Investigation of Genes that are Regulated During Erythroid Differentiation

By

Kendra L. Stetler

A THESIS SUBMITTED IN CONFORMITY WITH THE REQUIREMENTS OF THE DEGREE OF MASTER OF SCIENCE, GRADUATE DEPARTMENT OF MEDICAL BIOPHYSICS UNIVERSITY OF TORONTO

© Copyright by Kendra L. Stetler 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-58695-2
To my family, who always encouraged me to live life to the fullest, pursue my dreams and above all, taught me to never give up.
Abstract

Investigation of Genes that are Regulated During Erythroid Differentiation

Kendra Stetler

Master of Science, 2001

Department of Medical Biophysics

University of Toronto

In this thesis we attempted to identify novel genes that may play a role in erythroid proliferation and differentiation using the unique erythroleukemia cell line HB60-5. By utilizing the approach of subtractive hybridization, I have isolated clone 5, a gene whose expression is regulated during erythroid differentiation. Expression of clone 5 is high in HB60-5 cells and decreases as cells are induced to undergo terminal differentiation. Clone 5 appears to be widely expressed in murine and human hematopoietic and non-hematopoietic cell lines. However expression of clone 5 appears to be minimal in normal murine tissue with the exception of brain. A decrease in clone 5 expression was also observed during terminal differentiation of various hematopoietic precursors. Therefore clone 5 may represent a novel gene that plays a role in the transition from cellular proliferation to differentiation. Characterization of this gene may provide further insights into the mechanisms of hematopoiesis and malignant transformation.
Acknowledgements

I would like to thank Dr. Yaacov Ben-David for giving me the opportunity to grow and develop as a scientific researcher. I would like to acknowledge him for his insight and direction during the course of these studies. I would also like to thank the members of my committee, Dr. Cheryl Arrowsmith and Dr. Corrine Lobe, for their invaluable advice and guidance along the way. I would like to acknowledge the research efforts of Dr. Jeff Howard and Dr. Maria Rosa Bani for establishing the cell line and groundwork for this project. I wish to acknowledge and thank my laboratory colleagues for their never-ending support and assistance, particularly Dr. Brian Pak, Amandine Truong, Wendy Chu, Ken Wong and Dr. Nina Jones. Finally I would like to express my gratitude to my family and friends, especially Mohan Krishnamoorthy, Joanne Yu, Danielle Hanna, Adrienne Bartlett, Gail Hannah and the Drope clan for their constant support and encouragement during my time at Sunnybrook Hospital.
# Table of Contents

Dedication ii  
Abstract iii  
Acknowledgements iv  
Table of Contents v  
List of Figures viii  
List of Tables ix  
List of Abbreviations x  

1 Introduction 1  

1.1 Prevalence of Cancer ................................................................. 2  
1.2 Causes of Cancer ..................................................................... 3  
1.3 The Multistage Nature of Cancer ........................................ 4  
  1.3.1 Oncogenes .................................................................. 5  
  1.3.2 Tumor Suppressor Genes ................................................. 6  
1.4 Cellular Development and Homeostasis .............................. 6  
1.5 Cellular Growth and Proliferation ........................................ 7  
1.6 Apoptosis ........................................................................... 8  
1.7 Cellular Differentiation ............................................................ 10  
  1.7.1 Erythroid Differentiation ............................................... 13  
1.8 Growth Factors and Signal Transduction Pathways affecting Erythroid Development .................................................. 14  
  1.8.1 Erythropoietin Receptor ............................................... 16
2 Identification of a novel gene, Clone 5, that appears to be regulated during erythroid differentiation

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods
  2.3.1 Cell Lines
  2.3.2 PCR-Select cDNA Subtractive Hybridization
  2.3.3 cDNA Library Screening
  2.3.4 RNA Extraction and Northern Blotting
  2.3.5 Molecular Hybridization
  2.3.6 cDNA Probes
  2.3.7 Vectors and Transfection

2.4 Results and Discussion
  2.4.1 Isolation of differentially expressed genes
  2.4.2 Expression of Clone 5 during differentiation of erythroleukemia cells
  2.4.3 Clone 5 expression may be enhanced in transformed cell lines
  2.4.4 Clone 5 expression exhibits multiple transcripts
2.4.5 Isolation of cDNA corresponding to clone 5..........................45

2.4.6 Sequence Analysis of the clone 5 transcript...........................47

2.5 Acknowledgements......................................................................53

3 General Discussion and Future Perspectives....................................54

List of Publications............................................................................60

Bibliography......................................................................................61
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>View of the hematopoietic development system</td>
<td>12</td>
</tr>
<tr>
<td>1.2</td>
<td>The Friend retroviral complex</td>
<td>19</td>
</tr>
<tr>
<td>2.1</td>
<td>Expression of clone 5 in erythroleukemia cells</td>
<td>38</td>
</tr>
<tr>
<td>2.2</td>
<td>Expression of clone 5 in hematopoietic lineages</td>
<td>39</td>
</tr>
<tr>
<td>2.3</td>
<td>Expression of clone 5 in normal murine tissue</td>
<td>41</td>
</tr>
<tr>
<td>2.4</td>
<td>Expression of clone 5 in transformed murine hematopoietic cell lines</td>
<td>42</td>
</tr>
<tr>
<td>2.5</td>
<td>Expression of clone 5 in transformed human hematopoietic and human tissue tumor cell lines</td>
<td>44</td>
</tr>
<tr>
<td>2.6</td>
<td>Schematic view of cDNA fragments corresponding to clone 5</td>
<td>46</td>
</tr>
<tr>
<td>2.7</td>
<td>Comparison of clone 5 and Btf nucleotide sequences</td>
<td>48</td>
</tr>
<tr>
<td>2.8</td>
<td>Predicted amino acid sequences for clone 5 and protein domains</td>
<td>50</td>
</tr>
</tbody>
</table>
List of Tables

2.1 Summary of isolated cloned sequences from subtractive hybridization and corresponding gene sequences..........................................................35
List of Abbreviations

α-MEM ............................................................alpha minimal essential medium
ATPase ............................................................adenosine triphatase
BFU-E ..............................................................burst forming unit-erythroid
bp .................................................................base pair
Btf .................................................................Bcl-2 transcription factor
bZip ...............................................................basic leucine zipper
CdCl₂ .............................................................cadmium chloride
cDNA ............................................................complimentary deoxyribonucleic acid
CFU-E ............................................................colony forming unit-erythroid
cpm ...............................................................counts per minute
CO₂ .............................................................carbon dioxide
DEPC ............................................................diethylpyrocarbonate
DMSO ..........................................................dimethyl sulfoxide
DNA ............................................................deoxyribonucleic acid
dCTP ............................................................deoxycytosine triphosphate
E .................................................................erythrocyte
EDTA ............................................................ethylenediamine-tetra acetate
env .............................................................envelope
Epo  ................................................................. erythropoietin
Epo-R  ............................................................ erythropoietin-receptor
EST  ..................................................................... expressed tag sequence
FBS  ...................................................................... fetal bovine serum
Fli-1  ................................................................. Friend leukemia integration 1
F-MuLV  ............................................................ Friend murine leukemia virus
FV  ........................................................................ Friend virus
GAPDH  ............................................................. glyceraldehyde-3-phosphate dehydrogenase
GFP  ....................................................................... green fluorescence protein
gp55  ....................................................................... glycoprotein 55 kDa
HMG  ....................................................................... high mobility group protein
IAPs  ................................................................. inhibitors of apoptosis
IAP  ....................................................................... intracisternal A-particle
IGF-I  ................................................................. insulin-like growth factor 1
IL-3  ........................................................................ interleukin-3
Jak-2  .............................................................. Janus kinase/Just another kinase 2
kDa  ................................................................. kiloDaltons
kb  ........................................................................ kilobases
M  ........................................................................ molar
Mac  ................................................................. macrophage
MAP kinase ....................................................... mitogen activated protein kinase
Meg  .......................................................................... megakaryocyte
mF  .......................................................................... millifarads
ml ................................................................. millilitre
mM ................................................................. millimolar
mRNA .............................................................. messenger ribonucleic acid
MT ................................................................. metallothionein
NaCl ............................................................... sodium chloride
Neut ............................................................... neutrophil
PBS ............................................................... phosphate buffered saline
PCR ............................................................... polymerase chain reaction
PEG .............................................................. polyethylene glycol
PI3K .............................................................. phosphatidylinositol 3-kinase
pMac .............................................................. precursor of macrophage
pMeg .............................................................. precursor of megakaryocyte
pNeut ............................................................ precursor of neutrophil
RNA ............................................................... ribonucleic acid
RT-PCR ......................................................... reverse transcription-PCR
S ................................................................. subunit
SCF ............................................................... stem cell factor
SDS ............................................................... sodium dodecyl sulphate
SSC ............................................................... standard sodium citrate
SFFV ............................................................. spleen focus forming virus
SHP-1 ............................................................ Src-homology-2 phosphatase
Stat .............................................................. signal transducing and activators of transcription
µg ................................................................. microgram
μl ........................................................................................................... microlitre
μM ............................................................................................................ micromolar
UTR .......................................................................................................... untranslated region
V ............................................................................................................... volts
vol ........................................................................................................... volume
wt ........................................................................................................... weight
Zn$_2$SO$_4$ .............................................................................................. zinc sulphate
Chapter 1:

Introduction
1.1 Prevalence of Cancer

The fight against cancers continues to be an ongoing battle despite the remarkable discoveries and progress made over the last century. The Canadian and American Cancer Societies report that over the past seventy years the incidence rate of all cancers combined has risen continuously, and only in the last eight years have cancer incidence rates leveled off or decreased slightly (Wingo et al., 1998). The predominant forms of cancer are prostate, breast, lung and colorectal which alone account for approximately 50% of all new cases reported each year.

The mortality rate during the same time however, has remained relatively constant and more recently displays a slight decrease in trend, therefore demonstrating that the overall prevalence of cancer has increased (National Cancer Institute of Canada, 2000). How can we explain this increase in the prevalence of cancer along side the strides that have been made in cancer research?

Many factors may be attributable including increasing population size, longer life span of the human race; as well as increased sensitivity of detection methods and thus documentation. In accordance with the first two points, it is then surprising that the mortality rate has not risen along with incidence rates. This latter fact helps to illustrate the impact the scientific community has made in dissecting the events associated with the disease to provide a better understanding and thus course of action against cancer. This sustained mortality rate could perhaps be attributed to the heightened awareness of the general public resulting in decreased exposure to cancer causing agents such as tobacco. Once again increased sensitivity of detection methods allows doctors to discover lesions at an earlier and more treatable stage (Wingo et al., 1998). In some cases the success of
treatment has increased along side incidence rate, therefore demonstrating an overall decrease in the threat of certain types of cancers such as breast, prostate, colorectal and some lymphomas. Still other types of cancer including lung, ovarian, adult leukemias and melanomas have not followed this increase in therapeutic response. Therefore, researchers are still working to understand the molecular events leading to tumor progression and metastasis as well as finding adequate treatment.

The basis for all cancers is preclominantly the same and it is this basis of the genetic and molecular events associated with and leading up to tumor progression and development that scientists strive to understand. As we learn more about cancer and its complexity, the focus for treatment of this disease has shifted from finding “a cure” to one of prevention strategies (Hanahan and Weinburg, 2000, in review). It is the hope that further knowledge in turn will lead to increasing prevention strategies along with less invasive but more favorable treatment of the disease to improve the overall quality of life for cancer patients.

1.2 Causes of Cancer

The question of what causes a normal cell to develop into a malignant one has not been completely answered. It was Knudson in 1971 who initially proposed the theory of the “two hit” hypothesis suggesting that in order for a cell to develop into a pre-cancerous stage it had to acquire and sustain at least two genetic mutations that confer a selective growth advantage upon the cell. Furthermore, it has since been suggested that a number of factors could be attributable to causing these genetic mutations, some being genetically inherited while others involve exposure to certain cancer-causing agents.
One of the most predominant factors that is genetically innate, and therefore beyond one’s control, is age. In many cancers there is a direct correlation of incidence with age, therefore the longer a person lives, the more time they have to acquire genetic mutations. For this reason, cancer is predominantly a disease of the elderly (Ramel, 1992, in review). Hereditary factors include an inherited predisposition to certain genetic mutations that have been linked to some cancers. These can include mutations in particular genes giving rise to inappropriate expression, abnormalities in chromosome number or translocation and defects in genes responsible for DNA repair mechanisms (Yeung et al., 1999).

Exposure to certain cancer causing agents can be controlled thereby minimizing the risk of acquiring mutations and developing cancer. These agents are termed carcinogens and include chemicals (eg. tobacco) (Rice and Wilbourn, 2000), biological agents (eg. viruses) and physical mutagens (radiation) (Burkart et al., 1999). Recently, more speculative carcinogens include certain food additives and preservatives that may be linked to certain cancers (Ferguson, 1999). Inherited genetic mutations and exposure to carcinogens do not by any means assure development of cancer, they only increase one’s risk of acquiring initial and/or further genetic alterations that could lead to cellular transformation and tumor progression.

1.3 The Multistage Nature of Cancer

The nature by which a normal human cell progresses toward a malignant phenotype is complex and still not fully understood. It is known that the cancer disease is a multistage phenomenon in which the events allowing this progression to take place, involve both molecular and biochemical changes within the cell (Nowell, 1976). These changes cause a
disruption of the normal cellular mechanisms that provide a homeostatic balance between cell growth, cell differentiation and cell death or apoptosis (Hanahan and Weinburg, 2000, in review). Thus the transformation of a normal cell to a malignant one results from the accumulation of genetic mutations within genes controlling these processes and thereby altering the growth properties of the cell. Since there are a number of signaling pathways involved in regulating each of these cellular mechanisms there are numerous targets that can potentially acquire genetic mutations by cancer causing agents.

It has been shown that in order for a cell to progress towards a tumorigenic phenotype the cell requires at least two genetic alterations which cause irreversible DNA damage and thus provide the cell with a selective growth advantage (Feinberg, 1994; Jackson, 1985). Generally, these mutations cause activation of an oncogene and disruption of a tumor suppressor gene.

**1.3.1 Oncogenes**

One detectable alteration or mutation is usually associated with increased activation of a region of DNA within the genome which can result in constitutive expression of the affected loci that may contain one or more genes (Hunter, 1991, in review). In an inactivated state these genes are known as proto-oncogenic and upon activation are termed oncogenes. These genes may be naturally present in the genome but only cause uncontrollable growth when constant activation occurs due to a mutation. This provides the cell with a continual growth signal and the means to exit a quiescent state, entering into the cell cycle to divide indefinitely as with the extensively studied oncogenes c-myc and ras.
This mutation alone however is not sufficient to cause malignancy as a cell’s repair mechanism is still intact. Therefore any mutation generally would be detected and corrected. However, increased growth can hinder the efficacy of repair mechanisms and thus increase susceptibility of the cell to further mutations.

1.3.2 Tumor Suppressor Genes

Generally, a second detectable mutation thus targets the cell’s detection and repair mechanism and is generally associated with the inactivation of tumor suppressor genes. The function of such genes is to protect the cell from uncontrollable growth by temporarily suppressing entrance into cell cycle. This allows damaged DNA to be repaired before the next round of DNA replication so that mutations are not passed on to daughter cells and amplified (Yeung, et al., 1994).

The result of these two mutations is a cell with continuous growth signals and no way to retard cellular growth or repair damaged DNA before the cell enters the next cycle of replication. This provides mutated cells with the capacity to expand exponentially to surpass the general cell population to form a tumorigenic cellular mass.

1.4 Cellular Development and Homeostasis

Cancers develop as a result of mutations that arise hindering a cell’s growth and developmental kinetics. The ability of a cell to develop and function properly is no simple task. It requires the intricate control and coordination of many cellular processes including cell proliferation, differentiation and apoptosis. These three processes are carefully regulated in order to maintain a delicate balance between the gain of newly developing
progenitor cells and loss of cells due to differentiation and apoptosis (Cross and Dexter, 1991, in review).

This cellular balance, known as homeostasis, is an organism's way of ensuring that there are a controlled number of functionally competent cells at any time. Regulation of cellular processes are mediated by appropriately timed expression and coordination of numerous genes and their protein products in order to create a complex protein network. With so many genes involved in regulating normal cellular functions, this poses numerous targets for genetic mutations leading to tumorigenicity (Hanahan and Weinberg, 2000, in review).

Disruption of proliferation, differentiation and/or apoptosis, as a result of genetic mutations, causes an upset in the homeostatic balance resulting in fewer developmentally mature cells and/or an increase in progenitor cells. Tumorogenesis or cancer is a perfect example of this upset and therefore researchers strive to unravel the basic molecular events controlling these processes in order to obtain a better understanding of tumor progression.

1.5 Cellular Growth and Proliferation

Proliferation is perhaps one of the first cellular mechanisms that is evident during embryonic development. It is the process by which one cell can increase in size, divide and eventually give rise to daughter cells. Thus the cell enters the cell cycle in which its DNA is replicated and the cell then undergoes mitosis to produce two genetically identical cells. The cell cycle and thus proliferation is initiated in response to various external stimuli. Some of these factors provide positive growth signals including hormones and growth
factors, while others have an anti-proliferative effect. (Hanahan and Weinberg, 2000, in review).

Although the majority of cellular growth and proliferation occurs during embryonic development, in certain tissues it is an ongoing process throughout life. For instance, in adults, tissues or systems that have regenerative capabilities continue to undergo proliferation. Some occur on a continual basis including the blood system, dermis and intestinal lining (Cross and Dexter, 1991, in review) while others do so at specific times such as during wound healing or an immune response. In these scenarios cellular proliferation is beneficial to an organism, as it is required to maintain tissue homeostasis.

Cellular proliferation in adults can also occur in a number of diseases, including cancer, where cellular homeostasis is disrupted and the cell no longer responds to anti-proliferative factors. In this case, a single cell obtains the capability to proliferate continually despite growth and/or inhibitory signals, which can have detrimental effects. The result is uncontrolled proliferation and clonal expansion of cells that are tumorigenic. These cells continue to increase in mass and eventually invade others tissues, a process known as metastasis, which disrupts normal cellular and tissue functions (Hanahan and Weinberg, 2000, in review).

1.6 Apoptosis

Apoptosis is yet another process employed by all multi-cellular organisms in cellular development, homeostasis, defense mechanisms, and aging (Vaux and Strasser, 1996, in review). The most common scenario in which apoptosis is exercised is one in which a cell has completed its role and is no longer required. It has been proposed that cells possess
some sort of internal monitoring system to aid in detection of aged, non-functioning or damaged cells. The cell then activates pro-apoptotic genes in particular pathways providing a signal for the cell to undergo programmed cell death or apoptosis (Evan and Littlewood, 1998).

Characteristics of apoptosis includes membrane disruption, break down of cytoplasmic and nuclear skeletons, chromosomal degradation and nuclear fragmentation. This causes shrinkage and shriveling of the cell membrane, which is then engulfed by neighboring cells (Wyllie et al., 1980). This removal of cells is the body’s way to maintain a homeostatic balance in cellular number and tissue mass (Kasof, et al., 1999).

Another circumstance under which a cell will execute this process is to rid an organism of damaged cells that have been unable to repair themselves. This alleviates the potential for a cell with altered or damaged DNA to enter the next cell cycle and replicate thereby passing on and eventually amplifying the mutated cellular DNA as is the case with many diseases including cancer (Song and Steller, 2000, in review). Therefore apoptosis is a self-administered protection mechanism within the cell in order to maintain genetic integrity.

There are two main classes of genes involved in apoptosis; those that promote apoptosis aptly termed “pro-apoptotic”, and those that act to protect the cell from death, the “anti-apoptotic” genes. The list of apoptotic genes presently is fairly extensive and continues to increase. Many gene families have been isolated such as the inhibitors of apoptosis (IAPs), the caspases, Apafs and Bcl-2 family members. Perhaps one of the most well studied families of apoptotic genes is the Bcl-2 family. This family is unique in that it includes both pro-apoptotic genes such as Bad, Bax, Bak, Bid, Bim and anti-apoptotic genes including Bcl-2, Bcl-xL, and Bcl-W (Knudson and Korsmeyer, 1997).
At any given time within the cell, both pro-apoptotic and anti-apoptotic genes are being expressed. It is believed that the regulation of apoptosis depends on a precarious balance between these two types of apoptotic factors. Therefore, gene activation is not the significant factor governing cell fate but rather the proportion of pro-apoptotic versus anti-apoptotic proteins present that determines whether a cell will survive or die (Kasof et al., 1999). Hence, only when the ratio of pro-apoptotic versus anti-apoptotic proteins is high; will the cell be subjected to a fate of programmed cell death. The converse is also true (Knudson, and Korsmeyer, 1997; Pellegrini and Strasser, 1999, in review).

Not surprisingly then, mutations that inhibit genes involved in apoptosis and their respective signal transduction pathways can alter a cell’s innate monitoring system and thus their defense and survival mechanisms. A lack of control in this facet can cause the cell to become vulnerable to further mutations and uncontrolled growth such as in the case of cancer (Stambolic et al., 1999, in review).

1.7 Cellular Differentiation

The majority of cellular differentiation takes place during embryogenesis and shortly thereafter during tissue and organ development. As a cell develops and differentiates, certain molecules controlling cellular development act as signals to direct its progression along particular biochemical pathways towards its fated cell type. To allow this development to occur the cell must undergo certain genetic and molecular alterations, involving regulation of numerous genes, to accommodate these signals and thus allowing the cell to develop to a mature state.
As some tissues have the capability to continually regenerate in response to intracellular signaling, there is also a need for differentiation after embryonic development. Hematopoiesis is an excellent example of a system that is continuously undergoing self-renewal and differentiation to produce new progenitor cells that divide and give rise to a number of functionally mature blood cells (Socolovsky et al., 1998, in review). The hematopoietic system therefore, provides an excellent model in which cellular differentiation can be studied.

Definitive hematopoiesis occurs post-natally in the bone marrow as well as the thymus and spleen in mice (Shivdasani, and Orkin, 1996, in review). It begins with a single pluripotent stem cell that is capable of self-renewal but which can also divide and give rise to a totipotent lymphoid and myeloid stem cell. The lymphoid stem cell in turn develops into B-and T-lymphocytes whereas the myeloid stem cell can give rise to the remainder of the blood cells; monocytes-macrophage, basophils, neutrophils, eosinophils, platelets and erythrocytes or red blood cells (Figure 1.1) (Sachs, 1987, in review; Socolovsky et al., 1998, in review). As a cell progresses further and further along a developmental pathway it becomes more committed to a particular cell lineage thus its self-renewal capacity becomes more limited at each stage. Therefore, once a cell enters the lymphoid lineage it is predestined to develop into a mature lymphocyte and can no longer give rise to any cells of the myeloid lineage. The converse holds true as well, demonstrating that the process of differentiation is irreversible once a cell reaches a certain stage of commitment (Brady et al., 1995).
Figure 1.1 Schematic diagram of hematopoiesis. The hematopoietic cell lineage is an example of a cellular system that is constantly undergoing cellular proliferation, differentiation and apoptosis in order to maintain a homeostatic balance and defined number of functionally circulating cells.
Throughout hematopoiesis, the cell depends on certain growth factors and cytokines. It is somewhat controversial as to whether these factors provide signals, which direct the cell towards a certain lineage and cell type (Sieweke and Graf, 1998, in review; Socolovsky et al., 1998, in review) or simply maintain cell viability and proliferative capacity (Orkin and Zon, 1997, in review). Some of these factors are present in more than one lineage while others are lineage-specific factors. It has been widely proposed that lineage-specific growth factors or cytokines are essentially the final determinant that directs a cell to follow a specific pathway to a stage where it can only give rise to one cell type (Mayani et al., 1993).

### 1.7.1 Erythroid Differentiation

The process of erythropoiesis is the development of mature erythrocytes. It begins with the pluripotent stem cell giving rise to the myeloid stem cell and therefore is part of the myeloid lineage. During embryogenesis this process occurs in the fetal liver but in the adult it takes place in the bone marrow (Shivdasani and Orkin, 1996, in review). Later on, these progenitor cells migrate to and accumulate in the spleen where they are stored until needed. In order to keep these cells in their progenitor state, growth factors are secreted by the spleen. When these cells are needed they are then directed to develop further into erythroblasts in response to lineage-specific growth factors and finally become functionally mature enucleated erythrocytes that are released into the blood stream to carry out their role of transporting oxygen throughout the body.

Proliferation and differentiation of erythroid progenitor cells is regulated by certain cytokines including interleukin-3 (IL-3) and insulin-like growth factor I (IGF-I) which are common to many hematopoietic cell lineages. More predominantly, stem cell factor (SCF)
and erythropoietin (Epo) are two growth factors that have been shown to play a key role in erythropoiesis (Krantz, 1991, in review).

Initially the myeloid stem cell requires IL-3 and SCF, the ligand that binds to the c-Kit cell surface receptor. SCF provides a signal that directs the cell to proliferate and divide in order to produce a certain number of erythroid precursor cells. The cell proceeds through the pathway developing into an erythroid burst forming unit (BFU-E) then an erythroid colony forming unit (CFU-E). Later on in the pathway the cell responds to SCF, IGF-I and the lineage-specific growth factor Epo, which further directs the cell to develop into a pro-normoblast, normoblast, and a reticulocyte or erythroid progenitor cell (Dai, et al., 1997; Klingmüller, 1997, in review).

The complexity of differentiation therefore provides many opportunities for genetic mutations and dysfunction to occur. This process is often de-regulated in the development of cancer. Therefore a better understanding of the mechanism regulating both proliferation and differentiation of cells could be of great biological and clinical importance (Osawa et al., 1996).

1.8 Growth Factors and Signal Transduction Pathways affecting Erythroid Development

As described above, erythropoiesis is dependent upon stimulation with certain growth factors. These factors must transduce signals throughout the cell, at appropriate times to regulate as cell survival, proliferation and differentiation. These signals direct the cell as to which process to initiate in order to maintain a homeostatic balance between proliferating progenitor cells and functioning terminally differentiated cells (Miyajima et al., 1999, in review).
When a signaling pathway becomes activated and recruits proteins downstream of the receptor, these molecules in turn regulate and activate other target genes and proteins. Transcription factors are proteins that, once activated, can translocate to the nucleus of a cell and bind to DNA. The role of a transcription factor is to bind to the promoter region of target genes, thereby activating transcription of these genes. The increased expression of these target genes may affect other genes and/or signaling pathways. Conversely, activated proteins and transcription factors may also work to inhibit the expression and function of other genes and/or proteins that may in turn suppress a particular signaling pathway and cellular function thus allowing others to become dominant.

Activated signaling pathways, therefore, provides a signal to the cell to carry out a particular function such as to grow and proliferate, undergo differentiation and/or apoptosis. By determining which genes are regulated during various cellular processes we gain insight into the pathways and proteins that control each process and thus the genes that may be mutated when a process no longer functions properly as in tumor progression.

In the context of this thesis we are interested in the cell surface receptors and signal transduction pathways that are activated in erythroid progenitor cells during proliferation and differentiation. Two major players in the regulation of erythroid growth and differentiation are Epo and SCF along with their respective receptors (Quang et al., 1997). Mutations in these receptors are associated with a decreased number of erythroid progenitor cells and thus a decline in functionally mature erythrocytes (Ohneda et al., 1992; Wu et al., 1995). These two receptors and the subsequent role of their respective signaling pathways are described in further detail in the following sections.
1.8.1 **Erythropoietin Receptor**

The erythropoietin receptor (Epo-R) is one of the predominant cell surface receptors that can initiate intracellular signaling pathways within an erythroid cell including the Shc/Ras-MAP kinase, phosphatidylinositol 3-kinase (PI3K) and Stat (Lacombe and Mayeux, 1999, in review) signaling pathways. The Epo-R becomes activated upon the binding of its ligand Epo, which then facilitates homo-dimerization of two Epo-R molecules (Krantz, 1991, in review). The Epo-R has associated to its intracellular portion a molecule known as Janus kinase 2 (Jak2) which contains tyrosine kinase activation domains. Once homo-dimerization of the receptor occurs, the Jak2 molecules auto-phosphorylate one another, causing them to become activated. This allows recruitment of other molecules such as Stat5, which in turn becomes phosphorylated, initiating a signaling cascade. Stat5 is a transcription factor and upon its activation by Jak2, it translocates to the nucleus where it regulates transcription of certain genes (Witthuhn et al., 1993; Penta and Sawyer, 1995).

This cascade involving Jak2/Stat5 has been reported to be involved in initiating both cellular proliferation (Damon et al., 1995) and differentiation (Iwatsuki, 1997; Zochodne et al., 2000). While other pathways triggered by Epo induction have been shown to be involved in cell survival and proliferation (Damen et al. 1995; Miura et al., 1994). Therefore, the signaling cascades that may be responsible in controlling cellular differentiation in erythroid cells remains somewhat ambiguous at present.

1.8.2 **C-Kit Receptor**

The c-Kit receptor is another prominent receptor found on the surface of erythroid cells although its expression is not confined to the erythroid lineage. The c-Kit receptor
becomes activated upon binding of its ligand SCF. This interaction facilitates homodimerization of the receptor and intrinsic tyrosine kinase activity allows auto-phosphorylation in order to recruit and activate downstream target proteins (Majumder et al., 1988). The SCF/c-Kit receptor interaction has been shown to activate a downstream signaling cascade involving the Shc/Ras-MAP kinase pathway, which leads to cellular proliferation (Zochodne et al., 2000). In addition it can also activate the PI3K pathway (Linnekin, 1999, in review) which has been implicated to play a role in erythroid cell survival and development.

Developmental pathways regulated by growth factors such as Epo and SCF are often disrupted during transformation of erythroid progenitor cells by Friend virus as described below.

**1.9 Friend virus-induced Erythroleukemia**

Scientists have strived over the years to unravel the mystery of the cancer disease and tumor progression; hence many laboratory models have been discovered and utilized. Friend erythroleukemia is perhaps one of the best animal models allowing researchers to study and dissect the molecular events associated with the multi-stage nature of cancer and its disruption of cellular homeostasis. Friend virus-induced erythroleukemia infects mice by way of a retrovirus, the "Friend" virus, which targets cells of the hematopoietic and more specifically the erythroid lineage (Ben-David and Bernstein, 1991).

The Friend erythroleukemia virus was first isolated by Charlotte Friend in 1957. Two separate isolates of Friend virus termed FV-A and FV-P have been identified. Both FV-A and FV-P are complexes of two viral species, namely a unique replication defective spleen
focus-forming virus (SFFV-A and SFFV-P, respectively) and a common replication
competent helper virus known as Friend Murine Leukemia Virus (F-MuLV) (Figure 1.2A).
Both FV-A and FV-P strains display similar multi-stage malignancies when injected into
adult and newborn susceptible mice (Figure 1.2B).

The initial stage of FV-A/P-induced disease is characterized by the rapid polyclonal
expansion of immature erythroblasts, preferentially within the spleen. This is due to a 55
kDa fusion glycoprotein, gp55, encoded by SFFV (Li et al., 1990), that can bind to and
activate the Epo-R. The virus integrates into a host genome in one of two ways. Firstly,
insertion of the provirus upstream of a coding sequence can cause activation of a proto-
oncogene such as Spi-1, a member of the ets family of transcription factors (Moreau-
Gachelin et al., 1988). This allows emergence of clonal tumorigenic cells during the
malignant stage of disease. Conversely, integration of the provirus within a coding sequence
can lead to inactivation of that gene such as the tumor suppressor gene p53. p53 is an
extensively studied gene involved in DNA repair and cell cycle control whose function is
abrogated in many cancers including erythroleukemias (Ben-David et al., 1988; Mowat et
al., 1985). It is believed that mutations within the p53 gene confers an additional in vivo
selective growth advantage as well as survival of transformed erythroleukemic cells in
culture (Lavigueur and Bernstein, 1991).

In contrast to FV-P/A the F-MuLV helper virus alone can induce Friend
erythroleukemia when injected into susceptible newborn mouse strains resulting in anemia
and splenomegaly (Silver and Kozak, 1986). Erythroleukemias induced by F-MuLV lack a
gp55-like encoded env sequence and thus do not display a typical preleukemic, polyclonal
stage as seen in FV-A/P-induced tumors (Ben-David and Bernstein, 1991). F-MuLV-
Figure 1.2 Schematic representation of the Friend retrovirus. The FV-A and FV-P retroviruses are complexes of the helper F-MuLV virus and the SFFV-A/P virus capable of inducing erythroleukemia in adult mice. F-MuLV is also capable of inducing erythroleukemia in susceptible strains of newborn mice.
in 75% of F-MuLV induced-erythroleukemia cell lines (Ben-David et al., 1990). Fli-1 encodes for a transcription factor belonging to the ets family of transcription factors (Ben-David et al., 1991b). Interestingly, Fli-1 activation through a specific translocation is also detected in 85% of human Ewing sarcomas, demonstrating the importance of this gene in human malignancies (Delattre et al., 1992).

Primary erythroleukemias that have an activated Fli-1 gene undergo a massive program of apoptosis when directly explanted into culture. However, these cells, when injected into adult syngeneic mice, are able to grow in vivo to form secondary tumors. Many of these secondary tumors have given rise to Epo-dependent cell lines surviving in vitro. It has been shown that these cell lines bear mutations or deletions in the p53 gene, supporting the notion that inactivation of this tumor suppressor gene may be responsible for the immortalization of tumor cells in culture (Howard et al., 1993). Epo-independent cell lines were also established from tertiary tumors generated by transplantation of the p53 deficient, Epo-dependent cell lines. This growth factor independence is due to rearrangements in the Epo gene leading to autocrine production of Epo in these cell lines (Figure 1.2B) (Howard et al., 1996).

Mutations evident in Friend erythroleukemia cell lines lead to uncontrolled growth, immortalization and expansion of erythroid progenitor cells. This suggests that these specific mutations play a role in the progression of multistage leukemias induced by F-MuLV. Hence analysis of cell lines established from Friend virus-induced erythroleukemia and the genes targeted by mutations could provide important insight into the role of these genes in hematopoiesis and malignant transformation.
1.10 Establishment of the In Vitro Model System: HB60-5

Normally, in order to establish a transformed cell line that proliferates indefinitely from F-MuLV-induced erythroleukemias, serial transplants of an initial tumor into syngeneic mice must be performed. It is thought that with each transplantation, another genetic hit or mutation takes place thus allowing tumor progression to occur from an early stage to a late stage by conferring a selective growth advantage upon the cell (Howard et al., 1993).

Interestingly, the HB60-5 murine erythroleukemia cell line was established from a primary tumor in our laboratory approximately five years ago. Susceptible newborn mice injected with the F-MuLV virus formed enlarged spleens containing erythroid progenitor cells. These splenic cells were extracted from mice and cultured in vitro in α-MEM supplemented with fetal bovine serum (FBS) and SCF and/or Epo. Both Epo and SCF are known to be the key regulators in the survival, proliferation and differentiation of erythroid progenitor cells (Yee et al., 1994; Penta and Sawyer, 1995; Witthuhn et al., 1993). It was found that this cell line survives and proliferates in an exponential fashion when cultured in the presence of both SCF and Epo but can be induced to undergo terminal erythroid differentiation when cultured with Epo alone (Tamir et al., 1999). Both morphological and molecular studies were carried out to verify that these cells were indeed erythroid in nature and that they exhibit signs of differentiation when induced with Epo. The ability of HB60-5 cells to undergo Epo-induced differentiation provides an excellent physiological model to study the events associated with cellular proliferation and differentiation. Therefore it may aid in the identification and analysis of genes that play a role in these cellular processes and
thus serve as targets for the genetic mutations involved in malignant induction and development.

1.11 Subtractive Hybridization

As a cell carries out normal cellular processes such as proliferation, differentiation, apoptosis, or stress response, many genes are turned on or off to allow the cell to accommodate these changes (Oetting, 2000, in review). It is then interesting to determine which genes are activated or down regulated in each cellular process as this information could further elucidate to the genetic events contributing to tumor progression. Acquisition of this knowledge could then prove useful in developing innovative approaches for combating various diseases.

There are many methods for isolating genes that are enlisted to perform a particular role during the regulation of various cellular processes such as DNA microchip arrays, differential display and subtractive hybridization. PCR-select subtractive hybridization is one way to isolate differentially expressed sequences among two sub-populations of cells that differ in one characteristic such as drug or antibiotic resistance, stress tolerance or growth characteristics (Diatchenko et al., 1996). This technique has numerous advantages; it requires a small amount of RNA, differentially expressed sequences are amplified which allows detection of small changes in gene expression and low abundance transcripts, finally it provides a quick and efficient method of isolation.

In the context of this thesis, PCR-select subtractive hybridization was used to isolate gene sequences whose expression is altered as erythroid progenitor cells exit the proliferation stage and are induced to enter their terminal differentiation pathway. This can
be achieved by harvesting cells from each sub-population and isolating mRNA which is then converted to cDNA by way of reverse transcriptase polymerase chain reaction (RT-PCR). The differentially expressed cDNA sequences in each sub-population can then be isolated through a series of hybridization reactions with one another and finally amplified by PCR using nested primers. The isolated cDNA sequences can then be used to confirm differential expression during erythroid differentiation and for sequencing to aid in identification of known genes as well as potentially novel sequences warranting further investigation.

### 1.12 Overall Experimental Approach

In order to aid in the full understanding of the events associated with tumor progression, we first need to acquire a better comprehension of the genetic changes that take place when a cell switches from a proliferative state to one of differentiation and/or apoptosis. This in turn can lead to the identification of genes that are involved in the various signaling pathways regulating these processes and therefore may be potential targets for genetic mutations leading to malignancy. By identifying genes whose genetic alterations lead to tumorigenicity, we may also discover a gene or pathway to target for abrogation of tumor development. Thereby these finding may perhaps bring us closer to developing a novel therapeutic approach in the fight against cancer.
Chapter 2:

Identification of a novel gene clone 5 that appears to be regulated during erythroid differentiation.
2.1 ABSTRACT

Proliferation, differentiation and apoptosis are all naturally occurring cellular processes that are tightly regulated to maintain tissue homeostasis. Cancers can arise when one or more of these pathways are somehow de-regulated upsetting the homeostatic balance. This upset can result in increased survival and de-differentiation of cells that allows increased tumorigenic growth. We are investigating the molecular mechanisms involved in cellular proliferation and differentiation of erythroid progenitor cells using a novel Friend virus-induced murine erythroleukemia cell line HB60-5 recently established in our lab. These cells proliferate in the presence of erythropoietin (Epo) and stem cell factor (SCF) but undergo terminal erythroid differentiation upon withdrawal of SCF and culturing in the presence of Epo alone. In an attempt to identify novel genes that are regulated during erythroid differentiation, subtractive hybridization was performed on mRNA obtained from HB60-5 cells before and after Epo-induced differentiation. Using this method we have isolated a novel gene termed clone 5, which was later shown to be homologous to a recently reported human gene termed Btf (Bcl-2 associated transcription factor). Our results show that clone 5 expression gradually decreases in HB60-5 cells during the transition from proliferation to differentiation. Furthermore, we show that clone 5 is expressed in a number of hematopoietic cell types with expression being significantly higher in progenitor cells relative to their respective terminally differentiated cells. Our observations suggest that clone 5 expression decreases during erythroid differentiation and may play a role in regulating this process.
2.2 INTRODUCTION

The mammalian cell is capable of performing many functions, all of which are controlled and directed by a complex network of intracellular signaling pathways. A cell's proliferative ability initially provides an organism with the means for multi-cellular development. Later on, it also provides a constant supply of progenitor cells for various functions. The process of cellular development or differentiation directs the progenitor cell along a particular developmental pathway towards a fated cell type (Brady et al., 1995). This produces mature cells that are then capable of carrying out their respective function or role within the cell. Apoptosis is the operation of programmed cell death. This process is employed during embryogenesis for organ development. It is also used as a cell completes its life span and is no longer able to carry out its function such as blood cells or when a cell has sustained genetic damage that cannot be repaired (Singh, 2000). Apoptosis may therefore function to protect an organism from accumulating too many circulating cells as well as from passing on incorrect genetic information to newly replicating cells which can have severe consequences (Muta and Krantz, 1995).

Cellular proliferation, differentiation and apoptosis must be tightly regulated to maintain tissue homeostasis. Regulation of these mechanisms thus requires sophisticated timing and coordination, which can be mediated by the cell's response to external stimuli, such as hormones and growth factors. Binding of these factors to a transmembrane protein on the cell's surface initiates a cascade of activated proteins within a particular signal transduction pathway. These activated proteins in turn regulate expression of target genes, all of which provide the cell with a signal to grow, differentiate or die.
Regulation of any or all of these cellular mechanisms is often disrupted in human cancers by the acquisition of genetic mutations altering genes involved in cell growth management. This can result in increased cell division and expansion of tumor cells, which are incapable of self-destruction by apoptosis (Hanahan and Weinberg, 2000, in review). Dissection of these cellular mechanisms and isolation of their key regulators could therefore provide information that would be beneficial in treatment and perhaps even prevention of malignant progression.

Friend-virus induced erythroleukemia has served as an excellent model to study the molecular aspects associated with multi-stage malignancies. A unique erythroleukemia cell line, termed HB60-5, was recently established in our laboratory. These cells proliferate indefinitely by culturing in media containing stem cell factor (SCF), a ligand for the c-Kit receptor, and erythropoietin (Epo). However, HB60-5 cells can be induced to undergo terminal erythroid differentiation in response to Epo alone. Therefore, this provides an excellent model with which to investigate the molecular mechanisms associated with the switch between cellular proliferation and differentiation. We have previously shown that the activation of the c-Kit and Epo receptors can initiate downstream signaling pathways involved in cellular growth and differentiation, Ras-MAP kinase and Jak2/Stat5 respectively (Zochodne et al., 2000).

In this study we are interested in utilizing the HB60-5 in vitro system to identify genes with altered expression patterns during differentiation of erythroid cells. These differentially expressed genes could be implicated to play a role in regulating cellular proliferation and/or differentiation. Using the method of subtractive hybridization, we have isolated several clones, which displayed altered expression during Epo-induced
differentiation of erythroid cells. One particular clone appears to represent a novel murine gene whose function has yet to be determined.

2.3 MATERIALS AND METHODS

2.3.1 Cell Lines. HB60-5 cells were derived from a primary erythroleukemia induced after injection of BALB/C mice at birth with clone 57 of the F-MuLV helper virus, as described previously (Tamir et al, 1999). Cells were maintained in α-MEM supplemented with 10% FBS, 10% conditioned medium from SCF-producing cells BHK-MKL (Tsai et al, 1994), and 0.2 units Epo/ml (Boehringer Mannheim). To induce differentiation, HB60-5 cells were washed twice with PBS and cultured in α-MEM containing 10% FBS and 1.0 unit Epo/ml. Cells were then harvested at various time intervals (0-72 hrs). DP27-17 cells were maintained in α-MEM supplemented with 5% FBS and were induced to differentiate by washing twice with PBS and culturing in α-MEM containing 5% FBS and 1.5% DMSO. Cells were harvested at various intervals (0-72 hrs). Murine hematopoietic cell lines CB7, WeHI3, EL4, OCI/M2, MOLT-4 and human hematopoietic cell lines HEL, K562, Jurkat, HL60 were maintained in α-MEM supplemented with 5% FBS. Murine cell lines Da3, BaF3 were cultured in RPMI media and supplemented with 5% FBS and 10% conditioned medium from IL-3 producing cells WeHI3. Human cell lines ARH-77, WI-L2 were maintained in RPMI media supplemented with 5% FBS. All cell lines were cultured at 37°C under humidified conditions with 5% CO₂.
2.3.2 PCR-Select cDNA Subtractive Hybridization. Subtractive hybridization was performed using the PCR-Select cDNA Subtraction Hybridization kit purchased from CLONTECH (Palo Alto, California). Briefly, total RNA was harvested from HB60-5 proliferating cells before and after Epo-induced differentiation for 48 hours using TRIzol (GIBCO-BRL Laboratories) according to the manufacturer's protocol. Polyadenylated RNAs (mRNA) were selected by affinity chromatography on oligo (dT) cellulose (Pharmacia) as described by the supplier.

First and second-strand driver and tester double-stranded cDNA was synthesized from 2µg of poly(A)* RNA using the Great Lengths cDNA Synthesis Kit (CLONTECH) and 1ng of oligonucleotide Pr16 as a primer with T4 DNA polymerase. The resulting cDNA pellet was dissolved in 10µl of de-ionized water and digested with Rsal, followed by phenol extraction and ethanol precipitation. Half of the entire volume of digested tester cDNA (diluted in 5 µl of water) was ligated to adapter 1 and adapter 2R in separate ligation reactions using 0.5 units of T4 DNA ligase (Life Technologies, Inc.). The reactions were incubated at 16°C overnight, followed by heat inactivation the next day. Driver double-stranded cDNA (1.5µl) was added to each of two tubes containing 1.5µl of adapter 1- and adapter 2R- ligated tester cDNA. The samples were mixed, and 1µl of hybridization buffer [50mM Hepes, pH 8.3/0.5 M NaCl/0.02 mM EDTA, pH 8.0/10% (wt/vol) PEG 8000] was added. The solution was overlaid with mineral oil, the DNAs were denatured (1.5 min, 98°C), annealed (8 hrs at 68°C). After this first hybridization, fresh heat-denatured driver and hybridization buffer was added and the mixture was allowed to hybridize overnight at 68°C. This final hybridization was followed by heat inactivation at 72°C for 7 min and stored at -20°C.
Two PCR amplifications were conducted in a total volume of 25 μl, containing 1 μl of diluted subtracted cDNA, 5 μM of PCR primer P1, 5 μM of PCR primer P2R and 22 μl of PCR master mixture prepared using the Advantage cDNA PCR Core Kit (CLONTECH). PCR was performed with the following parameters: 75°C for 7 min; 30 cycles at (91°C for 30 sec, 68°C for 30 sec, 72°C for 2.5 min); and a final extension at 72°C for 7 min. Following a 10 fold dilution of the PCR product, 1 μl was then used as a template in secondary PCR for 10 cycles under the same conditions used for the primary PCR, except PCR primer P1 and P2R were replaced with nested PCR primer PN1 and PN2R respectively. The PCR products were analyzed by 2% agarose gel electrophoresis, acid-depurinated before denaturation and transferred to nitrocellulose. It was hybridized with the GAPDH (0.5kb) DNA probe. Products from the secondary PCRs were inserted into the pCR2.1 vector using a T/A cloning kit (Invitrogen).

DNA sequencing was performed by the chain termination reaction automatically at the York University facility (Toronto, Canada). Nucleic acid homology searches were performed using the BLAST algorithm through the National Center for Biotechnology Information (National Institutes of Health, Bethesda).

2.3.3 cDNA Library Screening. A λgt11 cDNA expression library was constructed from 5 μg of poly(A)+ RNA isolated from the erythroleukemia cell line CB7, using a Pharmacia cDNA synthesis kit as described previously (Ben-David et al., 1991a). The library (10^6 phages) was amplified once and stored at 4°C. To isolate cDNA clones, 5 x 10^5 phages were screened initially with the [α-^32P]dCTP labeled 400-bp PCR product obtained in the subtraction corresponding to the 5' end of the clone 5 cDNA. After four rounds of plaque
puration, nine clones were isolated. The library was screened a second time using a [α-\(^{32}\)P]dCTP labeled enzyme digested Eco RI fragment corresponding to the C-terminus of the coding region of clone 5. After three rounds of plaque purification, seven clones were isolated.

2.3.4 RNA Extraction and Northern Blotting. Total cellular RNA was isolated using TRIzol reagent as described by the supplier (Gibco, Life Technologies, Inc.). Total RNA (20μg) was dissolved in 2.2M formaldehyde, denatured at 65°C for 1 min, and electrophoresed in a 1% agarose gel containing 0.66M formaldehyde. After transfer to nylon membranes (Zetaprobe™, Bio-Rad Laboratories), RNA integrity as uniformity of RNA loading was assessed by staining the membrane with 0.5M sodium acetate, 0.04% methylene blue pH 5.2 prior to hybridization.

2.3.5 Molecular Hybridization. Radiolabelled DNA probes were synthesized using the Klenow fragment of DNA polymerase I with random hexadeoxyribonucleotides (Boehringer Mannheim) by the method of Feinburg and Vogelstein (1983). Briefly, 50ng of DNA was denatured at 100°C for 10 minutes and labelled with 10μl of oligo-mix solution, 5μl of [α-\(^{32}\)P]dCTP and 1μl of Klenow fragment of DNA polymerase I at 37°C for a minimum of 30 minutes. Labelled probes were purified using G50-packed columns.

The filters were hybridized with \(2 \times 10^6\) cpm/ml of G50-purified random-primed probe in a hybridization mixture containing 50% formamide, 10% dextran sulfate, 1.5X SSC (1X SSC = 0.15M NaCl/0.015M sodium citrate, pH 7.0), 5X Denhardt’s solution (1X Denhardt’s solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) at 42°C
over night. The filters were washed initially with two washes of 1X SSC and 0.2% SDS at room temperature for 30 minutes followed by two washes of 0.2X SSC and 0.1% SDS at 65°C for 30 minutes. Filters were exposed to Kodak XRP film at -80°C with an intensifying screen.

2.3.6 cDNA Probes. Cloned cDNAs from the subtractive hybridization were amplified for 20 cycles with nested PCR primers PN1 and PN2, using the same conditions as the secondary PCR described above. One microlitre of the PCR product was prepared for hybridization in Northern blot analysis.

The clone 5 probe was a 1.5-kb PstI-SacI fragment corresponding to the N-terminus of the coding region. The 500-bp EcoRI fragment of mouse α-globin was used to confirm terminal erythroid differentiation. Methylene blue staining of ribosomal RNA was used to verify equal RNA loading per lane.

2.3.7 Vectors and Transfection. The pEF-clone 5-His vector was constructed by cloning a 2321-bp KpnI-XhoI cDNA fragment, corresponding to the N-terminal portion of the clone 5 coding sequence, to a 225-bp XhoI-XbaI cDNA PCR product, corresponding to the C-terminus of the coding sequence, in frame into the KpnI-XbaI site of the plasmid pEF Tracer-A (Invitrogen) to create a clone 5-His fusion protein.

The pMT clone 5-His expression vector was constructed by subcloning an inducible metallothionein promoter into pCDNA3 vector in place of the CMV promoter. The 2639-bp KpnI-PmeI clone 5-His fragment from pEF-clone 5-His was excised and subcloned into the KpnI-EcoRV site of the pMT vector. For stable transfection, $5 \times 10^6$ HB60-5 cells were
mixed with 30µg of pEF clone 5-His or pMT clone 5-His expression vectors in 0.8ml PBS along with appropriate vector controls and then subjected to electroporation (Bio-Rad Laboratories) of 950 mF and 280 V. After 48 hr recovery in medium containing Epo and SCF, the cells were selected for resistance by 0.2 mg/ml zeocin (Invitrogen) or 0.8 mg/ml geneticin (G418; Gibco, Life Technologies), respectively.

Cells transfected with the pMT vector were induced to express the clone 5 gene by culturing in growth medium containing 100µM zinc (ZnSO₄) and 2µM cadmium (CdCl₂) at 37°C.

**2.4 RESULTS AND DISCUSSION**

**2.4.1 Isolation of differentially expressed genes**

During differentiation, many genes are turned on and off to facilitate the regulation of this process. In cancer, including leukemia, this process is somehow de-regulated. Hence, the identification of genes affecting differentiation could help to better understand the potential genetic pitfalls that lead to tumor progression. Recently we isolated an erythroleukemia cell line designated HB60-5 that proliferates in response to SCF and Epo stimulation but undergoes terminal differentiation upon SCF withdrawal (Tamir et al., 1999). This system therefore provides us with an excellent tool to identify genes associated with erythroid differentiation.

We employed the method of subtractive hybridization to isolate genes whose expression is altered during Epo-induced differentiation of HB60-5 cells. Toward this goal, mRNA was isolated from HB60-5 cells before and after Epo stimulation for 48 hours and subjected to subtractive hybridization. The subtractive hybridization reactions were carried
out both ways in order to identify genes that are up-regulated and down-regulated during differentiation of HB60-5 cells. To confirm their differential expression, isolated sequences were used as probes for Northern blots prepared using RNA from HB60-5 cells at various stages of Epo-induced differentiation. Our results revealed that out of thirty-three clones examined, nine displayed a detectable altered RNA expression pattern (Table 2.1).

Upon sequencing these clones and comparing to the Genbank database, six clones displayed a high level of similarity to previously identified genes suggesting that these isolated sequences represent a region within these genes. Many of these genes have been shown in the literature to be associated with differentiation. Expression of clone 2 was down-regulated during differentiation and showed 94% similarity to murine ribosomal protein S17. This protein has been shown to associate with ubiquitin genes and display abnormally high expression in a variety of hematopoietic malignant tumor cells with a marked decrease as cells enter terminal differentiation (Shimbara et al., 1993). Clone 3 expression decreased and displayed 98% similarity to the SHP-1 protein tyrosine phosphatase. It is believed that SHP-1 plays an inhibitory role in SCF signaling and function thus would be down-regulated after withdrawal of SCF (Avraham and Price, 1999, in review). Clone 7, which was up-regulated, exhibited 98% similarity to a murine high mobility group (HMG) protein 2. Other HMG proteins have been shown to be differentially regulated during erythroid differentiation possibly regulating the expression of β-globin and thought to be involved in cellular processes such as growth and differentiation (Crippa et al., 1991; Chase et al., 1999). Clones 9 and 25 were both shown to have increasing expression during differentiation and shared 99% and 89% similarity respectively with intracisternal Aparticle (IAP-IL3). Increased IAP expression has been shown previously to correlate with
<table>
<thead>
<tr>
<th>Clone</th>
<th>RNA Expression Pattern</th>
<th>Homologous Sequence in Genbank</th>
<th>Percent of Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Down-regulated</td>
<td>Murine ribosomal protein S17</td>
<td>94%</td>
</tr>
<tr>
<td>3</td>
<td>Down-regulated</td>
<td>Murine SHP-1 protein-tyrosine phosphatase</td>
<td>98%</td>
</tr>
<tr>
<td>5</td>
<td>Down-regulated</td>
<td>Human cDNA KIAA0164</td>
<td>92%</td>
</tr>
<tr>
<td>7</td>
<td>Up-regulated</td>
<td>Murine high mobility group protein 2</td>
<td>98%</td>
</tr>
<tr>
<td>8</td>
<td>Up-regulated</td>
<td>Human flow-sorted chromosome 6 HindIII fragment</td>
<td>75%</td>
</tr>
<tr>
<td>9</td>
<td>Up-regulated</td>
<td>Murine intracisternal A-particle IAP-IL3</td>
<td>99%</td>
</tr>
<tr>
<td>11</td>
<td>Up-regulated</td>
<td>Murine 26S proteosome subunit ATPase</td>
<td>94%</td>
</tr>
<tr>
<td>23</td>
<td>Down-regulated</td>
<td>Human 2-5A binding protein</td>
<td>67%</td>
</tr>
<tr>
<td>25</td>
<td>Up-regulated</td>
<td>Murine IL-3 with inserted intracisternal A-particle IAP-IL3</td>
<td>89%</td>
</tr>
</tbody>
</table>

Table 2.1 Isolated differentially expressed sequences from the subtractive hybridization were sequenced and compared to the Genbank database. Nine of the thirty-three clones screened displayed altered RNA expression during erythroid differentiation. Six clones correspond to previously known genes while clones 5, 8, and 23 only display homology to human sequences. While the percentage homology of clones 8 and 23 is quite low indicating, clone 5 exhibits 92% homology to a human cDNA sequence termed KIAA0164.
hematopoietic cells in late-stage differentiation (Wiest et al., 1989; Takayama et al., 1991). Clone 11 expression increased during differentiation and exhibited 94% similarity to subunit 4 of the 26S proteasome ATPase involved in cell cycle ubiquitination and protein degradation (Hoyle and Fisher, 1996). Differentiation is associated with exiting the cell cycle therefore it is not surprising that cell cycle genes are regulated to a certain degree with differentiation. The remaining three clones failed to exhibit similarity to any murine genes in the database, although clones 8 and 23 showed some similarity to the HindIII fragment of human flow-sorted chromosome 6 (75%) and human 2-5A binding protein (83%).

Initial comparison to the Genbank database revealed that clone 5 displayed 89% similarity to a previously cloned human cDNA sequence KIAA0164 (accession number D79986, Nagase et al., 1996), whose encoded protein sequence did not display homology to any known motif within the databases. Therefore this provided evidence to suggest that clone 5 represented a novel, uncharacterized gene rather than an artifact of the subtractive hybridization method. The KIAA0164 cDNA also provided a small amount of information to initially work with as well as a sequence for comparison during sequencing of clone 5. Based on this information as well as the ability of clone 5 expression to be down-regulated during differentiation, clone 5 was chosen for further investigation.

2.4.2 Expression of Clone 5 during differentiation of erythroleukemia cells

Northern blot analysis of Epo-induced differentiation of HB60-5 cells, using clone 5 as a probe, showed two different sized mRNA transcripts of 3- and 5-kb with the smaller transcript appearing to be the predominant form. The expression of both transcripts was shown to be down-regulated during Epo-induced differentiation of HB60-5 cells. While
high expression of clone 5 was maintained until 24 hours post-induction, a dramatic decline in gene expression was evident after this time point (Figure 2.1A). Down-regulation of clone 5 was associated with up-regulation of α-globin, a marker of erythroid differentiation.

We also looked for expression of clone 5 in other erythroleukemia cell lines to confirm that it is not a trait restricted to HB60-5 cells. Using DP27-17 cells, in which differentiation is induced by culturing with 1.5% DMSO, a similar expression pattern of clone 5 was demonstrated in these cells as well as a dramatic decrease in clone 5 expression after 24 hours of DMSO induction (Figure 2.1B). These results show a correlation between clone 5 down-regulation and commitment to differentiation in erythroleukemic cells.

Previously, Dr. Norman Iscove’s laboratory at the Ontario Cancer Institute, developed a PCR assay to isolate cDNA from cells derived from various stages of hematopoietic development (Brady et al., 1995). We obtained a lineage blot from Dr. Iscove in order to examine the pattern of clone 5 expression in hematopoietic cells. Although clone 5 expression was low in the earliest progenitor cells (E/Meg/Mac/Neut/Mast), expression became abundantly evident in subsequent precursor stages of most hematopoietic lineages such as E/Meg/Mac, Mac/Neut, pMac, pNeut, and pMeg. In accordance with our previous results, expression of clone 5 decreased as these cells progressed towards their respective terminally differentiated stage (Figure 2.2). Cells of the erythroid lineage aptly illustrated this decrease in expression. Multipotent precursor cells (E/Meg/Mac) initially demonstrated lower levels of clone 5, which swiftly increased to show abundant expression in the BFU-E progenitor cells. As differentiation progressed to the next stage of CFU-E, expression of clone 5 was dramatically decreased. Finally, once it reached the final stage of erythroid
Figure 2.1 The expression of Clone 5 appears to be regulated during differentiation of erythroid cells by either Epo or DMSO. (A) HB60-5 cells induced to undergo terminal differentiation in response to Epo, as shown by increasing α-globin expression, exhibit a decrease in Clone 5 mRNA expression. (B) DP27-17 cells also display a decrease in Clone 5 expression as they are induced to differentiate, also shown by increasing α-globin expression, with 1.5% DMSO. Two sized mRNA transcripts are evident in both cell lines which may be due to alternative splicing.
Figure 2.2 Hematopoietic stem cells were cultured with various cytokines and growth factors. The fate of these cells were then followed to determine their lineage potential. As seen in erythroid cells, cells of various hematopoietic lineages display substantial expression of Clone 5 at the progenitor stage and diminishes as these cells progress towards their terminally differentiated counterpart. Erythrocyte (E), megakaryocyte (Meg), macrophage (Mac), neutrophil (Neut, N), mast cell (Mast), burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), precursor macrophage (pMac), precursor neutrophils (pNeut), precursor megakaryocyte (pMeg), B-cell (B), T-cell (T) and various controls (air, NIH 3T3, S17 and 95/1.7).
differentiation, the erythrocyte, expression was undetectable. A similar pattern of clone 5 expression was also seen during the development of megakaryocytes, mast cells, neutrophils, and macrophages. These results demonstrate that the expression of clone 5 is abundant in hematopoietic progenitor cells and decreases as these cells undergo lineage-specific terminal differentiation.

Intrigued by the wide range of gene expression among hematopoietic cells, we then looked at the tissue distribution of clone 5 expression using normal mouse tissues. Total RNA was isolated from various mouse tissues and subjected to Northern blot analysis using clone 5 as a probe. As shown in Figure 2.3, low levels of the 3- and 5-kb transcripts were expressed in all tissues examined with the exception of the brain. This tissue showed higher levels of the 5-kb transcript. RNA from HB60-5 cells was included as a positive control. These results demonstrate that clone 5 is expressed in both hematopoietic and non-hematopoietic tissues and that the constitutive levels of expression are tissue-specific.

2.4.3 Clone 5 expression may be enhanced in transformed cell lines

We next investigated the expression of clone 5 in transformed cell lines of hematopoietic and non-hematopoietic origin. Toward this aim, total RNA was extracted from transformed murine hematopoietic cell lines of various lineages and hybridized with clone 5. As shown in Figure 2.4, clone 5 was highly expressed in a variety of murine hematopoietic cell lines originating from erythroid (HB60-5, DP17, CB7), myeloid (BaF3, Da3), monocytic (WeHI3) and lymphoid (EL4, B10, Yab3) lineages. Clone 5 was also abundantly expressed in embryonic stem (ES) cells while lower expression was seen in the
Figure 2.3 Expression of Clone 5 in various murine tissues. Tissues from various organs were isolated and total RNA extracted. It appears that Clone 5 is only marginally expressed in normal with the exception of brain tissue. Tissues displaying slightly higher levels of expression are comprised of cells with a higher self-renewal capacity.
Figure 2.4 A panel of murine cell lines representing a variety of blood cells were examined to determine the distribution of Clone 5 expression. It is apparent the Clone 5 is expressed in a wide variety of hematopoietic cells. HB60-5, DP17, and CB7 represent erythroid cells; BaF3, Da3 are from the myeloid lineage; WeHI3 is a myelomonocytic-macrophage cell line; EL4, B10, Yab3 belong to the T-cell lineage.
murine fibroblast cell line, NIH 3T3. Both the 3-kb and 5-kb transcripts were detected in each cell line.

We also examined the expression of clone 5 in cell lines derived from various hematopoietic and non-hematopoietic human tumors. Hematopoietic cell lines were derived from erythroid (K562, OCI/M2, HEL), and lymphoid (WI-L2, ARH-77, MOLT4, JURKAT) origins (Figure 2.5A). Cell lines derived from non-hematopoietic human tumors originated from colon (DLD-1, HT29), gastric (MKN-45), stomach (TMK-1), breast (HBL100), epidermoid carcinoma (A431), melanoma (MeWo), and ovarian (Skov3) tissue (Figure 2.5B). HB60-5 cells were included as a positive control in both cases. In contrast to murine derived tumor cell lines, expression of clone 5 in human cell lines showed little to no expression of the 3- and 5-kb transcripts. However, a third mRNA transcript of 1.5-kb was detected to varying degrees in all cell lines examined, which appeared to be the predominant form in human tissues. These results further demonstrate that clone 5 is widely expressed in a number of human hematopoietic and non-hematopoietic cell lines of various tissue origin.

In comparison to the normal murine tissue, clone 5 expression appeared to be significantly higher in both murine and human transformed cell lines. Also, clone 5 was expressed in all cells examined suggesting a fundamental role for this gene in normal cellular physiology. This may include a role in the transformation and survival properties of cells and thus could contribute to tumor progression.
Figure 2.5 Clone 5 expression in human hematopoietic and cancer cell lines. (A) A panel of human hematopoietic cell lines representing a variety of lineages including K562, OCI/M2, HEL (erythroid); WI-L2, ARH-77 (lymphoblastic), MOLT-4, Jurkat (T-cells). Clone 5 shows little expression at the 3-kb and 5-kb transcript regions but a third RNA transcript of 1.5-kb is present to some degree among the various hematopoietic cell types and seems to be the pre-dominant form in human cells. (B) A panel of human cancer cell lines including colon (DLD-1; HT29), gastric (MKN-45), stomach (TMK-1), breast (HBL100), epidermoid carcinoma (A431), melanoma (MeWo), and ovarian (Skov3) display little or no expression at 5-kb and 3-kb transcripts. Again Clone 5 is expressed to some degree in all tissues screened with a 1.5-kb mRNA transcript appearing as the predominant transcript in human.
2.4.4 Clone 5 expression exhibits multiple transcripts

As previously stated, hybridization with clone 5 resulted in three different sized mRNA transcripts. The 3- and 5-kb transcripts appear as the predominant form observed in murine cells while the 1.5-kb transcript seems to be predominant in human cell lines. The appearance of these three different sized transcripts is intriguing although, at present, there is no definitive explanation for this occurrence. It remains possible that these three transcripts represent alternatively spliced forms of clone 5 and that splicing may be species and/or tissue-specific. Another explanation may be that the three different transcripts represent three distinct genes comprising a multi-gene family containing a highly conserved homologous region that is detected by the clone 5 probe. It is possible that these three distinct genes are involved in the same cellular process but have opposing functions as is seen with the Bcl-2 family of apoptotic genes (Pelligrini and Strasser, 1999, in review).

2.4.5 Isolation of cDNA corresponding to clone 5

In order to characterize clone 5 and elucidate a possible role for this protein, we set out to isolate the full-length cDNA using a λgt11-CB7 erythroleukemia cDNA library. Using the previously isolated 500-bp PCR subtractive hybridization fragment as a probe, we obtained a 2.5-kb cDNA fragment corresponding to the 5' end of the KIAA0164 gene (Figure 2.6). This cDNA fragment was subcloned into the pCR 2.1 plasmid vector and sequenced. Additional screening of the same library using a 500-bp EcoRI/Xhol fragment corresponding to the region between 2-kb and 2.5-kb of this cDNA, yielded the 3' portion of this transcript that was approximately 4.5-kb with a region overlapping the first fragment of
Figure 2.6 Isolation of sequences from the λgt11-CB7 cDNA library. Clone 5-1 was isolated in the first screen using the PCR product corresponding to 500-bp in N-terminal region. Clone 5-6b was obtained in the second screen using the 500-bp Eco RI/Xho I fragment.
approximately 1kb. This 4.5 cDNA fragment was then subcloned into the pCR 2.1 plasmid vector and the remaining nucleotide sequence of the coding region was determined.

The clone 5 murine sequence displayed approximately 92% sequence similarity to the human KIAA0164 cDNA sequence at the nucleotide level and 91% at the predicted amino acid sequence level (Figure 2.7).

### 2.4.6 Sequence analysis of the clone 5 transcript

Sequence analysis showed that clone 5 was approximately 6-kb in length. This cDNA sequence included the coding region and a portion of the 3'-untranslated region. This clone did not include the 5' UTR and the 3' UTR terminal portions of the cDNA. The insert represents the 3-kb transcript containing a single long open reading frame of 2616 nucleotides and 872 amino acids with the ATG initiation site located at base pair 214 of the cDNA sequence. The 5' UTR does not contain a Kozak consensus sequence, ACCATGG, typically found in eukaryotic translation initiation sites (Kozak, 1986). Based on the predicted protein sequence, the clone 5 protein would have a molecular weight of 96 KDa.

Using the clone 5 sequence, we searched various databases for motifs that might provide evidence for a potential function. The amino acid sequence contains potential phosphorylation sites for many different signaling molecules and also contains a region resembling an ATP binding domain G-X-G-X-X-G at amino acids 373-378. These domains suggest that clone 5 may be phosphorylated by numerous molecules and therefore may be involved in one or more signal transduction pathways.

In a recent comparison of clone 5 sequence in GenBank databases, we found that the corresponding human cDNA was recently submitted by another group, who has termed it
Figure 2.7 Sequence homology between the murine Clone 5 gene and the human BtfL gene. It is evident that there is a high conservation of homology between the murine and human homologues.
Btf (Bcl-2 associated transcription factor) (Kasof et al., 1999). Briefly, in this study, Kasof et al. isolated Btf in a yeast two-hybrid screen while searching for binding partners of the adenovirus E1B 19,000-molecular-weight (19K) protein. E1B 19K is homologous to the mammalian anti-apoptotic gene Bcl-2 and both act by binding to and antagonizing pro-apoptotic proteins. E1B 19K also cooperates with E1A to transform primary rodent cells. The yeast two-hybrid screen initially yielded a 1.5-kb transcript while Northern analysis of hematopoietic and non-hematopoietic cell lines detected two transcripts of 3- and 5-kb. The appearance of the 1.5-kb transcript however, was not reported by this group. The sequence corresponding to the 3-kb transcripts was then isolated and cloned from a HeLa cDNA library while the sequence corresponding to the 5-kb transcript was deduced by comparisons to EST sequences in the database and the KIAA0164 human cDNA. Using construct reporter assays, they demonstrated that Btf binds DNA in vitro and represses the expression of Btf and therefore may act as a transcriptional repressor of certain genes. They also demonstrated that E1B 19K, Bcl-2 and Bcl-xL have the ability to bind and sequester Btf, blocking its transcriptional repression activity. Finally they suggested that Btf may possess a role in promoting apoptosis indirectly, or as a tumour suppressor gene as it maps to a chromosomal region that is often deleted in many cancers.

Sequence analysis shows that Btf contains a basic leucine zipper (bZip) motif and a Myb DNA binding domain characteristic of transcription factors and other proteins involved in DNA binding and transcriptional regulation (Figure 2.8). Both domains are conserved and therefore present in the clone 5 sequence supporting the notion that these two proteins may be able to bind to and affect expression of target genes.
Figure 2.8 Sequence analysis of Btf and Clone 5. (A) The predicted amino acid sequence of Clone 5 has conserved homology with the recently reported human gene Btf, in the Genbank database (accession no. AF249273). It is predicted to encode a complete 921-amino acid long (5Kb) transcript BtfL and a shorter (3Kb) transcript BtfS due to a 49 aa deletion 801-849 (bold) as well as differences in the 3’ UTR. Clone 5 was obtained in a CB7 cDNA library screen and represents the murine homologue of the 3Kb transcript. The underlined regions represent the positions of nuclear localization sequences. (B) A schematic representation of Btf and Clone 5 proteins.

Our results suggest that the murine clone 5 gene, by nature of its expression, may be involved in promoting cellular proliferation and survival and/or inhibiting cellular differentiation. However, the study by Kasof et al. (1999) has demonstrated that Btf expression in HeLa cells via transfection causes apoptosis. One explanation for such a discrepancy would be that the balance between Btf and its partners Bcl-2/Bcl-xL could determine whether cells survive or undergo apoptosis. For example, the pro-apoptotic action of Btf has been shown to be suppressed by overexpressing Bcl-2 in cells (Kasof et al., 1999). However, our laboratory recently demonstrated that Bcl-2 and Bcl-xL expression is detected at high levels in all of the Friend virus-induced erythroleukemia cell lines (manuscript in preparation). Therefore, it is possible that during differentiation, down-regulation of Btf results in increased availability of free Bcl-2/Bcl-xL and less Btf-Bcl2/Bcl-xL complex in the cells. The presence of free Bcl-2 in cells undergoing differentiation may increase their survival capability, a property that is necessary for the completion of maturation. Therefore, when the level of Btf exceeds the level of Bcl-2/Bcl-xL in cells, which has been tested by ectopic expression, these cells will have less free Bcl-2/Bcl-xL which results in them becoming more prone to apoptosis.

To aid in determining the function of Btf/clone 5, we subcloned the cDNA first into the pEF Tracer vector under control of the human elongation factor promoter with co-expression of a green fluorescence protein (GFP). After cells were transfected with the pEF-Btf vector, cells producing GFP were visible after two days of culturing. However, when an antibiotic resistant cell line was established from the Btf transfected cells, none of these cells were positive for GFP expression. Therefore it seemed that the positively transfected cells were not capable of surviving and expanding in culture. These results support the
hypothesis that overexpression of Btf within cells leads to cell death. To further prove these results, it is therefore necessary to generate an inducible system for Btf expression in erythroleukemic cells. Such inducible vector has been recently constructed and the outcome of its expression will be investigated in future experiments in Dr. Ben-David’s laboratory.

In summary, we report here that a novel murine gene, clone 5, has been isolated and appears to be the murine homologue to the KIAA0164 cDNA sequence and the recently reported human gene Btf. Clone 5 gene expression is down-regulated during differentiation of erythroid as well as other hematopoietic cells \textit{in vitro}. Clone 5 is a widely expressed gene in which three different sized mRNA transcripts of 5-, 3-, and 1.5-kb can be detected. The predicted amino acid structure contains basic leucine zipper and Myb DNA binding domains suggesting that it may function as a transcription factor and thus control expression of other target genes. We propose that clone 5 may be involved in the regulation of cellular processes such as enhancing proliferation and survival as well as inhibiting cellular differentiation. At present we are unable to elucidate a potential role for clone 5 within the cell with any certainty hence further experiments are required to aid in characterization of the clone 5 protein product.

Characterization of the clone 5 gene and its protein may provide information crucial in better understanding the mechanisms of proliferation, differentiation and apoptosis that control tissue homeostasis. This in turn may aid in elucidating the chain of events that occur allowing these processes to become altered and de-regulated during tumor development and progression.
2.5 ACKNOWLEDGMENTS

The authors would like to thanks Dr. Norman Iscove for providing us with the cDNA blot comprising hematopoietic precursor and developmentally mature cells. We would also like to thank Dr. Burton Yang for his assistance with sub-cloning Clone 5 and Dr. Dwayne Barber for supplying some of the erythroleukemia cell lines.
Chapter 3:
General Discussion and
Future Perspectives
Cancer and the multi-stage nature of tumor progression have been under investigation for much of the past century with great strides being made along the way. Over this time, we have gained insight into many facets of this disease including the discovery of potential cancer-causing agents, targets genes of genetic mutation, as well as the cellular pathways and processes that become disrupted. As more information is acquired about this cancer, it further reveals the complexity with which it occurs.

Attempting to study cancer as a whole may then be unrealistic and perhaps impossible. Instead, many researchers focus their efforts on investigating one particular aspect of the characteristics of cancer and tumor progression. Some investigate particular genes that are altered in tumor development, others look at signal transduction pathways that are enhanced or inhibited often as a result of altered gene expression. Still others choose to study survival mechanisms of malignant cells such as hypoxia and angiogenesis.

Findings in all these areas are then akin to pieces of an enormous puzzle that is slowly being put together in a coordinated effort by scientists world-wide. Once this puzzle is complete, hopefully it will provide a fuller understanding of why tumors arise and how malignant cells gain the capability to overcome mechanisms governing cellular homeostasis. Of course understanding tumor development and progression is only the beginning. The true goal lies in the ability to either treat or even prevent cancer from occurring, much like a common cold which was also life threatening at one time. This goal of finding a “cure” for cancer may be attainable only upon completion of the puzzle.

Investigation of the cellular processes that are involved in the regulation of cellular homeostasis and thus de-regulated during malignancy is a logical beginning. It is believed that malignant progression is a result of a cell losing the ability to fully differentiate into its
fated cell type and gaining the capacity to continually divide while also escaping mechanisms promoting cell suicide or apoptosis. These alterations change the growth kinetics of the cell and cause a disruption in the homeostatic balance. This results in cell growth surpassing cell death and thus the accumulation of genetically and functionally altered cells within a confined space. Determining which genes are key regulators of these cellular processes may provide potential targets for mutations causing cancer as well as providing targets for such treatments as gene therapy.

Cellular proliferation, differentiation and apoptosis are the major processes involved in regulating cell growth, development and homeostasis. Therefore genes involved in the signal transduction pathways governing these processes are prime targets for genetic mutations leading to tumor progression. The complexity of these pathways make it difficult to ascertain a complete picture of cellular processes individually as well as interactions between them. Identification of proteins involved in these pathways and their respective genes that aid in regulating cell growth and survival could better our understanding. In the context of this thesis we worked towards contributing to this goal by utilizing the method of subtractive hybridization for isolation and identification of genes that are differentially regulated during Epo-induced differentiation of erythroleukemia HB60-5 cells after 48 hours. We were successful in isolating a novel murine gene sequence, Clone 5, which we believe to be homologous to a recently reported human gene called Btf. As stated previously we propose that Clone 5 plays a role in promoting cell survival and proliferation and may also inhibit terminal differentiation. In our experiments to date we have been able to elucidate a possible role for Clone 5 through in vitro transfection studies therefore further investigation is pending. The fact that Clone 5/Btf is a novel gene in which little is known
makes it very exciting to be working on as there are numerous directions that could be taken in order to gain knowledge about its function as well as interactions with other molecules. Initial experiments could include designing a more effective transfection construct and expression system. Using an inducible system may add slight difficulty but is perhaps more conducive to observing a change in cell phenotype and behaviour especially if ectopic gene expression proves to be lethal.

It is still speculative as to whether Clone 5 acts to promote cellular proliferation, enhance cell survival mechanisms, relating to apoptosis, or inhibit terminal differentiation. Therefore by performing certain assays that focus on proliferative and differentiation or carrying out apoptotic studies, the process in which the Clone 5 protein is involved may become more apparent. It would be intriguing as well to look at cell cycle properties associated with Clone 5 expression as it may also play a role in this cellular mechanism with respect to inhibition of cell cycle arrest.

It would be imperative to produce an antibody for use in protein studies to determine if protein expression during Epo-induced differentiation of HB60-5 cells follows the same pattern as RNA expression. The phosphorylation status and activity of the protein may also change during differentiation and should be ascertained. Studies looking at protein-protein or protein-DNA interactions is an attractive aspect to follow as well as we speculate that Clone 5 contains domains for DNA binding and therefore may act as a transcription factor. The yeast two-hybrid screen or casting technique may be used to isolate and identify other proteins or nucleotides sequences respectively with which the Clone 5 protein has affinity for and therefore interacts with directly. These protein and gene interactions can be confirmed by immunoprecipitation and gel mobility shift assays respectively.
Of course, future directives for the study of any gene would not be complete without performing *in vivo* studies to confirm observations made with *in vitro* studies. Furthermore, the characterization of any new gene would not be complete in the scientific community without completing a "knock-out" mouse model. Knock-out experiments are an excellent tool which prove invaluable in providing phenotypic analyses of protein function due to absence of gene expression and protein function. One could also use an *in vivo* inducible system such as the Cre-loxP to determine physical and genetic detriments incurred by loss of Clone 5 expression and protein function. Again, the inducible system may be useful to look at changes if any that occur as a result of transcriptional inhibition post-natally as opposed to absence of expression during embryonic development and beyond.

In order to have a full understanding in the workings of any gene, many avenues of investigation are required to obtain information on how, when, and where it is expressed and it's protein has functional ability. Once we are able to elucidate the function of a protein, which pathways and processes it participates in, and which molecules the protein interacts with, we can begin to fit these pieces into the puzzle that is the cancer disease. As researchers continue to make discoveries, the wealth of knowledge and understanding of cancer increases. This in turn can aid in providing new strategies with increased efficacy for combating this disease.

It is a very realistic possibility that even if researchers do obtain a complete understanding of factors governing tumor development and progression as we see it today, that the cancer disease will evolve and add further complexity and uncertainty leading scientists back to the bench for more answers. Hence, many have come to the realization that, there may never be a "cure" for cancer. Instead the focus is on developing new
innovative methods that are less invasive while being more effective. While another focus of the medical community public awareness in prevention strategies for certain cancers such as no smoking to reduce lung cancer incidence, self-breast examination for earlier detection of breast cancer, and limiting sun exposure to reduce chances of contracting melanoma. Eliciting participation from general public in the form of education and reduction of risk factors is as much as part of fighting this disease as developing treatments.

Scientists have come along way in the past century in dissecting the events associated with tumor progression and increasing comprehension of malignancy. However, the combat is far from over at present resulting in the continuing diligence of research efforts around the globe.
List of Publications


Iwatsuki, K., T. Endo, H. Misawa, M. Yokouchi, A. Matsumoto, M. Ohtsubo, K.J. Mori, and A. Yoshimura. 1997. STAT5 activation correlates with erythropoietin receptor-
mediated erythroid differentiation of an erythroleukemia cell line. J. Biol. Chem. 272:8149-8152.


