to flank the β-globin SIN-LV cassette and preliminary reports now suggest that the cHS4 insulator may reduce vector genotoxicity in assays designed to test oncogenicity of vectors (Malik P. et al. and Neinhuis A. et al., unpublished results). It is to be noted, however, that although insulator elements improve safety by blocking enhancers from "oncogene-activating" insertions, can conceivably block cellular enhancers from activating tumor-suppressor genes. Therefore, the effects of insulator elements on transgene expression, silencing, and enhancer blocking need to be studied in depth.

Other groups have adopted a different approach to address safety, such as homologous recombination using AAV vectors or zinc-finger nucleases, to correct the specific mutation [48–50]. Although they are in their infancy, these targeted approaches may eventually be safer than the relatively randomly integrating viral vectors.

In summary, studies on lentiviral vectors for gene therapy for hemoglobin disorders, although experimental, have paved the way for preclinical studies on gene therapy for β-thalassemia and sickle cell anemia, so that safety and feasibility clinical trials can ensue. The challenges and hazards of gene therapy apply here, just as with any other gene therapy study, even as newer and better gene transfer and delivery methods are being discovered. However, this area of research offers much hope for future and a definitive cure of thalassemia and sickle cell disease through genetic correction of autologous hematopoietic stem cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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CREB is a critical regulator of normal hematopoiesis and leukemogenesis

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CREB is a critical regulator of normal hematopoiesis and leukemogenesis

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The cAMP-responsive element binding protein (CREB) is a 43-kDa nuclear transcription factor that regulates cell growth, memory, and glucose homeostasis. We showed previously that CREB is amplified in myeloid leukemia blasts and expressed at higher levels in leukemia stem cells from patients with myeloid leukemia. CREB transgenic mice develop myeloproliferative disease after 1 year, but not leukemia, suggesting that CREB contributes to but is not sufficient for leukemogenesis. Here, we show that CREB is most highly expressed in lineage negative hematopoietic stem cells (HSCs). To understand the role of CREB in hematopoietic progenitors and leukemia cells, we examined the effects of RNA interference (RNAi) to knock down CREB expression in vitro and in vivo. Transduction of primary HSCs or myeloid leukemia cells with lentiviral CREB shRNAs resulted in decreased proliferation of stem cells, cell-cycle abnormalities, and inhibition of CREB transcription. Mice that received transplants of bone marrow transduced with CREB shRNA had decreased committed progenitors compared with control mice. Mice injected with Ba/F3 cells expressing either *Bcr-Abl* wild-type or T315I mutation with CREB shRNA had delayed leukemic infiltration by bioluminescence imaging and prolonged median survival. Our results suggest that CREB is critical for normal myelopoiesis and leukemia cell proliferation. (Blood. 2008;111: 1182-1192)

Introduction

Hematopoiesis is regulated by transcription factors that drive bone marrow progenitor cells to proliferate and differentiate. Among the nuclear factors that control gene transcription is a leucine zipper transcription factor, cAMP-responsive element binding protein (CREB), which activates genes that control metabolism, cell cycle, signal transduction, and cell survival. CREB is a member of the activating transcription factor (ATF)/CREB family of transcription factors and requires phosphorylation of serine 133 for function.[1,2] We demonstrated previously that CREB is a downstream target of hematopoietic growth factor signaling activated by granulocyte-macrophage–colony stimulating factor and interleukin-3.[3-5] A role for CREB in oncogenesis has been suggested by its overexpression in the majority of bone marrow samples from patients with acute leukemia.[6] CREB is overexpressed at both the protein and mRNA levels in leukemic blasts and in leukemia stem cells.[7-9] Furthermore, CREB is amplified in blast cells from CREB-overexpressing patients.[6]

We also demonstrated previously that CREB overexpression in myeloid cells increases cell proliferation and survival. CREB transgenic mice that overexpress CREB in the myeloid lineage develop myeloproliferative disease/myelodysplastic syndrome but not acute leukemia, suggesting that CREB contributes to myeloid cell proliferation but is not sufficient for development of acute leukemia. Bone marrow progenitors from CREB transgenic mice demonstrate increased stem-cell self-renewal in replating assays and increased sensitivity to hematopoietic growth factors.[8] We demonstrated that CREB overexpressing myeloid cells also have increased expression of cyclin A associated with an increase in the number of cells in S phase. Therefore, CREB seems to play a role in hematopoietic stem cell (HSC) proliferation and survival through its effects on cell-cycle regulation.

To understand the requirement of CREB in hematopoietic stem cells and myeloid leukemia cells, we investigated the expression of CREB in normal mouse and human HSCs and studied the effects of CREB down-regulation on normal and leukemic cell proliferation and maturation. In this article, we report that CREB is highly expressed in normal lineage negative (lin^-) or uncommitted hematopoietic progenitor cells and that inhibition of CREB expression using shRNAs resulted in decreased proliferation and differentiation of normal and neoplastic hematopoietic cells in vitro and in vivo, respectively. We also demonstrate by expression profiling, potential mechanisms by which CREB may influence HSC fate. Our results suggest that CREB plays a critical role in normal HSC proliferation and leukemia progression.

Methods

Stem cells and preparation

Murine hematopoietic stem cells and progenitors were isolated from adult C57BL6 mice as described previously.[10-15] Mouse whole bone marrow cells were divided into lin^- and lineage-positive (lin^+) cells using the mouse

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lineage cell-depletion kit of the magnetic activated cell-separation system in combination with the auto MACS magnetic cell separator (Miltenyi Biotec, Auburn, CA). The lin^- population was sorted either on a FACSDiVa or a BD FACSAria cell sorter (BD Biosciences, Rockville, MD) into hematopoietic stem cells and progenitors. The lin^+ fraction was sorted into mature hematopoietic cells, including T cells, B cells, granulocytes, macrophages, and erythroid cells. Human cord blood cells were obtained from Cambrex Charles City (Charles City, IA). Human lin^- cord blood cells were separated into $CD34^-$ and $CD34^+$ cells using the human CD34 MicroBead kit in combination with the auto MACS separator. Human lin^- $CD34^+$ cord blood cells were sorted by FACS into hematopoietic stem cells and progenitors, and lin^+ $CD34^-$ cord blood cells were sorted into mature hematopoietic cells. Human peripheral blood stem cells (PBSCs) and bone marrow cells were sorted by FACS into hematopoietic stem cells ($CD34^+$ $CD38^-$) and progenitors ($CD34^+$ $CD38^+$). Human lin^+ PBSCs were sorted by FACS into matured hematopoietic cells, including T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($CD56^+$), granulocytes/macrophages ($CD14^+$), and erythroid cells (glycophorin-A^+). For cell-cycle experiments, samples were stained according to a hypotonic propidium iodide buffer–based protocol.[16,17]

Quantitative real-time and reverse transcription-polymerase chain reaction

Total RNA was extracted from sorted cells ($2\text{-}5 \times 10^4$ cells per sample) using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized using the Omniscript Reverse Transcriptase Kit (Qiagen) or Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). To detect the expression of transcription factors SCL, c-Mpl, Pu-1, Aiolos, and CEBPα in mouse HSC and progenitors, reverse transcription-polymerase chain reaction (RT-PCR) was performed using primers and PCR conditions described previously.[18,19] Quantitative real-time PCR (q-PCR) was performed in triplicate using the TaqMan probe system with CREB- and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific probe and primers on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. *CREB* expression data were standardized using *GAPDH* expression data. Primer sequences are available upon request. For shRNA experiments, cells (5×10^6) were lysed in TRIzol and stored at −80°C before RNA extraction. RNA extraction was performed according to a standard protocol supplied by the manufacturer (Invitrogen, Carlsbad, CA), and pellets were resuspended in RNAse-free water. The cDNA was transcribed with a Superscript RT III based-protocol. DNAse treatment was not performed as a result of the selection of intron-spanning primers. Quantitative real-time PCR was performed with the SyberGreen reagent (Bio-Rad Laboratories, Hercules, CA) in triplicate and analyzed by the standard curve method standardized to the housekeeping gene β-actin.[20,21]

shRNA sequence design and constructs

The *CREB*-specific shRNA sequences (*CREB* shRNA-1, *CREB* shRNA-2, *CREB* shRNA-3) were selected and validated based on accepted parameters established by Tuschl et al[22-24] (http://www.rockefeller.edu/labheads/tuschl/sirna.html). Sequence 2 was chosen for in vivo experiments based on maximal gene inhibition. Controls included empty vector, luciferase shRNA, and scrambled shRNA. shRNA sequences are *CREB* shRNA-1 (5′-GCAAATGACAGTTCAAGCCC-3′), shRNA-2 (5′-GTACAGCTGGCTAACAATGG-3′), shRNA-3 (5′-GAGAGAGGTCCGTCTAATG-3′), Luciferase shRNA (5′-GCCATTCTATCCTCTAGAGGA-3′), and scramble shRNA (5′-GGACGAACCTGCTGAGATAT-3′). Short-hairpin sequences were synthesized as oligonucleotides and annealed according to standard protocol. Annealed shRNAs were then subcloned into pSICO-R shRNA vectors from the Jacks laboratory at MIT (http://web.mit.edu/ccr/labs/jacks/index.html).[25] The second generation SIN vector HIV-CSCG was used to produce human shRNA vectors.[26]

Cell lines

The following human leukemia cell lines were transduced with shRNAs: K562 (Iscove medium + 10% fetal calf serum [FCS]), TF-1 (RPMI medium + 10% FCS + recombinant human granulocyte macrophage–colony-stimulating factor [rhGM-CSF]), and MV-411 (Iscove medium + 10% FCS). Murine leukemia cell lines included Ba/F3-*Bcr/Abl* wild type (RPMI medium + 10% FCS) and Ba/F3-*Bcr/Abl* T315I (RPMI medium + 10% FCS). All leukemia cell lines express CREB. Cells were cultured at 37°C, 5% CO_2 and split every 3 to 4 days. Proliferation and viability assays in Figure 4 were performed in triplicate by the trypan blue exclusion method. Several transductions were performed for each shRNA sequence to avoid clonal effects or selection. Each experiment was performed within a week after transduction.

Western blot analysis

Boiling SDS-Laemmli method was used for all Western blot analyses. Protein lysates were separated on a 10% SDS-polyacrylamide gel. Immunoblot was performed with anti-CREB (UBI, New York, NY), or β-tubulin antisera (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously.[8] All experiments were performed 3 times.

Lentiviral transduction of primary cells

One day before transduction, 7×10^5 cells were cultured in retronectin-treated 96-well plates and pre-stimulated with appropriate cytokines; for murine bone marrow, interleukin (IL)-3 (6 ng/mL), human IL-6 (10 ng/mL), and stem-cell factor (50 ng/mL) were used; for human peripheral blood, IL-3 (10 ng/mL), IL-6 (10 ng/mL), stem-cell factor (50 ng/mL), Flt-3 ligand (6 ng/mL), and thrombopoietin (6 ng/mL) were used.[27-30] All experiments were repeated at least 3 times. Human blood and bone marrow cells were obtained with institutional review board consent approved by the institution (UCLA IRB #98-09-036-21) in accordance with the Declaration of Helsinki.

Colony-forming assays

Transduced and GFP-sorted hematopoietic cells (2×10^4 murine bone marrow cells or 500 human $CD34^+$ cells) were plated in methylcellulose (Stem Cells, Palo Alto, CA) containing a full complement of cytokines (GM-CSF, IL-3, IL-6, stem-cell factor, erythropoietin [EPO]) or EPO alone for blast forming unit–erythroid (BFU-E)/colony-forming unit–erythroid (CFU-E) assays, and cultured for 21 days. Colony counts, cytospins, flow cytometric analysis, and Western blot analysis were performed in triplicate as described previously.[8] Fluorescence-activated cell sorting (FACS) antibodies used include CD150, CD135, Thy 1.1, CD117, Flk2, Sca-1, Mac-1, Gr-1, CD3e, B220, and Ter119.

In vivo bioluminescence

SCID mice (The Jackson Laboratory, Bar Harbor, ME) were tail vein-injected with 10^6 Ba/F3-*Bcr/Abl* T315I cells expressing luciferase that were transduced with either *CREB* shRNA or a control scrambled shRNA. Mice were followed over a period of approximately 4 weeks with serial examinations for disease. Luciferin (15 μg) was injected 10 to 15 minutes before imaging and repeated every 7 days for a total of 2 weeks. Relative intensity units for regions of interest were measured in triplicate and averaged,[31] with antilogs of log transform statistics used to estimate the geometric mean ratio of intensities and its confidence interval. These experiments were repeated twice. A similar approach was taken with Ba/F3-*Bcr/Abl* wild-type cells expressing luciferase that were either not transduced or transduced with *CREB* shRNA or a control, scrambled shRNA. SCID mice were injected with 5×10^6 cells, monitored, and images were acquired over 4 weeks as described previously in this paragraph.

Apoptosis experiments

Standard Western blot analysis methods were applied to lysates made from mouse bone marrow cells (10^6) that were transduced with *CREB* shRNA or

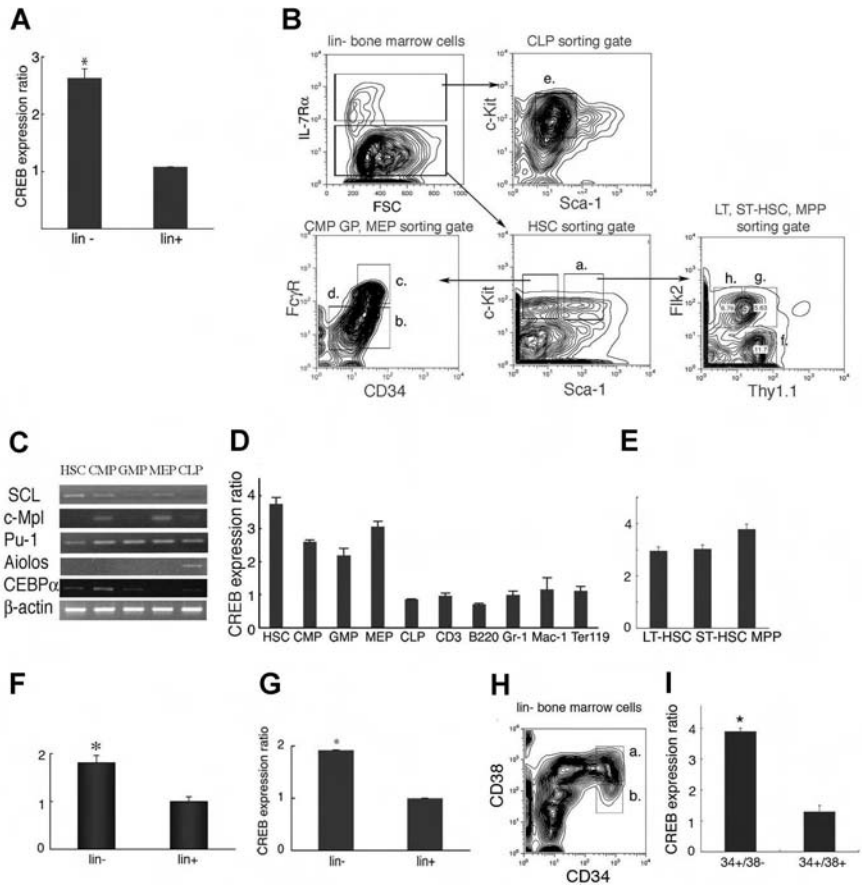

Figure 1. CREB expression in mouse and human hematopoietic progenitors. (A) CREB expression levels in lin$^-$ and lin$^+$ cells as measured by q-PCR. CREB expression was higher (2.6-fold) in the lin$^-$ population than the lin$^+$ population ($P = .01$). (B) Gating strategy for cell sorting of lin$^-$ cells to isolate HSC (Ba), CMP (Bb), GMP (Bc), MEP (Bd), CLP (Be), LT-HSC (Bf), ST-HSC (Bg), and MPP (Bh). (C) cDNA from HSC CMPs, CLPs, GMPs, and MEPs were confirmed by expression of SCL, c-Mpl, Pu-1, Aiolos, and CEBPα. (D) CREB expression levels in HSC, CMP, GMP, MEP, and differentiated cells. Compared with mature cells, CREB was 2.5- to 4-fold higher in HSC, CMP, GMP, and MEP. (E) CREB expression in LT-HSC, ST-HSC, and MPP. CREB was slightly higher (4- vs 3-fold) in the MPP fraction compared with the LT-HSC and ST-HSC populations, but this was not found to be statistically significant ($P = .06$). CREB expression in human lin$^-$ and lin$^+$ cells isolated from cord blood (F) or peripheral blood (G) stem cells. CREB expression was higher for both cell types in the lin$^-$ population than the lin$^+$ population ($P = .01$). (H and I) CREB expression in human bone marrow cells. CREB was also expressed at higher levels in CD34$^+$ CD38$^-$ cells (b) than CD34$^+$ CD38$^+$ cells (a). All experiments were performed in triplicate. Error bars represent SE.

control shRNA lentivirus. Blots were probed with anti-PARP antisera (Cell Signaling Technology, Danvers, MA) to assess for cleavage fragments confirmatory for apoptosis. In addition, flow cytometic methods were performed using an allophycocyanin-tagged annexin-V monoclonal antibody and propidium iodide to assay for apoptotic cells.

Murine bone marrow transplantation

C57BL/6j CD45.2 mice were purchased from The Jackson Laboratory and treated with prophylactic trimethoprim-sulfa therapy from four days prior to transplant in water. Recipient mice were sublethally irradiated on day 0 with a single dose of 9.5 Gy (950 rads) and subsequently injected through the tail vein with 2×10^5 green fluorescent protein-positive (GFP$^+$) bone marrow mononuclear cells from CD45.1 donors. Donor cells were transduced as described previously ("Lentiviral transduction of primary cells") and positive transductants were FACS sorted. Engraftment was monitored by serial eye bleeds and analysis of bone marrow with complete blood counts and lineage-specific monoclonal antibodies using flow cytometry.[32-34] Antibodies used included myeloid (Gr-1;Mac-1) and lymphoid (B220;CD3e). These experiments were performed twice.

Results

To characterize the expression of CREB during adult mouse hematopoiesis, we performed q-PCR on mRNA derived from lin$^-$ and lin$^+$ murine bone marrow cells, and distinct lin$^-$ and lin$^+$ subpopulations. CREB expression was higher (2.6-fold) in the lin$^-$ population than in the lin$^+$ population (Figure 1A, $P < .01$). Next, lin$^-$ cells were further fractionated into HSCs (Figure 1Ba), common myeloid progenitor (CMP; Figure 1Bb), common lymphoid progenitor (CLP; Figure 1Be), granulocyte-macrophage progenitor (GMP; Figure 1Bc), and megakaryocytic-erythroid progenitor (MEP; Figure 1Bd) using their characteristic cell surface antigen expression pattern and the gating and cell-sorting strategy as outlined in Figure 1B. After sorting, cDNAs from HSC, CMPs, CLPs, GMPs, and MEPs were confirmed by expression of SCL, c-Mpl, PU.1, Aiolos, and CCAAT/enhancer-binding protein α (C/EBPα; Figure 1C). As expected from the data we had obtained from lin$^-$ cells, CREB expression was higher in HSC, CMP, GMP, and MEP compared with differentiated cells (Figure 1D). To further define CREB expression in HSC, we examined the expression of CREB in long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs; Figure 1E). CREB was slightly higher in the MPP fraction compared with the LT-HSC and ST-HSC populations, but this was not found to be statistically significant (Figure 1E, $P = .06$).

To assess the mRNA CREB expression patterns in human hematopoiesis, lin$^-$ and lin$^+$ cells were isolated from human cord blood and peripheral blood stem cells and subjected to q-PCR. CREB expression was higher (2-fold) in the lin$^-$ population than in the lin$^+$ population for both cord and peripheral blood stem cells (Figure 1F,G, respectively, $P < .01$). CREB expression in human bone marrow cells was higher in CD34$^+$ CD38$^-$ cells than in CD34$^+$ CD38$^+$ cells (Figure 1H,I). Our results demonstrate that the lineage negative population consistently expressed higher levels of CREB regardless of the stem-cell source.

We examined the requirement of CREB for normal hematopoiesis by transducing normal murine bone marrow cells with CREB or control lentiviral shRNAs. Although 3 separate shRNAs were tested in cell lines initially (Figure 4 and data not

shown), shRNA sequence 2 was used to transduce primary bone marrow cells because it was the most effective in down-regulating *CREB*. Methylcellulose colony assays were performed to assess qualitative and quantitative effects of CREB on normal hematopoiesis in vitro (Figure 2A,B). Transduction efficiency varied between 5% and 34%, as assessed by measuring the GFP^+ fraction by flow cytometry (data not shown). We first analyzed CREB expression in primary mouse bone marrow cells transduced with CREB shRNA lentivirus by Western blot analysis and observed a significant inhibition of CREB expression (~80%) in *CREB* shRNA–transduced cells, compared with control cells (Figure 2Ai). After 21 days, we detected a statistically significant decrease (up to 5-fold) in the number of GFP^+ CFU-GM colonies with *CREB* shRNA-transduced mouse bone marrow cells compared with vector or luciferase control (Figure 2Aii). These colonies consisted of myeloid progenitor cells that were mostly Mac-1^+ (Figure 2Aiii). The Mac-1^+ Gr-1^+ progenitor cell fraction, an immature population of cells, was noted to be up to 4-fold higher in the *CREB* shRNA–transduced cells compared with control cells.[35] It is noteworthy that there were fewer differentiated Mac-1^+ cells in the *CREB* shRNA–transduced cells (30%) compared with vector control (50%), suggesting that CREB is critical for both hematopoietic cell proliferation and possibly terminal differentiation of monocytes. There was no statistically significant difference in the numbers of BFU-E or CFU-granulocyte/erythrocyte/monocyte colonies in methylcellulose with CREB and scrambled shRNA-transduced murine bone marrow progenitor cells. Likewise, long-term culture-initiating cell assays showed no difference between CREB and scrambled shRNA-transduced cells, suggesting that although CREB is a critical regulator of early myelopoiesis, it does not appear to be necessary for hematopoietic stem-cell proliferation and differentiation (data not shown).

The role of CREB in normal human hematopoietic cells was studied by knocking down *CREB* through lentiviral transduction of mobilized, $CD34^+$ normal peripheral blood mononuclear cells. Efficiency of transduction was as high as 34% by GFP expression (data not shown). *CREB* knockdown was 65% by Western blot analysis after 21 days in methylcellulose (Figure 2Bi). We observed a 3-fold decrease in CFU-GM colony formation in methylcellulose with *CREB* shRNA–transduced cells compared with control cells ($P < .05$; Figure 2Bii). Cytospin assays confirmed that CFU-GM were in the form of immature myeloid cells (data not shown). *CREB* shRNA–transduced stem cells had decreased numbers of mature $CD14^+$ monocytes (Figure 2Biii). This suggests that *CREB* down-regulation could be inhibiting terminal differentiation in addition to proliferation and survival in vitro.

CREB has been previously demonstrated to enhance cell proliferation and survival. To investigate the role of CREB in apoptosis of primary bone marrow progenitor cells, we transduced human and mouse bone marrow cells with *CREB*, scrambled, or vector control shRNA lentivirus. Identical sequences were used as described in Figure 2. In colony assays after 5 days, *CREB* shRNA–transduced cells showed a decrease in the human $CD34^+$ fractions and an undetectable $CD34^+$ $CD38^-$ HSC population compared with controls (Figure 3A and data not shown). For mouse bone marrow transductions, a relatively higher proportion of apoptotic cells in GFP^+ *CREB* shRNA–transduced cells with an increase in cells stained with annexin-V and propidium iodide (PI) (Figure 2B; $P < .05$). However, the overall percentage of apoptotic cells was less than 2%, suggesting that apoptosis occurs in a small proportion of the total number of transduced cells. Primary mouse BM progenitor cells transduced with *CREB* shRNA had increased PARP cleavage compared with control cells (Figure 3C). Therefore, our results suggest that *CREB* down-regulation in HSCs inhibits proliferation and survival and to a lesser extent induces apoptosis in vitro.

To study the requirement of CREB in normal hematopoiesis in vivo, we also analyzed hematopoietic reconstitution of sublethally irradiated B6/C7 CD45.2 mice at 5 and 12 weeks after infusion of marrow progenitor cells from B6/C7 CD45.1 transduced with *CREB* shRNA or scrambled shRNA lentivirus. We analyzed by flow cytometry those cells that were GFP^+ and $CD45.1^+$. Peripheral blood counts were comparable between both groups; however, there was a statistically significant increase (4-fold) in myeloid (Gr-1/Mac-1^+) progenitors and less dramatic increase in Mac-1^+ cells in the *CREB* shRNA group (Figure 4A and data not shown). At 12 weeks, very little difference was observed in lineage-specific cells (Gr-1/Mac-1^+, Mac-1^+, and Gr-1^+, $B220^+$, and $CD3e^+$) or hematopoietic stem cells ($CD135^+$, $CD150^+$, Thy1.1^+, Flk-2^+, $CD117^+$, Sca-1^+, $Ter119^+$) from bone marrow between *CREB* shRNA compared with scrambled shRNA (Figure 4B). These results suggest that CREB could also be playing a role in regulating myeloid differentiation of committed progenitor cells.

We next studied the effects of *CREB* down-regulation in proliferation of myeloid leukemia cells. All cell lines had strong basal CREB expression (Figure 5A). *CREB* was successfully knocked-down in human (TF-1 and K562) myeloid leukemia cell lines transduced with *CREB* shRNA at an efficiency of 45% to 92% confirmed by Western blot analysis and q-PCR (Figure 5A,B). In addition, phosphorylated CREB levels were lower in *CREB* shRNA–transduced cells (Figure 5A). The growth and viability of K562 and TF-1 leukemia cells were significantly decreased with *CREB* shRNA–transduced cells compared with control cells (Figure 5C,i-iii). Furthermore, we did not see off-target effects indicated by 2′,5′-oligoadenylate synthetase 1 (OAS-1) expression in cells treated with interferon (Figure 5D).[36] Multiple transductions were performed with the different shRNA sequences to avoid the effects of clonogenicity. We showed previously that overexpression of CREB in myeloid leukemia cells resulted in increased cyclin A promoter activity.[8] To examine the effects of *CREB* shRNA on *CREB* transcriptional activity, we transfected TF-1 cells with a construct containing the cyclin A promoter with the cAMP-responsive element and the luciferase reporter gene.[8] A significant decrease in cyclin A promoter activity was observed in cells transduced with *CREB* shRNA compared with vector control shRNA or untransduced cells (Figure 5E). We also analyzed the effects of *CREB* shRNA on cell-cycle regulation in TF-1 cells stimulated with GM-CSF for 12 hours after growth factor starvation (Figure 5F). Our results demonstrated decreased percentage of cells in S-phase and increased percentage of cells in G_1 and G_2/M when *CREB* was down-regulated with 2 different *CREB* shRNA sequences.

To study the requirement of CREB during progression of leukemia in vivo, Ba/F3 cells overexpressing Bcr-Abl wild type or Bcr-Abl with the imatinib-resistant T315I mutation were injected into SCID mice (Figures 6 and S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article.). These mice normally die within 21 days of injection from leukemic infiltration of liver and spleen. The Bcr-Abl–expressing Ba/F3 cells express CREB equal to the Ba/F3 wild-type cells. The T315I mutant cell line was chosen based on the 2-fold increased expression of CREB protein compared with *Bcr-Abl* or untransduced Ba/F3 cells (Figure

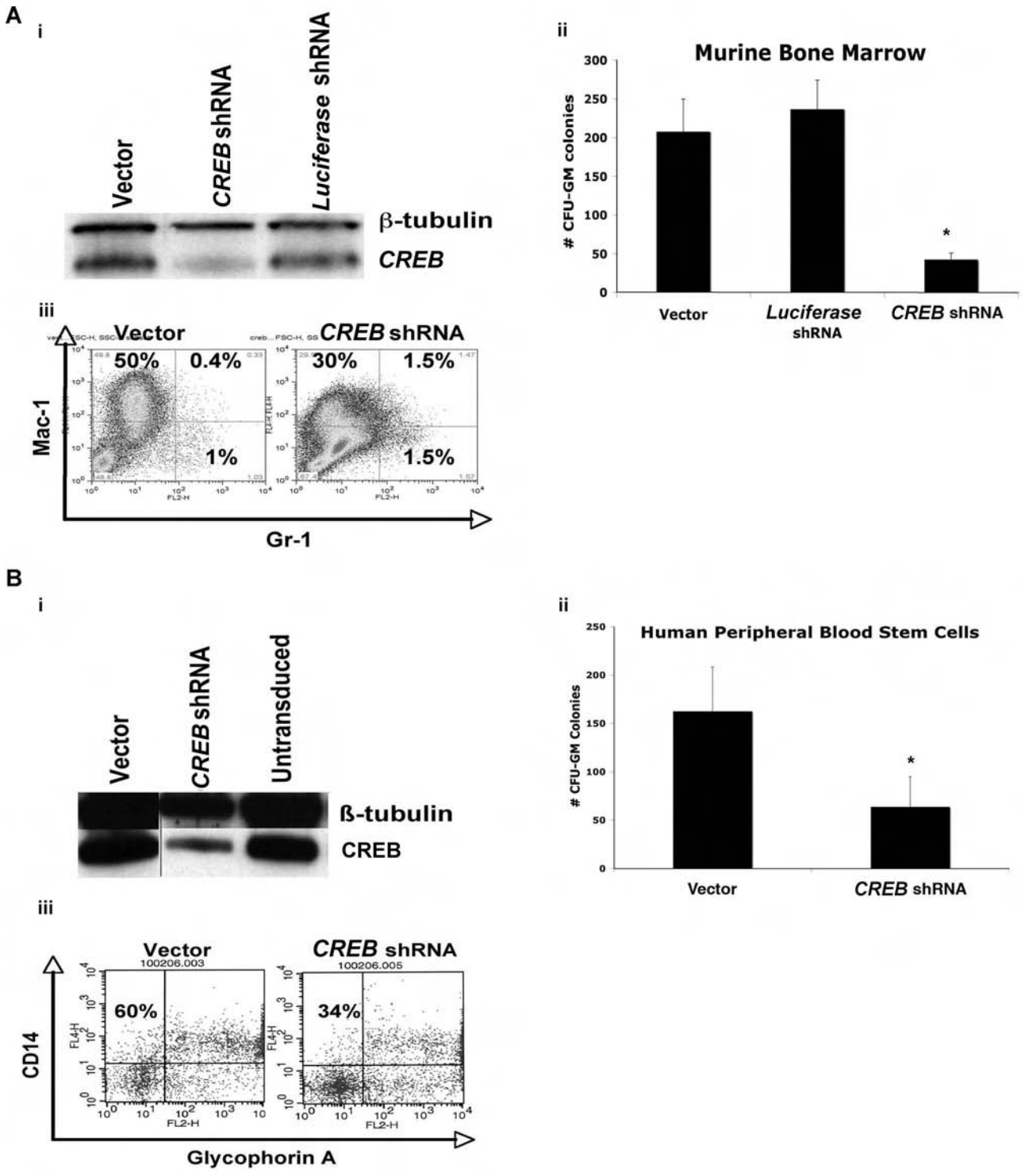

Figure 2. CREB is critical for normal myelopoiesis in vitro. (A) (i) Western blot analysis demonstrating knockdown of *CREB* approaching 80% compared with control cells. (ii) Total numbers of CFU-GM colonies after 21 days in methylcellulose for murine hematopoietic cells. (iii) Flow cytometric analysis of murine bone marrow transduced with *CREB* shRNA or control lentivirus and sorted for GFP$^+$ fraction, cultured in methylcellulose over 21 days. *CREB* knockdown cells had a lower fraction of mature granulocyte and monocytes compared with control cells. (B) (i) Western blot analysis demonstrating knockdown of *CREB* up to 65% compared with control cells. (ii) Total number of CFU-GM colonies after 21 days in methylcellulose for human peripheral blood stem cells. (iii) Flow cytometry analysis of transduced CD34$^+$ human peripheral blood stem cells cultured in methylcellulose over 21 days. All experiments were performed in triplicate. Error bars in Aii,Bii represent SE.

6A). Thus, we hypothesized that removing the CREB-dependent component of Bcr-Abl T315I-induced transformation pathways could affect leukemia progression. Transduction of Ba/F3 Bcr-Abl T315I cells resulted in an 80% decrease in CREB protein and mRNA expression (Figure 6B,C). Six-week-old SCID mice were injected with either 10^6 or 5×10^5 Ba/F3 *Bcr-Abl* wild-type or T315I transduced cells also expressing luciferase (kindly provided to us by Neil Shah, University of San Francisco, CA).[37,38] Mice were analyzed after each luciferin injection 15 minutes before imaging at days 7 and 14. A modest effect on proliferation was also noted in vitro after *CREB* knockdown in Ba/F3 T315I cells and analysis using trypan blue

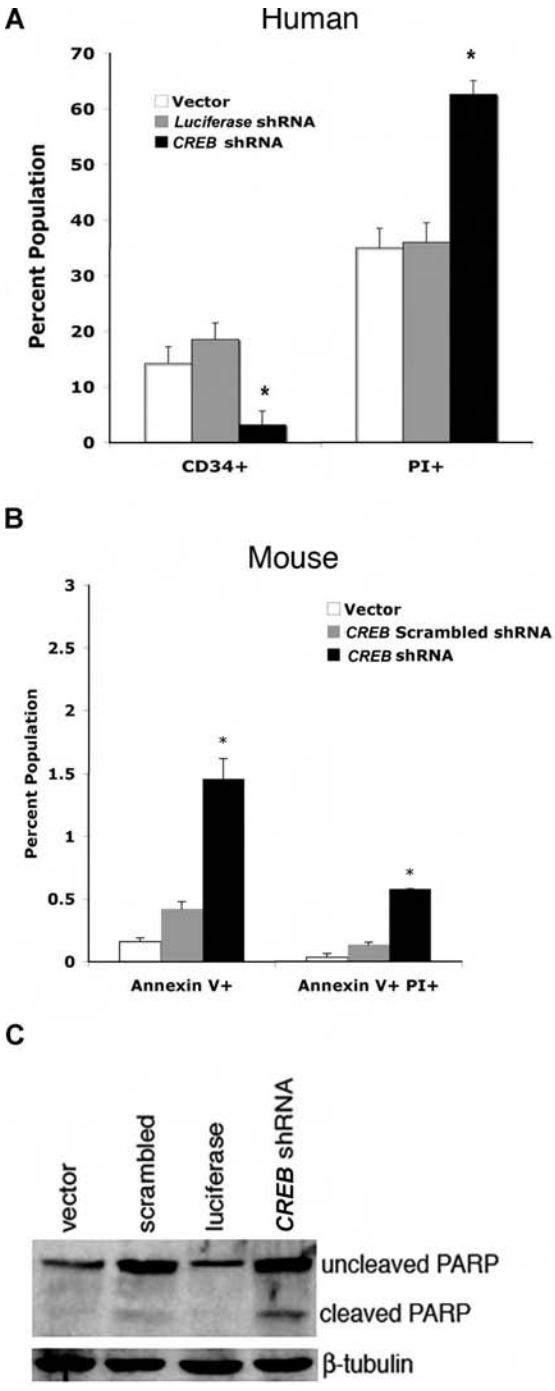

Figure 3. *CREB* shRNA induces apoptosis in HSCs and bone marrow progenitor cells. (A) Transduced human peripheral blood cells plated in methylcellulose for 3 weeks and stained with monoclonal antibodies for CD34 and CD38 expression. Cells were stained with PI to assess cell death. (B) Murine bone marrow cells were transduced at a density of 10^6 cells/mL with lentivirus expressing *CREB*, scrambled, or vector shRNA at a multiplicity of infection (MOI) of 100. After 2 days of culturing in media containing cytokines (mIL-3, 10ng/mL; mSCF, 25 ng/mL; and hIL-6, 10 ng/mL), cells were sorted using flow cytometry for GFP expression. Sorted calls were cultured in cytokine containing media for 5 days and stained for annexin-V and PI. All experiments were performed in triplicate. (C) Western blot analysis with lysates from mouse BM cells (10^6) transduced with *CREB* shRNA, scrambled, luciferase, and vector control lentivirus. Immunoblots were probed with anti-PARP or β-tubulin antisera. Error bars in panels A and B represent SE.

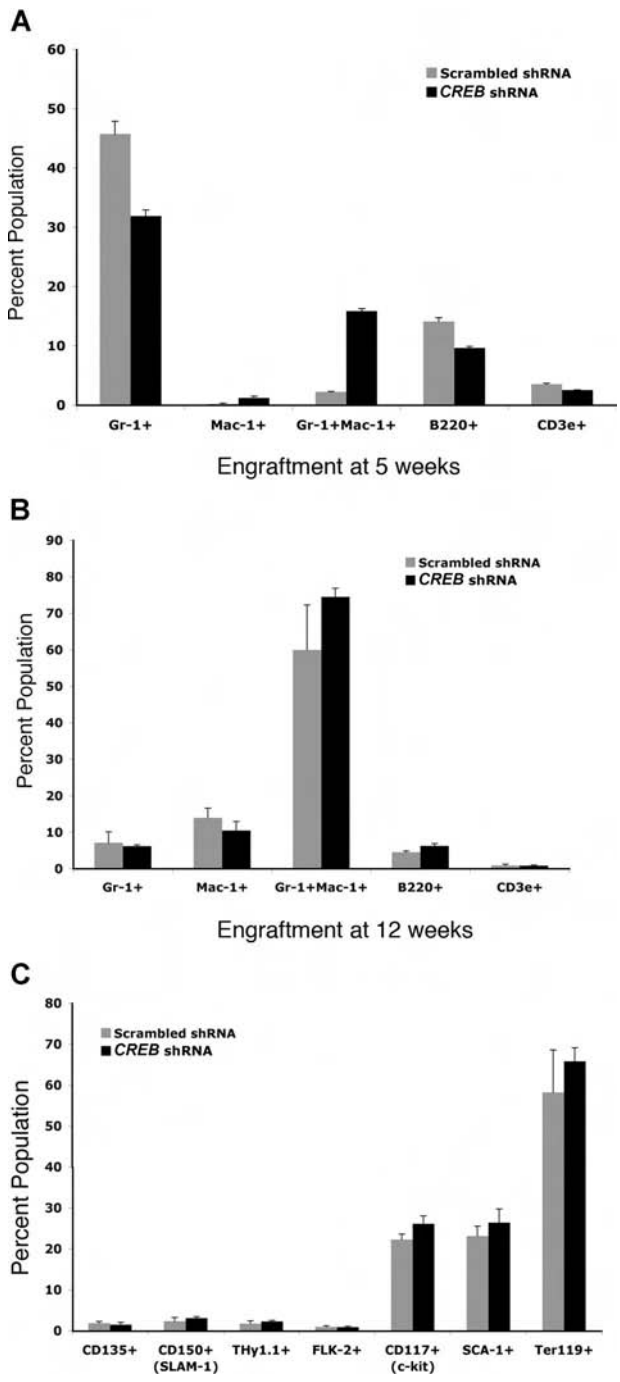

Figure 4. CREB is critical for myelopoiesis in vivo. Bone marrow from CD45.1 mice transplanted into CD45.2 mice were analyzed with lineage-specific and hematopoietic stem-cell markers using FACS analysis at 5 and 12 weeks after transplantation. (A) Myeloid engraftment as measured by staining of bone marrow cells from transplant-recipient mice at 5 weeks. (B) Myeloid engraftment as measured by FACs staining of bone marrow cells at 12 weeks. At least 5 mice in each group were analyzed. Experiments were performed in triplicate and repeated twice. Error bars represent SE.

exclusion (Figure 6D). Bioluminescence imaging performed on days 7 and 14 demonstrated a significant decrease in disease burden in *CREB* knockdown mice with Ba/F3 *Bcr-Abl* T315I cells compared with mice injected with scrambled shRNA construct or Ba/F3 *Bcr-Abl* wild-type cells transduced with control shRNA (Figures 6E,S1). This was confirmed by comparing quantified bioluminescence intensities between comparable regions of interest. For *Bcr-Abl* T315I-transduced cells, geometric mean bioluminescent intensity in control mice (scrambled shRNA) was 3.4-fold larger than the knockdown group at day 7 and 4.9-fold at day 14 (95% confidence intervals, 2.1- to

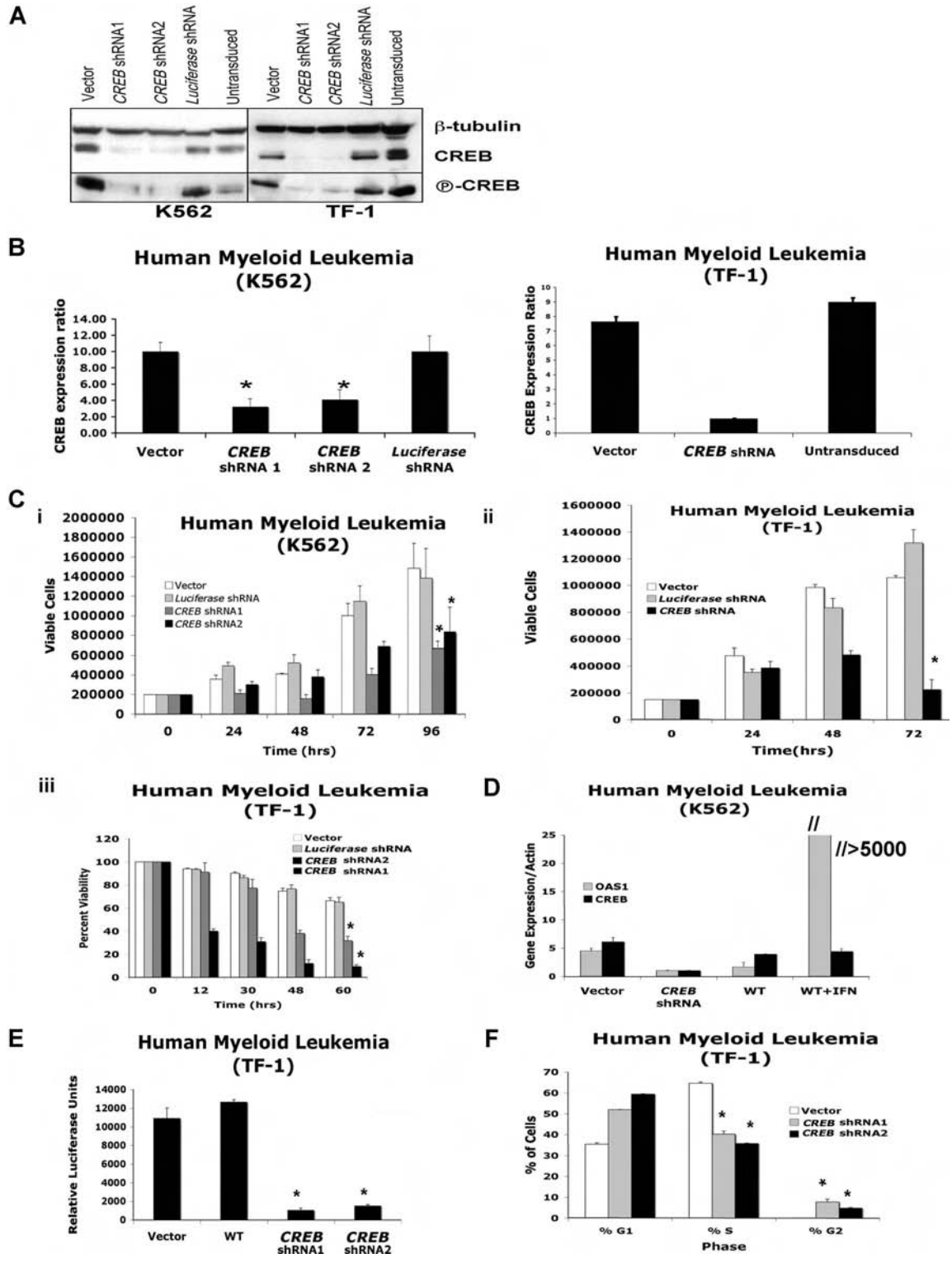

Figure 5. CREB is essential for leukemia cell proliferation and survival. (A) Human (K562, TF-1) leukemia cells were transduced with a lentivirus expressing no shRNA,[1] *CREB* shRNA-1,[2] *CREB* shRNA-2,[3] or *luciferase* shRNA[4] at a multiplicity of infection (MOI) of approximately 100. Wild-type cells[5] were also used as a control. Western blot analyses were performed with CREB, phospho-CREB, and β-tubulin antisera. (B) Five micrograms of total RNA were extracted from transduced leukemia cells, and q-PCR was performed to determine CREB expression. *CREB* was knocked down by up to 75% relative to control shRNA (vector) in human myeloid leukemia cells. (C) Trypan blue exclusion method was performed in triplicate to assess growth and survival of transduced leukemia cells. *CREB* knocked-down cells demonstrated diminished proliferation and viability 72 hours after transduction. (D) K562 cells were transduced and cultured for 48 hours before harvesting total RNA. Parental K562 cells were cultured in the presence of interferon-2α (100 units/mL) for 48 hours as a positive control. Quantitative reverse transcription-PCR was performed in triplicate with primers specific for *CREB*, *actin*, and *OAS-1*. (E) Luciferase reporter assays in human TF-1 leukemia cells transduced with *CREB* or control shRNAs. Decreased transcriptional activity was observed in *CREB* knocked-down cells and repeated in triplicate. (F) Cell-cycle analysis of *CREB* knocked-down TF-1 cells after synchronization by serum starvation overnight and stimulated for 12 hours with GM-CSF revealed decreased percentage of cells in S-phase. Experiment was performed in triplicate. Error bars in panels B-F represent SE.

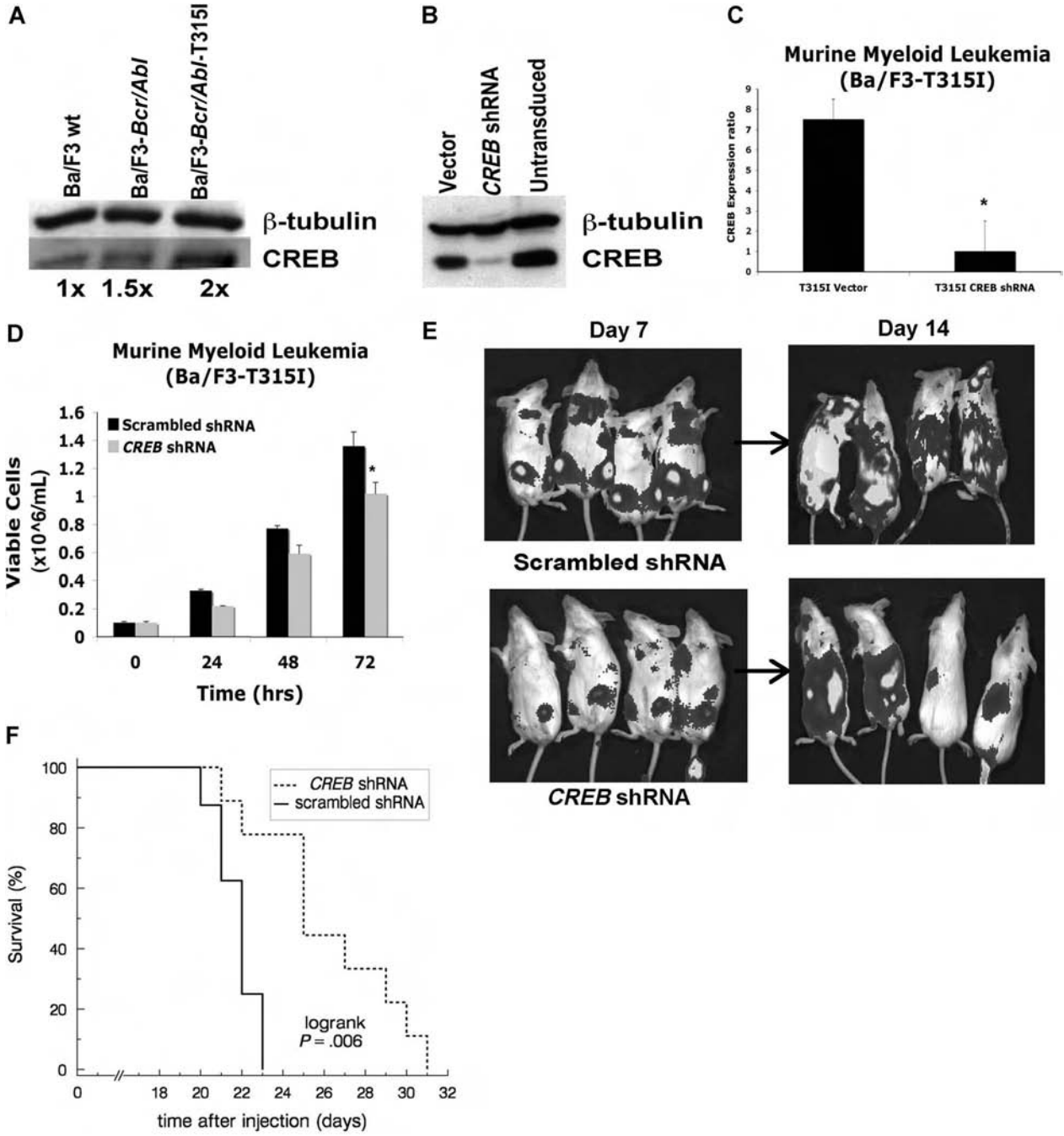

Figure 6. CREB inhibits progression of leukemia in vivo. (A) Western blot analysis with CREB and tubulin antisera, demonstrating 2-fold increase in expression of CREB in T315I mutant of *Bcr-Abl* in murine pro-B lymphocyte line (Ba/F3) compared with wild-type Ba/F3 cells. (B) Western blot analysis after lentiviral transduction with *CREB* shRNAs demonstrating 90% inhibition. (C) Quantitative reverse transcription-PCR showing diminished *CREB* mRNA levels in transduced Ba/F3 T315I cells. (D) Trypan blue exclusion method performed in triplicate shows diminished growth after transduction with *CREB* shRNA compared with empty vector. (E) Bioluminescence imaging of SCID mice injected with 10^6 cells transduced with *CREB* shRNA or *CREB* scrambled shRNA lentivirus. Mice were imaged at days 7 and 14. Tumor burden is lower in *CREB* shRNA–injected mice. (F) Kaplan-Meier survival analysis of mice injected with 5×10^5 cells showing longer survival with *CREB* knockdown ($n = 9$) compared with scrambled shRNA ($n = 9$). All deaths were due to leukemia, except for a day 7 handling event in the scrambled group treated as a censored observation. Error bars in panels C and D represent SE.

5.4-fold and 3.1- to 7.9-fold, respectively). Flow cytometric analysis of splenocytes from injected mice confirmed the presence of GFP^+ cells (data not shown). Bioluminescence in control cells was similarly elevated above knockdown using unmutated *Bcr-Abl* transduced cells (Figure S1). Furthermore, Kaplan-Meier analysis demonstrated a 3-day increase in median survival time for mice injected with Ba/F3 T315I cells transduced with *CREB* shRNA compared with scrambled shRNA at both 5×10^5 cells injected (Figure 6F, $P = .006$) and 10^6 cells (data not shown, $P = .014$ by logrank test). For mice injected with 10^6 Ba/F3 *Bcr-Abl* wild-type cells, there was a 4-day increase in median survival using *CREB* shRNA transduction compared with scrambled shRNA (Figure S1, $P = .09$ by Wilcoxon-Mann-Whitney test).

To understand possible downstream pathways mediating the effects of *CREB* down-regulation, including cell-cycle abnormalities, we sought to determine the levels of known CREB target genes, *cyclins D* and *A*, which both affect cycle progression

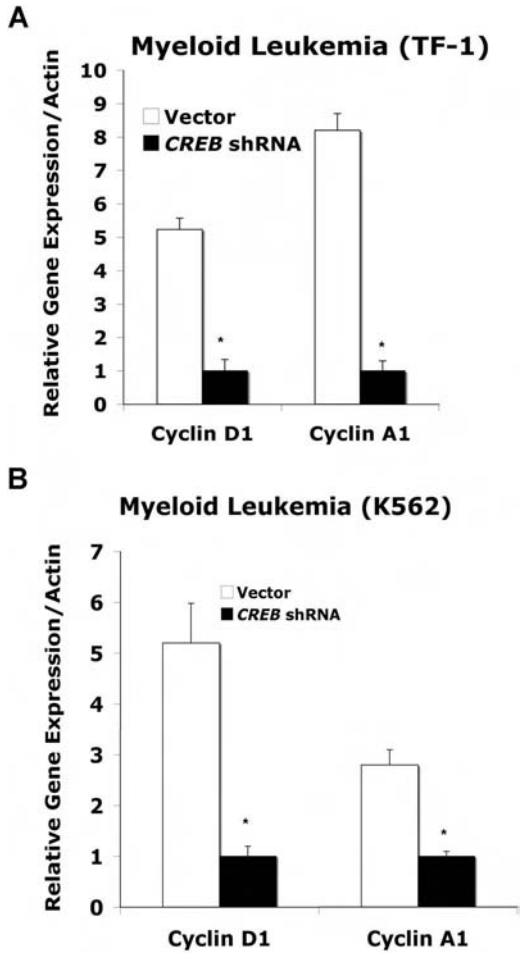

Figure 7. Expression of cyclins A and D in leukemia cells. TF-1 (A) or K562 (B) myeloid leukemia cells were transduced with control and *CREB* shRNA lentivirus and synchronized. At 12 hours, 5 μg of total RNA was isolated for q-PCR by SyberGreen method. Cyclin A1- and D1-specific primers were used, and expression was normalized to the house keeping gene β-actin. Experiments were performed in triplicate. Error bars represent SE.

from G_1 to S. Cyclin A also regulates the G_2/M transition.[1] *Cyclin D* is known to be a direct target of CREB by serial analysis of gene expression (SAGE).[39] Cyclin A has been reported to be induced by CREB and is a direct target gene of CREB.[40] We observed a 5-fold decrease in *cyclin D* mRNA expression in TF-1 and K562 cells transduced with *CREB* shRNA compared with control cells (Figure 7A,B, $P < .05$).[41] We also observed decreased *cyclin A* mRNA levels in *CREB* shRNA–transduced TF-1 and K562 cells (Figure 7A,B, $P < .05$). These results suggest that one possible reason for increased numbers of cells in G_1 and G_2/M in *CREB* shRNA–transduced leukemia cells is the inhibition of known target genes, such as cell-cycle proteins, *cyclin D*, and *cyclin A*.

Discussion

We report that *CREB* can be successfully down-regulated through lentiviral transduction in murine and human primary stem cells and leukemia cell lines in vitro and in vivo. Our work demonstrates that CREB is an important regulator of stem-cell proliferation, survival, and differentiation during normal hematopoiesis. These observations are consistent with previous work suggesting that CREB is critical for neuronal differentiation and survival.[42] Our results are also consistent with previous observations that when overexpressed, CREB acts as a proto-oncogene, resulting in increased myeloid cell proliferation and myeloproliferative disease.[8,43] In this article, we show that inhibition of *CREB* expression leads to abnormal proliferation, survival, and cell-cycle regulation of normal HSCs and leukemia cells.

Analysis of CREB expression in normal HSC populations support a role for CREB in uncommitted progenitors, because lineage-negative stem cells have higher levels of CREB expression compared with lineage-positive cells. In colony assays, *CREB* shRNA had a more dramatic effect on murine HSC proliferation than differentiation. Furthermore, there is an increase in less mature Gr-1$^+$ /Mac-1$^+$ cells compared with differentiated Gr$^+$ 1 or Mac-1$^+$ cells. It is noteworthy that the effects of *CREB* shRNA, as shown in colony assays, seemed to affect monocyte differentiation more than granulocyte differentiation. Reduced proliferation of human peripheral blood CD34$^+$ CD38$^-$ cells transduced with *CREB* shRNA suggests the dependence of these cells on CREB. *CREB* shRNA–transduced human peripheral blood CD34$^+$ CD38$^-$ HSCs also appeared to undergo apoptosis more readily than CD34$^+$ CD38$^+$ HSCs. Although *CREB* down-regulation resulted in increased apoptosis, this does not seem to be a major factor in the role of CREB in differentiation. Future studies will focus on analysis of target genes and pathways that regulate hematopoietic differentiation and proliferation downstream of *CREB*.

CREB also affected early engraftment of normal HSCs in lethally irradiated mice. There were more immature myeloid cells that were Gr-1/Mac-1$^+$ at 5 and 12 weeks after transplantation (although more dramatic at 5 weeks), which is consistent with in vitro colony assays. One explanation for this discrepancy is that the CFU-GM colonies were counted after 2 weeks, whereas cells from mice were analyzed after 5 and 12 weeks. The numbers of terminally differentiated Gr-1$^+$ or Mac-1$^+$ cells were the same in *CREB* and control shRNA-transduced HSCs at 5 and 12 weeks after transplant. It is possible that other CREB family members (eg, ATF2) are able to compensate for the decreased levels of CREB in myeloid cells transduced with *CREB* shRNA. Furthermore, the overall survival of mice that received transplants was not affected by *CREB* shRNA. These results suggest that, in vivo, additional mechanisms or redundant pathways could be responsible for regulating HSCs under stress conditions and that CREB is not required for early hematopoietic reconstitution.

CREB seems to regulate leukemia cell proliferation in vitro and in vivo. Our ability to successfully suppress but not completely inhibit the growth of several leukemia cell lines suggests that CREB is one of several transcriptional factors or signal transduction pathways driving leukemic proliferation. *CREB* shRNA not only affected proliferation, but also phosphorylation and transcriptional activity of *CREB* in acute myeloid leukemia cells. Our results demonstrate that CREB is necessary for maximal proliferation of myeloid leukemia cells in vitro and that *CREB* is probably one but not the only critical target of signaling pathways regulating growth of these cells. CREB may ultimately function in a role analogous to C/EBPα and PU.1, which have been shown to be critical in early myeloid hematopoiesis and leukemia.[44]

We observed that *CREB* shRNA in vivo inhibited early leukemic progression but did not significantly prolong maximal survival. This may be due in part to the aggressive nature of Bcr-Abl wild type and T315I mutation expressed in Ba/F3 cells injected into SCID mice. Our results also suggest that CREB-dependent signaling pathways are critical for *Bcr-Abl* T315I cell proliferation. The

time course of splenic and bone marrow infiltration was delayed with *CREB* shRNA–transduced cells compared with control cells. This suggests the possibility that CREB may be affecting initial homing or engraftment of leukemia cells in the spleen and bone marrow. However, the pattern of progression (ie, organs infiltrated) appeared to be the same between *CREB* shRNA and control cells. Alternatively, CREB may be slowing down the initial growth of cells in mice, resulting in a longer latency period for the leukemia to kill the mice. Experiments are in progress to identify the pathways regulating leukemic progression of Bcr-Abl–expressing cells.

Our results demonstrated that CFU-GM was decreased in mouse bone marrow progenitor colony assays transduced with *CREB* shRNA. In the transplantation experiments, the mice had increased Gr-1^+ /Mac-1^+ cells with *CREB* shRNA. Except for the Mac-1 population in colony assays, *CREB* shRNA had minimal effect on terminal differentiation of myeloid and lymphoid populations in vitro and in vivo (Figure 4 and data not shown). This could reflect the contribution of CREB in monocyte differentiation, under the specific culture conditions. The differences in the mouse bone marrow colony assays at day 14 and knockdown of *CREB* did not seem to affect hematopoietic reconstitution after 12 weeks and only minimally induced apoptosis in progenitor cells. In contrast, myeloid leukemia cells transduced with CREB shRNA had significant inhibition in growth and increased apoptosis. Taken together, our data suggest that CREB may be a viable target for leukemia therapy.

CREB knockdown resulted in aberrant progression from G_1 to S and exit from G_2/M to G_1, suggesting a critical link between CREB and the cell-cycle machinery in normal HSCs and leukemia cells. Known CREB target genes include *cyclin D1*, which activates G_1/S cyclin-dependent kinases Cdk4 and Cdk6.[39] Indeed, our results demonstrated that *CREB* knockdown inhibited cyclin D1 expression, which could provide a mechanism by which cells arrest in G_1 and fail to progress to S phase. Likewise, cyclin A1 has a CREB binding site in its promoter, suggesting that cells could fail to progress to S phase or exit from G_2/M as a result of decreased expression of cyclin A and failure to activate Cdk1 and Cdk2. It is noteworthy that CREB transgenic mice develop myeloproliferative disease with dysplastic myeloid cells in the spleen. CREB overexpression in myeloid leukemia cell lines and mouse spleens was associated with increased cyclin A expression and increased percentage of cells in S phase.[9] Thus, it is possible that by being a positive regulator of cyclins D and A, CREB overexpression could lead to genomic instability and ultimately transformation. In this article, we have shown that *CREB* down-regulation led to cell-cycle arrest and effects opposite of those observed with CREB overexpression. The key to understanding the role of CREB is to elucidate the target genes that mediate its effects in proliferation and survival. Future work will focus on the downstream pathways regulating CREB function during normal and aberrant hematopoiesis.

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Authorship

Contribution: J.C. wrote the manuscript and designed and performed experiments. K.K., W.S.W., D.J., and J.C. performed experiments. I.S. helped analyze data, supervised experiments, and edited the manuscript. D.B.S. helped design experiments. N.K. contributed reagents. R.B. edited the manuscript and consulted on project. E.M.L. performed statistical analysis and wrote the manuscript. K.M.S. supervised the research, designed experiments, contributed to analysis, and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Expression profile of CREB knockdown in myeloid leukemia cells

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Abstract

Background. The cAMP Response Element Binding Protein, CREB, is a transcription factor that regulates cell proliferation, differentiation, and survival in several model systems, including neuronal and hematopoietic cells. We demonstrated that CREB is overexpressed in acute myeloid and leukemia cells compared to normal hematopoietic stem cells. CREB knockdown inhibits leukemic cell proliferation *in vitro* and *in vivo*, but does not affect long-term hematopoietic reconstitution.

Methods. To understand downstream pathways regulating CREB, we performed expression profiling with RNA from the K562 myeloid leukemia cell line transduced with CREB shRNA.

Results. By combining our expression data from CREB knockdown cells with prior ChIP data on CREB binding we were able to identify a list of putative CREB regulated genes. We performed extensive analyses on the top genes in this list as high confidence CREB targets. We found that this list is enriched for genes involved in cancer, and unexpectedly, highly enriched for histone genes. Furthermore, histone genes regulated by CREB were more likely to be specifically expressed in hematopoietic lineages. Decreased expression of specific histone genes was validated in K562, TF-1, and primary AML cells transduced with CREB shRNA.

Conclusions. We have identified a high confidence list of CREB targets in K562 cells. These genes allow us to begin to understand the mechanisms by which CREB contributes to acute leukemia. We speculate that regulation of histone genes may play an important role by possibly altering the regulation of DNA replication during the cell cycle.

Background

Several proto-oncogenes have been demonstrated to be deregulated in human cancer. In particular, the development of the hematologic malignancies such as leukemia, is associated with aberrant expression or function of proto-oncogenes such as c-myc, evi-1, and c-abl. Many translocations with cytogenetic abnormalities that characterize leukemias involve rearrangement of transcription factors, including AML-ETO and Nup98-hox. Some of these leukemia-associated fusion proteins predict prognosis, e.g. t(8,21), t(15,17), and inv(16) are associated with a good prognosis in acute myeloid leukemia (AML) [1]. Approximately 50% of adult patients have been noted to have specific cytogenetic abnormalities. The overall survival of patients with AML is less than 50%. Since half of the patients diagnosed with AML have normal cytogenetic profiles, it is critical to understand the molecular pathways leading to leukemogenesis.

We identified that the cyclic AMP Response Element Binding Protein (CREB) was overexpressed in the majority of bone marrow samples from patients with acute leukemia [2, 3]. CREB is a leucine zipper transcription factor that is a member of the ATF/CREB family of proteins [4-6]. This transcription factor regulates proliferation, differentiation, and survival in a number of cell types, including neuronal and hematopoietic cells [4, 5]. CREB has been shown to be critical in memory and hippocampal development in mice [7, 8]. We previously described that CREB is phosphorylated at serine 133 downstream of signaling by the hematopoietic growth factor, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) in myeloid cells [9-11]. We further demonstrated that CREB phosphorylation results from the activation of the Mitogen Activated Protein

Kinase (MAPK) and pp90 Ribosomal S6 Kinase (pp90RSK) pathways in response to GM-CSF stimulation [9].

To understand the role of CREB in normal and neoplastic hematopoiesis we investigated the expression of CREB in primary cells from patients with acute lymphoblastic (ALL) and myeloid leukemia and found that CREB was overexpressed in the majority of leukemia cells from patients with ALL and AML at the protein and mRNA levels [2, 3, 12]. Furthermore, overexpression of CREB was associated with a worse prognosis. We created CREB transgenic mice that overexpressed CREB in myeloid cells. These mice developed enlarged spleens, high monocyte count, and preleukemia (myeloproliferative disease) after one year. Bone marrow progenitor cells from CREB transgenic mice had increased proliferative capacity and were hypersensitive to growth factors compared to normal hematopoietic stems cells (HSCs). Overexpression of CREB in myeloid leukemia cell lines resulted in increased proliferation, survival, and numbers of cells in S phase [12]. Known target genes of CREB include the cyclins A1 and D [4, 5, 12, 13]. Both of these genes were upregulated in CREB overexpressing cells from mice and human cell lines [4, 5]. Thus, CREB is a critical regulator of leukemic proliferation and survival, at least in part, through its downstream target genes.

CREB target genes have been published on the website developed by Marc Montminy http://natural.salk.edu/CREB/ based on ChIP/chip data [14]. Additional CREB target genes were described by Impey et al. [15]. In their studies, serial analysis of chromatin occupancy (SACO) was performed by combining chromatin immunoprecipitation (ChIP)

with a modification of Serial Analysis of Gene Expression (SAGE). Using a SACO library derived from rat PC12 cells, approximately 41,000 genomic signature tags (GSTs) were identified that mapped to unique genomic loci. CREB binding was confirmed for all loci supported by multiple GSTs. Of the 6302 loci identified by multiple GSTs, 40% were within 2 kb of the transcriptional start of an annotated gene, 49% were within 1 kb of a CpG island, and 72% were within 1 kb of a putative cAMP-response element (CRE). A large fraction of the SACO loci delineated bidirectional promoters and novel antisense transcripts [15]. These studies suggest that CREB binds many promoters, but only a fraction of the associated genes are activated in any specific lineage. We therefore set out to measure the functional targets of CREB in a hematopoietic model system.

Since CREB is overexpressed in bone marrow cells from patients with acute leukemia compared to normal HSCs, this provides a potential target for leukemia therapy. To this end, we stably transduced myeloid leukemia cells with CREB shRNA lentivirus [16]. CREB knockdown by 80% resulted in decreased proliferation and differentiation of both normal myeloid cells and leukemia cells *in vitro* and *in vivo* [16]. However, downregulation of CREB did not affect short-term or long-term engraftment of normal HSCs in bone marrow transplantation assays [16]. To understand the pathways downstream of CREB, we investigated genes that were differentially regulated in CREB shRNA transduced cells. In this paper, we report expression profiling of genes that weredifferentially regulated in CREB knockdown K562 myeloid leukemia cells and could be potential targets for development of new therapies for acute leukemia.

Methods

Cell lines. The following human leukemia cell lines were transduced with shRNAs: K562 (Iscoves + 10% FCS) and TF-1 (RPMI + 10%FCS + rhGM-CSF. Cells were cultured at 37^{o}C, 5% CO2 and split every 3 to 4 days. Primary AML bone marrow samples were processed as previously described [12]. All human samples were obtained with approval from the Institutional Review Board and consents were signed, according to the Helsinki protocol.

shRNA sequence design and constructs. The CREB specific shRNA sequences were selected and validated based on accepted parameters established by Tuschl et al. [17-19](http://www.rockefeller.edu/labheads/tuschl/sirna.html); CREB shRNA-1, CREB shRNA-2, CREB shRNA-3. Controls included empty vector, luciferase shRNA, and scrambled shRNA. shRNA sequences are: CREB shRNA-1(5'GCAAATGACAGTTCAAGCCC3'), shRNA-2 (5'GTACAGCTGGCTAACAATGG3'), shRNA-3 (5'GAGAGAGGTCCGTCTAATG3'), Luciferase shRNA (5'GCCATTCTATCCTCTAGAGGA3'), Scramble shRNA (5'GGACGAACCTGCTGAGATAT3'). Short-hairpin sequences were synthesized as oligonucleotides and annealed according to standard protocol. Annealed shRNAs were then subcloned into pSICO-R shRNA vectors from the Jacks laboratory at MIT [20]. The second generation SIN vector HIV-CSCG was used to produce human shRNA vectors [21].

Microarray analysis. Total RNA (10 μg) was extracted from K562 cells transduced with vector alone or CREB shRNA was submitted to the UCLA DNA Microarray Facility. RNA samples were labeled and hybridized by standard protocol to Affymetrix GeneChip Human Genome U133+ Array Set HG-U133A array. Gene expression values were calculated using the MAS5 software. The expression values are quantile normalized across all arrays. We obtained the expression profiles for a control set and CREB downregulated K562 cells. These are first quantile normalized, and then a t-test is performed between the two groups to identify significantly differentially regulated genes. The analysis was performed using Matlab (Mathworks, Inc.). We find a significant number of differentially expressed genes, which are either direct or indirect targets of CREB.

To further characterize the data we have aligned CREB binding data from chromatin immunoprecipitation studies with our expression data. The chromatin immunoprecipitation data was obtained from the website http://natural.salk.edu/CREB/[14]. To identify genes that are most significantly bound by CREB and differentially expressed in our knockdown experiment we first filtered genes by their fold change (greater than 1.5 or less than 0.7). Finally, we ranked genes according to the product of the binding and expression P value (jerry_bind_data.xls) (see Additional file 1).

We characterize these genes using three types of analyses: Ingenuity Pathway Analysis (IPA), Gene Ontology term enrichment analysis and tissue distribution. For the former

analysis, we used the Ingenuity Pathways Analysis tool on the lists of significant downregulated genes. We then identified functions that were overrepresented among these genes. For the second, we used the DAVID website (http://david.abcc.ncifcrf.gov/home.jsp) to identify Gene Ontology terms that were enriched in the list.

Finally, we show the tissue distribution of the 20 genes we identified as functional CREB targets. The tissue specific expression profiles of each gene are obtained from *HG_U133A/GNF1H and GNF1M Tissue Atlas Datasets.*[22].We display the results in figure clustergram.tiff (Figure 3). To obtain this figure we first compute the logarithm of the ratio of the expression intensity of each gene in each tissue, divided by its average intensity across all tissues. We then perform hierarchical clustering of both the genes and the tissues.

Quantitative Real time. K562 transduced with CREB shRNA (5×10^6) were lysed in Trizol and stored at -80^{o}C prior to RNA extraction. RNA extraction was performed according to a standard protocol supplied by the manufacturer (Invitrogen) and pellets were resuspended in RNAse free water. The cDNA was transcribed with a Superscript RT III based-protocol. DNAse treatment was not performed due to the selection of intron-spanning primers. Quantitative real-time PCR was performed with the SyberGreen reagent (Bio-Rad) in triplicates and analyzed by the standard curve method standardized to the housekeeping gene beta actin [23, 24].

Results and Discussion

Since CREB has pleiotropic effects on cell function and potentially activates several genes in hematopoietic and leukemia cells, we performed microarray analysis with total RNA isolated from K562 chronic myeloid leukemia cells transduced with CREB or control shRNA. The comparison of transcriptional profiles in wild type and CREB shRNA transduced K562 cells revealed a large number of differentially expressed genes (see Additional file 2). Among these genes, some are direct targets of CREB, while others are indirect targets. To infer which of these genes was potentially directly regulated by CREB, we combined the expression data with the ChIP-chip data of CREB bound promoters as demonstrated by Marc Montminy [14]. As was previously observed CREB binding sites are highly conserved across different tissues. However, these sites are activated by cAMP in a tissues specific manner. Therefore by combining these two datasets we attempted to uncover the functional CREB sites in hematopoietic tissues.

Our hypothesis for discovering functional CREB sites in hematopoietic cells is that if a gene is found to be differentially expressed in the CREB shRNA K562 transduced cells, and bound by CREB it is likely to be a direct target. To identify these genes we developed a metric that accounts for both the significance of the expression change and binding data for each gene (described in detail in Methods).

Since CREB has been described as both a transcriptional activator (when phosphorylated) and a repressor, we were interested in genes that were both up and downregulated in CREB shRNA transduced cells. The resulting rank ordered list allows us to sort genes by their likelihood of being functional CREB targets in K562 cells. It is difficult to

determine, however, where to draw a threshold between the true and false targets. We have decided to restrict our analysis to the top several hundred targets that had both significant changes in expression and binding, as we deemed these to be highly enriched for true versus false targets. However, we do not claim that these are the only functional CREB targets in K562 cells, as the exact number of true targets is difficult to determine. The top down and upregulated genes revealed by this analysis are listed in Tables 1 and 2, and the full list is found in the supplementary materials.

Genes withinthe downregulated list were BECLIN 1, UBE2B. Both these genes have a cAMP responsive element binding site(s) in their promoters. These genes were selected for further validation because they are known to be involved in autophagy/apoptosis (BECLIN 1), cell cycle/DNA repair (UBE2B)[25-28]. Quantitative real time-polymerase chain reaction (qRT-PCR) with mRNA from AML cell lines (K562 and TF-1) and primary leukemic blasts from a patient with M4-AML was performed. UBE2B expression was significantly reduced in CREB shRNA transduced TF-1 and K562 myeloid leukemia cells compared to controls (Figure 1, $p<0.05$). BECLIN and UBE2B were downregulated in primary AML cells transduced with CREB shRNA (Figure 1, $p<0.05$).

Having confirmed the validity of our microarray results in these two test cases we set out to characterize the function of the complete list of CREB target genes using two annotation schemes. The first utilizes the annotation contained in the Ingenuity Pathway Analysis software (IPA). This analysis showed that there is a significant enrichment for cell cycle ($P < 1e-3$) and cancer ($P < 1e-3$) genes. The full list of genes associated with

cancer is shown in Table 3. Many of these genes regulate cell cycle, signaling, DNA repair, or metabolism, which are consistent with previously published results [5, 15]. Furthermore, the role of CREB in the pathogenesis of leukemias has also been described in the literature [2, 3, 12, 29].

IPA also allows us to study CREB target genes in the context of protein-protein interactions networks. A network for downregulated genes interacting with CREB is shown in Figure 2, with a subset of the downregulated targets shown in grey, while other genes not in the target list that interact with these, shown in white. Here we see that there is prior literature supporting our analysis that CREB1 regulates PTGS2 (COX2), NR4A3 and TOM1, as depicted by the blue lines. Interestingly, COX2 is an important drug target, and suggests that commonly used COX2 inhibitors may provide a target for acute leukemia.

The second analysis that we performed used the terms from Gene Ontology to identify common characteristics among the top K562 CREB targets. Here we find the striking and unexpected result that ten percent of the downregulated targets code for histone genes ($P < 1e\text{-}10$, Table 4). We also performed an analysis of the top upregulated genes but did not find any significant GO terms.Although there is some prior literature indicating that CREB or CREB-related pathways may play a role in regulating histone modifications primarily through the histone deacetylase, CREB Binding Protein (CBP)[5, 30, 31], the fact that CREB directly regulates the transcription of histone genes in these cells is unexpected.

To further validate the hypothesis that CREB is an activator of these 20 histone genes, we utilized previously published analyses of the gene promoters to identify consensus CREB binding sequences. The results shown in Table 1 demonstrate that nearly all the histone genes contain CREB half sites along with a TATA box in the vicinity of these. Thus three lines of evidence support the assignment of these 20 histone genes as CREB targets in K562 cells: expression, binding and sequence based.

We examined the distribution of expression of these 20 histone genes across human tissues. The expression data were obtained from the GNF body atlas. We were able to extract expression profiles for 81 histone genes contained in the human genome. Fifteen of these overlapped with the 20 histone CREB targets. We show the expression of all 81 histone genes in Figure 3, where the identity of 15 CREB target genes is shown in the last row. We see that the 15 genes are clustered into two groups containing more than one gene, with a third group consisting of a single histone HIST1H1C. One of the groups contains histones that are broadly expressed across human tissues, and particularly in all hematopoietic tissues. The second group is instead expressed in a very narrow range of tissues including K562 cells, bone marrow, prostate and thymus.

We examined the expression of three histones that are putative targets of CREB by real time PCR with mRNA from K562, TF-1, and primary cells from patients with AML. The three histones selected were based on our microarray analyses. Our results demonstrated a statistically significant decrease in histones HIST1H2Bj, HIST1H3B, and

HIST2H2AA in K562 and TF-1 cells (Figure 4). Interestingly, in primary cells from a patient with AML, only HIST1H3B and HIST2H2AA, but not HIST1H2BJ expression was decreased with CREB knockdown. These results suggest that histones are differentially expressed in AML and that specific histones are potential targets of CREB.This analysis supports the hypothesis that CREB regulates a subset of histone genes that are normally expressed in a small set of rapidly dividing tissues. These genes are presumably aberrantly activated in K562 and other leukemia cells, and could potentially contribute to the malignant phenotype.

Conclusions

We have identified a high confidence list of CREB target genes in K562 myeloid leukemia cells. Several important CREB target genes that function in DNA repair, signaling, oncogenesis, and autophagy were identified. These genes provide potential mechanisms by which CREB contributes to the pathogenesis of acute leukemia. Expression of the genes beclin-1 and ube2b was found to be decreased in myeloid leukemia cell lines and primary AML cells in which CREB was downregulated. In addition, we speculate that CREB may have more global effects on transcription, primarily through the regulation of histone genes thereby altering the regulation of DNA replication during the cell cycle.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

MP and SFN analyzed the microarray data, performed the statistical analysis, and drafted the manuscript. JCC, JC, DJ, and JT performed the real-time PCR experiments. KMS supervised the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1.

Expression of potential target genes downstream of CREB in myeloid leukemia cells. Primers specific for the ube2b, beclin1, and creb genes were generated and utilized for quantitative real-time PCR by SyberGreen method (Bio-Rad Inc.). Relative gene expression normalized to the housekeeping gene actin is shown for the following transduced cells: (A) K562 myeloid leukemia cells, (B) TF-1 myeloid leukemia cells, and (C) Human AML-M4 blasts.

Figure 2.

A network depicting interactions between direct CREB targets (shown in grey) and proteins that these interact with (shown in white). PTGS2, NR4A3 and TOM1 are direct CREB targets whose regulation by CREB was previously described in the literature (clue lines). PTGS2 (COX2) emerges as a central player in this network, and is thus implicated as a potential regulator of leukemias.

Figure 3

The tissue specific expression of histone genes. Each row of the figure represents a tissue from the GNF Body Atlas (see methods). We show only the top 30 tissues with highest variance of expression of histone genes. Each column represents a histone gene. We use

hierarchical clustering to order the rows and columns according to their similarity. Red indicates that the gene is over expressed relative to its mean expression levels across all tissues, and green that it is under expressed. The histone genes that we identify as direct targets of CREB are shown in red in the last row of the figure. We see that many of these are only expressed in a small subset of rapidly dividing tissues along with K562 cells.

Figure 4

Expression of target histone genes is decreased in CREB knockdown myeloid leukemia cells. Primers specific for HIST1H2BJ, HIST1H3B, and HIST2H2AA were generated and utilized for quantitative real-time PCR by the SYBR Green method (Applied Biosystems). Relative gene expression normalized to the housekeeping gene actin is shown for the following transduced cells: (A) K562 myeloid leukemia cells, (B) TF-1 myeloid leukemia cells, and (C) primary AML cells.

Table 1.

Gene Name	Fold Changh	CREB bindin	CREB site		Gene Name	Fold Changh	CREB bindin	CREB site
DKFZP434G22	0.551725	3.883395	ht h		HSPC056	0.44548	1.892546	ht h
ABCG2	0.479066	2.244422	ht h		HSU79303	0.573524	1.812829	ht
ALDH2	0.5604	1.989872	none		ILVBL	0.675128	1.893295	ht h
ALDH7A1	0.62012	2.051646	h		KIAA0103	0.682528	2.620283	ht h
ALS2CR19	0.46208	1.788188	ht		HSU79303	0.573524	1.812829	ht
ANC_2H01	0.659044	1.991467	ht h		ILVBL	0.675128	1.893295	ht h
ANG	0.693535	3.287977	ht		KIAA0103	0.682528	2.620283	ht h
APLP2	0.636685	1.219917	h		KIAA0141	0.689536	3.479426	h
APPL	0.668234	1.391059	h		KIAA0408	0.595271	3.603389	none
ARFD1	0.524897	2.336962	ht		KIAA0494	0.67838	5.420821	F
BCL2L11	0.589894	3.191337	H h		KLF5	0.553523	2.062499	H
BECN1	0.600243	1.151217	H h		KNSL8	0.468603	7.854334	HT ft
BMX	0.315984	1.072006	none		KPNA5	0.562667	2.859517	none
C20orf133	0.635849	2.420642	h		LANCL1	0.647544	1.020319	none
C6orf67	0.610619	2.665053	h		LOC51668	0.500097	1.062053	ht h
CA2	0.592202	1.082939	ht		LOC51762	0.599397	3.307553	ht h
CALB2	0.671562	1.894443	h		LYPLA3	0.664078	2.379015	HT h
CCDC2	0.533032	1.529166	none		MAF	0.597194	2.383458	FT
CENPE	0.306986	3.736367	FT ht		MAPKAPK5	0.699356	2.053184	FH
CGI-77	0.664435	4.334985	H ht h		MDM2	0.468991	2.523732	none
CLDN18	0.566707	4.30699	ht h		MGC15419	0.617252	3.032433	h
CNN1	0.670957	1.150221	F ht h		MPHOSPH1	0.423771	3.535138	ht h
CREB1	0.382751	1.816762	HT H ht h		MSH2	0.592302	3.203985	h
CSPG6	0.573523	3.082765	h		MVD	0.632896	3.854905	ht h
CUL5	0.683117	2.073118	H ht h		MYL4	0.69963	1.010099	h
DBP	0.67969	2.805267	ft ht		NEFL	0.343403	2.413823	HT h
DES	0.521516	1.509794	ht h		NFKBIL1	0.695019	4.072353	ht
DIS3	0.692573	3.837304	HT ht		NIPSNAP1	0.679129	1.215594	h
DNCI1	0.673721	2.195167	none		NOX3	0.455479	2.60292	h
DNMT3A	0.679821	1.035348	h		NR4A3	0.543361	5.002146	HT H h
DSIPI	0.40458	2.546212	HT		NUDT5	0.673003	2.561752	h
DUSP19	0.674195	2.225933	none		NUMB	0.675667	1.014954	HT ht
EIF2S1	0.631867	1.075696	H ht h		PDE6B	0.66696	2.699363	h
EIF2S2	0.644661	3.313634	ht h		PEX12	0.694707	6.199684	h
ESRRBL1	0.67914	4.633352	FH h		PFDN4	0.507631	2.196535	none
FBXO22	0.688756	2.206273	ht		PHC1	0.672187	1.053985	HT
FECH	0.516446	1.045191	h		PKD2L2	0.513894	2.249593	h
FECH	0.658471	1.045191	h		PLAA	0.603854	9.235476	none
FLJ10853	0.622952	3.981514	H ht		PPP1R2	0.568734	2.04019	ft
FLJ10858	0.668758	1.523113	none		PRDX3	0.615229	1.847784	none
FLJ10904	0.54026	1.085341	none		PSAT1	0.47554	2.492965	ht
FLJ11011	0.610253	3.387879	ht h		PSMAL/GCP	0.68221	1.341117	none
FLJ11342	0.683482	2.617474	ht		PTGS2	0.684401	3.057276	ht h
FLJ11712	0.62618	2.776373	ht		RAB31	0.698664	1.12667	ht
FLJ13491	0.633125	3.268155	none		RB1CC1	0.533475	1.390318	none
FLJ20130	0.640787	2.766588	h		RFC3	0.577787	6.745001	FH ht
FLJ20331	0.681859	8.752576	H		RHEB	0.682202	3.47317	HT H h
FLJ20333	0.690542	1.946262	ht h		RNASE4	0.436168	2.975774	ht h
FLJ20509	0.691949	1.96435	none		SARS2	0.692149	5.455469	H h
FLJ23233	0.471676	1.517415	none		SBBI26	0.683312	6.75719	H
FOXD1	0.593522	5.160553	HT ht		SDP35	0.502432	2.320591	h
GCAT	0.656744	2.122675	ht h		SERPINI1	0.31594	3.277692	ht
GCHFR	0.676365	2.188753	ht h		SHMT1	0.658252	1.127084	ht h
GFI1B	0.671179	0.999255	h		SILV	0.662805	2.130617	H
GMPR	0.672975	1.149663	ht		SLC11A2	0.684325	1.842417	none
GOLGA4	0.567882	2.939327	ht h		SLC22A5	0.657746	1.64513	none
GPNMB	0.410992	1.004344	none		SLC27A6	0.547039	1.029816	ht
GRHPR	0.68706	2.454475	H ht		SLC2A4	0.507466	2.273185	ht h
H2BFS	0.591569	2.358423	ht		SLC39A8	0.201136	1.004832	none
HBE1	0.639376	0.947159	h		SLC4A7	0.532067	1.262531	ht
HDGFRP3	0.65013	1.208322	none		SMARCA1	0.519982	1.056916	HT ht
HDGFRP3	0.668211	1.208322	none		SMC2L1	0.596288	2.916083	ht h
HEXA	0.54467	2.622927	none		SRI	0.671893	0.826457	ht
HIST1H1C	0.590374	1.983514	h		STK16	0.680797	6.555535	H h
HIST1H2AD	0.66909	4.768013	ht h		SULT1C2	0.599235	3.511947	f h
HIST1H2AI	0.542518	2.801688	H ht h		SURB7	0.498245	1.598812	ht
HIST1H2AJ	0.696531	3.066865	ft ht h		SYN1	0.696375	3.016534	F h
HIST1H2AL	0.602018	2.600144	FHT ht h		TAF1A	0.589389	2.689618	none
HIST1H2BB	0.590821	1.782458	ht h		TBC1D7	0.692755	1.281463	ht
HIST1H2BD	0.674855	3.111055	HT ht h		TCTE1L	0.368312	2.475611	ht
HIST1H2BE	0.546621	2.34815	ht		TFDP2	0.670657	1.016413	ht
HIST1H2BF	0.543665	1.985466	ht		TGDS	0.67197	1.523411	none
HIST1H2BH	0.617917	2.04185	none		THRB	0.670555	2.256453	H ht h
HIST1H2BI	0.585897	1.443622	ht		TMEM14A	0.656093	1.175355	ht h
HIST1H2BJ	0.493823	5.335159	HT ht h		TOM1	0.64031	3.221137	h
HIST1H2BM	0.687469	3.533372	ft ht h		TXN2	0.689274	1.893339	H ht h
HIST1H2BO	0.618862	4.014214	ht h		UBE2B	0.663194	3.652863	H ht h
HIST1H3B	0.556438	4.260113	ft ht		VRK1	0.650583	1.000406	h
HIST1H3H	0.641946	2.647758	H ht h		WASPIP	0.572355	1.01892	none
HIST1H4E	0.608257	2.458831	FT h		WDHD1	0.624889	4.984045	H ht h
HIST1H4I	0.612088	2.068983	ht		WWOX	0.671866	1.882778	h
HIST2H2AA	0.560962	4.032876	ht		ZNF134	0.677481	2.726853	ht h
HLA-DRA	0.365141	3.086303	ht h		ZNF222	0.5618	4.09755	ht h
HLXB9	0.667926	1.006593	none		ZNF230	0.410725	3.76825	ht h
HS2ST1	0.694429	1.032562	ht h		ZNF235	0.38371	2.959812	none
HSBP1	0.671929	1.891961	ht h					

Potential CREB target genes. Top down-regulated genes that show significant CREB binding and changes in expression in the CREB knockdown cells. The detailed criteria for selecting these genes are described in the methods section. For each grouping of genes, from left to right, column 1 shows the gene symbols, column 2 the ratio of the expression change in wild type versus knockdown, column 3 the CREB binding ratioand column 4 the presence of CREB binding motifs. The key for column 4 is as follows: F is a full CREB motif (TGACGCTA) that is conserved from human to mouse, while f is not conserved, H is a conserved CREB half motif (TGACG or CGTCA), while h is not conserved, and T is the conserved presence of a TATA motif less than 300 basepairs downstream of the CREB motif, while t is not conserved.

Table 2.

Gene Name	Fold Changh	CREB bindin	CREB site		Gene Name	Fold Changh	CREB bindin	CREB site
ACOX1	2.110674	2.911283	H ht		LDLR	1.678587	1.525499	ht
ADAT1	1.410234	3.769574	ht f h		LGALS3BP	2.131291	3.615437	none
APEH	1.400261	2.527266	h		LIM	1.696177	1.097432	none
APPBP2	1.486616	2.151867	H ht h		LIM	1.849989	1.097432	none
ARHB	2.758453	2.77377	H ht		LRRFIP1	1.941595	1.122307	h
ATP6V1A	1.446867	3.016595	HT ht h		METAP2	1.916632	2.635425	ht
BCL6	1.640646	6.084626	HT ht		METTL2	1.593867	3.474639	none
BDKRB2	1.600927	2.601219	none		MGC2731	1.588545	2.80081	HT h
BTN3A2	1.465264	3.426679	ht		MGC4054	1.502743	2.777966	ht
C20orf12	1.511854	3.12999	h		MOCS3	1.796255	5.213295	none
C20orf121	1.456022	3.532969	H		MRPS10	1.410471	1.834794	ht f
C20orf172	1.463616	4.659037	H h		NCOA3	1.495237	2.715807	ht
C20orf23	1.528396	2.622103	none		NDRG1	2.030896	2.312257	ht h
CD44	9.531947	1.335178	ht h		NEDF	1.567662	4.268912	ft ht
CDH12	3.296441	1.178959	none		NPR2L	1.618864	6.397355	ht h
CDKAL1	1.735322	3.445022	none		ODZ1	1.448279	2.310975	ht
CDKN1A	2.216725	1.778747	H ht h		OPA3	1.474233	7.631458	FHT ht h
CELSR3	1.546375	3.175919	H ht		OTC	1.693003	4.881484	ht
CENPF	1.415064	2.654622	ht		PAFAH2	1.67217	4.584628	none
CHRNB1	1.55045	1.412576	H h		PAFAH2	1.631066	4.584628	none
CLECSF2	1.747573	1.251667	none		PHC3	1.42261	1.747154	ht
CML2	1.47905	3.427882	ht		PHLDA1	3.92008	2.003171	h
COL15A1	2.56792	1.394566	none		PLAT	1.668223	1.95203	none
CREM	1.793497	3.67068	H		PLEKHB2	1.568395	4.611748	f
CRKL	1.690269	3.051845	H h		PPARGC1	2.268458	2.972107	HT F ht h
CSMD1	1.647116	1.61907	ht		PPFIBP1	1.852526	2.550633	ht h
CTMP	1.548763	3.386235	none		PPP1R10	1.870902	2.447557	H h
DBT	1.518604	4.292329	none		PPP1R3B	1.693114	1.622596	h
DCLRE1C	1.41992	3.010944	none		PSMAL/GCP	1.506527	2.707076	none
DDOST	1.582101	2.508459	ht		RAB7L1	1.638378	1.15364	ht h
DDX3X	1.817009	3.42975	none		RABL2B	1.486054	2.496157	h
DEGS	1.488221	1.464348	none		RASSF1	1.431271	4.04395	none
DIAPH1	1.412484	2.96506	none		RBL1	1.529652	2.451247	h
DUSP1	1.578824	2.102797	FT HT ht h		REL	1.944847	1.143935	H h
EGR2	5.148023	2.036633	HT ht h		RHOBTB3	1.63057	2.813465	none
EIF5	1.422558	4.208549	ht h		RIOK3	1.40951	2.008376	none
ELK1	1.405171	4.088789	ht		RNASE6PL	1.561704	2.252099	ht
ENC1	1.957151	1.549567	h		RNF32	1.954396	1.603905	H ht
F2R	1.804785	1.098488	ht h		SAS	1.768493	7.735178	HT ht h
FAM13A1	1.780869	2.014276	none		SERPINB9	2.244605	1.418097	ht h
FAT	2.00051	1.816506	F ht		SFPQ	1.477265	3.428149	ht
FKBP14	1.78994	3.042488	ht		SHARP	1.558516	1.078188	H ht
FLJ10781	1.463332	1.113364	ht h		SLC31A1	1.491104	3.803168	FH ht
FLJ10803	1.726196	2.63943	ht		SLC35E3	1.716026	1.969928	ht
FLJ11029	1.422001	3.085667	ht h		SLC38A2	1.497716	1.914154	H ht
FLJ11151	2.413055	1.840398	h		SLC39A6	1.477678	3.119807	h
FLJ20507	1.730068	2.922871	H ht h		SMA3	1.414595	2.654203	ht
FOSL1	2.220086	1.929543	HT ht h		SMARCF1	1.537978	1.046929	none
FRSB	1.423607	2.982919	ht		SNAP29	1.521481	2.454502	h
FXC1	1.423019	5.02095	HT H ht		SON	1.42477	4.933417	H
GALNS	1.772331	2.592543	h		SPG4	1.413533	3.160161	none
GCA	1.690161	2.92801	H h		SUFU	1.661693	2.275704	ht h
GTF2H3	1.593421	10.587057	H		TAP1	1.435113	3.105625	H h
GYS1	1.418699	2.559154	h		TIGD6	1.772719	3.636168	h
HBS1L	1.475369	3.891767	ht		TIMP1	1.791155	1.848154	HT h
HIP1	1.537214	2.114631	ht h		TNFRSF21	1.498482	2.635088	ht
HLA-C	1.429002	3.2916	h		TP53AP1	1.527339	3.493111	ht h
HSPG2	1.708361	1.453039	none		TPM4	2.201468	1.33368	H ht
ICAM1	2.20462	1.198603	ht h		TRIM26	1.400065	6.12308	ht
ID1	1.521685	2.3068	FT ht		TSSC3	1.879281	2.01021	H ht h
IDS	1.508286	1.1848	h		TTF1	1.513382	3.461645	ht h
IER5	1.66867	2.847755	HT ht		TUBA3	1.481437	2.500545	none
IL10RA	1.64246	2.830231	f		U2AF1L1	2.758542	3.548509	ht
IL10RB	1.410005	1.192048	ht h		U5-116KD	2.223148	2.779884	h
IL1R1	1.812093	1.329947	ht		USP2	2.35423	3.920336	HT H h
IL6	1.980266	1.460112	HT ht		VPS4B	1.474465	6.693871	H ht
IL6ST	1.54702	3.418269	none		YME1L1	1.441837	1.843132	F ht h
INPP1	2.071508	1.550135	ht h		ZFP37	1.572207	4.659572	ht h
ITGA5	2.028008	1.315131	none		ZNF142	1.50914	3.028386	h
JM4	1.606813	2.392743	HT h		ZNF155	1.69746	4.195939	none
KIAA0266	1.504796	2.986155	none		ZNF189	1.625836	4.104303	ht h
KIF14	1.453888	4.181899	none		ZNF221	1.777122	3.569536	none
KIF3B	1.623133	1.560467	none		ZNF324	1.488601	4.205703	h
LCMT2	1.587221	2.338943	H ht h					

Potential CREB target genes. Top up-regulated genes that show significant CREB binding and changes in expression in the CREB knockdown cells. The detailed criteria

for selecting these genes are described in the methods section. The column descriptions are the same as in Table 1.

Table 3.

Name	Location	Type	Drugs
Downregulated Cancer Genes			
ABCG2	Plasma Membr	transporter	
ANG	Extracellular S	enzyme	
BCL2L11	Cytoplasm	other	
BECN1	Cytoplasm	other	
BMX	Cytoplasm	kinase	
CA2	Cytoplasm	enzyme	methazolamide, hydrochlorothiazide, acetazolamide, trichloromethiazide, dorzolamide, chlorothiazide, dorzolamide/timolol, brinzolamide, chlorthalidone, benzthiazide, sulfacetamide, topiramate
CENPE	Nucleus	other	
CNN1	Cytoplasm	other	
CREB1	Nucleus	transcription regulator	
CUL5	Nucleus	ion channel	
GFI1B	Nucleus	transcription regulator	
KLF5	Nucleus	transcription regulator	
MDM2 (include	Nucleus	transcription regulator	
MPHOSPH1	Nucleus	enzyme	
MSH2	Nucleus	enzyme	
MVD	Cytoplasm	enzyme	
NR4A3	Nucleus	ligand-dependent nuclear receptor	
NUMB	Plasma Membr	other	
PPP1R2	Cytoplasm	phosphatase	
PTGS2	Cytoplasm	enzyme	acetaminophen/pentazocine, acetaminophen/clemastine/pseudoephedrine, aspirin/butalbital/caffeine,
RB1CC1	Nucleus	other	
SILV	Plasma Membr	enzyme	
SMC2	Nucleus	transporter	
SMC3	Nucleus	other	
TFDP2	Nucleus	transcription regulator	
THRB	Nucleus	ligand-dependent nuclear receptor	3,5-diiodothyropropionic acid, amiodarone, thyroxine, L-triiodothyronine
UBE2B	Cytoplasm	enzyme	
VRK1	Nucleus	kinase	
WWOX	Cytoplasm	enzyme	
Upregulated cancer Genes			
ACOX1	Cytoplasm	enzyme	
ARID1A	Nucleus	transcription regulator	
BCL6	Nucleus	transcription regulator	
BDKRB2	Plasma Membr	G-protein coupled receptor	anatibant, icatibant
CD44	Plasma Membr	other	
CDKN1A	Nucleus	kinase	
COL15A1	Extracellular S	other	collagenase
CREM	Nucleus	transcription regulator	
CRKL	Cytoplasm	kinase	
DCLRE1C	Nucleus	enzyme	
DEGS1	Plasma Membr	enzyme	
DIAPH1	Cytoplasm	other	
DUSP1	Nucleus	phosphatase	
EGR2	Nucleus	transcription regulator	
ELK1	Nucleus	transcription regulator	
ENC1	Nucleus	peptidase	
F2R	Plasma Membr	G-protein coupled receptor	chrysalin, argatroban, bivalirudin
FOSL1	Nucleus	transcription regulator	
HIP1	Cytoplasm	other	
HSPG2 (includ	Plasma Membr	other	
ICAM1	Plasma Membr	transmembrane receptor	
ID1	Nucleus	transcription regulator	
IL6	Extracellular S	cytokine	tocilizumab
IL1R1	Plasma Membr	transmembrane receptor	anakinra
IL6ST	Plasma Membr	transmembrane receptor	
ITGA5	Plasma Membr	other	
KIF14	Cytoplasm	other	
METAP2	Cytoplasm	peptidase	PPI-2458
NCOA3	Nucleus	transcription regulator	
NDRG1	Nucleus	kinase	
PHLDA1	Cytoplasm	other	
PLAT	Extracellular S	peptidase	
RASSF1	Nucleus	other	
RBL1	Nucleus	other	
REL	Nucleus	transcription regulator	
RHOB	Cytoplasm	enzyme	
SERPINB9	Cytoplasm	other	
SUFU	Nucleus	transcription regulator	
TIMP1	Extracellular S	other	
TNFRSF21	Plasma Membr	other	
USP2	Cytoplasm	peptidase	

The subset of CREB target genes associated with cancer according to Ingenuity Pathways Analysis. Column 1 is the gene name, column 2 the localization, column 3 is a description of the protein function and column 4 are compounds that target the protein.

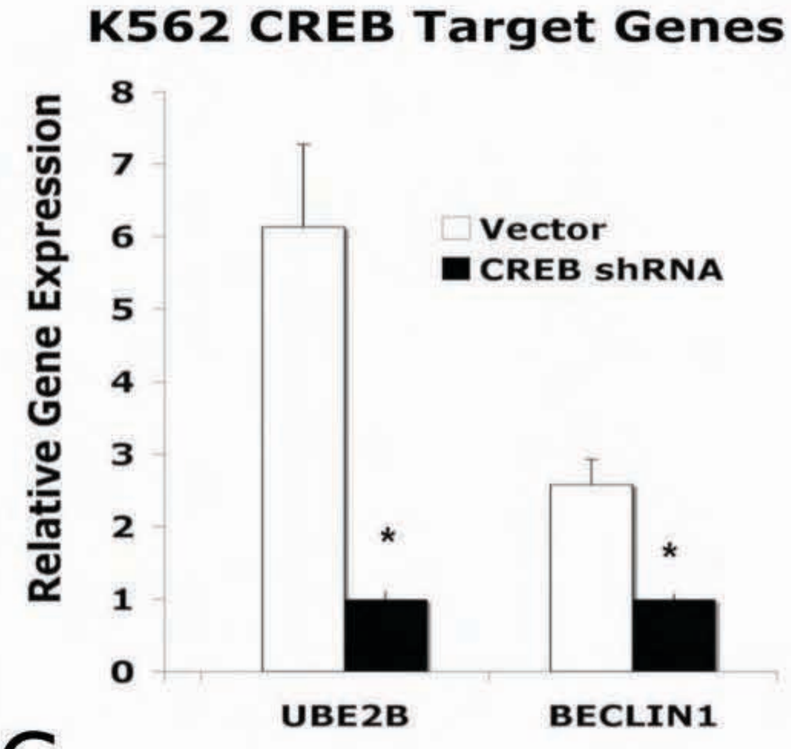
Table 4.

Category	Term	Count	%	PValue
GOTERM_CC_ALL	nucleosome	11	6.88%	6.22E-10
GOTERM_CC_ALL	chromosome	17	10.62%	2.39E-09
GOTERM_BP_ALL	nucleosome assembly	11	6.88%	6.60E-09
GOTERM_CC_ALL	chromatin	13	8.12%	7.56E-09
GOTERM_BP_ALL	chromatin assembly	11	6.88%	1.66E-08
GOTERM_BP_ALL	protein complex assembly	15	9.38%	2.19E-07
GOTERM_BP_ALL	chromatin assembly or disassembly	11	6.88%	3.84E-07
GOTERM_BP_ALL	chromosome organization and biogenesis	15	9.38%	5.56E-07
GOTERM_BP_ALL	chromosome organization and biogenesis (sensu Eukaryota)	14	8.75%	1.63E-06
GOTERM_CC_ALL	membrane-bound organelle	75	46.88%	1.93E-06
GOTERM_CC_ALL	intracellular membrane-bound organelle	74	46.25%	4.63E-06
GOTERM_CC_ALL	organelle	83	51.88%	5.39E-06
GOTERM_MF_ALL	DNA binding	38	23.75%	6.17E-06
GOTERM_BP_ALL	cellular physiological process	118	73.75%	8.86E-06
GOTERM_BP_ALL	establishment and/or maintenance of chromatin architecture	12	7.50%	1.02E-05
GOTERM_CC_ALL	intracellular organelle	82	51.25%	1.28E-05
GOTERM_BP_ALL	DNA packaging	12	7.50%	1.38E-05
GOTERM_BP_ALL	organelle organization and biogenesis	22	13.75%	1.59E-05
GOTERM_CC_ALL	nucleus	56	35.00%	2.46E-05
GOTERM_BP_ALL	DNA metabolism	19	11.88%	2.63E-05

Gene Ontology terms that are enriched among the top CREB targets. Column 1 is the ontology used (BP is biological process, CC is cellular localization and MF is molecular function), column 2 is the term, column 3 is the number of genes in the target list associated wit the term, column 4 is the percentage of genes in the target list associated with the term and column 5 is the P value for observing this number genes associated with the term.

Figure 1

A


K562 CREB Target Genes
Relative Gene Expression
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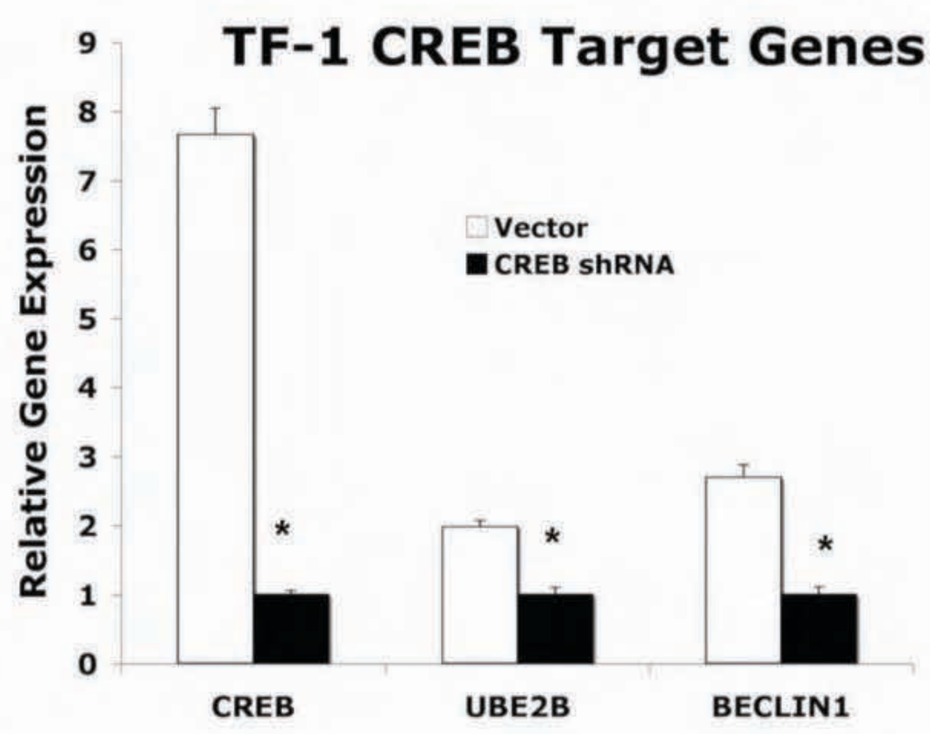
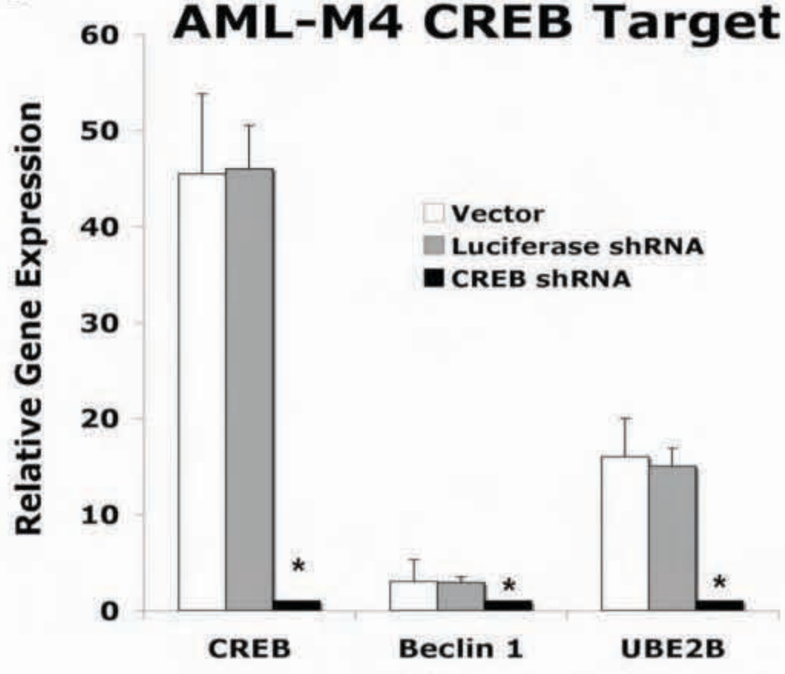
1
2
3
4
5
6
7
8
Vector
CREB shRNA
*
*
UBE2B
BECLIN1

B


TF-1 CREB Target Genes
Relative Gene Expression
0
1
2
3
4
5
6
7
8
9
Vector
CREB shRNA
*
*
*
CREB
UBE2B
BECLIN1

C


AML-M4 CREB Target Genes
Relative Gene Expression
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10
20
30
40
50
60
Vector
Luciferase shRNA
CREB shRNA
*
*
*
CREB
Beclin 1
UBE2B

Figure 2

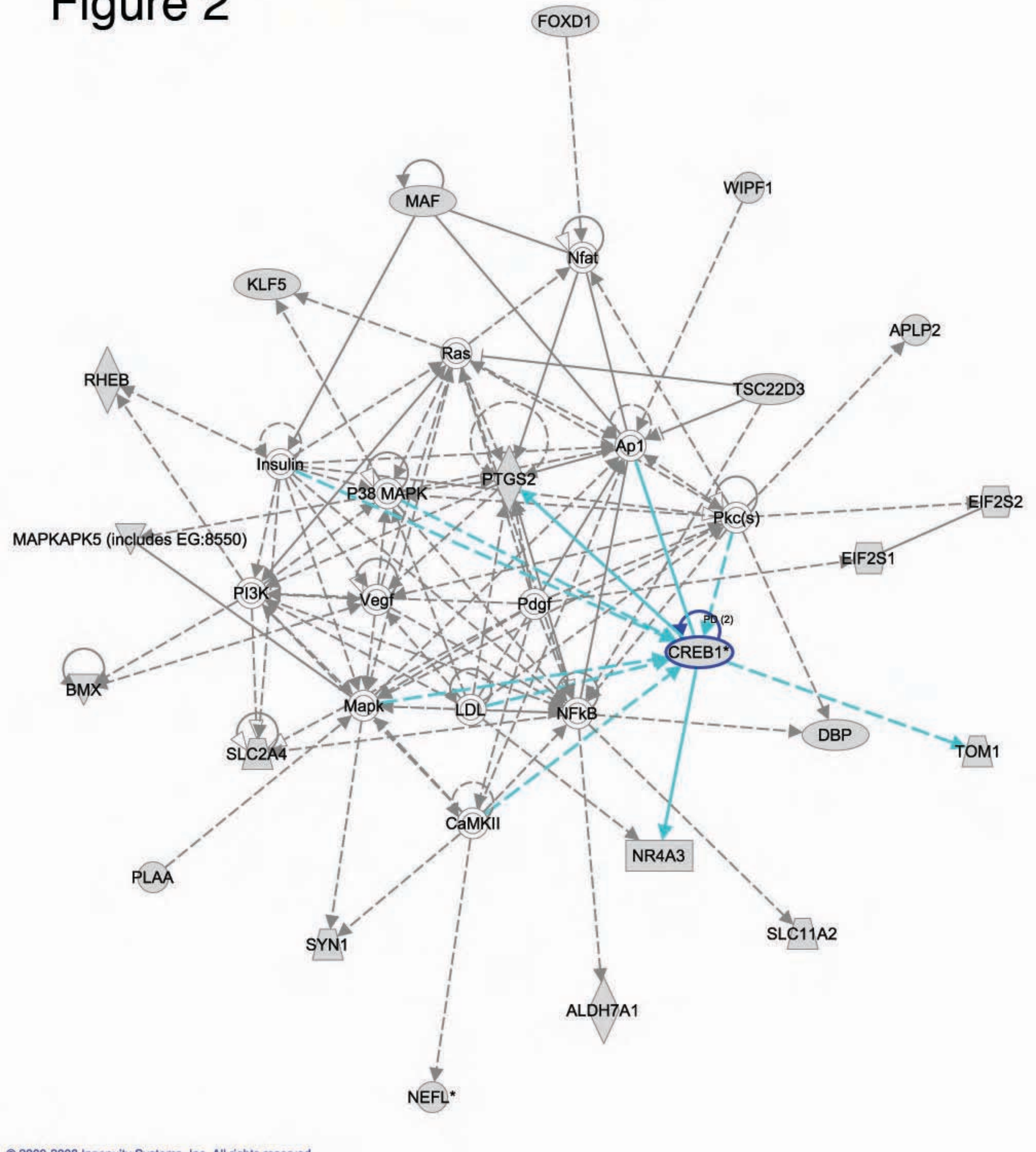
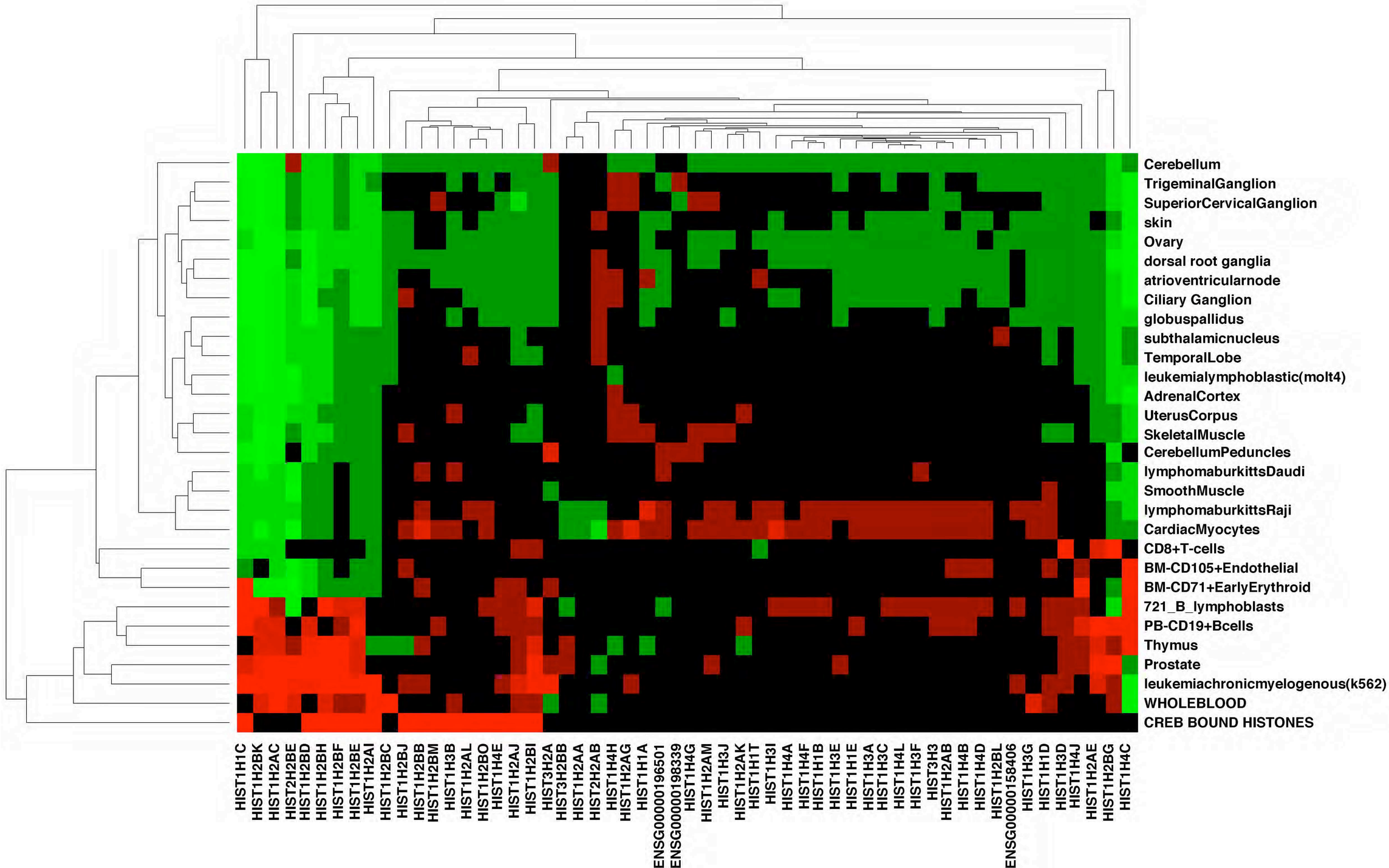


Figure 3


Cerebellum
TrigeminalGanglion
SuperiorCervicalGanglion
skin
Ovary
dorsal root ganglia
atrioventricularnode
Ciliary Ganglion
globuspallidus
subthalamicnucleus
TemporalLobe
leukemialymphoblastic(molt4)
AdrenalCortex
UterusCorpus
SkeletalMuscle
CerebellumPeduncles
lymphomaburkittsDaudi
SmoothMuscle
lymphomaburkittsRaji
CardiacMyocytes
CD8+T-cells
BM-CD105+Endothelial
BM-CD71+EarlyErythroid
721_B_lymphoblasts
PB-CD19+Bcells
Thymus
Prostate
leukemiachronicmyelogenous(k562)
WHOLEBLOOD
CREB BOUND HISTONES
HIST1H1C
HIST1H2BK
HIST1H2AC
HIST2H2BE
HIST1H2BD
HIST1H2BH
HIST1H2BF
HIST1H2BE
HIST1H2AI
HIST1H2BC
HIST1H2BJ
HIST1H2BB
HIST1H2BM
HIST1H3B
HIST1H2AL
HIST1H2BO
HIST1H4E
HIST1H2AJ
HIST1H2BI
HIST3H2A
HIST3H2BB
HIST1H2AA
HIST2H2AB
HIST1H4H
HIST1H2AG
HIST1H1A
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ENSG00000198339
HIST1H4G
HIST1H2AM
HIST1H3J
HIST1H2AK
HIST1H1T
HIST1H3I
HIST1H4A
HIST1H4F
HIST1H1B
HIST1H3E
HIST1H1E
HIST1H3A
HIST1H3C
HIST1H4L
HIST1H3F
HIST3H3
HIST1H2AB
HIST1H4B
HIST1H4D
HIST1H2BL
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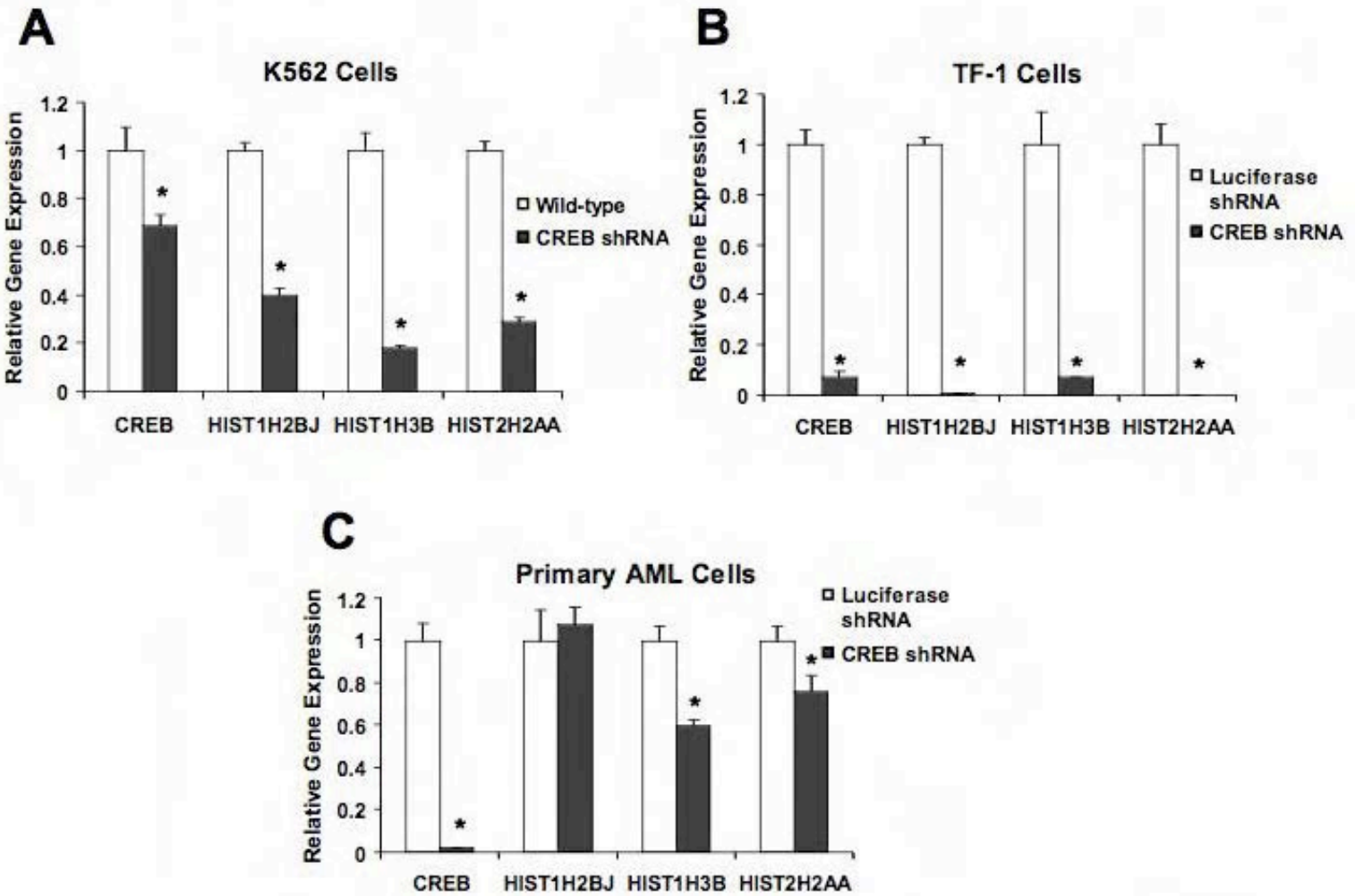
HIST1H3G
HIST1H1D
HIST1H3D
HIST1H4J
HIST1H2AE
HIST1H2BG
HIST1H4C

Figure 4


A
K562 Cells
Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
Wild-type
CREB shRNA
CREB
HIST1H2BJ
HIST1H3B
HIST2H2AA
B
TF-1 Cells
Relative Gene Expression
Luciferase shRNA
CREB shRNA
CREB
HIST1H2BJ
HIST1H3B
HIST2H2AA
C
Primary AML Cells
Relative Gene Expression
Luciferase shRNA
CREB shRNA
CREB
HIST1H2BJ
HIST1H3B
HIST2H2AA

Additional files provided with this submission:

Additional file 1: additional file 1.xls, 1924K
http://www.biomedcentral.com/imedia/2098497005214192/supp1.xls
Additional file 2: additional_file 2.xlsx, 7938K
http://www.biomedcentral.com/imedia/1188857053214193/supp2.zip

CURRICULUM VITAE

KATHLEEN MIHO SAKAMOTO, M.D., Ph.D.

CURRENT POSITION
Vice-Chair, Translational Research
Professor and Chief
Division of Hematology-Oncology
Mattel Children's Hospital
Department of Pediatrics and
Department of Pathology and Laboratory Medicine
David Geffen School of Medicine at UCLA

WORK ADDRESS
Mattel Children's Hospital at UCLA
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10833 Le Conte Avenue
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PHONE NUMBERS
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kms@ucla.edu or ksakamoto@mednet.ucla.edu

CITIZENSHIP
United States

SOCIAL SECURITY #
569-80-1510

EDUCATION

1975-1979	Williams College, Williamstown, MA; B.A. Cum Laude; Biology
1981-1985	University of Cincinnati, College of Medicine, Cincinnati, OH; M.D.
2000-2003	California Institute of Technology, Pasadena, CA; Ph.D. Biology Howard Hughes Medical Institute, Laboratory of Raymond J. Deshaies

LICENSURE

1986	California License, G58328
1994	DEA license, BS1361992

BOARD CERTIFICATION

1986	Diplomate, National Board of Medical Examiners
1989	Diplomate, American Board of Pediatrics (recertified 1999)
1994	Diplomate, American Board of Pediatrics, Hematology-Oncology (recertified 1999)

RESEARCH INTERESTS
Growth Factor Signal Transduction
Leukemogenesis
Cell Cycle Regulation and Hematopoiesis

EMPLOYMENT

1985-1986	Internship, Pediatrics, Children's Hospital of Los Angeles

1986-1988	Residency, Pediatrics, Children's Hospital of Los Angeles
1988-1989	Fellowship, Hematology/Oncology, Children's Hospital of Los Angeles
1991-1993	Clinical Instructor, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1993-1994	Visiting Assistant Professor of Pediatrics, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1994-1998	Assistant Professor of Pediatrics, Division of Hematology-Oncology, Department of Pediatrics, UCLA School of Medicine
1995-present	Joint appointment, Department of Pathology and Laboratory Medicine
1998-2003	Associate Professor of Pediatrics and Pathology, Mattel Children's Hospital at UCLA, UCLA School of Medicine
2003-present	Professor of Pediatrics and Pathology & Laboratory Medicine
2004-present	Visiting Associate, Division of Biology, California Institute of Technology
2005-present	Professor of Pediatrics and Pathology & Laboratory Medicine, David Geffen School of Medicine at UCLA
2005-present	Chief of Division of Hematology-Oncology, Mattel Children's Hospital, David Geffen School of Medicine at UCLA
2006-present	Vice-Chair of Translational Research, Mattel Children's Hospital, David Geffen School of Medicine at UCLA
2006-present	Co-Associate Director of Signal Transduction Program Area, Jonsson Comprehensive Cancer Center

RESEARCH EXPERIENCE

1978-1979	Senior Honors Thesis, Department of Biology, Williams College. "Effects of Centrifugation Time on Separation of Plant Organelles".
1979-1980	Research Assistant, Department of Biochemical Genetics, City of Hope Medical Center
1993-1996	Research Assistant, Department of Physiology, USC School of Medicine,
1980-1991	Postdoctoral Fellow, Division of Hematology-Oncology, in the laboratory of Judith C. Gasson, Ph.D., UCLA School of Medicine
1999	Visiting Associate, laboratory of Raymond Deshaies, Ph.D., Department of Biology, California Institute of Technology, Pasadena, CA
2000-2003	STAR program/Graduate Studies, Division of Biology, Laboratory of Raymond Deshaies, PhD, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA

HONORS

1988	Victor E. Stork Award, Children's Hospital of Los Angeles
1990-1993	Leukemia Society of America Fellowship Award
1991	Leukemia Society of America award as First Designated Researcher supported by the Leukemia Society staff
1992-1995	Jonsson Comprehensive Cancer Center/STOP CANCER Career Development Award
1996-2002	Leukemia Society of America Special Fellow Award
1994	Young Investigator Award in Oncology, American Society for Pediatric Hematology-Oncology
1995	UCLA Frontiers of Science Award
1996	Ross Award in Research By Young Investigators (Western Society for Pediatric Research)

1998-2003	Leukemia Society of America Scholar Award
1998	Elected Council Member, Western Society for Pediatric Research
1998	Participant, AAMC Workshop for Senior Women in Academic Medicine
1999	Invited Participant, American Cancer Society Professors Meeting, October, New York
1997	"Meet-the-Expert", Signal Transduction and Cell Cycle Control in Myeloid Cells, American Society of Hematology, New Orleans, LA
1998	Katherine E. Rogers Scholar for Excellence in Cancer Research, Jonsson Comprehensive Cancer Center, UCLA
1999	Member of Scientific Review Committee, CONCERN Foundation
2000	AACR-Novartis Scholar in Training Award, Oncogenomics meeting, Tucson, AZ
2001	Keystone Symposium on "Cell Cycle" - Travel Award, Keystone, CO.
2002	AACR-AFLAC Scholar-in-Training Award, meeting on Ubiquitination and Cancer meeting, Vancouver, Canada.
2002	Full member, Molecular Biology Institute, UCLA
2003-present	Children's Oncology Group, Myeloid Biology Subcommittee
2003-2007	Member, Grant Review Subcommittee on Leukemia, Immunology, and Blood Cell Development for American Cancer Society
2004	NIH Study Sections on Drug Discovery and Molecular Pharmacology and Basic Mechanisms of Cancer Therapy, and Special Emphasis Panel on Diamond-Blackfan Anemia and Bone Marrow Failure syndromes
2004	Grant Reviewer, UC Discovery Biotechnology Program
2004	Moderator, Leukemia Session at American Society for Pediatric Hematology-Oncology Annual Meeting
2004	Abstract Reviewer and Moderator for "Hematopoiesis: Regulation of Gene Transcription," ASH Meeting
2005-2009	Member, NIH Hematopoiesis Study Section
2004	Grant Reviewer, Susan G. Komen Breast Cancer Foundation
2005	"Ask the Experts" in Pediatric Cancer, AACR Public Forum, Anaheim, CA.
2005	Chair of Minisymposium, Modulation of Protein Stability. AACR, Anaheim, CA.
2005-present	Member, Translation Research Program Review Subcommittee for the Leukemia and Lymphoma Society of America
2005	Moderator, Pediatric Hematology-Oncology session, PAS/ASPHO meeting, Washington DC, May 2005
2005-2009	Grant Reviewer, California Research Cancer Committee (CRCC)
2005-2009	Member, ASH Scientific Committee on Myeloid Biology
2006	Reviewer, NIH Oncology Postdoctoral Fellowship Committee
2006-present	CDMRP (DOD) CML Grant Review Committee
2006	ASH abstract reviewer on "Hematopoiesis: Regulation of Gene Transcription" for annual meeting
2006	UCLA Finalist, Margaret Early Trust Award
2006	Benjamin Franklin High School Wall of Fame Award
2006	Member, American Pediatric Society
2007	Abstract Coordinating Reviewer, ASH meeting 2007
2007-present	Member, ASPHO Meeting Program Committee
2007-present	Member, ASPHO Career Development Task Force

2007-present	Member, Scientific Advisory Board, St. Balderick's Foundation
2007	Elected to become member of American Pediatric Society
2007	Stem Cells journal Lead Reviewer Award
2007	Nominated for Who's Who in America
2007	Best Doctors in America
2008-present	Chair, ASH Myeloid Biology Subcommittee
2008	"Meet the Expert" Transcription Factors in Myelopoiesis, ASH meeting
2008	Maryland Stem Cell Exploratory Grant Peer Review Committee
2008	NIH Special Emphasis Panel/CRG Loan Repayment Program Review Committee

EDITORIAL BOARD/REVIEWER

Editorial board: Stem Cells, Blood

Medical Editor, emedicine online textbook for Pediatrics (Hematology-Oncology section)

Current Drugs, panel of evaluators

Ad hoc reviewer for journals: Ad hoc reviewer for journals: Oncogene, Proceedings of the National Academy of Sciences, Molecular and Cellular Biology, Journal of Cellular Biochemistry, Leukemia, Biotechniques, Cytometry, Pediatric Research, Cancer Research, Molecular Cancer Therapeutics, American Journal of Hematology, Molecular Genetics and Metabolism, American Journal of Human Genetics, New England Journal of Medicine, Pediatric Blood and Cancer, Cancer Research, Clinical Cancer Research, British Journal of Hematology, Clinical Prostate Cancer, Pediatrics, Cancer Letters, Journal of Pediatric Hematology-Oncology

PROFESSIONAL SOCIETY MEMBERSHIPS

Candidate Fellow, American Academy of Pediatrics
Member, American Society of Hematology
Member, American Society of Pediatric Hematology-Oncology
Member, American Association for Cancer Research
Member, New York Academy of Science
Member, Western Society for Pediatric Research
Member, Society for Pediatric Research
Member, International Society for Experimental Hematology
Children's Oncology Group, AML Strategy Group
American Society for Biochemistry and Molecular Biology (ASBMB)

COMMITTEES ON CAMPUS

1994	Search Committee for Director of the Jonsson Cancer Center
1995	Search Committee for Nephrology Faculty Appointment
1996-1998	Admissions Committee, UCLA ACCESS program for graduate students
1996-1999	Admissions Committee, Medical Student Training Program, UCLA
1994-present	UCLA Cancer Committee
1998-1999	Chair of Tumor Cell Biology ACCESS Affinity Group for Graduate Students
1996-2002	Western Society for Pediatric Research (WSPR) Council member
2002-present	Search Committee for Pediatric Pulmonary
2002-present	Search Committee for Pediatric Nephrology

2002-present	Search Committee for Pediatric Hematology-Oncology
2006	Search Committee for Pediatric Cardiology
2006	Search Committee for Infectious Disease
2006	Committee for Loan Repayment, Department of Pediatrics
2006	Search Committee for Biostatistician, Department of Pediatrics
2006	Chair, Search Committee for Neonatology
2006-present	Pediatric Credentials Committee
2007	Member, Coordinating Committee for CNSI-CNBI Symposium on NanoBiotechnology
2008-2009	Membership committee, CNSI-CNBI

CAMPUS ACTIVITIES

1994-present	Faculty Mentor on the Medical Student Training Program
1994-present	Principal Investigator on the Tumor Cell Biology Training Grant
1995	Faculty Advisor Program for first year medical students
1995-present	Principal Investigator on the UCLA ACCESS program for graduate Students
2007-present	Organize the Pediatric Fellows Core Curriculum noon seminars, Science Day
2007-present	Organize the Basic Science Journal Clubs for Residents
2007-present	Organize the Pediatric Translational Research Program (seed grants, grant mentors, core equipment, symposium, seminar series)
2006-present	Organize seminars, lunch/business meetings, and roundtable discussions for the Jonsson Comprehensive Cancer Center
2007-present	Organizer for Hematopoiesis Journal Club for Jonsson Cancer Center

Teaching

1993-present	Pediatric Hematology-Oncology elective
1993-present	Advanced Clinical Clerkship in Pediatric Hematology-Oncology
1993-present	Laboratory course in Biochemistry for first year medical students
1993-present	Pediatric Clerkship
1993-present	Advanced Clinical Clerkship in Pediatrics
1995	Ethics and Accountability in Biomedical Research
1995-1997	Major Concepts in Oncology
1995	Molecular and Cellular Foundations of Disease
1993-1997	Organization of Pediatric Hematology-Oncology weekly clinic conferences
1995-1999	Organization of the Pediatric Departmental Monthly Research Seminars
1999-2004	M229 Course on Cell Biology and Pathogenesis for ACCESS Graduate Students on “Cell Cycle” (organized by Patricia Johnson)
1996-2003	Pathophysiology Course in Hematopathology (session on Lymphoma)
2005-present	Associate Director of the Signal Transduction Program Area, Jonsson Comprehensive Cancer Center
2005	MBI 298 seminar course on Ubiquitination
2005	Co-organizer, M294 Pathology course on Molecular Basis of Oncology

Clinical Activities

1993-present	Medical Staff, Pediatric Hematology-Oncology, UCLA School of Medicine and Santa Monica Hospital

COMMUNITY SERVICE

Leukemia and Lymphoma Society of America, Los Angeles Chapter,Board of Trustees and Executive Board

PATENTS

"Proteolysis Targeting Chimeric Pharmaceutical" (Raymond Deshaies, Craig Crews, and Kathleen Sakamoto), Ref. No. CIT3284.

"RNA inhibition of CREB" (Jerry Cheng, Kathleen Sakamoto), UC Case No. 2003-348

GRANTS

1989-1990 American Cancer Society Clinical Oncology Fellowship

1990-1993 5 F32 CA08974-04 Individual National Research Service Award Molecular Analysis of Target Cell Response to Human GM-CSF ($102,100); National Cancer Institute (Judith Gasson, Ph.D., P.I.)

1996-2002 Fellowship Award, Molecular Characterization of GM-CSF Action ($70,000) Leukemia Society of America (Judith C. Gasson, Ph.D., P.I.)

1993-1998 K08 CA59463, Clinical Investigator Award, Molecular Characterization of GM-CSF Action ($383,400), National Cancer Institute (Judith Gasson, Ph.D. P.I.)

1993-1996 3017-93, Special Fellow Award, Molecular Analysis of GM-CSF Action ($100,400), Leukemia Society of America (K. Sakamoto, M.D., P.I.)

1992-1995 Career Development Award, Molecular Characterization of GM-CSF Action ($150,000), STOP CANCER (K. Sakamoto, M.D., P.I.)

1992-1993 Seed Grant, Mutation Analysis of Structure-Function Relationships of Human GM-CSF Receptor Beta Subunit ($30,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)

1992-1993 Mutation Analysis of Structure-Function Relationships of the Human GM-CSF Receptor Beta Subunit ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (declined) (K. Sakamoto, M.D., P.I.)

1993-1995 Molecular Regulation of egr-1 by IL-3 and PIXY321 in Myeloid Leukemias ($100,000), Concern II (K. Sakamoto, M.D., P.I.)

1994 The Role of Cyclins in Myeloid Leukemias ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (K. Sakamoto, P.I.)

1995 UCLA Academic Senate Award ($1,500), "Stem Cell Factor Activation of Signal Transduction in Myeloid Leukemic Cells" (K. Sakamoto, M.D., P.I.)

1995	UCLA Frontiers of Science Award, The Regulation and Functional Role of p55CDC in Myeloid Leukemias ($28,000) (K. Sakamoto, M.D., P.I.)
1995	UCLA Prime Faculty Research Award, Molecular Regulation of Myeloid Cell Differentiation ($25,000) (K. Sakamoto, M.D., P.I.)
1995	Seed Grant, The Role of SRE-Binding Proteins During Signal Transduction in Myeloid Leukemias ($27,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)
1995	New Assistant Professor Grant, Transcriptional Regulation of egr-1 by Stem Cell Factor in Myeloid Leukemias ($35,000), Cancer Research Coordinating Committee (K. Sakamoto, M.D., P.I.)
1995-1997	Shannon Award, NIH (NCI) 1R55CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($80,000) (K. Sakamoto, M.D., P.I.)
1996	Concern II Foundation; Molecular Analysis of IL-3 and PIXY321 Signaling Pathways in Myeloid Leukemias ($50,000) (K. Sakamoto, M.D., P.I.)
1996-2002	First Award R29CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($350,000), NIH/NCI (K. Sakamoto, M.D., P.I.)
7/97-6/99	UC Biotechnology STAR Project, S97-03 "p55Cdc and Cell Cycle Regulation" ($40,000); Amgen, Inc. and University of California (K. Sakamoto, M.D., P.I.)
7/98-6/99	Contract with Eli Lilly, Inc. "Multiple Resistance Genes in Leukemias" ($32, 000), Co-PI with Leonard Rome, Ph.D. (K. Sakamoto, M.D., P.I.)
7/98-6/99	Jonsson Comprehensive Cancer Center Seed Grant, “Use of Low Molecular Weight Heparin in Cancer Patients Receiving Stem Cell Transplants,” ($30,000), Co-P.I. with Dr. Sinisa Dovat, M.D. (fellow)
7/98-6/2003	Leukemia Society of America Scholar Award, 1497-99 "The Role of p55Cdc during Myelopoiesis" ($350,000), Leukemia Society of America (K. Sakamoto, P.I.)
1/99-12/2001	Investigator initiated grant, California Cancer Research Program, “Cell Cycle Control and Cancer” ($400,000), California Department of Health Services (K. Sakamoto, P.I.)
7/99- 6/2000	Jonsson Comprehensive Cancer Center Seed Grant, “Development of a Novel Class of Protein-inhibiting Anti-cancer Therapeutics” ($15,000), K. Sakamoto (P.I.) and Raymond Deshaies (Co-P.I., Caltech)
1/2000	CaPCURE research award, “Development of a Novel Class of Protein-Inhibiting Therapeutics for Prostate Cancer” ($100,000). Raymond Deshaies (P.I., Caltech), K. Sakamoto, and Craig Crews (Co-P.I., Yale University).

1/99-12/02 Research Project Grant, "Molecular Analysis of Myeloid Cell Proliferation" ($300,000); American Cancer Society (K. Sakamoto, P.I.)

8/01-7/03 UC Biostar, "Targeting the estrogen receptor for Proteolysis", with Celgene, Inc. ($40,000), K Sakamoto, P.I.

1/02-12/02 CaPCURE research award, "Targeting the Androgen Receptor for Degradation in Prostate Cancer" ($75,000) K.Sakamoto (P.I.), Raymond Deshaies (Co-P.I., Caltech) and Craig Crews (Co-P.I., Yale University).

6/02-7/03 National Cancer Coalition, "Signal Transduction and Cell Cycle Analysis in Leukemia" ($5,000), K. Sakamoto (P.I.).

1/03-12/06 American Cancer Society, Research Scholar Award. "The role of CREB in Leukemogenesis," ($625,000). K. Sakamoto (P.I.).

1/03-6/04 Department of Defense, "Targeting the estrogen receptor for ubiquitination and proteolysis in breast cancer," ($222,819). K. Sakamoto (P.I.)

1/03-12/03 Diamond-Blackfan Anemia Foundation, "AML in Diamond-Blackfan Anemia: Molecular Basis and Therapeutic Strategies," ($25,000). K. Sakamoto (P.I.)

1/1/03-12/31/04 SPORE grant in Prostate Cancer Research, Seed Grant Award, "Targeting the Androgen Receptor for proteolysis in Prostate Cancer," $75,000. K. Sakamoto (P.I.)

4/1/03-3/31/04 Stein-Oppenheimer Award, "Targeting the Estrogen Receptor in Breast Cancer," $20,000. K. Sakamoto (P.I.)

6/1/03-5/30/04 Genomic Exploration Seed Grant, Jonsson Comprehensive Cancer Center, "CREB and Human Leukemias," $5,000, K. Sakamoto (P.I.)

7/1/03-6/30/04 Susan G. Komen Breast Cancer Thesis Dissertation Award," $20,000. K. Sakamoto, R. J. Deshaies (P.I.)

1/04-12/08 NIH/NHLBI R01 (HL 75826), "The Role of CREB in Leukemogenesis," ($200,000/year). K. Sakamoto (P.I.)

9/04-8/08 R21, "Ubiquitination and Degradation in Cancer Therapy," ($135,000/year). K. Sakamoto (P.I.)

7/04-7/05 Department of Defense, "Identification of small non-peptidic ligands that bind the $SCF^{beta\text{-}TRCP}$ ubiquitin ligase to target the ER for ubiquitination and degradation ($75,000). K. Sakamoto (P.I.)

7/05-5/07 Fulbright Fellowship/MEC (Spain) postdoctoral fellowship, "Targeting the Androgen Receptor for Ubiquitination and Degradation: A new strategy for Therapy in Prostate Cancer" ($60,000), K. Sakamoto and R. Deshaies (Co-P.I.).

5/05 Boyer/Parvin Postdoctoral Fellow Award ($5,000), awarded to Deepa Shankar,

Ph.D., K. Sakamoto (P.I.)

7/05 Stone Research Award ($1,000) award to undergraduate student Winston Wu, K. Sakamoto (P.I.)

7/05-6/07 Department of Defense postdoctoral fellowship, "Targeting the Androgen Receptor for Ubiquitination and Degradation: A New Strategy for Therapy in Prostate Cancer," ($80,000), K. Sakamoto (P.I.)

10/06-9/07 Diamond Blackfan Anemia Foundation, " Developing a zebrafish model of Diamond Blackfan Anemia." $25,000 (K. Sakamoto and S. Lin, P.I.)

10/05-9/09 NIH/NHLBI R01 (HL083077), " Molecular and Cellular Characterization of MPD." $225,000/ year (K. Sakamoto, P.I.).

7/06-6/08 Department of Defense, "The Role of CREB in CML," $45,800/year (K. Sakamoto, P.I.).

7/06-6/08 F32 HL085013 NRSA (NHLBI), "CREB and Hematopoietic Stem Cells," awarded to postdoctoral fellow Jerry Cheng, M.D. (K. Sakamoto, P.I.).

7/06-6/08 NCI T32 CA09056 Tumor Cell Biology Training Grant, "Studies in the Mechanisms of Targeted Therapy for Acute Myeloid Leukemia," for Alan K. Ikeda, M.D. (K. Sakamoto, P.I.).

10/06-9/09 Leukemia and Lymphoma Society Translational Research Grant, "Targeting Signaling Pathways in Pediatric AML." $200,000/year (K. Sakamoto, P.I. and Ted Moore, co-P.I.).

1/07-12/12 NHLBI, "Training in Developmental Hematology." $262,489/year (K. Sakamoto, P.I.).

1//08-12//08 Abbott Laboratories, Inc. "RTKIs in AML." $50,000 (K.Sakamoto, P.I.)

TRAINING FACULTY ON THE FOLLOWING TRAINING GRANTS (NIH T32 and K12 Programs)
Tumor Cell Biology
Tumor Immunology
Hematology
Vascular Biology
CHRCD
Medical Scientist Training Program (MSTP)
Gene Medicine
Stem Cell Research Institute
Training in Developmental Hematology (P.I.)

FACULTY MENTORSHIP
Faculty Mentor Ved Longhe, Assistant Professor In-Residence
Faculty Mentor Kek-Khee Loo, Assistant Professor In-Residence

TRAINEES

1991-1993	Hu-Jung Julie Lee, undergraduate student
1992-1993	Elana Lehman, medical student
1993-present	Kathy Hwain Shin, undergraduate student, Work/study and Lab Assistant
1994-1995	Robert C. Mignacca, M.D., postdoctoral fellow
1994-1995	Stephen Phillips, undergraduate student, Student Research Project
1995	Allison Wong, medical student; Short Term Training Program; Recipient
of	Howard Hughes NIH Research Scholar Award, 1996-1997
1995	Ramona Rodriguez, medical student; Short Term Training Program, Centers of Excellence
1995-2000	Evelyn Kwon, graduate student
1996	Michael Mendoza, medical student, Short Term Training Program;
Centers	of Excellence and FIRST/STAR Award recipient
1996-2002	Patricia Mora-Garcia (awarded Minority Supplement Award from NIH/NCI), Dept. Pathology and Laboratory Medicine
1996-2002	Michael Lin, graduate students (recipient of NIH/NCI Tumor Cell Biology Training Grant), Dept. Pathology and Laboratory Medicine
1997	Raymond Wang, medical student, Short Term Training Program
1995-1999	Wayne Chu, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of 1999 Merle Carson Lectureship, 1st Prize Southwestern Pediatric Society, The Tenth Joseph St. Geme, Jr. Research Award for UCLA Pediatric Trainees)
1999-2000	Kristin Baird, M.D. Pediatric Resident, Mattel Children's Hospital at UCLA, research elective
2000-2007	Deepa Shankar, Ph.D., Postdoctoral fellow (NIH Tumor Cell Biology Postdoctoral fellowship, JCCC fellowship).
2001-2002	Heather Crans, graduate student (recipient of NIH Tumor Immunology Training Grant), Dept. Pathology and Laboratory Medicine
2001-2003	Athena Countouriotis, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of Resident Research Award, American Academy of Pediatrics)
2002-2007	Jerry Cheng, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA (won SPR House Officer Award 2003, ASPHO/SPR meeting, Seattle, WA).
2002-2003	Tamara Greene, Medical Student, UCLA School of Medicine
2002-2003	Johnny Chang, M.D., Medical Oncology Fellow, Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine (recipient Of NIH Hematology Training Grant)
2003-2005	Noah Federman, M.D., Pediatric Resident, Mattel Children's Hospital, research elective
2003	Andy Liu, undergraduate student (Recipient of Undergraduate scholarship award for research performed in my laboratory)
2003	Ryan Stevenson, undergraduate student (now in medical school)
2004	Maricela Rodriguez, medical student
2005	Jenny Hernandez, Saul Priceman, Jose Cordero, Gloria Gonzales, Salemiz Sandoval
2005	Cid Sumolong, STTP, UCLA medical student
2005-2006	Winston Wu, undergraduate (recipient of John Stone Award for research performed in my laboratory)

2005-present	Salemiz Sandoval, graduate student (MBI)
2005-2007	Samuel Esparza, M.D., Pediatric Hematology-Oncology fellow, STAR/PhD graduate program
2005-2007	Jerry Cheng, M.D., Pediatric Hematology-Oncology fellow
2005-present	Tiffany Simms-Waldrip, M.D., postdoctoral fellow
2005	Katrin Rhodes, rotating ACCESS graduate student
2006	Sam Kaneko, first year UCLA medical student (STTP)
2006-2007	Kellie Lim, 4th year medical student mentor, UCLA Medical Specialties College Program
2006-present	Jenny Hernandez, graduate student (Pathology)
2006-present	Alan Ikeda, M.D., Pediatric Hematology-Oncology fellow
2006-present	Tara Lin, M.D., Adult Oncology, Postdoctoral fellow
2006	Andrew Goldsmith, ACCESS rotation student
2006-present	James Ch'ng, undergraduate student
2007-present	Chuck Gawad, Pediatric Resident, Mattel Children's Hospital
2007-present	Tiffany Chang, Pediatric Resident Mattel Children's Hospital
2007-present	AMA faculty mentoring program for medical students (Amanda Clauson and Supriya Bavisetty)
2007	Jo Chang, Pediatric Resident, Harbor-UCLA Medical Center
2007-present	Jessica Bushong, undergraduate student (recipient of CARE program award)

Ph.D. Thesis Committee for Graduate Students
Jason Christianson(P.I. A. Rajasekkaran, Pathology) 9/10/01
Robert Clipsham (P.I. Ed McCabe, M.D., Ph.D., Genetics) 7/01
Jared Goldstine (P.I. Harry Vinters, Pathology) 3/31/04
Jennifer Woo Tufts (P.I. Arnold Berk, Molecular Biology Institute) 3/07/06
Jin Xu (P.I. Charles Sawyers, Molecular Biology Institute) 3/04
Robert Signer (P.I. Ken Dorshkind, Pathology) 3/17/06
Alexandria Young (P.I. Debora Farber, Ph.D., Ophthalmology) 4/26/06
Katrin Rhodes (P.I. Hanna Mikkola, Ph.D., MCDB)
Graduate student, (P.I. Shuo Lin, Ph.D., MCDB)

BIBLIOGRAPHY

PEER-REVIEWED

1. Nagahashi, G and Hiraike (**Sakamoto**) KM. Effect of centrifugation time on sedimentation of plant organelles. Plant Physiol 69:546-548, 1982.

2. Yamamoto J, Yap J, Hatakeyama J, Hatanaka H, Hiraike (**Sakamoto**) **K**, Wong L: Treating Asian Americans in Los Angeles. Psychiatry 8:411-416, 1985.

3. **Sakamoto KM**, Bardeleben C, Yates KE, Raines MA, Golde DW, Gasson JC: 5' upstream sequence and genomic structure of the human primary response gene, EGR-1/TIS8. Oncogene 6:867-871, 1991.

4. **Sakamoto KM**, Nimer SD, Rosenblatt JD, Gasson JC: HTLV-I and HTLV-II tax *trans*-activate the human EGR-1 promoter through different *cis*-acting sequences. Oncogene 7:2125-2130, 1992.

5. **Sakamoto-K**, Erdreich Epstein A, deClerck Y, Coates T: Prolonged clinical response to vincristine treatment in two patients with idiopathic hypereosinophilic syndrome. Am J Ped Hemat Oncol 14:348-351, 1992.

6. **Sakamoto KM**, Fraser JK, Lee H-J J, Lehman E, Gasson JC: GM-CSF and IL-3 signaling pathways converge on the CREB-binding site in the human EGR-1 promoter. Mol Cell Biol, 14: 5920-5928, 1994.

7. Lee H-J J, Mignacca RM, and **KM Sakamoto**. Transcriptional activation of egr-1 by Granulocyte-Macrophage Colony-Stimulating Factor but not Interleukin-3 requires phosphorylation of CREB on Serine 133. J. Biol. Chem., 270: 15979-15983, 1995.

8. Wong A and **KM Sakamoto**. GM-CSF-Induces the Transcriptional Activation of Egr-1 Through a Protein Kinase A-Independent Signaling Pathway. J Biol Chem 270: 30271-30273, 1995.

9. Horie M, **Sakamoto KM,** Broxmeyer HC. Regulation of egr-1 gene expression by retinoic acid in a human growth factor-dependent cell line. Int J Hematology, 63: 303-309, 1996.

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47. **Sakamoto KM**, Crews C, Kumagai A, and RJ Deshaies. A novel approach to treat cancer. Accepted for poster presentation. Oncogenomics Meeting, Tucson AZ, January, 2001.

48. Deshaies RJ, **Sakamoto KM**, Seol JH, Verma R. Prospecting at the Cross-roads of ubiquitin-dependent proteolysis and cell cycle control. FASEB meeting, Orlando, FA, 2001.^

49. Crans HC, Landaw EM, Bhatia S, and **KM Sakamoto**. CREB as a prognostic marker in Acute Leukemia. Accepted for poster presentation, American Society of Hematology meeting, Orlando, FA, 2001.

50. Countouriotis A, Landaw EM, Moore TB, and **KM Sakamoto**. CREB expression in Acute Leukemia. Accepted for poster presentation and Pediatric Resident Travel Award, A. Countouriotis, Society for Pediatric Research/American Society of Pediatric Hematology/Oncology, May 2002.

51. Mora-Garcia P, Wei J, and **KM Sakamoto**. G-CSF Signaling induces Stabilization of Fli-1 protein in Myeloid Cells. American Society for Hematology, Philadelphia, PA, December 2002.

*52. Countouriotis AM, Landaw EM, Moore TB, Sakamoto KM. Comparison of bone marrow aspirates and biopsies in pediatric patients with acute lymphoblastic leukemia. Western Society for Pediatric Research, Carmel, CA. January 2003

*53. Cheng JC, Crans-Vargas HN, Moore TB, and **KM Sakamoto**. Aberrant CREB expression in Patients with Acute leukemia. Western Society for Pediatric Research. Carmel, CA. January 2003.

54. Countouriotis AM, Landaw EM, Moore TB, **KM Sakamoto**. Comparison of bone marrow aspirates and biopsies in pediatric patients with acute lymphoblastic leukemia. Society for Pediatric Research/ASPHO, Seattle, WA. January 2003.

55. Cheng JC, Crans-Vargas HN, Moore TB, and **KM Sakamoto**. Aberrant CREB expression in Patients with Acute leukemia. Western Society for Pediatric Research. Carmel, CA. January 2003. Won the SPR Resident's Research Award.

*56. Shankar DB, Cheng J, Headley V, Pan R, Countouriotis A, and **KM Sakamoto**. CREB is aberrantly expressed in acute myeloid leukemias and regulates myelopoiesis in vitro and in vivo. American Society for Hematology, San Diego, CA. December 2003.

*57. Shankar DB, Landaw EM, Rao N, Moore TB, and **KM Sakamoto**. CREB is amplified in AML blasts and is associated with an increased risk of relapse and decreased event-free survival. Oral presentation, American Society for Hematology, San Diego, CA. December 2004.

58. Shankar DB, Kinjo K, Cheng JC, Esparza S, Federman N, Moore TB, and **KM Sakamoto**. Cyclin A is a target gene of activated CREB downstream of GM-CSF signaling that regulates normal and malignant myelopoiesis. Poster presentation, American Society for Hematology, San Diego, CA. December 2004.

59. Kinjo K, Shankar DB, Cheng JC, Esparza S, Federman N, Moore TB, and **KM Sakamoto**. CREB overexpression in vivo results in increased proliferation, blast transformation, and earlier engraftment of myeloid progenitor cells. Poster presentation, American Society for Hematology, San Diego, CA. December 2004.

*60. Kinjo K, Shankar DB, Moore TB, and **KM Sakamoto**. CREB Regulates hematopoietic progenitor cell proliferation and myeloid engraftment. (AFMR Scholar Award and WSCI Travel Award Winner). WSPR, Carmel, CA, February, 2005.

*61. Menzel LP, Hummerickhouse R, Hagey A, Shah NP, Shankar DB, Moore TB, and **KM Sakamoto**. Analysis of a targeted receptor tyrosine kinase inhibitor in the treatment of acute myelogenous leukemia. WSPR, Carmel, CA, February, 2005.

*62. Shankar DB, Chang J, Parcells B, Sandoval S, Li J, Wei R, Tapang P, Davidsen SK, Albert DH, Glaser KB, Moore TB, and **KM Sakamoto**. The Multi-Targeted Receptor Tyrosine Kinase Inhibitor, ABT-869, Induces Apoptosis of AML cells both *in vitro* and *in vivo*. Accepted for an oral presentation at the American Society for Hematology, Atlanta GA, December 2005.

*63. Shankar DB, Kinjo K, Chang J, and **KM Sakamoto**. CREB Transgenic Mice Develop Myeloproliferative Disease/Myelodysplastic Syndrome after a Prolonged Latency. Accepted for an oral presentation, American Society for Hematology, Atlanta GA, December 2005.

*64. Parcells BW, Ikeda AK, Moore TB, Glaser KB, and **KM Sakamoto**. The Multi-Targeted Receptor Tyrosine Kinase Inhibitor ABT-869 Induces Apoptosis in Baf3 cells expressing the

FLT3 Internal Tandem Duplication Mutation. Accepted for an oral presentation, WSPR, Carmel, CA. February 2006.

*65. Simms-Waldrip T, Hernandez J, Shankar DB, Moore TB, Shoemaker A, and **KM Sakamoto.** Targeting Bcl-2 in acute myeloid leukemia cells. Accepted for an oral presentation, WSPR Carmel, CA. February 2006.

*66. Rodriguez-Gonzalez A, Kim KB, Crews CM, Deshaies RJ, and **KM Sakamoto**. Development of Protacs to target the estrogen receptor for ubiquitination and degradation in breast cancer cells. Accepted for an oral presentation. AACR meeting, Washington DC, April 2006.

67. Francisco Martinez F, Jimenez F, Machuca C, Villegas H, and **KM Sakamoto**. Transcriptional activation of krox-1 induced by sexual hormones in osteosarcoma cells. Accepted for a poster presentation. American Society for Gene Therapy Baltimore, Maryland. May 2006.

68. Cheng JC, Shankar D, and **KM Sakamoto**. Requirement of CREB in Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

69. Esparza SE, Shankar DB, and **KM Sakamoto**. Identification of Meis1 as a Target of CREB in Myeloid Leukemogenesis. Accepted for poster presentation. American Society for Hematology, Orlando FL, December 2006.

70. Sandoval S, Shankar DB, and **KM Sakamoto**. Acceleration of Leukemogenesis in CREB Transgenic mice by Retroviral Insertional Mutagenesis. American Society of Hematology, Orlando FL, December 2006.

71. Rodriguez-Gonzalez A, Ch'ng JH, Ikeda A, Lin T, Bahrami B, Mazitschek R, Bradner JE, Fu C, and **KM Sakamoto**. Targeting Histone Deacetylase 6 and the Aggresome Pathway in Acute Lymphoblastic Leukemia Cells. Accepted for poster presentation. American Society of Hematology, Atlanta, GA, December 2007.

72. Ikeda A, Judelson D, Li J, Wei RQ, Tapang P, Davidsen SK, Albert D, Glaser KB, Fu C, and **KM Sakamoto**. Inhibiting FLT3 phosphorylation and signaling in AML. Accepted for poster presentation. American Society of Hematology, Atlanta, GA, December 2007.

73. Cheng JC, Judelson D, Kinjo K, Chang J, Landaw EM, and **KM Sakamoto**. CREB Plays a Critical Role in the Regulation of Normal and Malignant Hematopoiesis. Accepted for poster presentation. American Society of Hematology, Atlanta, GA, December 2007.

74. Hernandez J, Li J, Wei RQ, Tapang P, Davidsen SK, Albert DH, Marcotte PA., Glaser KB, Fu C, and **KM Sakamoto**. Multi-Targeted Receptor Tyrosine Kinase Inhibitor, ABT-869, Induces apoptosis and Inhibition of Proliferation of Ba/F3 FLT-3 ITD mutant cells. Accepted for poster presentation. American Society of Hematology, Atlanta, GA, December 2007.

*75. Danilova N, **Sakamoto KM**, and S Lin. Imbalance of p53 family members as a new target of therapeutics for treatment of Diamond Blackfan Anemia. Accepted for oral presentation. American Society of Hematology, Atlanta, GA, December 2007.

76. Ikeda AK, Judelson DR, Federman N, Li J, Wei RQ, Tapang P, Davidsen SK, Alber DH, Glaser KB, Landaw E, and **KM Sakamoto**. ABT-869, a Multi-targeted Receptor Tyrosine Kinase Inhibitor, Suppresses Proliferation of Ewing's Sarcoma by inhibiting the PDGFRB and c-Kit Pathways. Accepted for poster presentation. American Society of Pediatric Hematology-Oncology meeting, Cincinnati Ohio, May 2008.

*oral presentation of abstract

INVITED PRESENTATIONS

1. **Sakamoto KM.** "Cytokine Signals and Cell Cycle Control During Myelopoiesis" Childhood Leukemia, Biological and Therapeutic Advances. April 17, 1998, Los Angeles, California.

2. **Sakamoto KM.** Serine/Threonine Phosphorylation in Cytokine Signaling Workshop sponsored by the National Cancer Institute. March 30, 1999, Washington, D.C.

3. **Sakamoto KM.** "Signal Transduction Pathways Activated by GM-CSF." October 29-30, 1999. ACS Professors Meeting, New York.

4.**Sakamoto KM.** "Signal Transduction and Cell Cycle Control in Myeloid Cells" for Meet-the-Experts Breakfast, American Society of Hematology, December 5, 1999, New Orleans, LA.

5. **Sakamoto KM.** CapCURE meeting, September 2000, Lake Tahoe. "Novel Approach to treat Prostate Cancer"

6. **KM Sakamoto and RJ Deshaies.** What SCF Ubiquitin Ligases Are and how they can be used to regulate cancer progression, 4/01

7. **Sakamoto KM**. Bone marrow cells regenerate infracted myocardium, Journal Club, 4/01

8. **Sakamoto KM.** Acute Leukemia for Pediatric Residents at UCLA School of Medicine, 7/01

9. **Sakamoto KM.** ITP, Olive View Grand Rounds, 8/01

10. **Sakamoto KM.** Childhood Leukemia: causes and treatment. American Cancer Society, Los Angeles Chapter, 10/01

11. **Sakamoto KM.** "The Role of SCF Ubiquitin Ligase in Human Disease: Implications for Therapy." Caltech Biolunch, March 6, 2002.

12. **Sakamoto KM**. "Targeting Cancer-Promoting Proteins for Ubiquitination and Degradation" Signal Transduction Program Area Seminar, Jonsson Comprehensive Cancer Center, UCLA. August 1, 2003.

13. **Sakamoto KM.** "Development of Approaches to Target Proteins for Ubiquitination and Degradation in Human Disease." Thesis Defense, Caltech. December 18, 2003.

14. **Sakamoto KM.** "Role of CREB in Human Leukemias." Gene Medicine Seminar Series. Jan 26, 2004.

15. **Sakamoto KM**. "Childhood Neutropenias." Pediatric Resident Noon conference. February 4, 2004.

16**. Sakamoto KM**, "The Role of CREB in human leukemias", Gene Medicine Research Seminar, January 26th 2004.

17. **Sakamoto, KM**. "CREB and Acute Myeloid Leukemia," Leukemia Research Group Meeting, March 4, 2004.

18. **Sakamoto, KM.** "The Role of CREB in Leukemogenesis," Pediatric Research Seminar, May 20, 2004.

19. **Sakamoto, KM**. Meet the Professors lunch for UCLA ACCESS graduate students. October 6, 2004.

20. **Sakamoto KM**. UCLA Training Program in Tumor Biology. Retreat for UCLA ACCESS graduate students. October 31, 2004.

21. **Sakamoto KM**. "Hematology Jeopardy" Pediatric Noon Seminar, December 13, 2004.

21. **Sakamoto KM**. "Transcriptional Regulators in Normal and Malignant Hematopoiesis," MBI Noon Seminar, November 30, 2004.

22. **Sakamoto KM**. "Targeting Proteins for Ubiquitination and Degradation in Prostate Cancer" GU SPORE seminar, December 21, 2004.

23. **Sakamoto KM**. "Writing your first NIH grant: an overview," Pediatric Research Seminar, April 7, 2005.

24. **Sakamoto KM**. "Targeting the Ubiquitin-Proteasome System for Cancer Therapy." Minisymposium on Modulation of Protein Stability, AACR, Anaheim, CA, April 20, 2005.

25. **Sakamoto KM**. "The Role of CREB in Myelopoiesis." Myeloid Workshop, Annapolis, MD, 2005

26. **Sakamoto KM**. "The Use of RNA Interference to Study and Treat Human Disease." Organizer, Cell Biology Methods workshop, PAS/SPR meeting, Washington, D.C., 2005.

27. **Sakamoto KM**. Young Investigators Workshop. American Society of Pediatric Hematology-Oncology meeting, Washington D.C. 2005.

29. **Sakamoto KM**. "Update on Acute Leukemia: Where we've been and where we are today." Pediatric Grand Rounds, Children's Hospital of Los Angeles. August 19, 2005.

30. **Sakamoto KM.** "Acute Myeloid Leukemia," Pediatric Resident Rounds, September 21, 2005.

30. **Sakamoto KM and Krasne S**. "Grants Writing Workshop," ACCESS graduate students, September 26, 2005.

31. **Sakamoto KM.** "COG Spring Meeting Report on Acute Leukemia." Tuesday 3pm Division Conference, March 28, 2006.

32. **Sakamoto KM.** "Successes and Challenges of Childhood Cancer: Leukemia as a Model." Life after Childhood Cancer, March 29, UCLA symposium sponsored by the Leukemia and Lymphoma Society of America.

33. **Sakamoto KM.** "The Cancer Problem." M294 Basic Concepts in Oncology Course, April 3, 2006.

34. **Sakamoto KM.** "Update on Childhood Leukemia." Pediatric Grand Rounds, Charles R. Drew University of Medicine and Science, April 4, 2006.

35. **Sakamoto KM.** "Choosing a Career in Basic Science Research." Young Investigator Workshop (organizer). ASPHO/SPR meeting. April 30, 2006.

36. **Sakamoto KM.** "RNA interference and Stem Cells," New Approaches in Stem Cell Technologies Workshop, SPR meeting, San Francisco, California. April 29, 2006.

37. **Sakamoto KM.** "Leukemia." M294 Basic Concepts in Oncology Course, May 10, 2006.

38**. Sakamoto KM**. " Update on Childhood Leukemias." Olive View Grand Rounds, May 17, 2006.

39. **Sakamoto KM.** "Leukemia" Pediatric Resident Noon Lecture, June 5, 2006.

40. **Sakamoto KM**. "State of Art lecture on AML/Myelodysplastic Syndromes" for fellows, Division Conference, Tuesday July 18, 2006.

41. **Sakamoto KM**. "Preparation of the RRC Site Visit." Division Conference, August 29, 2006.

42. **Sakamoto KM.** "Molecular and Cellular Characterization of MPD: The Role of CREB in Myelopoiesis." NIH/NHLBI grantees meeting on MPD and MDS, November 9, 2006.

43. **Sakamoto KM.** "How to write your first NIH grant: an overview." Pediatric Fellows Noon Seminar, February 29, 2007.
44. **Sakamoto KM.** "Promotions: Rising up the Academic Ladder." Young Investigator's Workshop, ASPHO meeting, May 2007.

45. **Sakamoto KM.** "Childhood Leukemia." Harbor-UCLA Pediatric Grand Rounds, May 24, 2007.

46. **Sakamoto KM.** "Introduction to Cancer." Pathology M294 Basic Concepts in Oncology, April 2, 2007.

47. **Sakamoto KM.** "Acute and Chronic Leukemia." Pathology M294 Basic Concepts in Oncology, June 1, 2007.

48. **Sakamoto KM.** "Update on Childhood Leukemia." Cedars-Sinai Hematology-Oncology Grand Rounds, August 14, 2007.

48. **Sakamoto KM.** "Role of CREB in normal and neoplastic myelopoiesis." Hematopoiesis Group seminar series/Journal Club, October 19, 2007.

49. **Sakamoto KM.** "New therapies for acute leukemia." Leukemia and Lymphoma Society of America fund raiser, Parents Against Leukemia, Woodland Hills, October 27, 2007.

50. **Sakamoto KM**. "Molecular Characterization of CREB in MPD." NHLBI Grantees meeting, November 6, 2007.

51**. Sakamoto KM.** "Molecular pathogenesis and Targeted therapies in leukemia," Pediatric Hematology-Oncology, NCI, November 8, 2007.

52. **Sakamoto KM.** "CREB downregulation in normal and neoplastic hematopoietic cells." Invited speaker, Gene Therapy Symposium, Nappa Valley, November 15, 2007.

53. **Sakamoto KM**. ASH meeting 2000 report. Tuesday 3pm conference. December 18, 2007.

54. **Sakamoto KM.** PCRF symposium: 25 years of Cancer Research, "Molecular Mechanisms of AML and targeted therapies." Invited speaker, Anaheim, California. January 11, 2008.

55. **Sakamoto KM**. University of Wisconsin Frontiers in Pharmacology, "Molecular Mechanisms of Leukemogenesis and Targeted Therapies." Invited speaker, March 25, 2008. Madison, Wisconsin.

56. **Sakamoto KM**. AACR Roundtable Session on "Careers in Clinical and Translational Cancer Research." San Diego, April 12, 2008.

57. **Sakamoto KM.** AACR Session Chair for Minisymposium on "Gene Regulation in Cancer," AACR meeting. San Diego, April 14, 2008.

58. **Sakamoto KM.** "Acute Leukemia and Targeted Therapies." Pathology M294 Basic Concepts in Oncology, May 5, 2008.

59. **Sakamoto KM.** "Molecular Mechanisms of Leukemogenesis and Targeted Therapies.' Cincinnati Children's Hospital, May 12, 2008.

60. **Sakamoto KM.** "How to write your first NIH grant." Young Investigator's Workshop, American Society of Pediatric Hematology-Oncology annual meeting, Cincinnati Ohio. May 15, 2008.

61. **Sakamoto KM and Joanne Hilden.** "Ethics and Writing." Young Investigator's Workshop, American Society of Pediatric Hematology-Oncology annual meeting, Cincinnati Ohio. May 15, 2008.

62. **Sakamoto KM and Jeff Lipton.** "Translational Research: how not to fall between the bench and the bedside". American Society of Pediatric Hematology-Oncology annual meeting, Cincinnati Ohio. May 17, 2008.

63. **Sakamoto KM**. "Childhood Leukemia." Pediatric Resident noon conference. UCLA, June 25, 2008.

64**. Sakamoto KM**. First year Fellow Orientation, Mattel Children's Hospital UCLA, July 2, 2008.

64. **Sakamoto KM.** "Introduction to Professionalism," Professionalism Workshop for Pediatric Hematology/Oncology fellows. July 30, 2008.

65**. Sakamoto KM**. Neutropenia. Division of Pediatric Hematology-Oncology Lecture, August 5, 2008.