Abstract
ENFORCED EXPRESSION OF HOXA9 IN B-LINEAGE ALL PROMOTES SURVIVAL AND PROLIFERATION OF LEUKEMIC CELLS
by Jarrett Thomas Whelan
March, 2009
Director: Fred E. Bertrand
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

HoxA9 is frequently overexpressed in acute myeloid leukemia, myelodysplastic syndrome and a subset of acute lymphoblastic leukemia. In mouse models, HoxA9 has been shown to promote leukemogenesis. In spite of a central role in initiating leukemia, and in acting as a cofactor to promote leukemic cell growth and survival, the mechanistic pathways altered by HoxA9 overexpression that promote the growth and survival of leukemia, are not well defined. We have developed a stromal cell dependent model of human B-lineage ALL with conditional HoxA9 activity. The HoxA9-negative pre-B cell ALL cell line, BLIN-2, was stably transduced with a retrovirus bearing HoxA9 fused to the hormone binding domain of the human estrogen receptor. HoxA9 activity is stimulated by the addition of 4-hydroxytamoxifen to the growth medium. BLIN-2 cells have an absolute dependence on stromal cell contact for growth and survival, which permits the testing of the HoxA9 mediated effects in the context of the tumor microenvironment. Induction of HoxA9 activity in BLIN-2 resulted in increased proliferation in the absence of stromal cell support and induction of surface expression of IGF-1R. Through the use of specific IGF-1R inhibitors we demonstrated that the proliferative response upon HoxA9 stimulation was the result of signaling through the induced IGF-1R. In addition to promoting stromal cell independent proliferation, enforced induction of HoxA9 activity promoted apoptotic resistance to stromal

cell/growth factor withdrawal. Inhibition of IGF-1R signaling did not abrogate the anti-apoptotic effects of HoxA9, but did result in decreased proliferation. Models of IGF-1R typically attribute anti-apoptotic effects of IGF-1R signaling to Akt signaling, whereas the proliferative effects are mediated via the ERK pathway. No changes in Akt phosphorylation were observed in BLIN-2 cells with activated HoxA9, but levels of phospho-ERK were increased. These results are consistent with a role for HoxA9 induction of IGF-1R promoting proliferation, but not apoptotic resistance. Overall, these data indicate that enforced expression of HoxA9 in leukemia promotes proliferation via an IGF-1R dependent pathway and that HoxA9 promotes apoptotic resistance to stromal cell/growth factor withdrawal through a pathway that is independent of IGF-1R signaling.

ENFORCED EXPRESSION OF HOXA9 IN B-LINEAGE ALL PROMOTES SURVIVAL AND PROLIFERATION OF LEUKEMIC CELLS

A Dissertation

Presented To

The Faculty of the Department of Microbiology and Immunology

Brody School of Medicine at East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Microbiology and Immunology

by

Jarrett Thomas Whelan

March, 2009

ENFORCED EXPRESSION OF HOXA9 IN B-LINEAGE ALL PROMOTES SURVIVAL AND PROLIFERATION OF LEUKEMIC CELLS

by

Jarrett Thomas Whelan

APPROVED BY:

DIRECTOR OF DISSERTATION:_____
Fred E. Bertrand, Ph.D.

COMMITTEE MEMBER:_____
James A. McCubrey, Ph.D.

COMMITTEE MEMBER:_____
Shaw M. Akula, Ph.D.

COMMITTEE MEMBER:_____
Everett C. Pesci, Ph.D.

COMMITTEE MEMBER:_____
Brett D. Keiper, Ph.D.

CHAIR OF THE DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY:

_____
C. Jeffrey Smith, Ph.D.

DEAN OF THE GRADUATE SCHOOL:

_____
Paul Gemperline, Ph.D.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4HT	4- Hydroxytamoxifen
A	Adenine
AF10	ALL1 Fused gene from chromosome 10
AF4	ALL1 Fused gene from chromosome 4
AF6	ALL1 Fused gene from chromosome 6
AF9	ALL1 Fused gene from chromosome 9
Akt	AKR mouse thymoma
ALL	Acute Lymphocytic/Lymphoblastic Leukemia
ALL1	Acute Lymphoblastic Leukemia-1
AML	Acute Myelogenous Leukemia
AML1	Acute Myeloid Leukemia-1
AP	Acute Phase
APC	Antigen Presenting Cell
APL	Acute Promyelocytic Leukemia
ATO	Arsenic Trioxide
ATRA	All-Trans Retinoic Acid
BAD	Bcl-2 Antagonist of cell Death
B-cells	Bursa derived cells
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-xl
BCR	B-cell Receptor
Bcr	Break point cluster region
BLIN-2	B-Lineage-2
BLIN-3	B-Lineage-3
BM	Bone Marrow
bp	base pairs
BP	Blast Phase
BSA	Bovine Serum Albumin
BTK	Bruton's tyrosine kinase
C	Cytosine
c-abl	cellular-Abelson
CD	Cluster of Differentiation
CD40LG	CD40 Ligand
CDA	2-Chlorodeoxyadenosine
cDNA	Complimentary DNA
c-kit	Cytokine stem cell factor receptor
CLL	Chronic Lymphocytic Leukemia
CLP	Common Lymphoid Precursor
CML	Chronic Myelogenous Leukemia
c-Myb	cellular-Myeloblastosis gene
CP	Chronic Phase
CR	Complete Response
C-region	Constant-region
DH	Heavy chain Diversity region

dic	Dicentric
DIG	Digoxigenin
DL	Light chain Diversity region
DNA	Deoxyribonucleic Acid
E2A	Transcription factor 3(E2A Ig enhancer binding factors E12/E47)
EBV	Epstein-Barr Virus
ELISA	Enzyme Linked Immunosorbent Assay
ELL	Eleven-Nineteen Lysine-Rich Leukemia gene
EMSA	Electrophoretic Mobility Shift Assay
ENL	Eleven-Nineteen Leukemia gene
ER	Estrogen Receptor
ERK	Extracellular signal Regulated Kinase
ETS	E26 Transformation-Specific
FAB	French, American, British
FACS	Fluorescence Activated Cell Sorting
FDC	Follicular Dendritic Cell
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germinal Center
GFP	Green Fluorescent Protein
gp91Phox	gp91 Phagocyte NADPH oxidase
Grb2	Growth factor receptor bound protein-2
HCL	Hairy Cell Leukemia
HD	Hodgkin's Disease
HEK-293	Human Endothelial Kidney-293
HIV	Human Immunodeficiency Virus
HnRNP70	Human small nuclear Ribonucleoprotein 70kDa
HOX	Homeobox containing gene
HRX	Human Trithorax
HSC	Hematopoietic Stem Cell
HTLV-1	Human T-cell Leukemia Virus-1
Ig	Immunoglobulin
IGF-1	Insulin-like Growth Factor-1
IGF-1R	Insulin-like Growth Factor-1 Receptor
IGF-2	Insulin-like Growth Factor-2
Igα	Immunoglobulin-alpha
Igβ	Immunoglobulin-beta
IL-3	Interleukin-3
IL-7	Interleukin-7
INF-γ	Interferon-gamma
IRES	Internal Ribosomal Entry Site
IRS 1 – 4	Insulin Receptor Substrates 1– 4
JAK	Janus protein tyrosine Kinases
JH	Heavy chain Joining region
JL	Light chain Joining region
LTR	Long Terminal Repeat

mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
mb-1	Immunoglobulin-alpha
Meis1	Myeloid ecotropic viral integration site 1
MHC	Major Histocompatibility Complex
MigR1	Murine stem cell virus-IRES-GFP-R1
MLL	Mixed Lineage Leukemia
MM	Multiple Myeloma
MSC	Myeloid Stem Cell
mTOR	Mammalian Target of Rampamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHL	Non-Hodgkin's Lymphoma
NK	Natural Killer
p	Short arm
PAGE	Polyacrylamide Gel Electrophoresis
PAMPs	Pathogen-Associated Molecular Patterns
PARP	poly (ADP-ribose) polymerase
Pax5	Paired Box 5
Pbx1a	Pre-B-cell leukemia homeobox 1a
Pc-G	Polycomb-Gene
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
Ph+	Philadelphia chromosome positive
PI	Propidium Iodide
PI3K	Phosphotidylinositol-3-Kinase
Pim-1	Proviral integration site for Moloney murine leukemia virus-1
PML	Promyelocytic Leukemia
pre-BCR	Pre-B Cell Receptor
Pro-B cell	Progenitor B-cell
PRRs	Pattern Recognition Receptors
PVDF	Polyvinylidene Fluoride
q	Long arm
RAG-1	Recombinase Activating Gene -1
RAG-2	Recombinase Activating Gene -2
RARα	Retinoic Acid Receptor-alpha
Ras	Rat sarcoma
RT-PCR	Reverse Transcription-PCR
SEER	Surveillance, Epidemiology and End Results
Shc	Sarc homology collagen adaptor protein
SHM	Somatic Hypermutation
siRNA	Small interfering Ribonucleic Acid
SOS	Son of Sevenless
STAT	Signal Transducers and Activators of Transcription
T	Thymidine
t-AML	Therapy induced Acute Myeloid Leukemia
TBST	Tris-Buffered Saline with Tween-20

T-cells	Thymus derived cells
TCR	T-cell Receptor
TdT	Terminal deoxynucleotidyl Transferase
TEL	ETS variant 6
TH1	T-helper 1
TH2	T-helper 2
TKI	Tyrosine Kinase Inhibitor
Trx-G	Trithorax-Gene
VH	Heavy chain variable region
VL	Light chain variable region
VpreB	Ig light chain variable region
WHO	World Health Organization
Wnt	Wingless/Int
XLA	X-linked agammaglobulinemia
αIR3	anti-IGF-I Receptor-3 antibody
λ5	Lambda-5
ΨLC	Pseudo-Light Chain

CHAPTER 1: Introduction

1.1 Hematopoiesis: A Brief Overview

Hematopoiesis is the formation of the blood's cellular components. Unlike embryogenesis, which occurs only once at the beginning of life, hematopoiesis is a self renewing process that occurs indefinitely throughout the life span of a healthy individual. All of the blood cells of an organism are derived from pluripotent hematopoietic stem cells (HSCs) residing in the bone marrow. HSCs give rise to all cells of the myeloid and lymphoid lineages (Fig. 1) (1). HSCs are categorized by a common protein expression profile. Most notably, HSCs express the cell adhesion molecule CD34 and lack expression of the cell surface molecule CD38 (1). CD34 is the ligand for L-selectin and CD38 is an NAD glycohydrolase that augments cell proliferation. As HSCs become committed to different blood cell lineages they lose expression of CD34 and gain expression of CD38 (1).

HSCs undergo two types of cellular division in the stem cell niche; symmetrical and asymmetrical. Symmetrical division gives rise to two identical daughter cells, both remaining in the niche as stem cells. Asymmetrical division gives rise to one HSC and one of two committed progenitor cells, the myeloid stem cell (MSC) or the common lymphoid progenitor (CLP) (1). The HSC daughter remains in the niche as a stem cell and the committed progenitor cell leaves the niche to produce a large number of progeny. The MSC differentiates through the myeloid lineage specific programs to give rise to all of the cells of the myeloid lineage, while the CLP gives rise to all of the cells of the lymphoid lineage.

Figure 1: An Overview of Hematopoiesis. Hematopoietic stem cells residing in the bone marrow differentiate into a myeloid stem cell or a common lymphoid progenitor. The myeloid stem cell gives rise to all the mature cells of the myeloid lineages, including: platelets (megakaryocytes), neutrophils, eosinophils, basophils, macrophages, and erythrocytes (not listed). The common lymphoid progenitor gives rise to cells of the lymphoid lineages, including: natural killer cells (NK), plasma cells, memory B-cells, helper T-cells, and cytotoxic T-cells. Dendritic cells (not shown) are derived from both the myeloid and lymphoid lineages. Leukemias can occur at any stage during hematopoiesis, leading to developmental arrest and uncontrolled proliferation. Acute and chronic myeloid leukemia (AML/CML) occur in cells of the myeloid lineages, and acute and chronic lymphocytic leukemia (ALL/CLL) occur in cells of the lymphoid lineages.

Platelets
Neutrophil
Eosinophil
Basophil
Hematopoietic
Stem cell
Myeloid stem cell
Common lymphoid precursor
Mø precursor
NK precursor
B cell precursor
T Cell precursor
Committed
Progenitors
Monocyte
NK cell
B cell
T Cell
Mature Cells
Macrophage
Activated
NK cell
Plasma
cell
Memory
cell
Helper
T cell
Cytotoxic
T cell
AML/CML
ALL/CLL

Myeloid lineages. The myeloid lineage consists of both red and white blood cells. Red blood cells, or erythrocytes, are involved in the transport of O_2 and CO_2, bound to hemoglobin, to and from the tissues of the body, respectively. The white blood cells, or leukocytes, are comprised of a diverse group of cell types that function primarily to combat infections and assaults to the immune system. Leukocytes can be classified into three main categories: granulocytes, monocytes, and lymphocytes.

There are three classes of granulocytes: neutrophils, basophils, and eosinophils. These cells are characterized by the presence of numerous granules within their cytoplasm which consist of lysosomes and secretory vesicles. Granulocytes are relatively short lived cells whose numbers increase during immune responses. Neutrophils are the most numerous of the granulocytes and play an important role in the innate immune response. The innate immune response defends the host from infection from organisms in a non-specific manner. Neutrophils eliminate many foreign pathogens by phagocytosis, a process involving internalization and degradation of foreign material by enzymes contained within the lysosomes. The function of eosinophils is not entirely understood. They are thought to play a major role in host defense against parasite infestation, although this role remains debatable (2). Basophils are the least abundant of the granulocytes. They act as the chief effector cells of allergic responses and as purveyors of various allergy-associated mediators such as histamine (3).

Along with neutrophils, macrophages are one of the three phagocytic cells of the immune system. Macrophages, or mononuclear phagocytes, are mature monocytes whose main function is to ingest and process senescent erythrocytes. This function is important for heme and bilirubin metabolism. They are also involved in the removal of cellular

debris generated during tissue remodeling and clearance of cells that have undergone apoptosis (4). Furthermore, macrophages are important immune effector cells. As part of the innate immune response, macrophages scavenge the tissues of the body for invading pathogens to ingest and kill. They also play important regulatory and effector roles in adaptive immune responses (5). During adaptive immune responses, peptides derived from microorganisms that have been engulfed and degraded by macrophages are presented by major histocompatibility complex (MHC) class II molecules to T-cells (5). Macrophages stimulate the clonal expansion of T-cells through antigen presentation.

Megakaryocytes give rise to circulating platelets involved in the formation of blood clots. Megakaryocytes are 10 – 15 times larger than a typical red blood cell, hence their name (6). Dendritic cells (not shown in Fig. 1), the last of the phagocytic cells of the immune system, are actually derived from both the myeloid and lymphoid lineages and are the most potent stimulators of the adaptive immune response (7). As with macrophages, once an immature dendritic cell recognizes a pathogen the dendritic cell phagocytoses the pathogen and degrades it into smaller fragments that can be presented to B and T-cells in the spleen and lymph nodes.

Innate immunity is the first defense against invading pathogens and depends on germ-line encoded pattern recognition receptors (PRRs) on immune cells that recognize highly conserved pathogen-associated molecular patterns (PAMPs) found within classes of microbes (8). The adaptive or “specific” immune response is mediated primarily by B and T-lymphocytes. B and T-lymphocytes have a diverse repertoire of antigen receptors due to immunoglobulin (Ig) gene rearrangements. Ig gene rearrangements give rise to a limitless number of receptors capable of recognizing the antigenic component of any

potential pathogen or toxin (8). Activation of B and T-cells via antigen-mediated binding to Ig receptors initiates cell-mediated (T-cell) and humoral (B-cell) immune responses, directed by secreted antibodies (8).

Lymphoid lineages. The lymphoid lineages are comprised of B (bursal-derived), T (thymus-derived), and natural killer (NK) cells. Unlike B and T-cells, NK cells form part of the innate immune response. NK cells are large granular cells that kill a target cell "naturally" in a spontaneous fashion that does not require priming and is not restricted by the target cell's expression of MHC molecules (9). NK cells recognize virally infected or transformed cells, due to a release of cytokines, and kill them via perforin/granzyme release or death receptor-related pathways (9). NK cells also secrete cytokines and chemokines that influence the host's immune response. Interferon-gamma (INF-γ) release by NK cells activates antigen presenting cells (APC) (i.e. dendritic cells, macrophages, and B-cells) to upregulate expression of MHC class I molecules, induces macrophage killing of intracellular pathogens, inhibits proliferation of virus infected cells, and shapes the cellular (i.e. T_H1; see below) immune response (9). Thus, NK cells are at the interface between innate and adaptive immunity.

T-cells develop from CLPs in the bone marrow (BM) and migrate to the thymus where they mature. Once positive selection of non-self-reactive T-cells has been completed in the thymus, the mature T-cells enter the bloodstream and recirculate between the blood and peripheral lymphoid tissue as naïve T-cells. When a naïve T-cell encounters its specific antigen, it is induced to proliferate and differentiate into a primed effector T-cell capable of contributing to the removal of antigen. Becoming a primed effector T-cell requires T-cell receptor (TCR)-mediated recognition of foreign antigen

bound to self MHC class I or II molecules with simultaneous co-stimulatory signals from professional APCs. A primed effector T-cell will differentiate into a cytotoxic T-cell, that kills infected target cells, or a helper T-cell. There are two types of helper T-cell, called T_H1 and T_H2. T_H1 cells activate macrophages to kill invading pathogens and induce cell-mediated immunity and cytotoxic T-cell activity. T_H2 cells initiate the humoral immune response by activating naïve antigen-specific B-cells to produce IgM antibodies that cause the destruction of extracellular microorganisms and prevent the spread of intracellular infections.

B-cells, like T-cells, are derived from CLPs in the BM. However, B-cell development (see B-cell development) occurs in the BM. As B-lineage cells mature they migrate out of the BM into lymphoid follicles of the spleen and lymph nodes, where they encounter and respond to T-cell dependent foreign antigens bound to follicular dendritic cells (FDC). Like T-cells, B-cells have specific cell surface receptors encoded by the Ig superfamily of genes known as the B-cell receptor (BCR). Antigen-mediated engagement of the BCR results in the transmission of signals directly to the interior of the cell, leading to rapid proliferation and differentiation of mature B-cells into antibody secreting plasma cells or memory B-cells. Additionally, binding of antigen to the BCR results in the internalization and degradation of antigen which is then presented on the surface of the cell as peptides bound to MHC class II molecules.

Antigen induced B-cell differentiation and proliferation result in a process known as the germinal center (GC) reaction. GCs are areas in follicular regions of secondary lymphoid organs where antigen primed B-cells proliferate extensively. Antigen specific B-cells must have bound, processed, and presented antigenic peptide-MHC class II

complexes to initiate the T_H-cell-dependent GC reaction (10). The main purpose of the GC reaction is development of B-cell memory. During GC reactions BCR diversification occurs via the processes of somatic hypermutation (SHM) and affinity maturation. Somatic hypermutation occurs due to individual point mutations in the variable regions of the Ig genes (11). This process results in the generation of variant Igs, some of which bind to antigen with greater affinity, allowing for increased affinity of the antibody response. Another phenomenon of the GC reaction, known as affinity maturation, leads to the development of antibodies with greater antigen affinity. Briefly, at the early stages of an immune response, B-cells with relatively low affinity for antigen become activated to produce antibodies and proliferate. The low affinity antibodies will bind to and clear antigen, leaving only those B-cells with receptors of the highest affinity to capture antigen. Consequently, the higher affinity B-cells out compete low affinity B-cells for antigen and are clonally selected for to dominate a secondary immune response. This process is repeated leading to the production of antibodies with even greater affinity in a tertiary response.

Another modification of the Ig genes that occurs during a humoral immune response is known as isotype or class switching. Isotype switching does not change the specificity of an antibody, but does change the effector function of the antibody. There are five distinct antibody isotypes: IgM, IgG, IgD, IgA, and IgE. The different isotypes result from recombination of DNA encoding constant regions (C-region) of Ig genes. IgM is the first class of antibody to appear during an immune response, IgG is the most abundant antibody in the serum and lymphatic system. IgA is involved in mediating

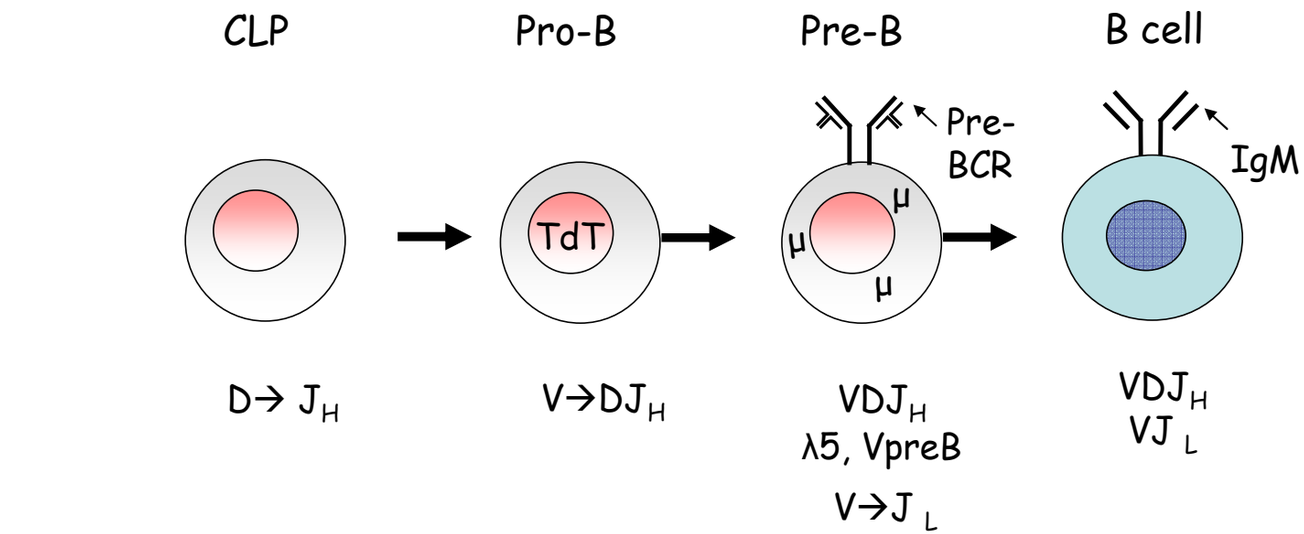
mucosal immunity, IgE is involved in allergic responses. The function of IgD in shaping a humoral immune response is still not completely understood (12).

1.1.3 B-Cell Development: An Overview

B-cell development is characterized by the expression of B-lineage restricted genes, the presence of cell surface markers and the ordered rearrangements of the heavy and light Ig genes to produce functional antigen receptors. The earliest recognizable B-lineage cell is the pro-B cell, or progenitor B-cell, derived from the multipotent CLP (Fig. 2). Maturation and lineage commitment of a CLP into a pro-B cell is dependent on upregulation of E2A and de novo expression of Pax5 transcription factors (13). Pro-B cells are characterized by the expression of the earliest B-lineage surface markers, CD19 and CD45R. The expression of CD19 and CD45R are maintained throughout B-cell development with the exception of mature plasma cells (14). Rearrangements of the Ig heavy chain variable (V_H), diversity (D_H), and joining (J_H) regions occur at the pro-B cell stage of development. D_H to J_H joining occurs in early Pro-B cells followed by V_H to DJ_H joining in late pro-B cells. Rearrangements of the heavy chain loci are mediated by recombinase activating gene 1 (RAG-1) and RAG-2 enzymes (15). Pro-B cells also express terminal deoxynucleotidyl transferase (TdT), a polymerase that adds nucleotides to the rearranging heavy chain segments. The addition of extra nucleotides generates additional diversity of the B-cell antigen receptor repertoire.

At the pro-B cell stage of development, rearrangement of the heavy chain is completed. In addition, during the pro-B cell stage, cells express Igα and Igβ chain components of the antigen receptor, as well as the components of the surrogate light chain (ΨLC), encoded by the VpreB and λ5 (mouse) or 14.1 (human) genes (16,17). On-

Figure 2: Early stages of B-cell development. B-cells are derived from multipotent common lymphoid progenitors (CLP) residing in the BM. During the transition from the CLP to the progenitor B-cell (pro-B cell) stage, cells are characterized by the expression of CD34, recombinase activating genes 1 and 2 (RAG-1, -2) and are actively undergoing D to J heavy chain rearrangement, mediated by RAG-1 and 2. At the pro-B cell stage cells have completed D to J_H rearrangement and are actively rearranging the V_H to DJ_H gene segments. Pro-B cells also express terminal deoxynucleotidyl transferase (TdT) and two of the first identifiable B-cell markers, CD19 and CD45R (B220 in mice). CD19 and CD45R expression are maintained throughout B-cell development. Once VDJ_H rearrangement has been successfully completed the cells are pre-B cells. pre-B cells are actively undergoing V-J light chain rearrangements, and are characterized by expression of cytoplasmic mu (μ) heavy chain and surface expression of the pre-B cell receptor (Pre-BCR), which consists of μ heavy chain and the surrogate light chain (λ5 and VpreB). Once successful light chain rearrangement has occurred the cells have become immature B-cells and express surface IgM.



CD19

CD34

CD45R

RAG-1
RAG-2

ce productive VDJ_H rearrangement has occurred, cells begin to express cytoplasmic μ heavy chains, most of which are retained in the endoplasmic reticulum (ER). However, some μ heavy chains associate with the ΨLC, Igα and Igβ to form a stable complex known as the pre-B cell receptor (pre-BCR). At this point the pro-B cell has transitioned from the pro-B cell stage to the pre-B cell stage of B-cell development (18). Surface expression of the pre-BCR is required for allelic exclusion of heavy chain gene expression (i.e. shutting down of rearrangement on the second chromosome) (19), and is absolutely required for pre-B cell progression (18). Ligand-independent oligomerization of the pre-BCR is the likely mechanism of proliferative stimulus for the expansion of pre-B cells with functional heavy chain rearrangements (11). However, some potential pre-BCR ligands that promote proliferation have been described (20,21).

During the pre-B cell stage of development, cells are also actively undergoing rearrangements of the V and J_L light chain loci, beginning with one of the two *kappa* light chain genes. If the rearrangement does not produce a functional *kappa* light chain, the cell will rearrange the second *kappa* light chain gene. If the *kappa* light chain rearrangement is successful the B-cell will make an Ig with a *kappa* light chain. If the *kappa* chain rearrangements are unsuccessful, the cell will attempt to rearrange the *lambda* light chain genes in succession until a functional rearrangement has occurred. As with heavy chain rearrangements, light chain rearrangements are also mediated by RAG-1 and RAG-2. Cells that fail to complete functional rearrangements of either the heavy or light chain loci will undergo apoptosis. Once a successful VJ_L rearrangement has occurred, a complete IgM molecule is expressed on the cell surface, and the cell is then defined as an immature B-cell.

Immature B-cells that have successfully expressed IgM on their surface are subject to both negative and positive selection via signals received through the antigen receptor. B-cells that express receptors that recognize auto-antigen, or self-reactive B-cells, undergo negative selection and have four possible fates: production of a new BCR by receptor editing, ignorance, anergy, or deletion (apoptosis) (13). If an immature B-cell expresses a receptor that recognizes self-antigen the cell is stimulated to commit apoptosis (deletion) or re-express RAG-1 and RAG-2 to undergo another round of receptor rearrangement (receptor editing) (22). Immunological ignorance of B-cells is the coexistence of self-reactive B-cells and their auto-antigen without any immunological response. This process results from 1) very low concentrations of self-antigen or 2) B-cells with low affinity to highly abundant self-antigen (23). Anergy occurs when an autoreactive B-cell binds to self-antigen in the periphery, this results in a reduction in surface IgM expression and cellular inactivation (23). The differential induction of ignorance, anergy and deletion, in that order, are directly proportional to the degree of receptor/self-antigen activation (23). These mechanisms prevent the development of autoimmunity. Non-self-reactive B-cells migrate into the periphery to secondary lymphoid organs where they undergo antigen specific positive selection (see GC reaction).

1.1.2 The Bone Marrow Microenvironment

An important aspect of B-cell development is the site where it takes place. During embryogenesis, hematopoiesis occurs in the fetal liver and BM, but as we age, hematopoiesis becomes restricted to the BM microenvironment (24). The ability of HSC to self-renew and differentiate is critical for tissue homeostasis. If differentiation were to

overwhelm self-renewal then the HSC compartment would become depleted. Conversely, if HSC self-renewal occurred excessively at the expense of the mature compartments then leukemia would likely develop. This balance between self-renewal and differentiation is tightly controlled by the stem cell niche within the BM microenvironment (25). An important function of the stem cell niche, therefore, is to regulate the balance between asymmetric/symmetric stem cell division.

The BM microenvironment is a spongy extracellular matrix containing fibronectin, collagen, laminin, and other proteoglycans that provide a scaffolding where stromal cells, such as osteoblasts, fibroblasts, endothelial cells, reticular cells, dendritic cells, or macrophages interact with different developing blood cells (25). Besides physical interactions, stromal cells also provide essential cytokines and growth factors required for proper development of B lineage cells. These factors are also absolutely required for the proper development and maintenance of the B-cell pool (25). For example, in mice, the cytokine IL-7 is required for B-cell development; without it cells become arrested at the Pro-B cell stage of development (26). In humans, the cytokine(s) that promote B-cell development are not quite as well understood (11). However, a common characteristic of leukemic disease is that transformed blood cells often lose stromal cell/growth factor dependency, leading to uncontrolled proliferation. This effect results in overcrowding of the BM microenvironment and disruption of normal blood cell development.

1.1.4 B-Cell Disorders

B-cell disorders are divided into two categories, 1) defects of Ig production (immunodeficiencies) and 2) excessive/uncontrolled proliferation (leukemias/lymphomas) (27). Rare defects in Ig production include X-linked agammaglobulinemia (XLA),

resulting from a mutation of the Bruton's tyrosine kinase (BTK) gene, which leads to developmental arrest at the pro-B cell stage (28) and hyper-IgM syndrome, which results from a mutation in the CD40 ligand (CD40LG) gene leading to a loss of class switching and overproduction of IgM (29). Both of these diseases are inherited on the X chromosome and, therefore, only affect males. However, acquired disorders in Ig production are far more common than inherited genetic mutations.

Leukemias corresponding to all stages of B-cell development have been found in humans, from the earliest stages to the terminally differentiated plasma cells. Acute B-cell leukemias occur at the earliest stages of B-cell development and involve the developmental arrest and uncontrolled proliferation of pro- and pre-B cells. Chronic B-cell leukemias involve immature, mature, or activated B-cells; multiple myelomas represent malignant outgrowths of plasma cells. Approximately 80% of all acute lymphocytic leukemias (ALL) are of the B-cell lineage (30), discussed in greater detail below.

1.2 Leukemia: An Overview

Leukemia, which literally means "white blood" in Greek, is an acute or chronic disease of the blood and blood-forming organs that originates in the BM and is characterized by an abnormal increase in the number of white blood cells in the tissues of the body. Abnormal increases in the number of white blood cells in the BM prevent normal blood cell development resulting in symptoms of anemia, fatigue, excess bleeding and infections. Each type of leukemia is categorized based on the white blood cell type that is predominately involved. In 2009 approximately 140,000 individuals in the United States will be diagnosed with leukemia or the leukemia-related diseases, lymphoma or

multiple myeloma (MM), and it is estimated that these diseases combined will account for approximately 53,000 deaths in the United States this year (Source: Surveillance, Epidemiology and End Results (SEER) Program 1975-2005, National Cancer Institute, 2008).

1.2.1 Causes and Risk Factors

Leukemias result from alterations in DNA resulting in the activation of oncogenes, the repression of tumor suppressor genes, or the generation of novel proteins with unique functions. The exact cause of these mutations is not well understood, but there is strong evidence supporting four likely causes. These include exposure to moderate to high doses of ionizing radiation, chemical exposure, viral infection, and genetic predisposition.

Ionizing radiation. Humans are constantly exposed to relatively low amounts of ionizing radiation (without harm) from naturally occurring radionuclides in the ground, in building materials, and in foods. However, exposure to moderate to high levels of ionizing radiation can cause cancer. Leukemias are especially sensitive to induction from exposure to moderate to high levels of radiation. The first evidence of this property came from studies of the Japanese survivors of the atomic bombings of Hiroshima and Nagasaki (31). Indeed, there was a one hundred-fold increase in the incidence of leukemia five years following exposure to atomic radiation compared with the unexposed population (32). Most leukemias were in excess and were especially pronounced at young ages following exposure, with the exception of chronic lymphoid leukemia (CLL), which is rare in the Japanese population (33).

Chemicals exposure. Exposure to certain chemicals as the result of indoor/outdoor air pollution, or occupational exposure, is also associated with an

increased risk of leukemia. Use of some insecticides and pesticides on interior and exterior plants, in particular frequent prenatal use, is associated with increased risk of leukemia (34). Furthermore, there is an increased risk of leukemia and lymphoma caused by exposure to volatile organic compounds such as benzene and 1,3-butadiene (35). Leukemias can also occur as the result of radiation therapy or chemotherapy for the treatment of primary tumors. These leukemias are referred to as secondary leukemias. Chemotherapeutic agents associated with secondary leukemias include: alkylating agents, topoisomerase inhibitors, or immunosuppressive agents such as azathioprine (36).

Viruses. Certain viruses can also cause leukemia. For example, Epstein-Barr virus (EBV) has been linked to the development of both Hodgkin's disease (HD) and Non-Hodgkin's lymphoma (NHL) (35). Human T-cell leukemia virus type 1 (HTLV-1) is associated with adult T-cell leukemia, and the human immunodeficiency virus (HIV) with B-cell lymphomas (35).

Genetic predisposition. Genetic predisposition plays a role in the risk for developing leukemia. Although only accounting for a small number of all leukemias, certain inherited diseases are responsible for an increased risk of developing leukemia. The diseases Fanconi anemia, Bloom syndrome, ataxia telangiectasia, Down syndrome, Shwachman syndrome, and neurofibromatosis are all associated with an increased risk of leukemia (37). These diseases are characterized by defects in DNA repair mechanisms, aneuploidy and chromosomal abnormalities. Other studies suggest that there is a increased risk of leukemia in children with siblings who develop leukemia (38,39). Finally, leukemia occurs more frequently in white individuals of European descent than

any other race or ethnicity, suggesting a genetic component to the disease in this population (37).

1.2.2 Classifications

Leukemias are grouped into four broad categories: acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myelogenous (AML), and chronic myelogenous (CML). Acute leukemias are characterized by a rapid onset and occur primarily in children and young adults. Subtyping of the various forms of acute leukemia are done according to the French-American-British (FAB) or the World Health Organization (WHO) classification systems. Leukemias are classified under either system based on the type of cell from which the leukemia developed, cellular cytogenetics and phenotyping to determine the degree of cellular maturity. Symptoms of acute leukemia will often appear suddenly and, based on the nature of the disease, aggressive treatments are generally required. Treatment strategies are tailored to the specific type of white blood cell involved.

Chronic leukemias are not at all common in children, occurring chiefly in older individuals. Chronic leukemias are characterized by a much slower progression, which can vary greatly from patient to patient. CML is divided into three phases; chronic phase (CP), accelerated phase (AP), and blastic phase (BP) (40). If left untreated CP lasts 2 – 5 years; AP can last up to a year; and BP lasts 3 – 6 months and is fatal (40). CLL is staged clinically based on the schemes proposed by Rai *et al*. (41), and Binet *et al*.(42); low-risk, intermediate-risk, and high-risk or stages A, B, and C, respectively (41-43). For both schemes, anemia and platelet counts $< 100 \times 10^9$/L are considered end stage CLL. The

treatments for both CLL and CML are dependent on the clinical stage and progression of disease.

1.2.3 Childhood Leukemias

In vertebrates, hematopoiesis is extremely active during fetal development as well as in the first few years of life; therefore, it is not surprising that leukemia is the most common childhood malignancy. In fact, although adults are diagnosed with leukemia about 10 times more frequently than children, leukemia is the number one disease of children (0 – 14 years) and accounts for 32.6% of all childhood cancers (Source: Cancer Facts and Figures 2007, American Cancer Society). Additionally, exceeded only by accidents, cancer is the second leading cause of death in children. Approximately one third of all childhood deaths result from leukemia (Source: Cancer Facts and Figures 2007, American Cancer Society). In the United States approximately 2,000 children are diagnosed with ALL each year, approximately 500 children are diagnosed with AML, and less than 100 are diagnosed with CML (44). Although CLL is the most common of leukemias diagnosed in adults, it is rarely diagnosed in children (44).

1.2.4 Treatment Options

There are numerous chemotherapeutic agents with differing modes of action available for the treatment of cancer. There are compounds that interfere with DNA replication and gene expression, such as anti-metabolites and alkylating agents, others that inhibit cellular signaling pathways, such as tyrosine kinase inhibitors (TKI), and tumor cell specific monoclonal antibodies (mAb). Leukemia treatment regimens are tailored to the type of leukemia that a patient has, as certain treatments may be highly effective for one type of leukemia and not at all effective for another (Table 1). The over-

Table 1. Common chemotherapeutic drugs used to treat leukemia

Drug	Type	Mechanism	Disease
Vincristine	Mitotic Inhibitor	Disrupts microtubules, arrests mitosis in metaphase	ND, NHL, ALL
Anthracyclines (Daunorubicin Doxorubicin)	Alkylating Agents	Intercalates into DNA, inhibits replication	ALL, AML, CLL
Cyclophosphamide	Alkylating Agent	Forms DNA intrastrand crosslinks, inhibits replication	AML, CLL, Childhood-ALL, HD, MM
ATRA (all-trans retinoic acid)	Retinoid	Induces cellular differentiation	APML
Imatinib	Tyrosine Kinase Inhibitor	Inhibits pro-survival cellular signaling pathways	Ph+ CML, Ph+ALL
Cladribine	Anti-metabolite	Purine analog, interferes with DNA processing	Hairy Cell Leukemia
Fludarabine	Anti-metabolite	Purine analog, interferes with DNA synthesis	CLL
Methotrexate	Anti-metabolite	Inhibits folate metabolism, blocks nucleoside synthesis	ALL, lymphomas
Mercaptopurine	Anti-metabolite	Inhibits purine synthesis	ALL
Cytarabine	Anti-metabolite	Cytosine analog, prevents DNA synthesis	ALL, AML, CML
Asparaginase	Catalytic enzyme	Deprives leukemic cells of circulating asparagine	ALL
Rituximab	Monoclonal Antibody	Binds to CD20, induces apoptosis of CD20+ B cells	CLL, NHL

all goal of treatment for leukemia is complete remission, and significant progress has been made over the past several decades to achieve this aim. However, conventional chemotherapeutics are still not always 100% effective at eradicating disease.

ALL. The greatest success story for treatment of leukemia is that for treatment of childhood ALL. Treatments for childhood ALL include: vincristine, steroids, anthracyclines, cyclophosphamide, asparaginase, methotrexate, mercaptopurine. The fine tuning of treatment schedules, dose-intensities, and delivery have increased the cure rate for pediatric ALL from approximately 30%, forty years, ago to 80% today (45). Using the same principle for the treatment of adult ALL, complete response (CR) rates or the disappearance of all signs of cancer are 80% to 90% today and cure rates are up to 40% (45).

AML. The most effective strategy for treating AML for the last 30 years is known as the "3 + 7 regimen" and consists of intravenous daunorubicin for 3 days followed by 7 days of continuous infusion with intravenous cytarabine (45). Presently, for patients with AML, the CR rate to daunorubicin and cytarabine is between 60% and 70% with a long term survival rate of 25% to 35% compared to 40 years ago when CR rates were between 20% and 30%, and long term survival was rare (45).

CML. Up to 95% of CML cases result from a reciprocal chromosomal translocation that fuses the long arm of chromosome 9 to the long arm of chromosome 22, known as the Philadelphia chromosome (Ph+) because it was first discovered and described by two scientists in Philadelphia, PA (46,47). This translocation results in the fusion of the break point cluster region (*Bcr*) gene with the Abelson (*c-abl*) gene, which encodes a non-receptor tyrosine kinase involved in cellular signaling. As a result, the Bcr-Abl chimeric protein has constitutive tyrosine kinase activity resulting in the

activation of down stream signaling pathways that promote leukemic cell survival and proliferation (46). CML used to be considered a poor prognosis leukemia until the discovery of imatinib mesylate (48,49). Imatinib mesylate (Gleevec ®/STI571/ Novartis compound CGP 57148) is a tyrosine kinase inhibitor that has high affinity for the Abl kinase domain, while being essentially inactive against most other tyrosine kinases with the exception of the platelet-derived growth factor (PDGF) receptor and c-kit (50). Since the introduction of imatinib for treatment of CML, the CML-specific annual mortality rate has dropped from between 10% and 20% to 1% today (45). Resistance to imatinib occurs in approximately 3% to 4% of CML patients and 50% are the result of mutations in the Abl kinase domain (45). The Bcr-Abl fusion is also the most frequent genetic aberration in adult ALL and is found in 20% – 30% of patients. Imatinib is also highly effective for the treatment of non-resistant, Ph^+, adult ALL, and the prognosis for these patients is excellent.(51)

CLL. The treatment of choice today for CLL in fludarabine. Fludarabine is an adenosine nucleoside analog that inhibits DNA polymerase (52). Before fludarabine, patients were treated with vincristine, steroids, cyclophosphamide, and anthracyclines. However, fludarabine has been demonstrated to be superior to these earlier treatments for CLL (53,54). Treatment with both fludarabine and cyclophosphamide has been shown to increase CR rates for CLL patients greater than fludarabine alone (55). More recently, in a study at the M. D. Anderson Cancer Center at the University of Texas, Houston, CLL patients were treated with a combination of fludarabine, cyclophosphamide and rituximab. Rituximab is a monoclonal antibody that recognizes CD20, a B-cell specific antigen that is expressed throughout B cell development beginning at the early pre-B cell stage (56).

Of the 300 patients in this study, 72 % had CR with an estimated 5 year survival of 79% (57). Other chemoimmunotherapy treatments still under investigation include fludarabine plus alemtuzumab, a CD52 specific mAb; or lumiliximide, a CD23 specific mAb (45).

Leukemia subsets. There are specific subsets of leukemia for which individual, highly effective treatments have been discovered. Acute promyelocytic leukemia (APL) is a subtype of AML characterized by a unique chromosomal translocation resulting in the fusion of the retinoic acid receptor alpha (RARα) encoding gene and the promyelocytic leukemia (PML) encoding gene (58). APL may be the most severe form of acute leukemia, with a fatal course of only a few weeks. Initially, chemotherapy for APL consisted of anthracyclines and cytarabine with CR rates of approximately 80% (58). However, the duration of remission was only 11 to 25 months with cures rates between 35% to 45% (58). The discovery of the anti-APL activity of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) was a major breakthrough for the treatment of APL. The mechanism of ATO-induced APL leukemic cell death is not entirely understood. However, ATRA, which was first used clinically in 1985 (59), strongly induces promyelocytes to terminally differentiate (58). Today, combination therapy of ATRA and ATO have improved CR rates to 90% and cure rates to 70% to 85% (45).

Hairy cell leukemia (HCL) is an uncommon form of CLL occurring primarily in older males. The name comes from the appearance of the cells under the microscope; cells appear to be covered with tiny hairs. The prognosis for HCL prior to the discovery of effective therapies was unfavorable. The mean survival for patients with HCL was 5 years (45). Since the advent of Cladribine (2-Chlorodeoxyadenosine; CDA), the 5 to 10 year survival of patients with HCL has increased to 90% (60). Today, HCL is associated

with excellent long-term outcome after 1 or 2, 5-day courses of a relatively nontoxic chemotherapy with Cladrabine (45).

1.3 Molecular Genetics of Leukemia

Unlike solid tumors, which are vastly heterogeneous in nature, leukemias are generally regarded as clonal malignancies characterized by the accumulation of somatic mutations in cellular DNA (61). These mutations lead to an arrest in differentiation and confer a proliferative and/or survival advantage for the cells affected. With the exception of CLL, chromosomal translocations account for the vast majority of leukemias (61-63). In fact, nonrandom chromosomal translocations account for approximately 70% of all acute leukemias (63). Chromosomal translocations that have been observed in leukemia can be grouped into several categories, including: translocations that activate tyrosine kinases, those that affect the function of transcription factors, and those that regulate the cells' transcriptional machinery (61).

The most commonly occurring translocation leading to the activation of a tyrosine kinase is the Bcr-Abl fusion. As mentioned, approximately 95% of CML cases result from the reciprocal chromosomal translocation that fuses chromosome 9 to chromosome 22, the Philadelphia chromosome (46,47). This translocation results in expression of a Bcr-Abl chimeric protein that has constitutive tyrosine kinase activity which alters signaling pathways that control the proliferation, survival, and self renewal of hematopoietic cells (46). In addition, 20% – 30% of adult ALL result from this same translocation (30).

The most frequent targets of chromosomal translocations in acute leukemia are genes encoding transcription factors. There are two main mechanisms by which

chromosomal translocations lead to the activation of transcription factors. The first mechanism involves a transcription factor-encoding gene translocated to the vicinity of a constitutively active promoter or enhancer element, such as those of the TCR or Ig encoding genes. The second mechanism involves two genes encoding transcription factors being incorporated into a single "fusion" gene generating a chimeric protein with altered function (63). Chimeric transcription factors activate transcriptional cascades that modify the normal pattern of gene expression within the cell.

Genes involved in chromosomal translocations in acute leukemia are highly conserved evolutionarily and are often directly or indirectly involved in controlling the earliest stages of embryonic development (63,64). For example, the chromosomal translocation t(12;21) fuses the 5' portion of the *TEL* gene, a member of the ETS family of transcription factors, with nearly the entire coding region of the *AML1* gene, a runt-related transcription factor and regulator of HSC commitment (65). This fusion results in the transcriptional silencing, instead of activation, of AML1 target genes due to transcriptional repression domains retained in the *TEL* protein (66). This results in the altered self renewal and differentiation capacity of HSCs (30,65). Furthermore, members of the HOX gene family (described below) may lie downstream of AML1 (67). HOX genes encode a family of transcription factors that play a critical role in embryogenesis and hematopoiesis.

The gene most frequently involved in chromosomal translocations in acute leukemia is the mixed lineage leukemia (*MLL;* a.k.a. *HRX* or *ALL1*) gene (63). To date, greater than 51 chromosomal translocation partners for *MLL* have been identified in acute leukemia (68). In decreasing frequency, the most frequent *MLL* fusion partners are *AF4*,

AF9, *ENL*, *AF6*, *ELL,* and *AF10* (69). Approximately 80% of infant pre-B cell ALL are associated with *MLL* translocations (70,71). Leukemias bearing *MLL* translocations are associated with a poor prognosis. Furthermore, *MLL* translocations are found in therapy induced AML (t-AML) in infants, children and adults (68). In leukemic cells downstream targets of *MLL* fusion proteins have not been entirely identified (72). However, leukemias bearing *MLL* translocations are profoundly associated with overexpression of specific members of the HOX gene family, including HoxA7 and HoxA9 (68).

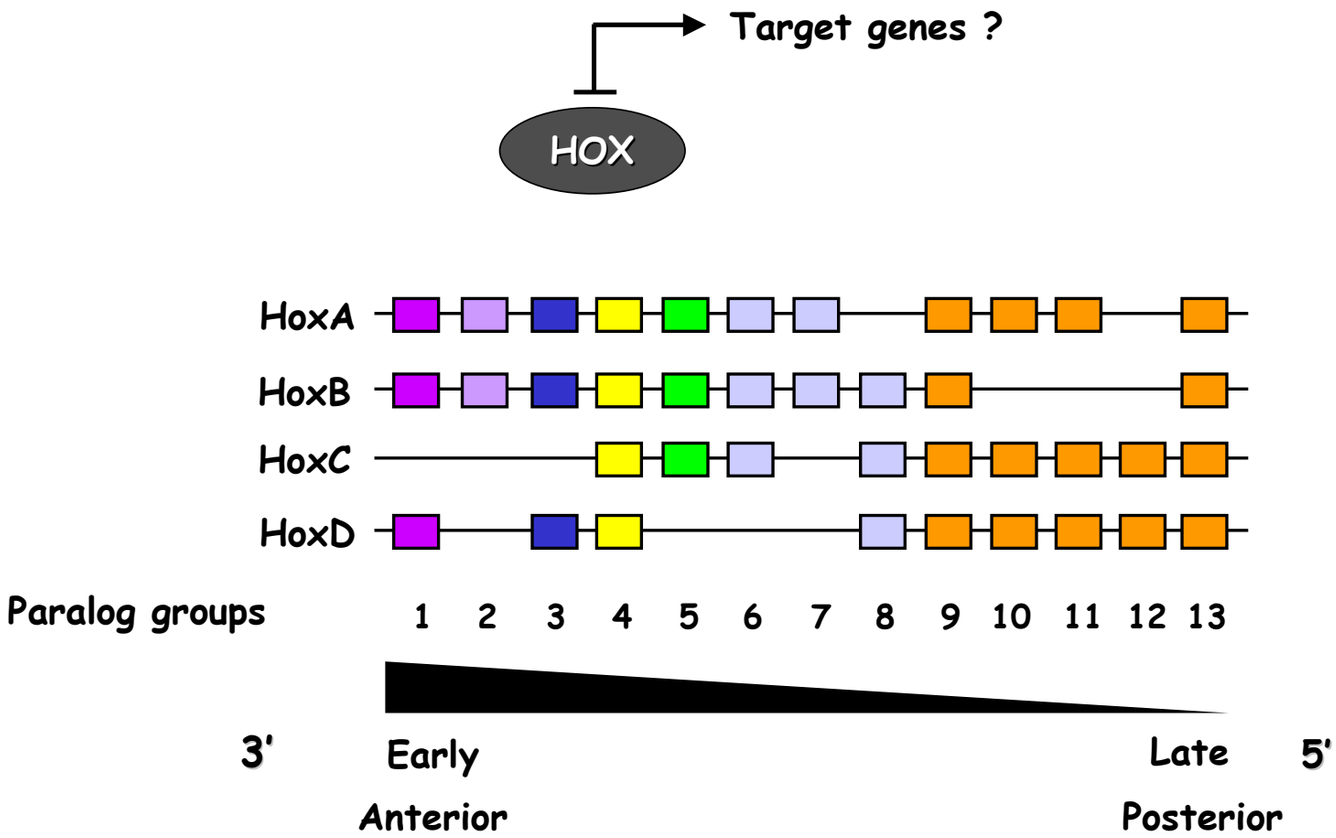
1.4 Human HOX Gene Family

Hematopoiesis is a intricate process regulated by the expression of multiple transcription factors that are either activated or inhibited as hematopoiesis proceeds. Deregulation of transcription factor expression and activity is believed to be required for malignant transformation (63). A specific set of evolutionarily conserved homeodomain containing genes, HOX genes, are responsible for encoding a family of transcription factors involved in early development that control pattern formation during embryogenesis. The HOX gene family contains conserved sequences of 183 nucleotides that encode a 61 amino-acid, helix-turn-helix, DNA-binding motif. The core homeodomain DNA-binding motif recognizes DNA sequences ranging from TGAT to TAAT to TTAT and TTAC (73).

In mammals, there are 39 different class I HOX genes organized into four individual clusters (HOXA, B, C, and D), covering distances of approximately 150 – 200 kb, located on four separate chromosomes (Fig. 3). These clusters may be further grouped into subclasses based on their primary sequence into 13 paralog groups within each individual cluster. Each HOX gene has a polyadenylation site, promoter, and a high-

Figure 3: The Human HOX Genes. There are 39 human HOX genes organized into four clusters (A – D) located on four separate chromosomes. The clusters are further divided into subclasses based on there primary sequence homology into 13 paralog groups. During embryogenesis the 3' HOX genes are expressed early in the anterior region of the embryo and the 5' HOX genes are expressed late in the posterior region of the developing embryo. The colored fields represent the anterior most limits of expression during embryogenesis i.e. HoxA1 is only expressed at the most anterior region of the developing embryo. Moving from the posterior to the anterior, expression levels fade and the boundaries of each expression domain overlap. Despite a role in embryogenesis and hematopoiesis, few HOX downstream targets have been identified.

Human Hox Genes



ly conserved order within the clusters (74). A critical role of the HOX transcription factors is the tight control and maintenance of HSC differentiation and proliferation.

1.4.1 Regulation of HOX Genes

Several HOX genes from the A, B, and C clusters are expressed in distinct patterns during hematopoiesis and embryogenesis (75). HOX genes exhibit a temporal spatial pattern of expression during both hematopoiesis and embryogenesis. In the embryo, the 3' HOX genes are expressed early in the anterior region of the developing embryo and the 5' HOX genes are expressed in the posterior region of the developing embryo late during embryogenesis. HOX genes are determinants of cell fate. The differentiation and stage-specific expression of each HOX gene programs cells to the appropriate developmental stage before advancing to the next stage of differentiation. During hematopoiesis, the 5' HOX genes are highly expressed in primitive stem/progenitor cells, and down-regulated as cells become committed to different lineage specific programs (76). Several mechanisms have been identified for the regulation of HOX gene expression. The identification of a TAAT retinoic acid response element sequence (RARE) in upstream promoter regions, common amongst HOX genes, allows for the binding of HOX proteins and subsequent activation through positive feedback (77). The genes involved in the maintenance of HOX gene expression in *Drosophila melanogaster* include the polycomb (Pc-G) gene, which encodes a repressor of HOX gene expression, and trithorax (Trx-G) gene, which encodes a protein responsible for the maintenance of HOX gene expression. Following the discovery of these genes in *Drosophila*, they were also identified in mammals. In humans, the *MLL* gene represents the human homologue of the Trx-G gene (77). Chromosomal translocations involving the

MLL gene almost invariably result in deregulation of HOX gene expression, and have been described in a number of human leukemias (75).

1.4.2 HOX in Normal and Malignant Hematopoiesis

Commitment and differentiation of HSCs is under precise genetic control, and there is evidence that HOX genes play a significant role in this regulatory process (75). Genes of the HOXA and HOXB clusters are preferentially expressed in $CD34^+$ human BM cells. Additional analysis of $CD34^+$ BM cells revealed two distinctive patterns of HOX gene expression in different sub-populations of $CD34^+$ cells. HOXA and HOXB genes located at the 3' ends of the clusters are expressed invariantly in all $CD34^+$ cells such as CLPs or pro-B cells. However, HOXA and HOXB genes located at the 5' ends of the clusters are expressed at high levels only in the most primitive sub-populations of $CD34^+$ cells (76).

Several experiments have been performed to illustrate the importance of HOX gene expression and regulation during hematopoiesis. For example, HoxA5 overexpression in human BM leads to an increased number of myeloid cells and lower numbers of erythroid progenitors (78). Conversely, limited expression of HoxA5 in BM cells results in higher numbers of erythroid progenitors and lower numbers of myelomonocytic cells (79). Overexpression of HoxA10 profoundly perturbs differentiation of $CD34^+$ hematopoietic cells into megakaryocytes, inhibits B-cell development, enhances proliferation of hematopoietic progenitors, and ultimately may induce AML (80). Disruption of HoxB6 expression leads to expansion of erythroid progenitors but a lack of erythroid differentiation (75). These examples illustrate how

deregulation of HOX gene expression can have severe effects on normal hematopoiesis, and underscore the importance of maintaining proper HOX gene expression.

Not surprisingly, deregulation of HOX gene expression, as a result of genetic mutation, is most highly associated with leukemia. The involvement of abnormal HOX gene expression in the development of leukemia was first identified in the WEHI-3 myeloid leukemic cell line. A block in the differentiation of myeloid leukemic cells results from a constitutively active HoxB8 gene due to a proviral insertion (81). Overexpression of IL-3 in this cell line led to the generation of IL-3 dependent myelomonocytic, megakaryocytes, and mast cells *in vitro*, and leukemia *in vivo*. The synergistic effects of the expression of both genes is highly transforming (81). More evidence demonstrating the involvement of aberrant HOX gene expression in leukemia came from the work of Nakamura and colleagues (82). Retroviral activation of HoxA7 and HoxA9 along with a retrovirally activated Meis1 gene in BXH-2 mice resulted in the development of myeloid leukemia. Coexpression of HoxA9, HoxA7 and Meis1 has been observed in human AML (83). Furthermore, overexpression of HoxA9 alone in murine BM results in AML, and coexpression of HoxA9 and Meis1b in mice, leads to the accelerated onset of leukemia compared to mice overexpressing HoxA9 alone, suggesting that cofactors of HOX may assist in the acceleration of the onset of leukemic transformation (83,84). Murine bone marrow transduction experiments using retroviral vectors provide a useful model for the study of human leukemia, and although it has not been shown for all HOX genes, overexpression of specific HOX genes has been shown to perturb normal hematopoiesis.

1.4.3 HOXA9 in Hematopoiesis and Leukemogenesis

Like other 5' members of the HOXA gene cluster, HoxA9 is strongly expressed in the most primitive $CD34^+$ population of hematopoietic precursors and is subsequently downregulated as cells differentiate into the various sub-populations of blood cell types. To determine the role of HoxA9 in hematopoiesis, Lawrence *et al*. used HoxA9 knockout mice to examine the physiological effects that this mutation would have on hematopoietic cell development (85). Mice bearing the HoxA9 mutation were physically similar to their wild-type littermates, with respect to health and weight, and were not predisposed to infection or leukemia following a year of observation (85). However, $HoxA9^{-/-}$ mice exhibited the most severe hematopoietic defects with a 33% reduction in the numbers of granulocytes, a 40% reduction in the numbers of $B220^+$ B-cells, and a 35% reduction in the number of $CD3^+$ T-cells. The homozygous HoxA9 knockout mice showed a percent distribution of myeloid/erythroid cell types similar to wild type mice; however, there was a global two-fold reduction in the total number of cell types in the mutant due to a decrease in the number of progenitor cells in the BM (85). As a result of the overall decrease in the number of progenitor cells, the homozygous HoxA9 mutant mice had smaller spleens and thymuses compared to wild type animals (85). These results demonstrate that HoxA9 plays a key role during the early stages of normal hematopoiesis.

The transforming effects of HoxA9 are enhanced by other oncogenic factors, including overexpression of Meis1a and expression of the E2A-Pbx1a fusion protein. The Sauvageau group showed that primary BM cells engineered to overexpress HoxA9 invariably led to the development AML within 3 to 10 months when transplanted into syngeneic mice (74,84,86). Moreover, when HoxA9 was overexpressed in primary BM

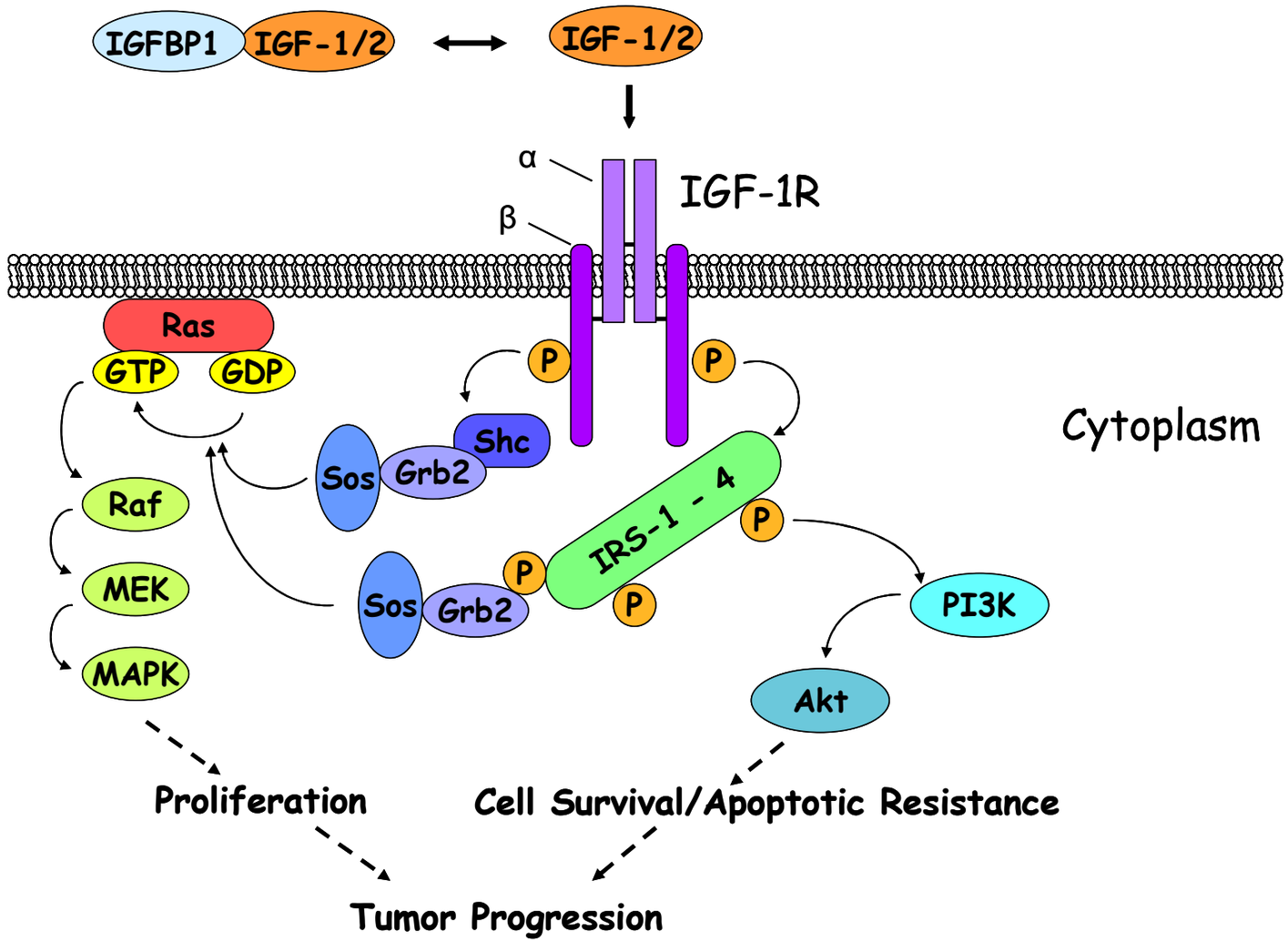
cells along with the HOX cofactor Meis1a or the E2A-Pbx1a fusion oncoprotein, they develop into AML when transplanted into syngeneic mice with a significantly shorter latency period than cells overexpressing HoxA9 or Meis1a alone (84,87). This same group demonstrated that overexpression of HoxA9 in more mature lymphoid cells partially blocked B lymphopoiesis at the pre-B-cell stage but had no detectable effect on T lymphoid development (86). Based on the seemingly central role that HoxA9 plays in normal hematopoiesis and leukemogenesis, it is not surprising that HoxA9 is the most frequently deregulated HOX gene in acute leukemia (88), and overexpression of HoxA9 is the single most highly correlated factor with poor prognosis for patients with AML (89).

Despite overwhelming evidence for the critical role that HoxA9 plays in normal and malignant hematopoiesis, very few direct downstream targets of HoxA9 have been empirically defined. These include: Pim-1 (90,91), c-Myb (92), gp91Phox (93), and some members of the Wnt gene family (94). Furthermore, an association between HoxA9 activity and growth receptor signaling has not been previously described.

1.5 Insulin-Like Growth Factor-1 Receptor

The insulin-like growth factor-1 receptor (IGF-1R) is a ubiquitous transmembrane receptor tyrosine kinase that is essential for the development and growth of normal tissues (Fig. 4). Signaling through IGF-1R stimulates cell proliferation and differentiation, changes in cell size, and can protect cells from apoptosis. IGF-1R is primarily the receptor for IGF-1, but can also bind IGF-2 and insulin, although the affinity for insulin is three orders of magnitude lower than that for IGF-1 or IGF-2 (95). IGF-1 is a growth factor produced primarily by liver and stromal cells of the BM microenvironment. IGF-1

Figure 4: The Insulin-like growth factor-1 receptor (IGF-1R). IGF-1R is a heterotetramer receptor tyrosine kinase consisting of two extracellular alpha chains, containing the ligand binding domain, and two intracellular beta chains, containing the catalytic domains. The half-life of the ligand, IGF-1, is prolonged in the plasma by IGF-1 binding protein 1. Binding of IGF-1 results in autophosphorylation of the beta subunits of the receptor. This results in the recruitment and subsequent activation of the auxiliary signaling proteins, insulin receptor substrates 1 – 4 (IRS-1–4) and Shc. Phosphorylation of IRS-1 results in activation of the PI3K/Akt pathway leading to signals promoting cell survival and protection from apoptosis. IRS-1 along with Shc can also induce signaling through the MAPK/ERK pathway through activation of Ras, leading to increased cellular proliferation. Both pathways converge to promote tumor growth.


IGFBP1
IGF-1/2
IGF-1/2
α
β
IGF-1R
Ras
GTP
GDP
P
P
Cytoplasm
Shc
Sos
Grb2
Raf
IRS-1 - 4
P
P
P
MEK
Sos
Grb2
PI3K
MAPK
Akt
Proliferation
Cell Survival/Apoptotic Resistance
Tumor Progression

is a potent mitogenic and anti-apoptotic molecule involved in the regulation of cell proliferation in renewing epithelial cell populations of organs including: breast, prostate, colon, and lung (95).

IGF-1 is a 6 – 8 kDa protein found in serum between 10 and 100 nmol/l and functions to stimulate a variety of metabolic processes (89). Endocrine expression of IGF-1 from the liver and BM is regulated by growth hormone (GH). Recent evidence also suggests that other tissue types are also capable of expressing IGF-1 (89). Thus, IGF-1 ligand is subjected to endocrine and paracrine regulation, as well as autocrine regulation in the case of many types of tumor cells (96).

The IGF-1 receptor is a pre-formed hetero-tetramer containing two alpha and two beta chains covalently linked by disulfide bonds. The receptor subunits are synthesized as part of a single polypeptide chain of 180 kD, which is proteolytically processed into alpha (130 kD) and beta (95 kD) subunits (97). The alpha chain is the extracellular domain and contains the site for ligand binding. The beta chain possesses the transmembrane domain, and the tyrosine kinase catalytic domain. Upon binding of IGF-1, the IGF-1R undergoes autophosphorylation at conserved tyrosine residues within the catalytic domain of the beta chain. Subsequent phosphorylation of additional tyrosine residues within the beta chain provides docking sites for molecules critical to the intracellular signaling, including the insulin receptor substrates (IRS) 1 – 4 and Shc. These substrates initiate phosphorylation cascades that transmit the IGF-1R signal (98).

The principle pathways for transduction of the IGF-1 signal are mitogen-activated protein kinase/extra cellular receptor kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathways (99). Phosphorylated IRS-1 activates PI3K, leading to

activation of Akt. Phosphorylated Akt enhances protein synthesis through mTOR and promotes cell survival. PI3K can also recruit Grb2/SOS by phosphorylated IRS-1 or Shc, which leads to the recruitment of Ras, and activation of the MAPK/ERK pathway. The MAPK/ERK pathway is primarily responsible for the mitogenic signal elicited following stimulation by IGF-1, and PI3K/Akt is responsible for the IGF-1R–dependent induction of anti-apoptotic or survival processes (96). Some studies suggest that signaling through the PI3K/Akt pathway can enhance and/or synergize with MAPK/ERK signaling, providing a more robust pro-survival signal (100). Thus, signaling through IGF-1R can promote both cellular proliferation and apoptotic resistance through two different cellular signaling pathways.

The caspase family of cysteine proteases plays a pivotal role in the execution of apoptosis (101). Stress-induced mitochondrial membrane instability results in the release of cytochrome C, which causes activation of the caspase cascade through caspase cleavage. This effect leads to collapse of the cellular infrastructure (101). The PARP protein is the best characterized substrate of caspase cleavage and PARP cleavage is often used as an indicator of apoptosis (102). IGF-1R signaling through PI3K/Akt can exert its anti-apoptotic effects through phosphorylation of the pro-apoptotic protein BAD (103,104). BAD influences the integrity of the mitochondrial membrane, and the release of cytochrome C, by associating with Bcl-2 and Bcl-xl, inhibiting their anti-apoptotic function (105). Phosphorylation of BAD by Akt blocks the pro-apoptotic activity of BAD (106). However, BAD is also phosphorylated, and inhibited, by the anti-apoptotic kinase Pim-1, a direct transcriptional target of HoxA9 (91).

1.5.1 IGF-1R and Cancer

Overexpression of growth factors and/or their receptors is a common event in many cancers, and it is therefore not surprising that many studies have implicated IGF-1R and its ligands in the development, maintenance, and progression of cancer (95). Early studies of the IGF-1R gene noted sequence homology between IGF-1R and tyrosine kinase oncogenes (97). Early studies also demonstrated that IGF-1 is mitogenic for breast cancer cells *in vitro* (107). Later it was demonstrated that IGF-1R expression was present on many human cancers (108), and that IGF-1 can stimulate the growth of multiple human neoplasms (98,109).

Overexpression of IGF-1, IGF-2, IGF-1R, or combinations thereof have been reported in glioblastomas, neuroblastomas, meningiomas (110), medulloblastomas (111), pancreatic carcinomas (112), and ovarian cancer (113). IGF-1R activity is also implicated in several solid tumors, such as those of the breast, prostate and colon, where aberrant HOX-gene expression has also been reported (114-116). Furthermore, multiple studies have demonstrated an increased risk of cancer associated with high levels of circulating serum IGF-1 (115-123). In fact, high serum levels of IGF-1 have been shown to correlate with childhood leukemia; an association between high infant birth weight and infant ALL that correlates with high levels of IGF-1, the so-called “big baby hypothesis” (124,125).

In leukemia, studies have shown that autocrine signaling through IGF-1R promotes the growth of AML cells and inhibition of IGF-1R signaling in these cells results in the apoptosis (126). It was also reported by this group that chemoresistance of AML cells correlated with IGF-1 expression (126). In other studies signaling through IGF-1R has been reported to relieve leukemic cells of IL-3 dependency, and blocking IGF-1R signaling in these cells inhibited proliferation and induced apoptosis (127,128).

Additionally, one study demonstrated that proliferation of the pre-B ALL cell line, REH, could be inhibited in a dose-dependent manner by monoclonal antibodies against IGF-I and IGF-1R (129).

The central role that the IGF-1R plays in promoting tumor survival and proliferation makes it an attractive target for cancer therapeutics. In 1989, the first evidence that an IGF-1R specific antibody (αIR3) could reduce tumor growth rate in animal models was demonstrated (130). Since then various strategies have been employed to inhibit IGF-1R expression and signaling both *in vitro* and *in vivo*. Among these strategies are: antisense oligonucleotides (131), antisense RNA (132,133), siRNA (134), triple helix-forming oligodeoxynucleotides (131), single chain antibodies (135), fully humanized monoclonal antibodies (136-138), and small molecule kinase inhibitors (139,140). Inhibitors of IGF-1R signaling are currently being used in clinical trials to treat solid tumors in humans. So far, the results have been promising and suggest that targeting IGF-1R *in vivo* may inhibit tumor growth and induce tumor cell death. Furthermore, the use of IGF-1R inhibitors along with other conventional therapies may increase the efficacy of treatment (141).

1.6 Experimental Objectives

1.6.1 Overview

The goal of this proposed research project was to elucidate the molecular mechanisms that contribute to survival and proliferation of pre-B ALL cells as a result of deregulated HoxA9 expression. Deregulation of HoxA9 expression is highly associated with acute leukemia; however, the role that HoxA9 overexpression plays in promoting leukemic cell growth and survival is not well understood. The proposed experiments

were designed to test the hypothesis that *HoxA9 overexpression promotes the growth and survival of acute leukemic cells through activation of specific downstream signaling pathways.* Although tremendous improvements have been made in the treatment of ALL over the past few decades (45), conventional chemotherapy is not 100% effective in the treatment of leukemia. In fact, leukemias bearing *MLL* translocations are associated with a poor prognosis. Moreover, approximately 80% of infant pre-B cell ALL are associated with *MLL* translocations (70,71). These leukemias represent a major subgroup of ALL with a particularly poor prognosis and require innovative treatment strategies (70,71). Leukemias bearing translocations of the *MLL* gene are profoundly associated with overexpression of HOX genes, particularly HoxA9 (68). Furthermore, HoxA9 overexpression also represents the single most highly correlated factor with treatment failure and poor prognosis for patients with AML. Therefore, identifying pathways altered by HoxA9 overexpression, which promote the survival and proliferation of acute leukemic cells, might provide valuable therapeutic targets for treatment of acute leukemia.

1.6.2 System

To determine how HoxA9 expression may contribute to the growth and survival of B-lineage acute leukemia, *in vitro* cell line models of pre-B cell ALL were utilized. Chiefly, the cell line that was used was the stromal cell-dependent pre-B ALL ($CD10^+$, $CD19^+$, pre-BCR^+) cell line, BLIN-2 (B-Lineage-2). BLIN-2 cells were derived from cyropreserved BM cells from a pediatric patient with newly diagnosed pre-B cell ALL (142). The karyotype of this cell line is 46,XX,18,dic(9;20)(p11;q11.1) and it shares clonal identity with the patient's original BM leukemic cells (142). BLIN-2 cells lack

HoxA9 expression and are absolutely dependent on stromal cell contact for survival and proliferation. As leukemic cells become more malignant they often lose dependency on growth factor/stromal cell support for survival and proliferation. Therefore, the BLIN-2 model allows us to test effects of HoxA9 overexpression on promoting growth factor/stromal cell independent growth of leukemic pre-B cells in the context of the tumor microenvironment.

CHAPTER 2: Materials and Methods

2.1 Cell Culture and Cell Lines

BLIN-2 and BLIN-3 cells have been described previously (142-144). BLIN-2/MigR1 or BLIN-2/HoxA9:ER cells were generated by retroviral transduction, followed by fluorescence activated cell sorting (FACS) for stable retroviral clones. BLIN-2 and BLIN-3 cells were maintained on foreskin fibroblast stromal cell layers as previously described (142-144). BLIN-3 cultures were supplemented with 10 ng/ml IL-7 (Peprotech, Rock Hill, NJ). BLIN-2 cells were maintained in serum free XVIVO-10 (Lonza BioScience, Walkersville, MD) without phenol red. RS4;11 (145) and SEMK2 (146) cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal calf serum, 100 U penicillin/ml, and 100 µg streptomycin/ml.

2.1.1 Cell Growth and Proliferation

Cell growth was evaluated via the MTT assay using CellTiter 96® reagent (Promega, Madison, WI). Briefly, 2×10^4 cells were washed 3 times in phenol red free XVIVO-10 medium and were seeded in triplicate into 96-well plates in phenol red free XVIVO-10 in the presence or absence of stromal cells. In some experiments cells were treated with 4HT (1 µM) (Sigma, St. Louis, MO), human recombinant IGF-1 (50 ng/ml) (Peprotech, Rocky Hill, NJ), AG1024 (1 µg/ml) (Calbiochem, La Jolla, CA) and/or the A12 mAb (15 ug/ml), at the indicated times, 20 µl of CellTiter 96® labeling reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl-2H-tetrazoli um, and phenazine ethosulfate] was added to each well and incubated at 37°C for 3 hr. The absorbance was measured at 492 nm and is expressed as relative proliferation. Unless indicated otherwise, all experiments were performed in triplicate.

2.2 Retroviral Vectors

The full length murine HoxA9 cDNA (87) (kind gift of G. Sauvageau, Institute for Research in Immunology and Cancer, Montreal, Canada) was fused in-frame with the hormone binding domain of the human estrogen receptor, and tagged with the 14 amino acid V5 epitope. The HoxA9:ER:V5 sequence was then cloned into the EcoRI and XhoI sites of the MigR1/IRES/GFP retroviral vector (147) to generate the HoxA9:ER construct.

2.2.1 Retroviral Infection

T75 flasks were coated with 0.1% gelatin for 1 hr at room temperature (RT). 6×10^6 HEK-293 cells were plated in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum, 100 U penicillin/ml, and 100 µg streptomycin/ml. Cells were cultured overnight at 37°C. 20 pmol of the expression vector and 20 µg of pCL-Ampho (Imgenex, San Diego, CA) were added to 1.5 ml of 0.25 M $CaCl_2$. The mixture was added to 1.5 ml of 2 × HBS (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4), pH 7.17 and incubated at RT for 30 min. The mixture was added to HEK-293 cell containing flasks and incubated at 37°C for 6 hr. Cells were then glycerol shocked with a 15% glycerol/phosphate buffered saline (PBS) solution, fresh DMEM was added and cells were incubated for 48 hr at 37°C prior harvesting of viral supernatant. Viral supernatant was filtered through a cellulose-acetate 0.45 µm filter (Millipore, Billerica, MA) and used immediately for retroviral transduction. For retroviral transduction, BLIN-2 cells were plated at a density of 1×10^6 cells/well in 6 well plates without stromal cell feeder layers in 1 ml of XVIVO-10 media, 4 µl of polybrene, and 1 ml of filtered viral supernatant per well. Following 6 hr incubation at

37°C, 3 ml of XVIVO-10 media was added to each well. Following 24 hr incubation at 37°C, cells were transferred to flasks with stromal cell feeder layers and cultured for 48 hr. Successfully transfected cells were FACS purified based on GFP expression. HoxA9 expression was confirmed by western blot analysis. Transfected cells were maintained in phenol red free XVIVO-10 media in the presence of stromal cell layers.

2.2.2 Flow Cytometry/Cell Sorting

Following retroviral infection, cells that were successfully transduced were purified on the basis of GFP expression by fluorescence activated cell sorting (FACS) using a FACS Vantage instrument (Becton-Dickenson, Moutainview, CA). For analysis of GFP expression, 1×10^6 cells were collected and resuspended in 300 µl of FACS buffer (1 × PBS, 2.5% fetal calf serum, and 0.02 NaN_3, pH 7.4) and analyzed using a FACSCalibur flow cytometer and CellQuest-Pro software.

2.3 Western Blot

Leukemic cells were lysed in ice cold RIPA buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, and 0.1 mM β-glycerophosphate) for 30 min at 4°C. The lysates were centrifuged for 30 min at 20,000 × g at 4°C. The supernatant was removed and the protein was quantitated using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Twenty to 40 µg of protein per sample was electrophoresed on a 10 % SDS-polyacrylamide gel electrophoresis (PAGE) gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The membranes were blocked with 5 % nonfat milk/TBST (50mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20) or 5 % bovine serum

albumin (BSA) /TBST. For some experiments, cytoplasmic and nuclear fractions were separated using the Pierce NE-PER Kit as per manufacture's instruction.

Membranes were incubated with anti-HoxA9 (Upstate Biotechnology, Charlottesville, VA); anti-c-Myb; anti-PARP, anti-caspase 7, anti-cleaved caspase-7, anti-IGF-1R, anti-ERK, anti-phospho ERK, anti-phospho Akt (ser473), anti-BAD, anti-phospho BAD (ser112), and anti-Pim-1 antibodies (Cell Signaling Technology, Beverly, MA); anti-Pax5 (BD Transduction Laboratories, San Diego, CA); anti-actin (Sigma, St. Louis, MO); anti-phosphotyrosine antibody (clone 4G10) (Upstate Biotechnology, Charlottesville, VA); anti-HnRNP70 (kind gift from Dr. Phillip Pekala, East Carolina University) or anti-β-tubulin primary antibodies, suspended in 5% nonfat milk/TBST or 5% BSA/TBST at the appropriate dilution for 2 hr to overnight. Membranes were washed in TBST 3 × for 10 min each then the membranes were incubated with the appropriate secondary antibody (1 : 5,000 dilution) suspended in 5% nonfat milk/TBST or 5% BSA/TBST, conjugated to horseradish peroxidase, for 1 hr. Membranes were washed 3 × for 10 min in TBST and visualized SuperSignal® West Pico chemiluminescent substrate according to manufacturer's instructions.

2.3.1 Immunoprecipitation

Cells were washed in phenol red free XVIVO-10 medium and then cultured in the presence or absence of A12 mAb (15 ug/ml) and 4HT (1 μM). Cells were treated with recombinant human IGF-1 (100 ng/ml) for 5 minutes followed by the addition of 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, and 0.1 mM β-glycerophosphate. Cells were washed in ice cold PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl

fluoride, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, and 0.1 mM β-glycerophosphate. 250 µg of cellular protein in 300 µl of lysis buffer was precleared for 2 hr at 4°C with 20 µl of protein A/G plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitation was performed overnight at 4°C with 2 µg of anti-IGF-1Rα antibody (clone 24-31) (Thermo Fischer Scientific, Fremont, CA). Twenty microliters of beads were added to samples and were incubated at 4°C for 2 hr. Beads were collected by centrifugation at 1000 × g for 5 min, washed twice in ice cold lysis buffer, and western blotting was performed as described using an anti-phosphotyrosine antibody (clone 4G10) (Upstate Biotechnology, Charlottesville, VA), per manufacturer's instructions, to detect phosphorylation of the co-immunoprecipitated IGF-1Rβ chain. IGF-1Rβ chain identity was confirmed by probing the blot with anti-IGF-1Rβ (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4 Electrophoretic Mobility Shift Assays

Complementary oligonucleotides (upper strand shown) containing a consensus binding site for HoxA9 (ctgcgATGATTTACGACcgc) (148) were synthesized (Invitrogen, Carlsbad, CA). EMSA assays were performed using the DIG Gel Shift Kit, 2nd Generation (Roche, Indianapolis, IN). Briefly, double-stranded, digoxegenin-labeled DNA and unlabeled probe were incubated with 10 µg of BLIN-2/HoxA9:ER or BLIN-2/MigR1 protein either in the presence or absence of 1 µg of anti-HoxA9 polyclonal antibody (Upstate Biotech) in labeling buffer [1 µg of d(I-C), 1 µg d(A-T) plus 2 µg BSA, in a final reaction volume of 20 µl]. Prior to the addition of DNA for EMSA, antibody and proteins were incubated together for 45 min at RT, prior to the addition of DNA. Labeled DNA targets were then incubated with each protein mixture for 30 min at

RT. Mixtures were run on a 6% non-denaturing polyacrylamide gel. Gel electrophoresis was performed in 0.25 × TBE (Tris-Borate-EDTA) buffer. Gels were transferred to PVDF membranes and signals were revealed using the DIG Nucleic Acid Detection Kit (Roche, Indianapolis, IN) per the manufacturer's instruction.

2.5 RT-PCR

Total RNA was extracted from 5 × 10^4 cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Oligo-dT primed cDNA was synthesized as follows: 10 µl of RNA was incubated with 1.6 µl of 5 × RT buffer, 0.5 µl of DNAse I, and 0.5 µl of RNase inhibitor (Invitrogen, Carlsbad, CA) at 37°C for 1 hr, followed by heat inactivation at 75°C. To this reaction was added 2.4 µl of 5 × RT buffer, 2 µl 0.1 M DTT, 1 µl of Oligo-dT, 1 µl of 10 mM dNTPs, and 1 µl of Reverse Transcriptase (Invitrogen, Carlsbad, CA). Following 1 hr incubation at 42°C and 10 min heat inactivation at 75°C, 1 µl of this preparation was used for each PCR reaction.

Each PCR reaction mixture contained 39 µl of H_2O, 5 µl of 10 × Buffer (Invitrogen, Carlsbad, CA), 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of each primer at 20 µM, 0.5 µl Taq polymerase (Invitrogen, Carlsbad, CA), and 1 µl of cDNA. For PCR reactions the primers and annealing temperatures were as follows: HoxA9 forward 5' - TGT GGT TCT CCT CCA GTT GAT AGA G - 3', reverse 5' - TCG GTG AGG TTG AAC AGT CGA G - 3' at 50°C; IGF-1R forward 5' - ACC ATT GAT TCT GTT ACT TC - 3', reverse 5' - ATA CTC TGT GAC ATT CTT AA - 3' at 51°C; c-Myb forward 5' - GTT TTC AGA ACA GTT CAA GT - 3', reverse 5' - TAC ACT TAG AGT AAT GCT TT - 3'at 55°C; mb-1 forward 5' - GCT CCC CTA GAG GCA GCG ATT AAG GGC TCA - 3', reverse 5' - AGG GTA ACC TCA CTC TTC TCC AGG CCA

GGC - 3’ 60°C; MLL/AF4 forward 5’ - CGT TCC TTG CTG AGA ATT TG - 3’, reverse 5’ - AAG CCC GTC GAG GAA AAG - 3’ at 55°C; and GAPDH forward 5’-TCC ATG CCA TCA GTG CCA CC - 3’, reverse 5’ - ATG AGC TTG ACA AAG TGG TC -3’ at 60°C.

2.5.1 Southern Blot Analysis

Ten microliters of each PCR product was separated on a 1.5 % agarose gel and transferred to a nylon membrane (Hybond-N, GE Healthcare, Piscataway, NJ). DNA was cross-linked to membranes by UV exposure followed by pre-hybridization with 25 ml of southern blot buffer (200 µM Tris-HCL, pH 7.5, 1 M NaCl), 0.1 % SDS (w/v) and 1 ml of boiled salmon sperm DNA (10 mg/ml) at 42°C for 3 hr in a hybridization oven. Following pre-hybridization the blots were hybridized at 42°C overnight with oligonucleotide probes internal to the PCR primers. Probes were labeled with digoxigenin (DIG) using the DIG Oligonucleotide 3’-End Labeling Kit, 2nd Generation (Roche, Indianapolis, IN) per the manufacturer’s instructions. The following probes were used: HoxA9, 5’ - TCA TGC GCG CTC CAC TCG GA - 3’; IGF-1R, 5’ - CTG CTC CTC TCC TAG GAT GA - 3’; c-Myb, 5’ - GAA TTG TAG CCA GTT GTT AAT - 3’; mb-1, 5’ - GCT CCC CTA GAG GCA GCG ATT AAG GGC TCA - 3’; MLL/AF4, 5’ - TGC AGG GCT AGC CCG CCT CAG CCA CCT ACT - 3’; and GAPDH 5’ - TTC GTT GTC ATA CCA GGA - 3’. Following hybridization, membranes were washed at 42°C in pre-warmed 1 × SSC buffer (150 mM NaCl, 15 mM Na-Citrate, pH 7.0) and 0.1 % SDS 3 × for 1 hr each. Hybridization signals were revealed using the DIG Nucleic Acid Detection Kit (Roche, Indianapolis, IN) per the manufacturer’s instruction.

2.6 Transfection with siRNA

HoxA9 siRNA targeted to the sequence 5'-AAT CAA CAA AGA CCG AGC AAA-3' was synthesized by Ambion (Austin, TX). Cells were transfected using an AMAXA Nucleofector per manufacturer's instructions and analyzed 24 hr later by RT-PCR for HoxA9 and IGF-1R expression.

2.7 ELISA Assays

Phosphorylated IGF-1R was detected using the STAR phospo-IGF-1R (TYR1135/1136) ELISA kit from Millipore (Billerica, MA). The kit was used as per manufacture's protocol, with 40 µg of whole cell lysates per well. Plates were read of 450 nm using a Thermo Electron Corporation Multiskan EX plate reader.

2.8 Propidium Iodide Cell Cycle Analysis

Cells were collected and resuspended in 1 ml of ice cold 1 × PBS and 2 ml of ice cold methanol. Cells were incubated on ice for 30 min then collected and resuspended in 500 µl of 1× PBS and 0.2 µg/ml RNase A. The mixture was incubated at 37° for 30 min. 200 µl of propidium iodide staining solution (0.25 mg/ ml propidium iodide, 0.25 mM EDTA, and 0.25% vol/vol Triton X-100) was added and incubated at room temperature for 30 min in the dark. Following incubation, flow cytometry was performed using a FACSCalibur flow cytometer and analyzed using CellQuest-Pro software and and ModFit LT™ 3.0, as described previously (143).

CHAPTER 3: HoxA9 Induces IGF-1R Expression In B-Cell ALL

3.1 Introduction

The homeobox (HOX) gene family encodes a group of transcription factors that are preferentially expressed during embryonic development and hematopoiesis and are commonly deregulated in acute lymphoblastic leukemia (ALL) (88). In hematopoiesis, the role of these transcription factors is to tightly control and maintain stem cell differentiation and proliferation (77). HOX gene family members contain conserved sequences of 183 nucleotides encoding a 61 amino-acid, helix-turn-helix, DNA binding motif. The core homeodomain DNA binding motif recognizes the DNA sequence (T/G)NA(T/C) (148). In mammals there are 39 different class I HOX genes organized into four clusters (A, B, C, and D), located on four separate chromosomes. The clusters may be further grouped into subclasses based on their primary sequence into 13 paralog groups within each individual cluster. The mixed lineage leukemia (*MLL*) gene encodes a protein responsible for maintenance of HOX gene expression, and translocations of the *MLL* gene are present at a high frequency in acute leukemia (73).

Deregulation of HoxA9 is associated with leukemia of both the myeloid and lymphoid lineages (74,149,150). Gene expression signatures of *MLL*-dependent T and B cell ALL show upregulated expression of HoxA9 (88). In normal hematopoiesis, HoxA9 is strongly expressed in the $CD34^{+}$ population of hematopoietic precursors and subsequently downregulated as cells differentiate into the various sub-populations of blood cell types (73). Overexpression of HoxA9 has been shown to perturb normal hematopoiesis in mice resulting in the development of leukemia (87).

Pim-1 (90,91), gp91Phox (93) and members of the Wnt gene family (94) have been reported to be targets of HoxA9 activity. However, a connection between HoxA9 and growth receptor signaling has not been previously described. In the present study, we have made use of a novel system to evaluate whether HoxA9 expression alters stromal cell dependent growth. The pre-B cell line, BLIN-2, requires direct contact with viable bone marrow stromal cells for optimal survival and proliferation (142) and lacks expression of endogenous HoxA9. Parental BLIN-2 cells were stably transduced with an inducible HoxA9 retrovirus. Induction of HoxA9 increased the survival and proliferative capacity of BLIN-2 cells in the absence of stromal cell support, and resulted in surface expression of IGF-1R. The HoxA9-mediated proliferative effects in BLIN-2 were abrogated with specific inhibitors of IGF-1R. Furthermore, siRNA "knock-down" of endogenous HoxA9 expression in cells bearing the MLL/AF4 chromosomal translocation resulted in loss of IGF-1R expression. These results are the first to functionally demonstrate a link between HoxA9 overexpression, induction of IGF-1R expression and proliferation of leukemic cells.

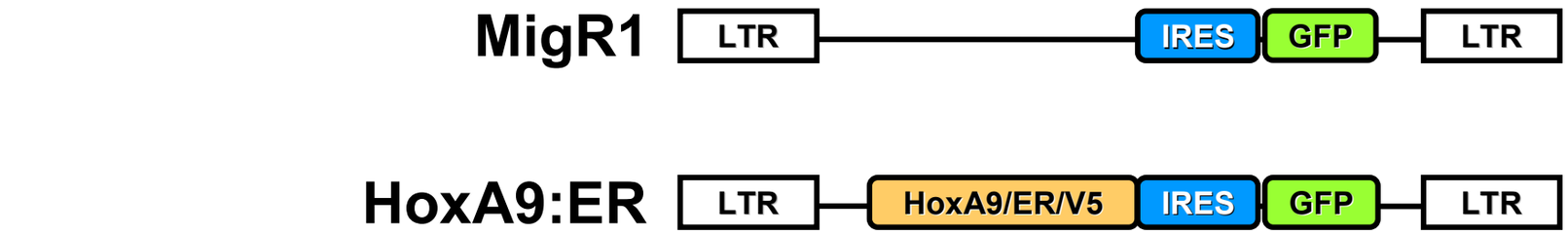
3.2 Results

3.2.1 Expression of the HoxA9:ER fusion protein

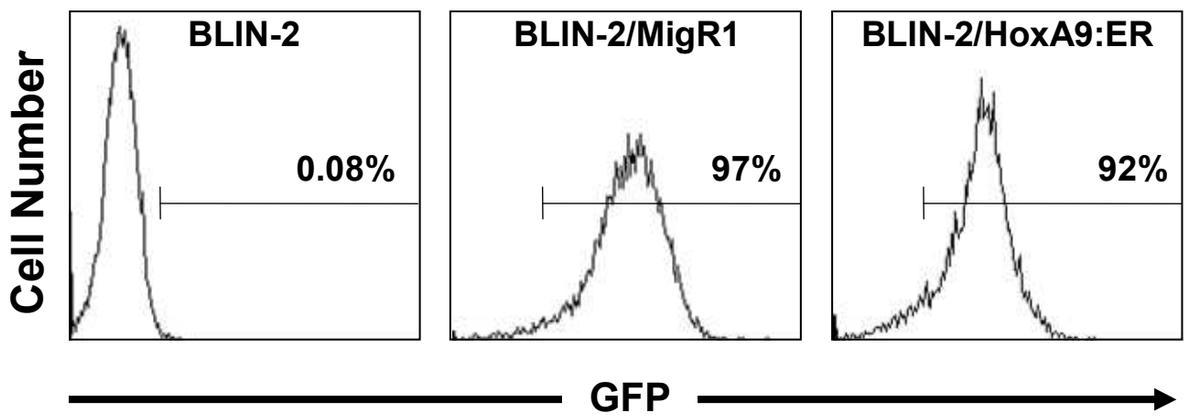
To determine how HoxA9 expression may contribute to the growth and survival of B-lineage acute leukemia, the stromal cell dependent pre-B ALL cell line, BLIN-2, was stably transduced with a 4-hydroxytamoxifen (4HT)-inducible HoxA9 retrovirus. This construct fuses the murine HoxA9 in frame with the hormone binding region of the human estrogen receptor and a 14 amino acid encoding V5 epitope tag (HoxA9:ER). GFP is expressed as a separately translated protein, under the control of an internal ribosomal

Figure 5: Retroviral transduction of BLIN-2 cells to generate stably transfected inducible BLIN-2/HoxA9:ER cells. A) Retroviral vector constructs. B) Post-sort FACS analysis of BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells**.**

A


MigR1
LTR
IRES
GFP
LTR
HoxA9:ER
LTR
HoxA9/ER/V5
IRES
GFP
LTR

B


BLIN-2
BLIN-2/MigR1
BLIN-2/HoxA9:ER
Cell Number
0.08%
97%
92%
GFP

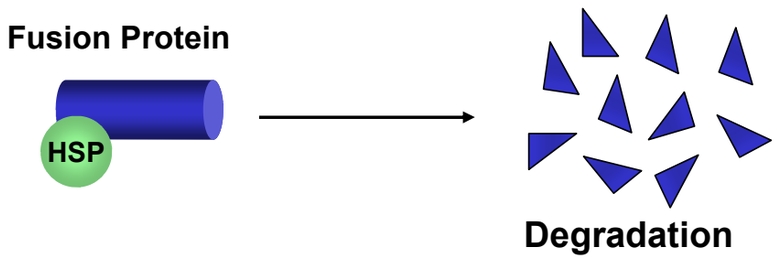
entry site (IRES), in order to facilitate the identification of transduced cells (Fig. 5A). Cells were also transduced with the retroviral vector lacking the HoxA9 fusion gene (MigR1). Following retroviral transduction, BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells were FACS purified to 97% and 92%, respectively (Fig. 5B).

The HoxA9:ER fusion gene encodes a chimeric hormone-inducible protein, that is regulated by addition of the estrogen analog 4HT to the growth media (Fig. 6). In the absence of the hormone inducer, the fusion protein is largely retained in the cytoplasm and is rapidly targeted for degradation via the proteasome pathway (151). Similar inducible systems have been successfully used by others to study the activity of transcription factors and signaling molecules (152,153). The HoxA9:ER fusion protein is stabilized in the presence of 4HT, which permits HoxA9 to localize in the nucleus. To test regulation of HoxA9:ER activity in BLIN-2 cells, BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells were grown in the presence or absence of 4HT and analyzed for HoxA9:ER protein expression in nuclear and cytoplasmic protein fractions. Little HoxA9:ER was observed in BLIN-2/HoxA9:ER cells cultured in the absence of 4HT. However, when cultured in the presence of 4HT, HoxA9:ER was readily detected in the nuclear protein fraction (Fig. 7). Pax5, a B-cell specific transcription factor, was included as a control for a nuclear specific protein (154). Actin was found almost entirely in the cytoplasm (155). This experiment confirms the stabilization and nuclear localization of the HoxA9:ER fusion protein in BLIN-2/HoxA9:ER cells treated with the estrogen analog 4HT.

Figure 6: Model for induction of the HoxA9:ER fusion protein. In the absence of estrogen, or the estrogen analog 4HT, the HoxA9:ER fusion protein is bound up by heat shock proteins (HSP) in the cytoplasm and rapidly targeted for proteolytic degradation. However, upon the addition of 4HT, the hormone analog out competes HSP for the binding domain of the ER because of a higher affinity for the binding site. This results in stabilization of the fusion protein which can subsequently translocates into the nucleus to activate the transcription of target genes.

HoxA9:ER “On/Off Switch”

No 4HT - HoxA9 “Off”



+ 4HT - HoxA9 “On”

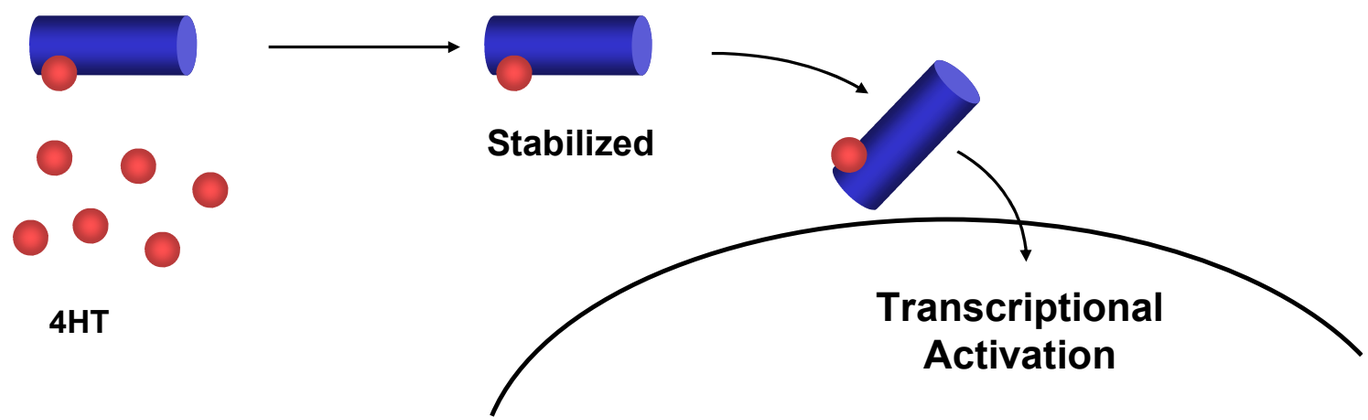
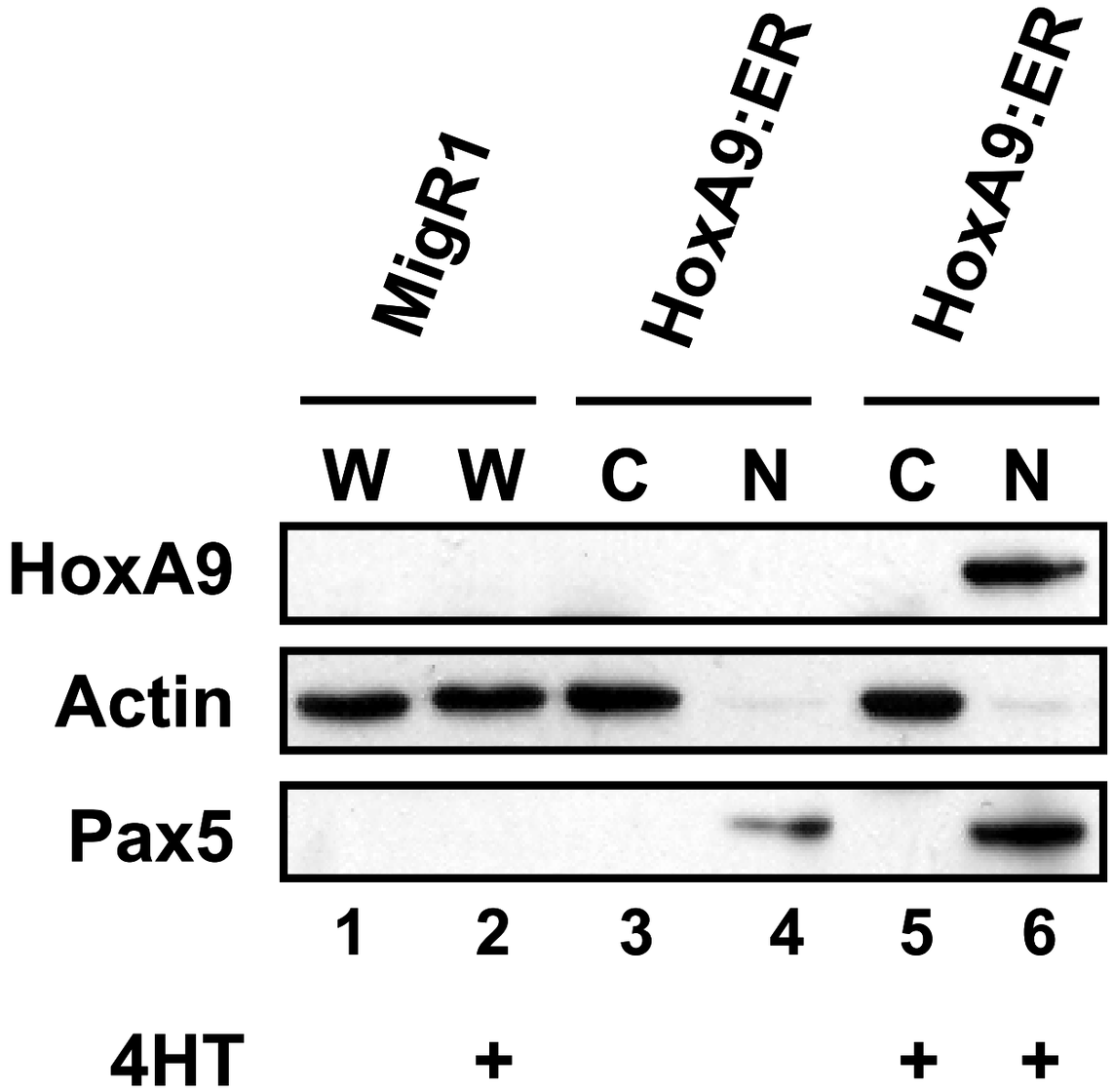


Figure 7: 4HT-mediated induction of HoxA9 in stably transduced BLIN-2/HoxA9:ER cells. Whole cell lysate (W) was prepared from BLIN-2/MigR1 cells (lanes 1 and 2), and cytoplasmic (C) and nuclear (N) proteins were prepared from BLIN-2/HoxA9:ER cells (lanes 3 – 6). 20 μg of protein per lane was separated on 8% SDS-PAGE gels followed by transfer to nitrocellulose membranes. Membranes were probed with the indicated antibodies. Cells were cultured in the presence or absence of 1μM 4HT for 24 hr.


MigR1
HoxA9:ER
HoxA9:ER
W W C N C N
HoxA9
Actin
Pax5
1 2 3 4 5 6
4HT + + +

3.2.2 HoxA9:ER binds to a HoxA9 DNA consensus binding sequence

EMSA analysis was performed to verify that HoxA9:ER was capable of binding to the HoxA9 DNA-binding consensus sequence. Protein lysates from BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells were incubated with a digoxegenin labeled probe (ctgcgATGATTTACGACcgc) and resolved through a non-denaturing polyacrylamide gel (Fig. 8). A shifted complex was observed when BLIN-2/HoxA9:ER lysate was incubated with the labeled probe (lane 6). This was competed away with excess unlabeled probe (lane 7). Pre-incubation of the lysate with anti-HoxA9 antibody resulted in a reduction in the intensity of the shifted band (lane 8), indicating that HoxA9 is part of this complex. No mobility shift was detected in lanes 2 – 5 using lysate from BLIN-2/MigR1, which lacks expression of endogenous HoxA9. These data indicate that the HoxA9:ER fusion protein is capable of binding the HoxA9 DNA binding sequence.

3.2.3 BLIN-2/HoxA9:ER cells exhibit increased proliferation in the absence of stromal cell support

To examine the effects of HoxA9:ER on proliferation and survival, BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells were cultured in the absence of stromal cell support with or without 1 μM 4HT for 5 days. Proliferation was determined by the MTT-assay, which measures mitochondrial respiration as a surrogate for cell number (Fig. 9). BLIN-2/MigR1 cells showed little change in proliferation over the course of the experiment in the presence or absence of 4HT. However, BLIN-2/HoxA9:ER cells, both in the presence and absence of 4HT, showed increased proliferation up to day three. After day three, only BLIN-2/HoxA9:ER cells cultured with 4HT continued to proliferate. Proliferation of BLIN-2/HoxA9:ER cells in the absence of 4HT may be due to leaky acti-

Figure 8: HoxA9:ER binds a consensus HoxA9 DNA binding sequence in BLIN-2/HoxA9:ER cells. EMSA was used to assess DNA binding of HoxA9:ER. 10 µg of total protein from BLIN-2/MigR1 or BLIN-2/HoxA9:ER cells was incubated with 4 ng of a labeled consensus binding sequence for HoxA9 (lanes 2 - 9) in the presence or absence of 250X excess unlabeled probe (Competitor) (lanes 3, 5, 7, and 9) and l µg of anti-HoxA9 antibody (lanes 4, 5, 8, and 9). The top arrow marks the migration of the HoxA9:ER/DNA complex (lane 6).

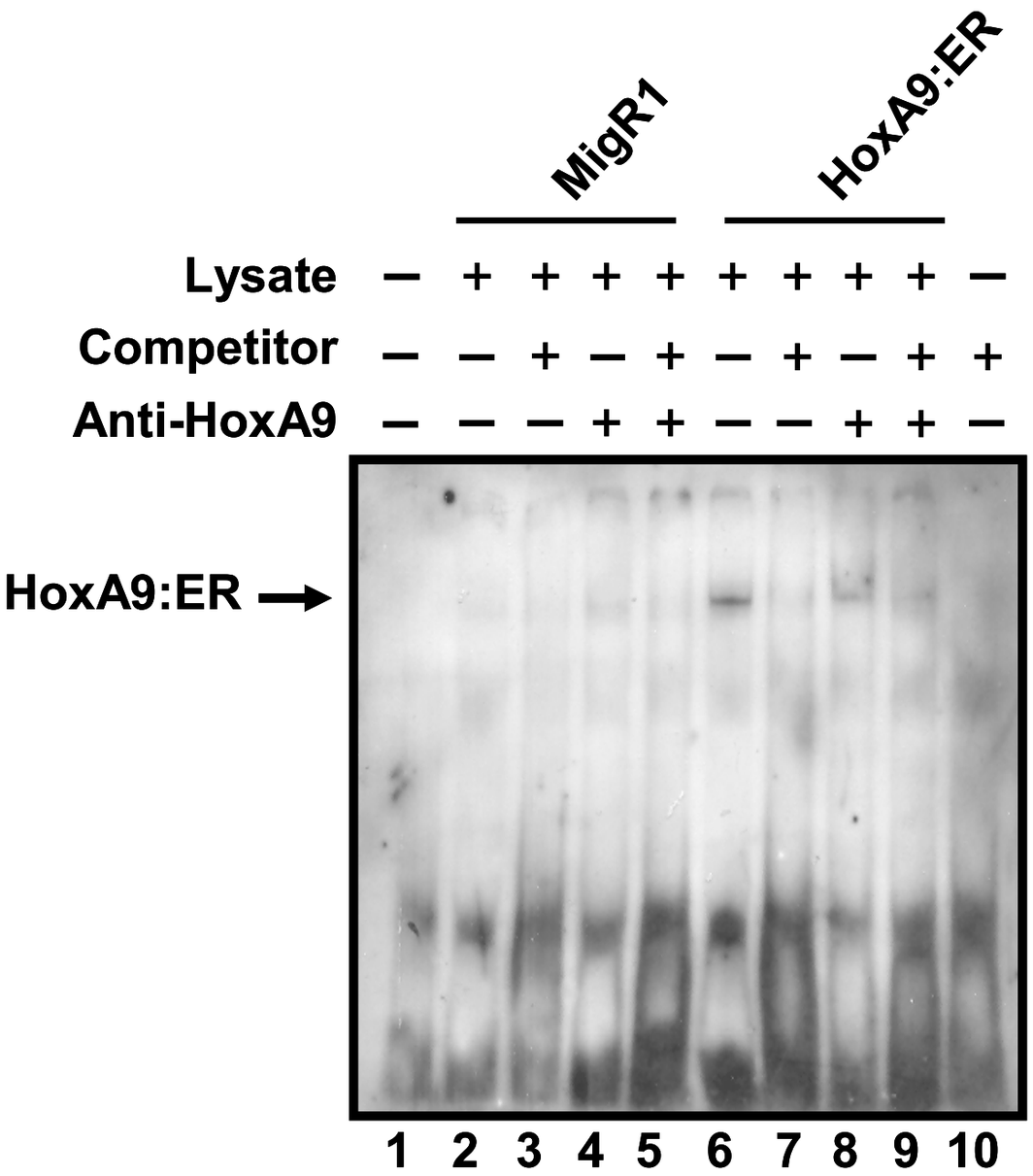
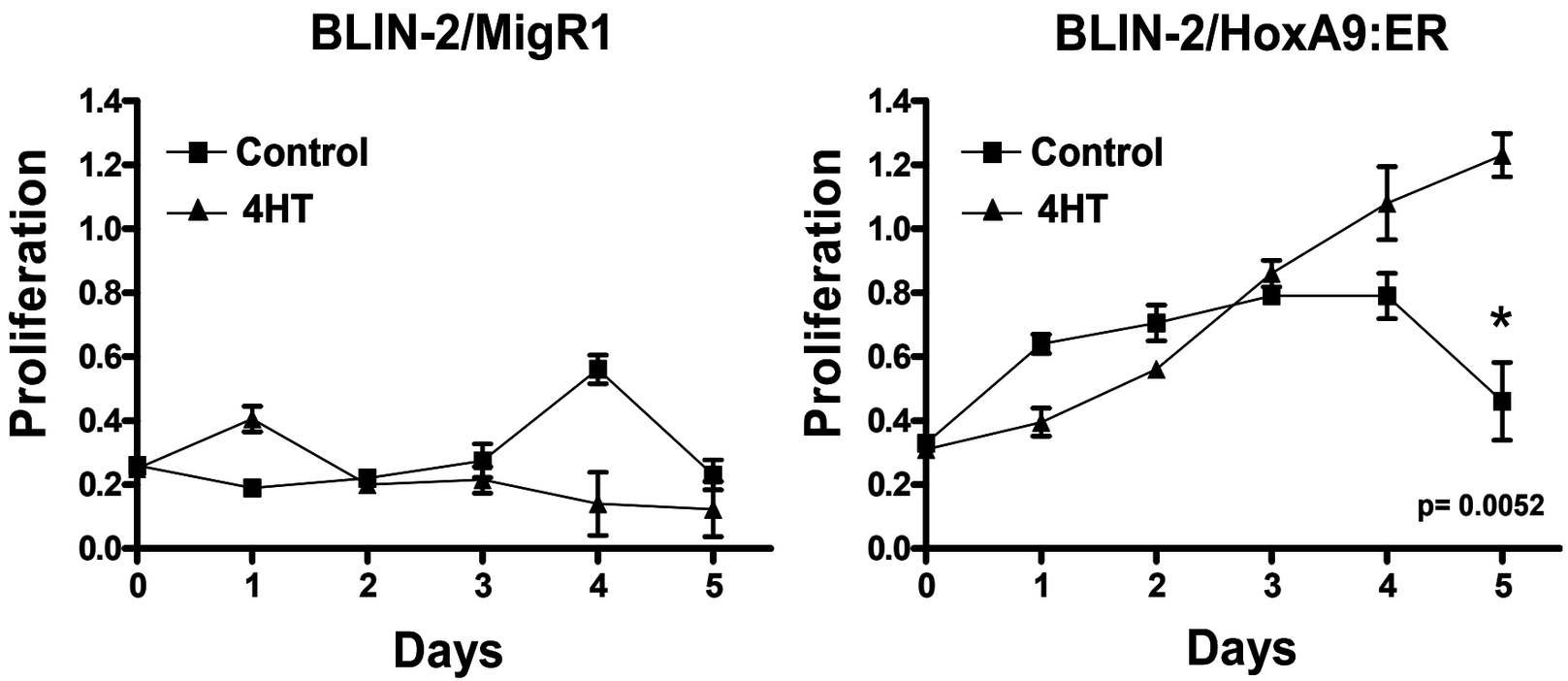

MigR1
HoxA9:ER
Lysate – + + + + + + + + –
Competitor – – + – + – + – + +
Anti-HoxA9 – – – + + – – + + –
HoxA9:ER
1 2 3 4 5 6 7 8 9 10

Figure 9: HoxA9 activity promotes BLIN-2/HoxA9:ER proliferation in the absence of stromal cells. Proliferation of BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells was evaluated by a quantitative colorimetric assay used to detect cell survival and proliferation, the MTT assay. Cells were cultured for five days in media alone (squares) or media containing 1μM 4HT (triangles). At the indicated time points cells were assayed for relative proliferation (expressed as absorbance at 492nm). Results are compiled from five independent experiments.


BLIN-2/MigR1
Proliferation
Control
4HT
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
BLIN-2/HoxA9:ER
Proliferation
Control
4HT
*
p= 0.0052
Days

vity of the HoxA9:ER fusion protein; however, 4HT is required for maintained proliferation. Thus, HoxA9:ER activity can promote stromal cell-independent proliferation of B-ALL cells.

3.2.4 HoxA9 activity alters protein levels of c-Myb

c-Myb has been reported to be a potential target of HoxA9 in leukemias expressing the MLL/ENL oncoprotein (92). c-Myb protein expression was evaluated in whole cell protein lysates prepared from parental BLIN-2 and BLIN-2/HoxA9:ER cells cultured with 4HT. c-Myb expression was increased in BLIN-2/HoxA9:ER cells as compared with parental BLIN-2 (Fig. 10A). To further verify increased c-Myb protein expression upon induction of HoxA9 activity, cytoplasmic and nuclear protein fractions were prepared from parental and BLIN-2/HoxA9:ER cells stimulated with 4HT and analyzed for c-Myb expression (Fig. 10B). c-Myb nuclear protein expression was increased in 4HT treated BLIN-2/HoxA9:ER cells compared with parental BLIN-2 cells, indicating that HoxA9 activity resulted in increased c-Myb protein levels.

3.2.5 HoxA9 activation induces surface expression of IGF-1R

As c-Myb has been reported to regulate IGF-1R expression (156-158), BLIN-2/HoxA9:ER cells were examined by RT-PCR for expression of IGF-1R. IGF-1R expression was detected in BLIN-2/HoxA9:ER cells treated with 4HT. No detectable IGF-1R expression was detected in untreated BLIN-2/HoxA9:ER cells or BLIN-2/MigR1 empty vector control cells (Fig. 11A). The RS4;11 cell line bears the MLL/AF4 translocation and has been reported to overexpress endogenous HoxA9 (159). IGF-1R gene expression was also observed in these cells.

Figure 10: c-Myb is increased in the presence of HoxA9 activity. A) Total cellular protein was prepared from parental BLIN-2 and BLIN-2/HoxA9:ER cells grown in the presence 1 μM 4HT for 24 hr. 20 μg of total cellular protein was resolved on 8% SDS-PAGE gels and transferred to PVDF membranes. Membranes were sequentially probed with antibodies against HoxA9, c-Myb and β-tubulin. B) Cytoplasmic (C) and nuclear (N) proteins were prepared from BLIN-2 and BLIN-2/HoxA9:ER cells cultured with 1 μM 4HT, followed by western blot analysis. Actin is found predominantly in the cytoplasm. Pax5 and p70 are nuclear proteins.

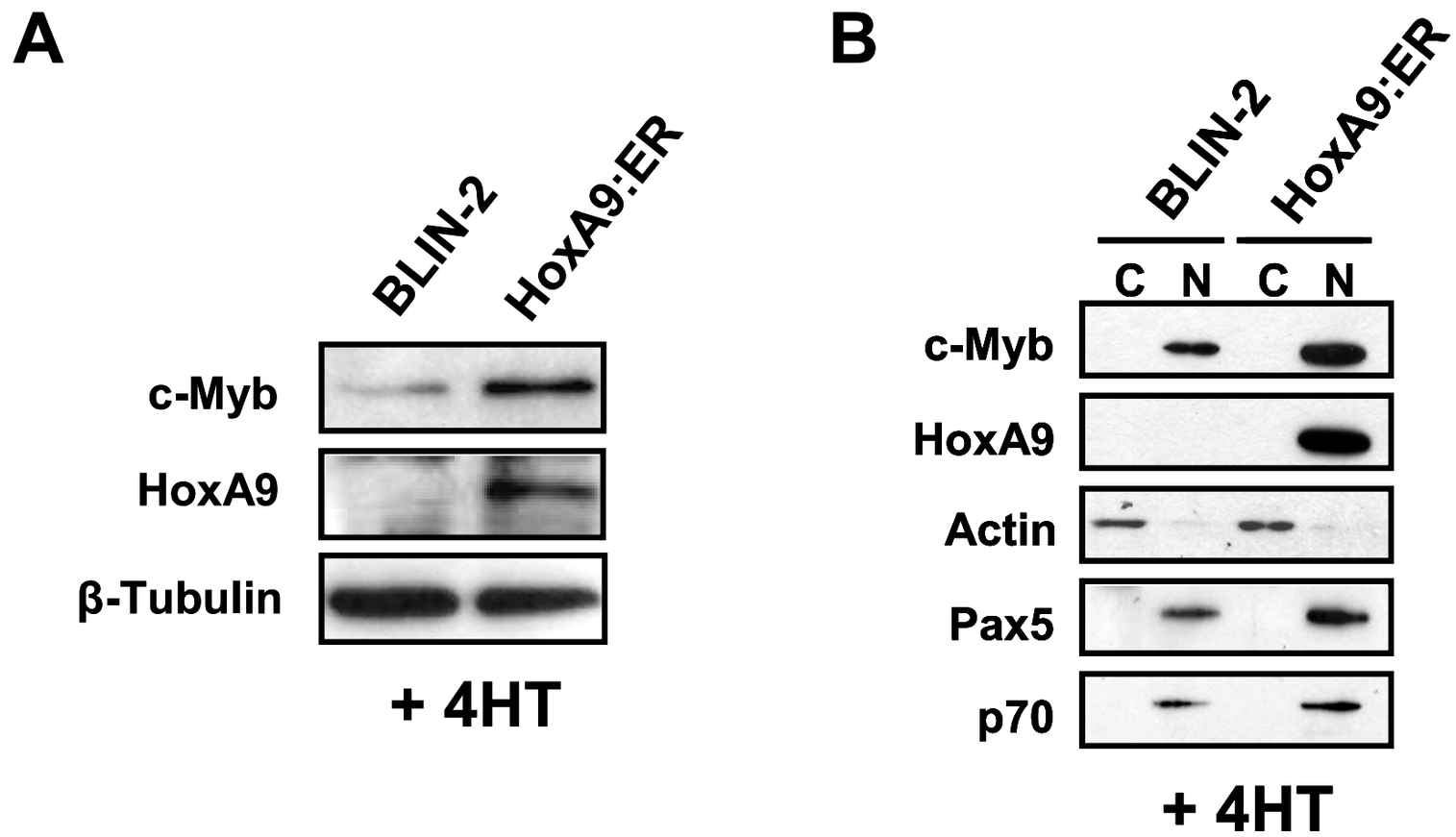
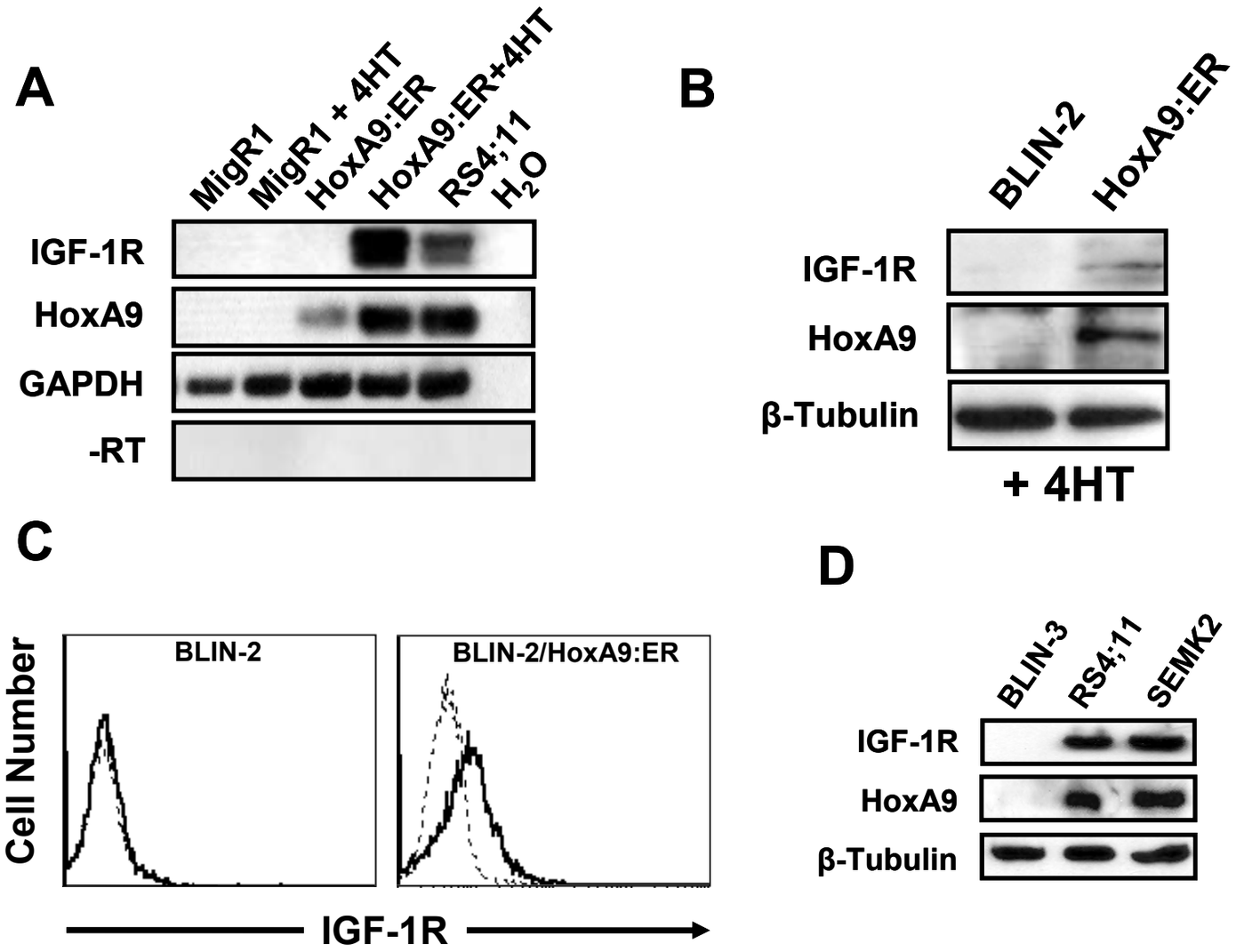

A
BLIN-2
HoxA9:ER
c-Myb
HoxA9
β-Tubulin
+ 4HT
B
BLIN-2
HoxA9:ER
C N C N
c-Myb
HoxA9
Actin
Pax5
p70
+ 4HT

Figure 11: HoxA9 induces expression of IGF-1R A) RT-PCR amplification of IGF-1R in BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells cultured in the presence of 1 μM 4HT. RS4;11 is a t(4;11) cell line that expresses endogenous HoxA9. -RT = no reverse transcriptase. H2O = no cDNA template. B) Western analysis of parental BLIN-2 and BLIN-2/HoxA9:ER cells cultured with 1 μM 4HT for 24 hr. C) FACS analysis of BLIN-2 and BLIN-2/HoxA9:ER cells cultured with 1 μM 4HT for 24hr. BLIN-2/HoxA9:ER cells exhibited low, but detectable levels of IGF-1R. Surface IGF-1R expression was not observed in parental BLIN-2 cells. Dotted lines are isotype matched negative controls; solid lines are anti-IGF-1R stained cells. D) IGF-1R expression in RS4;11 and SEMK2. Whole cell lysates from BLIN-3, RS4;11 and SEMK2 were analyzed for IGF-1R protein expression via western blotting. IGF-1R expression was only observed in the RS4;11 and SEMK2 cells lines known to express HoxA9. BLIN-3, that does not express HoxA9, did not express IGF-1R.


A
MigR1
MigR1 + 4HT
HoxA9:ER
HoxA9:ER+4HT
RS4;11
H2O
IGF-1R
HoxA9
GAPDH
-RT
B
BLIN-2
HoxA9:ER
IGF-1R
HoxA9
β-Tubulin
+ 4HT
C
Cell Number
BLIN-2
BLIN-2/HoxA9:ER
IGF-1R
D
BLIN-3
RS4;11
SEMK2
IGF-1R
HoxA9
β-Tubulin

IGF-1R protein expression was detected by western blot analysis in 4HT treated BLIN-2/HoxA9:ER cells, while no IGF-1R protein was detected in parental control cells (Fig. 11B). Surface expression of IGF-1R was next examined by flow cytometry (Fig. 11C). IGF-1R surface expression was detected on BLIN-2/HoxA9:ER cells but not on the parental BLIN-2 cells.

Detection of IGF-1R expression in RS4;11 cells raised the possibility that IGF-1R expression may correlate with HoxA9 expression in cells bearing the MLL/AF4 translocation. To test this hypothesis, three MLL/AF4 cell lines were examined by western analysis for IGF-1R expression. The SEMK2 and BLIN-3 cell lines both express the MLL/AF4 fusion protein. However, BLIN-3 cells lack expression of endogenous HoxA9 (143,144), have retained an absolute requirement for growth factor/stromal cell contact for optimal growth and proliferation, and are IL-7 dependent (143). In contrast, RS4;11 and SEMK2 cells do not require stromal cell contact or exogenous IL-7 for survival and proliferation. IGF-1R protein expression was observed in the growth factor-independent cell lines SEMK2 and RS4;11 (Fig. 11D). Interestingly, IGF-1R expression was not observed in the stromal cell dependent cell line, BLIN-3. Collectively, these results suggest that HoxA9 induces expression of IGF-1R, which in turn, promotes growth factor/stromal cell independent growth in leukemic cells.

3.2.6 Inhibition of IGF-1R blocks proliferation of BLIN-2/HoxA9:ER

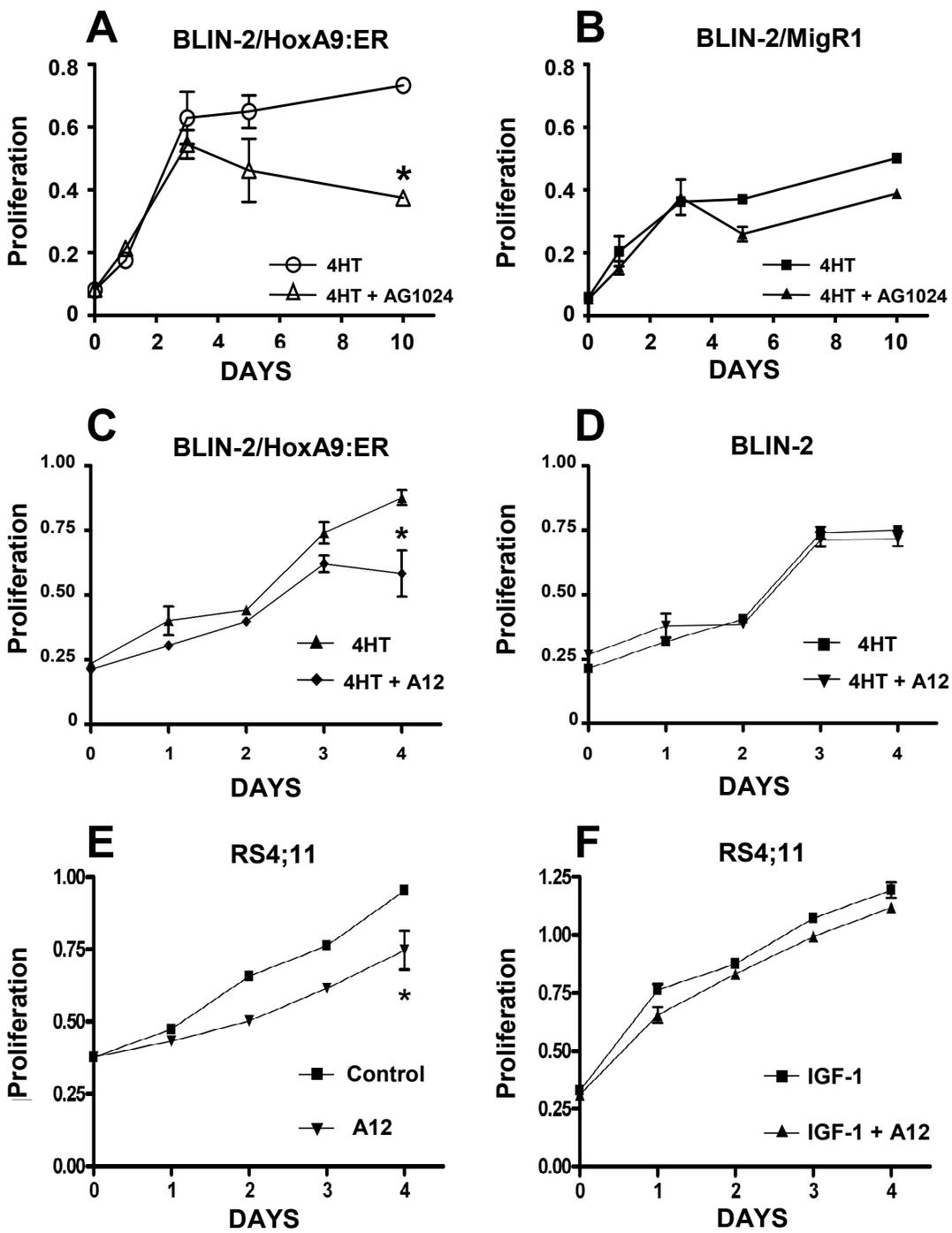
IGF-1R has been reported to relieve leukemic cells of growth factor dependency (127,128). To determine whether IGF-1R signaling promotes enhanced proliferation of pre-B cell ALL, BLIN-2/HoxA9:ER and BLIN-2/MigR1 cells were cultured on stromal cells with 4HT in the presence or absence of the IGF-1R tyrosine kinase inhibitor

AG1024 (1 μg/ml) (Fig. 12A, and B). BLIN-2/HoxA9:ER cells cultured in the presence of 4HT showed about an 8-fold increase in proliferation after 10 days of culture (Fig. 12A). BLIN-2/HoxA9:ER proliferation was decreased in the presence of AG1024. At day 5 there was nearly a 1.5-fold decrease in the proliferation of BLIN-2/HoxA9:ER cells treated with AG1024, and by day 10 these cells showed a 2-fold reduction in proliferation, as compared to cells treated with 4HT alone. BLIN-2/MigR1 cells cultured with 4HT exhibited nearly 2-fold less proliferation than the BLIN-2/HoxA:ER cells (Fig. 12B). These cells exhibited little decreased proliferation when treated with AG1024.

3.2.7 Monoclonal antibody inhibition of the IGF-1R receptor

In a series of separate and independent experiments, BLIN-2/HoxA9:ER cells were treated with the anti-IGF-1R monoclonal antibody, A12. A12 is an IGF-1R specific monoclonal antibody that possesses high affinity for the IGF-1R receptor and blocks binding of IGF-1 (136). Binding of A12 to the IGF-1R receptor results in the rapid internalization and degradation of the receptor, thus inhibiting IGF-1R signaling and reducing cell surface receptor levels (136). A12 has been reported to inhibit growth of various cancer cell lines, including multiple myelomas, and has shown strong antitumor activity in nude mouse models (136,160). 4HT-treated BLIN-2 and BLIN-2/HoxA9:ER cells were cultured in the presence of 15 μg of A12 mAb (Fig. 12C, and D). A12 significantly inhibited proliferation of BLIN-2/HoxA9:ER cells cultured with 4HT (Fig. 12C). A12 treatment had no effect on proliferation of parental BLIN-2 cells (Fig. 12D). These results suggest that HoxA9-mediated expression of IGF-1R is responsible for the increased proliferative capacity of BLIN-2/HoxA9:ER cells.

Figure 12: Inhibition of IGF-1R reduces proliferation. A–B) Inhibition of IGF-1R signaling reduces BLIN-2/HoxA9:ER proliferation in the presence of stromal cell support. BLIN-2/HoxA9:ER cells (A) cultured with stromal cells for 10 days in the presence of 1 μM 4HT (○) or in the presence of 1 μM 4HT plus 1 μg/ml of the IGF-1R inhibitor, AG1024 (△). BLIN-2/HoxA9:ER cells (B) were cultured as described, in the presence of 1 μM 4HT alone (■) or with 1 μM 4HT plus 1 μg/ml of AG1024 (▲). Proliferation was determined by colorimetric assay and is presented as absorbance at 492 nm. Data are from 5 independent experiments. * denotes statistical significance ($p \leq 0.0001$). C–D) Treatment with anti IGF-1R monoclonal antibody inhibits proliferation of BLIN-2/HoxA9:ER cells. BLIN-2/HoxA9:ER cells (C) were cultured on stromal cells with 1 μM 4HT to induce activity of HoxA9:ER, in the presence (♦) or absence (▲) of the anti-IGF-1R antibody, A12 (15 μg). Parental BLIN-2 cells (D) cultured with 1 μM 4HT with (▼) or without A12 mAb (■). Proliferation was determined via the MTT assay. * denotes statistical significance ($p < 0.02$). Data are from 5 independent experiments. E–F) Inhibition of IGF-1R signaling reduces proliferation of RS4;11 cells. RS4;11 cells were cultured in the presence or absence of 15 μg A12 monoclonal antibody (E: untreated, ■; A12 only, ▼) and 50 ng of IGF-1 (F: IGF-1 only, ■; IGF-1 and A12, ▲). Proliferation was determined via the MTT assay. Data are from 3 independent experiments. * denotes statistical significance ($p < 0.05$).


A
BLIN-2/HoxA9:ER
Proliferation
DAYS
4HT
4HT + AG1024
B
BLIN-2/MigR1
Proliferation
DAYS
4HT
4HT + AG1024
C
BLIN-2/HoxA9:ER
Proliferation
DAYS
4HT
4HT + A12
D
BLIN-2
Proliferation
DAYS
4HT
4HT + A12
E
RS4;11
Proliferation
DAYS
Control
A12
F
RS4;11
Proliferation
DAYS
IGF-1
IGF-1 + A12

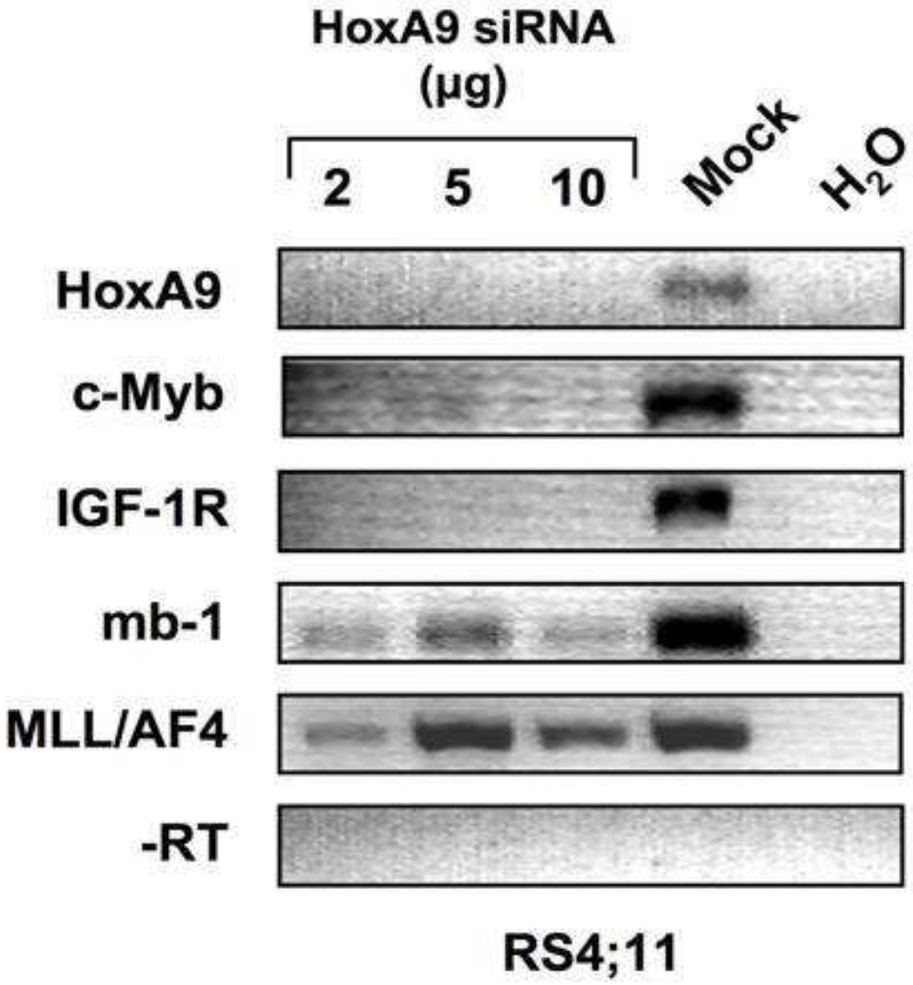
3.2.8 Blocking IGF-1R signaling in RS4;11 cells inhibits proliferation

To test whether blocking IGF-1R signaling would be effective at inhibiting the proliferation of an *MLL*-positive leukemia expressing endogenous HoxA9, RS4;11 cells were cultured in the presence or absence of A12 mAb. A12 treatment resulted in significant growth inhibition of RS4;11 cells (Fig. 12E). A12 growth inhibitory effects were observed as early as day 2. A12 treatment was not sufficient to completely inhibit the proliferation of RS4;11 cells thus there are likely other factors involved that contribute to the proliferation of these cells. To determine whether the observed RS4;11 growth effects were specific for IGF-1R inhibition, RS4;11 cells were cultured in the presence of IGF-1 or IGF-1 plus A12 (Fig. 12F). Treatment with IGF-1 abrogated the effects of the A12 antibody resulting in increased proliferation of the A12 treated cells. These results indicate that inhibition of IGF-1R has an anti-proliferative effect on cells overexpressing HoxA9.

3.2.9 Loss of endogenous HoxA9 expression reduces IGF-1R expression in MLL/AF4 positive leukemia

To test whether loss of endogenous HoxA9 affects IGF-1R expression in pre-B cell ALL, RS4;11 cells were transfected with increasing concentrations of HoxA9 siRNA and RT-PCR was used to evaluate HoxA9 mRNA levels 24 hr post-transfection (Fig. 13). Abrogation of HoxA9 gene expression with siRNA resulted in a loss of IGF-1R and c-Myb expression. Expression of the B-lineage specific gene mb-1 (Igα) and the MLL/AF4 fusion gene was not affected by the siRNA. This result provides strong evidence that endogenous HoxA9 is required for maintenance or induction of IGF-1R expression.

Figure 13: Loss of endogenous HoxA9 expression abrogates IGF-1R expression. RS4;11 cells were mock transfected or transfected with the indicated amounts of HoxA9 siRNA (μg), and RT-PCR was used to analyze mRNA levels from cells after 24 hr culture. Shown is the negative image of an ethidium bromide gel. –RT = no reverse transcriptase. Mock = mock transfected. H_2O = no cDNA template.


HoxA9 siRNA
(µg)
2
5
10
Mock
H_2O
HoxA9
c-Myb
IGF-1R
mb-1
MLL/AF4
-RT
RS4;11

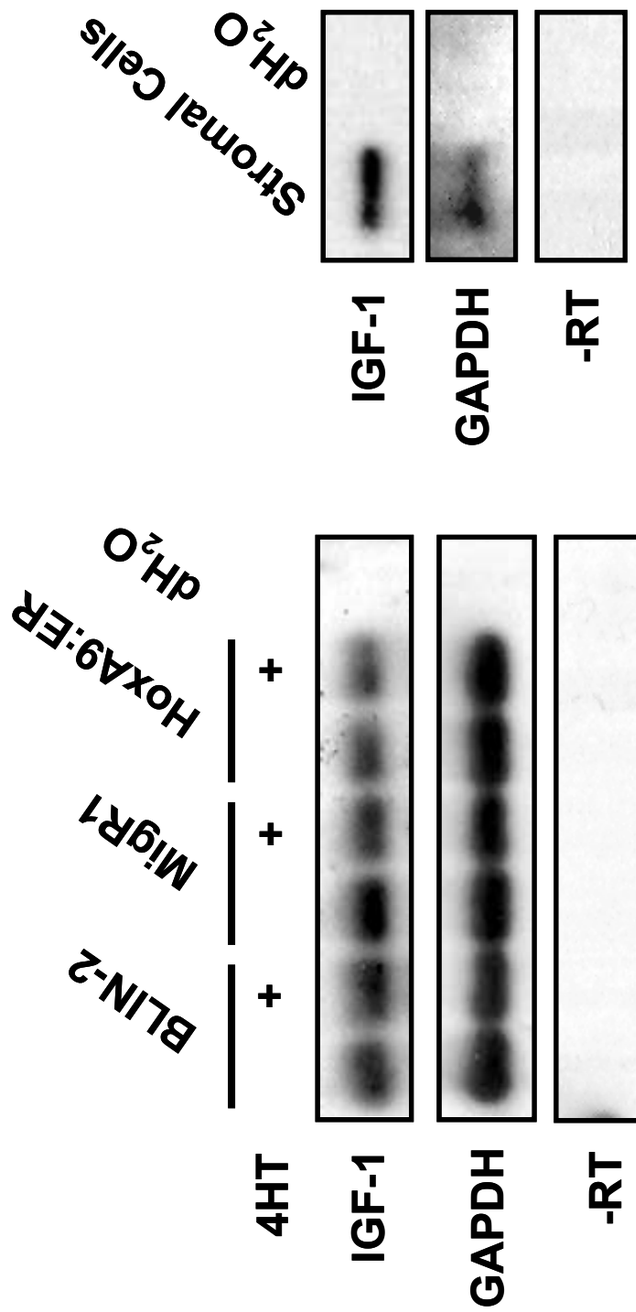
3.3.1 Expression of IGF-1.

RT-PCR was used to determine whether the transduced BLIN-2 cell lines, the parental cell line and the stromal cell monolayers express IGF-1. As shown in Fig. 14, the stromal cells express the IGF-1 gene. In addition, IGF-1 expression was observed in BLIN-2, BLIN-2/MigR1 and BLIN-2/HoxA9:ER. No alteration in IGF-1 expression was observed upon induction of HoxA9 activity with the addition of 4HT (lanes 5, and 6). IGF-1 expression was also observed in RS4;11 cells (data not shown). These results suggest that HoxA9 does not regulate the expression of IGF-1, although this growth factor is expressed by both the leukemic cells and the supporting stromal cells.

3.3.2 Phosphorylation of IGF-1R.

To determine if IGF-1R is phosphorylated in BLIN-2/HoxA9:ER cells, we performed immunoprecipitation of IGF-1R from cells subjected to the treatments shown if Figure 15. IGF-1Rβ was co-immunoprecipitated using an antibody against the IGF-1Rα chain. Phosphorylated IGF-1Rβ was detected with an anti-phosphotyrosine antibody. Western blot analysis for phosphotyrosine residues revealed phosphorylated IGF-1R was only detected in BLIN-2/HoxA9:ER cells in the presence of both 4HT and IGF-1 (Fig. 15A). Additional, treatment with the A12 mAb inhibited phosphorylation of the receptor in these cells. To verify these results, we performed an ELISA assay to detect IGF-1Rβ phosphorylation (Fig. 15B). There was a 4.5-fold increase in IGF-1R phosphorylation in BLIN-2/HoxA9:ER in the presence of both 4HT and IGF-1 as compared to parental controls. IGF-1R phosphorylation in BLIN-2/HoxA9:ER cells was inhibited by treatment with the A12 mAb. IGF-1R phosphorylation in BLIN-2/HoxA9:E-

Figure 14: IGF-1 Gene Expression. RT-PCR was performed to access IGF-1 gene expression in the indicated cells. Where indicated, cells were treated for 24 hours with 4HT (+4HT) prior to analysis. Shown is a Southern blot of the PCR products separated on a 1.5% agarose gel. H_2O = no cDNA template.


Stromal Cells
dH2O
IGF-1
GAPDH
-RT
4HT
BLIN-2
MigR1
HoxA9:ER
dH2O
IGF-1
GAPDH
-RT
1 2 3 4 5 6 7

R cells was not detected in the absence of IGF-1 treatment and this is likely due to the sensitivity of detection for the assays used.

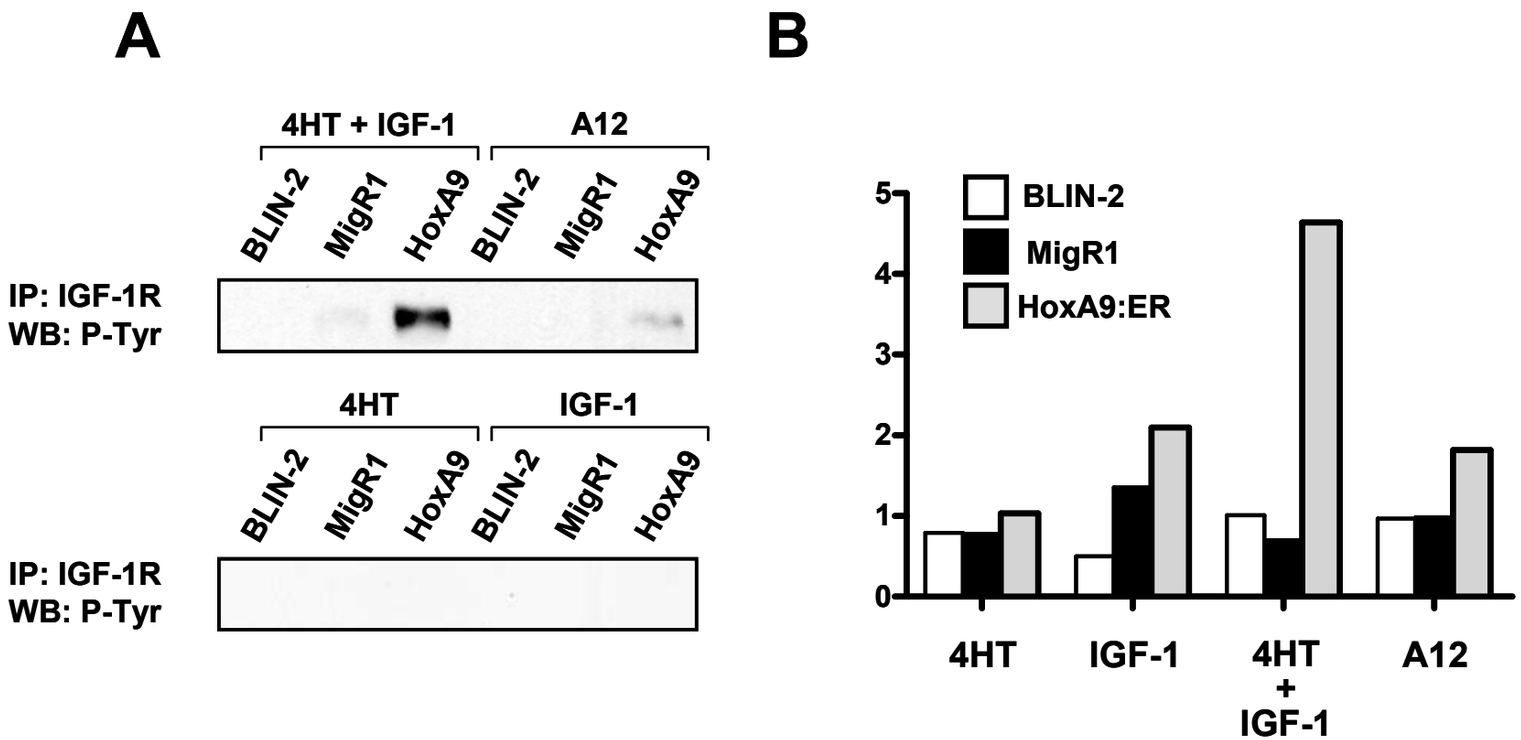
3.4 Conclusions

Overexpression of HOX genes has been widely associated with a variety of leukemias, including those bearing aberrations of the *MLL* gene (63). While several studies have indicated that enforced HOX gene expression in murine models promotes leukemogenesis, the specific HOX-regulated mechanism(s) and pathways that promulgate leukemic cell growth and survival are not known.

In this study, we have used a stromal cell dependent model of B-lineage ALL to determine how HoxA9 activity may promote stromal cell/growth factor independence. Using an inducible system, we found that HoxA9 activity resulted in prolonged proliferation in the absence of stromal cell support and that induction of HoxA9 activity resulted in IGF-1R expression. Inhibition of IGF-1R using either blocking antibodies (A12) or kinase inhibitors (AG1024) abrogated the proliferative effects in the presence of HoxA9 induction/activity. These findings were extended into cells that overexpress endogenous HoxA9 as a result of the MLL/AF4 oncoprotein. IGF-1R expression was found in SEMK2 and RS4;11 cells, both of which express MLL/AF4 and HoxA9. IGF-1R expression was not observed in BLIN-3 cells that express MLL/AF4, but lack endogenous HoxA gene expression (144). Proliferation of the factor independent cell line, RS4;11, was repressed upon inhibition of IGF-1R. siRNA “knock-down” of endogenous HoxA9 in RS4;11 cells resulted in loss of IGF-1R expression.

Several lines of evidence suggest a role for IGF-1 and IGF-1R in leukemia. High levels of serum IGF-1 correlate with childhood leukemia and high birth weight (124). Si-

Figure 15: A12 inhibits IGF-1R phosphorylation in BLIN-2/HoxA9:ER cells. A) BLIN-2, BLIN-2/MigR1, and BLIN-2/HoxA9:ER cells were cultured in the presence of 4HT (4HT), IGF-1 (IGF-1), 4HT and IGF-1 (4HT + IGF-1), or 4HT, IGF-1 and A12 (A12). Total protein was isolated and immunoprecipitation (IP) of the IGF-1Rα subunit of IGF-1R was performed followed by western blot analysis of phosphorylated tyrosine (P-Tyr) residues on the IGF-1Rβ chain. B) Phosphorylated IGF-1R was detected by ELISA. Cells were cultured as described above and ELISA was performed using 40 µg of total cellular protein. Plates were read at 450 nm and results are presented as fold increase in IGF-1R phosphorylation relative to untreated controls.


A
4HT + IGF-1
A12
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
IP: IGF-1R
WB: P-Tyr
4HT
IGF-1
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
IP: IGF-1R
WB: P-Tyr
B
BLIN-2
MigR1
HoxA9:ER
5
4
3
2
1
0
4HT
IGF-1
4HT + IGF-1
A12

gnaling via IGF-1/IGF-1R interactions have been shown to participate in the growth of survival of multiple myeloma cells (139,160). IGF-1R expression has been reported in AML blasts, and IGF-1 is capable of stimulating AML cell proliferation (126-128,161). Several studies have indicated that inhibition of IGF-1R signaling results in a reduction of cell proliferation and induction of apoptosis, particularly in AML (126-128).

Our data indicate that one mechanism of HOX-mediated cell proliferation is through expression and subsequent activation of the IGF-1R receptor. IGF-1R has been reported to relieve leukemic cells of cytokine dependency (127,128). IGF-1R activity is also implicated in promoting the growth of several solid tumors, such as breast, prostate and colon, in which aberrant HOX-gene expression has also been reported (114,115). IGF-1R has been reported to be regulated by the c-Myb transcription factor (156-158). In a recent report, expression of MLL/ENL resulted in increased c-Myb expression (92). In our study, c-Myb levels were increased upon induction of HoxA9 activity.

Our data suggest a model in which overexpression of HoxA9 results in increased expression of c-Myb and induction of IGF-1R expression. This effect would, in-turn, promote stromal cell/growth factor independent growth and survival, provided that IGF-1 was present. Our data indicate that IGF-1 is expressed by both the stromal cells and leukemic cells used in this system (Fig. 14), indicating autocrine and paracrine receptor activation.

Work from the laboratory of Martelli *et al.* (126) indicates that autocrine signaling from IGF-1 and IGF-1R promotes the growth of AML cells. These investigators reported that inhibition of IGF-1R in AML cells induced dephosphorylation of IGF-1R and apoptosis. In this study chemoresistance in AML cells correlated with IGF-1 secretion.

As HoxA9 is frequently overexpressed in AML (74), our results, documenting HoxA9 mediated induction of IGF-1R expression, is consistent with this study.

Therapeutics targeting IGF-1R have been successful in a variety of oncogenic settings (139,162). IGF-1R activation affects multiple cellular pathways leading to increased proliferation, loss of apoptotic sensitivity and anchorage-dependent growth (96). In addition, IGF-1R signaling is required for normal B-cell development, during the pro-B to pre-B cell transition (163,164). One study demonstrated that proliferation of the pre-B ALL cell line, REH, is inhibited in a dose-dependent manner by monoclonal antibodies against IGF-I and IGF-1R (129). Furthermore, treatment of IGF-1/insulin-dependent hematopoietic precursor cell lines with anti-IGF-1R monoclonal antibodies induced growth arrest (136,160). Our findings suggest that inhibition of IGF-1R may be an effective approach for treatment of leukemia or other cancers that exhibit elevated HOX-gene expression, either as a stand-alone therapy or in combination with other approaches. Eighty percent of infant ALL is associated with *MLL*-aberrations, for which overexpression of HoxA9 is a hallmark (149). In addition, high levels of serum IGF-1 and high birth weight have been reported to correlate with infant ALL, the so-called big baby hypothesis (124). Thus, one area in which inhibition of IGF-1R may be of benefit is as a potential therapeutic in *MLL* positive infant ALL.

In summary, our present data support a novel mechanistic role for HoxA9 activity in the promotion of leukemic cell growth; via induction of IGF-1R expression. This pathway will yield new potential targets for the treatment of leukemia and other cancers that involve HOX gene overexpression.

CHAPTER 4: HoxA9 Protects B-Lineage All Cells From Apoptosis Induced By Stromal Cell Withdrawal

4.1 Introduction

HOX genes, first described in *Drosophila melanogaster*, encode a family of transcription factors involved in cell fate determination during embryogenesis. These genes also play a critical role in hematopoietic stem cell differentiation and proliferation. However, the exact molecular pathways regulated by HOX gene expression in hematopoietic cell growth and differentiation have not been fully elucidated, but a variety of studies indicate that altered HOX expression can influence differentiation and growth factor responses (73). HoxA9 is the most highly expressed HOX gene in the hematopoietic stem cell compartment, and plays a fundamental role in hematopoietic stem cell self renewal (94). Several animal studies using knockout mice have demonstrated that aberrant HoxA9 expression can perturb normal hematopoiesis resulting in developmental defects in both myeloid and lymphoid lineages (84,85,165). Depending upon the availability of co-factors, such as Meis1, HoxA9 has been shown to be transforming in mice (87). Not surprisingly, the deregulation of HoxA9 gene expression, as the result of genetic mutation, is the factor most highly associated with human acute leukemia. Overexpression of HoxA9 is commonly found in a variety of acute leukemias and has been reported to result in proliferative expansion of hematopoietic stem/progenitor cells at the expense of mature compartments (86). HoxA9 may also act to promote tumor cell proliferation and survival in situations where other abnormalities are likely the initial transforming event, such as *MLL*-translocations (166-168).

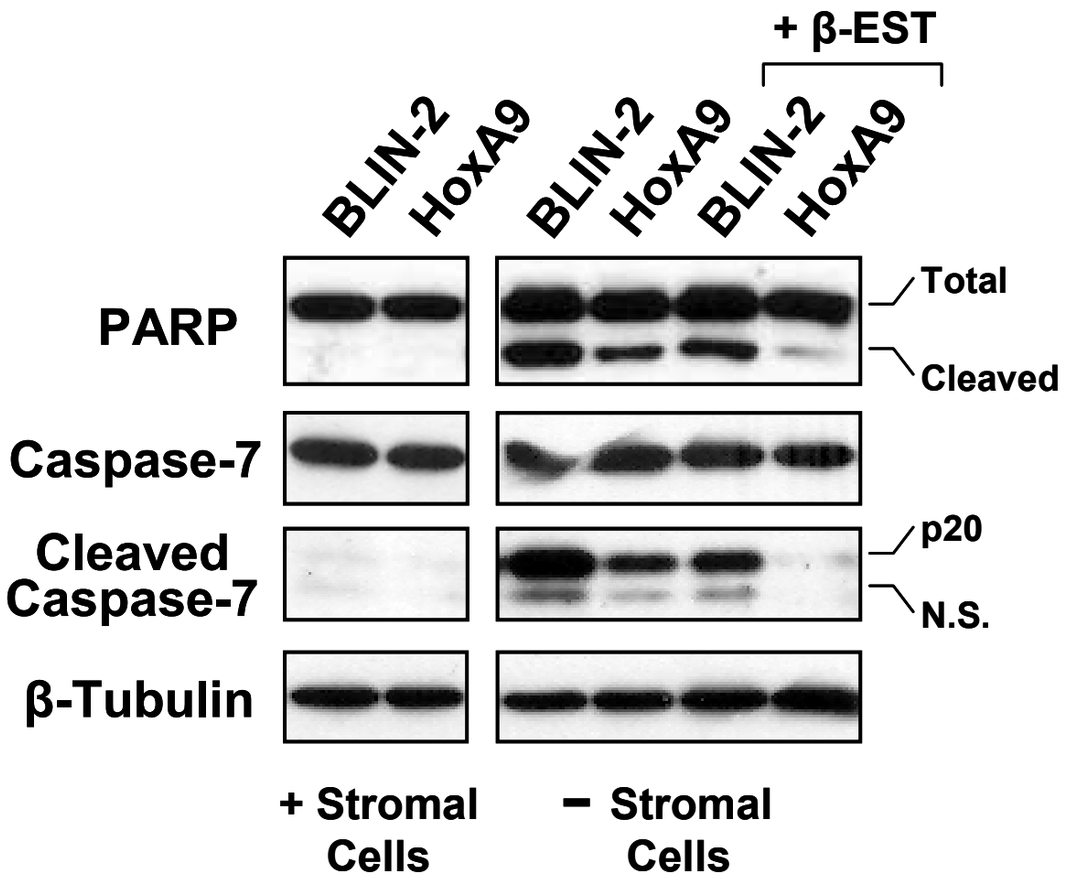
The data presented in Chapter 3 indicate that HoxA9 activity in BLIN-2 cells induces surface expression, and signaling through, the insulin-like growth factor-1 receptor (IGF-1R) resulting in increased cellular proliferation (169). Signaling through IGF-1R activates two major intracellular signaling pathways, the PI3K/Akt pathway and the MAPK/ERK kinase pathway (170). Signaling through the MAPK/ERK pathway is principally associated with regulation of cellular proliferation (95,171). For numerous cell types, signaling through the PI3K/Akt pathway is associated with survival and protection from apoptosis (95,172). Hence, we next sought to evaluate the apoptotic sensitivity of BLIN-2 cells in the context of HoxA9 activity and IGF-1R signaling. We report here that induction of HoxA9 activity in BLIN-2 cells results in protection from apoptosis induced by growth factor/stromal cell withdrawal. This effect was independent of IGF-1R signaling. Induction of HoxA9 activity increased expression of the Pim-1 proto-oncogene and increased BAD phosphorylation in the absence of growth factor/stromal cell support. Thus, providing an mechanism for apoptotic resistance independent of IGF-1R. Collectively, these data support a model in which HoxA9 promotes leukemic cell proliferation, through induction of IGF-1R expression and signaling, and resistance to apoptosis through a pathway independent of IGF-1R signaling.

4.2 Results

4.2.1 HoxA9 protects BLIN-2 cells for apoptosis

To determine whether BLIN-2 cells, stably transduced with an inducible HoxA9 retrovirus (BLIN-2/HoxA9:ER) (169), are protected from apoptosis induced by stromal cell/growth factor withdrawal, BLIN-2 and BLIN-2/HoxA9:ER cells were cultured in the

Figure 16: HoxA9 protects BLIN-2 from stromal withdrawal induced apoptosis. Total cellular protein from BLIN-2 and BLIN-2/HoxA9:ER (HoxA9) cells cultured in the presence (+Stromal Cells) or absence of stromal cell support (-Stromal Cells), and in the presence or absence of β-estradiol (β-EST) to induce HoxA9 activity was prepared. Total cellular protein (20 μg) was resolved on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were sequentially probed with antibodies against PARP, cleaved caspase-7, total caspase-7 and β-tubulin. β-tubulin is a loading control. Total = uncleaved PARP, cleaved = cleaved PARP, and p20 = cleaved caspase-7 product.


+ β-EST
BLIN-2
HoxA9
BLIN-2
HoxA9
BLIN-2
HoxA9
PARP
Total
Cleaved
Caspase-7
Cleaved Caspase-7
p20
N.S.
β-Tubulin
+ Stromal Cells
− Stromal Cells

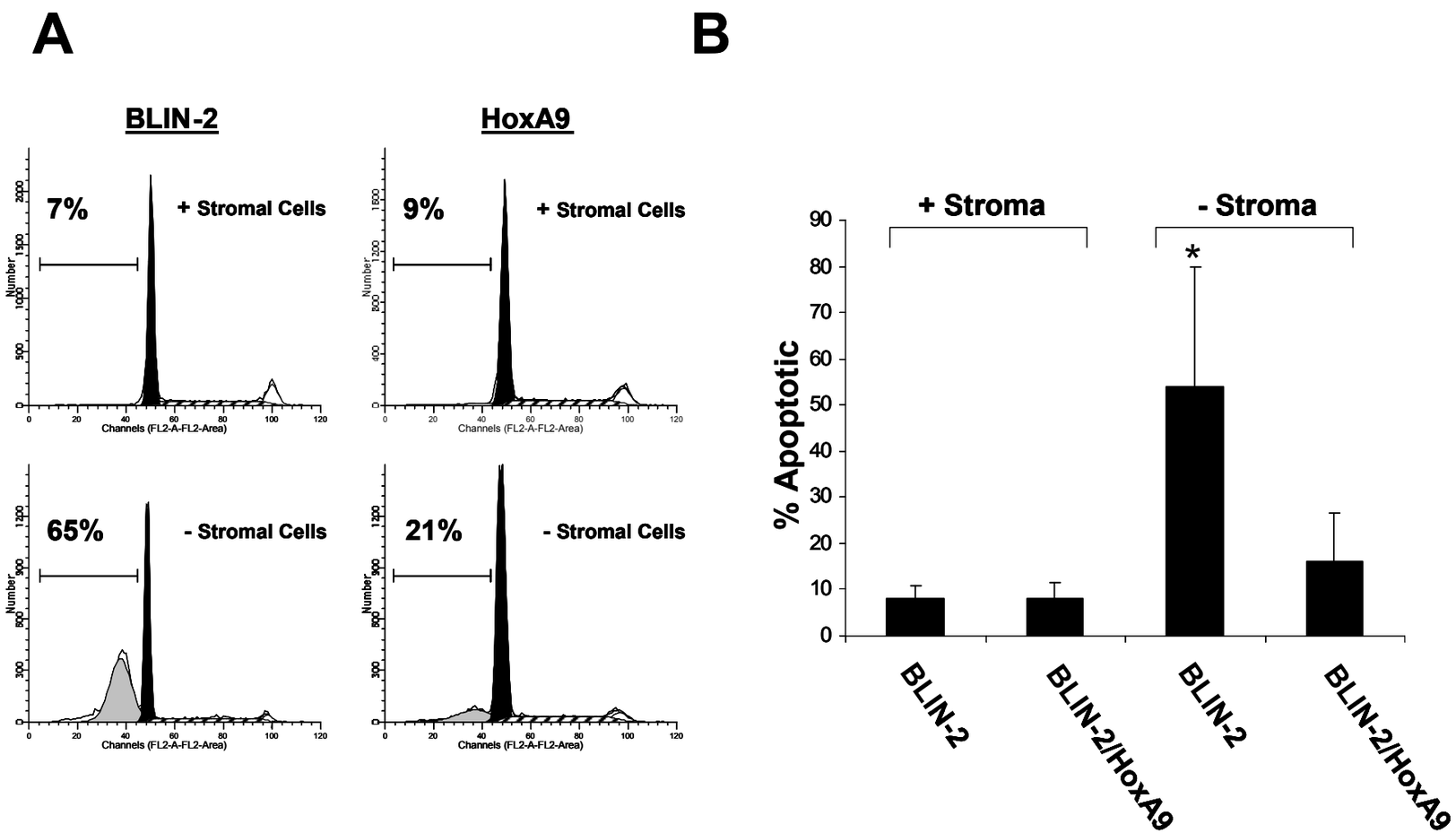
presence or absence of stromal cell support for 4 days. Additionally, cells were cultured in the presence or absence of β-estradiol to induce HoxA9 activity (Fig. 16). Western blot analysis was performed to evaluate the cleavage status of caspase-7 and the caspase-7 substrate, poly (ADP-ribose) polymerase (PARP). These markers indicate induction of apoptosis due to loss of pro-survival signals. BLIN-2 and BLIN-2/HoxA9:ER cells cultured in the presence of stromal cell support showed no detectable cleavage products of either caspase-7 or PARP. In the absence of stromal cell support, and β-estradiol, both BLIN-2 and BLIN-2/HoxA9:ER cells showed detectable caspase-7 and PARP cleavage products. However, upon treatment with β-estradiol to induce HoxA9 activity, caspase-7 and PARP cleavage products were reduced in BLIN-2/HoxA9:ER cells. β-estradiol produced a slight antiapoptotic effect in BLIN-2 cells in the absence of stromal cell support.

BLIN-2 and BLIN-2/HoxA9:ER cells were cultured as described above and stained with propidium iodide prior to flow cytometric analysis (Fig. 17A, and B). Consistent with the cleavage of caspase-7 and PARP, BLIN-2 cells showed a significant increase in sub-G1 events that represent the apoptotic population of cells. BLIN-2/HoxA9:ER cells had a slightly increased sub-G1 which was not statistically significant. Together these results suggest that HoxA9 protects BLIN-2 cells from apoptosis induced by stromal cell/growth factor withdrawal.

4.2.2 HoxA9-mediated protection from apoptosis is independent of IGF-1R

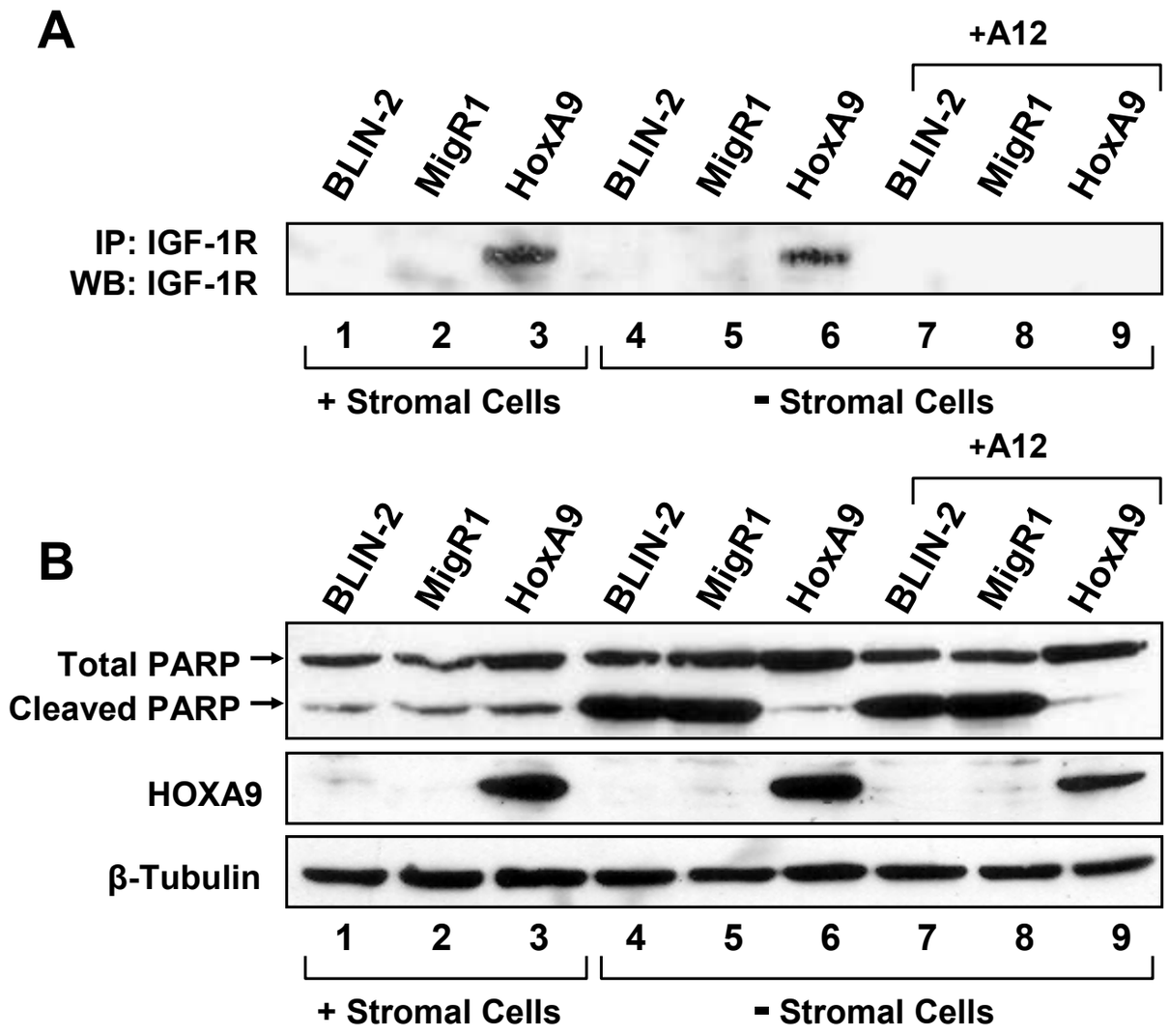
To determine whether signaling through the IGF-1R receptor is responsible for the resistance to apoptosis observed in BLIN-2/HoxA9:ER cells, BLIN-2 and the BLIN-2 derived cell lines were cultured in the presence or absence of stromal cell support. In the

Figure 17: Propidium iodide cell cycle analysis. A) A representative experiment of BLIN-2 and BLIN-2/HoxA9:ER cells cultured in the presence or absence of stromal cell support and analyzed for cell cycle analysis using propidium iodide DNA staining. B) Combined results from three separated experiments of propidium iodide cell cycle analysis of BLIN-2 and BLIN-2/HoxA9:ER cells cultured in the presence or absence of stromal cell support. Data is expressed as percent apoptotic cells. * denotes statistical significance ($p= 0.0388$).


A
BLIN-2
HoxA9
7%
+ Stromal Cells
9%
+ Stromal Cells
65%
- Stromal Cells
21%
- Stromal Cells
Number
Channels (FL2-A-FL2-Area)
B
+ Stroma
- Stroma
*
% Apoptotic
0
10
20
30
40
50
60
70
80
90
BLIN-2
BLIN-2/HoxA9
BLIN-2
BLIN-2/HoxA9

absence of stromal cell support, cells were additionally cultured in the presence or absence of the anti-IGF-1R monoclonal antibody, A12 (Fig. 18A, and B). Treatment with A12 results in inhibition of IGF-1R signaling, as well as internalization and degradation of the IGF-1R receptor (136). First, to determine whether A12 treatment was reducing IGF-1R expression on BLIN-2/HoxA9:ER cells, immunoprecipitation was performed followed by western blot analysis for IGF-1R expression (Fig. 18A). IGF-1R expression was detected only in BLIN-2/HoxA9:ER cells (lanes 3 and 6) and treatment with A12 resulted in a loss of detectable IGF-1R expression in BLIN-2/HoxA9:ER (lane 9). Next, western blot analysis was performed on the cells described above to evaluate PARP cleavage (Fig. 18B). Minimal PARP cleavage was detectable in all cells cultured in the presence of stromal cell support. In the absence of stromal cell support, BLIN-2 and BLIN-2/MigR1 cells showed high levels of cleaved PARP (lanes 4, 5, 7, and 8). BLIN-2/HoxA9:ER cells cultured in the absence of stromal cell support demonstrated little PARP cleavage (lane 6). Interestingly, this observation was not affected by addition of A12 (lane 9). These results suggest that protection from PARP cleavage in BLIN-2/HoxA9:ER cells was independent of IGF-1R signaling. Furthermore, BLIN-2/HoxA9:ER cells exhibited increased levels of phosphorylated ERK in the presence or absence of stromal cell support, but no change in Akt phosphorylation was observed (data not shown). These results are consistent with a model in which the HoxA9-mediated induction of IGF-1R expression, and subsequent activation of the receptor, promote proliferation of BLIN-2/HoxA9:ER cells (169) through activation of the MAPK/ERK signaling pathway, but not apoptotic resistance through the PI3K/Akt signaling pathway.

Figure 18: Protection from PARP cleavage is independent of IGF-1R expression. Total cellular protein was prepared from BLIN-2, BLIN-2/MigR1 (MigR1) and BLIN-2/HoxA9:ER (HoxA9) cells cultured in the presence (+Stromal Cells) or absence of stromal cell support (-Stromal Cells), and in the presence or absence of the A12 monoclonal antibody. Immunoprecipitation was performed for IGF-1R (IP) followed by western blot analysis (WB) for receptor expression, as described. Cells were also analyzed for PARP cleavage via western blotting. β-tubulin serves as a loading control.


A
+A12
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
IP: IGF-1R
WB: IGF-1R
1 2 3 4 5 6 7 8 9
+ Stromal Cells
- Stromal Cells
B
+A12
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
BLIN-2
MigR1
HoxA9
Total PARP
Cleaved PARP
HOXA9
β-Tubulin
1 2 3 4 5 6 7 8 9
+ Stromal Cells
- Stromal Cells

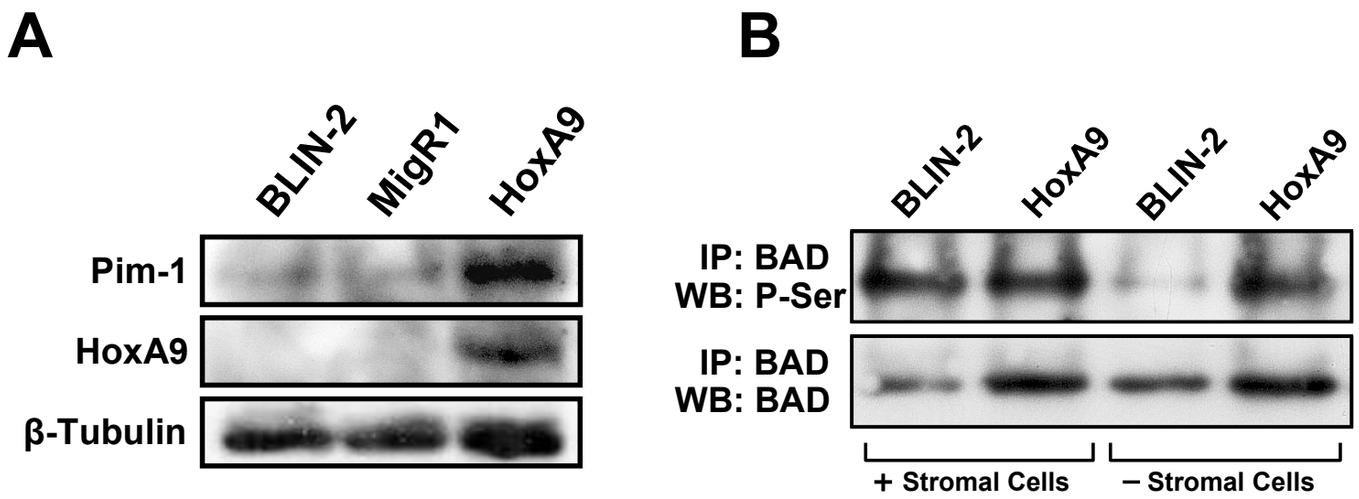
4.2.3 Pim-1 is a HoxA9 target in BLIN-2 cells

The Pim-1 proto-oncogene is reported to be a direct transcriptional target of HoxA9 in hematopoietic cells (90,91). The Pim-1 proto-oncogene is a serine/threonine kinase expressed in the hematopoietic and lymphoid tissues (173). Pim-1 plays a positive role in cell cycle progression at both the G1/S and G2/M transitions (173). To determine if BLIN-2/HoxA9:ER cells exhibit increased expression of Pim-1, western analysis was performed on BLIN-2, BLIN-2/MigR1, and BLIN-2/HoxA9:ER cells (Fig. 19A). Pim-1 expression was greatly increased in BLIN-2/HoxA9:ER cells compared to the parental cell lines.

4.2.4 BAD phosphorylation

One of the targets of Pim-1 is the pro-apoptotic protein BAD which is phosphorylated on Ser112. Thus, a functional role of Pim-1 is preventing apoptosis through the inactivation of BAD (174). BAD is also phosphorylated at Ser136 and phosphorylation at both of these sites promotes binding of BAD to 14-3-3 proteins to prevent association with Bcl-2 and Bcl-xl and induction of apoptosis. To determine whether BAD phosphorylation is increased in BLIN-2/HoxA9:ER cells, BLIN-2, BLIN-2/MigR1 and BLIN-2/HoxA9:ER cells were cultured in the absence of stromal cell support and in the presence of β-estradiol to induce HoxA9 activity. Immunoprecipitation was performed for total BAD protein followed by western analysis for the BAD phospho-(ser) 14-3-3 binding motif (Fig. 19B). Phosphorylation of BAD was undetectable in parental cell lines; however, phosphorylated BAD was readily detectable in BLIN-2/HoxA9:ER cells. These results suggest that HoxA9 protects BLIN-2 cells from apoptosis through Pim-1–mediated phosphorylation of BAD.

Figure 19: Pim-1 expression and BAD phosphorylation are increased in the presence of HoxA9 activity. A) Total cellular protein (20 μg) was resolved on 8% SDS-PAGE gels and transferred to PVDF membranes. Membranes were sequentially probed with antibodies against Pim1, HoxA9, and β-tubulin. B) Total cellular protein was isolated from BLIN-2 and BLIN-2/HoxA9:ER cells cultured in the presence and absence of stromal cell support. Immunoprecipitation (IP) of total BAD was performed followed by western blot analysis (WB) of phosphorylated serine 14-3-3 binding motif or total BAD.


A
BLIN-2
MigR1
HoxA9
Pim-1
HoxA9
β-Tubulin
B
BLIN-2
HoxA9
BLIN-2
HoxA9
IP: BAD
WB: P-Ser
IP: BAD
WB: BAD
+ Stromal Cells
– Stromal Cells

4.3 Conclusions

Overexpression of HoxA9 is highly associated with acute leukemia. However, molecular pathways activated by HoxA9 that promote survival and proliferation of leukemic cells have not been well defined. In Chapter 3 we reported that induction of HoxA9 activity in BLIN-2 cells induces surface expression and signaling through IGF-1R, resulting in an IGF-1R–dependent increase in cellular proliferation (169). IGF-1R has a wide distribution of expression and is essential for development and growth of normal tissues. Many studies have also implicated IGF-1R and its ligands, IGF-1 and IGF-2, in the development, maintenance, and progression of cancer (95). In tumor cells, overexpression of IGF-1R often leads to increased intracellular signaling through the PI3K/Akt and MAPK/ERK kinase pathways (170). Signaling through the PI3K/Akt pathway is associated with protection from apoptosis (95,172), and signaling through the MAPK/ERK pathway is associated with increased proliferation (95,171). Some studies also suggest that signaling through the PI3K/Akt pathway enhances and/or synergizes with MAPK/ERK signaling, providing a more robust pro-survival signal (100). Thus, signaling through IGF-1R can promote both cellular proliferation and apoptotic resistance through two different cellular signaling pathways.

Our previous findings addressed changes in the proliferative capacity of BLIN-2 cells in the absence of stromal cell/growth factor support; however, we did not address the apoptotic sensitivity of BLIN-2 cells under these conditions. Therefore, we sought to evaluate the apoptotic status of BLIN-2 cells in the presence of enforced HoxA9 activity, but in the absence of stromal cell/growth factor support.

In the absence of growth factor/stromal cell support, BLIN-2/HoxA9:ER cells exhibited less cleaved PARP and caspase-7 compared to parental cells, indicating that HoxA9 is protective against apoptosis. These results were further confirmed by cell cycle analysis. Interestingly, cells treated with a specific inhibitor of IGF-1R exhibited no changes in PARP cleavage, indicating that the HoxA9-mediated protection from apoptosis is independent of IGF-1R signaling. Furthermore, while phosphorylation of Akt remained unchanged, BLIN-2/HoxA9:ER cells exhibited increased phosphorylation of ERK. This is consistent with a model in which signaling through IGF-1R promotes proliferation of BLIN-2 cells through activation of the MAPK/ERK pathway, while protection from apoptosis is independent of IGF-1R signaling through PI3K/Akt.

The Pim-1 serine kinase has anti-apoptotic activity through phosphorylation of the pro-apoptotic protein BAD (174). BAD influences the integrity of the mitochondrial membrane, and release of cytochrome C, by associating with Bcl-2 and Bcl-xl and inhibiting their anti-apoptotic function (105). However, when phosphorylated, BAD is incapable of associating with Bcl-2 or Bcl-xl. Expression of Pim-1 is induced by a number of cytokines that signal through specific receptor tyrosine kinases, including PI3K/Akt (106,173). More recently, it was established that Pim-1 is a direct downstream target of HoxA9 in hematopoietic cells (91). Hu *et al*. showed that overexpression of HoxA9 in hematopoietic cells induces Pim-1–mediated phosphorylation of BAD, and partially rescued HoxA9$^{-/-}$ primitive mouse marrow cells from apoptosis (91). We examined BLIN-2/HoxA9:ER cells for Pim-1 expression and found that it was increased in the presence of HoxA9. As expected, BLIN-2/HoxA9:ER cells also exhibited increased phosphorylation of BAD upon growth factor/stromal cell withdrawal as

compared to the parental cell lines. These data provide an alternative pathway for the HoxA9-mediated resistance to apoptosis. Specifically, induction of HoxA9 in BLIN-2 resulting in increased Pim-1 expression followed by increased phosphorylation of BAD.

In Chapter 3 we reported that expression of the c-Myb proto-oncogene was increased upon induction of HoxA9 activity in BLIN-2 cells (169). Interestingly, Pim-1 has been shown to function downstream of Ras to stimulate c-Myb transcriptional activity in a p100 dependent manner (175), and c-Myb is a known regulator of IGF-1R expression (156-158). Therefore, Pim-1 may serve a dual role in promoting both survival and proliferation of leukemic cells through 1) BAD phosphorylation and 2) increased IGF-1R expression via enhanced transcriptional activation of c-Myb.

Pim-1 is involved in a number of cellular processes that control hematopoiesis and is frequently overexpressed or mutated in cancers (173). As such, Pim-1 serves as a promising target for chemotherapeutic intervention. Currently, highly selective and potent inhibitors of Pim-1 are in preclinical development (176), and at least one inhibitor is expected to begin phase I clinical trials later this year (205). Our data indicate that one mechanism of HoxA9-mediated cell proliferation is through expression and subsequent activation of the IGF-1R receptor. However, in our system IGF-1R signaling was not required for the survival of BLIN-2 cells in the absence of growth factor/stromal cell support. Therefore, targeting of IGF-1R alone in leukemic cells overexpressing HoxA9 may not be sufficient in causing cell death, but may inhibit leukemic cell proliferation. Pim-1 inhibitors alone or combinatorial therapy using IGF-1R inhibitors along with Pim-1 inhibitors may prove to be more efficacious in the treatment of leukemia characterized by the overexpression of HoxA9.

CHAPTER 5: DISCUSSION

5.1 Summary

Tumorigenesis is considered a stepwise process analogous to Darwinian evolution, in which the progressive acquisition of genetic mutations confers a selective growth advantage, causing cells to evolve from a state of normality to invasive cancers (177-179). Two key hallmarks of malignant transformation are uncontrolled proliferation and resistance to apoptosis (178). The means by which cancer cells obtain these traits is through defects in the regulatory networks that regulate normal cell proliferation and the apoptotic machinery. Understanding the mechanisms that govern these defects may lead to development of novel and more effective therapies.

Leukemias represent a broad class of tumors characterized by the outgrowth of immature white blood cells in the BM and lymphoid tissues of the body. As with other types of cancer cells, leukemic cells are characterized by uncontrolled proliferation and resistance to programmed cell death. Acute leukemias account for the majority of leukemia-related deaths, and approximately 30 to 40% of acute leukemias are characterized by deregulation of HOX gene expression (88). HoxA9 is one of the most frequently overexpressed HOX genes in acute leukemia, and several studies have indicated that aberrant HoxA9 expression can perturb normal hematopoiesis resulting in developmental defects in both myeloid and lymphoid lineages (73). Whether the initial transforming event, or acting as a cofactor, little is known regarding the identity of pathways activated by HoxA9 overexpression that directly contribute to proliferation and survival of tumor cells. Therefore, the studies of this research project were designed to

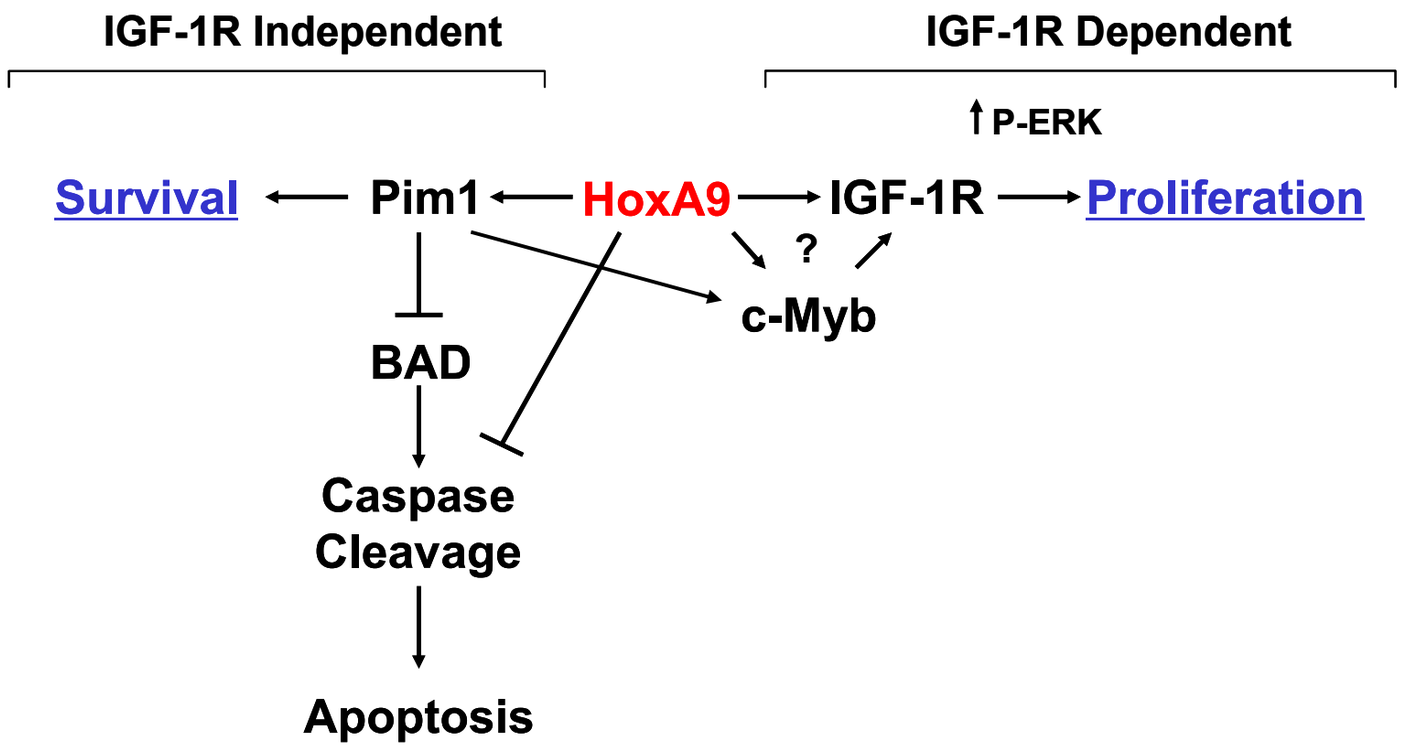
test the hypothesis that *HoxA9 overexpression promotes the growth and survival of acute leukemic cells through the activation of specific downstream signaling pathways.*

5.2 Model

Altogether these data support a model in which HoxA9 promotes the proliferation and survival of pre-B ALL cells through an IGF-1R–dependent and –independent pathway. Presented in Figure 20 is the proposed model for the HoxA9-mediated effects in acute leukemic cells. Overexpression of HoxA9 results in an increase in c-Myb expression, and induction of IGF-1R. Increased IGF-1R on the cell surface sensitizes cells to ERK phosphorylation and IGF-1R–dependent proliferation. c-Myb is a known direct regulator of IGF-1R expression (156-158), and is a downstream target of HoxA9 in MLL/ENL transformed hematopoietic cells (92). Overexpression of HoxA9 also results in a reduction in apoptosis due to growth factor/stromal cell withdrawal. This effect is likely the result of increased Pim-1 expression and subsequent BAD phosphorylation by the kinase. Interestingly, Pim-1 has been shown to cooperate with p100 to enhance the transcriptional activity of the c-Myb protein (175). Therefore, HoxA9 induction of IGF-1R expression may be enhanced through increased Pim-1 regulation of c-Myb.

c-Myb is the cellular homolog of v-myb, the transforming oncogene of the avian myeloblastosis virus, and encodes a transcription factor that plays a crucial role in the proliferation and differentiation of hematopoietic cells (180). Similar to the HoxA9, c-Myb is highly expressed in the immature compartment of hematopoietic cells and later downregulated as the cells mature through lineage specific developmental programs (180). c-Myb overexpression transforms cells in culture and results in acute leukemia in animals.(181) The c-Myb protein transactivates promoters with a specific DNA consens-

Figure 20: Model for HoxA9-mediated Proliferation and Protection from Apoptosis. Induction of HoxA9 activity results in IGF-1R dependent increase in proliferation and increased c-Myb expression. HoxA9 induction also increases Pim-1 expression and protects cells from growth factor/stromal cell withdrawal induced apoptosis through a pathway independent of IGF-1R signaling.

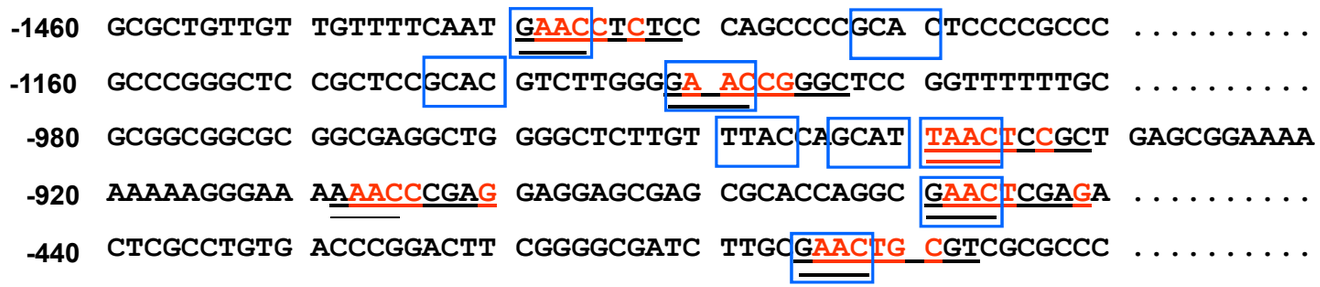

IGF-1R Independent
IGF-1R Dependent
P-ERK
Survival
Pim1
HoxA9
IGF-1R
Proliferation
?
c-Myb
BAD
Caspase
Cleavage
Apoptosis

us sequence, YAACBGYCR, where YAAC is absolutely required for DNA binding (182). Our results do not distinguish whether HoxA9 is directly regulating IGF-1R expression, or whether IGF-1R expression is induced indirectly through upregulation of c-Myb expression. The IGF-1R gene has a TATA-less promoter and most of the promoter activity is located in the proximal 416 bp upstream of the transcriptional start site (183,184). Further dissection of the 5'-flanking region outside of the core 416 bp has revealed cell type-specific patterns of promoter activity upstream of this core region (184). Sequence analysis of the IGF-1R 5'-flanking region revealed multiple putative HoxA9 consensus binding sequences, along with several c-Myb putative consensus binding sequences (Fig. 21). Thus, HoxA9 may directly regulate IGF-1R gene expression, through binding to the 5'-flanking region of the IGF-1R promoter, or indirectly through c-Myb upregulation. Furthermore, several of these consensus binding sequences have considerable overlap which might be important for synergistic activation of IGF-1R expression by HoxA9 and c-Myb. Interactions between HoxA9 and c-Myb have not been described. It would be interesting to investigate whether HoxA9 is capable of binding to, and transactivating the IGF-1R promoter alone. It would also be interesting to examine whether there is any physical interaction between HoxA9 and c-Myb, and whether these proteins can function together to increase IGF-1R expression.

5.3 Significance

These studies are the first to demonstrate a link between HoxA9 overexpression and growth factor receptor expression and signaling in acute leukemia. HoxA9 induction of IGF-1R expression and signaling represents one mechanism by which HoxA9 can promote leukemic cell survival and proliferation. Loss of responsiveness to microenviro-

Figure 21: 5'-Flanking sequence of the IGF-1R gene. Consensus DNA binding sequences for HoxA9 (blue boxes) and c-Myb (red letters, red underline). Doubled underlined c-Myb consensus sequence is absolutely required for c-Myb binding. Y = C or T; B = C or G or T; R = A or G; N = A or T or G or C.



c-Myb Consensus Sequence = YAACBGYCR

Hox Consensus Binding Sequence = (T/G)NA(T/C)

mental cues, such as growth factor or stromal cell-derived signals, is a critical step in leukemic progression (185,186). As leukemic cells lose the need for growth factor/stromal cell support for survival, there is no longer any selective pressure to retain those cells in discreet compartments of the BM. Thus, these cells can survive and continue to grow through the metastatic process.

One way cancer cells become self-sufficient is through upregulation of growth factors and growth factor receptors. IGF-1R signaling is associated with protection from apoptosis, enhanced cellular proliferation, drug resistance, and anchorage independent growth (98). IGF-1 is produced primarily by the liver and stromal cells of the BM microenvironment (95), and IGF-1 appears to have endocrine, paracrine, or autocrine effects on the immune system (187) In normal B-cell development little is known regarding the stage at which IGF-1 acts (188). *In vitro* studies have demostrated that IGF-1 promotes the differentiation of pro-B cells into pre-B cells (163). However, studies utilizing IGF-1 knockout mice revealed that there are no defects in B-cell development in these animals (189). Other studies suggest that IGF-1 may act on mature B-cells to promote immunoglobulin production (190,191), although IGF-1R is expressed at low levels on mature B-cells (164). In leukemia, overexpression of IGF-1R has been shown to relieve cells of cytokine dependency (127,128). Autocrine signaling through IGF-1R has also been reported to promote the growth of AML cells and inhibition of IGF-1R signaling induces apoptosis in these cells (126). Martelli *et al.* also reported that chemoresistance of AML cells correlated with IGF-1 secretion (126). Furthermore, daunorubicin resistant leukemic cells, $K562_{VCR}$, demonstrated an 11-fold increase in IGF-1R expression compared to parental $K562_{WT}$ cells (192). Interestingly, $K562_{VCR}$

cells also showed an 8-fold increase in HoxB4 gene expression (192). Therefore, overexpression or activation of IGF-1R signaling may not only be a major determinant of the tumorigenicity of leukemic cells, but also of drug resistance.

Activation of IGF-IR results in PI3K phosphorylation and subsequent downstream phosphorylation of Akt. Akt, in turn, can inhibit pro-apoptotic proteins, such as BAD, and inhibit initiation of apoptosis. IGF-1R receptor activation is also associated with induction of the MAPK/ERK signaling pathway, which is responsible for the mitogenic effects of IGF-1R signaling (98).

Induction of HoxA9 activity in BLIN-2 cells prolonged survival and proliferation in the absence of growth factor/stromal cell support. However, this factor alone was not sufficient to completely alleviate the requirement for stromal cell contact for long-term survival and proliferation. A role for IGF-1R receptor numbers has been described in which a minimum number of receptors, 15,000 to 22,000, were required to render mouse embryonic fibroblasts competent to grow in serum-free medium supplemented solely with IGF-I, and for growth in soft agar, 30,000 receptors per cell was the minimum requirement (193).Therefore, one possible explanation may be an insufficient number of receptors on the cell surface. Another possible explanation may be a defect in the PI3K/Akt signaling pathway, as no changes in Akt phosphorylation were observed upon induction of IGF-1R signaling, the primary signaling pathway activated by IGF-1R (95). This observation may also be explained by low concentrations of IGF-1R, or IGF-1. Hence, there may be a threshold for IGF-1R–mediated activation of Akt. To determine if high receptor numbers can promote long-term survival of BLIN-2 in the absence of

stromal cell support, future experiments should be performed in which IGF-1R is overexpressed in BLIN-2 cells.

Whereas the first and second possibilities rely on IGF-1R signaling, a third possibility is that one or more additional genetic aberrations are required, in addition to HoxA9 overexpression, to promote factor independent growth of BLIN-2 cells. Therefore, it is important that other models be used to validate findings in BLIN-2, and to rule out cell line specific effects.

We have previously reported that inhibition of PI3K/Akt, MAPK/ERK or the Janus protein tyrosine kinases/signal transducers and activators of transcription (JAK/STAT) signaling pathways resulted in reduced proliferation of BLIN-2 cells in the presence of stromal cell support (194). This effect was more robust when the inhibitors were used in combinations. In the same study, inhibition of the PI3K/Akt, MAPK/ERK, or JAK/STAT pathways did not result in induction of apoptosis; however, when pathways were inhibited simultaneously, there was a marked increase in the number of apoptotic events as compared to cells cultured in the absence of stromal cell support after 24 hr (194). Therefore, stromal cells appear to activate these signaling pathways to promote the survival and proliferation of BLIN-2 cells. However, inhibition of a single pathway was not sufficient to induce apoptosis, suggesting crosstalk between these pathways. Furthermore, these results indicate that activation of PI3K/Akt and MAPK/ERK through IGF-1R signaling may not be sufficient alone to alleviate stromal cell dependency of BLIN-2. Activation of the JAK/STAT pathway may also be a requirement. JAK(s) are non-receptor tyrosine kinases that phosphorylate engaged cytokine receptors, allowing for the docking and activation of STAT(s). Subsequently,

activated STATs form homo- or heterodimers and translocate to the nucleus to activate gene transcription (195). Indeed, constitutive signaling through JAK/STAT is common in AML, occurring in up to 70% of AML patients (196,197).

Leukemias characterized by overexpression of HoxA9 may benefit from IGF-1R targeted therapies, in combination with inhibitors of the JAK/STAT pathway, to inhibit proliferation and induce apoptosis of leukemic cells. In fact, IGF-1R and JAK/STAT targeted therapies may be of particular and immediate benefit in treating leukemias that bear *MLL* translocations. Ten percent of all acute leukemias have *MLL* translocations and 80% of infant ALL have *MLL* translocations (71). The presence of *MLL* translocations is associated with an extremely poor prognosis and there are no therapeutic options for this subset (71). Currently there are several IGF-1R inhibitors under clinical investigation, though none are approved by the FDA for general oncologic use (198). There are five monoclonal antibodies, including: CP-721,871, AMG-479, IMC-A12, R1507, and BIIB022; and three small-molecule inhibitors: XL-228, OSI-906, and Nordihydroguareacetic acid (NDGA). IMC-A12 is the clinical designation for the A12 mAb used in the studies presented here, and A12 is currently in phase II clinical trials for treatment of cancers of the breast, colon, head and neck, liver, pancreas, prostate, and connective tissues (198). Currently, no FDA approved JAK inhibitors are available for use in the clinic, although a few are being assessed in phase I and phase II clinical trials, including the small molecule inhibitors: AT9283, CEP-701 (lestaurtinib), and MK-0457 (tozasertib lactate) (199).

Pim-1 has been reported to be a direct target of HoxA9 transcriptional activity in hematopoietic cells (91). Pim-1 phosphorylation of BAD inhibits the initiation of

apoptosis (173). In our model, Pim-1 expression was also increased in the presence of HoxA9 activity, which was accompanied by increased BAD phosphorylation in the absence of growth factor/stromal cell support. This provides a second mechanism by which HoxA9 could promote the survival of acute leukemic cells. As such, leukemias identified as overexpressing HoxA9, such as those bearing *MLL* translocations, may benefit from inhibitors of Pim-1 activity. Inhibitors of Pim-1 are currently in development, and may be available for use in clinical trials in the very near future (176). Future experiments to be performed should include the use of Pim-1 siRNA or Pim-1 inhibitors on BLIN-2/HoxA9:ER cells to evaluate the effects on proliferation on apoptotic sensitivity.

There are likely hundreds of genes regulated by HoxA9 in leukemic cells, yet the identification of HoxA9 downstream targets has remained somewhat elusive. At least 220 candidate genes have been identified, via microarray analysis, to be either upregulated or repressed by at least two-fold by HoxA9 in leukemic cells (90). However, very few of these candidate genes have been empirically identified as direct downstream targets. Some of the targets upregulated by HoxA9 include: signaling molecules, such as EGFR pathway substrate 8 (EPS8); enzymes, such as aldehyde dehydrogenase-1 (ALDH1); oncogenes, such as v-jun avian sarcoma virus 17 oncogene homolog (c-JUN) and v-yes-1 Yamaguchi sarcoma viral oncogene 1 (YES1); and regulators of cell cycle progression, such as cyclin dependent kinase 7 (CDK7) (90). All of these molecules are associated with promoting carcinogenesis (200-204). Therefore, HoxA9 may promote the growth and survival of leukemic cells by multiple means. Despite this, identifying intracellular pathways activated as the result of HoxA9 overexpression in leukemic cells, rather than

specific HoxA9 targets, will provide valuable insight into the mechanisms by which HoxA9 promotes the survival and proliferation of leukemic cells. These pathways represent potential targets for more effective chemotherapeutic regimens.

REFERENCES

1. Fey MF. Normal and malignant hematopoiesis. Ann.Oncol. 2007 Jan;18 Suppl 1:i9-i13.
2. Afshar K, Vucinic V, Sharma OP. Eosinophil cell: pray tell us what you do! Curr.Opin.Pulm.Med. 2007 Sep;13(5):414-421.
3. Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. Curr.Opin.Allergy Clin.Immunol. 2006 Jun;6(3):226-233.
4. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat.Rev.Immunol. 2008 Dec;8(12):958-969.
5. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. Blood 2008 Aug 15;112(4):935-945.
6. Deutsch VR, Tomer A. Megakaryocyte development and platelet production. Br.J.Haematol. 2006 Sep;134(5):453-466.
7. McKenna K, Beignon AS, Bhardwaj N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J.Virol. 2005 Jan;79(1):17-27.
8. Pancer Z, Cooper MD. The evolution of adaptive immunity. Annu.Rev.Immunol. 2006;24:497-518.
9. Caligiuri MA. Human natural killer cells. Blood 2008 Aug 1;112(3):461-469.
10. McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG. Germinal center reaction. Curr.Opin.Hematol. 2001 Jan;8(1):52-59.
11. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood 2008 Sep 1;112(5):1570-1580.

12. Norvell A, Mandik L, Monroe JG. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J.Immunol. 1995 May 1;154(9):4404-4413.

13. Monroe JG, Dorshkind K. Fate decisions regulating bone marrow and peripheral B lymphocyte development. Adv.Immunol. 2007;95:1-50.

14. Krop I, de Fougerolles AR, Hardy RR, Allison M, Schlissel MS, Fearon DT. Self-renewal of B-1 lymphocytes is dependent on CD19. Eur.J.Immunol. 1996 Jan;26(1):238-242.

15. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell 1989 Dec 22;59(6):1035-1048.

16. Burrows PD, Cooper MD. B cell development and differentiation. Curr.Opin.Immunol. 1997 Apr;9(2):239-244.

17. Lassoued K, Illges H, Benlagha K, Cooper MD. Fate of surrogate light chains in B lineage cells. J.Exp.Med. 1996 Feb 1;183(2):421-429.

18. Papavasiliou F, Jankovic M, Gong S, Nussenzweig MC. Control of immunoglobulin gene rearrangements in developing B cells. Curr.Opin.Immunol. 1997 Apr;9(2):233-238.

19. Loffert D, Ehlich A, Muller W, Rajewsky K. Surrogate light chain expression is required to establish immunoglobulin heavy chain allelic exclusion during early B cell development. Immunity 1996 Feb;4(2):133-144.

20. Bradl H, Wittmann J, Milius D, Vettermann C, Jack HM. Interaction of murine precursor B cell receptor with stroma cells is controlled by the unique tail of

lambda 5 and stroma cell-associated heparan sulfate. J.Immunol. 2003 Sep 1;171(5):2338-2348.

21. Gauthier L, Rossi B, Roux F, Termine E, Schiff C. Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. Proc.Natl.Acad.Sci.U.S.A. 2002 Oct 1;99(20):13014-13019.
22. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. J.Exp.Med. 1993 Apr 1;177(4):1009-1020.
23. Goodnow CC. Transgenic mice and analysis of B-cell tolerance. Annu.Rev.Immunol. 1992;10:489-518.
24. Bertrand FE, Eckfeldt CE, Fink JR, Lysholm AS, Pribyl JA, Shah N, et al. Microenvironmental influences on human B-cell development. Immunol.Rev. 2000 Jun;175:175-186.
25. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. Nat.Rev.Immunol. 2006 Feb;6(2):107-116.
26. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J.Exp.Med. 1995 Apr 1;181(4):1519-1526.
27. Noonan K. Introduction to B-Cell disorders. Clin.J.Oncol.Nurs. 2007 Feb;11(1 Suppl):3-12.
28. Mohamed AJ, Yu L, Backesjo CM, Vargas L, Faryal R, Aints A, et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. Immunol.Rev. 2009 Mar;228(1):58-73.

29. Jesus AA, Duarte AJ, Oliveira JB. Autoimmunity in hyper-IgM syndrome. J.Clin.Immunol. 2008 May;28 Suppl 1:S62-6.

30. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N.Engl.J.Med. 2004 Apr 8;350(15):1535-1548.

31. FOLLEY JH, BORGES W, YAMAWAKI T. Incidence of leukemia in survivors of the atomic bomb in Hiroshima and Nagasaki, Japan. Am.J.Med. 1952 Sep;13(3):311-321.

32. Wintrobe MM. Blood Pure and Eloquent. : McGraw-Hill Inc.,US; 1980.

33. Wakeford R. The cancer epidemiology of radiation. Oncogene 2004 Aug 23;23(38):6404-6428.

34. Infante-Rivard C, Labuda D, Krajinovic M, Sinnett D. Risk of childhood leukemia associated with exposure to pesticides and with gene polymorphisms. Epidemiology 1999 Sep;10(5):481-487.

35. Irigaray P, Newby JA, Clapp R, Hardell L, Howard V, Montagnier L, et al. Lifestyle-related factors and environmental agents causing cancer: an overview. Biomed.Pharmacother. 2007 Dec;61(10):640-658.

36. Larson RA. Is secondary leukemia an independent poor prognostic factor in acute myeloid leukemia? Best Pract.Res.Clin.Haematol. 2007 Mar;20(1):29-37.

37. Belson M, Kingsley B, Holmes A. Risk factors for acute leukemia in children: a review. Environ.Health Perspect. 2007 Jan;115(1):138-145.

38. Draper GJ, Heaf MM, Kinnier Wilson LM. Occurrence of childhood cancers among sibs and estimation of familial risks. J.Med.Genet. 1977 Apr;14(2):81-90.

39. HEATH CW,Jr, MOLONEY WC. Familial Leukemia; Five Cases of Acute Leukemia in Three Generations. N.Engl.J.Med. 1965 Apr 29;272:882-887.

40. Sessions J. Chronic myeloid leukemia in 2007. J.Manag.Care.Pharm. 2007 Oct;13(8 Suppl A):4-7.

41. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975 Aug;46(2):219-234.

42. Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 1981 Jul 1;48(1):198-206.

43. Montserrat E, Rozman C. Chronic lymphocytic leukemia: present status. Ann.Oncol. 1995 Mar;6(3):219-235.

44. Pui CH. Childhood leukemias. N.Engl.J.Med. 1995 Jun 15;332(24):1618-1630.

45. Kantarjian H, O'Brien S, Cortes J, Wierda W, Faderl S, Garcia-Manero G, et al. Therapeutic advances in leukemia and myelodysplastic syndrome over the past 40 years. Cancer 2008 Oct 1;113(7 Suppl):1933-1952.

46. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. N.Engl.J.Med. 1999 Jul 15;341(3):164-172.

47. NOWELL PC, HUNGERFORD DA. Chromosome studies on normal and leukemic human leukocytes. J.Natl.Cancer Inst. 1960 Jul;25:85-109.

48. Yaish P, Gazit A, Gilon C, Levitzki A. Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science 1988 Nov 11;242(4880):933-935.

49. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N.Engl.J.Med. 2001 Apr 5;344(14):1031-1037.

50. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science 2000 Sep 15;289(5486):1938-1942.

51. Ottmann OG, Wassmann B. Treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. Hematology Am.Soc.Hematol.Educ.Program. 2005:118-122.

52. Riscoe MK, Brouns MC, Fitchen JH. Purine metabolism as a target for leukemia chemotherapy. Blood Rev. 1989 Sep;3(3):162-173.

53. Keating MJ, O'Brien S, Lerner S, Koller C, Beran M, Robertson LE, et al. Long-term follow-up of patients with chronic lymphocytic leukemia (CLL) receiving fludarabine regimens as initial therapy. Blood 1998 Aug 15;92(4):1165-1171.

54. Leporrier M, Chevret S, Cazin B, Boudjerra N, Feugier P, Desablens B, et al. Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. Blood 2001 Oct 15;98(8):2319-2325.

55. Catovsky D, Richards S, Matutes E, Oscier D, Dyer MJ, Bezares RF, et al. Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. Lancet 2007 Jul 21;370(9583):230-239.

56. Scott SD. Rituximab: a new therapeutic monoclonal antibody for non-Hodgkin's lymphoma. Cancer Pract. 1998 May-Jun;6(3):195-197.

57. Keating MJ, O'Brien S, Albitar M, Lerner S, Plunkett W, Giles F, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. J.Clin.Oncol. 2005 Jun 20;23(18):4079-4088.

58. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008 Mar 1;111(5):2505-2515.

59. Huang ME, Ye YC, Chen SR, Zhao JC, Gu LJ, Cai JR, et al. All-trans retinoic acid with or without low dose cytosine arabinoside in acute promyelocytic leukemia. Report of 6 cases. Chin.Med.J.(Engl) 1987 Dec;100(12):949-953.

60. Goodman GR, Burian C, Koziol JA, Saven A. Extended follow-up of patients with hairy cell leukemia after treatment with cladribine. J.Clin.Oncol. 2003 Mar 1;21(5):891-896.

61. Gilliland DG. Molecular genetics of human leukemia. Leukemia 1998 Sep;12 Suppl 1:S7-12.

62. Carney DA, Wierda WG. Genetics and molecular biology of chronic lymphocytic leukemia. Curr.Treat.Options Oncol. 2005 May;6(3):215-225.

63. Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997 Nov 7;278(5340):1059-1064.

64. Look AT. Oncogenic role of "master" transcription factors in human leukemias and sarcomas: a developmental model. Adv.Cancer Res. 1995;67:25-57.

65. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat.Rev.Cancer. 2002 Jul;2(7):502-513.

66. Sawinska M, Ladon D. Mechanism, detection and clinical significance of the reciprocal translocation t(12;21)(p12;q22) in the children suffering from acute lymphoblastic leukaemia. Leuk.Res. 2004 Jan;28(1):35-42.

67. Buske C, Humphries RK. Homeobox genes in leukemogenesis. Int.J.Hematol. 2000 Jun;71(4):301-308.

68. Basecke J, Whelan JT, Griesinger F, Bertrand FE. The MLL partial tandem duplication in acute myeloid leukaemia. Br.J.Haematol. 2006 Nov;135(4):438-449.

69. Meyer C, Schneider B, Jakob S, Strehl S, Attarbaschi A, Schnittger S, et al. The MLL recombinome of acute leukemias. Leukemia 2006 May;20(5):777-784.

70. Chen CS, Sorensen PH, Domer PH, Reaman GH, Korsmeyer SJ, Heerema NA, et al. Molecular rearrangements on chromosome 11q23 predominate in infant acute lymphoblastic leukemia and are associated with specific biologic variables and poor outcome. Blood 1993 May 1;81(9):2386-2393.

71. Pui CH, Behm FG, Downing JR, Hancock ML, Shurtleff SA, Ribeiro RC, et al. 11q23/MLL rearrangement confers a poor prognosis in infants with acute lymphoblastic leukemia. J.Clin.Oncol. 1994 May;12(5):909-915.

72. Daser A, Rabbitts TH. Extending the repertoire of the mixed-lineage leukemia gene MLL in leukemogenesis. Genes Dev. 2004 May 1;18(9):965-974.

73. Magli MC, Largman C, Lawrence HJ. Effects of HOX homeobox genes in blood cell differentiation. J.Cell.Physiol. 1997 Nov;173(2):168-177.

74. Thorsteinsdottir U, Kroon E, Jerome L, Blasi F, Sauvageau G. Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. Mol.Cell.Biol. 2001 Jan;21(1):224-234.

75. Owens BM, Hawley RG. HOX and non-HOX homeobox genes in leukemic hematopoiesis. Stem Cells 2002;20(5):364-379.

76. Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, et al. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc.Natl.Acad.Sci.U.S.A. 1994 Dec 6;91(25):12223-12227.

77. van Oostveen J, Bijl J, Raaphorst F, Walboomers J, Meijer C. The role of homeobox genes in normal hematopoiesis and hematological malignancies. Leukemia 1999 Nov;13(11):1675-1690.

78. Crooks GM, Fuller J, Petersen D, Izadi P, Malik P, Pattengale PK, et al. Constitutive HOXA5 expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. Blood 1999 Jul 15;94(2):519-528.

79. Fuller JF, McAdara J, Yaron Y, Sakaguchi M, Fraser JK, Gasson JC. Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. Blood 1999 May 15;93(10):3391-3400.

80. Thorsteinsdottir U, Sauvageau G, Hough MR, Dragowska W, Lansdorp PM, Lawrence HJ, et al. Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. Mol.Cell.Biol. 1997 Jan;17(1):495-505.

81. Kongsuwan K, Allen J, Adams JM. Expression of Hox-2.4 homeobox gene directed by proviral insertion in a myeloid leukemia. Nucleic Acids Res. 1989 Mar 11;17(5):1881-1892.

82. Nakamura T, Largaespada DA, Shaughnessy JD,Jr, Jenkins NA, Copeland NG. Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. Nat.Genet. 1996 Feb;12(2):149-153.

83. Lawrence HJ, Rozenfeld S, Cruz C, Matsukuma K, Kwong A, Komuves L, et al. Frequent co-expression of the HOXA9 and MEIS1 homeobox genes in human myeloid leukemias. Leukemia 1999 Dec;13(12):1993-1999.

84. Thorsteinsdottir U, Krosl J, Kroon E, Haman A, Hoang T, Sauvageau G. The oncoprotein E2A-Pbx1a collaborates with Hoxa9 to acutely transform primary bone marrow cells. Mol.Cell.Biol. 1999 Sep;19(9):6355-6366.

85. Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK, et al. Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood 1997 Mar 15;89(6):1922-1930.

86. Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J, Lawrence HJ, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 2002 Jan 1;99(1):121-129.

87. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. 1998 Jul 1;17(13):3714-3725.

88. Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. J.Pathol. 2005 Jan;205(2):154-171.

89. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999 Oct 15;286(5439):531-537.

90. Dorsam ST, Ferrell CM, Dorsam GP, Derynck MK, Vijapurkar U, Khodabakhsh D, et al. The transcriptome of the leukemogenic homeoprotein HOXA9 in human hematopoietic cells. Blood 2004 Mar 1;103(5):1676-1684.

91. Hu YL, Passegue E, Fong S, Largman C, Lawrence HJ. Evidence that the Pim1 kinase gene is a direct target of HOXA9. Blood 2007 Jun 1;109(11):4732-4738.

92. Hess JL, Bittner CB, Zeisig DT, Bach C, Fuchs U, Borkhardt A, et al. c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood 2006 Jul 1;108(1):297-304.

93. Bei L, Lu Y, Eklund EA. HOXA9 activates transcription of the gene encoding gp91Phox during myeloid differentiation. J.Biol.Chem. 2005 Apr 1;280(13):12359-12370.

94. Ferrell CM, Dorsam ST, Ohta H, Humphries RK, Derynck MK, Haqq C, et al. Activation of stem-cell specific genes by HOXA9 and HOXA10 homeodomain proteins in CD34+ human cord blood cells. Stem Cells 2005 May;23(5):644-655.

95. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat.Rev.Cancer. 2004 Jul;4(7):505-518.

96. Valentinis B, Baserga R. IGF-I receptor signalling in transformation and differentiation. Mol.Pathol. 2001 Jun;54(3):133-137.

97. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, et al. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 1985 Feb 28-Mar 6;313(6005):756-761.

98. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat.Rev.Cancer. 2008 Dec;8(12):915-928.

99. Le Roith D, Karas M, Yakar S, Qu BH, Wu Y, Blakesley VA. The role of the insulin-like growth factors in cancer. Isr.Med.Assoc.J. 1999 Sep;1(1):25-30.

100. McCubrey JA, Steelman LS, Blalock WL, Lee JT, Moye PW, Chang F, et al. Synergistic effects of pi3k/akt on abrogation of cytokine-dependency induced by oncogenic raf. Adv.Enzyme Regul. 2001;41:289-323.

101. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene 2003 Nov 24;22(53):8543-8567.

102. Cohen GM. Caspases: the executioners of apoptosis. Biochem.J. 1997 Aug 15;326 (Pt 1)(Pt 1):1-16.

103. Downward J. PI 3-kinase, Akt and cell survival. Semin.Cell Dev.Biol. 2004 Apr;15(2):177-182.

104. Samani AA, Brodt P. The receptor for the type I insulin-like growth factor and its ligands regulate multiple cellular functions that impact on metastasis. Surg.Oncol.Clin.N.Am. 2001 Apr;10(2):289-312, viii.

105. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 1995 Jan 27;80(2):285-291.

106. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997 Oct 24;278(5338):687-689.

107. Myal Y, Shiu RP, Bhaumick B, Bala M. Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T-47D) in culture. Cancer Res. 1984 Dec;44(12 Pt 1):5486-5490.

108. Pollak MN, Perdue JF, Margolese RG, Baer K, Richard M. Presence of somatomedin receptors on primary human breast and colon carcinomas. Cancer Lett. 1987 Dec;38(1-2):223-230.

109. Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. Endocr.Rev. 2007 Feb;28(1):20-47.

110. Wrobel G, Roerig P, Kokocinski F, Neben K, Hahn M, Reifenberger G, et al. Microarray-based gene expression profiling of benign, atypical and anaplastic meningiomas identifies novel genes associated with meningioma progression. Int.J.Cancer 2005 Mar 20;114(2):249-256.

111. Hartmann W, Koch A, Brune H, Waha A, Schuller U, Dani I, et al. Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. Am.J.Pathol. 2005 Apr;166(4):1153-1162.

112. Kwon J, Stephan S, Mukhopadhyay A, Muders MH, Dutta SK, Lau JS, et al. Insulin receptor substrate-2 mediated insulin-like growth factor-I receptor overexpression in pancreatic adenocarcinoma through protein kinase Cdelta. Cancer Res. 2009 Feb 15;69(4):1350-1357.

113. Gotlieb WH, Bruchim I, Gu J, Shi Y, Camirand A, Blouin MJ, et al. Insulin-like growth factor receptor I targeting in epithelial ovarian cancer. Gynecol.Oncol. 2006 Feb;100(2):389-396.

114. Giovannucci E, Pollak M, Platz EA, Willett WC, Stampfer MJ, Majeed N, et al. Insulin-like growth factor I (IGF-I), IGF-binding protein-3 and the risk of colorectal adenoma and cancer in the Nurses' Health Study. Growth Horm.IGF Res. 2000 Apr;10 Suppl A:S30-1.

115. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 1998 May 9;351(9113):1393-1396.

116. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 1998 Jan 23;279(5350):563-566.

117. Allen NE, Roddam AW, Allen DS, Fentiman IS, Dos Santos Silva I, Peto J, et al. A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. Br.J.Cancer 2005 Apr 11;92(7):1283-1287.

118. Stattin P, Bylund A, Rinaldi S, Biessy C, Dechaud H, Stenman UH, et al. Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and

prostate cancer risk: a prospective study. J.Natl.Cancer Inst. 2000 Dec 6;92(23):1910-1917.

119. Yu H, Spitz MR, Mistry J, Gu J, Hong WK, Wu X. Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. J.Natl.Cancer Inst. 1999 Jan 20;91(2):151-156.

120. Palmqvist R, Hallmans G, Rinaldi S, Biessy C, Stenling R, Riboli E, et al. Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. Gut 2002 May;50(5):642-646.

121. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J.Natl.Cancer Inst. 1999 Apr 7;91(7):620-625.

122. Zhao H, Grossman HB, Spitz MR, Lerner SP, Zhang K, Wu X. Plasma levels of insulin-like growth factor-1 and binding protein-3, and their association with bladder cancer risk. J.Urol. 2003 Feb;169(2):714-717.

123. Wu X, Tortolero-Luna G, Zhao H, Phatak D, Spitz MR, Follen M. Serum levels of insulin-like growth factor I and risk of squamous intraepithelial lesions of the cervix. Clin.Cancer Res. 2003 Aug 15;9(9):3356-3361.

124. Ross JA, Perentesis JP, Robison LL, Davies SM. Big babies and infant leukemia: a role for insulin-like growth factor-1? Cancer Causes Control 1996 Sep;7(5):553-559.

125. Vorwerk P, Wex H, Hohmann B, Mohnike K, Schmidt U, Mittler U. Expression of components of the IGF signalling system in childhood acute lymphoblastic leukaemia. Mol.Pathol. 2002 Feb;55(1):40-45.

126. Tazzari PL, Tabellini G, Bortul R, Papa V, Evangelisti C, Grafone T, et al. The insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 induces apoptosis in acute myeloid leukemia cells exhibiting autocrine insulin-like growth factor-I secretion. Leukemia 2007 May;21(5):886-896.

127. Bertrand FE, Steelman LS, Chappell WH, Abrams SL, Shelton JG, White ER, et al. Synergy between an IGF-1R antibody and Raf/MEK/ERK and PI3K/Akt/mTOR pathway inhibitors in suppressing IGF-1R-mediated growth in hematopoietic cells. Leukemia 2006 Jul;20(7):1254-1260.

128. McCubrey JA, Steelman LS, Mayo MW, Algate PA, Dellow RA, Kaleko M. Growth-promoting effects of insulin-like growth factor-1 (IGF-1) on hematopoietic cells: overexpression of introduced IGF-1 receptor abrogates interleukin-3 dependency of murine factor-dependent cells by a ligand-dependent mechanism. Blood 1991 Aug 15;78(4):921-929.

129. Baier TG, Jenne EW, Blum W, Schonberg D, Hartmann KK. Influence of antibodies against IGF-I, insulin or their receptors on proliferation of human acute lymphoblastic leukemia cell lines. Leuk.Res. 1992 Aug;16(8):807-814.

130. Arteaga CL, Kitten LJ, Coronado EB, Jacobs S, Kull FC,Jr, Allred DC, et al. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. J.Clin.Invest. 1989 Nov;84(5):1418-1423.

131. Resnicoff M, Coppola D, Sell C, Rubin R, Ferrone S, Baserga R. Growth inhibition of human melanoma cells in nude mice by antisense strategies to the type 1 insulin-like growth factor receptor. Cancer Res. 1994 Sep 15;54(18):4848-4850.

132. Burfeind P, Chernicky CL, Rininsland F, Ilan J, Ilan J. Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc.Natl.Acad.Sci.U.S.A. 1996 Jul 9;93(14):7263-7268.

133. Nakamura K, Hongo A, Kodama J, Miyagi Y, Yoshinouchi M, Kudo T. Down-regulation of the insulin-like growth factor I receptor by antisense RNA can reverse the transformed phenotype of human cervical cancer cell lines. Cancer Res. 2000 Feb 1;60(3):760-765.

134. Bohula EA, Salisbury AJ, Sohail M, Playford MP, Riedemann J, Southern EM, et al. The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript. J.Biol.Chem. 2003 May 2;278(18):15991-15997.

135. Sachdev D, Li SL, Hartell JS, Fujita-Yamaguchi Y, Miller JS, Yee D. A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. Cancer Res. 2003 Feb 1;63(3):627-635.

136. Burtrum D, Zhu Z, Lu D, Anderson DM, Prewett M, Pereira DS, et al. A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo. Cancer Res. 2003 Dec 15;63(24):8912-8921.

137. Wang Y, Hailey J, Williams D, Wang Y, Lipari P, Malkowski M, et al. Inhibition of insulin-like growth factor-I receptor (IGF-IR) signaling and tumor cell growth by a fully human neutralizing anti-IGF-IR antibody. Mol.Cancer.Ther. 2005 Aug;4(8):1214-1221.

138. Cohen BD, Baker DA, Soderstrom C, Tkalcevic G, Rossi AM, Miller PE, et al. Combination therapy enhances the inhibition of tumor growth with the fully human anti-type 1 insulin-like growth factor receptor monoclonal antibody CP-751,871. Clin.Cancer Res. 2005 Mar 1;11(5):2063-2073.

139. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer.Cell. 2004 Mar;5(3):221-230.

140. Scotlandi K, Manara MC, Nicoletti G, Lollini PL, Lukas S, Benini S, et al. Antitumor activity of the insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 in musculoskeletal tumors. Cancer Res. 2005 May 1;65(9):3868-3876.

141. Wen B, Deutsch E, Marangoni E, Frascona V, Maggiorella L, Abdulkarim B, et al. Tyrphostin AG 1024 modulates radiosensitivity in human breast cancer cells. Br.J.Cancer 2001 Dec 14;85(12):2017-2021.

142. Shah N, Oseth L, LeBien TW. Development of a model for evaluating the interaction between human pre-B acute lymphoblastic leukemic cells and the bone marrow stromal cell microenvironment. Blood 1998 Nov 15;92(10):3817-3828.

143. Bertrand FE, Vogtenhuber C, Shah N, LeBien TW. Pro-B-cell to pre-B-cell development in B-lineage acute lymphoblastic leukemia expressing the MLL/AF4 fusion protein. Blood 2001 Dec 1;98(12):3398-3405.

144. Bertrand FE, Spengeman JD, Shah N, LeBien TW. B-cell development in the presence of the MLL/AF4 oncoprotein proceeds in the absence of HOX A7 and HOX A9 expression. Leukemia 2003 Dec;17(12):2454-2459.

145. Stong RC, Korsmeyer SJ, Parkin JL, Arthur DC, Kersey JH. Human acute leukemia cell line with the t(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. Blood 1985 Jan;65(1):21-31.

146. Greil J, Gramatzki M, Burger R, Marschalek R, Peltner M, Trautmann U, et al. The acute lymphoblastic leukaemia cell line SEM with t(4;11) chromosomal rearrangement is biphenotypic and responsive to interleukin-7. Br.J.Haematol. 1994 Feb;86(2):275-283.

147. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 1999 Sep;11(3):299-308.

148. Shen WF, Rozenfeld S, Kwong A, Kom ves LG, Lawrence HJ, Largman C. HOXA9 forms triple complexes with PBX2 and MEIS1 in myeloid cells. Mol.Cell.Biol. 1999 Apr;19(4):3051-3061.

149. Imamura T, Morimoto A, Takanashi M, Hibi S, Sugimoto T, Ishii E, et al. Frequent co-expression of HoxA9 and Meis1 genes in infant acute lymphoblastic leukaemia with MLL rearrangement. Br.J.Haematol. 2002 Oct;119(1):119-121.

150. Rozovskaia T, Feinstein E, Mor O, Foa R, Blechman J, Nakamura T, et al. Upregulation of Meis1 and HoxA9 in acute lymphocytic leukemias with the t(4 : 11) abnormality. Oncogene 2001 Feb 15;20(7):874-878.

151. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. 1995 May 25;23(10):1686-1690.

152. Samuels ML, Weber MJ, Bishop JM, McMahon M. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Mol.Cell.Biol. 1993 Oct;13(10):6241-6252.

153. Sykes DB, Kamps MP. Estrogen-regulated conditional oncoproteins: tools to address open questions in normal myeloid cell function, normal myeloid differentiation, and the genetic basis of differentiation arrest in myeloid leukemia. Leuk.Lymphoma 2003 Jul;44(7):1131-1139.

154. Stuart ET, Gruss P. PAX: developmental control genes in cell growth and differentiation. Cell Growth Differ. 1996 Mar;7(3):405-412.

155. Hill MA, Schedlich L, Gunning P. Serum-induced signal transduction determines the peripheral location of beta-actin mRNA within the cell. J.Cell Biol. 1994 Sep;126(5):1221-1229.

156. Reiss K, Ferber A, Travali S, Porcu P, Phillips PD, Baserga R. The protooncogene c-myb increases the expression of insulin-like growth factor 1 and insulin-like growth factor 1 receptor messenger RNAs by a transcriptional mechanism. Cancer Res. 1991 Nov 1;51(21):5997-6000.

157. Reiss K, Porcu P, Sell C, Pietrzkowski Z, Baserga R. The insulin-like growth factor 1 receptor is required for the proliferation of hemopoietic cells. Oncogene 1992 Nov;7(11):2243-2248.

158. Travali S, Reiss K, Ferber A, Petralia S, Mercer WE, Calabretta B, et al. Constitutively expressed c-myb abrogates the requirement for insulinlike growth factor 1 in 3T3 fibroblasts. Mol.Cell.Biol. 1991 Feb;11(2):731-736.

159. Quentmeier H, Dirks WG, Macleod RA, Reinhardt J, Zaborski M, Drexler HG. Expression of HOX genes in acute leukemia cell lines with and without MLL translocations. Leuk.Lymphoma 2004 Mar;45(3):567-574.

160. Wu KD, Zhou L, Burtrum D, Ludwig DL, Moore MA. Antibody targeting of the insulin-like growth factor I receptor enhances the anti-tumor response of multiple myeloma to chemotherapy through inhibition of tumor proliferation and angiogenesis. Cancer Immunol.Immunother. 2007 Mar;56(3):343-357.

161. Shimon I, Shpilberg O. The insulin-like growth factor system in regulation of normal and malignant hematopoiesis. Leuk.Res. 1995 Apr;19(4):233-240.

162. Miller BS, Yee D. Type I insulin-like growth factor receptor as a therapeutic target in cancer. Cancer Res. 2005 Nov 15;65(22):10123-10127.

163. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood 1992 Sep 1;80(5):1207-1212.

164. Kooijman R, Willems M, De Haas CJ, Rijkers GT, Schuurmans AL, Van Buul-Offers SC, et al. Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. Endocrinology 1992 Nov;131(5):2244-2250.

165. Izon DJ, Rozenfeld S, Fong ST, Komuves L, Largman C, Lawrence HJ. Loss of function of the homeobox gene Hoxa-9 perturbs early T-cell development and induces apoptosis in primitive thymocytes. Blood 1998 Jul 15;92(2):383-393.

166. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat.Genet. 2002 Jan;30(1):41-47.

167. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer.Cell. 2002 Mar;1(2):133-143.

168. Hess JL. Mechanisms of transformation by MLL. Crit.Rev.Eukaryot.Gene Expr. 2004;14(4):235-254.

169. Whelan JT, Ludwig DL, Bertrand FE. HoxA9 induces insulin-like growth factor-1 receptor expression in B-lineage acute lymphoblastic leukemia. Leukemia 2008 Jun;22(6):1161-1169.

170. Kooijman R. Regulation of apoptosis by insulin-like growth factor (IGF)-I. Cytokine Growth Factor Rev. 2006 Aug;17(4):305-323.

171. Srinivasa SP, Doshi PD. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways cooperate in mediating cytokine-induced proliferation of a leukemic cell line. Leukemia 2002 Feb;16(2):244-253.

172. White MK, McCubrey JA. Suppression of apoptosis: role in cell growth and neoplasia. Leukemia 2001 Jul;15(7):1011-1021.

173. Bachmann M, Moroy T. The serine/threonine kinase Pim-1. Int.J.Biochem.Cell Biol. 2005 Apr;37(4):726-730.

174. Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ. Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. FEBS Lett. 2004 Jul 30;571(1-3):43-49.

175. Leverson JD, Koskinen PJ, Orrico FC, Rainio EM, Jalkanen KJ, Dash AB, et al. Pim-1 kinase and p100 cooperate to enhance c-Myb activity. Mol.Cell 1998 Oct;2(4):417-425.

176. Holder S, Zemskova M, Zhang C, Tabrizizad M, Bremer R, Neidigh JW, et al. Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase Mol.Cancer.Ther. 2007 Jan;6(1):163-172.

177. Nowell PC. The clonal evolution of tumor cell populations. Science 1976 Oct 1;194(4260):23-28.

178. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000 Jan 7;100(1):57-70.

179. FOULDS L. The experimental study of tumor progression: a review. Cancer Res. 1954 Jun;14(5):327-339.

180. Weston K. Reassessing the role of C-MYB in tumorigenesis. Oncogene 1999 May 13;18(19):3034-3038.

181. Lipsick JS, Wang DM. Transformation by v-Myb. Oncogene 1999 May 13;18(19):3047-3055.

182. Berge T, Bergholtz SL, Andersson KB, Gabrielsen OS. A novel yeast system for in vivo selection of recognition sequences: defining an optimal c-Myb-responsive element. Nucleic Acids Res. 2001 Oct 15;29(20):E99.

183. Cooke DW, Bankert LA, Roberts CT,Jr, LeRoith D, Casella SJ. Analysis of the human type I insulin-like growth factor receptor promoter region. Biochem.Biophys.Res.Commun. 1991 Jun 28;177(3):1113-1120.

184. Werner H, Bach MA, Stannard B, Roberts CT,Jr, LeRoith D. Structural and functional analysis of the insulin-like growth factor I receptor gene promoter. Mol.Endocrinol. 1992 Oct;6(10):1545-1558.

185. Gibson LF. Survival of B lineage leukemic cells: signals from the bone marrow microenvironment. Leuk.Lymphoma 2002 Jan;43(1):19-27.

186. LeBien TW. Fates of human B-cell precursors. Blood 2000 Jul 1;96(1):9-23.

187. Clark R, Strasser J, McCabe S, Robbins K, Jardieu P. Insulin-like growth factor-1 stimulation of lymphopoiesis. J.Clin.Invest. 1993 Aug;92(2):540-548.

188. Freund GG, Kulas DT, Way BA, Mooney RA. Functional insulin and insulin-like growth factor-1 receptors are preferentially expressed in multiple myeloma cell lines as compared to B-lymphoblastoid cell lines. Cancer Res. 1994 Jun 15;54(12):3179-3185.

189. Montecino-Rodriguez E, Clark RG, Powell-Braxton L, Dorshkind K. Primary B cell development is impaired in mice with defects of the pituitary/thyroid axis. J.Immunol. 1997 Sep 15;159(6):2712-2719.

190. Kimata H, Yoshida A. Effect of growth hormone and insulin-like growth factor-I on immunoglobulin production by and growth of human B cells. J.Clin.Endocrinol.Metab. 1994 Mar;78(3):635-641.

191. Baudler S, Baumgartl J, Hampel B, Buch T, Waisman A, Snapper CM, et al. Insulin-like growth factor-1 controls type 2 T cell-independent B cell response. J.Immunol. 2005 May 1;174(9):5516-5525.

192. Lehne G, Grasmo-Wendler UH, Berner JM, Meza-Zepeda LA, Adamsen BL, Flack A, et al. Upregulation of stem cell genes in multidrug resistant K562 leukemia cells. Leuk.Res. 2009 Apr 23.

193. Rubini M, Hongo A, D'Ambrosio C, Baserga R. The IGF-I receptor in mitogenesis and transformation of mouse embryo cells: role of receptor number. Exp.Cell Res. 1997 Feb 1;230(2):284-292.

194. Bertrand FE, Spengemen JD, Shelton JG, McCubrey JA. Inhibition of PI3K, mTOR and MEK signaling pathways promotes rapid apoptosis in B-lineage ALL in the presence of stromal cell support. Leukemia 2005 Jan;19(1):98-102.

195. Stirewalt DL, Meshinchi S, Radich JP. Molecular targets in acute myelogenous leukemia. Blood Rev. 2003 Mar;17(1):15-23.

196. Birkenkamp KU, Geugien M, Lemmink HH, Kruijer W, Vellenga E. Regulation of constitutive STAT5 phosphorylation in acute myeloid leukemia blasts. Leukemia 2001 Dec;15(12):1923-1931.

197. Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. Blood 2000 Jun 15;95(12):3765-3770.

198. Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. CA Cancer.J.Clin. 2009 Mar-Apr;59(2):111-137.

199. Sayyah J, Sayeski PP. Jak2 inhibitors: rationale and role as therapeutic agents in hematologic malignancies. Curr.Oncol.Rep. 2009 Mar;11(2):117-124.

200. Wang H, Patel V, Miyazaki H, Gutkind JS, Yeudall WA. Role for EPS8 in squamous carcinogenesis. Carcinogenesis 2009 Jan;30(1):165-174.

201. Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. Biochem.Biophys.Res.Commun. 2009 Jul 31;385(3):307-313.

202. Reno EM, Haughian JM, Jackson TA, Thorne AM, Bradford AP. c-Jun N-terminal kinase regulates apoptosis in endometrial cancer cells. Apoptosis 2009 Jun;14(6):809-820.

203. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, et al. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer.Cell. 2008 Mar;13(3):235-248.

204. Mani S, Wang C, Wu K, Francis R, Pestell R. Cyclin-dependent kinase inhibitors: novel anticancer agents. Expert Opin.Investig.Drugs 2000 Aug;9(8):1849-1870.

205. Liu X, Gourley ES, Lamb JD, Grand CL, Lloyd MA, Warner SL, Wolfe B, Bearss DJ, Vankayalapati H. Discovery of SGI-1776, a potent and selective Pim-1 kinase inhibitor. [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 2013.