DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

This proposal describes a 5 year training program for the development of an academic career in Laboratory Medicine. The principal investigator has completed structured residency training in Pathology at the University of _____ and now, will expand upon his scientific skills through an unique integration of interdepartmental resources.

This program will promote the command of transcriptional corepressor biology, as applied to hematopoietic disease. _____ will mentor the principal investigators scientific development. _____ is a recognized leader in the field of transcriptional repression. He is the Chief of Endocrinology and has trained numerous postdoctoral fellows and graduate students. To enhance the training, the program will enlist the expertise of _____, Assistant Professor of Pathology. _____ pioneered the *ex vivo* bone marrow transduction techniques that will be applied in the analyses of repression. In addition, an advisory committee of highly-regarded medical scientists will provide scientific and career advice.

Research will focus on transcriptional repression in myeloid progenitor cells. Recent work in _____ laboratory demonstrated that aberrant recruitment of corepressors may underlie the disruption of myeloid development in leukemia associated with the AML1-ETO fusion protein. The proposed experiments will entail introduction of AML1-ETO and derivatives into mouse bone marrow and myeloid cell lines. Subsequently, phenotypes will be examined using an assortment of biochemical, molecular, and cellular techniques. The specific aims include: 1) Establishing an *in vivo* model for the analysis of interactions between AML1-ETO and repression pathways, 2) Determining if corepressor recruitment by AML1 is sufficient to exert the pathogenic effects of AML1-ETO, and 3) Determining the role of ETO domains in the disruption of myeloid development by AML1-ETO. This will be the first detailed functional analysis of the mechanisms of transcriptional repression by AML1-ETO using models that attempt to mimic the pathways of the myeloid progenitor.

The Pathology department of the University of _____ provides an ideal setting for training physician- scientists by incorporating expertise from diverse resources into customized programs. Such an environment maximizes the potential for the principal investigator to establish a scientific niche from which an academic career can be constructed.

PERFORMANCE SITE(S) (*organization, city, state*)

Laboratory of _____
Department of _____
Hospital of the University of _____
_____, _____ _____

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
_____	University of _____	Principal Investigator
_____	University of _____	Sponsor
_____	University of _____	Collaborator
Advisory Committee:		
_____	University of _____	Advisory Committee
_____	University of _____	Advisory Committee
_____	University of _____	Advisory Committee
_____	University of _____	Advisory Committee

Use this substitute page for the Table of Contents of Research Career Awards

Type the name of candidate at the top of each printed page and continuation page.

RESEARCH CAREER AWARD

TABLE OF CONTENTS

(Substitute Page)

Page Numbers

Note: Type density and size for the entire application must conform to the instructions on page 6 of the general instructions.

*Include these items only when applicable.

CITIZENSHIP

[X] U.S. citizen or noncitizen national [ ] Permanent resident of U.S. [ ] If a permanent resident of the U.S., a notarized statement must be provided by the time of award

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: _____ laboratory consists of ~1500 square feet located on the 6th floor of the Clinical Research Building at the University of _____. The laboratory contains refrigerators, freezers, a microwave, gel electrophoretic equipment, microfuges, PCR machines, water baths, a Stratalinker, power supplies, and most of the small equipment required for the proposed studies. Across the hall is a tissue culture room with two laminar flow hoods, a phase contrast microscope, and four CO2 incubators, as well as a walk-in cold room.

Clinical: N/A

Animal: The animal facility is located in the _____ Building adjacent to the Clinical Research Building. The facility includes an irradiator and two procedure rooms suitable for bone marrow transplantation experiments. Mouse rooms are equipped with laminar flow hoods for non-surgical manipulations.

Computer: _____ laboratory is equipped with 3 Power Macintosh computers, zip drives, a scanner, and printers. Software is available for word processing, presentations, and sequence analysis. The laboratory has access to the University of _____ network, the Internet, Medline, and numerous online biomedical journals.

Office: Work will be conducted in the laboratory of _____ where the applicant has a desk and filing space.

Other: Secretarial assistance is available through the _____ Division. The proximity of the lab to investigators in the _____ Center (same floor) and _____, Department of _____ (same building) encourages frequent consultation and interchange of ideas.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

_____ has two -70 freezers, a gel drier, and Speedvac. _____ share common space with three ultracentrifuges, two superspeed centrifuges, a phosphoimager, ice machine, UV/visible spectrophotometer, and beta and gamma counters. The darkroom contains an X-O-Mat film processor and camera set-up. On the same floor are additional centrifuges and rotors available during periods of heavy use and/or repair of Endocrine Section equipment.

Section II: Specialized Information

1. Introduction to Revised Application

2. Letters of Reference

3. The Candidate

a. *Candidate's Background*

My career commitment from secondary school through residency has been the molecular genetics of human disease. I developed this focus prior to college, in the Secondary Science Training Program of the _____. I continued to study molecular genetics during college in the laboratory-oriented Honors Biology Program of the _____. Upon applying to _____ I declared my intent to pursue a career in pathology, specifically for the opportunities to combine science and medicine. Now, I have completed the structured clinical pathology rotations of residency, and I am poised to develop scientific skills in the analysis of transcriptional complexes and the molecular biology of leukemia. This broadening of skills will help me compete as an independent investigator, and will ultimately lead to a career in academic molecular pathology.

My specific scientific concentration is on the regulation of gene expression. I performed my thesis work in the laboratory of _____ at _____ studying the DNA elements controlling expression from the intricate beta-globin gene cluster. We selected a mouse model for our investigations and performed one of the earliest knockouts combining homologous recombination with site specific (Cre-lox) recombination technology(24). In addition to advancing the understanding of globin gene regulation, this work provided a universal lesson for mouse geneticists by emphasizing the often ignored potential for a single selectable marker to disrupt expression of multiple genes within a locus(45). I left the lab with a depth of understanding of locus control and mouse genetics.

After completing the M.D./Ph.D. program at _____, I entered residency in clinical pathology at the University of _____. Clinical rotations focused upon laboratory disciplines including hematopathology, flow cytometry, and molecular diagnosis. The goal of the program is to launch the careers of independent scientific investigators while promoting expertise in a clinical laboratory service. I have completed the structured, core rotations of residency, and I am initiating the research phase of the program.

b. *Career Goals and Objectives: Scientific Biography*

Ultimately, my goal is to attain a tenure-track position in a department of clinical pathology. I intend to devote approximately _____ of my time to the study of molecular pathways regulating gene expression in hematopoietic cells. The remaining time will be committed to the clinical discipline of molecular diagnosis: the diagnostic, prognostic, and therapy-determining molecular assessment of human disease. This allocation of time will foster a scientific career while elevating clinical performance.

In March, 1999, I entered the laboratory of _____, the _____ and an authority on transcriptional repression. The study of transcription factor complexes, under _____ supervision will serve multiple objectives in preparing me for an academic career in clinical pathology. My objectives include:

- acquiring a scientific background that will complement my clinical training.
- expanding my technical background with new biochemical and cellular skills.
- allowing me to update and enhance my skills in the discipline of molecular genetics; a field that has continued to explode in the three years since I completed graduate school.
- serving as a transitional period between postdoctoral training and an independent faculty position.

c. *Career Development Activities during Award Period*

My goal during the next five year period is to develop the skills necessary to succeed as an independent investigator. My strengths include a command of the scientific method and technical expertise in molecular genetics. However, in order to succeed as a scientist in the competitive field of transcription, I propose to acquire new perspectives and skills complementary to the discipline of clinical molecular pathology:

1) Acquire expertise in transcriptional repression.

The field of transcription is one of the most rapidly advancing in biology today. By studying corepressor transcription factors, I will acquire a background in the fundamental molecular pathways recently shown to be involved in human leukemias. In order to develop a working knowledge of today's issues and prime myself for independent activity, I propose the following:

- Research: Perform organized research under the guidance of _____, _____ and a leader in the field of transcriptional repression. Recently, transcriptional biology more commonly associated with nuclear hormone receptors converged with the field of hematopathology. _____ contributed to the recent revelations that linked corepressor complexes with both APL(16) and AML(15). This makes the _____ laboratory the ideal setting for acquiring expertise in the biology of corepressor complexes as they pertain to leukemia. By training in the _____ laboratory, I will be engrossed in the basic science of transcriptional repression. I will acquire new fundamental skills in biochemistry and cell biology including Western blot analysis, immunoprecipitation, and flow cytometry. Additionally, I will benefit from the lab meetings and departmental conferences in which the _____ laboratory participates.
- Lectures: Review developments in transcription and stay abreast of emerging topics through participation in a genetics lecture series (1 per week), journal review (1 per week) and transcription seminars (2 per week). My initial goal is to efficiently review the developments that have taken place in genetics during the three year period since I completed graduate school. I have begun this review by attending weekly genetics lectures. Additionally, I intend to follow current developments in the field of transcription through participation in a nuclear hormone receptor journal review and various transcription-related seminars hosted by both clinical departments and the graduate school.
- National meetings: Attend the nuclear hormone receptor Keystone meeting. The field of transcriptional repression is founded in nuclear hormone receptors. This meeting is likely to provide insights applicable to repression in general, including repression in hematopoietic cells.

2) Acquire experience in the analysis of hematopoietic disease.

Ultimately, I intend to study transcription as it applies to hematopoietic disease. As a graduate student, I acquired an understanding of embryonic stem cells and homologous recombination technology. This methodology is not consistently or easily used to analyze genes that produce embryonic lethal phenotypes. I, therefore, intend to develop skills in alternate approaches to modeling and analyzing hematopoietic disease.

- Research: Develop proficiency in somatic cell gene transfer and bone marrow transplantation in mice under the supervision of _____. _____ developed the cell lines(44) and transient transfection techniques(42, 44) for producing high titer retroviral cultures. He subsequently used bone marrow retroviral transduction methodologies to produce mouse models of chronic myelogenous leukemia(43) and T-cell acute lymphocytic leukemia(41). _____ has performed hundreds of bone marrow transplants in mice and continues to perform these procedures routinely. The _____ laboratory is, therefore, ideal for acquiring skills in mouse models of hematopoietic disease.
- Lectures: Stay abreast of developments in the laboratory analysis of hematologic disease through participation in resident's seminars (1 per week). The clinical pathology residents hold a weekly conference during which one member of the program reviews an emerging issue in laboratory medicine. Approximately half of these sessions focus on hematological issues. This will provide a regular update on developments in the analysis of blood diseases.
- National meetings: Attend the meeting of the American Society of Hematology. This conference is likely to be the most appropriate clinical forum for the presentation and discussion of the proposed hematopoietic model systems.

3) Acquire scientific and career guidance from an advisory committee.

In order to most effectively promote a career in the field of academic clinical pathology I have organized an advisory committee composed of physician-scientists with strengths in the fields of hematology and transcription. Each member of the committee is an outstanding investigator in his field.

The committee will meet twice yearly. The format of the meeting will include a formal presentation by the applicant followed by a critical review of the hypotheses, results, and future directions. Finally, the committee will provide career guidance. In addition to _____, the committee includes the following members.

- _____ is an Assistant Professor in _____ and a member of the Division of _____. _____ has extensive expertise in *ex vivo* retroviral transductions of mouse bone marrow(41-44). _____ is already providing valuable advice in the advancement of the mouse model described in specific aim 1.
- _____ is an Associate Professor in _____ and the Director of _____. _____ is an authority on mouse models of leukemia and has a background in transcription and molecular hematopathology.
- _____ is a Professor of _____ and _____. He has a depth of experience in clinical hematology, leukemia, and bone marrow transplantation.

4) Attend Miscellaneous Postdoctoral Sessions

The University of _____ offers an assortment of postdoctoral courses to strengthen the training program. These include:

- Bioethics: Sessions focus on issues appropriate for both basic and applied science.
- Orientation to laboratory animal research: Sessions provide mandated instruction in animal care, as well as, resources in the veterinary school for additional training, as needed.
- Safety training: Training includes mandatory radiation, biological, and chemical safety sessions. Training is also offered for the irradiator that will be used in performing bone marrow transplants in mice.

My plan for training in the responsible conduct of research is as follows. In year 1 of my K08, I will take Course # _____, Research Ethics and Integrity, 3 units, third term. This course introduces concepts inherent to the ethical conduct of research with human participants, issues of scientific integrity, and ethical theories and principles. This course will fulfill the _____ and the NIH requirements concerning training in the responsible conduct of research.

In the second year of my K08 I will take Course # _____, Research Ethics, 1 unit, second term. This course presents issues in the responsible conduct of research, such as research ethics, academic ethics, data management, data ownership, guidelines of professional conduct, research fraud, academic misconduct, and conflict of interest, and explains federal and institutional guidelines relating to research using human and animal models.

Timetable

The department of pathology recognizes the importance of focused scientific research during the transition from postdoctoral training to faculty status. Therefore, the initial years of this program will permit virtually all efforts to be devoted to the career development activities outlined above. Later years will include fellowship training in molecular diagnosis and faculty service work. Nevertheless, research will always receive _____ effort.

Year 1: _____
Year 2: _____
Year 3: _____
Year 4-5: _____

4. Statements by Sponsor(s), Consultant(s), and Collaborator(s)

5. Environment and Institutional Commitment to Candidate

a. *Description of Institutional Environment*

The Department of _____ at the University of _____ is the ideal setting for training physician scientists. Almost all residents pursue academic careers. The program advocates free scientific exploration within any laboratory of the university. This aspect permits the trainee to customize education using the diverse resources of the entire institution. I have exploited this strength of the program by entering the laboratory of _____ in the Division of _____. _____ contributed to the recent revelations linking corepressor complexes (typically associated with nuclear hormone receptors) to myeloid leukemias(15, 16). The _____ laboratory is, therefore, the ideal setting for acquiring expertise in the molecular pathogenesis of acute myelogenous leukemia. Furthermore, the _____ laboratory is optimally situated within the university to facilitate research. The _____ Building is located among a network of adjoined buildings. All of the pertinent contacts for this project are readily accessible. This includes the laboratory of our collaborator, _____ the animal facility, the histopathology laboratory, and the flow cytometry facility. Additional relevant scientific resources in the same building include laboratories in the Department of _____ and the Department _____ The environment is, therefore, an exceptional one for completing the proposed work.

b. *Institutional Commitment to Candidate's Research Career Development*

6. Research Plan

a. Statement of Hypothesis and Specific Aims

Aberrant recruitment of corepressor transcription factors (CoRs) underlies the pathogenic transcriptional activity of the AML1-ETO fusion protein. AML1-ETO is the product of a common translocation associated with acute myelogenous leukemia, t(8;21). A zinc finger domain in the C-terminus of ETO interacts with the nuclear corepressors, NCoR and SMRT. In the context of AML1-ETO, this domain is essential for repression of several AML1-responsive promoters and for inhibition of myeloid and monocytic differentiation in cell culture assays. We are interested in understanding how the CoRs and uncharacterized ETO domains contribute to the disruption of myeloid development.

Specific Aim 1: Establish an* in vivo *model for the analysis of interactions between AML1-ETO and corepressors. We hypothesize that AML1-ETO will be leukemogenic in mice when introduced into hematopoietic progenitor cells. Given the embryonic lethality of AML1-ETO "knock-in" mutations, we will perform *ex vivo* transduction of mouse bone marrow with AML1-ETO cDNA. We will transplant the marrow to lethally irradiated syngeneic recipients and monitor the animals for histopathological or flow cytometric evidence of leukemia. Successful disruption of the molecular pathways of the myeloid progenitor cell will provide the optimal system for functional analyses of AML1-ETO-corepressor interactions.

Specific Aim 2: Determine if corepressor recruitment by AML1 is sufficient to exert the pathogenic effects of AML1-ETO. We hypothesize that replacing ETO with a corepressor (CoR) will confer leukemogenic activity upon AML1. To test this, fusion proteins composed of the DNA-binding domain of AML1 and the repression domains of SMRT and NCoR will be constructed. Fusion proteins will be analyzed using well-established tissue culture assays and the mouse model described above. These experiments will reveal whether CoR recruitment is the sole pathogenic activity of the AML1-ETO fusion protein.

Specific Aim 3: Determine the role of ETO domains in the disruption of myeloid development by AML1-ETO. We hypothesize that ETO domains other than the CoR-binding domain contribute to the disruption of myeloid differentiation, possibly by participating in CoR recruitment. In order to test for activity, we will mutate ETO sequences in the context of AML1-ETO. We will systematically analyze biochemical interactions of fusion proteins with members of the corepressor complex, expression of AML1 target genes, and inhibition of myeloid differentiation. The properties of AML1-ETO mutants will identify biologically important regions of the ETO moiety.

Together, the above studies will illuminate the roles of ETO domains and the CoR complex in the pathogenesis of myeloid leukemia. This information will improve our understanding of acute myelogenous leukemia and could lead to new diagnostic markers, prognostic markers, or therapeutic targets in the ETO repression pathway.

b. Background, Significance, and Rationale:

Introduction

The t(8;21)(q22;q22) is the second most commonly occurring translocation associated with acute myelogenous leukemia. It occurs in approximately 20% of adult and 40% of pediatric leukemias of the French-American-British M2 subtype(37). The translocation creates a fusion between the AML1 gene on chromosome 21 and the ETO gene on chromosome 8. The fusion protein is leukemogenic, although, the pathways through which AML1-ETO act are just beginning to be understood. This application focuses on the interactions of AML1-ETO with the transcriptional repression pathways of the myeloid progenitor cell.

AML1

AML1 was identified by the cloning of the breakpoint involved in the recurring t(8;21) translocation of acute myelogenous leukemia (10, 37). AML1 is a well-characterized member of the core binding factor (CBF) family of transcription factors (also known as polyoma enhancer binding protein 2 family). It is expressed in hematopoietic cells and is essential for definitive hematopoietic development (40, 58). The protein has intrinsic DNA binding activity, conferred by the runt domain (named for its homology to a Drosophila transcription factor)(34). AML1 synergistically interacts with an assortment of other hematopoietic transcription factors including CBFβ, C/EBP, Ets family members, and myb to activate transcription of hematopoietic target genes (Reviewed (54)). AML1 also derives transcriptional activating activity from interactions with the histone acetyl transferase, p300/CBP. Interestingly, the product of the t(8;21) translocation lacks the C-terminal domain of AML1 (required for p300/CBP interaction) (27) but retains the runt domain (sufficient for DNA binding) (Figure 1). Thus, AML1 domains confer upon AML1-ETO a potential to disrupt expression of AML1 target genes.

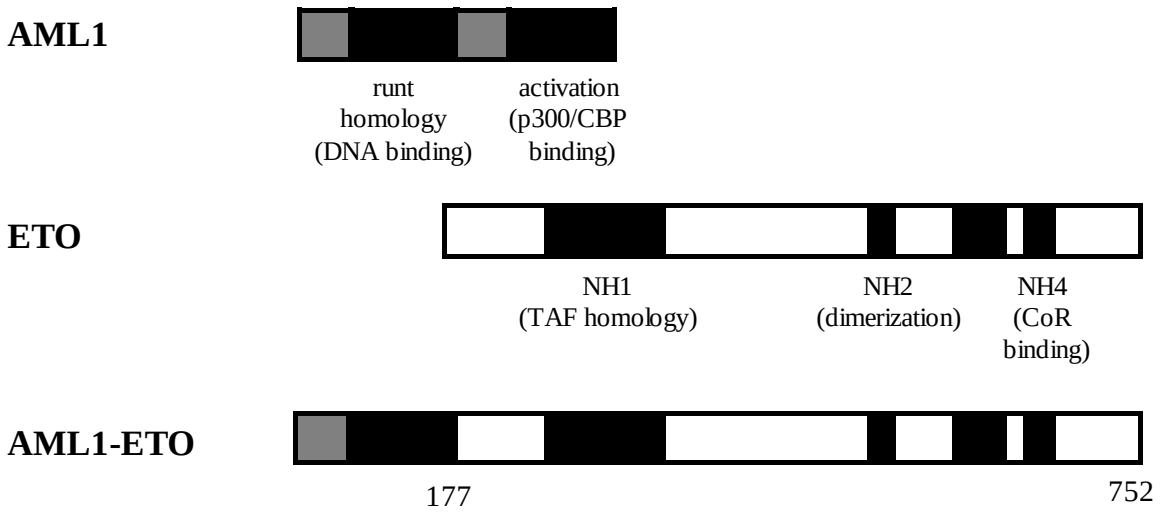

Figure 1. Maps of the AML1, ETO, and AML1-ETO gene products.
The 177 N-terminal amino acids of AML1 are fused to the 575 C-terminal residues of ETO. Domains of interest are highlighted including the DNA-binding domain and p300/CBP-interaction domain of AML1 and the Nervy homology (NH) domains of ETO.

ETO

In contrast to AML1, the activities of ETO are not clearly understood. ETO is a member of poorly characterized family of proteins including itself (also called MTG8), ETO-2(9), MTGR1(26), and MTG16 (14). The ETO gene encodes a putative transcription factor, with proline rich domains, candidate serine and threonine phosphorylation sites, a TAF110-homologous domain, a dimerization domain, and two zinc-fingers. Four domains are homologous to those of Drosophila Nervy, a poorly characterized gene expressed in the nervous system of the fly (Figure 1)(12). The ETO protein product is localized to the nucleus(11). Expression in

hematopoietic cells is debated(11, 36). The only demonstrated activity has been an ability to transform cells when overexpressed(52, 57). In fact, much of what is known about ETO is derived from the study of the AML1-ETO fusion protein. Because the function of ETO is unclear, it is difficult to know how ETO domains contribute to the activity of the AML1-ETO fusion protein.

Inhibition of Myeloid Differentiation by AML1-ETO

The t(8;21) creates a fusion between the AML1 gene on chromosome 21 and the ETO gene on chromosome 8. The chimeric gene product of the t(8;21) is comprised of the N-terminal 177 residues of AML1 and the C-terminal 575 residues of ETO (Figure 1) (36). Consistent with a role in leukemogenesis, AML1-ETO blocks differentiation of myeloid and monocytic tissue culture cells (1, 15, 26, 59). Likewise, in mice, AML1-ETO "knock-in" mutations are embryonic lethal(39, 60) due to the lack of definitive hematopoietic development. Interestingly, primitive hematopoiesis is normal and cells from hemizygous yolk sacs and livers can be passaged for several generations longer than wild-type cells. These findings are suggestive of a putative leukemogenic activity of the fusion protein. Unfortunately, investigation of this activity *in vivo* has been slow. The embryonic-lethal phenotype of the AML1-ETO fusion in mice prevents transmission through the germline. This hinders detailed functional analyses of AML1-ETO using reliable transgenic and gene targeting approaches. Specific Aim 1 will attempt to circumvent this obstacle using an *ex vivo* bone marrow transduction approach, under the guidance of an expert in the technique.

Disruption of expression of AML1 target genes by AML1-ETO

Aberrant expression of AML1 target genes undoubtedly underlies the leukemogenic activity of AML1-ETO. Numerous target genes involved in myelomonocytic development are regulated by AML1, including GM-CSF, IL-3, and M-CSF receptor, myeloperoxidase, and neutrophil elastase(13, 38, 53, 55, 63, 64) AML1-ETO retains the ability to interact with the promoters of AML1 target genes through the AML1 runt domain(34). Predictably, the AML1-ETO fusion protein represses transcription from selected AML1 target genes (13, 32, 35). It has been hypothesized that dominant negative interactions with AML1 contribute to repression(13, 32, 35). Nevertheless, the phenotype of the AML1-ETO "knock-in" mutation in mice is subtly different from that of the AML1 "knock-out," leaving open the possibility that the fusion protein has unique activities beyond its AML1-inhibitory function(39, 40, 58, 60). Indeed, the M-CSF receptor, an AML1 target gene, is paradoxically, upregulated by AML1-ETO(46). Similar, paradoxical, upregulation has been reported for Bcl-2(28), although it is controversial whether this is consistently observed in leukemic patients(3, 51). Clearly, the proposed mechanisms of AML1-ETO action are incomplete.

Recruitment of Corepressors by AML1-ETO

Recently, our laboratory(15) and others(33, 56), provided a novel explanation for the transcription-inhibiting activity of AML1-ETO: the C-terminus of ETO interacts both biochemically and functionally with corepressor proteins(15, 33, 56) including nuclear receptor corepressor (NCoR) (23) and silencing mediator for retinoid and thyroid hormone receptor (SMRT)(5). These molecules are components of multiprotein complexes that reduce gene expression to levels below baseline. Other members of the complexes include mammalian Sin3 (mSin3) and histone deacetylases (HDACs) (21). These molecules confer inhibitory activity on transcription factors such as unliganded nuclear hormone receptors(4, 6, 23, 49, 61, 62), orphan receptors(8, 50), and Mad(2, 48). Transcriptional inhibition may occur through chromatin remodeling or contact with the basal transcriptional machinery, but the mechanism is unproven. Importantly, the ability of AML1-ETO to inhibit transcription from the neutrophil protein 3 (NP-3) promoter(33) and to inhibit differentiation of U937 promonocytes(15) is dependent upon the C-terminal domain of ETO that interacts with CoRs. Therefore, the AML1-ETO fusion protein may interfere with myeloid differentiation by aberrantly recruiting corepressor complexes and histone deacetylase activity to the promoters normally activated by AML1 (Figure 2). Nevertheless, it remains unclear if this interaction is sufficient to cause leukemia or if additional activities of AML1-ETO are required. Specific Aim 2 will address this issue.

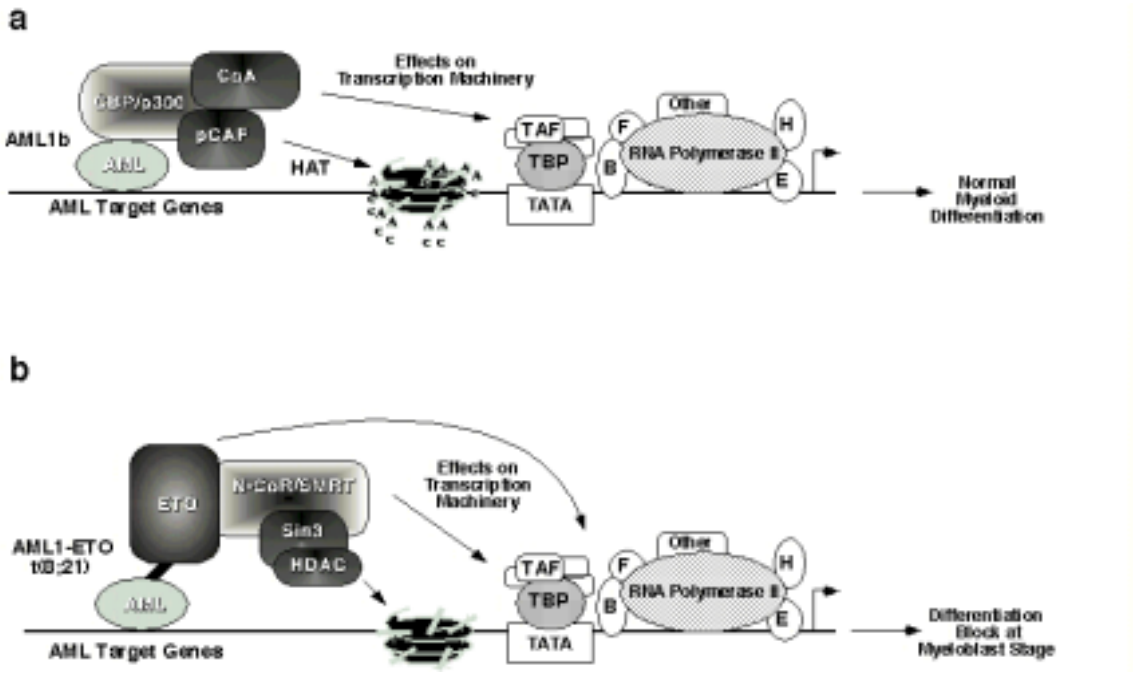

Figure 2. Model of the role of interactions of fusion protein AML1-ETO with CoRs (NCoR/SMRT) and the corepressor complex. (a) DNA-bound AML1 interacts with p300 and potentially with other coactivator molecules. This association results in transcriptional activation (possibly through histone acetylation and effects on transcriptional machinery) and normal myeloid development. (b) AML1-ETO recruits the corepressor complex. This results in transcriptional repression (and possibly activation of a subset of genes) and a block in differentiation. (15).

Precedent for Aberrant Recruitment of Corepressors in Leukemia

Intriguingly, this link between AML1-ETO and a corepressor complex is the second example of an interaction between a leukemogenic fusion protein and a transcriptional corepressor complex. The first example of such an interaction is that causing acute promyelocytic leukemia (APL). The common APL variant is a retinoic acid-responsive leukemia commonly associated with a t(15;17) translocation and PML-RARα fusion protein (Reviewed (17), 25). A rare, retinoic acid-resistant variant is associated with t(11;17) and the PLZF-RARα fusion protein(7, 17, 25, 30). Both of these fusion proteins are capable of associating with members of the corepressor complex including NCoR, SMRT, mSin3, and HDAC in the absence of retinoic acid. (These are the same factors that interact with AML1-ETO, Figure 2b). Amazingly, the responsiveness of disease to retinoic acid therapy is related to the persistence of the interaction of the fusion protein with the corepressor complex. (16-18, 20, 22, 31, 47) It, therefore, appears that an aberrant interaction with corepressors is a recurring mechanism whereby mutant transcription factors produce leukemia.

Corepressor recruitment may not be sufficient for leukemia

While recruitment of CoRs may be necessary for AML1-ETO (and PML-RARα) to inhibit myeloid differentiation it is not known if recruitment is sufficient. It is noteworthy that in both t(8;21) and the t(16;21) variant (between the AML1 gene and the ETO family member MTG16), almost the entire "ETO" protein is included in the fusion product(14, 36). No reported translocations with the AML1 locus truncate the ETO gene, increasing speculation that the entire protein may contribute to leukemogenesis. In fact, activity has been elicited from other domains of AML1-ETO in selected assays. For example, the Nervy-homology 2 domain (Figure 1) permits dimerization with ETO family members (26) and is necessary for repression of transcription (32). Furthermore, ETO domains may help explain the counterintuitive activation of certain AML1 target genes by AML1-ETO(46). Nevertheless, it is not known if ETO actively contributes to the leukemogenic process *in vivo*, or if it passively tethers CoRs to AML1 targeted promoters. Specific Aim 3 will investigate the roles of conserved ETO domains in the pathogenesis of leukemia.

Selection of model systems for analysis of AML1-ETO

Although the initial characterization of the interaction of AML1-ETO with corepressor pathways was elegantly described using an assortment of cell lines, additional information could be extracted from models amenable to myeloid differentiation assays. Selection of a model system can have a dramatic impact upon the outcome of transcriptional repression studies. For example, ETO functions as a repressor when tethered to a heterologous promoter in C33A (cervical carcinoma) cells but not 293T (renal epithelial) cells(65). Evidently, conclusions drawn from studies of a given cell line may not provide accurate information about AML1-ETO activity *in vivo*. Model systems suited to the evaluation of myeloid differentiation are available. Several groups have demonstrated that the intact AML1-ETO fusion protein inhibits myeloid differentiation in progenitor cell culture models(1, 26, 59), although detailed domain mapping has not been attempted. Likewise, *in vivo* analyses have suggested that AML1-ETO blocks hematopoietic development, but follow-up functional analyses have been slowed by the embryonic lethal phenotype of the AML1-ETO gene fusion(39, 60). Ideally, future studies of AML1-ETO function will be performed using model systems that recapitulate the repression pathways of the myeloid progenitor cell.

Significance

Simultaneously, our lab(15) and others(33, 56), made the link between AML1-ETO and corepressor pathways. Nevertheless, these initial observations have not been expanded with detailed functional analyses using models that mimic the molecular pathways of the myeloid progenitor cell. We, therefore, intend to employ a novel mouse model, and a well established myeloid cell culture model to probe the interactions between AML1-ETO and corepressor pathways.

First, we will attempt to develop the mouse model. Because AML1-ETO causes embryonic demise, we will deliver AML1-ETO somatically, by *ex vivo* transduction of mouse bone marrow. We hypothesize that transplanted marrow will cause leukemia in recipient mice. Such a model will provide, for the first time, an opportunity to evaluate interactions between AML1-ETO and pathways that are, in fact, active in the hematopoietic progenitor cell. Because the endpoint of the *in vivo* studies is the development of a myeloproliferative disorder, this model will be especially useful for distinguishing pathogenic activities from biological properties of AML1-ETO unrelated to disease.

Next, we will apply both *in vivo* and *in vitro* models to understand the importance of the interaction of AML1-ETO with CoRs. While CoR recruitment by AML1-ETO appears necessary, *in vitro,* for inhibition of development, it is not clear if it is sufficient to disrupt myeloid differentiation in the absence of ETO domains. Determining the importance of CoR recruitment is of fundamental significance to this field because it will guide investigation either upstream (i.e. to ETO domains) or downstream (i.e. to CoR-interacting proteins) of the CoR.

Finally, we will attempt to understand if ETO domains, other than the CoR-binding domain contribute to the leukemic process. These experiments will complement those of specific aim 2. Several ETO domains have activities *in vitro*. These domains may act by modifying interactions with the CoR complex or they may act through independent mechanisms. Clarifying the role of ETO in the inhibition of myeloid development is likely to provide insight into both the pathogenic activity of AML1-ETO and mechanisms of repression in general.

Recruitment of corepressor complexes may be a common mechanism in leukemogenesis. Examination of the pathways recruited by AML1-ETO is likely to improve the management of patients with not only t(8;21) leukemia, but other forms of myeloid leukemia as well. When pathogenic pathways are dissected using the appropriate model system, every molecular participant is a potential diagnostic marker, prognostic marker, or therapeutic target in patients with disease. It is, therefore, vital to learn which molecules are critical for AML1-ETO to disrupt myeloid gene expression and development.

c. Preliminary Studies and Any Results

The _____ laboratory recently published data indicating that the C-terminus of AML1-ETO interacts with corepressors (CoRs) and is necessary for the inhibition of differentiation of U937 monocytic cells(15) (See appendix). Based upon this data, we propose to evaluate the mechanisms of transcriptional repression by AML1-ETO using a mouse model and a myeloid cell culture model.

Efforts have been focused on producing a virus suitable for infection of mouse myeloid cells. This has entailed:

- construction of a retroviral vectors encoding AML1-ETO
- expression of the AML1-ETO fusion protein
- production of high titer retroviral stocks

We have completed these preliminary goals and are ready to begin infections of bone marrow and myeloid cell lines.

Vector Design

Two vectors have been constructed for the analysis of AML1-ETO *in vitro* and *in vivo*. The first, AML1-ETO MigR1 was assembled for *in vivo* analysis. The mouse stem cell retroviral vector (MSCV) was selected for its history of prior success in mouse leukemia models(43, 66). Advantages of the vector include the ability to infect mouse hematopoietic stem cells, an extended packaging site to maximize

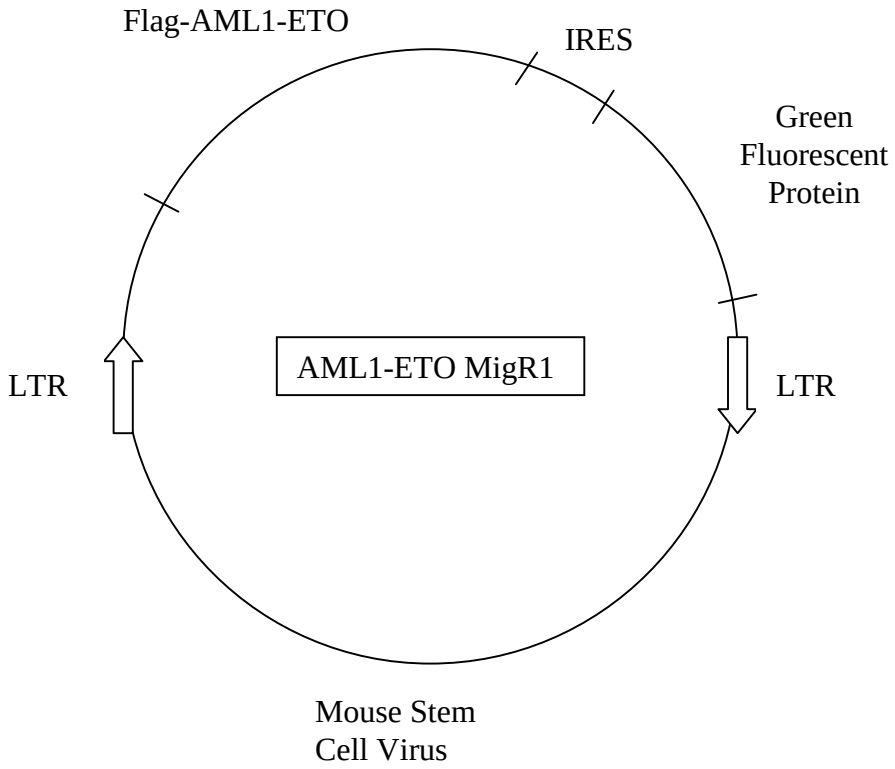

Figure 1. Map of AML1-ETO MigR1 retroviral vector for *in vivo* analyses.

retrovirus production, and long-term, high-level expression from the murine stem cell retroviral LTR(19, 43). The MigR1 vector, conveniently, expresses green fluorescent protein (GFP) to facilitate the analysis of infected cells by flow cytometry. A 2.3 kilobase, N-terminal, FLAG-tagged AML1-ETO cassette was produced by PCR.

The fidelity of PCR was confirmed by sequencing. The AML1-ETO cassette was inserted into the Xho1 site of the MSCV MigR1 multiple cloning site. AML1-ETO and GFP are expressed as a bicistronic message that includes and internal ribosomal entry site (IRES). A map of the vector is shown (figure1).

The second vector, AML1-ETO MSCV 2.2, was assembled for *in vitro* analysis. The 2.3 kilobase AML1-ETO cassette, described above, was inserted into the Xho1 site of the MSCV 2.2 multiple cloning site. The selectable PGK-neo gene is located downstream of AML1-ETO. A map of the vector is shown below.

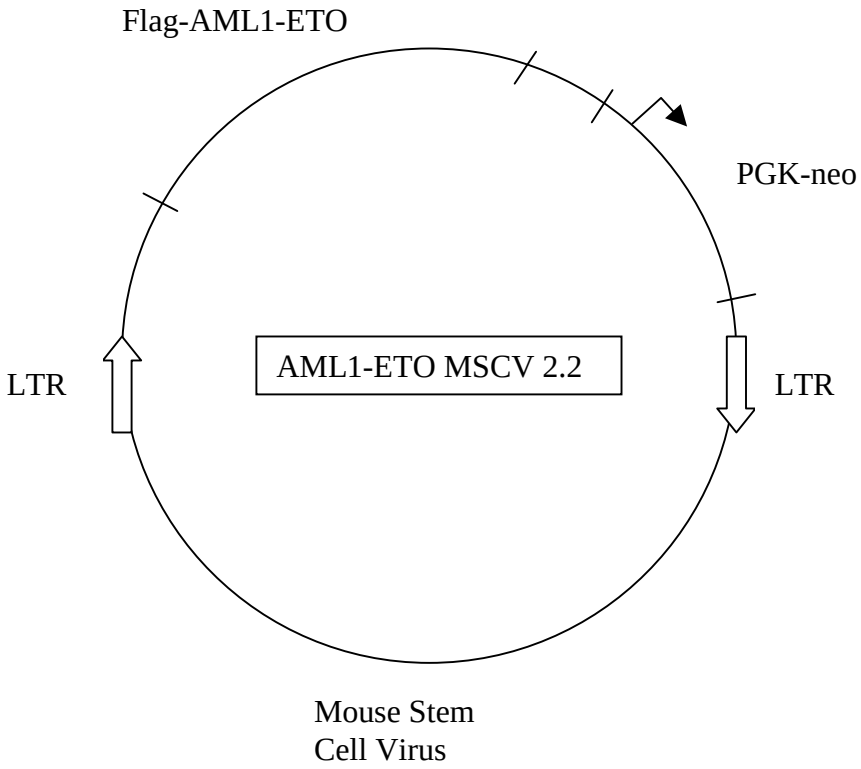

Figure 2. Map of AML1-ETO MSCV 2.2 retroviral vector for *in vitro* analyses.

AML1-ETO expression

To insure that the constructs described above, in fact, express the AML1-ETO fusion protein appropriately, Bosc23 packaging cells(44) were transfected with both AML1-ETO MigR1 and AML1-ETO MSCV 2.2. 48 hours post-transfection protein extracts were prepared and Western blot analysis was performed. Identical results have been obtained using anti-FLAG and anti-ETO (Pharmingen) primary antibodies. Both vectors, AML1-ETO MigR1 and AML1-ETO MSCV2.2 appropriately express the AML1-ETO fusion protein.

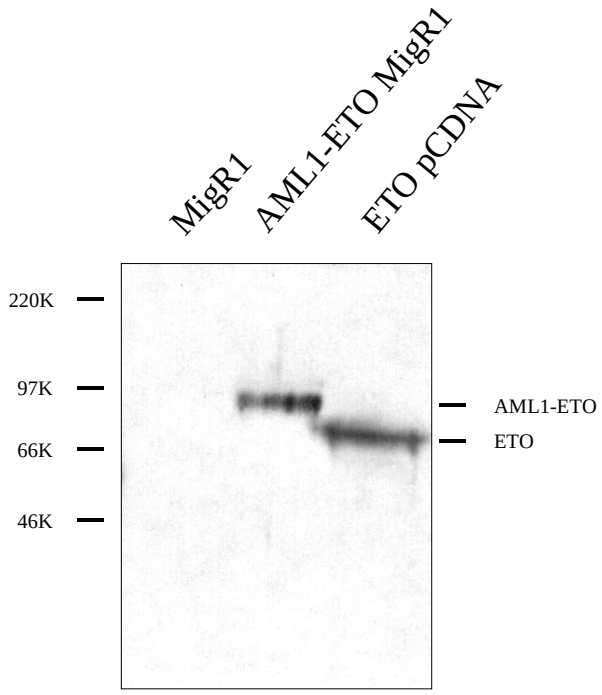

Figure 3: MSCV retroviral vectors encode AML1-ETO.
Extracts were prepared from Bosc23 cells transfected with AML1-ETO MigR1 and subjected to Western blot analysis using an anti-ETO primary antibody recognizing the C-terminus of ETO (Pharmingen). Extracts were prepared from Bosc23 cells transfected with 1) MigR1 retroviral vector, 2) AML1-ETO MigR1 retroviral vector, and 3) ETO pCDNA (positive control expression vector) (extract in lane 3 was diluted 1:100).

Production of high titer retroviral stocks

A critical parameter for the successful development of myeloid leukemia models using retroviral transduction is the titer of retrovirus used to infect bone marrow cells(43). The Bosc 23 packaging cell line was developed by Pear et al. to maximize the production of high titers of retrovirus using transient transfection techniques(44). 2 x 10^6 Bosc 23 cells were transfected with 2 μg of retroviral vector DNA using FuGENE6 (Boerhinger) in the presence of 25μM chloroquine. 48 hours after transfection, 0.1 ml of BOSC 23 supernatant was added to NIH3T3 cells as a cocktail of 0.1 ml supernatant, 2.9 ml Dulbeccos Modified Eagle Medium, 10% fetal calf serum (Gibco), 100 U/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco), plus polybrene (final concentration 4 μg/ml). Additional media was added 4 hours after infection. Two days after infection, NIH3T3 cells were harvested and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickenson). Titers were calculated as: (percentage of GFP positive cells) x (number of cells plated) / (volume of virus used for infection). As shown by the histogram and calculation below, titers of greater than 10^6 are produced routinely.

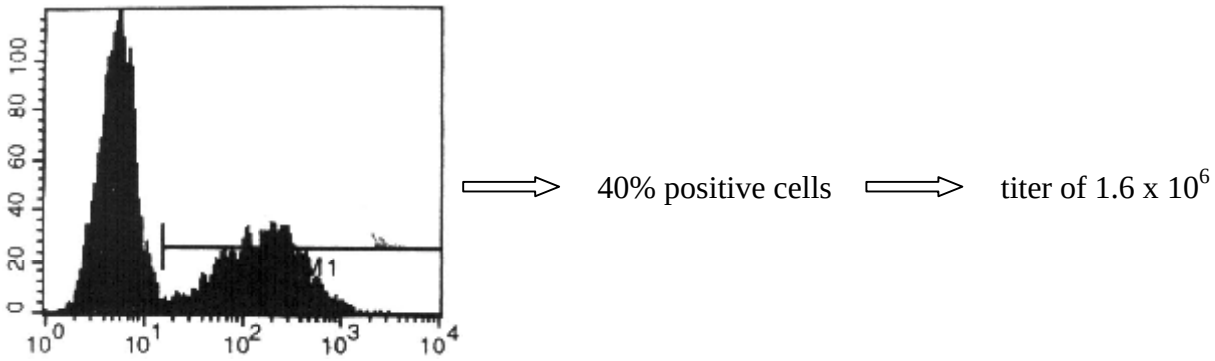

Figure 4. Production of High-Titers of Retrovirus.
The flow cytometry histogram of NIH3T3 cells transduced with GFP-expressing retroviral vector. Bosc 23 packaging cells were transfected with the MigR1 retroviral vector encoding GFP. 48 hours after transfection, 0.1 ml of supernatant was used to infect NIH3T3 cells. 48 hours later, the infected NIH3T3 cells were analyzed by flow cytometry. 40% of cells were infected. (40% of cells infected) x ($4x10^5$ cells plated) / (0.1 ml virus) = titer of 1.6 x 10^6. Similar results have been produced with AML1-ETO MigR1.

d. Research Design and Methods

We propose to examine the association of AML1-ETO with corepressor (CoR) pathways. We will attempt to perform these studies using two model systems appropriate for analysis of myeloid development. In specific aim 1, we will use retroviral transduction to introduce AML1-ETO into the bone marrow of mice. The proposed *ex vivo* transduction / transplant system was pioneered by our collaborator, _____, who is committed to providing the training, protocols, and reagents needed to establish the system in the _____ laboratory. Specific aims 2 and 3 will employ the mouse model as well as a complementary 32D3 myeloid progenitor cell model. The 32D3 model has been used previously to demonstrate the effects of AML1-ETO(1, 59). The _____ laboratory has extensive expertise in all proposed molecular analyses outlined below, including RT-PCR, immunoprecipitations, Western blotting, and histone deacetylase assays.

Specific Aim 1: Establish an* in vivo *model for the analysis of interactions between AML1-ETO and corepressors.

We hypothesize that expression of AML1/ETO in murine bone marrow progenitors will be sufficient to cause a myeloproliferative disorder. Analyses of AML1-ETO using different cell lines can give contradictory results(65), most likely due to cell type specific differences between repression pathways. Therefore, the ideal system for analyzing leukemogenic interactions between CoRs and AML1-ETO is that which most closely recapitulates the molecular pathways of the myeloblast. Development of a mouse model will permit the evaluation, for the first time, of interactions between AML1-ETO and CoRs *in vivo*. This approach has the potential to selectively reveal activities of AML1-ETO that are involved in disease. The approach would distinguish these pathogenic activities from those that are incidentally detected using *in vitro* models. An *in vivo* model, therefore, could be invaluable in determining the direction of future studies of disease mechanism.

We will attempt to produce leukemia in mice by *ex vivo* transduction of mouse bone marrow with vectors expressing AML1-ETO. Transduced bone marrow will be transplanted to lethally irradiated syngeneic recipient mice. These animals will be monitored for histopathological or flow cytometric evidence of leukemia. _____, will supervise development of the model. _____ has performed this procedure for seven years in several hundred mice and continues to perform the transductions / transplants routinely. Therefore, the necessary reagents and expertise are available to develop the mouse model for the analysis of AML1-ETO.

AML1-ETO MigR1 vector

A retroviral vector to be used for *in vivo* studies was constructed to express AML1-ETO in hematopoietic progenitor cells and to, subsequently, permit the flow-cytometric recognition of infected cells using green fluorescence protein (GFP) (see preliminary data). The mouse stem cell viral vector (MSCV), used in previous leukemia models(43, 66), was designed to express a bicistronic mRNA encoding N-terminal FLAG-tagged AML1-ETO and humanized green fluorescent protein (GFP) downstream of an internal ribosomal entry site (IRES) (43). The vector encodes the N-terminal 177 amino acids of AML1 fused to the C-terminal 575 amino acids of ETO. Appropriately, a 95 kD protein is encoded (see preliminary data).

Retrovirus production

A critical parameter for development of a successful retroviral transduction model is production of high titers (10^6/ml) of retrovirus. The Bosc23 packaging cell line was developed by Pear et al. to achieve this target (44). We can routinely achieve titers in excess of 10^6, as measured by GFP expression in infected NIH3T3 cells (see preliminary data).

Bone marrow infection

Bone marrow infections will be performed as described (41-43). $4x10^6$ bone marrow cells from Balb/c mice will be harvested from dissected long bones four to five days after treatment with 5-FU (5 mg / mouse). Cells will be transduced by spinoculation(42) on consecutive days in cocktails of 1 ml retroviral supernatant plus 3 ml DMEM, 10% fetal bovine serum (Gibco), 5% WEHI-conditioned medium, 6 U/ml recombinant mouse IL-3 (Genzyme), 10,000 U/ml recombinant mouse IL-6 (Genzyme), 5 U/ml recombinant mouse SCF

(Amgen), 2 μg/ml polybrene (Sigma), 100 U/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco), and 2 mM L-glutamine (Gibco).

Transplantation

2.5 x 10^5 to 5.0 x 10^5 cells cultured for 48 hours, as described above, will be injected into tail veins of lethally irradiated syngeneic Balb/c recipient mice.

Monitoring virus expression

To insure that virus is expressed in the periphery, 100 μl of blood will be collected monthly and analyzed by flow cytometry for GFP expression. Flow cytometry will be performed in the University of _____ _____ Center facility using a FACScan flow cytometer (Becton Dickenson).

Monitoring disease onset

Mice will be monitored for disease by observation daily (decreased activity, tachypnea-denoted by hunched posture, etc.), blood smear analysis weekly (see below), and flow cytometric analysis monthly (see below). Mice will be monitored for up to a year based upon the experience of the _____ laboratory.

Histopathology

Wright stained blood smears of mice will be analyzed weekly for histopathological evidence of leukemia. Animals with increased white blood cell counts (>50,000) or circulating blasts will be sacrificed and examined for gross or histopathological involvement of peripheral blood, bone marrow, spleen, liver, thymus, or lymph nodes. The applicant has training in hematopathology and is capable of routine analyses. Additionally, the expertise of _____, the Director of _____ and member of the advisory committee, will be sought if unexpectedly complex morphology is observed.

Flow cytometry

Flow cytometry will be used every other week to monitor animals for signs of disease. We will analyze 100 μl of blood for abnormal FSC/SSC, Gr1/Mac1, or B220/Thy1.2 profiles. Additionally, flow cytometry will be performed on peripheral blood, bone marrow, spleen, liver, thymus, and lymph nodes of mice with leukemia. Analyses will be performed using GFP to gate virus-infected cells. Second and third fluorochromes will be PE and PerCP. A comprehensive panel of antibodies will be used to determine the profile of myeloid and lymphoid markers (including Gr1, Mac1, Ter119, B220, Thy1.2, CD4, CD8) expressed on leukemic cells. Flow cytometry will be performed in the University of _____ _____ Center facility using a FacsCalibur dual laser flow cytometer (Becton Dickenson).

Determination of retrovirus induced disease

In order to demonstrate that the AML1-ETO retrovirus, in fact, causes disease in the involved animals, proviral integration will be confirmed by Southern blot analysis and expression of AML1-ETO protein will be confirmed by Western blot analysis.

Serial transplantation

The standard for declaring a leukemia model is the ability to serially passage disease from a donor to a recipient. We will therefore transplant bone marrow cells from diseased mice to sublethally irradiated syngeneic recipients. Recipient mice will be monitored for disease as described above.

Alternative approaches

We expect that the outlined retroviral transduction experiment is the most likely approach to successfully produce leukemia in mice. Although, AML1-ETO “knock-in” mutations completely disrupt definitive hematopoiesis, we expect to have better success because we will be infecting cells somewhat more differentiated than the primitive hematopoietic stem cells. (These stem cells are less likely to be infected because of their low proliferation rate). We have opted not to take conventional transgenic approaches or conditional “knock-in”

approaches because we do not know the exact stage of hematopoietic development during which AML1-ETO must be expressed to disrupt myeloid development.

We feel that the project has a high likelihood of success, given the availability of necessary reagents and technical expertise. Nevertheless, we recognize that producing myeloid leukemia in mice is subject to biological limitations. For example, mice may develop a myeloproliferative disorder that does not resemble acute myelogenous leukemia of the French-American-British M2 subtype. This outcome would not compromise our ability to perform the *in vivo* studies of corepressor biology. (However, it could limit the ability to draw general conclusions pertinent to myeloid development). It is also possible that disease will be produced with low penetrance. If this occurs, it may be possible to increase disease frequency using alternative genetic backgrounds such as p53 -/+ or -/-. If mice develop no evidence of a myeloproliferative disorder, we can satisfactorily address questions in specific aims 2 and 3 using well-established cell culture models.

Although numerous models are potentially available for *in vitro* studies (primary bone marrow cells, FDCP-mix, MEL, etc.) the differentiation of both U937 cells and 32D3 cells has previously been shown to be inhibited by AML1-ETO(1, 15, 59). While either cell line would be an acceptable alternative to the mouse model, we intend to use the 32D3 myeloid progenitor cell line for two reasons. First, we speculate that 32D3 cells may more closely represent myeloid precursors than the monocytic U937 cells. Additionally, we will be able to employ the same ecotropic virus produced for the *in vivo* studies. The remainder of this proposal will, therefore, focus on the 32D3 cell model system.

32D3 cells are a diploid, IL-3 dependent myeloid progenitor cell line that can be induced to differentiate into neutrophil-like cells when treated with G-CSF(29). Cells are cultured in Iscoves modified Dulbecco medium (IMDM; GIBCO),10% fetal bovine serum (Gibco), 100 U/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco) and 2 ng/ml recombinant murine IL-3 (R+D Systems). Differentiation is performed by washing cells twice in IMDM and plating cells at a concentration of 2 x 10^5 cells/ml in IMDM, 10% FBS, 100 U/ml streptomycin, 100 U/ml penicillin and 10 ng/ml recombinant murine G-CSF (R+D Systems). Cells are harvested after 7-12 days for Wright staining or flow cytometry (using a marker such as Gr1). 4-6 clones will be examined for each construct.

Specific Aim 2: Determine if CoR recruitment by AML1 is sufficient to exert the pathogenic effects of AML1-ETO.

We hypothesize that corepressor recruitment by AML1 may be sufficient to disrupt myeloid development in the absence of ETO domains. This is based upon the recent observation by the _____ laboratory(15) and others(33, 56), that AML1-ETO interacts with corepressors (CoRs), NCoR and SMRT. Addressing the issue of sufficiency is critical to guiding future studies either upstream or downstream of the CoR. We will attempt to determine if fusing the CoR repression domains to the AML1 DNA-binding domain can confer pathogenic activity upon AML1. We intend to examine fusion constructs for the ability to disrupt myeloid differentiation through transcriptional mechanisms.

Selection of mouse vs. cell culture model

AML1-CoR constructs will be evaluated using both *in vivo* and *in vitro* models. The *in vivo* mouse model developed in specific aim 1 is potentially the best assay of activity because it tests specifically for production of disease. (As discussed, if the mouse model is not produced successfully, constructs will be tested for the ability to inhibit myeloid differentiation in 32D3 cells). The model system used, subsequently, in specific aim 2 will depend on the phenotype of AML1-CoR constructs in the differentiation assay. Constructs that produce leukemia in mice will be amenable to follow-up studies *in vivo* because circulating blasts will be available for analysis. In contrast, analysis of constructs that do not produce leukemia will best be performed in 32D3 cells because this model can better provide adequate immature myeloid cells for experimentation. We will perform the expression analyses described below, using leukemic blasts from mice when possible, but we will not hesitate to employ the 32D3 system as necessary.

AML1-NCoR and AML1-SMRT Vectors

Fusion genes encoding the first 177 residues of AML1 (the same as those found in AML1-ETO) and the repression domains of NCoR (residues 1-1445) and SMRT (residues 1-483) will be constructed. (It is noteworthy that the MSCV retrovirus can effectively package inserted sequences greater than 7 kb)(43). The fusion genes will be cloned into two retroviral vectors. First, they will be cloned into MSCV MigR1 containing retroviral sequences, IRES, and GFP for *in vivo* analyses. Second, they will be cloned into MSCV 2.2 containing the selectable PGK-neo gene for *in vitro* studies(43). Constructs will be evaluated for errors by sequencing and for the ability to express stable, intact protein by Western blot analysis.

Retrovirus production and cell infection

Retrovirus will be produced using Bosc23 cells (44) and infections will be performed by spinoculation(42), as described in specific aim 1.

Inhibition of myeloid differentiation

Vectors expressing AML1-NCoR and AML1-SMRT will be tested for the ability to inhibit myeloid differentiation. Mice and 32D3 cells will be analyzed as discussed in specific aim 1 using microscopy and flow cytometry to monitor myeloid differentiation. The outcome of this experiment will answer the fundamental question of whether or not CoR recruitment by AML1 is sufficient to disrupt myeloid differentiation.

Disruption of AML1 target gene expression

AML1-ETO is hypothesized to disrupt myeloid differentiation by perturbing expression of AML1 target genes. It has been speculated that these specific downstream targets contribute to the leukemic phenotype. We, therefore, intend to examine expression of selected AML1 target genes in mouse myeloid cells and 32D3 cells transduced with AML1-CoR fusions. Infected cells from mice will be collected by sorting for GFP fluorescence whereas infected 32D3 clones will simply be expanded for analysis. Quantitative RT-PCR will be performed to detect expression of targets including IL-3, GM-CSF, and M-CSF receptor. Representative target genes with the potential to influence disease phenotype have been selected. These includes both target genes that are increased and decreased by AML1-ETO. Expression profiles will be compared and contrasted with those of cells transduced with the AML1-ETO construct. Results should confirm a transcriptional activity of AML1-CoR and may provide insight into the AML1 target genes participating in disease.

Alternative Approaches

We do not anticipate significant technical difficulties with specific aim 2. Cloned sequences necessary for construct design are available in the _____ laboratory and the proposed techniques are performed routinely.

Interpretation

The _____ laboratory has demonstrated that the CoR-binding domain is essential for the activity of AML1-ETO (15). This aim will address the fundamental issue of the sufficiency of CoR recruitment. If AML1-CoR fusions are sufficient to inhibit myeloid differentiation, then focus will, appropriately, be directed downstream of the CoR, to CoR-interacting molecules. Alternatively, if AML1-CoR does not inhibit myeloid development, additional efforts must be directed toward understanding the unique pathogenic functions of ETO domains. Completion of this aim is, therefore, critical to guiding future investigation in the field.

Expression studies are necessary to confirm that AML1-ETO acts through CoR recruitment. Our working hypothesis is that AML1-ETO recruits CoRs to exert its transcriptional effects and inhibit myeloid differentiation. We, therefore, anticipate that AML1-CoR will influence gene expression to the same degree as AML1-ETO. If the observed profile of gene expression is not correlated with the differentiation phenotype, the transcriptional activity of AML1-CoR must differ from the transcriptional activity of AML1-ETO (e.g. lack dominant negative activity, lack a transforming activity of ETO, etc.). Dissociation of the differentiation and expression phenotypes would suggest that the transcriptional effects of CoR recruitment are not identical to the pathogenic transcriptional effects. Differences in AML1 target gene expression may, then, provide insight into

the genes participating in disease. In summary, the results of expression studies will be interpreted in the context of the differentiation analysis.

Specific Aim 3: Determine the role of ETO domains in the disruption of myeloid development by AML1-ETO.

ETO has four conserved domains named Nervy-homology (NH) for similarity to the Drosophila factor, Nervy(12). The _____ laboratory recently demonstrated that NH4 corresponds to the CoR binding domain and is essential for inhibition of differentiation of U937 tissue culture cells(15). Deletions of other ETO domains, in the context of AML1-ETO, result in compromised interactions with CoRs or reduced transcriptional repression activity (32, 33). Nevertheless, it is unclear if such deletions affect myeloid development. The experiments described in specific aim 3 complement those of specific aim 2. Specific aim 3 has the potential to indicate whether specific domains of ETO have essential *CoR-dependent* or *CoR-independent* activities. We will systematically analyze AML1-ETO deletion mutants for the ability to

- inhibit myeloid differentiation
- disrupt AML1 target gene expression (IL-3, M-CSF receptor, and GM-CSF) and
- interact with members of corepressor complexes (NCoR, SMRT, mSin3, and HDAC)

This approach will provide insight into the activities of ETO domains and reveal whether or not they contribute to disease.

AML1-ETOΔNH1, AML1-ETOΔNH2 vectors

We will attempt to produce deletions in two conserved domains of the AML1-ETO cassette using PCR. The NH1 (TAF110 domain) deletion will lack AML1-ETO residues 277-344. The NH2 (dimerization domain) deletion will lack AML1-ETO residues 500-520. The FLAG AML1-ETO cDNA will be released as a Xho1 cassette and cloned into MSCV MigR1 containing retroviral sequences, IRES, and GFP(43) for *in vivo* experiments and MSCV 2.2 containing PGK-neo for *in vitro* studies. Constructs will be evaluated for errors by sequencing and for the ability to express stable, intact protein by Western blotting.

Inhibition of myeloid differentiation

Experiments will be performed as described in specific aim 2.

Disruption of AML1 target gene expression

Experiments will be performed as described in specific aim 2.

Interactions with members of the corepressor complex

CoRs function in repression complexes to produce their transcriptional effects. AML1-ETO interacts not only with CoRs, but also with other members of the corepressor complex, including mSin3 and HDAC(33). We will attempt to determine if AML1-ETO mutants are capable of recruiting corepressor complexes as effectively as intact AML1-ETO. Protein extracts will be prepared from leukemic blasts or 32D3 cells, as previously described. Coimmunoprecipitations will be performed using antibodies directed against the FLAG epitope of the fusion proteins followed by immunoblotting using antibodies directed against NCoR, SMRT, mSin3, HDAC. Additionally, immunoprecipitates will be tested for histone deacetylase activity. Undoubtedly, during the course of these studies, new members of the repression complexes will be published. (Some have already been identified in the _____ laboratory.) As appropriate, attempts will be made to evaluate interactions with these proteins, as well as those listed. The findings of the coimmunoprecipitation experiments will be interpreted in light of both differentiation and expression studies. These experiments will provide clues to the ETO domains participating in repression complex recruitment and to the domains contributing to pathology.

Alternative Approaches

One area of potential difficulty with the techniques described in specific aim 3 relates to the coimmunoprecipitation analyses. While the detection of protein-protein interactions is the forte of the _____ laboratory, it may be difficult to demonstrate such interactions using the systems described. One potential problem is the level of expression of fusion constructs. If AML1-ETO mutants are not expressed in cells at a level adequate for coimmunoprecipitation studies, the model may have to be adjusted. For example, it may be necessary to design vectors with the potent CMV promoter driving expression and perform transfections of 32D3 cells by electroporation. An additional area of potential difficulty is related to the source of starting material. Protein analysis in myeloid cells can be compromised by proteolysis if the cells show a degree of differentiation. If such a difficulty arises, we anticipate that we can circumvent it using the 32D3 cell model.

Interpretation

The systematic approach to analyzing deletion mutations of AML1-ETO will help clarify the role of AML1-ETO in leukemogenesis. These experiments will complement those of specific aim 2. In specific aim 2 we simulated constitutive recruitment of CoR in order to look for an effect of an ETO deficiency. In specific aim 3, we are altering ETO and looking for deficiencies in either CoR recruitment or other undescribed activities. The activity of AML1-ETO mutants in myeloid differentiation, gene expression, and complex recruitment assays will provide clues to the importance of the NH domains, themselves, and to the role of ETO in transcription.

Differentiation analyses address the ultimate question of whether or not a given domain contributes to inhibition of myeloid differentiation. These assays are likely to be the most sensitive measures of activity because they do not exclude any specific mechanism of inhibition. Results will be interpreted in conjunction with expression studies and complex recruitment analyses.

Expression studies will indicate if AML1-ETO mutants can disrupt AML1 target gene expression. As in specific aim 2, the activity of AML1-ETO mutants is likely to be correlated with the differentiation phenotype, if in fact, AML1-ETO exerts its leukemic effects solely through corepressor recruitment. A discrepancy between the differentiation and expression phenotypes would suggest that we revealed a unique, non-transcriptional activity of ETO. This will potentially be significant in unraveling the mechanism by which AML1-ETO produces leukemia.

Coimmunoprecipitation studies indicate the degree to which AML1-ETO mutations affect recruitment of the corepressor complex. Domains outside of NH4 have been implicated in recruitment of the repression complex(33). These results will be compared to those from differentiation and expression studies. If AML1-ETO acts through CoR recruitment, then corepressor complex recruitment should correlate exactly with gene expression and differentiation studies. Dissociation between activities could signify CoR-independent transcriptional effects or unique, non-transcriptional activities of ETO. These experiments will be crucial to examining the intricacies of CoR recruitment by AML1-ETO.

In summary, these studies will provide insight into the molecular pathogenesis of acute myelogenous leukemia. The project combines the expertise in transcriptional repression of the _____ laboratory and the expertise in leukemia modeling of the _____ laboratory. This collaboration not only advances the proposed science, but also strengthens the proposed training program. In the process of performing these experiments, I expect to develop perspectives and skills necessary for my transition to an independent physician-scientist.

Timetable

The development of an *in vivo* model for the study of AML1-ETO is the first priority of the proposed work. We are ready to begin transduction/transplantation experiments, although, the time to leukemia development is unpredictable. Experience in _____ laboratory suggests that leukemia should develop within several weeks to a year. We, therefore, anticipate that specific aim 1 will be completed within the first year.

Constructs to be used in specific aims 2 and 3 will be assembled over years 1 and 2. The analysis of constructs described in specific aim 2 will be initiated prior to those in specific aim 3. Constructs will be tested in functional assays as they are completed. Assays for inhibition of development will be performed first. They

will be completed over years 2-4. Expression studies and coimmunoprecipitation studies will be performed over years 3-5.

e. Human Subjects N/A

f. Vertebrate Animals

The University of _____ and _____ has approved a protocol regarding the bone marrow transplant procedure in mice. The approval letter is attached.

This system was selected because, historically, the murine hematopoietic system has served as an excellent model for studying cellular development and proliferation. Tissue culture models may not accurately replicate the hematopoietic stem cell or provide definitive insight into the molecular pathways involved in the pathogenesis of leukemia. Furthermore, tissue culture models do not provide insight into phenotype at the level of the organism.

Bone marrow transplant experiments will entail harvest of marrow, *ex vivo* transduction, and transplant to lethally irradiated syngeneic recipients. Balb/C mice (6-12 weeks old and greater than 20g) will receive a lethal dose of irradiation (900 Rads) from a Cs source to be received in two 450 Rad doses given over a 24 hour period. After the second radiation dose, the mice will receive 1-2 x 10^6 bone marrow cells from a syngeneic donor via tail vein injection. This quantity of bone marrow cells results in 100% survival. Because the mice will be immunocomprimised for approximately 14 days, the recipient mice will be maintained on autoclaved food and water.

Groups of 14 mice (6 donor mice for 8 recipient mice) will be used in these studies and experiments will be repeated at least once (12 and 16 mice per group, respectively.) The bone marrow and spleen of diseased animals will be used in serial passage to determine the malignant potential of the cells (maximum 16 mice per group, if all recipients develop leukemia.) IACUC has approved the use of 444 mice for the proposed studies.

Care will be taken to minimize animal distress. Annex II IACUC Guidelines for Tumor Load Studies will be followed. Recipients will be monitored for physical evidence of leukemia (cachexia, decreased activity, etc.) or peripheral histopathological evidence of leukemia (using a drop of tail blood). Mice with evidence of leukemia or other illness will be euthanized using CO2.

g. Literature Cited

h. Consortium/Contractual Arrangements N/A

i. Consultants

7. Appendix