ELUCIDATING THE ROLE OF FLI-1 IN NORMAL DEVELOPMENT & MALIGNANT TRANSFORMATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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General Abstract

Previous studies of genes associated with retroviral-induced neoplasia have provided the foundation for much of our current knowledge of both tumor suppressor and oncogenes, and have contributed to our understanding of both gene function and malignant transformation. The study of Friend virus-induced erythroleukemia, a well-studied example of multistage malignancy, has led to the identification of several oncogenes, including the Ets transcription factor, fli-1. Fli-1 plays a vital role in hematopoiesis, and vasculogenesis through the transcriptional regulation of its target genes, some of which are critical for the control of cellular proliferation, differentiation, and survival. The aberrant regulation of Fli-1 is associated with a number of cancers and human diseases, including erythroleukemia, Ewing’s sarcoma, lupus, and Jacobsen or Paris Trouseau syndrome. The essential goal set out to be achieved by the research presented herein is to establish a better understanding of both the oncogenic and developmental roles of Fli-1 by investigating the molecular basis by which its deregulated expression leads to fundamental aberration in the fine balance between proliferation and differentiation.
This thesis is dedicated in loving memory of

ANGELAROSA DE FRANCESCO

November 16, 1935 – September 19, 2009

Nonna, I can only aspire to be the person you were. I was so fortunate to experience your inspirational strength, love, wisdom, beauty, faith and courage. Your unconditional love, life lessons and our memories together will live on forever in our family.

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May 17, 1975 – July 24, 2010

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\(\alpha\text{MEM}\) – \(\alpha\)-minimum essential medium
aa – amino acid
AGM – aorta-gonad-mesonephros
AML – acute myelogenous leukemia
ATA – amino-terminal transcriptional activation
BAC – bacterial artificial chromosome
B-CLL – B-cell chronic lymphocytic leukemia
BFU-E – erythroid burst-forming units
bGHpA – bovine growth hormone polyadenylation signal
bp – base pair
C/EBP – CCAAT/enhancer binding protein
CFC – colony-forming cell
CFU-E – erythroid colony-forming units
ChIP – chromatin immunoprecipitation
CSD – consortium; Children’s Hospital Oakland Research Institute, the Wellcome Trust Sanger Institute and the University of California at Davis
CTA – carboxy-terminal transcriptional activation
DMEM – Dulbecco’s modified eagle’s medium
DMSO – dimethyl sulfoxide
E – embryonic day
EBS – Ets binding site
EGFP – enhanced green fluorescence protein
EKLFLF – erythroid Kruppel-like factor
En - engrailed
Epo – erythropoietin
EpoR – erythropoietin receptor
ERK – extracellular signal-regulated kinase
ES – embryonic stem cell
EMSA – electrophoretic mobility shift assay
Ets – E26 transformation-specific sequence
EUCOMM – European Conditional Mouse Mutagenesis Program
EWS – Ewing’s sarcoma
FBS – fetal bovine serum
F-MuLV – Friend murine leukemia virus
Fli-1 – Friend leukemia integration-1
Flp – Flip recombinase
FLS – Fli-1-specific region
FOG-1 – Friend of GATA-1
FV-A – Friend virus anemia-inducing strain
FV-P – Friend virus polycythemia-inducing strain
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
G-CSF – granulocyte colony-stimulating factor
GFP – green fluorescence protein
GP – glycoprotein
gp55 – 55 kDa envelope glycoprotein
GM-CSF – granulocyte-macrophage colony-stimulating factor
HEL – human erythroleukemia cell line
H-L-H – helix-loop-helix
HSC – hematopoietic stem cell
HSV-TK – herpes simplex virus thymidine kinase
IKMC – International Knockout Mouse Consortium
IL – interleukin
IMDM – Iscove’s Modified Dulbecco’s Medium
IRES – internal ribosomal entry site
Jak2 – Janus kinase 2
Jnk – c-Jun NH2-terminal kinase
kb - kilobase
KOMP – Knockout Mouse Project
LIF – Leukemia Inhibitory Factor
LMO2 – Lin-1, Isl-1, Mec-3 (LIM)-domain only 2
MAPK – mitogen activated protein kinase
MFI – mean fluorescence intensity
miRNA - microRNA
Myb – myeloblastosis oncogene
Neo – neomycin
NorCOMM – North American Conditional Mouse Mutagenesis Project
NT – non-transfected
NF-κB – nuclear factor-κ B
PBS – phosphate-buffered saline
PGK – phosphoglycerate kinase
PI – propidium iodide
PI3-K – phosphatidylinositol-3 kinase
Rb - retinoblastoma
RLU – relative luciferase units
RNAi – RNA interference
SCF – stem cell factor
SDS – sodium-dodecyl sulfate
SFFV-A – spleen focus forming virus anemia-inducing strain
SFFV-P – spleen focus forming virus polycythemia-inducing strain
SH2 – Src homology 2
SHIP-1 – SH2-containing inositol phosphatase-1
shRNA – short hairpin RNA
siRNA – small interfering RNA
SLE – systemic lupus erythematosus
Sfpi-1 or Spi-1/PU.1 – SFFV proviral integration-1
STAT – signal transducer and activator of transcription
Tal1 – T-cell acute lymphocytic leukemia 1
Tel – translocation Ets leukemia
TIGM – Texas A&M Institute for Genomic Medicine
T-L-T – turn-loop-turn
TPO – thrombopoietin
TPOR – thrombopoietin receptor
VSVG – vesicular stomatitis virus G glycoprotein
Chapter 1
General Introduction

1.1 Erythropoiesis

Hematopoiesis, the synthesis of blood cells from pluripotent stem cells and committed lineage progenitors, is initiated within the yolk sac and fetal liver early in embryonic development, and continues within the bone marrow throughout adulthood. Erythropoiesis, the production of red blood cells or erythrocytes, is the first form of blood cell differentiation in mammalian development. The presence of erythrocytes is vital since their primary function is to facilitate oxygen and carbon dioxide transport between tissues and cells, and consequently is required for growth and survival.

Primitive erythropoiesis, characterized by the transient circulation of large, nucleated embryonic erythrocytes, originates within the yolk sac blood islands. These primitive erythroid cells synthesize several forms of globin, adapted to attract oxygen from maternal blood, and persist in circulation until mid to late gestation (Brotherton et al., 1979). Recent studies have revealed that primitive erythroblasts, derived from the yolk sac, mature, undergoing a loss of proliferative capacity, progressive decrease in cell size, globin gene switching, accumulation of hemoglobin, nuclear condensation and ultimately enucleation (Kingsley et al., 2004), (Kingsley et al., 2006). This enucleation seemingly occurs by nuclear extrusion, with the support of liver-derived macrophages and associated integrins (McGrath et al., 2008).

Definitive erythropoiesis constitutes the second course of erythroid differentiation. During mid gestation, definitive erythroid progenitors or erythroid burst-forming units (BFU-Es), also originating within the yolk sac, enter and mature in the fetal liver at the
onset of embryonic circulation (Palis et al., 1999), (Lux et al., 2008). At the same time, embryonic hematopoietic stem cells (HSCs) also enter and mature in the fetal liver, which are presumed to sustain long-term generation of red blood cells. Prior to the formation of the bone marrow cavity, these early BFU-Es expand within the fetal liver and differentiate into rapidly dividing erythroid colony-forming units (CFU-Es) and eventually enucleate to become mature definitive erythroid cells expressing adult forms of globin. CFU-Es undergo cell division and mature through several morphologically defined stages; proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatophilic erythroblasts and mature erythrocytes. As these erythroid cells differentiate they display a gradual decrease in cell size, increase in chromatin condensation, and increase in hemoglobin concentration (Wong et al., 1986), (Palis et al., 1999). Developing erythroid progenitor and precursor cells surround a central macrophage, forming an erythroblastic island, where differentiation is regulated by adhesive interactions, central macrophage function, and various cytokines and chemokines (Chasis & Mohandas, 2008). During late gestation, differentiation of definitive erythroid progenitors shifts to the bone marrow, where they remain to mature throughout adulthood (Zhang & Hill, 2004), (Palis et al., 1999).

Overall, current data support a model of erythroid ontogeny in the mammalian embryo where three divergent populations of erythroid progenitors mature in three separate environments. The first course of erythroid differentiation, primitive erythropoiesis, arises within the yolk sac, whereby primitive erythroid cells continue to mature in circulation. The second course, definitive erythropoiesis, also originates within the yolk sac, and is distinguished by BFU-E colonization of the fetal liver, where they mature attached to macrophages to generate the first circulating mature erythrocytes. BFU-Es derived from embryonic long-term HSCs constitute the third course of erythroid differentiation. HSCs give rise to a common myeloid progenitor that gives rise to
bipotential progenitors restricted to the granulocyte/macrophage or erythroid/megakaryocyte lineages. BFU-Es initially mature in the fetal liver and later in the bone marrow to generate adult erythrocytes (McGrath & Palis, 2008), (Palis, 2008). A commonality between these divergent populations is that they arise as a consequence of precisely regulated signaling involving several key cytokines, growth factors and transcription factors.

**Figure 1.1 – Erythropoiesis.** Schematic diagram indicating the various stages of erythropoiesis. The morphologically defined stages include; proerythroblasts (BFU-E and CFU-E), basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatophilic erythroblasts, reticulocytes, and mature erythrocytes.
1.1.1 **Signaling in erythropoiesis**

Erythropoiesis is controlled by several cytokines, growth factors and their receptors such as granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-6, stem cell factor (SCF), IL-1, IL-3, IL-4, IL-9, IL-11, granulocyte-macrophage (GM)-CSF, insulin growth factor and erythropoietin (Epo). Among them, Epo, and its transmembrane receptor (EpoR), play a central role in the regulation of erythroid cell proliferation, differentiation and survival (Krantz, 1991). Epo acts on the later committed stages of erythroid development, primarily beginning at the CFU-E stage. Epo synthesis occurs predominantly in the kidneys in response to hypoxia, or low oxygen levels in tissues, and the circulating concentration of Epo is the main regulator of erythroid progenitor numbers. Primitive erythroblasts express higher levels of the EpoR, and display heightened sensitivity to Epo signaling. Mice carrying a targeted disruption of *epo* or the *EpoR* display a significant decrease in the number of primitive erythroid cells, and die around embryonic day 13 (E13) due to defects in definitive fetal liver erythropoiesis. Interestingly, BFU-E and CFU-E progenitors are present in the fetal livers of these mice (Wu et al., 1995), (Lin et al., 1996), (Kieran et al., 1996). Thus, while the expression of Epo, and the EpoR, is not critical for the commitment of the erythroid lineage, it is vital for the progression of definitive erythroid development, as it is essential for CFU-E proliferation, survival and terminal differentiation. Moreover, the function of other endogenous ligands or receptors cannot compensate for the loss of Epo and its receptor.

The binding of Epo to its receptor leads to dimerization of the EpoR and triggers several downstream signaling pathways, mainly through activation of the EpoR associated tyrosine kinase, Janus kinase 2 (Jak2). Once activated by autophosphorylation, Jak2 phosphorylates eight tyrosine residues within the cytoplasmic domain of the receptor.
These phosphorylated tyrosines provide docking sites for various intracellular Src homology 2 (SH2)-domain containing proteins, such as signal transducer and activator of transcription (STAT) proteins, SHIP-1 (SH2-containing inositol phosphatase-1), the p85 subunit of phosphatidylinositol-3 kinase (PI-3K) and Shc, which upon binding can be subsequently phosphorylated and activated. The stimulation by Epo and thereby activation of Jak2 results in activation of the STAT5/Bcl-xl, PI-3K/AKT and the Shc/Ras/mitogen activated protein kinase (MAPK) pathways (Longmore et al., 1998), (Wojchowski et al., 1999).

The cytokine SCF also plays an important role in erythroid cell development. Through binding to the tyrosine kinase receptor, c-kit, SCF potentiates Epo signaling to promote proliferation and expansion of definitive erythroid progenitors (Nocka et al., 1989), (Russell, 1979), (Zochodne et al., 2000). The c-kit receptor can associate with the box-2 cytoplasmic region of the EpoR, and activate the EpoR through phosphorylation (Wu et al., 1995). The requirement for SCF/c-kit interaction and activity in erythroid development became evident from studies performed utilizing White spotting (W) and Steel (Sl) mutant mice carrying inherited mutations within the c-kit and SCF genes. Targeted disruption of SCF/c-kit signaling leads to defects in definitive hematopoiesis, severe anemia, and lethality (Nocka et al., 1989), (Russell, 1979). Interestingly, the lethality of c-kit mutant mice can be rescued by the transgenic expression of Epo, demonstrating that Epo signaling can compensate for the absence of c-kit signaling in erythroid development (Waskow et al., 2004). Similar to Epo, the binding of SCF to c-kit leads to dimerization and phosphorylation of several cytoplasmic tyrosine residues, providing docking sites for intracellular proteins containing SH2 domains and subsequent activation of various signal transduction pathways such as PI-3K/AKT and Src/Lyn (Munugalavadla & Kapur, 2005). Therefore, SCF/c-kit signaling acts in synergy with that of
Epo/EpoR, by regulating common signal transduction pathways necessary for erythropoiesis.

### 1.1.2 Transcriptional regulation of erythropoiesis

Erythroid development involves the complex control of several cytokine and growth factor-induced signal transduction pathways that govern proliferation, survival and differentiation. Downstream transcription factors are responsible for the intricate regulation of these imperative processes. Broad-spectrum transcription factors such as Tal1 (T-cell acute lymphocytic leukemia 1), GATA-2, LMO2 (LIM-only protein 2), and c-Myb (myeloblastosis oncogene) are essential for promoting properties of self-renewal and/or differentiation of hematopoietic progenitors. Erythroid-specific development is dependent upon the expression of several transcription factors, including GATA-1, FOG-1 (Friend of GATA-1), EKLF (Erythroid Kruppel-like factor), Sfpi-1 or Spi-1/PU.1 and Fli-1 (Cantor & Orkin, 2002), (Perry & Soreq, 2002), (Kim & Bresnick, 2007), (Tsiftsoglou et al., 2009).

GATA-1 is expressed in erythroid cells, megakaryocytes, eosinophils, mast cells, and multipotential hematopoietic precursor cells. The critical role of GATA-1 in erythropoiesis was revealed through gene targeting analysis performed using GATA-1 null mice. Mice carrying a targeted disruption of gata-1 are embryonic lethal around midgestation due to severe anemia. These mice display complete ablation of erythropoiesis owing to the apoptosis and blocked maturation of proerythroblasts (Fujiwara et al., 1996). In addition to direct DNA binding to most erythroid genes (Weiss & Orkin, 1995), GATA-1 is associated with critical protein-protein interactions with other factors including, FOG-1 (Tsang et al., 1997), ELKF (Merika & Orkin, 1995), and Spi-1/PU.1 (Zhang et al., 1999), (Nerlov et al., 2000). GATA-1 upregulates the erythroid transcription program (Ney, 2006).
and suppresses hematopoietic precursor multipotentiality, as well as alternative myeloid cell fates (Bresnick et al., 2005). FOG-1, also highly expressed in erythroid cells and megakaryocytes, closely interacts with GATA-1 to direct erythroid differentiation, but does not bind DNA directly. Similar to GATA-1 null mice, FOG-1 knockout mice are embryonic lethal due to severe anemia and blocked maturation at the proerythroblast stage (Tsang et al., 1998). Together with GATA-1, ELKF, an erythroid-specific zinc-finger transcription factor, promotes erythroid differentiation and is essential for the regulation of globin gene expression. ELKF knockout mice display embryonic lethal anemia and failure to activate globin gene expression in the fetal liver (Drissen et al., 2005).

Members of the Ets family of transcription factors, Spi-1/PU.1 and Fli-1, promote the proliferation and self-renewal of erythroid progenitors. Spi-1/PU.1 expression promotes differentiation of lymphoid, granulocytic and monocytic cells (Scott et al., 1994) and blocks differentiation of erythroid cells (Ben-David & Bernstein, 1991), likely through the repression of GATA-1 mediated transcriptional activation (Rekhtman et al., 1999). Spi-1/PU.1 transgenic mice develop erythroleukemia (Moreau-Gachelin et al., 1996), and sustained Spi-1/PU.1 overexpression in erythroid cells blocks terminal differentiation (Moreau-Gachelin et al., 1989), (Moreau-Gachelin et al., 1988). Spi-1/PU.1 deficient mice die from hematopoietic failure, in both lymphoid and myeloid lineages, by late gestation. These mice display normal numbers of both yolk sac and fetal liver-derived megakaryocyte and BFU-E/CFU-E erythroid progenitors, however some mice also display an impairment of erythroblast maturation (Scott et al., 1994). Accordingly, fetal liver-derived Spi-1/PU.1 deficient hematopoietic progenitors fail to proliferate in response to the presence of Epo and SCF, IL-3, or GM-CSF, indicating a role for Spi-1/PU.1 in supporting the proliferation of immature erythroid progenitors (Fisher et al., 2004). Similar to Spi-1/PU.1, sustained Fli-1 overexpression in erythroid progenitors blocks terminal differentiation and is associated with viral erythroleukemogenesis. Fli-1
expression influences properties of self-renewal, reduces the levels of GATA-1, and is thereby downregulated during differentiation of pluripotent hematopoietic cells (Athanasiou et al., 2000), (Howard et al., 1993). Transcription factors play an integral role in the determination of blood cell fate, and therefore their dysregulation, associated with chromosomal translocation, mutation or viral insertion, often leads to hematological malignancy. The effects of Fli-1 expression in hematopoiesis and malignant transformation are described in greater detail in Section 1.3.

1.2 Friend virus-induced erythroleukemia as a model of leukemogenesis

During normal hematopoietic development stem cells abide by a distinctive differentiation program. Various cytokines, and their cognate receptors and signal transduction pathways, are essential for the formation of lineage-committed progenitors and various blood cell types. The decision of self-renewal or maturation of hematopoietic progenitors requires a fine balance between the tightly regulated processes of proliferation, differentiation, and survival. However, the intrinsic control systems that exist to maintain this balance, occasionally fail. Cells begin to exhibit a block in terminal differentiation, uncontrolled proliferation, enhanced survival and the accumulation of oncogenic mutations. Eventually these events lead to transformation of hematopoietic progenitor cells, causing various types of leukemia. Oncogenic mutations linked to this uncoordinated balance, and the induction of cancer, are often associated with dysregulated expression of tumor suppressor genes and oncogenes. The discovery, and much of our current knowledge of both tumor suppressor and oncogenes can be attributed to studies of genes associated with retroviral-induced neoplasia that have contributed to our understanding of both gene function and malignant transformation. Prime examples, fli-1 and spi-1/PU.1, Ets family proto-oncogenes, were discovered through studies of retroviral-
induced disease like so many other Ets transcription factors. Integration at the \textit{fli-1} or \textit{spi-1/PU.1} locus occurs upon infection with distinct components of the Friend leukemia virus, originally discovered by Charlotte Friend in 1957 (Friend, 1957).

\subsection*{1.2.1 FV-P/A-induced erythroleukemias}

Friend virus (FV)-induced murine erythroleukemia is a well-characterized animal model of multi-step leukemogenesis (Ben-David \& Bernstein, 1989), (Tambourin et al., 1981) that has been used as a powerful tool to identify several oncogenes such as \textit{fli-1}, \textit{spi-1/PU.1}, \textit{p53}, \textit{fli-2/p45 NFE2}, and \textit{fli-3/miR-17-92} (Lee et al., 2003). Two separate isolates of Friend virus, anemia- and polycythemia-inducing strains, termed FV-A and FV-P, respectively, have been identified. Both isolates represent complexes of two distinct viral species, namely a unique replication defective spleen focus forming virus (SFFV-A or SFFV-P) and a common replication competent friend murine leukemia virus (F-MuLV). The FV-A and FV-P strains generate similar multistage malignancies within 1-3 weeks upon injection into susceptible strains of newborn or adult mice (Mager et al., 1981). The preleukemic stages of FV-P/A-induced disease are characterized by the rapid polyclonal expansion of immature erythroblasts, usually within the spleen and liver. This proliferation can be attributed to the ability of gp55, a 55 kDa fusion envelope glycoprotein encoded by SFFV (Li et al., 1990), to mimic the effect of Epo by binding to and activating the EpoR (Li et al., 1990), (Berger et al., 1985), (Hoatlin et al., 1990), (Li et al., 1987). The emergence of clonal tumorigenic proerythroblastic cells during the malignant stage of the disease is dependent upon retroviral insertional activation of the \textit{spi-1/PU.1} Ets transcription factor (Moreau-Gachelin et al., 1988), (Gobel et al., 1990). Proviral integration at the \textit{spi-1/PU.1} locus exclusively occurs 5' of the transcription start site, where transcription of viral sequences are oriented in the opposite direction of \textit{spi-1/PU.1} transcription. The localization and
orientation of viral integration allows the viral enhancer to mediate viral activation of the 
\textit{spi-1/PU.1} promoter. Additional mutations within the tumor suppressor genes \textit{p53} (Ben-
David et al., 1990b), (Chow et al., 1987), (Mowat et al., 1985), (Munroe et al., 1990) or \textit{p45 NFE2} (Li et al., 2001) confer additional selective growth advantages \textit{in vivo}, as well as 
increased survival of transformed erythroblasts in culture, thereby contributing to the 
progression of FV-induced erythroleukemia (Ben David et al., 1988), (Lavigneuer & 
Bernstein, 1991), (Ben-David & Howard, 1995), (Li et al., 2001), (Prasher et al., 2001). In 
accordance with the study of Friend disease, the overexpression of Spi-1/PU.1 alone is 
adequate to transform erythroblasts, irrespective of p53 mutational status (Barnache et al., 
1998), since transgenic mice similarly develop erythroleukemia (Moreau-Gachelin et al., 
1996). Leukemic proerythroblasts, isolated from Spi-1/PU.1 transgenic mice, grow 
independently of Epo and are incapable of undergoing differentiation. The study of Friend 
disease has also revealed the dependence of erythroleukemic cells on Spi-1/PU.1 
overexpression to continuously proliferate (Delgado et al., 1994), (Juban et al., 2009). 
The exact mechanisms by which Spi-1/PU.1 overexpression results in erythroid cell 
transformation have yet to be precisely defined, however evidence suggests that Spi-
1/PU.1 blocks erythroid differentiation and promotes erythroid proliferation.

\section*{1.2.2 F-MuLV-induced erythroleukemia}

The common replication competent viral species of the Friend virus complex, F-
MuLV, is also capable of inducing erythroleukemia in susceptible strains of newborn mice. 
Upon injection with F-MuLV alone, strain-specific neonates develop a multistage disease 
within 3-6 weeks, the pathology of which closely mimics the stepwise genetic changes seen 
in multistage leukemia (Silver & Kozak, 1986). This F-MuLV-induced erythroleukemia is 
characterized by proerythroblastic expansion leading to anemia, splenomegaly, and
eventually death within eight weeks of viral injection ([Figure 1.2]). However, the rapid polyclonal expansion of proerythroblasts is not observed since F-MuLV lacks the sequence encoding the envelope glycoprotein, gp55. Integration of the provirus at the fli-1 locus drives enhancer-mediated Fli-1 overexpression, and has been identified as the pivotal genetic event of this disease (Ben-David, 1990a), (Ben-David et al., 1991). Fli-1 viral integration is followed by further expression changes in anti-apoptotic genes, namely bcl-2
and bcl-xl, tumor suppressor genes, like p53, p45 NFE2, as well as downregulation of rb (Retinoblastoma) and modification of the Epo gene, resulting in Epo-independent proliferation of erythroblasts and fully leukemic clones (Ben-David et al., 1990a), (Ben-David et al., 1990b), (Ben-David & Bernstein, 1991), (Tamir et al., 1999). The rapid induction and decreased survival rates of F-MuLV-induced erythroleukemia in p45 NFE2 and p53 deficient mice suggests that these tumor suppressor genes play an important role during in vivo cancer progression (Li et al., 2001), (Silver & Kozak, 1986). While these secondary mutations occur at the genetic level, Fli-1 also directly upregulates the transcription of bcl-2 (Pereira et al., 1999), (Lesault et al., 2002), and downregulates p53 via an MDM2-mediated mechanism (Truong et al., 2005). Recent studies have provided substantial evidence to suggest that Fli-1 overexpression is essential for proerythroblast proliferation, survival and maintenance of the malignant phenotype (as discussed in Chapter 2), (Juban et al., 2009). Thus the F-MuLV-induced erythroleukemia mouse model has served as an important tool in identifying fli-1 as an important proto-oncogene, and has provided functional data on the role of fli-1 in malignant transformation in this disease. This functional data will enable us to gain a better understanding of the molecular mechanisms and pathways responsible for pathogenesis of human diseases associated with aberrant expression of fli-1.

While fli-1 is activated in approximately ninety percent of F-MuLV-induced erythroleukemias, our laboratory has shown that a small percentage of infected mice display retroviral insertion within the fli-3 locus, leading to activation of a microRNA (miRNA) cluster termed miR17-92 (Cui et al., 2007). Similar to Fli-1, miR17-92 is also overexpressed in various cancer cell types (Ota et al., 2004), (Hayashita et al., 2005), (He et al., 2005), (Wang et al., 2006) and activates similar signaling pathways associated with erythroid transformation.
1.3 Ets family of transcription factors

It is well known that the transcriptional regulation of key hematopoietic genes, regulating the biological processes of proliferation, differentiation and survival, is a fundamental control mechanism for the formation and subsequent activity of HSC's (Cantor & Orkin, 2001). Many of the genes proven to be indispensable through the course of hematopoiesis contain binding sites for the Ets (E26 transformation-specific sequence) family of transcription factors (Gottgens et al., 2002), (Swiers et al., 2006). The graded expression of Ets gene family members in various hematopoietic precursors and mature lineage-committed cell types indicate a role for these transcription factors in lineage commitment and stage progression from the earliest stages of development through to adulthood.

The Ets family of transcription factors are found in the genomes of diverse organisms including *Drosophila, Xenopus, Danio rerio, Gallus, Mus musculus* and *Homo sapiens*, establishing this as one of the largest transcription factor families (Seth et al., 1992), (Papas et al., 1997), (Mavrothalassitis & Ghysdael, 2000), (Sementchenko & Watson, 2000). All Ets proteins share a highly conserved winged helix-loop-helix DNA binding Ets domain, a minimal 85 aa region generally located at the carboxyl terminal end, which specifically interacts with DNA sequences containing a consensus GGAA/T core motif. The binding sequence specificity of Ets proteins to the consensus sequence is dependent upon flanking sequences of the core motif, and is facilitated by synergistic interactions with other transacting factors (Seth et al., 1992), (Watson et al., 1992), (Papas et al., 1997), (Hsu et al., 2004). Therefore, several Ets proteins have the ability to bind to the same target sequence or can bind to multiple Ets binding sites, with different affinities. There are approximately 30 members of the Ets superfamily, which include *ets-1* (Watson et al., 1985), *ets-2* (Watson et al., 1988), *sfpi-1* (*spi-1/PU.1*) (Goebl, 1990), *spi-1B* (Ray et al.,
1992), *erg* (Rao et al., 1987), *elk1* and *elk2* (Rao et al., 1989), *elk4* (sap-1) (Dalton & Treisman, 1992), *etv4* (pea3/E1AF) (Xin et al., 1992), *gabpα* (LaMarco et al., 1991), *elf-1* (Thompson et al., 1992), *elk3* (sap-2) (Lopez et al., 1994), *etv6* (tel) (Golub et al., 1994), and *fli-1* (Ben-David et al., 1990a), (Ben-David et al., 1991). Ets proteins function as transcriptional activators or repressors to regulate the expression of genes that control the signaling pathways governing various cellular processes and invariably, transformation. Thus, deregulated Ets transcription factor function can lead to malignancy and indeed, many Ets proteins are associated with different cancers. Understanding their diverse roles can therefore provide insight into the molecular changes involved in cancer progression.

The founding member of the Ets family, *ets-1*, provided the name and prototype by which this transcription factor family is characterized. The *ets-1* proto-oncogene was originally discovered in a study of retroviral-induced neoplasia as the cellular gene overexpressed as a result of the viral fusion protein (gag-myb-ets) encoded by the avian replication-defective retrovirus, E26, causing acute erythroid-myeloid and lymphoid leukemia (Leprince et al., 1983), (Nunn et al., 1983), (Nunn et al., 1984). Subsequent discoveries of other Ets factors can also be attributed to studies of retroviral-induced leukemia, specifically *spi-1/PU.1*, activated in SFFV-induced erythroleukemia (Moreau-Gachelin et al., 1988), and *fli-1*, activated in F-MuLV-induced erythroleukemia (Ben-David et al., 1990a), (Ben-David et al., 1991), as a result of site-specific viral integrations, as mentioned above in Section 1.2. Retroviral integration at the *fli-1* locus also occurs in early hematopoietic cells infected with the 10A1 viral isolate of MuLV (Ott et al., 1994), in granulocytic leukemia induced by Graffi virus (Denicourt et al., 1999), and in non-T and non-B lymphomas induced by the Cas-Br-E virus (Bergeron et al., 1991). Given that aberrant regulation of Fli-1 is associated with an array of retroviral-induced tumors, further studies have focused on characterizing the obvious functional role of this transcription factor in normal hematopoietic development and malignant transformation.
Table 1.1 – Ets Transcription Factor Family

Members of the Ets transcription factor family share a highly conserved DNA binding (Ets) domain, a minimal 85 aa region generally located at the carboxy terminal end, which interacts with DNA sequences containing a GGAA/T core motif. The founding member, ets-1, was discovered as the avian E26 leukemia virus oncogene. Ets proteins are involved in the regulation of various cellular processes and many are associated with malignant transformation. Several Ets family members and their associated gene function are listed. References represent studies associated with their discovery/characterization.

<table>
<thead>
<tr>
<th>Ets Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>elf-1</td>
<td>Blood and endothelial cell development</td>
<td>Liu et al, 2008</td>
</tr>
<tr>
<td>elk1/elk2</td>
<td>inflammatory response/MAPK signaling</td>
<td>Rao et al., 1989</td>
</tr>
<tr>
<td>elk3 (sap-2)</td>
<td>angiogenesis/hypoxia</td>
<td>Lopez et al., 1994</td>
</tr>
<tr>
<td>elk4 (sap-1)</td>
<td>MAPK signaling/T cell development</td>
<td>Dalton &amp; Treisman, 1992</td>
</tr>
<tr>
<td>erg</td>
<td>endothelial cell differentiation/megakaryopoiesis</td>
<td>Rao et al., 1987</td>
</tr>
<tr>
<td>ets-1</td>
<td>angiogenesis/cardiac neural crest development/lymphopoiesis</td>
<td>Watson et al., 1985</td>
</tr>
<tr>
<td>ets-2</td>
<td>angiogenesis/thymocytes proliferation &amp; survival/inflammatory response</td>
<td>Watson et al., 1988</td>
</tr>
<tr>
<td>etv4 (pea3/E1AF)</td>
<td>Neuron development</td>
<td>Xin et al., 1992</td>
</tr>
<tr>
<td>etv6 (tel)</td>
<td>erythropoiesis/hematopoietic progenitors</td>
<td>Golub et al., 1994</td>
</tr>
<tr>
<td>fli-1</td>
<td>erythropoiesis/megakaryopoiesis/hematopoiesis/vasculogenesis/immune response</td>
<td>Ben-David et al., 1990a, Ben-David et al., 1991</td>
</tr>
<tr>
<td>gabc-α</td>
<td>cell cycle control/protein synthesis</td>
<td>LaMarco et al., 1991</td>
</tr>
<tr>
<td>sfpi-1 (spi-1/PU.1)</td>
<td>myeloid, B &amp; T cell development</td>
<td>Goebl, 1990</td>
</tr>
<tr>
<td>spi-1B</td>
<td>plasma cell differentiation</td>
<td>Ray et al., 1992</td>
</tr>
</tbody>
</table>
1.4 Friend Leukemia Integration-1 (Fli-1)

Friend leukemia integration-1 (\textit{fli-1}), a well-studied member of the Ets family, is often associated with oncogenesis (Ben-David et al., 1991), (Delattre et al., 1992), (Watson et al., 1992), (Truong & Ben David, 2000) as it is activated in the majority of F-MuLV-induced erythroleukemias (Ben-David et al., 1991) (Section 1.2). The aberrant regulation of Fli-1 has been implicated in various human cancers (Delattre et al., 1992), (Kwiatkowski et al., 1998), (Mhawech-Fauceglia et al., 2006) and disease (Hart et al., 2000), (Favier et al., 2003), (Zhang et al., 2004), (Wenger et al., 2006). Genetic loss of function studies in mice have established that Fli-1 plays a critical role in hematopoiesis, vasculogenesis and immune function, and its expression is vital for early development (Hart et al., 2000), (Spyropoulos et al., 2000). As a transcription factor, its oncogenic properties arise from aberrant transcriptional regulation of its target genes, some of which are critical for the control of cellular proliferation, differentiation, cell adhesion, apoptosis, vascular development and hematopoiesis. Fli-1 is preferentially expressed in vascular endothelial cells and all hematopoietic tissues, including the thymus and spleen, and expressed at a lower level in the lungs, heart and ovaries (Ben-David et al., 1991), (Melet et al., 1996), (Truong & Ben David, 2000). Accordingly, Fli-1 regulates genes important in the development of these tissues (Ben-David et al., 1991), (Klemsz et al., 1994). Given its breadth of expression, it is expected that changes in Fli-1 expression would inevitably result in vascular and/or hematopoietic abnormalities. Indeed its discovery is attributable to its role in erythroleukemogenesis in mice since \textit{fli-1} is a common site for retroviral integration, and activation, in Friend virus-induced erythroleukemia (Section 1.2).

\textit{Fli-1} is located on mouse chromosome 9 and human chromosome 11q24, a region of several abnormalities in human disease. Both the murine and human homologs of the \textit{fli-1} gene are approximately 120 kb, consist of nine exons, and encode two protein isoforms,
p51 (452 aa) and p48 (419 aa) (Ben-David et al., 1991), (Watson et al., 1992). Two highly conserved in-frame translation initiation codons, localized to nucleotide 161 within exon 1 (AUG +1), and 261 within exon 2 (AUG +100) of the mouse fli-1 mRNA sequence (NM_008026.4) are responsible for the synthesis of the two Fli-1 protein isoforms (Sarrazin et al., 2000). Transcription of the mouse fli-1 gene produces a full-length mRNA transcript of 3087 bp, and a processed length of 1359 bp. The final exon, 9, containing several functional domains is the largest and is approximately 530 bp in length, while the first intron is the largest at approximately 64 kb in length (Figure 1.3). At least two major transcription initiation (CAP) sites have been localized to 143 and 114 nucleotides upstream the defined mouse fli-1 cDNA 5’ end (Barbeau et al., 1996). Comparison of the nucleotide sequences revealed that the mouse and human isoforms of fli-1 share 94% homology in the sequences adjacent to these CAP sites (Barbeau et al., 1996). Moreover, the fli-1 promoter contains a potential TATA box element, and several conserved regulatory elements. These include GATA, STAT, and Ets binding sites, GT-rich, GC-rich, AP-2, AP-3 and CTC elements, some of which are conserved between mouse and human. The fli-1 promoter also contains binding sites for Sp-1, c-Myc, Gata-1, Ets-2, Oct-3, TBP, Etv-4, EBP, ATF/CREB, E2A-PBX1, and Fli-1 itself (Barbeau et al., 1996), (Dhulipala et al., 1998). Additionally, the highly conserved 5’ non-translated region of exon 1 is predicted to form a very stable hairpin structure, capable of post-transcriptional autoregulation (Barbeau et al., 1996).
Figure 1.3 – Genomic organization of the murine fli-1 gene. Fli-1 is located on mouse chromosome 9 and contains nine exons extending over approximately 120 kb, with a processed mRNA transcript length of 1359 bp, and encodes two protein isoforms of 452 aa (p51) and 419 aa (p48).

A pseudogene has not been identified for the fli-1 gene, however comparison of the amino acid sequence of Fli-1 has revealed an 81% homology to the Ets-related protein Erg, localized adjacent to the ets-2 gene on chromosome 16 (Prasad et al., 1992), (Watson et al,
Fli-1, Erg, Ets-1 and Ets-2 similarly contain 5' and 3' Ets domains. The 5' Ets domain of Fli-1 shows 82% conservation with the amino acid sequence of Erg and approximately 60% conservation with that of Ets-1 and Ets-2. The 3' Ets domain of Fli-1 shows 98% conservation with the amino acid sequence of Erg (Truong & Ben David, 2000). Moreover, the fli-1 genetic locus is within 155 kb of the ets-1 proto-oncogene in mouse and 106 kb in human, suggesting that these Ets transcription factors arose by gene duplication from a common ancestral gene (Ben-David et al., 1991). The fli-1 gene is conserved in human, mouse, chimpanzee, dog, cow, rat, chicken and zebrafish.

Alternative transcription initiation sites result in the production of two protein isoforms encoded by fli-1, p51 and p48. Gene knockout studies have provided evidence to suggest that both the p51 and p48 isoforms retain the same functional domains and activity (Melet et al., 1996). The functional domains located within the Fli-1 protein include the 5' Ets domain, and a Fli-1-specific region (FLS) referred to as the amino-terminal transcriptional activation (ATA) domain, and a 3' Ets domain and carboxy-terminal transcriptional activation (CTA) domain (Figure 1.4). The 5' Ets domain is located within amino acids 121-196 and the FLS, which is absent in the Erg protein, is localized within amino acids 205-292. The 3' Ets domain is located within amino acids 277-360 and is responsible for sequence specific DNA-binding activity of Fli-1. The CTA domain, located within amino acids 402-452, is also involved in transcriptional activation and protein-protein interaction. Both the 5' and 3' Ets domain contain sequences of helix-loop-helix (H-L-H) secondary structures that are also present in Erg (Rao et al., 1993), while the FLS and CTA domains contain sequences, which resemble turn-loop-turn (T-L-T) secondary structures. The structures of the ATA and CTA domains contribute to the transcriptional activity of Fli-1. It has been suggested that the CTA region may serve simultaneously as a transcriptional activator and repressor (Rao et al., 1993). Mice engineered to lack the CTA domain of Fli-1 express negligible to low levels of the mutant of Fli-1 mRNA and protein
(Hart et al., 2000), (Spyropoulos et al., 2000). The participation of the CTA domain in transcriptional activation has been further substantiated by these recombinant studies since recombinant Fli-1 protein, lacking the CTA domain, display only 50 - 60% of the transcriptional activity of wildtype Fli-1. The reduction in the level of mutant Fli-1 protein in these mice also indicates that the CTA domain may additionally function to autoregulate Fli-1 expression. NMR spectroscopy analyses have shown that the 3' Ets domain of Fli-1 is comprised of three alpha-helices and a four stranded beta-sheet, resembling the structures of the class of helix-turn-helix DNA-binding proteins found in the catabolite activator protein of Escherichia coli, as well as those of several eukaryotic DNA binding proteins including H5, HNF-3/forkhead, and the heat shock transcription factor (Liang et al., 1994a), (Liang et al., 1994b). Interestingly, comparison of the Fli-1 3’ Ets domain to other structures revealed that this 3’ Ets domain employs a new variation of the winged helix-turn-helix core motif for binding to DNA (Liang et al., 1994a), (Liang et al., 1994b).

**Figure 1.4 – Functional domains of the Fli-1 protein.** Both the murine and human forms of Fli-1 contain several functional domains; the amino terminal transcriptional activation domain (ATA), which consists of the 5’ Ets domain and the Fli-1-specific region (FLS), the 3’Ets domain and the carboxy-terminal transcriptional activation (CTA) domain.
Phosphorylation of both Fli-1 protein isoforms has been predominately detected on serine residues. This phosphorylation is modulated by the concentration of intracellular calcium, similar to Ets-1 and Ets-2, and dephosphorylation is controlled, at least in part, by the phosphatase PP2A. Post-translational modification by phosphorylation affects Fli-1 DNA binding, protein-protein interaction and transcriptional activation, thereby contributing to the overall control of gene function (Zhang & Watson, 2005).

Fli-1 binds to DNA in a sequence-specific manner, and it has been determined that the optimal DNA binding sequence for Fli-1 is ACCGAAG/aT/c (Mao et al., 1994). The bases flanking the core GGA Ets DNA-binding motif synergistically contribute to the binding specificity among different Ets transcription factors. Gene promoters containing these Ets sequences have shown to be transcriptionally regulated by Fli-1, including bcl-2 (Lesault et al., 2002), MDM2 (Truong et al., 2005), gplX, gpllb (Bastian et al., 1999), mpl (Deveaux et al., 1996), and recently SHIP-1 (as discussed in Chapter 3).

1.4.1 Fli-1 plays an important role in erythropoiesis & erythroleukemogenesis

The overexpression of fli-1 in F-MuLV-induced erythroleukemia also provided insight into its nonpathological role in erythropoiesis, the production of red blood cells. As discussed in Section 1.1, cellular differentiation requires timely and appropriate expression of specific genes, which is carried out via transcription factors. During erythropoiesis, primitive BFU-E cells respond to the cytokines, SCF and Epo, to undergo specific changes to become mature red blood cells, as discussed in Section 1.1. However, F-MuLV-infected mice show a block in erythroid differentiation. To further understand this, our laboratory isolated a unique erythroleukemia cell line termed HB60-5, which proliferates in the presence of Epo and SCF but undergoes differentiation in response to Epo alone. Preliminary experiments revealed that Epo-induced differentiation of these
cells requires a transient, drastic decrease in Fli-1 levels and that constitutive expression of Fli-1 blocks this process (Tamir et al., 1999). Furthermore, overexpression of Fli-1 dramatically shifts the effects of the Epo/EpoR signal transduction pathway (Zochodne et al., 2000). For example, while Epo-induced differentiation of HB60-5 cells correlates with Jak-2/Stat5 phosphorylation, ectopic expression of Fli-1 in these cells appears to activate the Shc/Ras pathway resulting in proliferation. These results suggest that constitutive activation of fli-1 in erythroblasts alters their responsiveness to Epo, switching the signaling event(s) associated with terminal differentiation to proliferation.

Recently, a study in our laboratory, as well as that of another laboratory, demonstrated that Fli-1 inhibition suppresses growth and induces cell death in both murine and human erythroleukemias (Juban et al., 2009), (as discussed in Chapter 2). RNAi-mediated downregulation of Fli-1 in both murine and human erythroleukemic cell lines resulted in a marked growth inhibition and apoptosis, as well as downregulation of an important Fli-1 target gene, bcl-2 (Lesault et al., 2002). These results demonstrate the importance of Fli-1 not only in the progression, but also in the maintenance of the malignant phenotype. This study will be discussed in detail in Chapter 2.

1.4.2 Clinical relevance of Fli-1

While aberrant Fli-1 expression is associated with viral integration in mice, it is sometimes associated with chromosomal translocations in human diseases. The human fli-1 gene is commonly deleted in the relatively infrequent congenital disorder, known as Jacobsen or Paris-Trousseau syndrome. These chromosomal deletions result in Fli-1 deficiency, and often include clinical abnormalities such as growth and mental retardation, cardiac defects, dysmorphogenesis of the digits and face, pancytopenia, and thrombocytopenia (Krishnamurti et al., 2001), (Favier et al., 2003), (Wenger et al., 2006).
However, in Ewing's sarcomas and primitive neuroectodermal tumors, translocations between chromosome 11 and 22, t(11;22) (q24;q12) results in a chimeric EWS/Fli-1 fusion protein detectable in 85% of these tumors. This fusion protein contains the carboxy-terminal region or Ets domain of Fli-1 and the amino terminal region of EWS (Ewing's Sarcoma), and functions as a transcriptional activator that plays a significant role in this tumor progression (Delattre et al., 1992), (Ohno et al., 1993). Recently, Fli-1 overexpression has also been implicated in other human diseases, such as systemic lupus erythematosus (SLE) (Zhang et al., 1995), (Georgiou et al., 1996), (Zhang et al., 2004).

SLE is an autoimmune disease with a broad range of clinical and immunological abnormalities. The cause remains unclear, however the combination of genetic predisposition and environmental triggers is considered to contribute the development of this disease. Fli-1 expression is detected in the developing spleen and thymus, and in mature B cells, and throughout T cell development in pro-T, pre-T and mature T cells (Melet et al., 1996), (Anderson et al., 1999) although its expression is downregulated in activated T cells (Zhang & Watson, 2005). Interestingly, Fli-1 overexpression occurs in several murine lupus models (Georgiou et al., 1996), (Zhang et al., 2004), as well as in peripheral blood leukocytes of SLE patients, as compared with normal healthy controls (Georgiou et al., 1996). Fli-1 transgenic mice display abnormal production of autoreactive lymphocytes and autoantibodies that leads to the development of an autoimmune kidney disease similarly observed in the murine model of lupus (Zhang et al., 1995). Additionally, a 50% reduction of Fli-1 expression in the MRL/MpJ-Fas$^{lpr}$ (MRL/lpr) murine lupus model markedly prolongs survival and significantly reduces renal disease with decreased autoantibody production and proteinuria (Zhang et al., 2004). Taken together, these observations suggest that Fli-1 overexpression is an important factor in the development of this disease due to the apparent role of Fli-1 in lymphocyte function. Fli-1 overexpression may also play a role in the development of human leukemias.
Acute myelogenous leukemia (AML) is a common cancer that frequently involves the loss of the *tel* (translocation Ets leukemia) gene by chromosomal translocation (Lopez et al., 1999). Tel, an Ets transcription factor, is known to interact with Fli-1 on a protein-protein basis (Kwiatkowski et al., 1998), (Lopez et al., 1999). *In vitro* and *in vivo* analyses demonstrate that binding of wildtype Tel to Fli-1 inhibits Fli-1’s regulatory function, without affecting its DNA binding properties (Kwiatkowski et al., 1998). In most leukemias, where one *tel* gene is lost by translocation, the remaining allele is deleted (Lopez et al., 1999), thereby eliminating its normal regulation of Fli-1. This essentially results in Fli-1 overexpression, which is thought to contribute to the malignant phenotype in this human leukemia. Recent immunohistochemical and tissue microarray studies have revealed the expression of Fli-1 in a wide variety of benign and malignant tumors and leukemias, such as capillary hemangioma, neuroblastoma, small cell lung carcinoma, glioblastoma, non-Hodgkin’s lymphoma, lymphoblastic lymphomas angiosarcoma, Kaposi’s sarcoma, and medullar breast carcinoma (Mhawech-Fauceglia et al., 2006). Furthermore, breast cancer culture studies have revealed the ability of Fli-1 to contribute to the malignancy of breast cancer by inhibition of apoptosis through upregulating the expression of the antiapoptotic gene, *bcl-2* (Sakurai et al., 2007). Immunohistochemical analysis has also revealed that Fli-1 expression is a valuable tool in the diagnosis of benign and malignant vascular tumors.

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the western world and is characterized by an accumulation of long-lived mature B cells positive for the abnormal co-expression of cell surface markers CD5, CD19, CD23, and low expression of IgM (immunoglobulin M subunit). These cells are refractory to immune clearance/apoptosis, and are arrested in the *G0/G1* phase of the cell cycle. Clonal expansion of the B cell leukemic population is attributed to chromosomal abnormalities and intrinsic defects in apoptotic machinery due to increased expression of Bcl-2, ZAP70,
cyclinD1 and c-Myc, and decreased expression of p53 and Rb. While this disease may have a long latency period, it can progress very rapidly, and unfortunately, most patients develop drug resistance and are incurable by conventional chemotherapy (Zwiebel & Cheson, 1998). Recently, I have shown that most B-CLL patients also express high levels of Fli-1, which correlate to elevated expression Bcl-2 levels in these cells (unpublished data). Fli-1 is known to transactivate bcl-2, as well as MDM2, a negative regulator of p53. It is quite plausible that Fli-1 may play a direct role in the evasion of apoptosis. Further investigation into the role of Fli-1 in the development and maintenance of B-CLL will be presented in the first aim of my project. Fli-1 gain of function studies provide evidence to suggest that Fli-1 is an important regulator of lymphoid cell function, development and programmed cell death.

1.4.3 Fli-1 gain of function studies

Transgenic mice overexpressing Fli-1 under the control of the H2K promoter have previously been generated in the laboratory of Dr. Alan Bernstein (Zhang et al., 1995). These mice display 2-to-3 fold overexpression of Fli-1 protein in the spleen and thymus, and develop a lupus-like, severe autoimmune disease culminating in glomerulo-nephritis, ultimately resulting in death. These mice exhibit increased lymphopoiesis, splenomegaly, accumulation of abnormal T and B cells in peripheral lymphoid tissues, develop autoimmunity due to an increase in B-cell survival and immunoglobulin production, as a result of Fli-1 overexpression. In addition, B cells isolated from these mice are hyperresponsive to mitogens and exhibit a significant reduction in activation-induced cell death in vitro. Oddly, other lineages were not ostensibly affected in these mice, lower levels of expression due to promoter selectivity could explain this phenomenon. Phenotypically, these fli-1 transgenic mice exhibit a B cell dysfunction similar to bcl-2
overexpressing transgenic mice (Strasser et al., 1991). Given that Fli-1 is a direct transcriptional regulator of bcl-2 (Pereira et al., 1999), (Lesault et al., 2002) it is possible that Fli-1 overexpression interferes with programmed cell death in B cells by this mechanism, resulting in a similar autoimmune disease.

Our lab has also recently generated a transgenic mouse model in which fli-1 is overexpressed. Fli-1 cDNA was inserted into a vector immediately upstream of an internal ribosomal entry site (IRES) fused to the enhanced green fluorescence protein (EGFP) gene. A floxed lacZ/neo cassette, residing upstream the Fli-1-IRES-EGFP sequences, is cleaved as a result of an in vivo recombination event mediated by Cre expression, thereby initiating Fli-1/EGFP expression. Fli-1 transgenic mice were crossed to Tie-2/Cre mice, allowing expression of Fli-1/EGFP within blood vessels and hematopoietic tissues. Interestingly, with the exception of B and T cells, the level of green fluorescence detected in hematopoietic tissues of these fli-1 transgenic mice is much lower in comparison to control mice (unpublished data, Lee et al). Consistent with observations made by Zhang et al. (Zhang et al., 1995), results indicate that Fli-1 expression is tightly regulated and aberrant expression may not be tolerated in some cell types. Also, the capacity of the lymphoid lineage to tolerate high levels of Fli-1 may suggest a developmental role for Fli-1 in these cells and hence additional studies required to further elucidate the role of fli-1 in lymphoid development.

1.4.4 Fli-1 loss of function studies

Many Ets family members share homologous sequences in their DNA binding domain, and so it is possible that other family members might compensate for an absent, or non-functional Ets protein, in knockout mice. However, most Ets transcription factor knockout mice, including fli-1, display a detectable phenotype and die in utero (Bassuk &
Leiden, 1997), suggesting that their expression is vital for early development. Several groups have generated and analyzed the effects of targeted disruptions at the fli-1 locus to examine the effects of Fli-1 deficiency. The first targeted disruption of fli-1 generated a non-lethal minor phenotype, including a reduction in thymus size and in the total number of thymocytes (Melet et al., 1996). This mild phenotype was found to be due to the targeting construct used to generate these mice. A neomycin resistance cassette was placed within exon II, to delete the first translational start site and disrupt all but the first ten amino acids of Fli-1. However, alternative splicing around the neomycin resistance cassette generated a truncated form of the protein that retained all of the known functional domains located mainly at the 3’end or N-terminus of Fli-1. As this was not a genuine knockout model, two separate groups (Hart et al., 2000), (Spyropoulos et al., 2000), performed additional targeting experiments with similar observations further validating a role for Fli-1 in hematopoietic development.

The study presented by Hart et al. (Hart et al., 2000), demonstrates that Fli-1 expression is vital for embryogenesis and its absence results in embryonic lethality on embryonic day 11.5 (E11.5). The targeting construct was designed to disrupt Fli-1 activity by replacing the 3’ DNA-binding Ets domain and CTA domain, located within exon 9, with a lacZ reporter gene and positive selection cassette. Embryos homozygous for the fli-1 deletion display a complete loss of detectable Fli-1 protein and fli-1 transcripts at day E9.5. Fli-1 null mice display reduced vascular integrity of the vascular plexus and hemorrhaging in the mid-brain. This was concurrent with specific downregulation of the angiopoietin receptor-1, tek/tie2, and increased numbers of megakaryocyte progenitors. Similarly, the study presented by Spyropoulos et al. (Spyropoulos et al., 2000) also describes profound hematorrhachitic and hematopoietic phenotypes in mouse embryos homozygous for a targeted disruption in flt-1 with embryonic lethality. Similarly, Spyropoulos et al. (Spyropoulos et al., 2000) designed a targeting construct to contain an insertional
disruption immediately upstream the transcriptional activation domain, comprising of the 3’ Ets domain and the CTA domain of exon 9, whereby resultant targeted cells expressed a truncated Fli-1 protein with significantly reduced transcriptional activation activity. Fli-1 targeted mutants displayed dramatic hemorrhaging from the dorsal aorta to the lumen of the neural tube and ventricles of the brain on E11.0 and die by E12.5. In addition, reduced numbers of proerythroblasts and basophilic erythroblasts, and a drastic reduction in the number of colony-forming cells observed at E11.0 in fetal livers, demonstrates abnormal hematopoiesis in these mice.

Fli-1 chimeric mice have been generated to rescue the embryonic lethal phenotype and further study Fli-1-associated cell-autonomous defects (Masuya et al., 2005). Lineage expression analysis in the peripheral blood and bone marrow exhibited a decrease in the number of neutrophils, granulocytes, monocytes and early erythroid progenitors as well as an increase in the number of natural killer (NK) cells and granulocyte/macrophage progenitors. Additionally, isolated bone marrow cells lacking Fli-1 expression display reduced expression of hematopoietic genes. These include reduced expression of *gata-1*, and *c-mpl*, important for megakaryocytic development, CCAAT/enhancer binding protein (C/EBP)α, C/EBPε, G-CSFR, and GM-CSFRα/β1 known to play a critical role in granulopoiesis, and *tal1*, important for the development of both primitive erythropoiesis and definitive hematopoiesis. Transplantation studies of the bone marrow from chimeric mice recapitulated the findings of the donor *fli-1* chimeric mice, with a prominent reduction in the number of granulocytes. Clonal bone marrow cell culture studies also revealed a significant increase in the number of primitive erythroid progenitors, BFU-Es. Taken together, these results confirm the role of Fli-1 in erythroid and megakaryocytic differentiation and suggest that Fli-1 plays a physiologic role in the production or maturation of leukocytes (Masuya et al., 2005).
Fli-1 is expressed in hematopoietic cells, including lymphocyte subsets, and increasing evidence suggests that modulation of Fli-1 expression impacts lymphocyte development and function. Moreover aberrant Fli-1 regulation is associated with the progression of various leukemias and autoimmune disorders, suggesting that Fli-1 may act as an important mediator in lymphocyte transformation or dysfunction. In order to assess the role of Fli-1 in lymphocyte development, mice carrying a targeted disruption of the Fli-1 protein were generated, lacking only the CTA domain (Zhang et al., 2008). As mentioned above, previously generated fli-1 knockout mice express a truncated form of Fli-1, whereby both the 3' Ets and CTA domains within exon 9 are deleted (Hart et al., 2000), (Spyropoulos et al., 2000). Homozygous mutant mice, lacking the CTA domain alone, display a significant reduction in the number of B cells within the peripheral blood, pre-B and immature B cells in the bone marrow, and follicular B cells of the spleen, as well as a rise in the number of transitional and marginal zone B cells. Moreover, B cells isolated from Fli-1 CTA deficient mice display a reduced proliferative capacity. The effects of Fli-1 deficiency on B cell development are cell autonomous, and are associated with changes in the expression of genes implicated in B cell development, including a reduction in Pax-5, E2A, and Egr-1 expression, and an increase in Id1 and Id2 expression (Zhang et al., 2008). Fli-1 CTA deficient mice also exhibit an altered humoral immune response to immunization, with significantly lowered concentrations of serum immunoglobulin. Therefore, Fli-1 is a regulator of both central and peripheral B cell development, and plays an influential role on the in vivo immune response.

The above-mentioned studies imply a role for Fli-1 in both hematopoietic and endothelial cell development, although fli-1 loss of function studies performed in the Xenopus and zebrafish embryos have provided conclusive evidence indicating that Fli-1 expression is vital for the development of these lineages (Liu et al., 2008). In the absence of Fli-1, the induction of both primitive and definitive hematopoiesis is perturbed. This is
associated with reduced expression of *tal1, lmo2, flk1, mpo, spi-1B* and *runx1*, and the eventual loss of the hematopoietic and endothelial progenitor population by apoptosis. The induction or regulation of Fli-1 expression is induced by bone morphogenic protein (Bmp) signaling or *Cloche*, important regulators of blood and endothelium, depending on the origin of the progenitor population. This study has finally permitted the conclusion that Fli-1 acts at the top of the transcriptional network as a master regulator of blood and endothelial cell development within the cells of the mesoderm and Fli-1 function is indispensable in *Xenopus* and zebrafish embryos (Liu et al., 2008).

The ubiquitous expression of Fli-1 in all endothelial cells (Hewett et al., 2001) suggests a role for Fli-1 in endothelial cell fate and angiogenesis. It has been suggested that Fli-1 is the first nuclear marker of endothelial cell differentiation (Rossi et al., 2004), is essential for embryonic vascular development (Hart et al., 2000), (Spyropoulos et al., 2000) and acts as a master regulator, establishing the blood and endothelial programmes in the early embryo (Liu et al., 2008). Fli-1 also plays a central role in the regulation of extracellular matrix genes, such as type 1 collagen, and inhibits collagen biosynthesis (Czuwara-Ladykowska et al., 2001), (Kubo et al., 2003), (Jinnin et al., 2005), (Asano et al., 2009). Recently, mice carrying a conditional targeted deletion of Fli-1 in endothelial cells have been generated to further characterize the role of Fli-1 in the vasculature. Consistent with the phenotype of the previous *fli-1* knockout mice, displaying a loss in vascular integrity, these *fli-1* conditional knockout mice display abnormal skin vasculature, markedly reduced vessel integrity and increased permeability. This phenotype is associated with altered expression of genes involved in the maintenance of blood vessel integrity, governing endothelial cell interactions, and basement membrane remodeling, such as VE-cadherin, PECAM-1, MMP-9, PDGFB, and S1P1 receptor. Thus, Fli-1 may play a significant role in the regulation of vascular homeostasis, likely governing vessel maturation and stabilization (Asano et al., 2010).
THESIS OBJECTIVES

The progression of cancer is a multi-stage process arising from the activation of proto-oncogenes, inactivation of tumor suppressor genes, and the additive or cooperative activities of multiple signaling pathways. Erythroleukemia induced by Friend virus is a well-studied example of multi-stage malignancy in mice and is perhaps one of the best animal models available to study the onset and progression of hematological malignancy. As such, the study of Friend disease has provided significant insight into the genetic changes that promote hematopoietic proliferation and survival, and impair differentiation. Proviral insertional activation of the Ets transcription factor, fli-1, is the most critical genetic event in the induction of erythroleukemia induced by the Friend murine leukemia virus (F-MuLV). The essential goal set out to be achieved by the research presented herein was to establish a better understanding of both the oncogenic and developmental roles of Fli-1 by investigating the molecular basis by which its deregulated expression leads to a fundamental aberration in the fine balance between cellular proliferation and differentiation.

CHAPTER TWO

Elucidating the role of Fli-1 in F-MuLV-induced erythroleukemia and human erythroleukemia

In order to ascertain a correlation between the aberrant regulation of Fli-1 with the malignant and anti-apoptotic phenotype in various cancer cell lines, Fli-1 protein levels and function were attenuated using RNA interference (RNAi) and expression of a Fli-1 dominant negative protein. The overall findings of this study suggest, for the first time, that
continuous Fli-1 overexpression plays an essential role in the maintenance and survival of both murine and human erythroleukemia.

CHAPTER THREE

Identification of a novel target gene: The inositol phosphatase SHIP-1 is regulated by both Fli-1 and Spi-1/PU.1

The oncogenic effects of the Ets transcription factors, Fli-1 and Spi-1/PU.1, recurrently deregulated in Friend virus-induced erythroleukemia, are mediated through the transcriptional regulation of target genes directing signaling pathways that govern erythroid differentiation, proliferation and survival. This chapter describes the identification of a novel target gene, the inositol phosphatase, SHIP-1, that is negatively regulated by Fli-1 and positively regulated by Spi-1/PU.1. Overall this study has provided evidence to suggest that while the deregulated expression of both Fli-1 and Spi-1/PU.1 leads to erythroid transformation, this contribution may not result from a common mechanism.

CHAPTER FOUR

Fli-1 overexpression in SFFV-induced erythroleukemia increases hematopoietic progenitor activity

Virtually all Friend murine leukemia virus (F-MuLV)-induced tumors display proviral integration at the fli-1 locus, which drives normal Fli-1 protein overexpression. Recent evidence has established that the aberrant regulation of Fli-1 is critical for the initiation and progression of F-MuLV-induced erythroleukemia and contributes to the survival and proliferation of erythroid progenitors. Yet it remains unclear how deregulated
Fli-1 expression alters the balance between erythroid differentiation and proliferation. In an effort to understand the precise consequences of Fli-1 overexpression in erythroid transformation, we examined the effects of enforced Fli-1 expression in an erythroblastic cell line harboring activation at the spi-1/PU.1 locus. This study demonstrates that Fli-1 overexpression may contribute to the initiation and progression of erythroid transformation by promoting the expansion and proliferation of erythroid progenitors, possibly potentiating properties of self-renewal.

CHAPTER FIVE

Generation of a fli-1 conditional knockout mouse to study the effects of Fli-1 deficiency on hematopoietic development

Mice carrying a targeted disruption at the fli-1 locus display abnormal hematopoiesis and vasculogenesis, and dramatic hemorrhaging within the midbrain. The hematopoietic and hematorrhachitic phenotypes ultimately result in death at midgestation, a time that comprises a critical transition period when the site of hematopoiesis changes from the yolk sac to the liver. In order to further delineate the role of Fli-1 in normal hematopoietic development, the generation of a conditional knockout mouse is necessary to bypass the embryonic lethal phenotype of the previously generated Fli-1 knockout mice. This model will facilitate the identification of Fli-1 target genes, as well as to unveil additional hematopoietic defects beyond midgestation, and perhaps shed light on the molecular pathology of disorders associated with Fli-1 aberrant regulation, such as Paris-Trousseau and Jacobsen syndrome.
Chapter 2
Elucidating the role of Fli-1 in F-MuLV-induced erythroleukemia and human erythroleukemia

Chapter 2 is a modified version of the following publication:

Continuous Fli-1 expression plays an essential role in the proliferation and survival of F-MuLV-induced erythroleukemia and human erythroleukemia

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2.1 ABSTRACT

Erythroleukemia induced by Friend Murine Leukemia Virus (F-MuLV) serves as a powerful tool for the study of multistage carcinogenesis and hematological malignancies in mice. Fli-1, a proto-oncogene and member of the Ets family, is activated through viral integration in F-MuLV-induced erythroleukemia, and is the most critical event in the induction of this disease. Fli-1 aberrant regulation is also observed in human malignancies including Ewing’s sarcoma, which is often linked to expression of the EWS/Fli-1 fusion oncoprotein. Here we examined the effects of Fli-1 inhibition to further elucidate its role in these pathological occurrences. The constitutive suppression of Fli-1, through RNA interference, inhibits growth and induces death in F-MuLV-induced erythroleukemia cells. Expression of a dominant negative protein Engrailed (En)/Fli-1 reduces proliferation of EWS/Fli-1 transformed NIH-3T3 cells and both F-MuLV-induced and human erythroleukemia cells. F-MuLV-induced erythroleukemia cells also display increased apoptosis, associated with reduced expression of Bcl-2, a known fli-1 target gene. Introduction of En/Fli-1 into an F-MuLV-infected erythroblastic cell line induces differentiation, as shown by increased α-globin expression. These results suggest, for the first time, an essential role for continuous Fli-1 overexpression in the maintenance and survival of the malignant phenotype in murine and human erythroleukemias.
2.2 INTRODUCTION

*Fli-1* is a member of the Ets family of transcription factors. Similar to other Ets proteins, Fli-1 functions as both a transcriptional activator and repressor with a highly conserved DNA-binding sequence called the Ets domain recognizing a consensus GGA(A/T) core motif. Additionally, deregulated expression of Ets proteins is associated with aberrant development and malignant transformation (Oikawa & Yamada, 2003). Fli-1 is preferentially expressed in vascular endothelial cells and hematopoietic tissues, including the thymus and spleen, and has been shown to play an important role in their development (Ben-David et al., 1990a), (Klemsz et al., 1994). Given the expression profile of Fli-1, its aberrant regulation would be expected to result in vascular and/or hematopoietic abnormalities and indeed, the discovery of Fli-1 is attributed to its role in the initiation and progression of erythroleukemogenesis in mice.

Earlier studies of genes associated with retroviral-induced neoplasias have contributed to our understanding of gene function, as well as to the processes of differentiation and transformation. A prime example of this is erythroleukemia induced by Friend Murine Leukemia Virus (F-MuLV). Upon injection of F-MuLV, strain-specific neonates develop a multistage disease characterized by erythroblastic expansion leading to anemia, splenomegaly, and eventually death within eight weeks of injection. Viral integration at the *fli-1* locus drives Fli-1 overexpression, and has been identified as the pivotal genetic event associated with this disease (Ben-David et al., 1990a). *Fli-1* viral integration is followed by further changes in the expression of anti-apoptotic and tumor suppressor genes, as well as the modification of *Erythropoietin* (*Epo*), resulting in Epo-independent proliferation of erythroblasts and fully leukemic clones (Lee et al., 2003). Moreover, Fli-1 has been reported to regulate an array of genetic targets, such as *Rb*, *MDM2* and *bcl-2*, whose roles in malignancy have been well established (Tamir et al.,
The study of F-MuLV-induced erythroleukemia has also provided insight into the non-pathological role of Fli-1. Constitutive activation of fli-1 in erythroblasts alters their responsiveness to Epo, switching the signaling events associated with terminal differentiation to proliferation (Pereira et al., 1999), (Tamir et al., 1999), (Zochodne et al., 2000).

While aberrant Fli-1 expression is associated with viral integration in mice, it is also associated with several human disorders. In Ewing’s sarcomas and primitive neuroectodermal tumors, a translocation between chromosome 11 and 22, t(11;22) (q24;q12) results in a chimeric EWS/Fli-1 fusion protein detectable in 85% of these tumors. This fusion protein contains the carboxy-terminal Ets domain of Fli-1 with the amino-terminal RNA binding domain of EWS (Delattre et al., 1992). The EWS/Fli-1 fusion protein, functions as a transcriptional activator causing the initiation and progression of this disease, and possesses a strong transforming ability in fibroblasts mediated through the Ets domain (May et al., 1993). Additionally, a group examining the expression of Fli-1 in several benign and malignant neoplasms has further implicated the importance of fli-1 aberrant regulation in tumor progression (Mhawech-Fauceglia et al., 2006).

These observations emphasize a role for aberrant fli-1 regulation in the initiation of malignancies such as erythroleukemia and Ewing's sarcoma. However, the molecular mechanisms underlying such transformations remain unclear. Modulating Fli-1 expression, such as by RNA interference (RNAi) and dominant-negative studies, should provide a better understanding of fli-1 function and help to further elucidate its role in pathological occurrences.

We hypothesized that modulating Fli-1 expression, would impact the survival, proliferation and differentiation of erythroleukemia cells. Here we provide evidence to
suggest that continuous flt-1 expression is necessary for the maintenance and survival of several malignant phenotypes.

2.3 MATERIALS AND METHODS

2.3.1 Cell lines

The murine erythroleukemia cell lines, CB3 and HB22.2 were maintained in α-minimum essential medium (α-MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco). The human erythroleukemia cell line, HEL, and the human myelogenous leukemia cell line, K562, were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco). The murine erythroleukemia cell line, HB60-5, was maintained in the same medium supplemented with 0.2 U of Epo and 100 μg of stem cell factor (SCF) per ml. To induce differentiation, HB60-5 cells were washed twice with phosphate-buffered saline (PBS) (Gibco) and incubated in the presence of 10% FBS and 1 U of Epo per ml. The mouse fibroblast cell line, NIH-3T3, and the human embryonic kidney cell line, 293T, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco).

2.3.2 RNA interference (RNAi)

Predesigned flt-1 small interfering RNA (siRNA) sequences were purchased from Ambion (Ambion/Applied Biosystems, Foster City) to generate small hairpin RNA (shRNA) oligonucleotides targeting the 3’ end of exon 2 following the second ATG start site. shRNA oligonucleotides also contain a hairpin loop sequence (5’-TTCAAGAGA-3’), and an RNA
Polymerase III terminator sequence consisting of a six-nucleotide poly(T) tract. Additionally a 5'-BamHI restriction site was inserted in the top strand and a 5'-EcoRI restriction site was inserted in the bottom strand of the shRNA oligonucleotides to enable directional cloning into the pSIREN RetroQ ZsGreen retroviral vector (Clontech, Takara Bio Inc., Mountain View, CA). A unique XbaI restriction site was also inserted immediately downstream the terminator sequence to confirm the presence of shRNA oligonucleotide inserts. Complementary PAGE-purified shRNA oligonucleotides, synthesized by Sigma Aldrich/Sigma Genosys Canada (Oakville, ON, Canada), were annealed in a thermal cycler (95°C for 30 sec, 72°C for 2 min, 37°C for 2 min, 25°C for 2 min). Additionally a negative control shRNA annealed oligonucleotide, provided by Clontech, was cloned into the same retroviral vector. The negative control shRNA contains the same nucleotide composition as the fli-1 shRNA oligonucleotide, but lacks sequence homology to the genome. The annealed double stranded oligonucleotides were ligated into the pSIREN vector (Clontech), using T4 DNA ligase (New England Biolabs, Pickering, ON, Canada).

2.3.3 RNAi virus production

The RNAi-ready retroviral vectors were triple-transfected with Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) into 293T cells, following the manufacturer's protocol (Invitrogen). The vesicular stomatitis virus G glycoprotein (VSVG) expressing vector, as well as the vector expressing the gag and pol viral packaging signals, were kindly provided by Dr. Dwayne Barber (University of Toronto, Canada). Viral supernatant was collected 48, 72 and 96 hours post transfection and concentrated by ultracentrifugation at 25 000 rpm for 2 hours. The virus was resuspended in approximately 1/50 of the original volume. CB3 cells (2.5 x 10^6) were infected with 2ml of concentrated virus, in the presence of polybrene (10μg/ml final concentration), as described previously (Cui et al., 2007).
2.3.4 Construction of Engrailed (En)/Fli-1

The *Drosophila* Engrailed (En) repressor domain encoding 298 amino acids of the En 298 N-terminus was removed from the En/Erg plasmid (a gift from Dr. Marina Ina Arnone, Stazione Zoologica, Naples, Italy) by digestion with the *BamHI* and *BglII* restriction enzymes (New England Biolabs). The En 298 fragment was then cloned upstream of the *BamHI* site in the MigR1-Fli-1 vector, also expressing GFP for the detection of transduced cells. This construct was designated En/Fli-1 in which the ATG start was inherent from the repressor domain of Engrailed.

2.3.5 Cell proliferation assay

The growth rate of each erythroleukemic cell line was examined in low serum media, containing 1% FBS. NIH-3T3 (5 x 10³ cells) were seeded in triplicates in 3 cm wells with low serum media and cell proliferation was measured by performing trypan blue exclusion assay.

2.3.6 Soft agar assays

In each experiment, 5 x 10³ cells of each cell line were trypsinized and resuspended in 2ml of complete medium in 0.3% agarose (Promega, Madison, WI). The agar-cell mixtures were plated in triplicate on plates containing a 1% complete medium agar mixture. After 2-3 weeks, the agar assays were scored for viable colonies.
2.3.7 RNA extraction and reverse transcription PCR

Total RNA was extracted from cultured cells by using TRIZOL (Gibco) according to the manufacturer’s protocol, and treated with amplification grade DNasel (Invitrogen) to avoid amplification of contaminated genomic DNA. Complementary DNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen). PCR primers were as follows; Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), forward primer: 5’-AACTTTGGCATTGTGGAAGG-3’, reverse primer: 5’-TGTGAGGGAGATGCTCAGTG-3’; *bcl-2*, forward primer: 5’-GATTCTGACAGACTCAGGAAGAAAC-3’, reverse primer: 5’-TCAGTCATCCACAGGGCGAT-3’; *α-globin*, forward primer: 5’-GATTCTGACAGACTCAGGAAGAAAC-3’, reverse primer: 5’-CCTTTCAGGGCTTCAGCTCCATAT-3’. Gel images were scanned using Quantity One software (Bio-Rad, Mississauga, Canada).

2.3.8 Flow cytometric cell cycle analysis

Single parameter analysis of DNA content was performed on cells fixed in 70% ethanol, washed twice with PBS (Gibco) and resuspended in staining solution containing 500ul RNaseA (200U/ml) (Sigma Aldrich, Oakville, ON, Canada) and 500ul propidium iodide (PI) (50g/ml) (Molecular Probes, Eugene, OR). Linear fluorescence signals of PI (area and width) were assessed on a Becton Dickinson FACS Calibur (BD Biosciences, San Jose, CA, USA) with dye excitation by a 488nm laser light. Data were stored as mode files of at least 5 x 10^3 single cell events for subsequent off-line analysis using Modfit and WinList software (Verity Software, Topsham, ME). DNA cell cycle analysis was accomplished using the DIP_N2 and DIP_N3 algorithms in Modfit.
2.3.9 Immunoblotting and antibodies

Cells were lysed with radioimmunoprecipitation assay buffer (0.5% Nonidet P-40, 50 mM Tris HCl [pH 8.0], 120 mM NaCl, 50 mM NaF, 10g of aprotinin per ml, 100g of leupeptin per ml, 10mM phenylmethylsulfonyl fluoride). Lysates were resolved on sodium-dodecyl sulfate (SDS)–10% polyacrylamide gels, and immunoblotted as described elsewhere (Howard, Berger, Bani, Hawley, & Ben-David, 1996). Antibodies to Fli-1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Sana Cruz, CA). Images were scanned using Quantity One software (Bio-Rad).

2.3.10 Immunohistochemistry

293T cells were plated at a density of 1 x 10⁶ cells per poly-L-leucine-coated glass coverslip (Assistent, Germany) in six-well plates. After 24 hours, cells were transfected with the MigR1 empty control vector, MigR1-Fli-1, or MigR1-En/Fli-1, using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Immunocytochemical staining was performed 48 hours post transfection. Coverslips were fixed with cold (4°C) 4% paraformaldehyde at room temperature for 10-20 minutes, permeabilized with absolute methanol at –20°C for 7 min, washed three times with PBS (Gibco) and blocked in 10% normal horse serum (PAA Laboratories GmbH, Germany). After blocking cells were incubated with Fli-1 polyclonal antibody (Santa Cruz Biotechnology) overnight at 4°C, washed three times with PBS (Gibco), incubated with biotinylated goat anti-rabbit IgG antibody and avidin-biotinylated peroxidase solution (Santa Cruz Biotechnology) and washed again. Antibody binding was revealed using 3,3’-diaminobenzidine-glucose oxidase for 15 minutes. Counterstaining was performed with hematoxylin. For immunohistochemical controls, the primary antibody was omitted.
2.3.11 Luciferase reporter assay

A DNA fragment containing the Fli-1 Ets DNA binding site was cloned upstream the luciferase gene, vector designated pGL4.28-FB. 293T cells (10^6 cells) were plated in six-well plates and transfected 24h later with the indicated amount of plasmid DNA using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. The DNA mixture used for transfection includes the indicated amounts of reporter gene constructs (pGL4.28 vector and pGL4.28-FB) and expression plasmids (MigR1 empty control vector, MigR1-Fli-1, MSCV empty control vector and MSCV-EWS/Fli-1). Cell lysates were prepared 48h after transfection and assayed for luciferase activity by using the luciferase assay system kit (Promega). The results shown are the mean of at least three independent transfection experiments. To account for transfection efficiency, β-gal concentrations were used to normalize results (Cui et al., 2007).
2.4 RESULTS

2.4.1 Fli-1 downregulation in erythroleukemic cells by RNAi leads to a marked growth inhibition and cell death

Retroviral vectors were cloned to express functional fli-1 shRNAs or negative control shRNAs under the control of the U6 RNA Polymerase III-dependent promoter. These shRNA retroviral expression plasmids were triple-transfected into 293T cells with plasmids expressing either the gag and pol packaging signals or the vesicular stomatitis virus glycoprotein (VSVG) envelope protein. Concentrated viral supernatants were used to infect the F-MuLV-induced erythroleukemia cell line, CB3, expressing high endogenous levels of Fli-1. Initial microscopic observation and cell counting with trypan blue demonstrated that fli-1 shRNA-treated cells exhibited significantly reduced proliferation rate (Figure 2.1A) and increased cell death compared to the appropriate controls (Figure 2.1B).

To validate the efficacy of RNAi-mediated Fli-1 downregulation as well as to correlate growth inhibition and cell death with this downregulation, we analyzed an unsorted population of CB3 cells 24 hours post-infection. Indeed, Western blot analysis confirmed the efficacy of Fli-1 knockdown (Figure 2.1C). These data provide evidence to suggest that continuous Fli-1 overexpression is essential for the survival and proliferation of erythroleukemic cells. However, constitutive suppression of Fli-1 through RNAi is extremely toxic to erythroleukemia cells and the rapid onset of cell death has made further examination difficult. Therefore additional analyses were necessary to investigate this phenomenon.
Figure 2.1 – Effects of RNAi-mediated Fli-1 downregulation in erythroleukemic cells. Growth rate kinetics of non-transfected (NT), polybrene control, negative control shRNA (NC) or fli-1 shRNA (F) treated CB3 cells. Cells were stained with trypan blue and cell numbers were counted each day as indicated. Downregulation of Fli-1 in F-MuLV induced erythroleukemia cell line, CB3, inhibits (A) proliferation and (B) induces cell death. (C) Fli-1 protein expression was reduced in fli-1 shRNA (F) treated CB3 cells.
2.4.2 En/Fli-1 expression inhibits both Fli-1 and EWS/Fli-1 activity

To support the phenomenon observed with RNAi-mediated Fli-1 downregulation, dominant negative strategies were employed to modulate Fli-1 expression. This dominant negative approach inhibits the transactivation activity of Fli-1 through introduction of specific transcriptional suppressors that bind and suppress the Ets domain of Fli-1. Engrailed (En) is a Drosophila homeodomain protein required for proper segmentation and maintenance of the posterior compartment identity. The repressor activity of En lies in the sequence containing amino acids 2-298 which are distant from the DNA binding homeodomain (Wang et al., 2004). Accordingly, translational fusions with the En repressor domain have efficiently converted plant transcription factors (Markel et al., 2002), β-catenin (Montross et al., 2000), and c-myb (Lyon & Watson, 1996), into dominant-negative proteins. Thus we generated a retroviral vector expressing a dominant negative Fli-1 protein, designated En/Fli-1. This vector, as well as similar retroviral vectors expressing Fli-1 and EWS/Fli-1, were transiently transfected into 293T cells. To determine the efficiency of transgene expression, Western blot analysis was performed and revealed 48 and 51, 68, and 78 kDa bands corresponding to the two protein isoforms of Fli-1, EWS/Fli-1 and En/Fli-1 proteins, respectively (Figure 2.2A). Immunohistochemical analysis of 293T cells transfected with Fli-1 or En/Fli-1 and stained with a Fli-1 specific antibody, revealed identical localization of exogenous Fli-1 protein (data not shown).

To investigate the efficacy of En/Fli-1 to inhibit the transactivating ability of Fli-1, luciferase assays were performed using a vector with the Fli-1 Ets DNA binding site cloned upstream of the luciferase gene, vector designated pGL4.28-FB. Figure 2.2B shows that exogenous expression of both Fli-1 and EWS/Fli-1 in 293T cells increased the luciferase activity of the pGL4.28-FB reporter in a dose-dependent fashion, compared to the empty vector, pGL4.28. As expected, EWS/Fli-1 expression displayed more robust luciferase
activity than Fli-1 (Figure 2.2B) (Watson et al., 1992), (Rao et al., 1993), (Zhang et al., 1993). Co-transfection of the En/Fli-1 expression vector with either the Fli-1 or EWS/Fli-1 expression vectors decreased luciferase activity, compared to cells transfected with either Fli-1 or EWS/Fli-1 expressing vectors alone (Figure 2.2B).
Figure 2.2 – En/Fli-1 suppresses Fli-1 and EWS/Fli-1 activity and inhibits the transforming ability of EWS/Fli-1. (A) Expression of murine Fli-1 (48 kDa/51 kDa), EWS/Fli-1 (68 kDa) and En/Fli-1 (78 kDa) proteins following transient transfection into 293T cells, as shown by Western blot. 293T and CB3 cells transfected with the control vector were used as negative and positive controls respectively. (B) Fli-1 and EWS/Fli-1 increases the luciferase activity of the reporter, pGL4.28-FB, in a dose-dependent manner. En/Fli-1 inhibits the luciferase activity of both Fli-1 and EWS/Fli-1. (C) Expression of Fli-1, EWS/Fli-1, and En/Fli-1 in NIH-3T3 cells infected with the corresponding retrovirus, using a Fli-1 polyclonal antibody. (D) Soft agar analysis of EWS/Fli-1-transformed NIH-3T3. EWS/Fli-1-expressing cells formed large macroscopic colonies, as shown. The graph displays the average number of colonies observed in experiments performed in triplicate. (E) En/Fli-1 reduces the growth rate of EWS/Fli-1 transformed NIH-3T3 cells in low serum media. Cells were seeded in triplicate, grown in 1% FBS and counted at 3 day intervals.
2.4.3 En/Fli-1 represses the transforming ability of EWS/Fli-1 in transformed NIH-3T3 cells

Since EWS/Fli-1 has the same Ets binding site as Fli-1, En/Fli-1 was examined for its ability to repress or inhibit EWS/Fli-1-mediated NIH-3T3 cell transformation. To do so, we generated a stable NIH-3T3 cell line overexpressing mouse EWS/Fli-1, En/Fli-1, or Fli-1 (Figure 2.2C). It is well known that one of the hallmarks of a transformed phenotype is the ability of cells to grow in semi-solid medium. Soft agar assays demonstrated that empty vector transfected NIH-3T3 cells grew poorly in this condition as only 15±10 small colonies per 5000 cells plated were counted (Figure 2.2D). In contrast, EWS/Fli-1-expressing NIH-3T3 cells grew efficiently in soft agar, forming large macroscopic colonies of greater than 200 cells, indicating anchorage-independent growth (Figure 2.2D). One of the EWS/Fli-1-transformed clones (designated EWS/Fli-1) was infected with the En/Fli-1, Fli-1 or empty vector retrovirus to test their ability to form colonies in the soft agar assay. EWS/Fli-1 cells infected with the Fli-1 retrovirus or vector control displayed no change in their ability to grow on soft-agar. In contrast, infection of these cells with the En/Fli-1 retrovirus significantly reduced the number and size of colonies (Figure 2.2D). These data indicate that the En/Fli-1 repressor protein impairs anchorage-dependent growth of transformed NIH-3T3 cells, thereby altering the transforming ability of EWS/Fli-1, suggesting a central role for the Fli-1 Ets binding site in malignant transformation.

2.4.4 En/Fli-1 reduces the proliferation rate of EWS/Fli-1 transformed NIH-3T3 cells in low serum media

Under normal culture conditions of 10% FBS supplemented medium, no significant difference could be detected in the growth rate of both wildtype or EWS/Fli-1 transformed NIH-3T3 cells co-transfected with the En/Fli-1 retrovirus (data not shown). However,
under low serum culture conditions (1% FBS), the EWS/Fli-1 transformed cells grew rapidly, while proliferation in the control cells remained unchanged (Figure 2.2E). EWS/Fli-1 transformed cells co-transfected with En/Fli-1 displayed a significantly reduced proliferation rate, similar to the rate of empty vector control transfected cells (Figure 2.2E). Thus, En/Fli-1 expression was able to inhibit the proliferation of EWS/Fli-1-transformed cells in low serum growth conditions.

2.4.5 En/Fli-1 suppresses proliferation of erythroleukemic cells overexpressing Fli-1

The aforementioned results suggested the ability of En/Fli-1 to inhibit the transactivating activity of Fli-1 and EWS/Fli-1 as well as to suppress the transforming ability of the latter. Therefore, in order to further establish the critical role of continuous Fli-1 overexpression in malignant transformation, En/Fli-1 was introduced into an F-MuLV-induced erythroleukemia cell line termed CB3, which overexpresses Fli-1 (Ben-David et al., 1991) (Figure 2.3A). Three days post infection, with either En/Fli-1 or control vector retroviruses expressing GFP, transduced cells were sorted by flow cytometry based on green fluorescence prior to immunoblotting and growth rate analysis (Figure 2.3B and C). Similar to our previous RNAi experiments, the proliferation rate of CB3 cells was attenuated significantly after infection with the En/Fli-1 retroviral expression vector (Figure 2.3C). Accordingly, cell cycle analysis revealed that CB3 cells expressing En/Fli-1 display a decreased proportion of cells in S phase (Figure 2.3D). In addition, cell cycle analysis revealed an apoptotic population in these cells, as evidenced by the appearance of a sub-G1 peak (Figure 2.3D). It is likely that Fli-1 overexpression contributes to the anti-apoptotic phenotype through direct regulation of its target genes.
Figure 2.3 – En/Fli-1 expression suppresses proliferation of murine erythroleukemia (A) Fli-1 expression in the indicated cell lines as determined by Western blot analysis. GFP positive CB3 cells, infected with the control or En/Fli-1 expressing virus, were sorted by flow cytometry and subjected to (B) Western blot analysis and (C) trypan blue exclusion assay. (D) Cell cycle analysis of GFP positive CB3 cells infected with En/Fli-1. (E) En/Fli-1 reduces bcl-2 expression in GFP positive CB3 cells, as determined by RT-PCR. F-MuLV-induced erythroleukemia cell lines HB60-5 and HB22.2 infected with the control or En-Fli-1 expressing vector were subjected to (F) Western blot and (G) trypan blue exclusion assay.
The anti-apoptotic gene, \( bcl-2 \), overexpressed in F-MuLV-induced erythroleukemias (Howard et al., 2001), has previously been shown to be a direct Fli-1 target gene, and Fli-1 mediated Bcl-2 upregulation contributes to the enhanced survival of erythroblasts (Lesault et al., 2002). Consequently the direct transcriptional regulation of Fli-1 target genes such as \( bcl-2 \) is thought to contribute to the transforming ability of Fli-1 in erythroleukemic cells. Therefore, since En inhibits the ability of Fli-1 to act as a transcriptional activator we examined \( bcl-2 \) expression in En/Fli-1 expressing CB3 cells. Indeed, RT-PCR analysis revealed that the RNA level of \( bcl-2 \) was significantly reduced in these cells as compared to the control vector infected cells (Figure 2.3E).

The role of Fli-1 inactivation was further examined in other F-MuLV-induced erythroleukemia cell lines overexpressing Fli-1, termed HB60-5 and HB22.2 (Figure 2.3A). As expected, En/Fli-1 expression in both cells lines (Figure 2.3F) also results in a marked growth inhibition (Figure 2.3G). Interestingly, this phenomenon is rescued over time through the loss of En/Fli-1 expression (Figure 2.4). HB60-5 cells, infected with the control or En/Fli-1 retroviral expression vector, were sorted by flow cytometry at three and twelve days post-infection. En/Fli-1 protein expression and growth rates were examined in each population. The expression of the dominant negative fusion protein was lost 12 days post-infection and was associated with a proliferation profile identical to that of the control cells (Figure 2.4). Together these results confirm a similar phenomenon observed with RNAi-mediated Fli-1 downregulation, suggesting that continuous Fli-1 overexpression is required for the proliferation and survival of murine erythroleukemic cells.
2.4.6 En/Fli-1 inhibits proliferation of human erythroleukemic cells in culture

Similar to F-MuLV-induced erythroleukemia cell lines, the human erythroleukemia cell line, HEL, also displays aberrant fli-1 expression (Figure 2.3A). The importance of continuous Fli-1 overexpression was examined in HEL cells by introduction of the En/Fli-1 retrovirus (Figure 2.5A). Expression of En/Fli-1 in HEL cells (Figure 2.5A), similar to

**Figure 2.4** – The gradual loss of En/Fli-1 expression is associated with increased proliferation of erythroleukemic cells. Erythroleukemic HB60-5 empty vector control and En/Fli-1 cell populations were sorted by flow cytometry at (A) three and (B) twelve days post infection, subjected to Western blot analysis, and stained with trypan blue, and cell numbers were counted each day as indicated. (A) En/Fli-1 expression results in a marked growth inhibition. (B) The loss of En/Fli-1 expression is associated with an increased growth rate, similar to that of the control cell population.
murine erythroleukemic cells (Figure 2.3), resulted in a reduced proliferation rate (Figure 2.5B). Moreover, similar to the murine erythroleukemic cells, the expression of En/Fli-1 was lost after 12 days of culture (data not shown). These data for the first time display the importance of Fli-1 expression in the maintenance of human erythroleukemia.

![Graph](https://via.placeholder.com/150)

**Figure 2.5** – En/Fli-1 inhibits growth of human erythroleukemic cells, HEL, in culture. (A) The GFP positive HEL cells infected with the control or En/Fli-1 expressing vector were sorted by flow cytometry and subjected to cell growth analysis. (B) Western blot analysis displaying expression of En/Fli-1 as detected by the Fli-1 antibody.

### 2.4.7 En/Fli-1 promotes differentiation of erythroid progenitors transformed by Fli-1

The F-MuLV-induced erythroleukemia cell line, HB60-5, proliferates in the presence of erythropoietin (Epo) and stem cell factor (SCF), but undergoes terminal differentiation in the presence of Epo alone (Tamir et al., 1999). We have previously revealed that ectopic expression of Fli-1 in these cells blocks erythroid differentiation by switching Epo-induced differentiation to Epo-induced proliferation (Tamir et al., 1999). To further verify the role
of Fli-1 in erythroid differentiation, we introduced En/Fli-1 in HB60-5 cells overexpressing Fli-1 (Figure 2.6A). These cells have been designated HB60-5-Fli-1. Additionally, our previous studies have shown that the expression of \( \alpha \)-globin, a differentiation marker for erythroid cells, is elevated in response to Epo in HB60-5 cells. However, Fli-1 overexpression in these cells inhibits this response (Tamir et al., 1999). When both HB60-5-Fli-1 and En/Fli-1 expressing HB60-5-Fli-1 cells were grown in the presence of Epo, an increased level of \( \alpha \)-globin was detected in En/Fli-1 expressing cells (Figure 2.6B), indicating that En/Fli-1 expression reversed the inhibitory effect on erythroid differentiation caused by Fli-1 overexpression in HB60-5 cells.

**Figure 2.6 – En/Fli-1 reduces inhibited differentiation in an erythroleukemia cell line infected with Fli-1.** Erythroleukemic HB60-5 Fli-1 cells were infected with the control empty vector or En/Fli-1 expressing vector and induced to undergo differentiation with the addition of 1 unit/ml of Epo for the indicated time period. **(A)** Expression of Fli-1 and En/Fli-1 in the indicated cells. **(B)** RT-PCR analysis of \( \alpha \)-globin expression in the control and En/Fli-1 expressing HB60-5-Fli-1 cells, at the indicated time points.
2.5 DISCUSSION

F-MuLV-induced erythroleukemia is a well-established tumor model that is aptly suited for studying the mechanisms governing the proliferation, maturation and transformation of erythroid progenitor cells (Ben-David & Bernstein, 1991). The activation of Fli-1 through retroviral insertion in erythroleukemia induced by F-MuLV is a critical initial step in the multistage development of this leukemia (Ben-David et al., 1990a). F-MuLV studies involving examination of the role of fli-1 in erythropoiesis have suggested that constitutive Fli-1 expression dramatically increases the self-renewal potential of proerythroblasts and blocks erythroid differentiation (Tamir et al., 1999). Conversely, transgenic mice generated to overexpress Fli-1 do not develop erythroleukemia but instead exhibit increased lymphopoiesis, splenomegaly, accumulation of abnormal B and T cells and develop a severe lupus-like autoimmune disease, ultimately resulting in death (Zhang et al., 1995). Mice carrying a targeted deletion in the Ets domain at the fli-1 locus display abnormal hematopoiesis and vasculogenesis and die between embryonic day 11.5-12.5 (Hart et al., 2000), (Spyropoulos et al., 2000). The first targeted disruption at the fli-1 locus, deleting the first ATG start site, resulted in the expression of a truncated protein and generated a non-lethal minor phenotype, including a reduction in thymus size and in the number of total thymocytes (Melet et al., 1996). Interestingly, these mice remain susceptible to the development of erythroleukemia following infection with F-MuLV, however, the latency period is significantly increased (Melet et al., 1996). Although these observations implicate that Fli-1 overexpression is vital for the initiation and progression of erythroleukemia, the molecular mechanisms underlying the role of aberrant fli-1 regulation in oncogenesis remains poorly understood.

To assess the role of Fli-1 overexpression in the maintenance and survival of murine erythroleukemia, we downregulated Fli-1 expression using RNAi. RNAi-mediated
suppression of Fli-1 in F-MuLV-induced erythroleukemia cells results in reduced proliferation associated with rapid cell death. Due to the toxicity of constitutive Fli-1 suppression and to further investigate this phenomenon we generated a dominant negative form of Fli-1, termed En/Fli-1. In this system, ectopic expression of this dominant-negative fusion protein mimics a loss-of-function allele. This approach has also been successful in inhibiting the function of the Ets-related gene Erg (F. Rizzo and M.I. Arnone unpublished data/personal communication), plant transcription factors (Markel et al., 2002), as well as β-catenin (Montross et al., 2000), and c-myb (Lyon & Watson, 1996). A luciferase assay confirmed the ability of En/Fli-1 to act as a transcriptional repressor, capable of inhibiting the transactivation activity of Fli-1, as well as that of the oncogenic fusion protein EWS/Fli-1. This chimeric oncogene, generated by chromosomal translocation in Ewing’s sarcoma, is a potent transcriptional activator containing the strong transactivating domain of EWS and the Ets DNA binding domain of Fli-1, with a more powerful transforming ability than Fli-1 (Watson et al., 1992), (Rao et al., 1993), (Zhang et al., 1993), (May et al., 1993). We show that expression of the En/Fli-1 repressor impairs anchorage-dependent growth of EWS/Fli-1 transformed NIH-3T3 cells and reduces the proliferation rate of these cells in low serum media. Although En/Fli-1 does not completely inhibit the transforming ability of EWS/Fli-1, these experiments imply that the oncogenic potential of EWS/Fli-1 at least, in part, is attributed to the transactivating activity of the Fli-1 Ets DNA binding domain. However, the strong transactivation domain from EWS may permit enhanced or novel interactions with genes not normally responsive to Fli-1 in order to disrupt normal growth and differentiation (May et al., 1993), (May et al., 1997). These results support a previous study demonstrating that the expression of siRNAs targeting the fusion between EWS and fli-1 slows the proliferation rate of a Ewing’s sarcoma cell line and expression of fli-1 siRNAs in these cells further potentiates this effect (Dohjima et al., 2003). Moreover, a recent study has demonstrated that mice expressing
EWS/Fli-1 develop a rapid onset of myeloid/erythroid leukemia that closely resembles F-MuLV-induced erythroleukemia (Torchia et al., 2007). Furthermore, cell cycle analysis revealed the ability of En/Fli-1 to suppress growth and induce apoptosis in an F-MuLV-induced erythroleukemia cell line. Interestingly, Fli-1 suppression also attenuates the growth rate of the human erythroleukemia cell line, HEL. This study, for the first time, provides evidence to suggest that Fli-1 plays an essential role in the proliferation of human erythroleukemia.

Fli-1 downregulation, mediated through RNAi or expression of the dominant-negative En/Fli-1 fusion protein, confirmed the essential role of fli-1 in the regulation of cellular proliferation and survival (Yi et al., 1997), (Goltze et al., 2000). To uncover the molecular mechanism responsible for this phenomenon, we have shown that En/Fli-1 expression is only tolerated for a short period after its introduction into erythroleukemia cells. Additionally, loss of Fli-1 in these cells is associated with apoptosis. Previously, Fli-1 has been shown to activate transcription of the anti-apoptotic gene, bcl-2 (Lesault et al., 2002). Accordingly, the expression of bcl-2 in CB3 cells infected with En/Fli-1 was much lower than cells infected with the control vector alone. These data suggest that the fli-1 proto-oncogene contributes to cell survival, at least in part by the upregulation of bcl-2 in erythroleukemic cells (Howard et al., 2001), resulting in evasion of the normal apoptotic program.

In addition to inhibition of apoptosis, Fli-1 overexpression in erythroid cells has also been shown to inhibit erythroid differentiation leading to massive proliferation in response to Epo (Tamir et al., 1999). Indeed, ectopic expression of Fli-1 in the HB60-5 erythroleukemia cell line switches Epo-induced differentiation to Epo-induced proliferation. To verify the role of Fli-1 in differentiation, we have shown that the
expression of En/Fli-1 in HB60-5 cells overexpressing Fli-1 increases their differentiation status as measured through the expression of $\alpha$-globin.

In summary, the experiments described here suggest, for the first time, an essential role for continuous Fli-1 overexpression in the proliferation and survival of murine and human erythroleukemias. It appears that Fli-1 has the potential to alter these cellular processes, likely through the regulation of several target genes. The approach used in this study could eventually provide potential avenues for the elucidation of $fli-1$ function in malignant transformation through identification of additional genes regulated by this transcription factor. Furthermore, these findings reveal that Fli-1 downregulation may have important implications in the pathogenesis of diseases, such as erythroleukemia, and perhaps provide clues to novel treatment options.
Chapter 3

Identification of a novel target gene: The inositol phosphatase SHIP-1 is regulated by both Fli-1 and Spi-1/PU.1

Chapter 3 is a modified and extended version of the following publication:

The inositol phosphatase SHIP-1 is negatively regulated by Fli-1 and its loss accelerates leukemogenesis

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**Author Contribution:** L.M.V-F performed the experiments in Figure 3.1A,B,D, Figure 3.2A,B,D, Figure 3.4A,B,C, Figure 3.5A,B,C,D. G.K.L performed the experiment in Figure 3.1C, Figure 3.3A,B. Y-J.L performed the experiment in Figure 3.2C, Figure 3.3A,B, Figure 3.4B. J-W.C performed the experiment in Figure 3.3C
3.1 ABSTRACT

Erythroleukemia induced by the Friend Virus complex serves as a powerful tool for the study of multistage carcinogenesis and hematological malignancies in mice. Proviral integration within the fli-1 or spi-1/PU.1 locus leads to their deregulated production, and is the critical genetic event in the progression of Friend disease. Overexpression of these proto-oncogenes induces proliferation, enhanced survival, and inhibits terminal differentiation of erythroid progenitors through activation of several signal transduction pathways, such as that of Shc/Ras. Fli-1 and Spi-1/PU.1 share the same core DNA binding site, and the mechanisms by which Fli-1 and Spi-1/PU.1 cooperate and activate this signaling pathway remain unclear. The inositol phosphatase, SHIP-1, is associated with phosphorylated Shc and regulation of the MAPK and PI3-K/AKT pathways in erythroleukemic cells. We demonstrated, through chromatin immunoprecipitation and electrophoretic mobility shift assay, that both Fli-1 and Spi-1/PU.1 directly bind to a conserved Ets DNA binding site within the SHIP-1 promoter. Interestingly, luciferase assays confirmed that SHIP-1 transcription is suppressed by Fli-1 and enhanced by Spi-1/PU.1. Accordingly, enforced expression of Fli-1 in erythroleukemic cells reduces SHIP-1 expression, while enforced expression of Spi-1/PU.1 increases SHIP-1 transcription, and enhances proliferation associated with activation of significant erythroid signaling molecules such as Jak2, c-myc, MAPK and AKT. This study has provided evidence to suggest that while SHIP-1 is a direct target of both Fli-1 and Spi-1/PU.1, these transcription factors do not contribute to erythroleukemia by a common mechanism.
3.2 INTRODUCTION

The progression of cancer is a multi-step process in which oncogenes and tumor suppressor genes mediate changes in gene expression required for malignant transformation. Transcription factors, often described as oncogenes or tumor suppressor genes, play a pivotal role within signal transduction pathways governing cellular proliferation, differentiation, and apoptosis (Darnell, Jr., 2002). Friend erythroleukemia has served as a valuable model of multistage malignancy in mice that has led to the discovery of both tumor suppressor and oncogenes, and has contributed to our understanding of both gene function and malignant transformation.

The activation and deregulated expression of the transcription factors, fli-1 and spi-1/PU.1, as a result of proviral integration, are known to contribute to murine erythroleukemia induced by Friend Virus. The Friend virus complex consists of a replication-defective spleen focus-forming virus (SFFV) and a helper replication-competent Friend murine leukemia virus (F-MuLV). Inoculation of Friend virus induces the stepwise progression of clonal erythroleukemia in susceptible mice (Ben-David et al., 1991), (Ben-David & Bernstein, 1991), (Lee et al., 2003), (Moreau-Gachelin, 2008). SFFV encodes the envelope glycoprotein, gp55, which binds and activates the erythropoietin receptor (EpoR), resulting in the constitutive activation of signaling pathways that allow for erythropoietin (Epo)-independent proliferation of erythroblasts (Moreau-Gachelin, 2008). The second stage of the disease, characterized by the clonal expansion of erythroleukemic clones incapable of differentiation, is triggered by the aberrant overexpression of Spi-1/PU.1. While the activation of Spi-1/PU.1, resulting from proviral integration, is imperative in SFFV-induced disease, the activation of Fli-1 is the critical genetic event in F-MuLV-induced multistage erythroleukemia (Ben-David et al., 1991), (Ben-David & Bernstein, 1991), (Lee et al., 2003). Fli-1 viral integration is followed by
further changes in the expression of anti-apoptotic and tumor suppressor genes, such as \textit{bcl-2} and \textit{p53} respectively, as well as modification of the \textit{Epo} gene, resulting in Epo-independent clonal outgrowth of erythroblasts.

Both proto-oncogenes and members of the Ets family of transcription factors, \textit{fli-1} and \textit{spi-1/PU.1}, play critical roles in normal development, hematopoiesis and oncogenesis (Moreau-Gachelin et al., 1988), (Ben-David et al., 1990a), (Ray et al., 1990), (Ben-David et al., 1991), (Moreau-Gachelin et al., 1996), (Hart et al., 2000), (Spyropoulos et al., 2000), (Fisher et al., 2004), (Liu et al., 2008). Accordingly, a recent study from our group, and that of another laboratory, has demonstrated that inhibition of Fli-1, as well as Spi-1/PU.1 suppresses growth and induces cell death in murine and human erythroleukemias (Delgado et al., 1994), (Cui et al., 2009), (Juban et al., 2009). Interestingly, Fli-1 and Spi-1/PU.1 recognize the same core Ets DNA binding domain, GGAA/G, and it has also been suggested that \textit{fli-1} is a direct target of Spi-1/PU.1 (Barbeau et al., 1999), (Starck et al., 1999). Therefore the deregulated expression of both Fli-1 and Spi-1/PU.1 leads to erythroid transformation, a contribution that is likely mediated through the transcriptional regulation of a common set of target genes directing erythroid-specific signal transduction pathways that govern erythroid differentiation, proliferation and survival.

Epo-independent proliferation and enhanced survival of erythroblasts is associated with phosphorylation of Janus kinase 2 (Jak2), and activation of several signaling pathways, such as of the phosphatidyl inositol 3-kinase (PI3-K), extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), and Shc/Ras pathways (Zochodne et al., 2000), (Ghaffari et al., 2003). The importance of Shc/Ras signaling in erythropoiesis is evidenced by the severe anemia and embryonic lethality observed in \textit{ras} deficient mice (Johnson et al., 1997). The Src homology 2 (SH2) domain-containing
inositol-5-phosphatase-1 (SHIP-1) is associated with phosphorylated Shc and activation of Ras in Epo-stimulated erythroleukemic cells (Zochodne et al., 2000), and correlates with increased proliferation of transformed erythroid cells (Tamir et al., 1999). Since SHIP-1 is a key regulator of the ERK/MAPK and PI3-K signaling pathways (Helgason et al., 2000), (Liu et al., 1998), we hypothesized that both Fli-1 and Spi-1/PU.1 promote Epo-induced proliferation, and activation of the above mentioned pathways, in part, through regulation of a common target gene, SHIP-1.

SHIP-1 plays a role in the activation and proliferation of myeloid cells, macrophages and mast cells. This phosphatase also negatively regulates c-Jun NH2-terminal kinase (JNK) (Robson et al., 2004), and nuclear factor-κB (NF-κB) activity (Kalesnikoff et al., 2002). SHIP-1 is expressed exclusively in hematopoietic cells and developing spermatogonia, and is activated following cytokine, growth factor or immunoreceptor activation in hematopoietic cells (Liu et al., 1998), (Kalesnikoff et al., 2002), (Kalesnikoff et al., 2003). SHIP-1 is expressed in mature T cells, granulocytes, monocytes/macrophages, mast cells and platelets (Sly et al., 2003). Interestingly, it has been documented that SHIP-1 expression is turned off during erythropoiesis, specifically in TER119 positive erythroid cells (Kalesnikoff et al., 2003). Expression of a short form of SHIP-1 (termed s-SHIP) modulates signaling important for the maintenance of primitive stem cell populations (Tu et al., 2001), (Desponts et al., 2006). SHIP-1 knockout mice are viable but have a shortened lifespan (Helgason et al., 2000). These mice overproduce granulocytes/macrophages, develop splenomegaly, display extramedullary hematopoiesis, exhibit increased numbers of erythroid progenitors and suffer from massive myeloid infiltration of the lungs (Helgason et al., 2000), (Harder et al., 2004). SHIP-1 is involved in various leukemias (Sattler et al., 1997), (Siegel et al., 1999), (Helgason et al., 2000), (Luo et al., 2003), (Horn et al., 2004), particularly, it acts as a negative regulator in chronic myelogenous leukemia and other leukemias containing the BCR-ABL fusion protein (Sattler et al., 1997). Recently, our
group has demonstrated that F-MuLV-infected SHIP-1 knockout mice display accelerated erythroleukemia progression, suggesting that decreased levels of SHIP-1, associated with greater activation of the Shc/Ras, MAPK and PI3-K pathways, provide an advantage to erythroleukemia development.

Here, I have shown that Fli-1 transcriptionally represses SHIP-1 expression in Friend virus-induced erythroleukemias by binding to the Ets consensus DNA binding sequence located within the SHIP-1 promoter. Spi-1/PU.1 promotes SHIP-1 expression by binding to the identical Ets sequence within the SHIP-1 promoter. Enforced expression of Fli-1 in an SFFV-induced erythroleukemic cell line reduces SHIP-1 expression, however enforced expression of Spi-1/PU.1 in a F-MuLV-induced erythroleukemic cell line induces the transcription of SHIP-1, while protein expression remains undetectable. Increased Spi-1/PU.1 levels markedly enhances the proliferation of erythroleukemic cells and is associated with activation of erythroid-related proteins, Jak2, c-myc, MAPK and AKT. This study has provided evidence to suggest that while SHIP-1 is a direct target gene of both Fli-1 and Spi-1/PU.1, these transcription factors may not contribute to the development of erythroleukemia by a common mechanism.

3.3 MATERIALS AND METHODS

3.3.1 Cell lines

The Friend erythroleukemia cell lines CB3, CB7, HB22.2, DP16-1, and DP17-17, were maintained in alpha-minimum essential medium (α-MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). The erythroleukemia cell
line HB60-5 was maintained in (α-MEM) (Gibco) supplemented with 10% FBS (Gibco), 0.2 U/ml erythropoietin (Epo) (Janssen-Ortho, Toronto, ON, Canada) and 100 ng/ml of stem cell factor (SCF) (R & D Systems, Minneapolis, MN, USA), as previously described (Tamir et al., 1999). NIH 3T3 and 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco).

3.3.2 Enforced expression of Fli-1 and Spi-1/PU.1

The MigR1 Fli-1, empty vector control plasmid, MigR1, the LZRS Spi-1/PU.1, or empty vector control plasmid, LZRS (provided by Dr. M Anderson, University of Toronto), expressing GFP, was triple-transfected with Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) into 293T cells, following the manufacturer's protocol. The vesicular stomatitis virus G glycoprotein (VSVG)-expressing vector, as well as the gag and pol virus packaging signals were provided by Dr. D Barber, University of Toronto. Viral supernatant was collected 48 hours post-transfection. DP17-17 or CB3 cells (2.5 x 10^6) were infected with virus, and incubated 16 hours with virus in the presence of polybrene (8µg/ml final concentration), as previously described (refer to Chapter 2). Two days post-infection, cells were sorted by flow cytometry based on the intensity of green fluorescence.

3.3.3 Immunoblotting

Cells were lysed with radioimmunoprecipitation assay buffer (0.5% Nonidet P-40, 50 mM Tris HCl (pH 8.0), 120 mM NaCl, 50 mM NaF, plus 1 mM Na3VO4, 10 g/ml aprogin, 100 g/ml leupeptin and 10 mM phenylmethylsulfonyl fluoride). 40 µg lysates were fractioned by SDS/PAGE and transferred to a polyvinylidene fluoride membrane
The following antibodies were used: SHIP-1, Fli-1, Spi-1/PU.1, Jak2, c-Myc, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma-Aldrich, Oakville, ON, Canada); PTEN, phospho-p44/42 MAPK, phospho-Akt (Ser473), Akt (Cell Signaling, Danvers, MA, USA); MAPK (BD Transduction Laboratories, San Jose, CA, USA); goat-anti-mouse, and goat anti-rabbit HRP-conjugated (Promega, Madison, WI, USA).

3.3.4 RNA extraction and Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from cells using TRIZOL (Gibco), according to the manufacturer’s protocol and treated with amplification grade DNaseI (Invitrogen) in order to avoid amplification of contaminated genomic DNA. cDNA was synthesized with Super Script II (Invitrogen). PCR primers were as follows: β-actin, forward primer: 5'-AACTTTGGCATTGTGGAAGG-3', reverse primer: 5'-TGTGAGGGAGATGCTCAGTG-3'; spi-1/PU.1, forward primer: 5'-CCCGCTTTGCCTCCCACCAG-3', reverse primer: 5'-GCCAGGCAGACTCCAACCAG-3'; SHIP-1, forward primer: 5'-CTCCCTGCAAGAAGTCACC-3', reverse primer: 5'-CGGGTCAGTCTTTCAAATCG-3'; fli-1, forward primer: 5'-GAAACTGCCACAGCTGGATC-3', reverse primer: 5'-ATATGAACTCTGGCCTCAAC-3'; BIC/miR155, forward primer: 5'-AAACCAGGAAGGGGAAGTGT-3', reverse primer: 5'-ATCCACAGGCTGGACTCTTG-3'; gata-1, forward primer: 5'-GGCGGAGGGACGGACAGGT-3', reverse primer: 5'-CAAGGATGGCGTCAAGTACC-3', reverse primer: 5'-ACAGTAATGGCGACCGACAAG-3'; eklf, forward primer: 5'-ACGCGTACGGCTGCGCACCAGTAA-3', reverse primer: 5'-ACGCGTACGGCTGCGCACCAGTAA-3'; SHP-1, forward primer: 5'-AGGAGGAGGACGTGGCTTCTTT-3', reverse primer: 5'-GACAGCCCTGGCTGGCGATG-3'; Rb, forward primer: 5'-TGACACAAACCAGCTGCGT-3', reverse primer: 5'-CCGAGCGCTCCTGTCTGACC-3'; PTEN,
forward primer: 5’-CCAATGTTCAGTGGCGGA-3’, reverse primer: 5’-GAACTTGTCTTCCGTCGTG-3’; bcl-xl, forward primer: 5’-TTGGACAATGGACTGGTTGA-3’, reverse primer: 5’-TGAGTGGACGGTCAGTGTCT-3’; TRAIL, forward primer: 5’-GTGCTCCTGCAGGCTGTGTC-3’, reverse primer: 5’-GTCTTCCACCTCTGGGCAAG-3’; bcl-2, forward primer: 5’-GATTCTGACAGACTCAGGAAGAAAC-3’, reverse primer: 5’-TCAGTCATCCACAGGGCGAT-3’. Gel images were scanned using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

3.3.5 Cellular proliferation assay

Cells, 1x10^4, were plated in triplicate, removed at 24 hour intervals, and cellular proliferation was measured by performing Trypan-blue exclusion assay. Briefly, cells were stained with Trypan-blue (Invitrogen) to exclude dead cells, and total viable cells were counted using a hemocytometer.

3.3.6 SHIP-1 luciferase reporter assay

A 988 bp region of the SHIP-1 promoter was amplified using PCR (forward: 5’-GTGCGTGCATGTGTGTAG-3’; reverse: 5’-AATTGCCTCTGCTGCTCCTA-3’; genomic DNA isolated from a Balb/c mouse), and cloned into the luciferase pGL3-enhancer vector (Promega), designated pGL3-SHIP-1. A 2 bp mutation within the Ets binding site was created using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), designated pGL3-mut-SHIP-1. Mutagenesis primers; forward: 5’-CTGAGTGCCTGAAACAGGTTGTCAGTGAAGCTG-3’; reverse: 5’-CAGCTTAACTGACTGACAACCTGTGTTCCAGGACTCAG. All vectors were verified by
sequencing at the TCAG facility (MaRS, Toronto, ON, Canada) using the ABI 3730XL sequencer. 293T cells, plated in triplicate, were transfected with the indicated amounts of DNA (0.2µg pGL3-SHIP-1 or pGL3-mut-SHIP-1, 0.2, 0.4, 0.6µg MigR1 Fli-1 or LZRS Spi-1/PU.1 and 0.01µg of pRL-SV40 containing the *Renilla* luciferase gene) using Lipofectamine 2000 (Invitrogen), following manufacturer’s protocol. Cells were assayed 48 hours post-transfection for luciferase activity using the Dual-Glo™ Luciferase Assay System kit (Promega).

### 3.3.7 Chromatin Immunoprecipitation and quantitative PCR

CB3, HB60-5, and KH16 cells (1 x 10⁸) were washed with phosphate-buffered saline (PBS) (Gibco) twice, crosslinked with 1% formaldehyde in PBS at 37°C for 15 minutes, followed by the addition of 125mM glycine for 5 min at room temperature. Fixed cells were harvested by centrifugation, washed in PBS, and incubated on ice for 50 minutes in swelling buffer (20mM HEPES pH 7.9, 10mM KCl, 1mM EDTA, 10% glycerol, 1mM DTT, 0.5mM PMSF, 0.1mM Na₃VO₄). Cells were broken by dounce homogenization, and nuclei were pelleted by brief centrifugation and resuspended in lysis buffer (10mM Tris-HCl pH 8.0, 140mM NaCl, 0.025% NaAzide, 1% Triton X-100, 0.1% SDS, 1mM DTT, 0.5mM PMSF, 0.1mM Na₃VO₄, 1% deoxycholate) and sonicated for 3 minutes using a Branson 250 Sonifier, followed by centrifugation. Fragmented chromatin was pre-cleared by incubation with protein A sepharose beads for 1 hour at 4°C. A 50μl aliquot of chromatin was removed for the input control. Immunoprecipitations were performed overnight at 4°C with either 2µg Fli-1, or Spi-1/PU.1 (Santa Cruz Biotechnology), or non-specific normal rabbit IgG (Promega) antibody, or without antibody (mock control). After immunoprecipitation, 120μL of 20% protein A sepharose beads was added and incubation continued for 1 hour. Precipitates were washed once in 0.1% SDS, 1% Triton X-100, 2 mM
EDTA, 150 mM NaCl, 20 mM Tris-HCl, four times in 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, and once in 250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, three times in TE buffer (10 mM Tris-HCl, 1 mM EDTA), and extracted by adding 200 µL each of 1% SDS and 100 mM NaHCO₃. 5 M NaCl was added to the eluates, final concentration 300 mM, and heated at 65°C overnight to reverse crosslinking. DNA fragments were incubated with proteinase K at 50°C for two hours, purified with phenol chloroform, and resuspended in TE buffer. PCR was performed to amplify SHIP-1 on the recovered DNA. Primers; SHIP-1 S1 forward: 5'-CATGCTTTTGGCCTATTCAC-3', S2 reverse: 5'-TGAGTGCTGAAACAGGAAGT-3', β-actin forward: 5'-TTCTACAATGAGCTGCCTGTG-3', reverse: 5'-GGGGTGTAGGTAGTCTCAAA-3', and Upstream-SHIP-1: 5'-CTGTCAGATGTGGTAGGCGGCGG-3', reverse: 5'-CCCAGCTAACCAGAATACACAAT-3'. The primers used for MDM2 amplification have been described previously (Truong et al., 2005). Chromatin immunoprecipitation was quantified using the real time PCR Qiagen QuantiFast SYBR green PCR kit (Qiagen, Mississauga, ON, Canada). Results are based on the relative proportion of input and chromatin precipitated by the Fli-1 or control IgG antibodies, where the input is equal to 1.

### 3.3.8 Electrophoretic mobility shift assay (EMSA)

Nuclear extract was isolated from CB3 cells using the method described in our previous publication (Li et al., 2001). Oligonucleotide sequences; 5'-CCTGAAACAGGAAGTCAGTCAG-3' (EBS1), 5'-CTATAATGAGGAAGTTCTTG-3' (EBS2), 5'-CCCTCGTGTGAGGGGTCCTGTG-3' (EBS3), 5'-TGCGGCTTCCGGGACGGG-3' (MDM2), 5'-CCTGAAACAGGTGGTCAGTCAG-3' (MUT-EBS1). Single-stranded oligonucleotides were radioactively labeled \([\gamma^{32}P]ATP\) (Perkin Elmer, Waltham, MA, USA) with T4 polynucleotide kinase (New England Biolabs). Single-stranded oligonucleotides were purified using
NUCTrap probe purification columns (Stratagene), annealed to two-fold excess cold complementary oligonucleotides by boiling for 2 minutes and cooling at room temperature for 1 hour. For competition assays, 10-fold and 100-fold excess cold single stranded oligonucleotides were added to the reaction. 2 µl of Fli-1, c-Myc, or Spi-1/PU.1 antibody (Santa Cruz Biotechnology) was added for the supershift assay. Addition of the c-Myc antibody was used as a negative control, since c-Myc does not bind to SHIP-1. Samples were electrophoresed on a 5% acrylamide gel in 0.5X TBE buffer. The gel was dried in a vacuum for 1 hour at 80°C.

3.3.9 Immunostaining and flow cytometric analysis

Freshly isolated cells were washed twice in PBS (Gibco) and immunostained for 15 min with the appropriate antibody; phycoerythrin (PE)-conjugated anti-TER119, PE-conjugated anti-c-kit, APC-conjugated anti-CD71, APC-conjugated anti-Sca1 (1:200) (eBioscience, San Diego, CA, USA). Following antibody incubation cells were washed once in PBS and resuspended in 500 ul PBS. Cell sorting and analysis of stained cells were performed using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA).

3.3.10 Cytospin preparation and histological staining

Cells (2 x 10⁴ per slide) were cytospun onto glass slides for 15 min at 1000rpm (Cytospin; Thermo Shandon, USA). Cells were fixed at room temperature in methanol for 5 minutes and air-dried. Fixed cells were stained with May-Grunwald stain followed by Giemsa stain according to the manufacturer’s protocol (Sigma). Light microscopy images
were obtained using a Leica DM LB2 microscope, Leica DFC 300FX camera and Leica Application Suite software version 3.1.0 (Leica Microsystems, Switzerland).

3.4 RESULTS

3.4.1 Expression of SHIP-1, Fli-1 and Spi-1/PU.1 in erythroleukemic cells

To fully understand the role of SHIP-1 in erythroleukemia cells, we first examined the expression of this phosphatase in erythroleukemic cell lines induced by various strains of Friend virus. As shown in Figure 3.1A and B, the expression of SHIP-1 protein is completely absent in F-MuLV-induced erythroleukemic cell lines overexpressing Fli-1, termed CB7, CB3, and HB22.2. However, SHIP-1 expression can be detected in SFFV-induced erythroleukemia cell lines termed HB60-5, DP16-1 and DP17-17, which have acquired insertional activation at the spi-1/PU.1 locus (Moreau-Gachelin et al., 1988), in lieu of fli-1 (Ben-David et al., 1990a), (Tamir et al., 1999). Further examination of these F-MuLV-induced erythroleukemia cell lines demonstrated that loss of SHIP-1 is exclusive to this group of cancer (data not shown) and this negative correlation does not occur between Fli-1 and PTEN, another inositol phosphatase that negatively regulates the PI3-K/Akt pathway (Figure 3.1B).

The expression of SHIP-1 in primary F-MuLV-induced erythroleukemia cells was also examined. Spleens of F-MuLV-induced erythroleukemic mice, which have acquired fli-1 insertional activation (Ben-David et al., 1990a), were isolated in order to determine the expression and relationship of Fli-1 and SHIP-1. Unlike F-MuLV-induced erythroleukemic cell lines, splenic tumors display SHIP-1 expression, albeit at lower levels than that observed in normal spleens. Western blot analysis has revealed that the primary
erythroleukemic cells retain a similar negative correlation between the expression of SHIP-1 and Fli-1 (**Figure 3.1C**).

The aforementioned results suggested the ability of Fli-1 to negatively suppress the expression of SHIP-1. Therefore to further establish the negative relationship between Fli-1 and SHIP-1, exogenous expression of Fli-1 was introduced into the SFFV-induced erythroleukemia cell line, termed DP17-17, expressing high levels of SHIP-1 and Spi-1/PU.1 and low levels of Fli-1 (**Figure 3.1A**). DP17-17 cells, transduced with either the empty vector control or Fli-1 GFP expressing retrovirus, were sorted by flow cytometry based on green fluorescence two days post infection. The ectopic expression of Fli-1 results in the downregulation of SHIP-1 expression, when compared to controls (**Figure 3.1D**). However, it is apparent that the qualitative level of SHIP-1 downregulation is not proportionate to the level of exogenous Fli-1 expression. It is likely that the limited capacity of Fli-1 to inhibit SHIP-1 is due to the presence of other transcription factors that are involved in the regulation of SHIP-1.
Figure 3.1 - Expression of SHIP-1, Fli-1 and Spi-1/PU.1 in erythroleukemic cells. SHIP-1, Fli-1, Spi-1/PU.1 (A) and PTEN (B) expression levels in HB60-5, DP16-1, DP17-17 cells, derived from SFFV-induced erythroleukemia, and CB3, CB7, HB22.2 cells derived from F-MuLV-induced erythroleukemia. (C) SHIP-1 and Fli-1 expression levels in the spleens of 8-week old F-MuLV-infected mice (B2-B4) and normal mice. (D) Exogenous expression of Fli-1 in DP17-17 cells. MigR1 Fli-1 infection results in decreased expression of SHIP-1 compared to non-transduced (N/T) and empty vector control (MigR1) transduced cells. β-actin was used as a loading control for all samples.

3.4.2 Fli-1 binds to the promoter of SHIP-1

The tightly coupled negative relationship observed between SHIP-1 and Fli-1, suggested that SHIP-1 might be directly downregulated by Fli-1. To determine if SHIP-1 is under the transcriptional control of the Fli-1 Ets transcription factor, the mouse promoter region was examined for Ets consensus ACCGGAAG/aT/c DNA-binding sites (Mao et al., 1994). Upon examination of 1000 bp adjacent to the transcriptional start site of the mouse SHIP-1 promoter, three possible Ets DNA binding sites were located, termed EBS1-3 (Figure 3.2A). Sequence analysis demonstrated a greater than 90% homology between
the mouse, human and rat SHIP-1 promoters in the nucleotides bounded by EBS1 and EBS2 (Figure 3.2B). Furthermore, the 100% conservation of nucleotide sequences EBS1 and EBS2 in all three species implies their significance in the regulation of SHIP-1.

To confirm the regulation of SHIP-1 by Fli-1, chromatin immunoprecipitation (ChIP) experiment was performed with three erythroleukemic cell lines, termed CB3, HB60-5 and KH16, expressing various levels of this Ets transcription factor. While CB3 cells express high levels of Fli-1, HB60-5 cells express lower levels and KH16 cells lack Fli-1 expression (Cui et al., 2009), (Wong et al., 1999). Chromatin was isolated and subjected to immunoprecipitation using a Fli-1 antibody (Truong et al., 2005). The DNA bound to Fli-1 was analyzed by PCR amplifying a 294 bp region within the SHIP-1 promoter using primers S1 and S2 (Figure 3.2C, Lane 4). The results in Figure 3.2C shows that while no amplification of SHIP-1 promoter is detected in KH16 cells, the levels of Fli-1 product are higher in CB3 cells compared to HB60-5 cells. This result suggests that Fli-1 binds directly to the SHIP-1 promoter, as indicated by the presence of the 294 bp fragment. As expected, this fragment cannot be detected in DNA samples where chromatin was immunoprecipitated using the control non-specific IgG antibody (Lanes 2 and 5). As a positive control, DNA bound to Fli-1 was PCR amplified in CB3 and HB60-5 cells using primers specific to a region within the promoter of MDM2, a known Fli-1 target gene, generating a 193 bp fragment (Lane 1), as described previously (Truong et al., 2005). ChIP quantitative-PCR was also performed on CB3 cells, using the indicated antibodies, to illustrate Fli-1 chromatin occupancy of the SHIP-1 and MDM2 promoters (Figure 3.2D). The β-actin promoter, as well as a 3 kb region upstream the SHIP-1 promoter (UP-SHIP-1), were used as negative controls. It is interesting that while SHIP-1 protein expression is undetectable in CB3 cells (Figure 3.1A-B), ChIP analysis has shown that Fli-1 is capable of binding to the SHIP-1 promoter, indicating an open chromatin structure within the SHIP-1 proximal promoter region of these cells.
Figure 3.2 – *in vitro* binding of Fli-1 to the SHIP-1 promoter. (A) The 988 bp promoter region upstream the start codon of murine SHIP-1 was cloned into the pGL3-enhancer vector. Three possible Ets binding sites (EBS) are highlighted. S1 and S2 indicate the locations of the primers used to amplify 294 bp of the SHIP-1 promoter. (B) The human, mouse, and rat SHIP-1 regions of conservation, EBS1 and EBS2 are fully conserved. (C) Binding of Fli-1 to the promoter of SHIP-1 (Lane 4) or MDM2 (Lane 1, positive control) as determined by ChIP in CB3, HB60-5 and KH16 cells using the Fli-1 or rabbit IgG antibody. (D) ChIP quantitative-PCR in CB3 cells illustrating Fli-1 chromatin occupancy of the indicated gene promoters, as well as a region 3kb upstream the SHIP-1 promoter (UP-SHIP-1). UP-SHIP-1 and βactin are negative controls. Results are based on the relative proportions of input and chromatin precipitated by Fli-1 and IgG antibodies, where the input is equal to 1.
3.4.3 Fli-1 binds to a specific ETS DNA binding sequence and negatively regulates SHIP-1 expression

Since it was established that Fli-1 is capable of binding to the *SHIP-1* promoter *in vivo*, it was logical to determine which Ets DNA binding site Fli-1 preferentially binds. To determine this, nuclear extracts from erythroleukemic CB3 cells were evaluated by EMSA for binding radioactively labeled probes corresponding to the putative Ets binding sites. Labeled EBS1-EBS3 oligonucleotides generated multiple bands indicating several probe-protein binding interactions (*Figure 3.3A*). However, with the addition of a Fli-1-specific antibody, a supershifted band is only observed with EBS1 (*Figure 3.3A*), thereby identifying an EBS1-Fli-1 binding complex. While the other interacting proteins with EBS-1 have not been identified, it is likely that additional transcription factors may cooperate with Fli-1 in the regulation of SHIP-1 expression.

To confirm the specificity of Fli-1 binding to the EBS1 sequence, this binding site was mutated at two nucleotides (ACAGGAAGTCA to ACAGGGTTGTCA), designated MUT-EBS-1. When the EMSA was repeated using the labeled MUT-EBS1 probe, a Fli-1 supershifted band could not be detected (*Figure 3.3B*). A labeled probe corresponding to the known Fli-1 binding site within the *MDM2* promoter (Truong et al., 2005) was used as a positive control (*Figure 3.3B*). These results demonstrate that Fli-1 regulates SHIP-1 expression through preferential binding to EBS-1.

Fli-1 acts as a repressor and activator of transcription in erythroleukemic cells (Tamir et al., 1999), (Athanasiou et al., 2000), (Lesault et al., 2002), (Truong et al., 2005). In order to ascertain that the identified Fli-1 binding sites are transcriptionally active a luciferase reporter assay was performed. Position -988 bp from the *SHIP-1* transcriptional start site (*Figure 3.2A*) was cloned into the pGL3 luciferase reporter vector, termed pGL3-SHIP-1. Co-transfection with the indicated amounts of a Fli-1 expression vector and the
pGL3-SHIP-1 vector into 293T cells, lacking both Fli-1 and SHIP-1 expression, resulted in a dose-dependent suppression of luciferase activity (Figure 3.3C and D). Using the same promoter containing the mutated EBS1, we tested the specificity of Fli-1 binding to the SHIP-1 promoter. This mutation significantly reduced luciferase activity when compared to the wild-type pGL3-SHIP-1 construct (Figure 3.3C) indicating the importance of this site in the regulation of SHIP-1 transcription. In contrast to the wild-type SHIP-1 promoter, Fli-1 expression in 293T cells was unable to cause a dose-dependent suppression of the SHIP-1 mutant luciferase reporter vector (Figure 3.3C and D). Therefore, Fli-1 suppresses the SHIP-1 promoter by specific binding to EBS-1.
Figure 3.3 – Fli-1 binds to EBS1 in the SHIP-1 promoter and negatively regulates its expression. (A) Nuclear extract isolated from CB3 cells was incubated with labeled EBS1-3 (A) or mutated (MUT-EBS-1) (B) oligonucleotides in the presence or absence of Fli-1 antibody and non-specific competitor poly (dl-dC) and subjected to EMSA. Competition assays were performed in the presence of 10-fold excess unlabelled oligonucleotides (cold competitor). (C) Luciferase assays were performed in 293T cells co-transfected with the indicated amounts of a Fli-1 expression vector, either the pGL3-SHIP-1 or pGL3-mut-SHIP-1 vector, and the Renilla luciferase vector. The pGL3-SHIP-1 luciferase reporter vector contains a 988 bp region within the SHIP-1 promoter (Figure 3.3A). Site-directed mutagenesis was used to alter nucleotides ACAGGAAAGTCA to ACAGGTTGTCA in EBS1 of pGL3-mut-SHIP-1. The relative luciferase units (RLU) are representative of the Renilla luciferase signal (x100). Luciferase assays were performed in triplicate. (D) Fli-1 protein expression in 293T cells transfected with the indicated amounts of the Fli-1 expression vector, relative to HB60-5 and CB3 cells. The two Fli-1 protein products are observed as a result of two isoforms, 48 and 51 kDa, synthesized by alternative translation initiation sites.
3.4.4 Spi-1/PU.1 binds directly to the SHIP-1 promoter to positively regulate its expression

Fli-1 and Spi-1/PU.1 recognize similar core Ets DNA binding sites, and may contribute to erythroleukemia development through the direct regulation of a similar set of target genes governing various signaling pathways involved in the development and progression of Friend virus induced-erythroleukemia. Microarray analysis of Spi-1/PU.1-/- erythroid cells revealed a significant reduction in the expression of SHIP-1, identifying SHIP-1 as a candidate Spi-1/PU.1 target gene (Fisher et al., 2004). Therefore, we hypothesized that SHIP-1 may also be deregulated by Spi-1/PU.1 overexpression, as a result of direct binding to its promoter. As mentioned previously, the SHIP-1 promoter contains three highly conserved Ets consensus ACCGGAAG/aT/c DNA-binding sites (Mao et al., 1994) (EBS1-3) within approximately 350 bp of the known transcription initiation site (Figure 3.2A-B). To determine if SHIP-1 is similarly under the transcriptional control of the Spi-1/PU.1 Ets transcription factor, a ChIP assay was performed using the SFFV-induced erythroleukemic cell line, HB60-5 and the F-MuLV-induced erythroleukemic cell line, CB3. HB60-5 erythroleukemic cells display expression of SHIP-1 and Spi-1/PU.1, as well as lower levels of Fli-1, while in CB3 cells SHIP-1 and Spi-1/PU.1 expression is absent, and Fli-1 expression is elevated (Figure 3.1A). Isolated chromatin was subjected to immunoprecipitation using the Spi-1/PU.1 antibody. Similar to the ChIP assay presented in Figure 3.2C for Fli-1, Spi-1/PU.1 chromatin occupancy of the 294bp region within the SHIP-1 promoter, containing EBS1-3, was determined by PCR. The results in Figure 3.4A, confirm Spi-1/PU.1 occupancy in HB60-5 cells as observed by amplification of the 294bp region of the SHIP-1 promoter. However, as expected, this amplification is absent in CB3 cells and cannot be detected in DNA samples where chromatin was immunoprecipitated using the control non-specific IgG antibody (Figure 3.4A Lanes 2). These results suggest that Spi-1/PU.1 directly binds to the SHIP-1 promoter to regulate its expression.
We investigated whether Spi-/PU.1 binds directly to the SHIP-1 promoter in vivo through EMSA assays using the Spi-1/PU.1 antibody. The Fli-1 and c-Myc antibodies were used as a positive, and negative control, respectively. Nuclear extracts from erythroleukemic HB60-5 cells were incubated with labeled probe corresponding to the EBS1 sequence. Similar to Fli-1, the addition of a Spi-1/PU.1 specific antibody results in the presence of a supershifted band (Figure 3.4B), thus detecting the presence of an EBS1-Spi-1/PU.1 binding complex demonstrating the in vitro binding of Spi-1/PU.1 to the EBS1 within the SHIP-1 proximal promoter. Addition of the c-Myc specific antibody did not result in a supershift, since c-Myc does not bind to SHIP-1.

Analogous to Fli-1, Spi-1/PU.1 acts as both a repressor and activator of transcription in erythroleukemic cells. Both the ChIP and EMSA analyses confirmed the ability of Spi-1/PU.1 to directly bind to the EBS1 within the SHIP-1 promoter, in the same way as Fli-1. Accordingly, we examined the ability of Spi-1/PU.1 to directly activate or repress the transcription of SHIP-1 through a luciferase reporter assay. The pGL3-SHIP-1 vector, containing a 988 bp region of the SHIP-1 proximal promoter, was co-transfected with a Spi-1/PU.1 expression vector into 293T cells, which lacks both SHIP-1 and Spi-1/PU.1 expression (data not shown). Enforced expression of Spi-1/PU.1 unexpectedly resulted in enhanced luciferase activity compared to the empty vector control, pGL3-SHIP-1 alone-transfected cells, and previously reported suppression by co-transfection with a Fli-1 expression vector (Figure 3.4C). The Spi-1/PU.1-dependent increase of luciferase activity was significantly abrogated with co-transfection experiments utilizing the pGL3-SHIP-1 mut vector, containing a mutated form of EBS1 (Figure 3.4C). Mutation of EBS1 confirmed the specificity of both Fli-1 and Spi-1/PU.1 binding within the same region of the SHIP-1 proximal promoter, designating the conserved EBS1 as a significant element of SHIP-1 regulation. Thus SHIP-1 is a direct target of both Fli-1 and Spi-1/PU.1, although
these Ets transcription factors bind to the same region, they have opposing effects on the transcriptional regulation of SHIP-1.

Figure 3.4 – *in vitro* and *in vivo* binding of Spi-1/PU.1 to EBS1 within the SHIP-1 promoter activates transcription. (A) Binding of Spi-1/PU.1 to the SHIP-1 promoter as determined by ChIP in HB60-5 and CB3 erythroleukemic cells using 2 µg of Spi-1/PU.1, or control rabbit IgG antibody. (B) Nuclear extract isolated from HB60-5 cells was incubated with γ-32P-labelled EBS1 probe in the presence or absence of the Spi-1/PU.1, c-Myc, or Fli-1 antibody and non-specific competitor poly (dl-dC). Competition assays were performed in the presence of 10-fold excess-labeled oligonucleotide (cold competitor). (C) Luciferase assays were performed in 293T cells co-transfected with the Spi-1 or Fli-1 expression vector, pGL3-SHIP-1 or pGL3-SHIP-1 mut luciferase reporter vector, and Renilla luciferase vector. The RLUs are representative of the Renilla luciferase signal (x100). Luciferase assays were performed in triplicate.
3.4.5 Enforced expression of Spi-1/PU.1 in F-MuLV-induced erythroleukemic cells induces SHIP-1 transcription and markedly increases cellular proliferation

Taken together the above-mentioned data suggests the ability of both Fli-1 and Spi-1 to regulate the expression of SHIP-1, where Fli-1 is a repressor and Spi-1/PU.1, an activator of transcription. It has been shown that Spi-1/PU.1 can also act as a repressor of Fli-1 transcription in Friend virus-induced erythroleukemic cells (Starck et al., 1999). In order to further establish the relationship between Spi-1/PU.1 and SHIP-1, as well as Fli-1, exogenous expression of Spi-1/PU.1 was introduced into an F-MuLV-induced erythroleukemia cell line, CB3, harbouring a fli-1 activated locus. CB3 erythroleukemic cells express high levels of Fli-1 and both Spi-1/PU.1 and SHIP-1 expression is absent. (Figure 3.1A-B). CB3 cells, transduced with either the empty vector control or Spi-1/PU.1 expressing retrovirus, were sorted by flow cytometry based on green fluorescence two days post-infection. Interestingly, enforced expression of Spi-1/PU.1 in CB3 cells increases the level of SHIP-1 transcription as expected, but has no effect on the protein levels of SHIP-1, while the transcript and protein levels of Fli-1 remain unaffected, compared to the appropriate controls (Figure 3.5A-B).

Deregulated expression of Spi-1/PU.1 contributes to erythroid differentiation by inducing proliferation and inhibiting differentiation. Trypan blue exclusion assay was performed to determine the effects of exogenous Spi-1/PU.1 expression on the proliferation of F-MuLV-induced erythroleukemia. The number of CB3 cells was significantly elevated in response to enforced Spi-1/PU.1 expression, compared to the appropriate controls (Figure 3.5C). May-Grunwald-Giemsa staining and flow cytometric analyses using Sca-1, c-kit, CD71 (transferrin receptor), and TER119 (erythroid-specific marker) cell surface markers were used to determine the effects of Spi-1/PU.1 expression on the differentiation status of the cells. Morphologically, Spi-1/PU.1 expressing
erythroleukemic cells were similar to the vector control and non-transduced cells. Accordingly, no significant difference in the expression of erythroid differentiation surface markers was detected (Figure 3.5D).

3.4.6 Spi-1/PU.1 expression alters the expression of erythroid-related genes

It is well known that Spi-1/PU.1 contributes to the development of erythroleukemia, through the direct transcriptional regulation of erythroid-related genes, such as *gata-1*. Enforced expression of Spi-1/PU.1 in F-MuLV-induced erythroleukemic cells results in a markedly increased growth rate, although it is still unclear how Spi-1/PU.1 contributes to the increased proliferation of erythroleukemic cells. The transcript levels of several genes involved in the signaling pathways governing erythroid differentiation and proliferation were analyzed by semi-quantitative RT-PCR. No significant changes were observed in the transcription of erythroid-related genes, such as *Rb, bcl-2, bcl-xl, TRAIL, gata2, eklf, PTEN*, and *SHP-1* (data not shown). Although, enforced expression of Spi-1/PU.1 led to the suppression of *gata-1* transcription (Figure 3.5A), a known Spi-1/PU.1 target gene (Rekhtman et al., 1999), (Zhang et al., 1999) that plays a critical role in the control of erythroid differentiation, proliferation and survival (Pervny et al., 1991), (Weiss & Orkin, 1995), (Gregory et al., 1999), (Zheng et al., 2006). Spi-1/PU.1 overexpression also led to enhanced phosphorylation of AKT and overall expression of MAPK, c-Myc and Jak2 erythroid-related proteins (Figure 3.5B).
Figure 3.5 – Enforced Spi-1/PU.1 expression induces SHIP-1 transcription, increases proliferation and induces changes in the expression of erythroid-related genes. (A) Semi-quantitative RT-PCR and (B) Western blot analysis of CB3 cells transduced with a Spi-1/PU.1 expressing retrovirus. (C) Increased proliferation rate of Spi-1/PU.1 transduced cells as determine by Trypan-blue exclusion assay, compared to the appropriate controls (D) May-Grunwald-Giemsa staining and FACS analyses for erythroleukemic surface markers of transduced CB3 cells reveals no significant changes in erythroid differentiation status.
3.4.7 Elevated expression of miR155 and the regulation of SHIP-1 in erythroleukemic cells

Enforced expression of Spi-1/PU.1 in F-MuLV-induced erythroleukemic cells resulted in increased transcript levels of SHIP-1, however SHIP-1 protein levels remained undetected, suggesting a form of SHIP-1 post-transcriptional regulation. miRNA-155 (miR-155) is a potent inhibitor of hematopoietic progenitor cell differentiation, and its overexpression results in increased granulocyte/monocyte proliferation, a marked reduction of erythroid differentiation and splenomegaly (Georgantas, III et al., 2007), (O'Connell et al., 2008). BIC, the precursor of miR-155, is activated by proviral integration in avian leukemia virus-induced lymphomas (Tam et al., 1997), and is highly expressed in a subset of cancer cells of lymphoid and myeloid origin (Eis et al., 2005), (Kluiver et al., 2005), (Garzon et al., 2008), (O'Connell et al., 2008). miR155 has also been shown to block the protein expression of Ets transcription factors Spi-1/PU.1 and Ets-1 (Georgantas, III et al., 2007), (Vigorito et al., 2007), as well as SHIP-1 (O'Connell et al., 2009) through direct 3'UTR interactions. Therefore we predicted that miR155 expression plays a role in the post-transcriptional regulation of SHIP-1 in Friend erythroleukemia and examined the expression of miR-155/BIC in relation to the expression of SHIP-1 and Spi-1/PU.1. Semi-quantitative RT-PCR analysis revealed that the expression of BIC (miR155 precursor)/miR155 is elevated in CB3 cells in response to enforced expression of Spi-1/PU.1 (Figure 3.5A). Therefore the absence of SHIP-1 protein in CB3 cells overexpressing Spi-1/PU.1 may be, at least in part, explained by the post-transcriptional regulation of SHIP-1 by miR-155.
3.5 DISCUSSION

The pivotal genetic event occurring in Friend virus-induced erythroleukemia is proviral insertional activation at the fli-1 or spi-1/PU.1 locus. Overexpression of these transcription factors leads to Epo-independent proliferation, inhibition of differentiation, and activation of several signaling pathways such as that of Shc/Ras, MAPK and Akt. During normal erythroid differentiation, Epo stimulation is associated with downregulation of both Fli-1 and Spi-1/PU.1 (Schuetze et al., 1993), (Moreau-Gachelin et al., 1996), (Tamir et al., 1999). In Friend virus-induced erythroleukemia the overexpression of Fli-1 or Spi-1/PU.1 in erythroblasts switches Epo-induced differentiation to Epo-induced proliferation (Schuetze et al., 1993), (Pereira et al., 1999), (Starck et al., 1999), (Tamir et al., 1999), (Fisher et al., 2004). The oncogenic effects of Fli-1 and Spi-1/PU.1 overexpression are likely mediated through the transcriptional regulation of their target genes that modulate cellular signaling events necessary for erythroleukemogenesis. Among such target genes, the inositol phosphatase SHIP-1 acts as a negative regulator of signaling by growth factors and receptors, such as that of the EpoR. In the present study, we discovered that SHIP-1 is commonly regulated by both Fli-1, and Spi-1/PU.1, albeit with opposing effects.

Upon examination of the transcriptional regulatory role of Fli-1 and Spi-1/PU.1, it was shown that both Ets transcription factors bind to the same conserved region within the proximal promoter of SHIP-1. We confirmed the ability of Fli-1 to act as a transcriptional repressor and conversely Spi-1/PU.1 to act as a transcriptional activator of SHIP-1, since enforced expression of Fli-1 in an SFFV-induced erythroleukemic cell line reduced the SHIP-1 expression, while enforced expression of Spi-1/PU.1 in an F-MuLV-induced erythroleukemic cell line increased SHIP-1 transcription. These data are in accordance with our earlier findings, in that SHIP-1 protein expression is undetectable in
established F-MuLV-induced erythroleukemic cell lines, harbouring a \textit{fli-1} activated locus, and present in primary tumors, as well as established SFFV-induced erythroleukemic cell lines, harbouring a \textit{spi-1}/\textit{PU.1} activated locus. Similarly, we have previously demonstrated that while the level of p53 in primary erythroleukemias is significantly reduced, the loss of this tumor suppressor gene is only detected in certain established erythroleukemic cell lines (Howard et al., 1993). Moreover, analogous to \textit{SHIP-1} (-/-) mice (Lakhanpal et al., 2010), F-MuLV-induced erythroleukemia is also accelerated in \textit{p53} (-/-) mice (Wong et al., 1999).

Deregulated \textit{SHIP-1} expression has an implication in erythroleukemia progression since \textit{SHIP-1} connects the C-terminal region of the EpoR to the Shc/Ras pathway (Zochodne et al., 2000), and is involved in the regulation of the MAPK and Akt pathways that are downstream the EpoR (Mason et al., 2000). In erythroid progenitors, MAPK signal transduction is mainly involved in proliferation (Miura et al., 1994) and PI3-K/Akt activation protects against apoptosis (Bouscary et al., 2003). Consequently, mice with a ubiquitous \textit{SHIP-1} deficiency develop a myeloproliferative disorder with increased numbers of erythroid progenitors and decreased B lymphocyte numbers (Helgason et al., 2000), (Leung et al., 2009). Lymphocytes derived from \textit{SHIP-1} (-/-) mice display elevated levels of MAPK and Akt that correlates with increased proliferation (Helgason et al., 2000). Thus the subsequent loss of \textit{SHIP-1} in late-stage Friend erythroleukemic cells may contribute to erythroid transformation induced by providing a selective advantage to allow for Epo-independent proliferation and immortalization.

In the absence of Epo, SFFV-transformed cells display constitutive activation of most signaling pathways normally induced in erythroid cells by Epo. Activation of Akt and MAPK signaling is imperative for Epo-independent erythroleukemic growth of proerythroblasts derived from \textit{spi-1}/\textit{PU.1} transgenic mice (Barnache et al., 2001).
Moreover, it has been suggested that a positive feedback loop exists between constitutive PI3-K/Akt signaling and elevated expression of Spi-1/PU.1 in Friend erythroleukemia (Breig et al., 2010). Consistent with the above-mentioned data, enforced expression of Spi-1/PU.1 in an F-MuLV-induced erythroleukemic cell line led to enhanced proliferation associated with increased phosphorylation of AKT, expression of MAPK, c-myc, Jak2, gata-1, and SHIP-1. The positive regulation of SHIP-1 by Spi-1/PU.1 unlikely contributes to enhanced proliferation since only SHIP-1 transcription, and not protein levels, increased. Although, the induced expression of the erythroid-related signaling molecules may explain the proliferative advantage provided by Spi-1/PU.1 overexpression. It is known that excess levels of Spi-1/PU.1 disrupt the function of GATA-1, a transcription factor that participates in the expression of various genes involved in erythropoiesis (Welch et al., 2004). The Ets domain of Spi-1/PU.1 binds to the gata-1 promoter to antagonize GATA-1 expression, thereby repressing its activity (Rekhtman et al., 1999), (Zhang et al., 1999). GATA-1 expression increases during erythropoiesis and acts to repress proliferation through the direct regulation of genes, such as c-myc, a proto-oncogene that in turn regulates the expression of cell cycle inhibitors like p18INK4C and p27Kip1 (Rylski et al., 2003). The precise mechanism by which Jak2 is activated by Spi-1/PU.1 overexpression is unknown, however several studies have shown that the activation of Jak2, leading to constitutive activation of Epo signaling, contributes to the pathogenesis of erythroleukemia and myeloproliferative disorders (Kaushansky, 2005), (Levine et al., 2005). This data has provided evidence to suggest that Spi-1/PU.1 impacts proliferation through activation of Epo signaling, likely in part, through elevated expression of Jak2. Further future investigation of the relationship between Jak2 and Spi-1/PU.1 expression may provide insight into the molecular mechanisms underlying Epo-independent growth leading to erythroid transformation.

Interestingly, Spi-1/PU.1 behaves as an agonist of Fli-1 expression through direct interaction with its promoter in SFFV-induced erythroleukemic cell lines, and the
constitutive expression of Fli-1 in these cells inhibits their ability to undergo chemically induced differentiation (Starck et al., 1999), indicating that the positive regulation of Fli-1 by Spi-1/PU.1 contributes to SFFV-induced erythroleukemia. Although in our studies, we have shown that levels of Fli-1 expression remained unchanged in Spi-1/PU.1 overexpressing F-MuLV-induced erythroleukemic cells. Thus the effects induced by Spi-1/PU.1 in these cells are not associated with further dysregulation of Fli-1.

miRNAs have been implicated in the differentiation, survival, and proliferation of hematopoietic cell lineages, and thus are also involved in hematopoietic transformation (Georgantas, III et al., 2007). It has been shown that miR-155 is a potent inhibitor of hematopoietic progenitor differentiation, and transduction of miR-155 into a leukemic cell line, followed by treatment of chemical inducers of differentiation, results in decreased erythroid and megakaryocytic differentiation. Sustained expression of miR-155 in mice causes a myeloproliferative disorder and impaired erythropoiesis (O’Connell et al., 2008). In addition, predicted targets of miR-155 include several hematopoietic differentiation-associated molecules, such as Spi-1/PU.1 and SHIP-1 (Georgantas, III et al., 2007), (Vigorito et al., 2007), (Costinean et al., 2009), (O’Connell et al., 2009). We have shown that enforced expression of Spi-1/PU.1 results in the increased transcription of SHIP-1, as expected, although protein levels remain undetectable. This phenomenon may be partially explained by the induction of miR-155 expression, since SHIP-1 is a known target of miR-155, although the induction and importance of miR-155 expression has yet to be elucidated in this erythroleukemic setting. The positive regulation of SHIP-1 by Spi-1/PU.1 may be more relevant in the lymphoid lineage and immune cell function since deficiency of both SHIP-1 and Spi-1/PU.1 results in decreased numbers of B lymphocytes (DeKoter & Singh, 2000), (Leung et al., 2009). Spi-1/PU.1 also upregulates the expression of another phosphatase SHP-1 (Wlodarski et al., 2007) that interferes with STAT1 and STAT3 signaling to inhibit erythroid differentiation (Nishigaki et al., 2006). Therefore the effects
of Spi-1/PU.1-mediated transcriptional regulation of specific target genes may be dependent upon the cell context or setting of cancer induction.

Fli-1 and Spi-1/PU.1 recognize the same core Ets DNA binding domain, and it is has been suggested that these transcription factors promote erythroid transformation through the regulation of a common set of target genes necessary to alter the balance of erythroid proliferation and differentiation. We have demonstrated that *SHIP-1* is a direct target gene of both Fli-1 and Spi-1/PU.1, although it is clear that these Ets transcription factors have opposing effects on the transcriptional regulation of *SHIP-1*. In erythroid cells, many factors are involved in the events leading to Epo-independent growth that is associated with transformation. Although the transcriptional dysregulation directed by Spi-1/PU.1 or Fli-1 overexpression ultimately leads to the same outcome, they may not contribute to the development of erythroleukemia by a common mechanism.
Chapter 4
Fli-1 overexpression in SFFV-induced erythroleukemia increases hematopoietic progenitor activity

4.1 ABSTRACT

Ets transcription factors play a major role in the hematopoietic cell proliferation and differentiation. The Ets oncogene, Fli-1, is highly expressed in both murine and human erythroleukemia, as well as Ewing's sarcoma and lupus and is commonly deleted in Jacobsen and Paris-Trousseau syndrome. Viral integration within the fli-1 locus is the pivotal genetic event in F-MuLV-induced erythroleukemia producing the overexpression of this transcription factor that is required for the maintenance and survival of these cells. Yet it remains unclear how deregulated Fli-1 expression alters the balance between erythroid differentiation and proliferation. In order to determine the effects of Fli-1 aberrant regulation in erythroid transformation, Fli-1 was exogenously expressed in a Friend erythroleukemic cell line, DP17-17, harboring activation at the spi-1/PU.1 locus. While the proliferation of these cells in culture remains unaffected, Fli-1 overexpression imparts morphological and immunohistochemical characteristics of a more immature erythroid progenitor, although it does not affect the potential of these cells to undergo erythroid maturation upon stimulation with DMSO. Fli-1 overexpression increased the numbers of erythroid colonies on methylcellulose, and reduced tumorigenicity in vivo, since inoculation of Fli-1 overexpressing DP17-17 cells increased the latency of erythroleukemia progression, compared to control mice. Although all recipient mice developed dramatic enlargement of the spleen and liver, Fli-1 overexpression altered the hematopoietic phenotype, significantly increasing the expression of c-kit, Sca1 and CD71. This study demonstrates that Fli-1 overexpression may contribute to the initiation and progression of erythroid transformation by increasing hematopoietic progenitor activity.
4.2 INTRODUCTION

The progression of cancer is a multi-step process in which oncogenes and tumor suppressor genes mediate changes in gene expression. In hematological malignancies, such genetic changes promote hematopoietic progenitor proliferation and survival, and impair differentiation. Normal hematopoiesis is a complex and dynamic process of development, where proliferation and differentiation are intimately linked cellular events. Hematopoietic differentiation occurs as a result of a distinct gene expression program, whereby the self-renewal of pluripotent hematopoietic stem cells and sequential commitment of intermediate progenitors, with a decreased capacity to proliferate, is governed by specific combinations of lineage-specific transcription factors. Transcription factors, such as those in the Ets gene family, play an integral role in hematopoiesis by coordinating the balance between proliferation and differentiation and influencing properties of self-renewal. Therefore, unsurprisingly the dysregulation of normal Ets transcriptional machinery plays a causal role in several human and murine hematological malignancies associated with chromosomal translocations or viral insertions.

Multi-stage erythroleukemia induced by Friend virus has served as an excellent mouse model to study the effects of specific Ets transcription factors associated with the impairment of hematopoietic lineage development and ultimately hematological pathogenesis. Friend virus-induced erythroleukemia is characterized by a marked expansion of erythroid progenitors. The two strains of the Friend virus complex, polycythemia- and anemia-inducing isolates, consist of a replication defective spleen-focus forming virus (SFFV) and a replication-competent Friend murine leukemia virus (F-MuLV) (Mager et al., 1981), (Moreau-Gachelin, 2008). The emergence of clonal tumorigenic erythroblasts is dependent upon retroviral insertional activation of the Ets transcription factors, spi-1/PU.1 in SFFV-induced erythroleukemia (Moreau-Gachelin et al., 1988), and
*fli-1*, in F-MuLV-induced erythroleukemia (Ben-David et al., 1990a), (Ben-David et al., 1991). In both cases, tumorigenic erythroid progenitor cells are blocked in differentiation at the proerythroblast stage, with self-renewal capacities. SFFV induces enhancement of proerythroblasts that exhibit properties of erythroid colony-forming (CFU-E) cells, whereas F-MuLV-induced erythroleukemic cells exhibit properties of erythroid burst forming (BFU-E) cells (Shibuya & Mak, 1983). Leukemic cells grown in methylcellulose have given rise to several established erythropoietin (Epo)-independent cell lines, some of which retain the ability to undergo erythroid maturation upon treatment with chemical inducers of differentiation, such as dimethyl sulfoxide (DMSO). Recent evidence suggests that the maintenance of the malignant phenotype in these cell lines is dependent upon the aberrant regulation of *fli-1* (Cui et al., 2009), and *spi-1/PU.1* (Juban et al., 2009).

Fli-1, an Ets transcription factor expressed in all hematopoietic lineages (Ben-David et al., 1991), (Truong & Ben David, 2000), is critical for embryonic development (Hart et al., 2000), (Spyropoulos et al., 2000) and is considered to be at the top of the transcriptional blood network (Liu et al., 2008). Deregulated expression of Fli-1 has detrimental effects on both myeloid and lymphoid development (Zhang et al., 1995), (Bastian et al., 1999), (Hart et al., 2000), (Spyropoulos et al., 2000), (Masuya et al., 2005), (Zhang et al., 2008) and is also associated with malignant transformation of these lineages in mice and humans (Athanasiou et al., 1996), (Kwiatkowski et al., 1998), (Mhawech-Fauceglia et al., 2006), (Torchia et al., 2007). Fli-1 expression is required for megakaryocytic development, as observed in *fli-1* loss of function studies (Hart et al., 2000) and Jacobsen or Paris-Trousseau syndrome, caused by a chromosomal translocation resulting in the deletion of *fli-1* (Hart et al., 2000), (Wenger et al., 2006). Fli-1 overexpression in Friend disease has provided evidence for the role of Fli-1, as well as Spi-1/PU.1 in erythroid development. During the progression of normal erythropoiesis, Fli-1 (Lee et al., 2003) and Spi-1/PU.1 protein levels are downregulated (Quang et al., 1997),
Spi-1 supports proliferation of immature erythroid progenitors (Fisher et al., 2004), and exogenous expression of this transcription factor immortalizes erythroblasts (Schuetze et al., 1993). Overexpression of Spi-1 in normal mice recapitulates a similar Friend virus-induced erythroleukemic phenotype (Moreau-Gachelin et al., 1996). However, overexpression of Fli-1 in normal mice causes an autoimmune disorder, resembling lupus. We have previously reported the importance of Fli-1 overexpression in the maintenance of both murine and human erythroleukemia (Cui et al., 2009), and it is obvious that Fli-1 contributes to transformation through its role in directing hematopoiesis, yet the molecular mechanisms by which Fli-1 expression alters the balance between erythroid differentiation and proliferation remains unclear.

Previous knockout and transgenic studies have failed to identify a more specific role for Fli-1 in erythroid development, although it is clear that Fli-1 is a pivotal player in erythroleukemogenesis. Surprisingly, unlike Spi-1/PU.1, overexpression of this Ets transcription factor alone is insufficient to cause erythroleukemia in mice. Studies of Friend virus-induced erythroleukemia have implied that activation of Fli-1 inhibits the commitment of erythroid progenitors to differentiate through disruption of critical erythroid signaling pathways, such as that of Epo and stem cell factor (SCF). Indeed, Fli-1 has been shown to alter the expression of erythroid lineage-associated genes, such as \( Rb \) (Tamir et al., 1999), \( bcl-2 \) (Lesault et al., 2002) and recently \( SHIP-1 \) (Lakhanpal et al., 2010).

In order to directly assess the role of Fli-1 in erythroid transformation, an SFFV-induced erythroleukemia cell line was generated to ectopically express Fli-1 along with green fluorescent protein (GFP) reporter. Using this erythroleukemic cell line, we show that Fli-1 overexpression alters the state of erythroid differentiation, but does not affect the ability of these cells to undergo chemically-induced differentiation in culture.
Methylcellulose cultures revealed that Fli-1 overexpression enhances erythroid colony formation. Furthermore, we find that exogenous expression of Fli-1 in these erythroleukemic cells reduces their tumorigenicity when transplanted into syngeneic mice, although recipient mice nonetheless develop dramatic splenomegaly and hepatomegaly. Further characterization of the resultant erythroid malignancy revealed alterations in the expression of several hematopoietic cell surface markers, suggestive of a more primitive erythroid progenitor phenotype.

Here, we report valuable data using the Friend virus-induced erythroleukemia model to provide direct evidence suggesting that Fli-1 overexpression increases hematopoietic progenitor activity. These data may provide important insight into the pathology of human disease associated with the aberrant regulation of Fli-1.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture and treatments

The murine SFFV-induced erythroleukemia cell line, DP17-17, derived from DBA/2 mice, was maintained in alpha-minimum essential medium (α-MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics (penicillin and streptomycin) (Gibco). 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). To induce erythroid differentiation, FACS sorted DP17-17 cells were treated for two days with 2% dimethyl sulfoxide (DMSO) (Sigma Aldrich, Oakville, ON, Canada). Differentiation assays were performed in triplicate by seeding (1 x 10^5) cells/well in 3 ml of a 6 well plate.
After 48 hours of induction with DMSO, adherent cells were removed from the culture dish using a cell scraper for cytospin preparation and histological analysis.

4.3.2 Enforced expression of Fli-1

The MigR1 Fli-1, or empty vector control plasmid, MigR1, was triple-transfected with Lipofectamine 2000 (Invitrogen, Burlington, Canada) into 293T cells, following the manufacturer’s protocol. The vesicular stomatitis virus G glycoprotein (VSVG)-expressing vector, as well as the gag and pol virus packaging signals were provided by Dr. D Barber, University of Toronto. Viral supernatant was collected 48 hours post-transfection. DP17-17 (2.5 x 10⁶) were infected with virus, and incubated 16 hours with virus in the presence of polybrene (8µg/ml final concentration), as previously described (Cui et al., 2009). Two days post-infection, cells were sorted by flow cytometry based on the intensity of green fluorescence. Sorted cell populations were allowed to expand in culture, and sorted a second time based on high intensities of green fluorescence.

4.3.3 Immunoblotting

Cells were lysed with radioimmunoprecipitation assay buffer (0.5% Nonidet P-40, 50 mM Tris HCl (pH 8.0), 120 mM NaCl, 50 mM NaF, plus 1 mM Na₃VO₄, 10 g/ml aprotinin, 100 g/ml leupeptin and 10 mM phenylmethylsulfonyl fluoride). 40 µg lysates were fractioned by SDS/PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The following antibodies were used: SHIP-1, Fli-1, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma-Aldrich, Oakville,
ON, Canada); goat-anti-mouse, and goat anti-rabbit HRP-conjugated (Promega, Madison, WI, USA).

4.3.4 Cellular proliferation assay

Transduced and double-sorted DP17-17 cells, $1 \times 10^4$, were plated in triplicate, removed at 24 hour intervals, and cellular proliferation was measured by performing Trypan-blue exclusion assay. Briefly, cells were stained with Trypan-blue (Invitrogen, Burlington, ON, Canada) to exclude dead cells, and total viable cells were counted using a hemocytometer.

4.3.5 Cytospin Preparation and histochemical staining

Cells ($2 \times 10^4$ per slide) were cytospun onto glass slides for 15 min at 1000rpm (Cytospin; Thermo Shandon, USA). Cells were fixed at room temperature in methanol for 5 minutes and air-dried. Fixed cells were stained with May-Grunwald stain followed by Giemsa stain according to the manufacturer’s protocol (Sigma). Light microscopy images were obtained using a Leica DM LB2 microscope, Leica DFC 300FX camera, and Leica Application Suite 3.1.0 software (Leica Microsystems, Switzerland). Blinded erythroid differential counts were performed by Dr. Michael Rauh, hematopathologist, Sunnybrook Health Sciences Centre, University of Toronto. A total of 6 cytospin slides with May-Grunwald Giemsa stains were prepared during two separate experiments for each non-transduced, double-sorted MigR1, and MigR1 Fli-1 DP17-17 cell groups. Approximately 100-200 cells were counted on each slide and categorized into one of the three defined
stages; R1=proerythroblast, R2=early basophilic erythroblast, R3=late basophilic erythroblast. Data is presented as the percentage of total cells analyzed.

4.3.6 Immunostaining and Flow cytometric analysis

Freshly isolated cells were washed twice in PBS (Gibco) and immunostained for 15 min with the appropriate antibody; phycoerythrin (PE)-conjugated anti-TER119 (erythroid marker), PE-conjugated anti-c-kit (SCF receptor), PE-conjugated anti-CD41 (glycoprotein IIb), PE-conjugated anti-CD61 (glycoprotein IIIa), PE-conjugated anti-Gr-1 (granulocytic marker), PE-conjugated anti-MAC-1 (monocytic marker), APC-conjugated anti-CD71 (transferrin receptor), APC-conjugated anti-Sca1 (primitive hematopoietic cell marker) (1:200) (eBioscience, San Diego CA, USA). Following antibody incubation, cells were washed once in PBS and resuspended in 500 ul PBS. Cell sorting and analysis of stained cells were performed using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA), and the FlowJo flow cytometry analysis software (Flowjo TreeStar Inc, Ashland, OR, USA). Relative mean fluorescence intensity (MFI) values were based on the unstained population controls and calculated using the Geometric Mean statistic (average of log fluorescence). Statistical analyses were performed using the two-tailed Student’s t-test, where significance was considered when p < 0.05.

4.3.7 Colony-Forming Cell Assay

DP17-17 cells, transduced with the MigR1 empty vector control or MigR1 Fli-1 expressing vector and double sorted, were suspended in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco) supplemented with 2% FBS (Gibco) and added to methylcellulose
medium (M334, or M3434, Stem Cell Technologies, Vancouver, BC, Canada) to assay for the presence of erythroid colony-forming units (CFU-E) and mature erythroid burst-forming units (BFU-E) (M3334 formulation consists of 15% FBS, 1% BSA, 10 µg/ml Insulin, 200 µg/ml Transferrin, 3 U/ml rh EPO, and M3434 formulation consists of 15% FBS, 1% BSA, 10 µg/ml Insulin, 200 µg/ml Transferrin, 50 ng/ml rm SCF, 10 ng/ml rm IL-3, 10 ng/ml IL-6, 3 U/ml rh EPO), according to the manufacturer’s protocol. Colonies were counted after 12 days of culture. CFU-E and BFU-E colonies were detected by staining with benzidine solution, 0.4% benzidine (Sigma-Aldrich) in 12% acetic acid, with the addition of 0.3% hydrogen peroxide (Sigma). Individual colonies were isolated from methylcellulose cultures, cells were resuspended, and prepared for cytospins and histochemical staining, as described above.

4.3.8 Transplantation assay

DP17-17 cells, transduced with the MigR1 empty vector control or MigR1 Fli-1 expressing vector, were sorted by flow cytometry based on high intensities of green fluorescence, as described above. Double-sorted DP17-17 were suspended in phosphate-buffered saline (PBS) (Gibco), in a total volume of 200 µl, and administered intravenously into the tail veins of female recipient eight-week old DBA/2J mice, at concentrations of 1.0 x 10^6 (n=4), 1.0 x 10^5 (n=4) or 1.0 x 10^4 (n=2) cells. Injected mice were sacrificed if they presented with symptoms of disease progression, such as paleness, hunched posture, enlarged abdomen, and paralysis or difficulty breathing. In some mice, blood samples were collected from tails using a 200 µl EDTA-coated capillary tube (Drummond Scientific, Broomall, PA, U.S.A). Body, spleen, and liver weights were measured at time of death, as indicators of disease progression. Spleen, liver and bone marrow samples were isolated from recipient mice, cultured for two days, and subjected to Western and flow cytometric
analyses, as described above. The contribution and presence of the transduced DP17-17 cells injected was evaluated based on the detection of green fluorescence.

4.4 RESULTS

4.4.1 Enforced expression of Fli-1 in erythroleukemic cells

Friend virus-induced erythroleukemic cells have been used extensively as a model to study the molecular and genetic changes necessary for the control of erythroid differentiation and proliferation. The Friend virus complex consists of the spleen focus forming virus (SFFV) and the Friend murine leukemia virus (F-MuLV). SFFV induces erythroid malignant transformation through integration of the provirus at the spi-1/PU.1 locus producing the overexpression of a normal spi-1/PU.1 mRNA transcript and protein in 95% of Friend tumor cells (Moreau-Gachelin et al., 1988), (Paul et al., 1989). Moreover, overexpression of Spi-1/PU.1 alone is sufficient to induce erythroid transformation in mice (Moreau-Gachelin et al., 1996), (Barnache et al., 1998). The critical genetic event of F-MuLV-induced erythroleukemia is proviral integration at the fli-1 locus, causing its overexpression (Ben-David et al., 1990a), (Ben-David et al., 1991), although Fli-1 transgenic mice fail to develop erythroleukemia (Zhang et al., 1995), and the exact mechanisms by which Fli-1 alters erythroid proliferation and differentiation remains unclear. As shown in Figure 4.1A (modified Figure 3.1A), Spi-1/PU.1 is overexpressed in SFFV-induced erythroleukemia cell lines termed HB60-5, DP16-1, and DP17-17, however its expression is absent in F-MuLV-induced erythroleukemia cells, CB7, CB3 and HB22.2, overexpressing Fli-1. The SFFV-induced erythroleukemic cell lines express Fli-1, albeit at
significantly lower levels, compared to F-MuLV-induced erythroleukemia cell lines (Figure 4.1A).

In order to decipher the role of Fli-1 in erythroid proliferation and differentiation, exogenous expression of Fli-1 was introduced into the SFFV-induced-erythroleukemia cell line, termed DP17-17. DP17-17 cells, transduced with either the MigR1 empty vector control, or Fli-1 expressing retrovirus, were sorted by flow cytometry based on the intensity of green fluorescence two days post-infection. Sorted cell populations were isolated, and subjected to Western blot analysis to confirm enforced expression of Fli-1 (Figure 4.1B modified Figure 3.1B). Trypan blue exclusion assay was performed to reveal the effects of Fli-1 overexpression on the proliferation of the SFFV-induced erythroleukemic cells. The non-transduced (N/T), MigR1 and MigR1 Fli-1 transduced cell populations retained a comparable proliferation rate (Figure 4.1C), indicating that Fli-1 overexpression has no additional effect on proliferation in these cells. However, upon microscopic observation of transduced DP17-17 cultures, a clear distinction was observed in the gross morphology of the transduced populations. Typically, DP17-17 cells growing in culture have both an adherent and suspension population, and upon cell differentiation, using a chemical inducer, these cells become mainly adherent. Under normal culture conditions, exogenous Fli-1 expression in DP17-17 cells resulted in a remarkable shift in the proportion of these populations, increasing the numbers of suspension cells, compared to the MigR1 empty vector control (Figure 4.1D). This observation suggested to us that Fli-1 overexpression might have an affect on the differentiation of this erythroleukemic cell line.
Figure 4.1 – Exogenous expression of Fli-1 in an SFFV-induced erythroleukemic cell line. Fli-1 and Spi-1/PU.1 expression levels in HB60-5, DP16-1, DP17-17, CB7, CB3 and HB22.2 Friend erythroleukemic cells (A). Exogenous expression of Fli-1 in DP17-17 cells (B). β–actin was used as a loading control for all samples. Unchanged proliferation rate of transduced DP17-17 cells as determined by Trypan-blue exclusion assay (C). Gross morphological examination revealed that Fli-1 overexpression increases the proportion of the non-adherent cell population under normal culture conditions, compared to the MigR1 empty vector transduced cells (D).

4.4.2 Exogenous expression of Fli-1 alters the state of erythroid differentiation

Proliferation assays and initial microscopic observations of Fli-1 overexpressing DP17-17 cells revealed that while the proliferation rate of these erythroleukemic cells remained unchanged, the increased proportion of non-adherent cells in culture suggests that altered levels of Fli-1 expression may influence erythroid differentiation and
erythroid progenitor activity. To confirm this hypothesis, histochemical staining of transduced DP17-17 cells was performed to distinguish between different stages of erythroid development. Histology can detect the morphologically defined stages of erythropoiesis including, proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatophilic erythroblasts, reticulocytes and mature erythrocytes. Successive erythroid differentiation is characterized by a decrease in cell size, increase in condensation of nuclear chromatin, more abundant cytoplasm and increase in hemoglobinization as indicated by paler or less saturated dye. In two separate experiments, May-Grunwald Giemsa stains revealed that Fli-1 overexpressing DP17-17 cells exhibit darker staining of the nuclei, more dense appearance of the nuclei chromatin (indicating immature chromatin), and a scant, deeply basophilic cytoplasm, compared to control cells (Figure 4.2A). Overall, the morphological characteristics of Fli-1 overexpressing DP17-17 cells was indicative of a more immature cell type compared to the control cells. To confirm our findings, a clinical hematopathologist performed blinded erythroid differential counts of transduced DP17-17 cell cytospin preparations stained with May-Grunwald/Giemsa. Erythroblasts were characterized into three groups, where R1 represents the proerythroblast; R2 represents the early basophilic erythroblast, and R3 the late basophilic erythroblast stage (Zhang et al., 2003) (Figure 4.2B). DP17-17 cells overexpressing Fli-1 comprised 82%, 14%, and 4% of the R1, R2, and R3 groups, respectively. Control DP17-17 cell populations approximately comprised 60%, 25%, and 15% of the R1, R2, and R3 groups, respectively (Figure 4.2B). Therefore blinded histochemical analysis confirmed that exogenous expression of Fli-1 resulted in an enhanced proportion of cells in of a more immature erythroid phenotype, compared to the appropriate controls (Figure 4.2B).
Figure 4.2 – Exogenous expression of Fli-1 alters the state of erythroid differentiation, but does not affect chemically-induced differentiation of erythroleukemic cells. May-Grunwald Giemsa stained cytospin preparations of double-sorted DP17-17 cells transduced with the MigR1 empty vector control and Fli-1 expressing vector (A). Blinded erythroid differential counts from stained cytospin preparations of transduced DP17-17 cells performed by a clinical hematopathologist. The bar graph represents percentages of total cells analyzed (B). Stained cytospin preparations (C) and flow cytometric analysis (D) of untreated and DMSO-treated non-transduced and double sorted, transduced DP17-17 cells following two days of induction.
4.4.3 Exogenous Fli-1 expression does not affect the ability of Friend erythroleukemic cells to undergo erythroid differentiation in culture

DP17-17 cells are capable of undergoing part of erythroid differentiation upon treatment with dimethyl sulfoxide (DMSO) or other specific chemical-inducing agents, although they generally do not enucleate upon treatment. The above-mentioned data suggest the ability of Fli-1 expression to induce features of a less-differentiated erythroid phenotype. In order to determine if exogenous Fli-1 expression also affects the potential or capacity of Friend erythroleukemic cells to differentiate along the erythroid lineage, DP17-17 cells transduced with the MigR1 empty vector control or Fli-1 expressing vector were grown in the presence of DMSO to induce erythroid differentiation. Double sorted cell populations were seeded in triplicate and grown in culture medium supplemented with 2% DMSO. Following 48 hours of induction, most of the cells became adherent to the culture dish (data not shown). To examine changes in the morphology of the DMSO-treated cells, suggestive of erythroid differentiation, DP17-17 cells were removed from the culture dish for cytopsin preparation and histochemical staining. The morphology of May-Grunwald Giemsa stains was examined, observing the above-mentioned features of successive erythroid differentiation. DMSO-induced cultures of non-transduced and MigR1 empty control DP17-17 cells consisted predominantly of cells resembling the orthochromatophilic stage, with small, condensed nuclei, and paler blue, more abundant cytoplasms (Figure 4.2C). Comparison with un-induced cultures of the same population revealed succession through later stages of erythropoiesis following treatment with DMSO (Figure 4.2C). DMSO-induced cultures of DP17-17 cells with exogenous Fli-1 expression consisted predominantly of cells resembling the polychromatophilic stage, with slightly larger nuclei and more deeply stained, less abundant cytoplasm compared to the control DP17-17 cells treated with DMSO (Figure 4.2C). Further comparison with noninduced cultures overexpressing Fli-1 also revealed succession of erythropoietic development (Figure 4.2C). Erythroid maturation, and DMSO stimulation, coincides with a decrease in the expression of the transferrin receptor (CD71) and a slight increase in the erythroid
lineage cell surface marker (TER119). Flow cytometric analyses of DMSO-induced cultures with CD71 and TER119 also suggests increased commitment to the erythroid lineage, compared to noninduced cultures (Figure 4.2D). Therefore, while exogenous expression of Fli-1 induces a less differentiated phenotype, it does not affect the potential of Friend erythroleukemic cells to undergo chemically-induced erythroid differentiation.

4.4.4 Fli-1 overexpression increases the number of colony-forming cells

In order to further investigate the role of exogenous Fli-1 overexpression in erythroid differentiation, a colony-forming cell (CFC) assay was performed to quantify changes in the number of lineage-restricted progenitors of the erythroid or megakaryocytic lineage. Double-sorted DP17-17 cells transduced with the MigR1 empty vector control or Fli-1 expressing retrovirus, were suspended in semi-solid methylcellulose medium supplemented with Epo, or Epo plus other additional cytokines. CFU-E and BFU-E colonies were enumerated by staining with benzidine solution. Individual colonies were quantified and the lineage composition was classified based on morphological recognition by light microscopy, and cytochemical staining of cellular cytospins with May-Grunwald/Giemsa staining. Fli-1 overexpressing DP17-17 cells produced increased numbers of erythroid colonies, approximately 2.5x more colonies compared to the appropriate controls (Figure 4.3A). Typical morphologies of benzidine-stained cells from MigR1 or MigR1 Fli-1 colonies are presented in Figure 4.3B. Benzidine positive staining revealed that the vast majority of colonies generated by both groups of DP17-17 cells are of erythroid origin. Gross morphological examination by light microscopy also revealed that the majority of Fli-1 overexpressing colonies grew larger in size, compared to the appropriate controls (data not shown), suggesting that Fli-1 may enhance the proliferation of erythroid progenitors. Isolation of individual colonies, and subsequent cytospin preparation for May-Grunwald Giemsa staining confirmed the
erythroid composition, as stained cells displayed characteristic morphologies of mid to late stage erythroblasts (Figure 4.3C). This data indicates that while overexpression of Fli-1 does not alter the differentiation potential of erythroid progenitors in vitro, it endows features of a less-differentiated state and increases the number of colony-forming cells, suggesting that Fli-1 enhances hematopoietic progenitor cell activity.

Figure 4.3 – DP17-17 cells overexpressing Fli-1 display increased numbers of erythroid colonies. Transduced DP17-17 cells were plated in triplicate on methylcellulose media in the presence of cytokines. Erythroid colonies were quantified by staining with benzidine following 12 days of culture (A). Representative data indicating benzidine positive staining of methylcellulose cultures on day 12 (B). Analysis of individual methylcellulose colonies (C). Cellular cytospins and May-Grunwald-Giemsa stains of sampled colonies from transduced DP17-17 cells display characteristic morphologies of mid to late stage erythroblasts.
4.4.5 Fli-1 overexpression in SFFV-induced erythroleukemia increases the latency of disease progression and alters the hematopoietic phenotype

In order to determine whether Fli-1 overexpression, and the concurrent alteration of differentiation status, affects the progression of FV-P-induced erythroleukemia, DP17-17 cells were transplanted into syngeneic DBA/2J mice. Transduced and double-sorted DP17-17 cells (1 x 10^6, 1 x 10^5, 1 x 10^4) were intravenously injected into the tail veins of eight-week old DBA/2J mice, and monitored for physiological signs of FV-P-induced erythroleukemia progression, such as paleness, hunched posture, enlarged abdomen, and paralysis or difficulty breathing. No appreciable difference was observed in the time of appearance of erythroleukemia progression in mice receiving 1 x 10^6 cells (Figure 4.4A). However, mice injected with 1 x 10^5 and 1 x 10^4 Fli-1 overexpressing cells displayed a statistically significant increase in disease latency (Figure 4.4A). In mice inoculated with MigR1 Fli-1 transduced DP17-17 cells, two of the four mice with the 1 x 10^5 concentration and one of the two mice with the 1 x 10^4 concentration failed to develop erythroleukemic tumors (MigR1 Fli-1; n= 7). Additionally, one out of the two mice injected with the 1 x 10^4 concentration of MigR1 transduced cells also failed to develop erythroleukemia (MigR1; n=9). Friend virus-induced erythroleukemia is marked by the emergence and expansion of transformed erythroblasts in the spleen and liver, forming colonies (Moreau-Gachelin, 2008). As expected, a noticeable increase in size, and a reduction in the number of macroscopic spleen and liver colonies was observed in mice injected with lower cell concentrations, of both MigR1 and MigR1 Fli-1 injected groups (Figure 4.4B). All diseased recipient mice, irrespective of DP17-17 cell concentration, displayed overt splenomegaly (MigR1, 430 ± 80 mg; Fli-1, 500 ± 60 mg) and hepatomegaly (MigR1, 4.2 ± 0.5 g; Fli-1, 3.3 ± 0.14 g), compared to normal, non-injected mice of comparable age (Figure 4.4C and D). Remarkably, detailed anatomic analysis also revealed an enlarged thymus of a Fli-1 recipient mouse (1 x 10^4 cells injected), indicating extramedullary erythroblast expansion within the thymus (data not shown). Moreover, measured hematocrit values were indicative of a polycytemic state of disease (data not shown).
Figure 4.4 – Increased disease latency in mice inoculated with Fli-1 overexpressing DP17-17 erythroleukemic cells. The mean survival rate of eight-week old DBA/2J mice i.v injected with $1 \times 10^6$ (n=4), $1 \times 10^5$ (n=4), or $1 \times 10^4$ (n=2) DP17-17 cells transduced with MigR1 or MigR1 Fli-1 expressing retrovirus, where * indicates p<0.05 (A). Representative examples of spleens and livers isolated from mice injected with $1 \times 10^5$ (left panel) and $1 \times 10^4$ (right panel) DP17-17 cells (B). Mean spleen (C) and liver weights (D) of diseased recipient mice (MigR1, n=9; Fli-1, n=7). Uninfected mice of comparable age were used as the normal controls. Values represented as the mean ± standard deviation.
As indicated by dramatic enlargement of the spleen and liver, these organs were massively infiltrated by a population of tumorigenic DP17-17 erythroid progenitors. In order to further characterize the erythroid malignancy generated in recipient mice, tumor spleen and liver cultures were subjected to flow cytometric immunophenotyping. The contribution and presence of intravenously injected DP17-17 cells, transduced with MigR1 (n=9) or MigR1 Fli-1 (n=7), was detected through green fluorescence. Thus, the frequencies of several hematopoietic cell surface markers were determined as a percentage of the total GFP positive cell population in diseased recipient mice (summarized results in Table 4.1). In summary, the enlarged spleens and livers of both MigR1 and Fli-1 injected mice contained similar percentages of cells positive for CD71 and TER119, the typical erythroleukemic population (Table 4.1). Although the percentages of cells positive for CD41, CD61, c-kit, and MAC-1 in the spleen, as well as CD41 and c-kit in the liver, were significantly higher in Fli-1 injected mice compared to MigR1 injected mice (Table 4.1). Further statistical analysis of this data, by calculation of mean fluorescence intensity (MFI) (summarized results in Table 4.2), demonstrated an obvious increase in the expression of the transferrin receptor CD71 in both the spleen and liver of Fli-1 injected mice, although the overall percentage of positive cells remained similar in both groups of recipient mice (Table 4.2). Moreover, MFI values also revealed a significant shift in the fluorescence intensity of c-kit, Sca-1, MAC-1, and Gr-1 expression in Fli-1 recipient mice. Taken together, this study suggests that increased latency of FV-P disease progression caused by Fli-1 overexpression imparts changes in the malignant properties, possibly resulting from an inherent change in the hematopoietic phenotype of erythroid progenitors.
**Table 4.1** – Frequency of hematopoietic cell surface marker expression, as a percentage (% ± standard deviation) of total GFP positive cells isolated from the spleens and livers of recipient DBA/2J mice, where Fli-1; n=7, MigR1 control; n=9, and * indicates p<0.05

<table>
<thead>
<tr>
<th></th>
<th>Fli-1</th>
<th>MigR1</th>
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<td></td>
<td>Spleen</td>
<td>Spleen</td>
<td>Liver</td>
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<tr>
<td>CD41</td>
<td>33.8 ± 5.9*</td>
<td>19 ± 3.8</td>
<td>28.5 ± 10.9*</td>
<td>15.2 ± 4.8</td>
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<tr>
<td>CD61</td>
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<td>46.2 ± 4.3</td>
<td>55.2 ± 10.1</td>
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<td>CD71</td>
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<td>95.8 ± 2.8</td>
<td>98.8 ± 0.5</td>
<td>93.5 ± 1.9</td>
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<tr>
<td>TER119</td>
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<td>3.25 ± 1.9</td>
<td>1.1 ± 0.8</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>c-kit</td>
<td>86 ± 4.8*</td>
<td>71 ± 3.6</td>
<td>79.8 ± 3.9*</td>
<td>61.9 ± 3.5</td>
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<tr>
<td>Sca-1</td>
<td>40.2 ± 32.6</td>
<td>12.6 ± 9.5</td>
<td>32.5 ± 42.4</td>
<td>12 ± 8.1</td>
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<tr>
<td>MAC-1</td>
<td>31.5 ± 9.3*</td>
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<tr>
<td>Gr-1</td>
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<td>0.4 ± 0.2</td>
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**Table 4.2** – Mean fluorescence intensity values of hematopoietic cell surface marker expression in GFP positive cells isolated from the spleens and livers of recipient DBA2/J mice. Values are based on the unstained population controls and calculated as the geo mean ± standard deviation, where Fli-1; n=7, MigR1; n=9, and * indicates p < 0.05

<table>
<thead>
<tr>
<th></th>
<th>Fli-1</th>
<th>MigR1</th>
<th>Fli-1</th>
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<td></td>
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<td>Spleen</td>
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<tr>
<td>CD41</td>
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<td>3.0 ± 1.5</td>
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<tr>
<td>CD61</td>
<td>20.3 ± 4.8</td>
<td>12.0 ± 1.8</td>
<td>15.9 ± 3.8</td>
<td>13.2 ± 6.0</td>
</tr>
<tr>
<td>CD71</td>
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<td>57.1 ± 9.4</td>
<td>231.1 ± 12.6*</td>
<td>50.3 ± 6.9</td>
</tr>
<tr>
<td>TER119</td>
<td>0.9 ± 0.3</td>
<td>3.3 ± 2.3</td>
<td>0.8 ± 0.7</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>c-kit</td>
<td>32.1 ± 2.4*</td>
<td>24.2 ± 1.9</td>
<td>28.8 ± 5.3*</td>
<td>20.1 ± 4.1</td>
</tr>
<tr>
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<td>15.9 ± 9.2</td>
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<tr>
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<td>6.5 ± 4.3</td>
<td>16.1 ± 3.9*</td>
<td>7.6 ± 2.1</td>
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<td>0.65 ± 0.2*</td>
<td>0.3 ± 0.2</td>
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4.5 DISCUSSION

Transcription factors, such as those in the Ets family, play an integral role in hematopoiesis by coordinating the balance between proliferation and differentiation, and by influencing properties of self-renewal. The Ets family member, fli-1, is aberrantly regulated in a number of cancers, including erythroleukemia and Ewing's sarcoma (Ben-David et al., 1990a), (Ben-David et al., 1991), (Delattre et al., 1992). The phenotype of mice homozygous for a targeted deletion of fli-1, indicate that Fli-1 function is indispensable during embryonic development and Fli-1 plays various roles in hematopoiesis, specifically in the megakaryocytic lineage. Fetal livers of homozygous fli-1 mutants appear pale and small, and contain a significant reduction in the number of multilineage, erythroid, and myeloid progenitors compared to those of the wildtype and heterozygous embryos (Spyropoulos et al., 2000). Loss of function studies performed in the Xenopus and zebrafish embryos have provided conclusive evidence indicating that Fli-1 acts at the top of the transcriptional network as a master regulator of blood and endothelial cell development within the cells of the mesoderm. Further evidence for the role of Fli-1 in the hematopoietic stem cell compartment, derives from the association of Fli-1 in the transcriptional regulation of several important stem cell genes (Pimanda et al., 2007), (Gottgens et al., 2002). To explore a more specific contribution of Fli-1 aberrant regulation in malignant transformation, we compared the phenotypes of an SFFV-induced erythroleukemia cell line, with and without exogenous Fli-1 expression. Consistent with previous studies involving the function of Fli-1 in stem and progenitor cell populations, our analyses have identified a distinct role for Fli-1 in hematopoietic progenitor cell activity.

Exogenous expression of Fli-1 was introduced into DP17-17 cells, the SFFV-induced erythroleukemic cell line harbouring a spi-1/PU.1 activated locus. Although Fli-1 did not
affect the proliferation rate of these cells in culture, a clear distinction was observed in the gross morphology of Fli-1 transduced DP17-17 cells. Histochemical staining, as well as blinded erythroid differential counts performed by a hematopathologist, confirmed that Fli-1 overexpression resulted in a remarkable shift in the state of erythroid maturation. Overall, exogenous expression of Fli-1 in DP17-17 cells imparts morphological characteristics, including darker staining of the nuclei, more dense immature chromatin, and scant deeply basophilic cytoplasm, indicative of a more immature erythroid cell progenitor, compared to the appropriate controls. Although Fli-1 overexpression leads to alteration of the erythroid phenotype, it does not inhibit the ability of this erythroleukemic cell line to differentiate along the erythroid lineage in response to a chemical inducer of differentiation, DMSO.

Transcriptional regulation is a key mechanism controlling hematopoietic precursor cell fate decision, function and behaviour. Lineage determination or plasticity can be induced by inappropriate transcription factor expression that leads to alteration in the gene expression program that defines hematopoietic cell identity (Zhu & Emerson, 2002). Indeed, this knowledge has led to the possibility for the manipulated differentiation of stem cells. Therefore, it is plausible that Fli-1 overexpression induces changes in the expression of factors such as Tal1, GATA-2, GATA-1, LMO2 and FOG-1 (Cantor & Orkin, 2002), (Pimanda et al., 2007), (Liu et al., 2008) that promote properties of self-renewal and favour the transition of a more immature erythroid phenotype. Future studies focusing on the expression pattern of such gene candidates will be valuable in determining the interaction of Fli-1 with other transcription factors, and their role in lineage plasticity, hematopoietic cell activity, specifically growth and self-renewal.

A role for Fli-1 in the promotion of hematopoietic progenitor activity was also supported by culture studies on methylcellulose. Fli-1 overexpression in DP17-17 cells led to enhanced erythroid colony formation on methylcellulose in the presence of cytokines, compared to the appropriate controls. Generally, Fli-1 overexpression also produced
colonies that grew larger in size, suggesting enhanced proliferation. The ability of Fli-1 to induce proliferation is not surprising in view of the fact that Fli-1 is known to affect erythroid proliferation and survival through several known target genes, such as Rb (Tamir et al., 1999), bcl-2 (Lesault et al., 2002), and SHIP-1 (Lakhanpal et al., 2010). Moreover, the ability of Fli-1 overexpression not only to promote proliferation but also to enhance self-renewal of hematopoietic progenitors has been evidenced by recent data collected from studies performed in our laboratory using inducible in vitro ES cell differentiation assays. In this system, Fli-1 overexpression induces an increase in the number of hematopoietic cell precursors, including erythroid and myeloid progenitors, in the presence of various cytokines including Epo and SCF (unpublished data Ben-David lab, J. Zhang 2009).

DP17-17 SFFV-induced erythroleukemia cells are transplantable in syngeneic mice, and generate symptoms analogous to the original disease, such as grossly enlarged livers and spleens. In this study, the overexpression of Fli-1 led to increased latency of disease progression, upon transplantation of transduced DP17-17 cells in DBA/2J syngeneic mice. However, mice injected with both groups of transduced DP17-17 cells, MigR1 empty vector control and MigR1 Fli-1, displayed overt splenomegaly and hepatomegaly, compared to normal, non-injected mice of comparable age. Notably, SFFV induces the enhancement of proerythroblasts that exhibit properties of erythroid colony-forming (CFU-E) cells, whereas F-MuLV-induced erythroleukemic cells exhibit properties of erythroid burst forming (BFU-E) cells (Shibuya & Mak, 1983). Our observation that Fli-1 overexpression increases disease latency of SFFV-induced erythroleukemia seems to support the notion that Fli-1 overexpression imparts the expansion of a more immature erythroid phenotype. Since SFFV-induced erythroleukemia is characterized by the accumulation of CFU-E proerythroblasts, increased disease latency may be explained, at least in part, by the additional timing or incubation period required of Fli-1 overexpressing
DP17-17 cells to successively differentiate and accumulate CFU-E proerythroblasts, when the population of cells initially present at time of injection retained a more immature phenotype compared to the original DP17-17 erythroid cell population.

The cell surface antigens CD41 (integrin αIbb) and CD61 (integrinβ3) are expressed in cells of the megakaryocytic lineage, and these two antigens form the fibronectin receptor/complex glycoprotein (GP) IIb/IIIa. The expression of these integrins is a characteristic of the megakaryocytic lineage as it progresses from progenitor to megakaryocytes and then platelets (Levene et al., 1985). Although, it has been shown that megakaryocytes and erythroblasts originate from a common myeloid (Akashi et al., 2000), (Ceredig et al., 2009), and accordingly cells derived from megakaryocytic leukemia or erythroleukemia often display traits of both erythroid and megakaryocytic progenitors (Hassan & Freund, 1995), (Drexler et al., 2004), (Tallack & Perkins, 2010). The molecular mechanisms regulating differentiation of either lineage remain unclear, however it is likely that activation of signaling pathways regulating self-renewal, survival, and proliferation of the erythroid/myeloid progenitor are governed through alterations of gene expression profiles. The expression of Fli-1, along with the RUNX-1 transcription factor, is associated with megakaryocytic differentiation (Yamada et al., 2007), (Jackers et al., 2004). The differentiation of megakaryocytes is primarily driven by thrombopoietin (TPO), and several genes known to play an important role in megakaryopoiesis, including the TPO receptor (TPOR) or c-mpl (Edvardsson et al., 2006), glycoprotein (GP) IX and IIb, and cyclin D1 (Sun et al., 2001), all of which contain Ets binding sites and are regulated by Fli-1 (Hart et al., 2000), (Pang et al., 2006). In the present study, exogenous expression of Fli-1 in the SFFV-induced erythroleukemia cell line, overexpressing Spi-1/PU.1, seems to result in a “dedifferentiation effect”, whereby this erythroid progenitor cell takes on features of a less differentiated cell type, as shown by histological staining and methylcellulose assays. Flow cytometric analysis of cells isolated from the organs of mice injected with these cells
revealed a statistically significant increase in the percentage cells positive for CD41 and CD61 expression. This data indicates that Fli-1 overexpression imparts changes in the hematopoietic cell surface marker profile with enhanced acquisition of megakaryocytic features while maintaining erythroid features, indicative of a megakaryocytic/erythroid progenitor phenotype.

The ectopic expression of Fli-1 also stimulated an increase in the percentage and intensity of the early cell-surface marker c-kit expression, compared to mice injected with the vector control transduced cells. As the receptor for stem cell factor (SCF), c-kit is expressed in erythroid progenitors and is downregulated during the progression to mature erythrocytes. The binding of SCF to its receptor c-kit leads to the activation of signaling pathways that enhance erythroid cell proliferation and deter differentiation (Munugalavadla & Kapur, 2005). Accordingly, mice devoid of c-kit expression display reduced numbers of erythroid progenitors and die *in utero* of anemia (Russell, 1979), (Nocka et al., 1989). Interestingly, the aberrant regulation of Fli-1 in erythroleukemia and Ewing’s sarcoma has been associated with increased c-kit expression. Examination of the mouse promoter region of *c-kit*, revealed eight possible Ets binding sites within 1kb of the transcriptional start (data not shown). It has also been previously reported that both myb and Ets proteins are candidate regulators of c-kit expression since the human *c-kit* promoter contains potential binding sites for these transcription factors (Ratajczak et al., 1998). It is possible that enhanced c-kit expression results from the transcriptional regulation of this receptor by direct binding of Fli-1, resulting in alteration of the signaling pathway governing erythroid cell proliferation and self-renewal. Therefore, a positive relationship between c-kit and Fli-1 expression may partially explain the ability of this Ets transcriptional regulator to increase progenitor cell activity and induce changes that resemble an earlier stage of erythroid development. Future studies involving the regulation of the c-kit cell surface receptor by Fli-1 may further uncover the role of Fli-1 in
malignant transformation, and its ability to alter the balance between hematopoietic differentiation and proliferation.

As previously mentioned, Fli-1 overexpression imparts changes in the hematopoietic cell surface marker profile as observed in a transplantation assay. While exogenous expression of Fli-1 did not alter the percentage of CD71 positive cells, calculation of MFI values revealed a shift in the fluorescence intensity, demonstrating an increase in CD71 expression. CD71, the transferrin receptor, is essential for iron transport into proliferating cells, and its expression diminishes through the progression of erythroid development. Several studies have indicated that during erythropoiesis the expression of this receptor is highly dependent on its transcriptional regulation. The Ets transcription factor, Ets-1, has been shown to bind to a consensus Ets DNA binding site within a region located immediately upstream the transcription start site of the transferrin receptor (Marziali et al., 2002). Fli-1 and Ets-1 similarly contain 5’ and 3’ Ets domains and share conserved sequence homology within these domains (Truong & Ben David, 2000). Taken together, this data suggests that the transferrin receptor may possibly represent an additional Fli-1 target gene, whose altered expression can partially account for the effects of Fli-1 aberrant expression on erythroid proliferation and differentiation.

In conclusion, we have shown that Fli-1 overexpression in erythroid progenitors can induce changes in erythroid developmental potential, and increase hematopoietic progenitor activity. We have demonstrated that this phenomenon may be partially mediated through direct activation of distinct Fli-1 target genes, including erythroid lineage regulatory signaling molecules such as c-kit and CD71, representing a novel mechanism by which Fli-1 contributes to malignant transformation. Our future studies will focus on addressing the specific role of Fli-1 in promoting hematopoietic progenitor proliferation and/or self-renewal, as well as the identification of the direct transcriptional
regulation of these candidate genes by Fli-1, since their dysregulation serves to further dissect the role of Fli-1 in hematopoietic progenitor activity and transformation. Most importantly, the discovery of a role for Fli-1 in self-renewal may also shed light on the pathogenesis of diseases associated with Fli1 aberrant regulation including erythroleukemia and Ewing’s sarcoma.
5.1 ABSTRACT

The Ets transcription factor, Fli-1, is a key regulator of hematopoiesis and vasculogenesis and aberrant regulation of this proto-oncogene is often associated with malignant transformation. Fli-1 is activated by proviral insertion in Friend virus-induced erythroleukemia, translocated in Ewing’s sarcoma, deleted in Paris-Trousseau and Jacobsen Syndrome, and overexpressed in various forms of leukemia and autoimmune diseases. We have recently shown that downregulation of Fli-1 results in a marked decrease of proliferation and the induction of cell death by apoptosis. Embryos carrying a targeted disruption of the fli-1 locus display aberrant hematopoiesis, hemorrhaging within the midbrain and are dead by embryonic day 11.5-12.5, suggesting a vital role of fli-1 in development and the regulation of hematopoiesis and hemostasis. The embryonic lethality necessitates the generation of a conditional fli-1 knockout mouse model to further elucidate the role of this Ets transcription factor in late-stage definitive hematopoietic development and facilitate the identification of fli-1 target genes and molecular pathways critical for these processes. Such knowledge may provide insight into the pathogenesis of disease associated with aberrant regulation of Fli-1 and perhaps provide access to novel and possibly more efficient therapeutic intervention strategies.
5.2 INTRODUCTION

The Ets family of transcription factors are found in the genomes of diverse organisms including Drosophila, Xenopus, Gallus, sea urchin, Mus musculus and Homo sapiens (Seth et al., 1992), (Papas et al., 1997), (Mavrothalassitis & Ghysdael, 2000), (Sementchenko & Watson, 2000). Members of the Ets family function throughout development to regulate the expression of key hematopoietic genes that govern the biological processes of cellular proliferation, differentiation, survival, and migration. Ets proteins function as both activators and repressors of transcription and the graded expression of Ets family members in various hematopoietic precursors and mature lineage-committed cells indicate a role for these transcription factors in hematopoietic lineage commitment and stage progression from the earliest stages of development through to adulthood. Friend leukemia integration-1 (Fli-1), a well-studied member of the Ets family, was originally discovered as the proto-oncogene activated in Friend murine leukemia virus (F-MuLV)-induced erythroleukemia (Ben-David et al., 1990a), (Ben-David et al., 1991). Similar to all other Ets factors, Fli-1 contains a highly conserved Ets DNA-binding domain that binds DNA sequences containing a consensus GGA(A/T) core motif. Fli-1 protein isoforms, p51 and p48, contain a 5′ and 3′ Ets domain responsible for the sequence specific DNA-binding activity of Fli-1. The 5′ Ets domain is located within exons 3 and 4 between amino acids 121-196, and the 3′ Ets domain is located within exon 9 between amino acids 277-360, immediately upstream the carboxy-terminal transcriptional activation domain (CTA). The CTA domain also contributes to the transcriptional activation and protein-protein interactions of Fli-1 (Rao et al., 1993).

During embryogenesis Fli-1 is expressed transiently in endothelial and mesodermal tissues from embryonic day 8.5 to 17 (Vlaeminck-Guilleme et al., 2000). Fli-1 is preferentially expressed in vascular endothelial cells and all hematopoietic tissues,
including the thymus and spleen, and is expressed at a lower level in the lungs, heart, and ovaries (Ben-David et al., 1991), (Melet et al., 1996), (Truong & Ben David, 2000). Given its breadth of expression, it is expected that changes in Fli-1 expression would inevitably result in vascular and hematopoietic abnormalities. Indeed Fli-1 overexpression, occurring as a result of proviral insertional activation, leads to hematopoietic transformation. Activation of fli-1, by F-MuLV leads to erythroid transformation (Ben-David et al., 1991), and integration of both the 10A1 viral isolate of MuLV, as well as the Cas-Br virus, induces non-T and non-B cell lymphomas (Bergeron et al., 1991), (Ott et al., 1994). Global overexpression of Fli-1 in transgenic mice leads to splenomegaly, increased lymphopoiesis, and a progressive immunological renal disease, associated with an increased number of autoreactive T and B-lymphocytes, which ultimately results in death. Previous studies have indicated that Fli-1 also plays an important role in the regulation of megakaryopoiesis and platelet production (Athanasiou et al., 1996), (Deveaux et al., 1996), (Bastian et al., 1999), (Szalai et al., 2006) through the transcriptional activation of several megakaryocytic genes, such as glycoprotein (gp) IIb (Zhang et al., 1993), (Wang et al., 2002) gpVI (Holmes et al., 2002), gplX, gplb (Eisbacher et al., 2003), and c-MPL (Deveaux et al., 1996), (Kawada et al., 2001). Accordingly, Fli-1 overexpression induces megakaryocytic differentiation in the K562 human hematopoietic cell line (Athanasiou et al., 1996). Aberrant Fli-1 expression is also associated with the progression of Ewing’s sarcoma (Delattre et al., 1992), (Ohno et al., 1993), lupus (Georgiou et al., 1996), and AML (Lopez et al., 1999). The human fli-1 gene is commonly deleted in a relatively infrequent congenital disorder known as Paris-Trousseau or Jacobsen syndrome. Clinical abnormalities resulting from this Fli-1 deficiency include growth and mental retardation, cardiac defects, pancytopenia, and thrombocytopenia due to abnormal megakaryocytic development (Wenger et al., 2006). Taken together, these results imply that Fli-1 is an active player in human disease and have provided substantial evidence for the role of Fli-1 in
hematopoiesis. To examine the effects of Fli-1 deficiency in normal development, several groups have generated mice carrying a targeted disruption within the ETS domain of the fli-1 locus (Hart et al., 2000), (Spyropoulos et al., 2000).

Homologous recombination strategies have been employed to disrupt Fli-1 function in murine embryonic stem cells. Mice carrying a targeted disruption at the fli-1 locus, thereby expressing a truncated protein lacking the functional CTA and 3’ Ets domains, display abnormal hematopoiesis and vasculogenesis at embryonic day (E) 11.0 (E11.0), and die around E12.0-12.5 (Hart et al., 2000), (Spyropoulos et al., 2000). Fetal livers of homozygous fli-1 mutants appear pale and small, and contain a significant reduction in the number of erythroid (BFU-E and CFU-E), granulocyte-macrophage (CFU-GM) and multilineage (CFU-mix) progenitors, proerythroblasts and basophilic erythroblasts compared to those of the wildtype and heterozygous embryos (Spyropoulos et al., 2000). Yolk sacs of homozygous mutants display a more moderate loss in erythroid progenitors and severe loss in granulocyte, macrophage and multilineage progenitors (Spyropoulos et al., 2000). Moreover, cell culture studies of cells from the aorta-gonad-mesonephros (AGM) of Fli-1 knockout embryos revealed a marked reduction in the number of megakaryocytes compared to wildtype and heterozygous embryos (Kawada et al., 2001). Heterozygous fli-1 knockout mice develop normally and display normal platelet counts and peripheral blood lymphocyte differential counts. Multifocal hemorrhage within the midbrain between E11.0 and E12.5 is associated with disruption of the vascular network and weakened cell-cell adhesion. Embryonic death due to fli-1 deletion can be attributed to incomplete vascular development, hemorrhage into the neural tube, defects in hematopoiesis, and thrombocytopenia due to a block in megakaryocyte development (Hart et al., 2000), (Spyropoulos et al., 2000). Impaired hematopoiesis and hemorrhaging occur at a time that comprises a critical transition period when the site of hematopoiesis changes from the
yolk sac or AGM to the liver, and death occurs prior to the beginning of definitive lymphopoiesis.

The embryonic lethal phenotype observed in Fli-1 knockout studies creates a necessity for additional targeting experiments to unveil the effects of Fli-1 downregulation beyond midgestation in late-stage hematopoietic development. Therefore, the generation of a conditional \textit{fli}-1 knockout mouse is critical to bypass embryonic lethality and provide our laboratory with an essential tool to further elucidate the role of \textit{fli}-1 in hematopoietic development, function and ultimately malignant transformation. Conditional knockout studies should also facilitate the identification of novel Fli-1 target genes, and perhaps shed light on the molecular pathology of Paris-Trousseau and Jacobsen syndrome to allow for the development of more efficient therapeutic options.

5.3 MATERIALS AND METHODS

5.3.1 Vector Construction

\textit{Fli}-1 genomic clones were isolated from the RPC1-22 BAC library (BAC clone 264 M8), derived from 129S6/SvEvTac mouse (Genome Resource Facility, The Hospital for Sick Children, Toronto, Canada), using a Fli-1 cDNA probe containing the CTA and 3' Ets domain and sequences from exon 5 to exon 9. A vector to target \textit{fli}-1 was designed (\textbf{Figure 5.1A}) to disrupt the transactivation activity of Fli-1 by deleting exon 9. Long distance PCR was used to amplify specific \textit{fli}-1 genomic regions within the \textit{fli}-1 BAC clone to generate the targeting vector. The targeting vector contains a selectable \textit{Neo} cassette, containing the neomycin phosphotransferase gene under the transcriptional control of the
phosphoglycerate kinase (PGK) promoter and the bovine growth hormone polyadenylation signal (bGHpA) for termination, which is flanked by two FRT Flp recombinase sequences. The Neo cassette is flanked by approximately 5.5 kb and 3 kb of \textit{fli-1} genomic sequences at the 5’ and 3’ end, respectively, containing homology arms to facilitate homologous recombination. The 5’ homology arm is approximately 3.9 kb in length and contains exons 7 and 8. This region is located immediately upstream the sequence corresponding to exon 9 of \textit{fli-1}, approximately 1.5 kb containing the Ets and CTA domains, which is flanked by \textit{loxP} Cre-recombinase recognition sequences. The 3’ homology arm is approximately 3.1 kb in length and contains the \textit{fli-1} 3’ untranslated region (3’ UTR). The targeting vector also contains the herpes simplex virus thymidine kinase gene (HSV-TK), under the transcriptional control of the MC1 promoter. The HSV-TK cassette, located outside the 3’ homology arm, was used for negative selection. The nucleotide sequence of the construct was confirmed prior to electroporation of linearized targeting vector into 129/Sv murine embryonic stem (ES) cells, termed R1. The \textit{fli-1} targeting vector was linearized by digestion with \textit{KpnI} (New England Biolabs, Pickering, ON, Canada), and purified by phenol chloroform extraction. Resultant targeted ES cells will have a recombination signal positioned to delete the critical exon 9, thereby generating a Fli-1 protein that lacks the regulatory transactivation domain. This recombination event would recreate a comparable \textit{fli-1} deletion generated in both the previous \textit{fli-1} knockout studies resulting in embryonic death (Hart et al., 2000), (Spyropoulos et al., 2000).

### 5.3.2 Polymerase Chain Reaction (PCR)

BAC clone 268 M4 DNA was isolated using Qiagen suspension (P1), lysis (P2), and neutralization (P3) buffers, following the Children’s Hospital Oakland Research Institute: BACPAC Resources Center protocol (www.chori.org/BACPAC). The 3.9 kb 5’ homology
arm, 1.5 kb targeted exon 9, and 3.1 kb 3’ homology arm fragments were amplified using Platinum® Taq DNA Polymerase High Fidelity, following the manufacturer’s protocol (Invitrogen). PCR primers were as follows; 3.9 kb 5’ homology arm (Figure 5.1B), forward primer: 5’ – GTCGTCGACACAAGTCTTACCATGGAGCCATC – 3’, reverse primer: 5’ – GTCGAATTCAAGACAGGGGCTGCTAC – 3’; 1.5 kb exon 9 fragment (Figure 5.1C), forward primer: 5’ – GTAGTCGACAGATCTTGAACTCCTAGACCAGGCTAACC – 3’, reverse primer: 5’ – GTCGAATTCAAGTTCTGGGACTCAGTTC – 3’, 3.1 kb 3’ homology arm (Figure 5.1D), forward primer: 5’ – AGAGGATCCCAGAACTTTGAAAAGTCATGG – 3’, reverse primer: 5’ – ATAGGATCCTGTCAGGGGATTACGCGTTTTG – 3’. These long distance PCR primers were designed to contain various embedded restriction enzyme sites to allow for subsequent directional cloning into the targeting vector. PCR products were electrophoresed on a 0.8% ethidium bromide-stained agarose gel, and isolated by gel extraction using the Qiagen Gel Extraction kit, following the manufacturer’s protocol. Scanned images were quantified and evaluated using Quantity One® Software (Bio-Rad Laboratories, Hercules, CA, USA).

5.3.3 Cell Culture

R1 embryonic stem (ES) cells, derived from mouse strain 129/sv x 129/sv-cp (Nagy et al., 1993) were grown on a feeder layer of mouse fibroblasts and maintained in Dulbecco’s modified Eagle’s medium high glucose (Gibco, Grand island NY, USA), supplemented with 15% ES cell qualified fetal bovine serum (FBS) (Gibco), and Leukemia Inhibitory Factor (LIF) (Chemicon/Millipore, Billerica, MA, USA), and made complete with GlutaMAX™ (Gibco), MEM Non-essential amino acids (Gibco), sodium pyruvate (Gibco), Penicillin/Streptomycin (Gibco), and 2-mercaptoethanol (Sigma Aldrich, Oakville, ON, Canada). Prior to nucleofection, R1 ES cells were transferred to culture on 0.1% gelatin-
coated plates. R1 ES cells (2 x 10^6) suspended in pre-warmed Mouse ES cell Nucleofector® solution (Amaxa Biosystems, Walkersville, MD, USA) were combined with 10 µg linear fli-1 targeting vector, transferred to a cuvette, and nucleofected using the Amaxa Nucleofector® device (Amaxa Biosystems) set to program A-23. Nucleofected cells were simultaneously treated with G418 (200 µg/ml) (Sigma Aldrich) and ganciclovir (2 µM) (Sigma Aldrich) for positive and negative selection, respectively. Drug-resistant ES cell clones were expanded and screened for homologous recombination by southern blot analysis of their genomic DNA.

5.3.4 Southern blot screening

Genomic DNA from ES cell clones was prepared in 96-well plates by cell lysis with lysis buffer (10 mM Tris-HCl pH7.5, 10 mM EDTA, 10 mM NaCl, 0.5% sarcosyl) and proteinase K, and DNA precipitation using cold NaCl/ethanol (150 µl of 5 M NaCl per 10 ml cold 100% ethanol) prepared fresh. Genomic DNA was digested with EcoRI (New England Biolabs), electrophoresed on a 0.8% agarose gel, blotted onto a nitrocellulose membrane (Zeta-Probe; Bio-Rad Laboratories, Hercules, CA, USA) in 0.4 M NaOH, and UV cross-linked. Hybridization was performed using radioactively labeled [α-^32P] dCTP (Perkin Elmer, Waltham, MA, USA) probe (2 x 10^6 cpm/ml), incubated overnight, washed and exposed to film. The Neo probe was generated by amplifying an 800 bp fragment from the fli-1 targeting vector using standard PCR methods (forward primer: 5′ – AGACAATCGGCTGCTCTGAT – 3′; reverse primer: 5′ – ATGGGTCACGACGAGATCAT – 3′). Similarly, the fli-1 genomic probe was generated by amplifying a 700 bp genomic fragment from the fli-1 BAC clone, using standard PCR methods (forward primer: 5′ – AGCACAGATCATCTCAACGC – 3′; reverse primer: 5′ – ATCTGATGTCGCTCGAGCATTG – 3′).
Scanned images were quantified and evaluated using Quantity One® software (Bio-Rad Laboratories).

5.4 RESULTS

5.4.1 Homologous Recombination, Cre/loxP and Flp/FRT Recombination Systems

Gene targeting, the designed genetic alteration to an endogenous sequence by a homologous recombination event, has provided a powerful method to study gene function in mammalian systems. The successful generation of gene targeted mice requires conquering two major obstacles, homologous recombination and germ line transmission. The frequency of homologous recombination is dependent upon several parameters, including the length of shared homology between the recombining sequences (Deng & Capecchi, 1992), quantity of vector DNA copies delivered to the nuclei of the targeted cells, and cell proliferation rate or cell cycle (Bollag et al., 1989). Nonetheless, gene targeting strategies have proven to be an efficient method to elucidate in vivo gene function. Recently, site-specific recombination has been combined with homologous recombination-based gene targeting strategies to create conditional genome alterations. At present, two site-specific recombination systems are being employed, the Cre/loxP and Flp/FRT system. The popular P1 phage-derived Cre recombinase is a 38 kDa protein that catalyzes efficient recombination between two loxP recognition sites, unique 34-base pair consensus sequences (Sauer & Henderson, 1988), (Nagy, 2000). The yeast-derived Flip (Flp) recombinase is a protein that catalyzes recombination between two FRT recognition sites (Dymecki, 1996). Recombination recognition sequences are inserted into introns, so as not to disrupt sequences that function in splicing and transcription, and flank essential coding
exons. Therefore the conditional targeted allele retains wild type function in the absence of Cre or Flp recombinase, but mutates to a null allele when these recombinases are expressed.

Site-specific recombination is also a very valuable application in gene targeting strategies to remove a selectable marker, such as the Neomycin (Neo) resistance cassette, from the targeted allele. Previous studies (Fiering et al., 1995) have provided evidence to support the idea that the presence of a selectable marker cassette may influence the expression of neighbouring genes, thereby potentially influencing the resultant knockout phenotype. This interference leads to interpretation of phenotype that does not accurately reflect the functional role of the targeted gene of interest. Therefore, in order to avoid such misinterpretation, the selectable marker is removed with recombinase in ES cell culture. Three loxP sites can be inserted in the targeting vector, where two loxP sites flank the selectable marker cassette, located downstream the loxP site placed 5' of the essential coding exon (Nagy, 2000). An alternative strategy to the triple loxP approach is the use of a second recombinase system, Flp, to remove the selectable marker. The advantage of this strategy is that it circumvents the problem of having to screen for the correct or desired partial recombination event, specifically, when the essential exon remains and the selectable cassette is excised. Instead, this approach only necessitates the screening of the presence or absence of Neomycin (Neo) resistance (Nagy, 2000). Additionally, a recently developed enhanced Flp recombinase, Flpe, assures site-specific recombination efficiency similar to that of the Cre/loxP system (Buchholz et al., 1998). Accordingly, both recombination systems have been included into the design and construction of the fli-1 gene-targeting vector.

The proper insertion of loxP sites around a functionally active component of the gene of interest is critical to achieve a null allele upon Cre excision. Ideally, flanking the
entire gene with loxP sites would be the safest strategy to ensure that no functional protein is produced. However, this strategy, in most cases, is technically impossible. A typical strategy involves the selection of an essential coding exon or translational start to be floxed (Nagy, 2000). Both of the previous fli-1 knockout studies designed their targeting vectors to remove the CTA or Ets domain located within exon 9, which is required for transcriptional activation. These studies report that the removal of this domain results in efficient loss of Fli-1 function (Hart et al., 2000), (Spyropoulos et al., 2000). Therefore the fli-1 conditional targeting vector was designed accordingly. Two loxP sites were inserted within non-coding sequences to flank the essential coding exon, 9, containing the CTA and Ets domains. This disruption results in minimal change that leaves the targeted fli-1 gene completely functional. Therefore mice homozygous for the floxed or targeted allele will have completely normal gene function outside of Cre expression.

5.4.2 Design and Construction of a fli-1 Conditional Targeting Vector

In order to disrupt the fli-1 gene and create a null allele, traditional cloning techniques have been utilized to generate a fli-1 conditional gene targeting construct. The targeting vector contains 5’ and 3’ homology arms required for homologous recombination, and a floxed exon 9 (Ets domain) to direct Cre recombinase-mediated excision of the critical exon. Additionally, the herpes simplex virus thymidine kinase gene (HSV-TK) and an FRT-flanked selectable Neo cassette, containing the Neo resistance gene and polyadenylation signal under the transcriptional control of the phosphoglycerate kinase (PGK) promoter, were utilized to allow for the identification of negative and positive ES cell clones respectively (Figure 5.1A). These selectable cassettes were obtained from cloning vectors kindly supplied by Dr. M. Puri (University of Toronto, Toronto Canada).
Figure 5.1 – Generation of a fli-1 conditional targeting vector. (A) Schematic diagram of the fli-1 conditional targeting vector containing a floxed exon 9, an FRT flanked Neo resistance cassette, and the thymidine kinase gene for positive and negative selection, respectively. Long distance PCR was used to amplify BAC sequences to generate the (B) 5’ arm of 3.9 kb, (C) exon 9 fragment of 1.5 kb, and (D) 3’ arm of 3.1 kb in length, that were subsequently cloned into the targeting vector.

Successful homologous recombination is partially dependent upon the degree and length of sequence homology. Several manuscripts (Hasty et al., 1991), (Thomas et al., 1992), (te Riele et al., 1992) have provided evidence to suggest that efficient and accurate gene targeting can be ensured by a targeting construct containing at least 1 kb of targeted homology flanking any embedded heterology. To ensure sequence homology, fli-1 genomic
DNA used to clone the *fli-1* conditional targeting vector was isolated from the RPC1-22 BAC library (Genome Resource Facility, The Hospital for Sick Children, Toronto, Canada), which was screened for the presence of exons 5 through 9 by Southern blot analysis. This BAC library, derived from the 129S6/SvEvTac mouse strain, is of the same strain as the R1 ES cells used for gene targeting. The *fli-1* conditional targeting vector contains a 3.9 kb 5’ homology arm (Figure 5.1B), comprised of exons 7 and 8, and introns 6, 7 and 8. As well as a 3.1 kb 3’ homology arm (Figure 5.1D), comprised of the 3’ UTR, to ensure the occurrence of efficient homologous recombination between the wildtype and targeted Fli-1 allele.

Several mini-targeting vectors were generated for each of the homology arms and critical floxed exon (refer to Figure 5.2 for a schematic diagram of the cloning strategy). All vectors were sequenced at each point of ligation to ensure sequence integrity. These vectors were digested and ligated together to generate the final *fli-1* targeting vector, of approximately 16 kb in total length (Figure 5.2). Finally, the entire *fli-1* conditional targeting vector was sequenced to ensure proper insertion of functional recombination sites, complete homology with published sequences and that no unintended sequence alterations were introduced.
Figure 5.2 - Schematic diagram outlining the cloning strategy for the generation of the \textit{fli-1} conditional gene targeting vector. Several “mini” gene targeting constructs were cloned and subsequently combined to generate the final targeting vector. Position of the \textit{KpnI} site, used to linearize the vector prior to electroporation, is indicated.

5.4.3 Generation and Identification of ES cells carrying the targeted Fli-1 allele

The \textit{fli-1} targeting vector was linearized outside of the arms of homology by digestion with \textit{KpnI}. The \textit{KpnI} recognition site is a single and unique sequence for the restriction enzyme that does not exist within the \textit{fli-1} genomic DNA of the targeting vector. Following enzyme digestion, the linearized targeting vector was purified by phenol
chloroform extraction and electroporated into R1 ES cells (kindly provided by Dr. M Puri) using the AMAXA nucleofection protocol. Recall that a homologous recombination event is sensitive to the amount of DNA delivered into the nuclei of each cell. However, a high number of DNA copies can increase the frequency of non-homologous recombination. The advantage of the AMAXA protocol, is that it significantly reduces the quantity of DNA required for efficient delivery, and thus may also lower the probability of random integration events. Electroporated R1 ES cells were grown on G418 (neomycin)-resistant mouse embryonic fibroblasts (kindly provided by Dr. M Puri), mitotically inactivated by irradiation, and ES cell clones were screened by their ability to grow in medium containing G418 and ganciclovir for positive and negative selection, respectively. Drug-resistant ES cell clones were plated in duplicate and expanded. Recombined and targeted transformants were identified by Southern blot analysis of genomic DNA from the expanded ES cell clones. Genomic DNA was digested with EcoRI, and Southern blots were hybridized with a 5' fli-1 genomic probe (containing sequences upstream the 5' homology arm) or Neo probe (containing sequences within the Neo cassette) (Figure 5.3). These probes will not hybridize to the fli-1 conditional targeting vector DNA. Hybridization with the 5' fli-1 genomic probe and Neo probe provide two distinct hybridization patterns detecting the wildtype and targeted fli-1 alleles (Figure 5.3). The change in hybridization pattern results from the introduction of an additional EcoRI site, inserted upstream the 5’ loxP site adjacent to exon 9, in the course of the cloning strategy (Figure 5.3). Therefore, in the case of the 5’ flanking genomic probe, non-targeted ES cell clones will be identified by the presence of a single hybridizing band of 16.5 kb, representing the expected wild type allele fragment. ES cell clones heterozygous for the targeted alleles will be identified by the presence of two bands, with an additional hybridizing band of 10.6 kb (Figure 5.3, and Figure 5.4A). In the case of the Neo probe, clones heterozygous for the targeted disruption will be identified by the presence of a single hybridizing band of 6.5 kb. Non-
targeted clones may not display hybridizing bands or hybridizing bands may be of random sizes, since multiple random insertions may occur (Figure 5.3 and Figure 5.4B). Figure 5.4 shows representative data for the analysis of the R1 ES cell clones that were screened by this method.

Figure 5.3 – Targeted disruption of murine fli-1. Schematic representation of the fli-1 conditional gene targeting vector, genomic organization of the wild type, targeted, and recombined fli-1 alleles. Positions of the 5' flanking and Neo probes, diagnostic EcoRI sites and predicted fragment sizes are indicated for each allele.
Figure 5.4 – Southern blot analysis for the detection of positively recombined ES cell clones. Representative data of R1 ES cell clone genomic DNA digested with EcoRI and subjected to Southern blot using the 5’ flanking probe. Wild type fli-1 alleles are detected by the presence of a single 16.5 kb band, and the recombined allele, by the presence of both the wild type and 10.6 kb band (A). Representative data of R1 ES cell clone genomic DNA digested with EcoRI and subjected to Southern blot using the Neo probe. Correctly recombined alleles are detected by the presence of a 6.1 kb band. Non-homologous recombination events are identified by the presence of random band sizes (B). Possible recombined clones of approximately 6.1 kb were analyzed a second time using the wildtype 5’ probe to verify a correct recombination event.
The sequences of the neomycin phosphotransferase gene, encoding Neo resistance, and its PGK promoter, can have inadvertent consequences on the targeted gene or flanking genes if they are not removed from the targeted allele (Meyers et al., 1998), (Nagy et al., 1998), (Ren et al., 2002). In order to keep the targeted fli-1 allele fully functional, the conditional targeting vector was designed with FRT recognition sequences flanking the Neo positive selection cassette. Following identification of positively recombined ES cell clones, the Neo cassette can be removed by transient expression of yeast-derived Flp DNA recombinase (Dymecki, 1996), leaving behind two loxP recognition sites flanking the critical exon, exon 9. Efficient Flp-mediated excision of the Neo positive selection cassette will render the ES cells sensitive to the presence of neomycin or G418. Therefore, targeted ES cell clones grown on replica plates in medium with and without G418 can be screened for the recombination event resulting in the deletion of the Neo cassette.

Correctly recombined targeted ES cell clones will be used in morula aggregation to generate chimeric mice identifiable by agouti coloured coats. Male chimeric mice will be mated with wildtype females to generate heterozygotes and Southern blot analysis will be used to confirm germ line transmission of the targeted allele to successive progeny (Figure 5.3). Heterozygous offspring will be intercrossed to generate homozygous mice. Mice homozygous for the targeted fli-1 allele should display a normal or wildtype phenotype since the floxed Ets domain results in a minimal change that leaves the gene completely functional. The normal phenotype of these mice also ensures that the targeting strategy has not interfered with the regulation and expression of a neighbouring gene, hence mice homozygous for the floxed or modified allele should have completely normal gene function outside of Cre expression. Mice homozygous for the targeted fli-1 allele can be mated with an inducible Cre transgenic mouse line allowing for spatial temporal regulation of the targeted fli-1 gene.
5.5 DISCUSSION

The analyses of genetic alterations using embryonic stem (ES) cell-mediated gene targeting technology has provided a means to gain a better understanding of essential gene function that underlie normal development and disease mechanisms. Site-specific DNA recombination is a critical and efficient tool that has been combined with homologous recombination-based gene targeting approaches to provide the possibility of conditional genome alterations. This strategy has become useful since mutations in numerous genes have shown to cause embryonic lethality, consequently obscuring the study and analysis of potential later gene functions. Additionally, site-specific recombinases can be expressed under the control of temporal and/or tissue specific promoters in order to direct the expression of mutant proteins during particular developmental periods and cell lineages of interest. Hence, the combination of such strategies has allowed for the generation of conditional transgenesis or gene knockouts that permit the analyses of in vivo gene function.

Ets proteins function as both activators and repressors of transcription and play an intricate role in hematopoiesis and angiogenesis and invariably transformation. Fli-1 plays a critical role in hematopoiesis, vasculogenesis and immune function, and its expression is vital for early embryonic development (Hart et al., 2000), (Spyropoulos et al., 2000). Mice carrying a targeted disruption at the fli-1 locus display abnormal hematopoiesis and vasculogenesis at embryonic day 11.0 (E11.0), a time that comprises a critical transition period when the site of hematopoiesis changes from the yolk sac or aorta-gonad-mesonephros (AGM) region to the liver, and die around E12.0-12.5 (Hart et al., 2000), (Spyropoulos et al., 2000), prior to the beginning of definitive lymphopoiesis. The phenotypes of mice carrying a fli-1 targeted deletion, although they display blood and endothelial defects, have precluded the assumption that fli-1 is essential for late-stage
hematopoietic development, specifically of the myeloid and lymphoid lineages. Therefore the goal of this project was to generate a fli-1 conditional knockout mouse, necessary to bypass embryonic lethality and provide our laboratory with a powerful tool to study the in vivo effects of fli-1 deletion beyond midgestation. A cloning strategy, utilizing BAC genomic DNA fragments was employed to generate a fli-1 conditional gene targeting vector. The latest ES cell electroporation technique, using the AMAXA nucleofection protocol, led us to pick ES cell colonies that were screened using Southern blot. However, a properly recombined ES cell clone was not identified.

A major limitation of this gene targeting approach is the low proportion of homologous recombination events, compared to random integration of electroporated targeting vector DNA. Therefore random integration is one of the major barriers to successful gene targeting. Although it has been over twenty years since the first mutant mice have been generated by gene targeting (Doetschman et al., 1987), (Thomas & Capecchi, 1987), the parameters that affect the frequency of homologous recombination and germ line transmission are not well-known. Nonetheless, several criteria can be met to ensure success according to the parameters that are known. Efficient and accurate gene targeting is partially dependent upon the degree of sequence homology, and the fli-1 targeting vector was designed to contain at least 1 kb of targeted homology (Hasty et al., 1991), (Thomas et al., 1992), (te Riele et al., 1992). However, it is plausible that these homology arms, amplified by long distance PCR of BAC DNA, contain sequence errors. Since, a 0.5% sequence divergence has been reported to result in a 20-fold decrease in gene targeting frequency (te Riele et al., 1992), errors likely present within these large homology arms may serve to reduce the rate of successful targeting.

ES cell screens using Southern blot resulted in the detection of numerous random, or non-homologous recombination events. Such substantial numbers may indicate the
inefficiency of the thymidine kinase gene for negative selection of recombination events. Additionally, the use of ganciclovir for negative selection can induce DNA mutations, therefore use of another negative selection cassette, such as that of the diphtheria toxin fragment A (DTA), may help to circumvent this issue in the future.

Since the beginning of this project, several manuscripts have been published describing studies that have provided further insight into the role of Fli-1 in hematopoiesis and vasculogenesis (Zhang et al., 1995), (Masuya et al., 2005), (Liu et al., 2008), (Asano et al., 2010). The first, published by Masuya and colleagues (Masuya et al., 2005), utilized morula-stage aggregation to generate fli-1 chimeric mice in order to rescue the embryonic lethal phenotype of the fli-1 knockout. Chimeras were generated by aggregation of Ly-5.2 fli-1 mutant heterozygote embryos with Ly-5.1 fli-1 wild-type embryos. Comparison of lineage expression by Ly-5.1 and Ly-5.2 cell populations revealed that chimeric mice display a significant reduction in the number of granulocytes, neutrophils, monocytes and early erythroid progenitors as well as an increase in the number of natural killer cells and granulocyte/macrophage progenitors. Consistent with the hematopoietic cell phenotype, bone marrow cells lacking Fli-1 expression display altered expression of several key hematopoietic and lineage-specific genes. These include reduced expression of megakaryocytic genes gata-1 and c-mpl, myeloid-specific genes C/EBPα and C/EBPε, granulocyte-specific genes G-CSFR, and GM-CSFRα/β1, as well as tal1, important for the development of both primitive erythropoiesis and definitive hematopoiesis. Clonal bone marrow cell culture studies also revealed a significant increase in the number of primitive erythroid progenitors. Taken together, this study suggests that Fli-1 transcriptional regulation plays a role in both myeloid and erythroid proliferation and differentiation (Masuya et al., 2005).
Fli-1 is expressed in hematopoietic cells, including lymphocyte subsets, and increasing evidence suggests that modulation of Fli-1 expression impacts lymphocyte development and function. Fli-1 transgenic mice develop a severe autoimmune disease, closely resembling lupus. A two-fold overexpression of Fli-1 results in increased lymphopoiesis, B cell survival and immunoglobulin production, accumulation of abnormal T and B cells, and a progressive immune complex-mediated renal disease, ultimately resulting in death (Zhang et al., 1995). Aberrant Fli-1 regulation is associated with the progression of various leukemias and autoimmune disorders in humans, suggesting that Fli-1 may act as an important mediator in lymphocyte transformation or dysfunction. Mice carrying a targeted deletion of the fli-1 locus display abnormal hematopoiesis, and die prior to the beginning of definitive lymphopoiesis (Hart et al., 2000), (Spyropoulos et al., 2000), therefore precluding the assumption that fli-1 is essential for lymphoid development. In order to assess the role of Fli-1 in lymphocyte differentiation and function, mice carrying a targeted disruption of the Fli-1 protein were generated, lacking only the CTA domain (Zhang et al., 2008). As mentioned above, previously generated fli-1 knockout mice express a truncated form of Fli-1, whereby both the 3’ ETS and CTA domains within exon 9 were deleted (Hart et al., 2000), (Spyropoulos et al., 2000). Homozygous mutant mice, lacking the CTA domain alone, display a 40-50 percent reduction in Fli-1 transcriptional activation activity, and express a stable truncated Fli-1 protein in splenocytes, thymocytes and isolated splenic B cells. Fli-1 CTA deficient mice exhibit a significant reduction in the number of B cells within the peripheral blood, pre-B and immature B cells in the bone marrow, follicular B cells in the spleen, as well as an increase in the number of transitional and marginal zone B cells. Moreover, B cells isolated from these mice display a reduced proliferative capacity. The affects of fli-1 deficiency on B cell development are cell autonomous, and are associated with changes in the expression of genes implicated in B cell development, including a reduction in Pax-5, E2A, and Egr-1
expression, and an increase in Id1 and Id2 expression. Fli-1 CTA deficient mice also exhibit an altered humoral immune response to immunization, with significantly lowered concentrations of serum immunoglobulin. Therefore, Fli-1 appears to play a regulatory role in both central and peripheral B cell development, and plays an influential role in the in vivo immune response (Zhang et al., 1995).

Recently, fli-1 loss of function studies performed in the Xenopus and zebrafish embryos have provided conclusive evidence indicating that Fli-1 plays a vital role in both early hematopoietic and endothelial cell development (Liu et al., 2008). The induction of both primitive and definitive hematopoiesis is perturbed in the absence of Fli-1 expression. Fli-1 deficiency is associated with reduced expression of tal1, lmo2, flk1, mpo, spi-1B and runx1, and the eventual loss of the hematopoietic stem and endothelial cell progenitor population by apoptosis. Moreover, bone morphogenic protein (Bmp) and Cloche, important regulators of blood and endothelium, were shown to induce or regulate the expression of Fli-1, depending on the origin of the progenitor population. This study has finally permitted the conclusion that Fli-1 acts at the top of the transcriptional network as a master regulator of blood and endothelial cell development and Fli-1 function is indispensable in Xenopus and zebrafish embryos (Liu et al., 2008).

The vascular defects exhibited by previous fli-1 knockout mice (Hart et al., 2000), (Spyropoulos et al., 2000), the ubiquitous expression of Fli-1 in all endothelial cells (Hewett et al., 2001), the regulation of extracellular matrix genes and the inhibition of collagen biosynthesis (Czuwara-Ladykowska et al., 2001), (Kubo et al., 2003), (Jinnin et al., 2005), (Asano et al., 2009), all suggest a role for Fli-1 in endothelial cell fate and angiogenesis. Accordingly, a recent publication from Asano et al. (Asano et al., 2010), has further characterized the role of this Ets transcription factor in the vasculature by generating mice carrying a conditional targeted fli-1 deletion in endothelial cells. In this
study, *loxP* sites were placed flanking exons 3 and 4, the location of the 5’ Ets domain. Mice homozygous for the *fli-1* floxed allele were crossed with mice expressing Cre recombinase under the control of the Tie2, endothelial-specific promoter. Cre-mediated excision resulted in approximately a 50 percent reduction in Fli-1 protein levels (Asano et al., 2010). Consistent with the phenotype of previous *fli-1* knockouts displaying a loss in vascular integrity (Hart et al., 2000), (Spyropoulos et al., 2000), these *fli-1* conditional knockout mice display abnormal skin vasculature, markedly reduced vessel integrity and increased permeability. This phenotype is associated with altered expression of genes involved in the maintenance of blood vessel integrity, governing endothelial cell interactions, and basement membrane remodeling, such as VE-cadherin, PECAM-1, MMP-9, PDGFB, and S1P<sub>1</sub> receptor (Asano et al., 2010). Thus, the recently generated Fli-1 conditional knockout mouse has provided additional insight into the critical role of Fli-1 in the regulation of vascular homeostasis, as well as the control of vessel maturation and stabilization.

Gene targeting and the ability to generate gene knockouts has provided a powerful and successful research tool for biologists to decipher gene function. Over the past twenty years, almost 4000 targeted gene knockout mice have been generated using a variety of approaches (MGI; [http://www.informatics.jax.org](http://www.informatics.jax.org)), although less than one quarter of these knockouts are publicly available to researchers from a repository. The International Knockout Mouse Consortium (IKMC) was created to provide a standardized public resource for a collection of mouse knockouts to accelerate the understanding of gene function and of human health and disease, while reducing expenses and saving time (Austin et al., 2004), (Auwerx et al., 2004). The IKMC includes the collaboration of multiple programs, including the European Conditional Mouse Mutagenesis Program (EUCOMM), North American Conditional Mouse Mutagenesis Project (NorCOMM), Texas A&M Institute for Genomic Medicine (TIGM), and the Knockout Mouse Project (KOMP), which includes
the VelociGene division of Regeneron Pharmaceuticals, and a consortium (CSD) consisting of the Children’s Hospital Oakland Research Institute, the Wellcome Trust Sanger Institute and the University of California at Davis. Searching the IKMC database (http://www.knockoutmouse.org/search) several attempts have been made to generate a fli-1 conditional knockout mouse. Both the CSD and Regeneron have completed fli-1 targeting vectors, Regeneron also has ES cell colonies picked. The CSD has used a targeted-trapping approach, whereby an essential exon is flanked by loxP sites and an FRT flanked reporter cassette containing a promoterless β-galactosidase reporter gene, neomycin-resistance gene, and polyadenylation signal, is located immediately upstream the 5’ loxP site. Expression of Cre recombinase alone results in deletion of the floxed exon, creating a lacZ-tagged null allele. The combination of Flp and Cre recombinase expression allows either to restore wildtype expression by removal of the reporter cassette or to delete the essential exon. This approach can also be used in conjunction with the temporal or tissue-specific Cre transgenic mice. Conversely, Regeneron has developed a gene targeting strategy that does not depend on gene structure or exons deemed critical for proper gene function. The VelociGene’s null allele targeting approach utilizes BacVectors, extremely large gene targeting vectors containing an average of more than 100 kb of sequence homology. Through BAC recombineering strategies (Copeland et al., 2001), null mutant alleles are generated by deleting all, or a large proportion, of the coding sequence in a gene after inserting an expression-selection cassette. This cassette contains the β-galactosidase reporter gene and polyadenylation signal driven by the targeted gene’s endogenous promoter, followed by a floxed neomycin or hygromycin resistance gene and polyadenylation signal driven by the promoter from the human ubiquitin C gene. Taken together, the above-mentioned strategies, in combination with the implementation of robotics and high-throughput ES cell screening (Valenzuela et al., 2003), allow for the rapid construction of targeting vectors and generation of gene knockouts for the entire
mouse genome. The IKMC has provided researchers with an invaluable tool for elucidating the function of thousands of genes. Currently, it appears unadvisable for individual research groups to focus on the generation of gene knockouts; instead laboratories ought to focus on specialized phenotyping and gene expression analysis of the mice generated by the IKMC.
Chapter 6
General Discussion

6.1 DISCUSSION

Normal hematopoiesis is a complex and dynamic process of development, where proliferation and differentiation are intimately linked cellular events. The differentiation of the pluripotent hematopoietic stem cell into various types of functional blood cells is accompanied by a gradual restriction in differentiation potential, as well as a decrease in the capacity of the cell to proliferate. The balance between proliferation and differentiation is a tightly regulated cellular process, however the intrinsic control systems that exist to maintain this balance occasionally fail, leading to cellular transformation.

It is well known that human malignancies develop as a result of mutational events in oncogenes and tumor suppressor genes; this is not surprising as their gene products play key roles in the signaling pathways that govern cellular proliferation, differentiation and apoptosis. At the nuclear level, these imperative processes are controlled by transcription factors, and hence they are often considered to have proto-oncogenic potential. Disruption in the homeostatic balance that exists between proliferation and differentiation ultimately results in aberration of cellular behaviour, and is often due to altered expression of transcription factors. It has become increasingly evident that the timing, levels and ratio of altered expression, not simply of the transcription factor, but also of its target genes, modulates the fate of the cell and results in malignancy. As such, understanding the full spectrum of transcription factor function and the events leading to deregulation of cellular proliferation and differentiation is crucial in elucidating the underlying mechanisms of hematopoietic malignancy, as well as providing indications for potential therapeutic targets.
Transcription factors aberrantly regulated in leukemia often play an influential role in the development of hematopoietic cells, other than that of malignancy. Many of the genes proven to be indispensable through the course of hematopoiesis contain binding sites for the Ets family of transcription factors (Gottgens et al., 2002), (Swiers et al., 2006). A growing number of transgenic and knockout studies have shown that deregulated expression of numerous Ets transcription factors results in dramatic hematopoietic defects and underscore the importance of their expression during embryonic development (Moreau-Gachelin et al., 1996), (Hart et al., 2000), (Spyropoulos et al., 2000), (Vlaeminck-Guillen et al., 2000), (Masuya et al., 2005), (Zhang et al., 2008). The graded expression of Ets gene family members in various hematopoietic precursors and mature lineage-committed cell types indicate an integral role for these transcription factors in self-renewal and lineage commitment from the early stages of development and through to adulthood. Accordingly, deregulated Ets transcription factor function can lead to malignancy, and many Ets proteins are associated with various hematopoietic disorders and malignancies (Nunn et al., 1983), (Moreau-Gachelin et al., 1988), (Georgiou et al., 1996), (Lopez et al., 1999), (Mueller et al., 2002), (Mhawech-Fauceglia et al., 2006).

Studies involving retroviral-induced neoplasia have contributed to our understanding of transcription factor function and have been instrumental in the identification of numerous tumor suppressor and oncogenes, as well as the characterization of normal and malignant functions. The proto-oncogene and founding member of the Ets family, ets-1, was originally discovered as the cellular gene overexpressed as a result of the E26 avian viral fusion protein causing acute erythroid-myeloid and lymphoid leukemia (Leprince et al., 1983), (Nunn et al., 1983). Erythroleukemia, induced by the Friend virus, is a well-studied example of multi-stage malignancy in mice and is one of the best animal models to study the onset and progression of hematological malignancies. Discovered in 1957 by Dr. Charlotte Friend,
Friend disease is a rapidly progressive retroviral erythroleukemia characterized by the expansion of erythroid progenitors, splenomegaly, and death (Friend, 1957). The versatile model system of Friend virus-induced erythroleukemia has served as a valuable research tool for the molecular analysis of erythroid differentiation and erythroid signal transduction. Over the past two decades, the study of Friend Disease has led to the identification of several oncogenes and tumor suppressor genes including \textit{fli-1}, \textit{spi-1}/\textit{PU.1}, \textit{p53}, \textit{fli-2}/\textit{p45 NFE2}, and \textit{fli-3}/\textit{miR-17-92} whose functions have been shown to play important roles in hematopoiesis, and malignant transformation in mice and humans (Delattre et al., 1992), (Li et al., 2001), (Mueller et al., 2002), (Oren, 2003), (Mhawech-Fauceglia et al., 2006), (Cui et al., 2007).

Remarkably, Friend virus itself is able to influence the differentiation and self-renewal of erythroid progenitors through the expression of the envelope protein gp55. Gp55 is capable of binding directly to the EpoR, thereby activating downstream signaling pathways in the absence of the natural ligand. Thus, irrespective of the levels of Epo concentration, the binding of gp55 to the EpoR leads to uncontrolled cell division (Berger et al., 1985), (Hoatlin et al., 1990), (Li et al., 1990). However, fully leukemic transformation requires genetic changes, that is, activation of the proto-oncogenes, \textit{spi-1}/\textit{PU.1} (Moreau-Gachelin et al., 1988), (Gobel et al., 1990) or \textit{fli-1} (Ben-David et al., 1990b), (Ben-David et al., 1991). The Friend virus complex consists of two components, a replication competent helper virus F-MuLV, and a replication defective SFFV. Injection of F-MuLV alone also induces a multistage disease in strain-specific neonates. Conversely, F-MuLV does not express the gp55 envelope protein and therefore deregulated Epo signaling solely results from genetic changes imparted by the activation of Fli-1 expression. Since the insertional activation of \textit{fli-1} is the most critical event in the induction of F-MuLV-induced erythroleukemia, a major focus in our laboratory is to study the molecular mechanisms by which this transcription factor alters the responsiveness of erythroid progenitor cells to Epo and triggers malignant transformation. Our laboratory and others have demonstrated
that Fli-1 has the capacity to alter the expression of oncogenes such as *MDM2* (Truong et al., 2005), *bcl-2* (Lesault et al., 2002) and tumor suppressor genes such as *Rb* (Tamir et al., 1999) that play a critical role in normal hematopoiesis, vasculogenesis, immune function and leukemogenesis. **Table 6.1**, summarizes the growing body of experimental evidence revealing the regulation of numerous blood and endothelial cell-specific genes that have contributed to our understanding of Fli-1 function. The major objective of this proposal is to determine the molecular mechanisms by which Fli-1 is involved in the imperative cellular processes of proliferation and differentiation.
Table 6.1 – Summary of Fli-1 transcriptional target genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcriptional Regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-2</td>
<td>positive</td>
<td>Lesault et al, 2002</td>
</tr>
<tr>
<td>C/EBPα/ε</td>
<td>positive</td>
<td>Masuya et al, 2005</td>
</tr>
<tr>
<td>c-mpl</td>
<td>positive</td>
<td>Deveaux et al, 1996; Kawada et al, 2001</td>
</tr>
<tr>
<td>c-myc</td>
<td>positive</td>
<td>Dhulipala et al, 1998</td>
</tr>
<tr>
<td>Egr-1</td>
<td>positive</td>
<td>Zhang et al, 2008</td>
</tr>
<tr>
<td>gata-1</td>
<td>negative</td>
<td>Watson et al, 1992; Seth et al, 1993</td>
</tr>
<tr>
<td>GM-CSFR</td>
<td>positive</td>
<td>Masuya et al, 2005</td>
</tr>
<tr>
<td>gpIIb</td>
<td>positive</td>
<td>Zhang et al, 1993; Wang et al, 2002</td>
</tr>
<tr>
<td>gpIb, gpIX, gpVI</td>
<td>positive</td>
<td>Eisbacher et al, 2003; Holmes et al, 2002</td>
</tr>
<tr>
<td>lmo2</td>
<td>positive</td>
<td>Liu et al, 2008</td>
</tr>
<tr>
<td>MDM2</td>
<td>positive</td>
<td>Truong et al, 2005</td>
</tr>
<tr>
<td>pax-5</td>
<td>positive</td>
<td>Zhang et al, 2008</td>
</tr>
<tr>
<td>Rb</td>
<td>negative</td>
<td>Tamir et al, 1999</td>
</tr>
<tr>
<td>runx-1</td>
<td>positive</td>
<td>Liu et al, 2008</td>
</tr>
<tr>
<td>Tal1</td>
<td>positive</td>
<td>Masuya et al, 2005; Pimanda et al, 2007</td>
</tr>
<tr>
<td>SHIP-1</td>
<td>negative</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>tie 2</td>
<td>positive</td>
<td>Dube et al, 1999; Hart et al, 2000</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>positive</td>
<td>Asano et al, 2010</td>
</tr>
</tbody>
</table>
The data presented in Chapter Two provided conclusive evidence that the acquisition of secondary mutations is not sufficient to maintain the transformed erythroid phenotype in F-MuLV-induced erythroleukemia and human erythroleukemia. The constitutive suppression of Fli-1 using RNAi and dominant negative studies leads to a marked growth inhibition and apoptotic cell death of several erythroleukemic cell lines. These results have provided evidence to suggest that continuous Fli-1 expression is essential for the continued proliferation and survival of malignant erythroid progenitors. This study has also provided clues to the function of Fli-1 in the block of erythroid differentiation, since downregulation of Fli-1 provided the ability, albeit limited, of erythroid progenitor cells to undergo maturation upon stimulation with cytokines. We observed that Fli-1 downregulation reverses the inhibitory effect on erythroid differentiation, thereby allowing for successive erythroid maturation, as marked by increased expression of a globin gene, in response to treatment with Epo. It is clear that Fli-1 plays a role in erythroid differentiation and that deregulated Fli-1 expression alters the Epo signaling cascade, however the precise manner by which this transcription factor modulates Epo signal transduction pathways to enhance proliferation remains unclear. Further investigation into this role for Fli-1 was described in Chapters Three and Four.

The ability to induce rapid apoptosis in erythroleukemic cell lines simply through the suppression of Fli-1 was interesting, however not predicted. The concurrent downregulation of Bcl-2 upon inhibition of Fli-1 activity may explain, at least in part, the induction of apoptotic cell death. It has been previously shown that transcription of the anti-apoptotic gene, bcl-2, is directly regulated by Fli-1 (Lesault et al., 2002). However it is likely that additional factors contribute to this described phenomenon, whether it be a direct or indirect consequence of transcriptional regulation. Additional studies aimed at the identification of novel Fli-1 target genes would be valuable in identifying the mechanisms by which Fli-1 dysregulation is critical for survival and provide a definitive
role for Fli-1 in the protection against programmed cell death in erythroid cells. Future studies involving expression profiling with cDNA microarray strategies could possibly lead to the identification of genes, other than bcl-2, involved in erythroid survival signaling that are regulated by Fli-1.

Fli-1, like all transcription factors, exerts its effects through the transcriptional regulation of target genes. Direct interaction of Fli-1 with Ets domains present within the gene promoters may activate or repress gene transcription. Therefore, the actions of Fli-1 may be revealed through the identification of target genes and provide a better understanding of the pathways affected by Fli-1 aberrant regulation. Overexpression of Fli-1 is able to disrupt the responsiveness of erythroid progenitors to the presence of Epo through a yet undefined mechanism. Downstream signaling molecules involved in the Epo and SCF signaling pathways are obvious candidates. Indeed, the experiments presented within Chapter Three identified SHIP-1 as a direct target gene of Fli-1. Epo-independent proliferation and enhanced survival of erythroblasts is associated with phosphorylation of the tyrosine kinase Jak2 and activation of PI3-K, MAPK and Shc/Ras pathways (Zochodne et al., 2000), (Ghaffari et al., 2003). Deregulated SHIP-1 expression has an implication in the progression of erythroleukemia since it connects the cytoplasmic region of the EpoR to the Shc/Ras pathway (Zochodne et al., 2000), and is involved in the regulation of MAPK and Akt (Mason et al., 2000). Recently, our laboratory has shown that the loss of SHIP-1 results in the accelerated progression of F-MuLV-induced erythroleukemia (Lakhanpal et al., 2010). Therefore the negative regulation of SHIP-1 by Fli-1 provides a selective advantage to erythroleukemia development, and serves as a prime example of how altered expression of Fli-1 target genes leads to alteration of the EpoR signaling cascade.

The absence of SHIP-1 expression in F-MuLV-induced erythroleukemic cell lines and the presence of SHIP-1 expression in SFFV-induced erythroleukemic cells lines
prompted us to investigate the relationship between Spi-1/PU.1 and SHIP-1. Fli-1 and Spi-1/PU.1 recognize the same core Ets DNA binding domain, thus it has been postulated that erythroid transformation associated with the activation of both Fli-1 and Spi-1/PU.1 is mediated through the transcriptional regulation of a common set of target genes directing erythroid differentiation and proliferation. Interestingly, Chapter Three also describes the binding of Spi-1/PU.1 to the same conserved Ets domain within the SHIP-1 promoter, however this interaction results in positive transcriptional regulation. The differential regulation of SHIP-1 expression suggests that although Fli-1 and Spi-1/PU.1 may share similar target genes, their contribution to erythroid transformation is through distinct genetic and signaling pathways.

The overexpression of Spi-1/PU.1, led to enhanced erythroleukemic proliferation, associated with increased phosphorylation of AKT, and elevated expression of MAPK, c-myc, and Jak2. The enhanced expression of these erythroid-related signaling molecules may explain the proliferative advantage provided by Spi-1/PU.1 overexpression. Further investigation of this relationship utilizing drug treatment strategies to inhibit the function of these signaling molecules, such as the Jak2 inhibitor AG490, or the PI3-K inhibitor Ly294002. The interpretation of such studies will provide a better understanding by which Spi-1/PU.1 overexpression is able to alter the signaling events leading to erythroblast expansion.

Unexpectedly, enforced expression of Spi-1/PU.1 induced the transcription but did not affect the protein levels of SHIP-1. This data implies the occurrence of SHIP-1 post-transcriptional regulation. We observed that Spi-1/PU.1 overexpression also resulted in elevated expression of miR-155. The induction of miR-155 expression may explain the lack of SHIP-1 protein expression since SHIP-1 is a known target of this miRNA (O'Connell et al., 2009). According to several recent publications, the positive regulation of SHIP-1 by Spi-
1/PU.1 and the connection with miR-155 may be more relevant in the lymphoid lineage and immune cell function (DeKoter & Singh, 2000), (O’Connell et al., 2008), (Leung et al., 2009). Therefore, the effects of Spi-1/PU.1 dependent regulation of specific target genes may rely upon the cell context or setting of cancer induction. Nonetheless, it would be interesting to study the effects of miR-155 overexpression on the levels of SHIP-1 and Spi-1/PU.1 and the proliferative potential of several Friend erythroleukemic cell lines. Similar to Fli-3, encoding the miR-17-92 cluster (Cui et al., 2007), elevated expression of miR-155 may provide a selective advantage and contribute to erythroleukemic transformation.

As mentioned above, it is evident that the level and timing of Fli-1 expression modulates the signaling events required for the orchestrated development of the erythroid lineage. Yet the ability of Fli-1 to influence the balance between proliferation and differentiation is undefined and requires further investigation. It is important, not only to study the effects of flt-1 gene dysregulation in a wildtype environment during normal development, but also in a malignant setting. Fli-1 aberrant regulation is vital for F-MuLV erythroleukemogenesis. Although it may not be the critical genetic event in the induction of all associated malignancies, deregulated Fli-1 expression undoubtedly contributes to pathogenesis. Consequently the goal of the study presented in Chapter Four was to increase the levels of Fli-1 expression and perturb the normal transcriptional activation ability of Fli-1 in an oncogenic setting.

Fli-1 was exogenously expressed in an SFFV-induced erythroleukemia cell line, DP17-17. DP17-17 cells, harbouring activation at the spi-1/PU.1 locus, grow in the absence of Epo, and upon transplantation in syngeneic mice generate symptoms analogous to the original disease. Histochemical staining and blinded erythroid differential counts revealed that Fli-1 overexpression leads to a remarkable shift in the state of erythroid maturation, while the ability of these cells to differentiate in the presence of DMSO is unaffected. The
ability of Fli-1 to influence hematopoietic progenitor activity was also supported by the results of a colony-forming assay, whereby Fli-1 overexpression led to enhanced colony formation on methylcellulose. These colonies generally grew larger in size, possibly suggesting that although Fli-1 overexpression does not affect the overall growth rate of this cell line, it induces proliferation of a specific erythroid colony-forming cell population. Transplantation assays revealed that the resultant hematopoietic phenotype of these cells reduces their tumorigenicity in vivo, since exogenous expression of Fli-1 led to increased disease latency, and several mice injected with lower cell concentrations failed to develop disease at all. However, mice injected with the control and Fli-1 overexpressing cells similarly display disease characteristics such as overt splenomegaly and hepatomegaly. In the case of SFFV-induced erythroleukemia, increased latency may be explained by the additional timing or incubation required for Fli-1 overexpressing DP17-17 cells to successively differentiate and accumulate CFU-E proerythroblasts since the cells initially present at time of injection retained a more immature phenotype.

Once Fli-1 is activated it alters signals emanating downstream the Epo and SCF receptor. Therefore its oncogenic properties likely stem from the ability of this transcription factor to “switch on” of a gene program that is a prerequisite for specifying progenitor self-renewal and proliferation, allowing for the expansion of leukemic clones. Therefore Chapter Four presents intriguing data suggesting that Fli-1 acts as a positive regulator of hematopoietic progenitor activity by initiating changes in the gene expression profile. The potential or progression of erythroid differentiation is defined by the decision of multipotent and lineage-committed progenitors to proliferate, differentiate or undergo apoptosis, as determined through differential Epo and SCF signaling cascades. Our data suggests that perhaps the timing of Fli-1 expression is critical in determining the downstream events and sequential expression of erythroid-specific genes, thereby affecting the decision to proliferate or differentiate. Indeed earlier studies performed in
our laboratory using the Friend virus-induced erythroleukemia model have determined that normal progression of erythropoiesis, after the proerythroblast stage, requires a decrease in Fli-1 expression (Tamir et al., 1999). The constitutive activation of Fli-1 in F-MuLV-induced erythroleukemia blocks differentiation and appears to shift the effects of Epo signaling through activation of the Shc/Ras pathway resulting in proliferation (Zochodne et al., 2000). Therefore a role for Fli-1 in supporting the proliferation and self-renewal of hematopoietic progenitors would also support this notion. Accordingly, a growing body of experimental evidence also substantiates a similar role for Fli-1 (Masuya et al., 2005), (Liu et al., 2008), (Zhang et al., 2008) and recent studies performed in our laboratory using ES cell differentiation assays revealed the ability of Fli-1 overexpression to induce the expansion of erythroid and myeloid progenitor populations (unpublished data, J. Zhang 2009). Confirmation of this concept allows for a better understanding of how the deregulated expression of Fli-1 is required to sustain the proliferation and survival of both murine and human erythroleukemias.

The ectopic expression of Fli-1 also stimulated an increase in the percentage and intensity of the early cell-surface marker, c-kit. As the receptor for SCF, c-kit is expressed in erythroid progenitors and is downregulated during the progression of erythropoiesis. The prospect of c-kit as a direct target gene of Fli-1 is an exciting one. C-kit has the ability to interact with and phosphorylate the EpoR (Wu et al., 1995). This interaction induces the activation of the Shc/ras pathway promoting proliferation. Examination of the mouse promoter region of c-kit revealed eight possible Ets DNA binding sites within 1 kb of the transcriptional start. The possible direct regulation of c-kit by Fli-1 warrants further investigation. To test this hypothesis, classical methods can be used to assess whether c-kit is a direct Fli-1 target gene. Similar to the methods used to validate the relationship between Fli-1 or Spi-1/PU.1 and SHIP-1, an electrophoretic mobility shift assay (EMSA), in which radioactively labeled oligonucleotide sequences corresponding to the putative Ets
binding sites will establish the capability and binding specificity of Fli-1 on the c-kit promoter. Moreover, ChIP and luciferase assays will be useful in confirming the presence of in vivo binding interactions, and ascertain that Fli-1 acts as an activator of c-kit expression. Further elucidation of this relationship may also be provided by additional colony-forming cell assays. Splenocytes, hepatocytes and bone marrow cells isolated from transplanted mice added to methylcellulose medium, supplemented with various cytokine combinations, or in the absence of cytokines, may reveal differences on cytokine responsiveness and dependence.

The embryonic lethality of previously generated fli-1 knockouts created a necessity for additional targeting experiments to unveil the effects of Fli-1 downregulation beyond midgestation in late-stage hematopoietic development. Chapter Five describes the strategies utilized by our laboratory to generate a fli-1 conditional knockout mouse. Unfortunately our efforts were unsuccessful, however since the onset of this project several manuscripts have been published describing studies that have provided further insight into the role of Fli-1 in hematopoiesis and vasculogenesis (Zhang et al., 1995), (Masuya et al., 2005), (Liu et al., 2008), (Asano et al., 2010). The studies reported by Zhang XK et al. (2008) and Asano Y et al (2010) have revealed novel roles for Fli-1 in B cell development and function, and vascular maturation and homeostasis, respectively. Nonetheless, the fundamental flaw of these studies is the elucidation of Fli-1 function based solely on the deletion of a single functional domain. The Fli-1 protein contains four major functional domains, the 5’ Ets domain, the Fli-1 specific region (FLS), the 3’ Ets domain and the carboxy-terminal transcriptional activation (CTA) domain, spanning exons three to nine. Therefore the presence of Fli-1 protein retaining several functional domains may mask the genuine effects of Fli-1 deletion. Velocigene of Regeneron Pharmaceuticals has developed a gene targeting strategy that does not rely on gene structure or exons deemed critical for proper gene function. The availability of a conditional Fli-1 knockout
mouse from Velocigene is a better representation of Fli-1 deletion, resulting in a true null allele since all functional domains are absent. Currently, a fli-1 targeting vector has been successfully generated and ES colonies have been picked. Future studies utilizing the Velocigene fli-1 knockout, in conjunction with the temporal or tissue-specific Cre expression, will be useful in characterizing the phenotype of Fli-1 deletion since the graded expression and timing of Fli-1 deletion in various cell types will have different outcomes. The original discovery of Fli-1 and its involvement in Friend virus-induced erythroleukemia defined a role for this Ets transcription factor in the erythroid lineage. However subsequent investigations of Fli-1 function have revealed additional goals in other blood lineages as well as interaction and cooperation with other transcription factors. Perhaps the most interesting study would involve a cross with a mouse line capable of expressing Cre under the control of the interferon-inducible myxovirus resistance-1 (Mx-1) promoter, capable of efficient widespread Cre expression upon induction by administration of interferon or polyinosinic/polycytidic acid. Modulated levels of Fli-1 at different time points may reveal that graded expression in specific hematopoietic progenitors and timing of Fli-1 expression specify the maturation of one lineage at the expense of the other, elucidating an active and more significant role for Fli-1 in hematopoietic cell self-renewal, fate or lineage selection and specificity of development.
Concluding Remarks

Since the initial discovery of the proto-oncogene, Fli-1, by Dr. Ben-David almost two decades ago using the Friend erythroleukemia mouse model, a growing number of publications have described experimental and clinical evidence to support a role for Fli-1 in hematopoietic development and transformation. The ultimate goal of the project described herein was to establish a better understanding of both the oncogenic and developmental roles of Fli-1 by investigating the molecular basis by which its deregulated expression leads to fundamental aberration in the fine balance between proliferation and differentiation ultimately leading to transformation. We have shown that continuous Fli-1 overexpression is essential for erythroleukemic cell proliferation and survival and propose that the negative regulation of a novel target gene, SHIP-1, contributes to the malignant phenotype. Studies of Fli-1 overexpression have also shed light on the basis of Epo-independent erythroid hyperproliferation and hematopoietic progenitor activity and underscore the importance of Friend disease to uncover the molecular basis of oncogenesis. The versatile role of Fli-1 in governing the differentiation and proliferation of various blood lineages and association with various blood cancers and disorders underscore the notion that inhibition of Fli-1 function may serve as an efficient therapeutic option for the treatment of various neoplastic disorders.
References


the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis. *Molecular and Cellular Biology, 13*, 2929-2941.


Appendix 1

**Vascular Endothelial Growth Factor – A Positive and Negative Regulator of Tumor Growth**

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ABSTRACT

Over the past decade, the well-documented role of vascular endothelial growth factor (VEGF) in tumor angiogenesis has led it to become one of the leading therapeutic targets for the treatment of cancer. Emerging evidence from genetically modified animal models, however, suggests that elevated levels of VEGF, or a proangiogenic phenotype, may impede, rather than promote early tumor development and progression. For example, hypermorph VEGF transgenic mice display delayed progression of a retroviral-induced murine leukemia, and knockdown of VEGF expression within the myeloid compartment accelerates tumor progression. Several mechanisms have been proposed to explain this paradox, whereby VEGF induces changes within the hematopoietic compartment and tumor microenvironment through recruitment of tumor inhibitory monocytic cells and the negative regulation of tumor angiogenesis. Thus, it is apparent that the levels of VEGF expression in both tumor, and non-tumor tissues, as well as the timing of its modulation relative to cancer induction, play an important role in determining the effects of VEGF expression on tumorigenicity. In light of these recent findings, the various mechanisms underlying the negative role of VEGF during early tumor development, progression and metastasis will be discussed.
INTRODUCTION

A growing body of experimental and clinical evidence has emerged in the last decade to support the concept that mediators of angiogenesis, particularly vascular endothelial growth factor (VEGF), contribute to the development and progression of various malignancies and the formation of metastases. Numerous studies have reported high levels of VEGF expression in a wide variety of tumors that have been associated with poor prognosis (Schoenleber SJ et al. 2009), (Podar K et al. 2005). This evidence has led to the principle that the inhibition of VEGF can block tumor angiogenesis, remove the main nutrient supply, and thereby incapacitate tumor growth. However, evidence from recent clinical trials targeting VEGF challenges this well-accepted principle since monotherapy with anti-VEGF agents has only modestly improved overall survival of cancer patients and tumor resistance is often observed (Kerbel RS et al. 2008), (Mayer EL et al. 2007), (Hurwitz HI et al. 2006). Accordingly, recent experimental evidence from Stockmann et al. and Greenberg et al. (2008) suggests that, while anti-VEGF therapy has been modestly successful at inhibiting tumor growth, VEGF can act as a negative regulator of angiogenesis and tumor progression (Greenberg JI et al. 2008), (Stockmann C et al. 2008). This work questions the idea that maximally sustained levels of VEGF and potentially other pro-angiogenic factors are required for tumor growth (Greenberg JI et al. 2008), (Stockmann C et al. 2008). From these and other findings it is apparent that manipulation of the host towards a proangiogenic phenotype, either systemically or within the hematopoietic compartment, manifests into a protective role against tumor growth in vivo. Although evidence exists in the literature to support the idea that VEGF is a positive regulator of tumor growth, what we would like to elaborate upon here, is the concept that VEGF also acts as a negative regulator of tumor growth.
VEGF ACTS AS A NEGATIVE REGULATOR OF TUMOR PROGRESSION

Emerging evidence from genetically modified animal models suggests that elevated levels of VEGF, or a proangiogenic phenotype, may impede, rather than promote early tumor development and progression. Intriguingly, Stockmann and colleagues observed that, deleting VEGF in myeloid cells accelerates tumorigenesis in a mouse model of breast and lung cancer (Stockmann C et al. 2008), depicting the protective role of VEGF. This study suggests that VEGF, derived from innate immune cells, plays an intricate role in orchestrating angiogenesis and tumorigenesis. The study by Greenberg et al. also suggests that VEGF, upon binding to its receptor, VEGF-R2, acts as a negative regulator of angiogenesis by impeding the function of vascular smooth muscle cells (VSMCs) and pericytes through induction of a receptor complex with the platelet-derived growth factor receptor (PDGF-Rβ), leading to the loss of vessel stabilization (Greenberg JL et al. 2008). Consistent with these works, our group reported a tumor inhibitory role for VEGF by demonstrating that a two-fold overexpression of systemic levels of VEGF, in mice heterozygous for a VEGF “hypermorphic” allele, decelerates tumorigenesis in a retroviral-induced, spontaneous murine leukemia model (Cervi D et al. 2007). Alterations in the innate immune function, specifically enhanced natural killer cell activity, and increased hematopoietic progenitor cell survival, were identified as acquired phenotypes which strongly correlated with and were likely responsible for this leukemic inhibition (Cervi D et al. 2007). Similarly, in the same study, the capacity of erythropoietin (Epo) to confer deceleration of leukemia progression was also demonstrated (Cervi D et al. 2007). This correlation was not unexpected since Epo, like VEGF, is known to be a proangiogenic hormone and exhibits pleiotropic properties (Chen J et al. 2009), (Hardee ME et al. 2006), (Ribatti D et al. 1999). Recently, Nagy et al. (2009, manuscript submitted) have similarly utilized VEGF transgenic mice to demonstrate an inhibitory role for VEGF in a murine breast cancer model (Sung H-K et al. 2009). Taken together these studies have provided
evidence to support several mechanisms whereby VEGF inhibits the growth and progression of various cancer types through recruitment of tumor inhibitory monocytic cells and the negative regulation of tumor angiogenesis.

**VEGF and the recruitment of tumor inhibitory myeloid cells**

Over the recent past, the contribution of various bone marrow-derived cells, primarily from the myeloid lineage, to tumor angiogenesis has been the subject of intense investigation. In tumors, infiltrating monocytes differentiate into tumor-associated macrophages (TAMs) that are persuaded by the tumor microenvironment to secrete proangiogenic and prometastatic cytokines stimulating cell migration, invasion and metastasis (Condeelis J et al. 2006), (Murdoch C et al. 2005). Additionally, these recruited monocytes are capable of undergoing trans-differentiation into endothelial-like cells, expressing the angiopoietin receptor Tie2, that directly participate in the formation of blood vessels required for tumor growth (Schmid MC et al. 2007), (De Palma M et al. 2005). Mouse tumor models that are refractory to anti-VEGF therapy display a CD11b+/Gr1+ myeloid cell population within the tumor microenvironment (Shojaei F et al. 2007). These cells, which are associated with tumor refractoriness, carry alterations in the gene expression of both pro and antiangiogenic factors, such as downregulation of the angiogenic inhibitor, thrombospondin-1 (Shojaei F et al. 2007).

Interestingly, unlike the tumor promoting ability of the above-mentioned monocytic cells or TAMs, in a murine leukemia model it was recently identified that induction of a proangiogenic phenotype, achieved through in vivo Epo administration, results in the accumulation of a different monocytic cell population (Sca1+/c-Kit/Mac1+) capable of leukemia inhibition, in an NO-dependent manner (Usenko T et al. 2009). These tumor inhibitory monocytic cells differentiate into macrophages and dendritic cells
capable of nitric oxide (NO) production (Usenko T et al. 2009). The production of NO has been implicated in the destruction of tumor cells by macrophages (Ignarro LJ et al. 1999) as well as induction and accumulation of hypoxia inducible factor-1α (HIF-1α) (Kasuno K et al. 2004), (Metzen E et al. 2003). These findings suggest that recruitment of different subsets of myeloid-lineage cells into the tumor microenvironment leads to the acceleration or inhibition of tumor growth.

It is clear that tumor infiltrating monocytes can have separate and contradictory functions, although the mechanisms regulating the recruitment and activation of these cells in the tumor microenvironment remains to be fully understood. Indeed, several proangiogenic cytokines such as granulocyte-colony stimulating factor and monocyte chemoattractant protein-1 have been implicated in the mobilization of TAMs and myeloid cells to the tumor microenvironment (Murdoch C et al. 2005), (Shojaei F et al. 2007). However, additional studies are necessary to provide evidence of direct correlation between the activity of these chemoattractants and the recruitment of tumor infiltrating myeloid cells. Conceivably, the recruitment of monocytic cells with opposing functions is dependent upon the concentration of oxygen within the tumor microenvironment. This notion can be supported by studies reporting that TAMs, which exert proangiogenic and prometastatic phenotypes, are attracted to and accumulate under hypoxic conditions (Murdoch C et al. 2005). The state of hypoxia can be induced by the modulation of VEGF such as anti-VEGF therapy. Conversely, normoxic conditions, maintained by a proangiogenic phenotype, may explain the accumulation of tumor inhibitory myeloid cells (Cervi D et al. 2007), (Usenko T et al. 2009).

As mentioned above, we provided evidence to suggest that the tumor inhibitory role of this myeloid population is attributed, at least in part, to its ability to produce NO (Usenko T et al. 2009). In turn, this induces the activation of HIF-1α (Kasuno K et al. 2004).
and prevents the degradation of these tumor inhibitory myeloid cells (Metzen E et al. 2003). Recent studies have discovered that HIF-1α expression promotes myeloid cell survival, inflammatory and immune function (Walmsley SR et al. 2009), (Zinkernagel AS et al. 2007), potentially enhancing the antitumoral phenotype. Moreover, HIF-1α has been implicated in the regulation of growth factor induced VEGF expression as well as Epo expression (Semenza GL et al. 2001). The accumulation of HIF-1α thereby sustains the proangiogenic conditions in the tumor microenvironment, through promoting the expression of VEGF or other mediators of angiogenesis, which in turn maintains the normoxic conditions therefore leading to further recruitment of tumor inhibitory myeloid cells. The interpretation of these findings has led us to propose this intriguing positive feedback loop model, which may explain the inhibitory role of tumor associated myeloid cells recruited to the tumor microenvironment under normoxic conditions maintained by increased levels of VEGF or a proangiogenic phenotype (Figure 1).
**Figure 1 – Positive and negative regulation of tumor growth.** In an antiangiogenic phenotype, provided by anti-VEGF therapies, monocytes, capable of promoting tumor progression, are recruited to the hypoxic tumor microenvironment. This myeloid population responds to hypoxia by upregulating prometastatic and proangiogenic factors, thereby enhancing tumor progression. Conversely, in a proangiogenic phenotype, maintained by increased levels of angiogenic factors, tumor inhibitory cells are recruited to the normoxic tumor microenvironment. The tumor inhibitory role of these cells is partly attributed to their ability to produce NO. NO production leads to the accumulation of HIF-1α, which enhances immune cell function, and sustains the proangiogenic phenotype by promoting the expression of mediators of angiogenesis, such as VEGF. This, in turn, maintains the normoxic conditions and leads to further recruitment of immunoregulatory cells.
VEGF and the regulation of tumor angiogenesis

The key regulatory role of VEGF in angiogenesis that leads to suppression of early tumour growth may not arise from alterations within the hematopoietic compartment alone. Angiogenesis is dependent upon the formation of vascular sprouts, which requires the proliferation and invasion of endothelial cells and subsequent vessel stabilization that is provided by vascular smooth muscle cell (VSMC) and pericyte coverage of the newly formed vessels (Greenberg JI et al. 2008). Interestingly, Stockmann et al. (2008) reported that the cell-lineage-specific targeted deletion of VEGF in myeloid cells accelerates tumor progression in multiple subcutaneous isograft models of lung cancer and an autochthonous transgenic model of breast cancer (Stockmann C et al. 2008). In the absence of myeloid cell-derived VEGF, an atypical high-density vessel network is formed. Quantitative analysis demonstrated that these mice display decreased intratumoral blood vessel length, tortuosity, and permeability, as well as increased levels of pericyte coverage. The lack of this malignancy-associated increased vascularization facilitates more rapid tumor development. This study suggests that myeloid-derived VEGF plays a unique role in the management of the tumor “angiogenic switch”, facilitating changes in tumor vessel function and normalization, thereby indicating the ability of VEGF to act as a negative regulator of tumor progression.

In an accompanying study published simultaneously with that of Stockmann et al. (2008), Greenberg et al. (2008) presented intriguing evidence also suggesting that VEGF acts as a negative regulator of angiogenesis by disrupting the function of VSMCs and pericytes (Greenberg JI et al. 2008). Vessel stabilization and maturation is dependent upon the migration of VSMCs and pericytes to vessel walls, which is mediated by binding of platelet-derived growth factor (PDGF) to its cognate receptor, PDGF-Rβ (Greenberg JI et al. 2008). The capacity of VEGF to act as a negative regulator of angiogenesis can be
attributed to the formation of a previously undescribed VEGF-R2/PDGFRβ receptor complex resulting in the suppression of PDGF-Rβ signaling. Upon binding to its receptor, VEGF-R2, present on the surface of VSMCs and pericytes, VEGF blocks PDGF-mediated phosphorylation of PDGF-Rβ, and in turn ablates vessel stabilization (Greenberg Ji et al. 2008). The deletion of tumor cell-derived VEGF blocks the induction of this receptor complex and thereby increases pericyte coverage and tumor vessel maturation (Greenberg Ji et al. 2008). Thus, while VEGF is known to promote sprouting angiogenesis by endothelial cell activation, it can also modulate vessel integrity and/or vascular tone by interfering with accessory cell function.

CONCLUSIONS

VEGF may act as a negative regulator by initiating changes in the microenvironment, or tumor niche. Such changes include alterations in the host innate inflammatory response (Stockmann C et al. 2008), (Cervi D et al. 2007), and even the function of mesenchymal cells, such as pericytes (Greenberg Ji et al. 2008), that are crucial for orchestrating tumor growth and angiogenesis. Accordingly, Ebos and colleagues (2009) have recently demonstrated that blockade of the VEGF pathway, prior to tumor induction, leads to a more aggressive metastasis and shortened survival, possibly by the resulting establishment of a “tumor-promoting niche” (Ebos JM et al. 2009). Also in support of this notion, Xue et al. (2008) demonstrated that the function of multiple organs is systemically impaired by tumor cell-produced VEGF (Xue Y et al. 2008). This effect can be counteracted by treatment with VEGF antagonists, leading to a survival advantage. Thus, levels of VEGF in both tumor and non-tumor tissues play important and differential roles in determining the effects of VEGF on tumorigenicity. However, additional analyses of the consequences of VEGF deletion or activation after tumor initiation, using conditional alleles, are necessary.
to provide critical insight into the effect of this angiogenic factor on tumor progression and metastasis.

Overall, the evidence presented above suggests that VEGF plays an important role in governing the mechanisms that control tumor growth and confer a survival advantage to the host. Consequently, it may be an oversimplification that tumor growth is largely dependent on maximally sustained levels of VEGF and potentially other proangiogenic factors, particularly during the early stages of disease. Moreover, the ability of VEGF to act as either an enhancer or inhibitor of tumor growth is dependent upon its concentration in the host microenvironment, as well as the context and timing of its modulation relative to cancer induction. This is apparent since the studies described above suggest that VEGF acts as an inhibitor of tumor growth when its levels are modulated through genetic modification prior to cancer induction. The study by Stockmann et al. (2008) has established a critical role for VEGF derived from tumor infiltrating immune cells, in the formation of malignancy-associated high-density vasculature, signaling to the tumor endothelium, and the sensitization and efficacy of chemotherapeutic agents (Stockmann C et al. 2008). The study by Greenberg et al. (2008) has confirmed the ability of VEGF to modulate tumor vessel maturation by interfering with pericyte function. Recently, pre-clinical studies have shown that mice treated with VEGF-targeted therapies, prior to tumor induction, display increased tumor invasion and metastasis and decreased overall survival (Ebos JM et al. 2009), (Pàez-Ribes M et al. 2009). Moreover, clinical evidence also challenges this well-accepted theory since monotherapy with VEGF-targeted agents have resulted in only modest objective responses with short-term survival benefits (Mayer EL et al. 2007), (Hurwitz H et al. 2006), whereas the combination of anti-angiogenic agents with chemotherapy improves overall survival rates (Miller K et al. 2007), (Hurwitz H et al. 2004). As such, the combination of anti-VEGF therapy with anti-metastatic therapy, would likely suppress the role of VEGF in tumor growth to provide a more effective treatment.
option and ameliorate clinical outcomes.

These above findings also implicate that the hematopoietic compartment is a pivotal player in the control of tumor growth through the regulation of VEGF. A better understanding of the proangiogenic phenotype of the host, particularly with respect to the myeloid compartment and accessory cells of the vasculature, as presented herein, may likely lead to improved cancer therapies and/or preventative interventions in the future. Therefore further experimental and clinical studies are required to clarify the controversy surrounding the dichotomous roles of VEGF in tumor angiogenesis.


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