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Gene Repression and Cell Cycle Regulation by PU.1 in Acute Myeloid Leukemia

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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GENE REPRESSION AND CELL CYCLE REGULATION BY PU.1 IN ACUTE MYELOID LEUKEMIA

by

Rachel Ziliotto

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Rachel Ziliotto 2013
ABSTRACT

Acute myeloid leukemia (AML) is associated with mutations or chromosomal translocations in genes encoding transcription factors. PU.1 is a transcription factor that is required for the development of nearly all white blood cell types of the immune system, including B cells, granulocytes, and monocytes. Mutation of the gene encoding PU.1, SPI1 in humans and Sfpi1 in mice, is associated with AML. We hypothesized that reduced expression of PU.1 in Sfpi1BN/BN myeloid cells will result in the development of AML in transplanted mice due to reduced repression of E2F1, leading to deregulation of the cell cycle. Results indicate that NOD/SCID/γc−/− mice transplanted with Sfpi1BN/BN splenocytes become sick with disease resembling AML. Induction of PU.1 expression results in repression of the cell cycle regulator, E2F1, suggesting PU.1 represses E2F1 in order to enable cell cycle exit and differentiation. Understanding the pathways controlled by PU.1 can be used in therapies for the treatment of AML.

Keywords: PU.1, AML, transcription factor, myeloid cell, cell cycle, E2F1
ACKNOWLEDGMENTS

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I would also like to thank Kristin Chadwick for her assistance with flow cytometry work.
DEDICATION

I would like to dedicate this monograph to my parents, Grant and Irene, and my sister, Danielle. Without your support and encouragement throughout the years my success would not be possible.
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LIST OF ABBREVIATIONS

7-amino-actinomycin (7-AAD)
Acute Myeloid Leukemia (AML)
Bromodeoxyuridine (BrdU)
CDK inhibitor (CKI)
Chromatin Immunoprecipitation (ChIP)
Common Lymphoid Progenitor (CLP)
Common Myeloid Progenitor (CMP)
Cyclin-dependent kinase (CDK)
Fluorescence Activated Cell Sorting (FACS)
Granulocyte colony-stimulating factor (G-CSF)
Granulocyte macrophage colony-stimulating factor (GM-CSF)
Granulocyte/Monocyte Progenitor (GMP)
Green Fluorescent Protein (GFP)
Hematopoietic Stem Cell (HSC)
Macrophage colony-stimulating factor (M-CSF)
Macrophage/Dendritic cell Progenitor (MDP)
Megakaryocyte/Erythroid Progenitor (MEP)
MicroRNA (miRNA)
Multipotential progenitor (MPP)
Non-obese diabetic/severe combined immunodeficient/IL-2 receptor gamma chain null (NOD/SCID/γc−/) (NSG)
Polymerase Chain Reaction (PCR)
Purine-Rich Box Binding-1 (PU.1)
Quantitative PCR (qPCR)
Retinoblastoma protein (Rb)
Reverse transcriptase quantitative PCR (RT-qPCR)
CHAPTER 1: INTRODUCTION

1.1 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a cancer of the myeloid lineage of white blood cells. It is a disease characterized by a block in differentiation of myeloid cells and their uncontrolled proliferation in the bone marrow (1). The projected number of new diagnoses of AML in the United States in 2012 was nearly 14,000, up from 12,000 in 2010 (2). The estimated number of deaths from these diagnoses was 10,200, surpassing the number of deaths in 2010 by over 2000 (2). Until the 1970s, five-year survival rates for AML were less than 15% (1). During the 1980s and 1990s refinements in the diagnosis and advances in therapeutic approaches improved the prognosis for patients with AML. Nevertheless, the survival rate among patients less than 65 years of age is still only 40% (1) and the prospects for elderly AML patients is significantly worse, with median survival times of only a few months (3). The dismal outlook for older patients is a result of not only the deteriorating health of the patient but also the nature of the disease (4). When taken with the fact that AML is the most common acute leukemia affecting adults (4), these discouraging statistics highlight the importance of study of the disease. AML is diagnosed by identification of leukemic myeloblasts in bone marrow exceeding 30% of marrow aspirate (1). Recently, the relevance in classifying the subtype of AML and determining course of treatment based on the specific subtype has become appreciated (1, 5). The most commonly used method of classification (known as the FAB classification system) divides AML into nine distinct subtypes based on the particular myeloid lineage involved and the degree of differentiation of the cells (1). These distinctions are determined by the morphological appearance of the blasts and their reactivity with histochemical stains (1). The FAB classification system was named after the group that developed it, the French-American-British (FAB) group, and is listed in Table 1.1. The FAB classification system also incorporates genetic trends, such as chromosomal rearrangements involving certain genes that are present in a large percentage of AML patients (1). Specifically, genes that
Table 1.1 FAB Classification of AML and associated genetic abnormalities/transcription factor mutations.

Table 1.1 adapted from Lowenberg et al. 1999 (1) and Rosenbauer & Tenen 2007 (6)

<table>
<thead>
<tr>
<th>FAB Subtype</th>
<th>Common Name (% of Cases)</th>
<th>Results of Staining</th>
<th>Transcription Factor (Frequency in AML)</th>
<th>Mutations and Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Acute myeloblastic leukemia with minimal differentiation (3%)</td>
<td>- - -</td>
<td>RUNX1 (9%)</td>
<td>Missense, nonsense or frameshift mutations</td>
</tr>
<tr>
<td>M1</td>
<td>Acute myeloblastic leukemia without maturation (15–20%)</td>
<td>+ + -</td>
<td>C/EBPα (7-9%)</td>
<td>Amino-terminal dominant negative; carboxy-terminal loss of DNA binding</td>
</tr>
<tr>
<td>M2</td>
<td>Acute myeloblastic leukemia with maturation (25–30%)</td>
<td>+ + -</td>
<td>RUNX1-ETO t(8;21) (12-15%)</td>
<td>RUNX1 DNA-binding domain fused to the transcriptional corepressor ETO</td>
</tr>
<tr>
<td>M3</td>
<td>Acute promyelocytic leukemia (5–10%)</td>
<td>+ + -</td>
<td>PML-RARα t(15;17) (6-7%)</td>
<td>PML gene fused to RARα</td>
</tr>
<tr>
<td>M4</td>
<td>Acute myelomonocytic leukemia (20%)</td>
<td>+ + +</td>
<td>MLL fusions t11q23 (4-7%)</td>
<td>MLL gene fused with one of 30 distinct genes encoding partner proteins</td>
</tr>
<tr>
<td>M4E</td>
<td>Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)</td>
<td>+ + +</td>
<td>CBFβ-MYH11 inv16 (8-10%)</td>
<td>Inversion of breaks in chromosome 16; joins CBFβ with the myosin gene MYH11</td>
</tr>
<tr>
<td>M5</td>
<td>Acute monocytic leukemia (2–9%)</td>
<td>- - +</td>
<td>MLL fusions t11q23 (4-7%) MOZ-CBP t(8;16)</td>
<td>Amino-terminal dominant negative; carboxy-terminal loss of DNA binding</td>
</tr>
<tr>
<td>M6</td>
<td>Erythroleukemia (3–5%)</td>
<td>+ + -</td>
<td>PU.1 (&lt;7%)</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>Acute megakaryocytic leukemia (3–12%)</td>
<td>- - +</td>
<td>t(1;22)</td>
<td></td>
</tr>
<tr>
<td>M7 with Down’s syndrome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*AMKL, acute megakaryoblastic leukemia; CBFβ, core-binding factor-β; C/EBPα, CCAAT/enhancer binding protein-α; GATA1, GATA-binding protein 1; MLL, mixed lineage leukemia; MYH11, myosin heavy chain 11; PML, promyelocytic leukemia; RARα, retinoic acid receptor-alpha; RUNX1, runt-related transcription factor 1
encode DNA-binding transcription factors or the regulatory components of transcriptional complexes are frequently subject to mutation (1). For example, the t(8;21) translocation of the transcription factor acute myeloid leukemia 1 protein (AML1) (also known as runt-related transcription factor 1, RUNX1 or core-binding factor subunit alpha-2, CBFα2), which regulates a number of hematopoiesis specific genes (1), is found in 12-15% of AML cases (6). The consequence of this chromosomal translocation is AML1 fusing to the transcriptional corepressor ETO. The resultant chimeric protein, represses instead of activating AML1-regulated target genes, including the genes encoding the transcription factors Purine-Rich Box Binding (PU.1), CCAAT/enhancer binding protein alpha (C/EBPα) and RUNX1 (6). Interestingly, mutation of AML1’s normal partner, core binding factor-β (CBFβ) is associated with a different subtype of AML that involves chromosome 16 inversions (6-7). This transcription factor mutation is present in 8-10% of AML cases (6). Examples of genetic mutations associated with the various FAB system subtypes of AML are included in Table 1.1. Due to the fact that transcription factors are essential for normal development of the hematopoietic system (6), it is not surprising that mutation results in deregulation of hematopoiesis (1,6), and ultimately, in some cases, cancer. The more recent World Health Organization (WHO) classification takes into account such leukemia-associated molecular parameters (6).

Due to the important role that deregulated transcription factor activity can play in leukemia, such genes are currently being targeted for therapeutic intervention in myeloid and other cancers (6). For example, in acute promyelocytic leukemia (APL; FAB system subtype M3) a chromosomal translocation (t(15;17)) joins the gene encoding the transcription factor promyelocytic leukemia (PML) to the gene encoding retinoic acid receptor-α (RARα) (6, 8). It is well known that RARs play a role in the differentiation of myeloid cells (9); Tsai and Collins have demonstrated that inhibition of RARα blocks granulocytic differentiation (10). It has been shown that the PML- RARα fusion blocks myeloid transcription factors, such as PU.1 and C/EBPα, resulting in a block in myeloid
differentiation (6). The differentiation block can be relieved by treatment with all-trans retinoic acid (ATRA), increasing the survival of patients with APL (6, 11). Given that the hallmark of AML is a severe block in hematopoiesis, specifically myeloid differentiation (6), understanding normal hematopoiesis and myeloid development is critical.

1.2 Hematopoiesis

Hematopoiesis is the development of all blood lineages from a hematopoietic stem cell (HSC). HSCs exist as a small, rare population of cells in the bone marrow of adult mammals (12). HSCs are derived from the mesodermal layer of the embryo, which becomes specialized to a hematopoietic fate (12). Hematopoiesis, then, occurs in two major waves, at four sequential locations, in mammals: 1) the yolk sac, 2) an area surrounding the dorsal aorta known as the aorta-gonad mesonephros (AGM) region, 3) the fetal liver, 4) the bone marrow (13). The placenta has been acknowledged as an additional site for hematopoiesis, during the AGM to fetal liver period (13). In the mouse, the first or “primitive” wave of blood production in the mammalian yolk sac yields red blood cells (erythroid cells) that enable tissue oxygenation as the embryo grows (13). The primitive wave begins at embryonic day 7.5 in the blood islands of the yolk sac and shortly after (~ embryonic day 8.5) in the AGM region. The AGM region produces hematopoietic progenitor cells. The primitive hematopoietic system is rapidly replaced by adult-type hematopoiesis, known as “definitive” hematopoiesis, that begins at ~ embryonic day 10 (13). This second wave, in the mouse, involves the fetal liver, thymus, spleen, and ultimately the bone marrow; the second wave generates blood cells of specific lineages, such as B cells and T cells (13). Definitive hematopoiesis occurs first in the fetal liver, with maximal hematopoietic activity occurring embryonic day 14.5. Following embryonic day 15, hematopoiesis decreases in the fetal liver, presumably as hematopoiesis begins to occur in other immune organs such as the bone marrow and spleen (14). By embryonic day 20, hematopoiesis is underway in the bone marrow where it will occur for the remainder of
the animal's life (14). As indicated, the developmental time periods for hematopoiesis mentioned, are for that of mice. Notably, there are differences between humans and mice, but the mouse model provides foundational information for understanding the development of the hematopoietic system (15).

The hematopoietic stem cell has two defining characteristics: self-renewal and multipotency (16). Self-renewal is the ability to undergo many cellular divisions while remaining undifferentiated. This includes the option of dividing asymmetrically, which allows one daughter cell to maintain the HSC population while allowing the second daughter cell to differentiate. Multipotency is the ability to differentiate into cell types of multiple different lineages. The HSC has the ability to generate progenitors, which differentiate into all lineages of the blood in a hierarchical fashion (17). Multipotent stem cells go on to divide to produce a common lymphoid progenitor which gives rise to the lymphoid lineage, or a common myeloid progenitor which gives rise to the myeloid lineage. Terminally differentiated cell types of the lymphoid lineage include natural killer (NK) cells and the T and B lymphocytes, while terminally differentiated cells of the myeloid lineage include macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells, erythrocytes (red blood cells) and megakaryocytes that generate platelets (18).

More specifically, then, hematopoiesis is the acquisition of defining blood cell phenotypes as a result of coordinated, cell-specific gene expression (19). Molecular pathways, including cytokine receptors and cell specific transcription factors, have been identified as regulators controlling the different blood cell lineages (19-21). Zhu and Emerson have proposed that it is the effects of both intrinsic transcription factors and external signaling pathways initiated by cytokines that govern stem cell fate decisions (21).

Multicolour flow cytometry has permitted the identification of phenotypically distinct stem-cell and intermediate-precursor populations (6). The Weissman laboratory has proposed an ordered sequence of hematopoiesis, beginning with a subpopulation of long-term HSCs (defined phenotypically as LIN-IL-7RαSCA1 KIT FLT3 Thy1lowCD34)
The LIN marker represents a group of antigens on mature hematopoietic cells; the LIN panel includes CD11b, Gr-1, B220, TER119, CD4, CD8, CD5, and CD3. These cells have the ability for life-long self-renewal and multilineage differentiation (6).

Long-term HSCs give rise to short-term HSCs (defined phenotypically as LIN-IL-7Rα-SCA1+KIT+FLT3lowThy1lowCD34+), which retain the ability for multilineage differentiation potential, but have less self-renewal potential (6). The short-term HSCs give rise to the multipotential progenitors (MPPs) (defined phenotypically as LIN-IL-7Rα-SCA1+KIT+FLT3lowThy1lowCD34+), which have lost self-renewal potential but are still able to differentiate into all blood-cell types (6). From this point, the MPPs give rise to either the common lymphoid progenitor (CLP) (defined phenotypically as LIN-IL-7Rα-SCA1lowKITlow) or the common myeloid progenitor (CMP) (defined phenotypically as LIN-SCA1-KIT'CD34+FcyRIIFcγRIII') (23, 24). The Weissman model proposes that all myeloid cells arise from CMPs. The CMP goes on to give rise to more specified progenitors including granulocyte/monocyte progenitors (GMPs) (defined phenotypically as LIN-SCA1-KIT'CD34+FcγRIIFcγRIII'), megakaryocyte/erythroid progenitors (MEPs) (defined phenotypically as LIN-SCA1-KIT'CD34+FcγRIIFcγRIII'), and as has recently been shown, basophil progenitors (25), as well as a shared macrophage and dendritic cell progenitor (MDP) (26). From these various progenitors, the terminally differentiated cell types are established. The hematopoietic lineage diversification system proposed by the Weissman group is summarized in Figure 1.1A.

The Weissman model has recently been challenged by Jacobsen and colleagues, who propose that the erythroid lineage diverges much earlier, without going through a shared CMP stage (27). The Jacobsen model states that following differentiation from a long-term HSC into a short-term HSC the short-term HSC differentiates into either a MEP, or a lymphoid-primed multipotent progenitor (LMPP). The LMPP then differentiates into the GMP, leading to granulocytes and macrophages, or the CLP, leading to NK cells, B cells and T cells (27). The Jacobsen model is illustrated in Figure 1.1B. The
Figure 1.1 Current Models of Hematopoiesis.

Figure 1.1 adapted from Rosenbauer & Tenen 2007 (6) and Reya et al. 2001 (22)

(A) Classical/Weissman model of hematopoiesis. CLPs are thought to generate T and B-cells, while CMPs give rise to GMPs, MEPs, MDPs, and mast-cell and basophil progenitors. (B) Jacobsen model of hematopoiesis. The Jacobsen model states that MEPs are the direct progeny of ST-HSCs, while all myeloid and lymphoid lineages are the progeny of LMPPs.

Long-term (LT) and short-term (ST) hematopoietic stem cell (HSC); Multipotent progenitor (MPP); Common lymphoid progenitor (CLP); Common myeloid progenitor (CMP); Granulocyte/macrophage progenitor (GMP); Megakaryocyte/erythroid progenitor (MEP); Macrophage/dendritic cell progenitor (MDP); Lymphoid-primed multipotent progenitor (LMPP).
Jacobsen model is most compatible with the finding that PU.1 deficient mice are devoid of CLPs and CMPs, but have relatively normal numbers of erythroid cells (6). Furthermore, several other alternative models of hematopoiesis have been proposed including but not limited to the stochastic model, the sequential restriction model, myeloid-based models, as well as models based on studies of transcription factors (28). In conclusion, while much progress has been made in understanding hematopoiesis, more research is still required to establish a complete pathway to the development of blood cells.

1.3 Transcriptional regulation of myeloid cell development

Myelopoiesis is the developmental process of producing differentiated cells of the myeloid lineage from HSCs. Transcription factors play a pivotal role in myeloid cell differentiation (29-35). The myeloid lineage comprises most of the cells of the innate immune system and includes monocytes/macrophages, neutrophils, eosinophils, basophils, mast cells and dendritic cells (18). Erythrocytes (red blood cells) and megakaryocytes have also been traditionally considered part of the myeloid lineage, though that view has recently been challenged with the Jacobsen model (27). Myeloid cells, specifically granulocytes (collective term for neutrophils, eosinophils, and basophils) (18) and monocytes, are key mediators of the inflammatory response (32). Myeloid cells mediate recognition, ingestion, and destruction of foreign organisms, antigen presentation, cytokine production, and other functions of the immune and inflammatory reactions (32). HSCs in fetal liver or in adult bone marrow give rise to GMPs, which in turn give rise to granulocyte, monocyte, and granulocyte/monocyte-colony forming units (CFU-G, CFU-M, CFU-GM) (33). Of note, GMPs share with CLPs the ability to generate myeloid dendritic cells (33).

Cell development and differentiation is defined by gene expression patterns (33). Accordingly, transcription factors play a pivotal role by inducing the expression of lineage-specific markers (33). While there is no single regulator of myelopoiesis, the formation of cells of the myeloid lineage is controlled by a relatively small number of
transcription factors that regulate expression of myeloid specific genes, such as those encoding receptors for macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF)(6, 16, 30-32). Table 1.2 illustrates transcriptional regulators of granulocytic and monocytic genes. Notably, numerous reports have identified two key, non-redundant, transcription factors as absolutely critical for normal myeloid cell development: PU.1 and C/EBPα (30-32). Knock-out studies of these factors reveal major distortion of myeloid development (36-41). Furthermore, aberrant expression of these two transcription factors has been shown to contribute to the pathogenesis of AML (6, 32, 42-47). As mentioned, in addition to transcriptional regulation, cytokine activity has also been shown to influence hematopoiesis (32). While signaling through cytokine receptors modulates factor activity, whether or not they can actually determine cell fate has been contested (32, 33).

Although myelopoiesis cannot occur in the absence of either C/EBPα or PU.1, normal myeloid cell development also requires the cooperation of several other transcription factors. The very first transcription factors to play a role, are those that orchestrate the formation of HSCs from earlier stem cells of the mesoderm: RUNX1 (AML1) and stem-cell leukemia factor, SCL (also known as T-cell acute lymphocytic leukemia protein, TAL1, encoded by the gene TAL1) (6). Confirmation of the importance of these two transcription factors, come from studies that illustrate that both RUNX1−/− and TAL1−/− mice are embryonic lethal and have no detectable hematopoiesis, (48, 49).

C/EBPα is necessary for production of GMPs from CMPs (6). Interestingly, C/EBPα is expressed by HSCs, myeloid progenitors (CMPs, GMPs), and granulocytes, but not by macrophages (24, 50). Several CEBPA gene knockout studies have demonstrated that lack of C/EBPα results in a block of the CMP to GMP transition (31). CEBPA−/− mice lack GMPs and granulocytes (neutrophils and eosinophils) but retain monocytes (36, 37). It is also necessary to highlight the early differentiation block of granulocytes in CEBPA−/− mice; C/EBPα is no longer required for granulocytic differentiation beyond the GMP
Table 1.2 Regulation of Granulocytic and Monocytic Genes.

Table 1.2 adapted from Friedman 2002 (26)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcriptional Regulators of gene encoding protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early-stage granulocytes</strong></td>
<td></td>
</tr>
<tr>
<td>mim-1</td>
<td>C/EBPs, c-Myb</td>
</tr>
<tr>
<td>Myeloperoxidase (MPO)</td>
<td>C/EBPs, PU.1, CBF, c-Myb</td>
</tr>
<tr>
<td>Neutrophil elastase (NE)</td>
<td>C/EBPs, PU.1, CBF, c-Myb, Sp1</td>
</tr>
<tr>
<td>Myeloblastin (MBN)</td>
<td>C/EBPs, PU.1, c-Myb</td>
</tr>
<tr>
<td>G-CSF receptor</td>
<td>C/EBPs, PU.1</td>
</tr>
<tr>
<td>GM-CSF receptor</td>
<td>C/EBPs, PU.1</td>
</tr>
<tr>
<td>CD13</td>
<td>c-Myb, Ets-1 or Ets-2, c-Maf</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>C/EBPs, PU.1</td>
</tr>
<tr>
<td>c-fes</td>
<td>PU.1, Sp1</td>
</tr>
<tr>
<td><strong>Early-stage monocytes</strong></td>
<td></td>
</tr>
<tr>
<td>M-CSF receptor</td>
<td>C/EBPs, PU.1, CBF, c-Jun</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>C/EBPs, PU.1</td>
</tr>
<tr>
<td><strong>Late-stage granulocytes</strong></td>
<td></td>
</tr>
<tr>
<td>gp91phox</td>
<td>PU.1, IRF-8, CDP</td>
</tr>
<tr>
<td>Lactoferrin (LF)</td>
<td>C/EBPs, Sp1, CDP</td>
</tr>
<tr>
<td>Fc\gamma RI</td>
<td>PU.1</td>
</tr>
<tr>
<td><strong>Late-stage monocytes</strong></td>
<td></td>
</tr>
<tr>
<td>Macrosialin</td>
<td>PU.1, c-Jun</td>
</tr>
<tr>
<td>Scavenger receptor (type I)</td>
<td>PU.1, c-Jun</td>
</tr>
<tr>
<td>Scavenger receptor (type II)</td>
<td>PU.1, c-Jun</td>
</tr>
<tr>
<td>CD14</td>
<td>C/EBPs, Sp1</td>
</tr>
<tr>
<td>gp91phox</td>
<td>PU.1, IRF-8, CDP, Hox10A</td>
</tr>
<tr>
<td>CD11b</td>
<td>PU.1, Sp1</td>
</tr>
<tr>
<td>CD18</td>
<td>PU.1, Sp1</td>
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<tr>
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<td>PU.1</td>
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<tr>
<td>Fc\gamma RIIIA</td>
<td>PU.1</td>
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</table>
stage, as conditional deletion of CEBPA in GMPs allows for normal granulopoiesis (37). In addition to its role in myeloid differentiation, and regulation of myeloid specific genes (see Table 1.2), C/EBPα also controls stem-cell self-renewal properties (37) and coordinates cell-cycle exit (51).

The transcription factor c-Myb has also been implicated as a requirement for early granulopoiesis (33). Like MYC, MYB is a proto-oncogene that promotes proliferation and thus must be down-regulated for terminal myeloid differentiation to occur (34). Consistent with c-Myb’s role in the early stages of differentiation is the fact that c-Myb activates early granulocyte genes including the genes encoding CD13, neutrophil elastase, myeloperoxidase, and myeloblastin (34) (Table 1.2). C-Myb is expressed in immature myeloid, lymphoid, and erythroid cells, and MYB−/− mice lack each of these lineages (30, 33).

RARα is also connected with early granulocytic differentiation. While RARs are widely expressed, RARα is preferentially found in myeloid cells (52). Dominant inhibition of RARα arrests granulocytic differentiation at the promyelocytic stage (10). Importantly, RARα activates the CCAAT/enhancer binding protein epsilon (C/EBPε) gene promoter linking RARα to granulocyte development (53).

From the GMP stage, the next major milestone is designation of granulocytic versus monocytic/macrophage fate. PU.1 binding partners have been shown to play a role in terminal monopoiesis. PU.1 interaction with the interferon-γ (IFN-γ)-responsive transcription factor IRF8 (interferon regulatory factor 8) (also known as interferon consensus sequence binding protein, ICSBP) has been implicated in monopoiesis (31). In the myeloid lineage, IRF8 is expressed by progenitors and macrophages, but not by granulocytes (54). IRF8−/− mice have reduced macrophages and increased granulocytes (55).

Other transcription factors, including c-Jun and c-Fos, also cooperate with PU.1 to regulate myeloid genes and monocytic differentiation (56-58). C-Jun assists PU.1 by either binding to adjacent DNA elements or by direct physical interactions. Physical interaction occurs between PU.1 and c-Jun via their DNA binding domains; this interaction
permits the pair to activate gene transcription (59). Jun proteins (c-Jun, JunB, JunD) are part of the bZIP, AP-1 subfamily of transcription factors that heterodimerize with c-Fos, Fos B, Fra1 or Fra2 (31). Intriguingly, c-Jun and c-Fos can also heterodimerize with C/EBPα to induce monocyte commitment (60).

Furthermore, the Maf transcription factor family (MafB, c-Maf) can zipper with Fos or Jun family members (30). MafB is expressed in monocyte/macrophage cells and exogenous MafB or c-Maf leads to monocytic differentiation (31). A role for MafB in late but not early monocyte lineage development has been suggested (31).

In regards to late granulocytic specification, transcription factors involved include C/EBPε, and growth-factor independence-1 (GFI1). While C/EBPα is the predominant isoform in immature granulocytes, C/EBPε predominates mature granulocytes (32). A clear role for C/EBPε in the regulation of terminal granulopoiesis is evident from the phenotype of C/EBPε deficient mice, which have immature granulocytes (neutrophils and eosinophils) that fail to develop normally past the promyelocyte stage and lack secondary granules (61-63). As well, the presence of C/EBPε regulatory sites in the promoters of genes expressed in neutrophils is highly suggestive of C/EBPε as a key player in granulocytic differentiation (30). Similarly, GFI1−/− mice lack neutrophilic granulocytes (6).

Notably, the development of early myeloid progenitors, including GMPs, was normal in GFI1−/− mice, but neutrophilic differentiation was blocked beyond the promyelocyte stage (64). This finding confirms the role of C/EBPα in early granulocyte development, and illustrates the significance of other factors, such as GFI1, in later stages of granulopoiesis. Finally, GFI1−/− mice accumulated neutrophil precursors that expressed monocyte-specific genes in addition to early granulocyte markers. The abnormal neutrophil population present in GFI1−/− mice correlates with the fact that GFI1 contains a transcriptional repressor and may function to repress monocyte/macrophage lineage traits during granulocytic maturation (64).

The transcription factor Specificity Protein 1 (Sp1), has also been implicated in
terminal granulopoiesis (as well as monopoiesis). This implication is based on the fact that Sp1 regulates several granulocytic and monocytic genes, including the genes encoding lactoferrin (LF) (granulocytic), CD14 and CD11b (monocytic) (see Table 1.2) (30). Furthermore, Sp1 is expressed at particularly high levels in maturing granulocytes (30).

Like GFI1, recent studies have shown that the transcription factors early growth response proteins 1 and 2 (EGR1, EGR2), NGF1-A-binding protein 2 (NAB2), CCAAT displacement protein (CDP), and Homeobox protein Hox-A10 (HoxA10) repress terminal differentiation (31). Laslo et al. have identified the EGR proteins and NAB2 as possible repressors of granulopoiesis, while stimulating monopoiesis (65). Evidence for the notion that EGR:NAB2 complexes repress granulopoiesis stems from the fact that EGR2 or NAB2 knockdown induces neutrophilic genes, including GFI1, and EGR2:NAB2 represses the GFI1 promoter (65). GFI1 represses the EGR2 promoter (65), providing further support for GFI1 as a transcriptional repressor of monopoiesis. In maturing neutrophils, the gp91phox promoter region, (gp91phox is a late stage granulocyte and monocyte gene) is repressed by both CDP and HoxA10 (66-67). CDP represses transcription by competing with transactivators for the same site (DNA elements that resemble the 5’-CCAAT-3’ motif) (68). Work by Skalnik et al., as well as Eklund et al., suggest that levels of CDP and HoxA10 decrease during terminal neutrophil and monocyte differentiation (66-67). Due to the fact that HoxA10 is preferentially expressed in immature myeloid cells (33), it has been postulated that HoxA10 plays a role in maintaining an earlier stage in myelopoiesis and inhibiting terminal differentiation (33). In conclusion, myeloid cell development is achieved via cooperative gene regulation, protein-protein interaction, autoregulation, regulation of factor levels, and induction of cell cycle arrest (33).

1.4 Purine-Rich Box Binding-1 (PU.1)

PU.1 is a member of the family of E26 transformation specific (ETS) transcription factors. Members of this family of proteins all contain a characteristic winged-helix-turn-
helix DNA binding domain that binds DNA sequences with a GGA core (“GGAA”) (69-70). PU.1 binds as a monomer to the purine-rich consensus DNA sequence (from which it derived its name) 5’GAGGAA-3’ (PU box) via its C-terminal ETS DNA binding domain (71).

In 1988, PU.1 was first discovered as a putative oncogene isolated from a murine Friend virus-induced erythroleukemia (72). The murine erythroleukemia was induced by a proviral insertion of the retroviral spleen focus forming virus (SFFV) (72). The gene encoding PU.1 became known as SPI1 (SFFV proviral integration site-1), in humans, and Sfpi1, in mice (72). Interestingly, Paul et al. found that overexpression of PU.1 blocks erythroid differentiation (73). The murine PU.1 protein contains 266 or 272 amino acids due to two potential translation start codons, and is ~42 kilodaltons (kDa) in size (74). The human PU.1 protein is quite similar, demonstrating 85% homology with murine PU.1 (75). As indicated, PU.1 expression is restricted to blood cells (71), where it plays a fundamental role in the development of both myeloid and lymphoid lineages (41).

In addition to DNA binding, the C-terminal ETS DNA binding domain of PU.1 can also interact with other proteins, including C/EBPα, CCAAT/enhancer binding protein beta (C/EBPβ), c-Jun and GATA-binding factor 1 (GATA-1) (59, 76-78) (Figure 1.2). PU.1 contains two other functional domains: an N-terminal transactivation domain, incorporating an acidic and a glutamine rich region, and a PEST domain (79). While PU.1 activates transcription via its N-terminal domain (79), this region also allows for interaction with other proteins such as GATA-1, TATA box binding protein (TBP) and Retinoblastoma protein (pRb) (78, 74) (Figure 1.2). TBP is a subunit of the basal transcriptional complex TFIID, which increases RNA polymerase II activity, and pRb is a regulator of cell cycle progression (74). The PEST domain is located between the transactivation domain and the ETS domain; PEST was named after its high content of proline (P), glutamic acid (E), serine (S), and threonine (T). This domain is involved in protein stability/degradation (74, 80). Furthermore, phosphorylation of PU.1 within its PEST domain, allows it to
Figure 1.2 Protein-protein interactions involving PU.1. The transcription factor PU.1 is illustrated, with its three domains: N-terminal transactivation domain (blue), PEST domain (red), and C-terminal ETS DNA binding domain (green). The proteins that interact with each domain within PU.1 are also labeled within the corresponding domain.
The image contains a diagram of protein domains and their interactions.

**N-Terminal Transactivation domain**
- TBP
- Rb
- GATA-1

**C-Terminal ETS domain**
- C/EBP alpha
- C/EBP beta
- c-Jun
- GATA-1

**PEST domain**
- IRF-4
- IRF-8

**ETS domain**
interact with IRF4 and IRF8 (31) (Figure 1.2).

1.5 The expression and regulation of PU.1 in myeloid development

PU.1 is widely expressed among the various blood lineages. PU.1 is expressed in HSCs, CMPs, CLPs, GMPs, monocytes, granulocytes, and B cells (6). Recent analysis has shown that the absence of PU.1 impairs the ability of HSCs to self-renew and prevents differentiation into CMPs and CLPs (38, 81). Importantly, PU.1 expression levels appear to be a crucial determinant in cell-fate decisions of both myeloid and lymphoid progenitors. PU.1 is expressed at similar levels in HSCs, CMPs, CLPs, and B cells, but its expression increases substantially during terminal myeloid (granulocytic and monocytic) differentiation (47, 82-83). Furthermore, Singh’s group illustrated how high levels of PU.1 induce commitment to the myeloid lineage, while lower levels induce B cell development (84). By contrast, PU.1 expression is downregulated early on during erythroid and T cell differentiation (72, 85-86). Recent evidence, however, has shown that certain subsets of T cells must re-express PU.1 during terminal differentiation. This includes a subset of IL-9 producing helper T cells (87). In cell lines, PU.1 is expressed by myeloid and B cells, but not by T cell lines (71, 88). When examining the myeloid lineage, specifically, it has been observed that high PU.1 levels support the production of macrophages, whereas low PU.1 levels support granulocyte production (47, 89-90). For example, one study found that expression of low levels of PU.1 in Sfpi1<sup>-/-</sup> cells induces granulopoiesis, while high levels induce monopoiesis (89). As well, expression of PU.1 at 20% of wild-type levels resulted in a loss of monopoiesis, but maintenance of granulopoiesis (47). In short, it is clear PU.1 expression is absolutely critical throughout myeloid cell development, beginning from the HSC stage (6).

Due to the significance of PU.1 levels in hematopoietic lineage commitment, regulation of PU.1 expression is essential. Many groups have shown that inappropriate expression of PU.1 will lead to anomalous development of the hematopoietic system and
may result in leukemia (91). Several different transcription factors regulate PU.1 expression, including Octamer (Oct-1 and 2), Sp1, GATA-1, C/EBPα, RUNX1, and PU.1 itself. Chen et al. have reported an octamer binding site 55 bp upstream of the transcriptional start site (TSS) (92). While both Oct-1 and 2 can bind specifically to the site -55 bp of the Sfpi1 promoter, Oct-2 expression is limited to B cells, while Oct-1 is expressed ubiquitously (92). Interestingly, a recently identified octamer coactivator, known as Bob1 (93), OBF-1 (94), or OCA-B (95), is expressed exclusively in B cells (92). This may explain why the octamer site is relatively more important for PU.1 expression in B cells than myeloid cells, where PU.1 itself is most important (92). The same group detected both a Sp1 and PU.1 binding site within the Sfpi1 promoter at -40 bp and +20 bp, respectively (92, 96). In myeloid cells, the PU.1 binding site (+20 bp) is located in the 5’-untranslated region of the Sfpi1 promoter and has been shown to be functionally important, leading to an autoregulatory loop (96). In addition, Okuno et al. recently proposed a second potential autoregulatory mechanism involving an upstream regulatory element (URE). Okuno et al. demonstrate that PU.1 binds a distal site located 14 kb upstream of the TSS (in mice) and mutation of this site, abolishing PU.1 binding, results in a decrease in PU.1 expression (97). Intriguingly, deletion of the URE in mice reduces PU.1 expression to 20% of wild-type levels in HSCs, myeloid cells, and B cells (47). The same distal element is located 17 kb upstream of the TSS in humans (6). Thus, PU.1 may be able to positively regulate its own expression through both the URE and the PU.1 promoter. RUNX1 also binds to the same distal enhancer as PU.1 itself, thereby controlling PU.1 expression (98). RUNX1 binds to three sites within the URE of PU.1 and regulates PU.1 both positively and negatively in a lineage dependent manner (98). The mutual antagonism between PU.1 and GATA-1 is another well-known example of PU.1 gene regulation. A GATA-1 binding site 15 bp upstream of the TSS has been observed (52, 92, 96). Furthermore, Zhang et al. demonstrate that interaction between PU.1’s ETS domain and the C-terminal zinc finger of GATA-1 inhibits PU.1 activation of myeloid target genes (99). They also
show that GATA proteins inhibit binding of PU.1 to c-Jun, a coactivator of PU.1 transactivation (99). The ability of GATA-1 to repress myeloid gene expression via interaction with the PU.1 ETS DNA binding domain and subsequent interference of PU.1 function has been confirmed by Nerlov et al. (100). Similarly, C/EBPα blocks the function of PU.1 through protein-protein interaction (73). As well, the leucine zipper in the DNA binding domain of C/EBPα interacts with the β3/β4 region in the ETS domain of PU.1, resulting in displacement of c-Jun. (78). Finally, PU.1 expression is also upregulated by the Notch1 signaling pathway (101).

1.6 The role of PU.1 during myeloid development

Knock-out, knock-in, overexpression, reduction, and restoration studies clearly indicate the problems that arise in the absence of normal PU.1 levels. Two labs, in particular, have played pivotal roles in our understanding of PU.1 by creating an Sfpi1−/− mouse model. The first lab, of Dr. Harinder Singh, replaced PU.1’s ETS DNA binding domain with the neomycin resistance gene. In their model, Sfpi1 deletion was embryonically lethal between days 16.5 and 18.5 of gestation. Upon analysis of PU.1 (−/−) fetal liver, no myelocytes (or lymphocytes) were detected (40), indicating an early and severe differentiation block on myelopoiesis. The second group, Dr. Richard Maki’s, created their Sfpi1−/− mouse by inserting the neomycin-resistance gene into the ETS domain. Dr. Maki’s group was able to generate pups, though they died of septicemia within 2 days, and again no mature granulocytes or macrophages were detected (41). Interestingly, Sfpi1−/− pups were able to survive up to 2 weeks with antibiotics and these older mice contained a small population of abnormal neutrophils and macrophages (41). Thus, both models demonstrate obvious defects in myeloid cell development. While a complete absence of PU.1 expression has severe effects, other groups have looked at a reduction in PU.1 expression and found similar problems. Dr. Daniel Tenen’s group was the first to report that AML could be induced by a decrease in PU.1 expression (47). Tenen’s group generated a hypop-
morphic Sfpi1 allele, by replacing the -14 kb URE with a neomycin resistance gene. Mice homozygous for this ΔUREneo allele expressed PU.1 at 20% of normal levels (47). These mice had an accumulation of abnormal, immature myeloid precursors in the bone marrow and spleens, that were responsive to G-CSF, but not M- or GM-CSF (47). Furthermore, myeloid differentiation was blocked in mice homozygous for the Sfpi1 allele that reduces PU.1 expression, and these mice eventually developed AML (43).

Our laboratory has generated a novel hypomorphic allele of Sfpi1 termed BN, which also produces PU.1 at 20% of wild-type levels (102). The allele was generated by mutation of the first coding exon (102). Within this region, two ATG translation start sites were replaced with a β-lactamase gene/neomycin resistance cassette (102). A third translational start codon exists within Sfpi1 enabling Sfpi1BN alleles to be transcribed, but resulting in a slightly truncated protein. Due to altered transcriptional regulation, it also results in PU.1 being expressed at a reduced level. Nonetheless, the protein itself is fully functional (102). Neonatal mice homozygous for the mutated Sfpi1 allele (Sfpi1BN/BN mice) are characterized by a hyperproliferation of immature myeloid cells in their bone marrow and spleen, as well as a complete absence of B-cells (98). Sfpi1BN/BN mice survive only 1-3 weeks following birth and demonstrate severe phenotype abnormalities, including osteopetrosis and reduced physical size (102).

1.7 Transcriptional targets of PU.1

The importance of PU.1 in myeloid cell development is highlighted by the presence of PU.1 binding motifs in the regulatory sequences of almost all myeloid-specific genes, as illustrated in Table 1.2 (6, 32). Notable PU.1 target genes include the genes encoding the receptors for essential cytokines as M-CSF, GM-CSF, and granulocyte colony-stimulating factor (G-CSF) (103-106). The growth factors that the M-CSFR, GM-CSFR, and G-CSFR bind are required for the proliferation and differentiation of myeloid cells. PU.1 also activates genes necessary for the myeloid phenotype, including the genes en-
coding CD11b, CD18, FcγRI/IIA, and scavenger receptors, type I and II (107-111) (see Table 1.2). CD11b is a cell surface marker expressed on mature monocytes, macrophages, and granulocytes (107); the β₂ leukocyte integrin CD18, together with CD11b forms the complex macrophage-1 antigen, Mac-1 (complement receptor 3, CR3), which plays several important roles in immune and inflammatory responses (108). The high-affinity Fc gamma receptor I (FcγRI) and the low-affinity Fc gamma receptor IIIA (FcγRIIIA) are both expressed exclusively in the myeloid lineage and are receptors for the Fc domain of immunoglobulin G (IgG). They play an important role in innate immunity through their ability to induce phagocytosis and trigger antibody-dependent cellular cytotoxicity (109-110). Expression of type I and II scavenger receptors (SRs) is highly restricted to monocytes/macrophages; SRs are maximally expressed during monocyte to macrophage differentiation (111). Finally, recent studies by Ghisletti et al. demonstrate that PU.1 helps control expression of inflammatory genes, such as the genes encoding interleukin-1 beta (IL-1β), interleukin-18 (IL-18), interleukin-12 subunit p40 (IL12p40 subunit), and tumor necrosis factor (TNF), in macrophages (112).

1.8 The Cell Cycle

Cell cycle exit is necessary for terminal differentiation to occur (33). Without cell cycle arrest, immature cells will continue to proliferate, possibly leading to the development of cancer. One of the hallmarks of cancer is cell cycle deregulation (113-114). Cell cycle deregulation can occur at different levels of the cell cycle control network (115).

The cell cycle is a process by which DNA is replicated and then separated into two new cells (115). The process is divided into four distinct stages: G1, S, G2 (collectively known as interphase) (116), and mitosis (M) (115). DNA replication occurs during S phase and is preceded by G1 phase, in which the cells are preparing for DNA synthesis. Following synthesis, the cells enter a second gap phase (G2), in which the cell prepares for mitosis. The actual segregation of chromosomes into two separate cells occurs during
**Figure 1.3 Control of the cell cycle.** The stages of the cell cycle and sites of activity of regulatory CDK/cyclin complexes are presented. Cyclin dependent kinase inhibitors (CKIs), p15 and p27 are also indicated. As well, the G1 to S phase transition involving pRb and E2F1 is illustrated.

P (phosphorylation); * demonstrates the CDK7-cyclin H complex throughout all phases of the cell cycle; → (activation); ← (repression)
mitosis, which is divided into prophase, metaphase, anaphase, telophase (115). A resting state, known as G0, also exists within the cell cycle. G0 is a departure from the G1 stage and cells can enter G0 before making the commitment to S phase. Cells in the G0 stage are non-growing and non-proliferating (115) (Figure 1.3).

Transition through the various phases of the cell cycle is highly regulated; it is controlled by numerous mechanisms including cyclin-dependent kinases (CDKs), cyclins, CDK inhibitors (CKIs), phosphorylating events, and CDK activating enzymes. CDKs are key regulators of the cell cycle. They are a family of serine/threonine protein kinases that are activated at specific points of the cell cycle (115). Once activated CDKs phosphorylate other proteins to induce downstream processes necessary for cell cycle progression (116-117). Five of the nine CDK family members are active during the cell cycle at various points. Figure 1.3 illustrates the five CDK family members (CDK4, CDK6, CDK2, CDK1, CDK7) that are active during the cell cycle and the particular stage at which their presence is required. CDK7 acts in combination with cyclin H as CDK activating kinases (CAK) (118); the CAK complex is required throughout all cell cycle phases (115). Furthermore, CDKs require activation from another group of regulatory proteins, known as cyclins, that complex with CDKs in order to regulate the cell cycle. While CDK protein levels remain stable during the cell cycle, cyclin levels fluctuate; when the level of a cyclin protein rises it is able to activate its particular CDK (115). As illustrated, different cyclins act at different stages of the cell cycle to activate different CDKs. D cyclins (D1, 2, and 3) bind to CDK4 and CDK6 to permit entry into G1 phase. Cyclin E associates with CDK2 to regulate progression from G1 to S phase. A CDK2-cyclin A is required during S phase. In late G2 and early M, a CDK1-cyclin A complex is formed to promote entry into M. The remainder of M is regulated by a CDK1-cyclin B complex (115) (Figure 1.3). In addition to cyclins, CDK activity is further regulated by phosphorylation. Some CDKs, such as CDK1, require phosphorylation to become fully active 115) (Figure 1.3). Phosphorylation induces conformational changes that en-
able CDKs to better bind cyclins (119). While CDK activating kinases, such as the CAK complex act on CDKs, so do CDK inactivating kinases. Similarly, cell cycle inhibitory proteins, known as CDK inhibitors (CKI) also regulate CDK activity (115). There are two relevant families of CKIs: the INK4 family, which includes p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d), and the Cip/Kip family, which includes p21 (Waf1, Cip1), p27 (Cip2), and p57 (Kip2) (115). The INK4 family inactivates G1 CDKs (CDK 4 and 6) by preventing cyclin binding (120) (Figure. 1.3). The Cip/Kip family inactivate CDK-cyclin complexes (121), specifically the G1 CDK-cyclin complexes, and to a lesser extent the CDK1-cyclin B complex (122) (Figure 1.3).

As mentioned, once active, CDKs are able to phosphorylate target proteins, which ultimately leads to cell cycle progression. The pathway controlling the progression of cells from G1 into S phase is well established (123-124) and involves the CDK4/6-cyclin D complex and its substrate, the product of the retinoblastoma tumour suppressor gene, pRb (115). In this scenario, CDK4/6 phosphorylates pRb in early G1 phase, resulting in disruption of the pRb-HDAC (histone deacetylase) complex and release of the transcription factors E2F1 and DP1 (115) (Figure 1.3).

E2F1 and DP1 are members of two different groups of proteins that heterodimerize to induce transcription (123, 125). The two different groups are the E2F gene family consisting of six E2F genes (E2F1-E2F6), and the DRTF (differentiation regulated transcription factor) protein (DP) gene family, which consists of two DP genes (DP1 and DP2) (123). Thus, heterodimers contain a subunit encoded by the E2F gene family and a subunit encoded by the DP gene family (123). E2F1-DP1 heterodimers positively regulate transcription of genes whose products are required for S phase progression, including cyclin A and cyclin E (115).

1.9 E2F1, PU.1 and AML

It is now well-established that the transcription factor E2F1 can promote entry
into S phase from G1 (126-127). As indicated above, E2F1 (when complexed with a DP) controls the transcription of genes essential for cell division; these include genes encoding cell cycle regulators (such as the cyclins indicated above), enzymes involved in nucleotide biosynthesis (such as thymidine kinase), and the main components of the DNA-replication machinery (126). The potency of E2F1 as a transcriptional activator has been shown through overexpression of the protein, which induces quiescent cells to re-enter the cell cycle (127-129). Furthermore, deregulation of E2F1 activity appears to be a characteristic of human cancers (125, 130). E2F1 is regulated via association with pRb; once pRb is phosphorylated by CDK-cyclin complexes, E2F1 is released. Thus, pRb restricts cell cycle progression by maintaining E2F1 and the disassociation of E2F1 from pRb drives proliferation (123). The idea that defects in regulation and inappropriate release of E2F1 might induce cancer, are illustrated by the fact that overexpression of E2F1 can induce transformation of primary cells (131-133). Moreover, Gibbs et al. have shown that deregulated expression of E2F1 blocks terminal myeloid differentiation, resulting in proliferation of immature myeloid cells (134). In addition to inducing proliferation, deregulated E2F1 activity can also trigger apoptosis (122, 126). This functional paradox of E2F1 has been investigated and the results demonstrate that E2F1 can have both oncogenic (through its role in cell cycle progression) and tumor-suppressive effects (through apoptosis induction) (130).

Certain hematopoietic transcription factors, such as, c-Myb, and c-Myc, promote proliferation, and are down-regulated upon differentiation (34), while others, such as C/EBPα promote cell-cycle arrest (6). Several different mechanisms by which C/EBPα acts on the cell cycle have been reported. The most important mechanism, however, appears to be repression of E2F1 activity. This is suggested by the fact that targeted mutation in the gene encoding C/EBPα that results in defective repression of E2F1 failed to support granulocytic differentiation (6). On the other hand, mice lacking the CDK2/CDK4-binding domain of C/EBPα (inhibition of cyclin-dependent kinase 2 activity is another
mechanism by which C/EBPα acts on the cell cycle) were normal (6). The importance of PU.1 in terminal myeloid differentiation suggests that a similar role for PU.1 as C/EBPα, in regulating the cell cycle, may exist.

1.10 Hypothesis

The increased proliferation and impaired differentiation of immature myeloid cells in Sfpi1^BN/BN^ mice implies that PU.1 is necessary for cell cycle exit during myeloid differentiation. In this scenario, when PU.1 levels are decreased, the cell cycle is no longer regulated and myeloid progenitors continue to expand uncontrollably, resulting in AML. We have found that PU.1 transcript levels are inversely correlated with the cell cycle regulator, E2F1. E2F1 functions to promote G1 to S phase progression, and deregulation of E2F1 is associated with cancer. This suggests that PU.1 regulates cell cycle at least in part by repression of E2F1 expression. Consistent with the idea that PU.1 represses E2F1 is the fact that PU.1 levels increase during myeloid terminal differentiation, when cell cycle exit must occur (6, 50, 87, 88).

In conclusion, I intend to investigate the precise mechanism of how PU.1 works to regulate proliferation and differentiation of hematopoietic stem cells (HSCs) and its role in the development of AML. In order to conduct these investigations, I have formed the following hypothesis:

*We hypothesize that PU.1 represses E2F1 to regulate cell cycle exit in myeloid cells.*
CHAPTER 2: MATERIALS AND METHODS

2.1 Mouse Strains

Our laboratory has generated a novel hypomorphic allele of \textit{Sfpi1} termed BN, which produces PU.1 at 20% of wild-type levels (102). \textit{Sfpi1}\textsuperscript{+/BN} mice were maintained as an \textit{Sfpi1}\textsuperscript{+/BN} colony in the West Valley Barrier facility at Western University (London, Ontario, Canada). \textit{Sfpi1}\textsuperscript{+/BN} mice were mated to breed \textit{Sfpi1}\textsuperscript{BN/BN} neonates. Animal husbandry and breeding were conducted in compliance with the University of Western Ontario Animal Care and Veterinary Services Standard Operating Procedures. Polymerase Chain Reaction (PCR) was used to genotype mice in colony using primers listed Table 2.1.

2.2 Cell Culture

Complete media contained 10% fetal bovine serum (FBS) (Wisent, St-Bruno, QC), penicillin (100 U/ml)/streptomycin (100 \textmu g/ml) (Mediatech, Manassas, VA), L-glutamine (0.292 mg/ml) (Mediatech), 2-mercaptoethanol (5 \times 10^{-5} M) (Sigma-Aldrich, St. Louis, MO), and HEPES (5 mM) (Sigma-Aldrich). GP + E-86 packaging cells and platinum-E (Plat-E) retroviral packaging cells were both grown in complete Dulbecco’s Modification of Eagle’s Medium (DMEM) medium (Mediatech). BN cells were grown in complete Iscove’s Modification of DMEM medium with L-glutamine and 25 mM Hepes (Mediatech). The cytokine GM-CSF (1 ng/ml) was added.

2.3 Generation of Inducible System

The Retro-X Tet-On 3G Inducible Expression System was purchased from Clontech (Mountain View, CA). In order to clone our gene of interest (PU.1) into the Clontech response vector (pRetroX-TRE3G vector), an additional \textit{EcoR}1 site was first added to the PU.1 cDNA sequence part of the MIG-PU.1 vector using PCR (primer sequences listed in Table 2.1). Following amplification, the PCR product was cloned into the StrataClone
### Table 2.1 PCR and RT-qPCR primer sequences.

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<td>5’ CGGCCAGAGACTTCTGTAG-3’</td>
</tr>
<tr>
<td>Sfpi1 Primer 2</td>
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</tr>
<tr>
<td>Sfpi1 Primer 3</td>
<td>5’-GCTCTTCGTCCAGATCATCC-3’</td>
</tr>
<tr>
<td>Sfpi1 Primer 4</td>
<td>5’-ATGGTCACACATCCCAAAGC-3’</td>
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<tr>
<td><strong>PCR – addition of EcoR1 site</strong></td>
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<td>3XFLAG EcoR1 forward</td>
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</tr>
<tr>
<td>3XFLAG EcoR1 reverse</td>
<td>5’-GGAATTCTGGGGCCCTCTAGATC-3’</td>
</tr>
<tr>
<td><strong>PCR – addition of Bg1II and EcoR1 sites</strong></td>
<td></td>
</tr>
<tr>
<td>TET3Gfwd-ATG-Bg1II</td>
<td>5’-ACGTAGATCTATGTCTAGACTGGACAAAGACAAAGTC-3’</td>
</tr>
<tr>
<td>TET3Grev-TAA-EcoRI</td>
<td>5’ACGTGAATTCTTTACCAGGGGGAGCATGTTC-3’</td>
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<tr>
<td><strong>qPCR</strong></td>
<td></td>
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<tr>
<td>E2f1 cDNA forward</td>
<td>5’-TCCCTGGGAGGATCACAGC-3’</td>
</tr>
<tr>
<td>E2f1 cDNA reverse</td>
<td>5’-CTAATGCCCTACCCCTCCTCG-3’</td>
</tr>
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PCR Cloning Vector (pSC-A-amp/kan) (Agilent Technologies, La Jolla, CA) in order to enhance the efficiency of restriction enzyme cutting and thus the generation of EcoR1 5’ overhangs. The PCR product is able to be cut out of the pSC-A-amp/kan vector with 100% efficiency due to the supercoiled nature of the vector and the PCR product’s location within the vector. StrataClone SoloPack competent bacteria cells were used in the transformation to clone the PU.1 PCR product into the pSC-A-amp/kan vector. Following confirmation that the PU.1 PCR product had been successfully cloned into the pSC-A-amp/kan vector, the entire product was retransformed in preparation for maxi-prep. The entire product was then maxi-prepped in order to amplify and produce high DNA yields. The pRetroX-TRE3G vector was then digested with EcoR1 in preparation for ligation of insert. Following maxi-prep and using EcoR1, PU.1 was excised from the pSC-A-amp/kan vector and ligated into the pRetroX-TRE3G vector using T4 DNA ligase. Ligated vector and insert were transformed and bacteria colonies were assessed for successful cloning of insert into vector using sequencing (to ensure insert was in correct orientation). Once successful cloning had been established the pRetroX-TRE3G vector with PU.1 cDNA insert was once again retransformed, maxi-prepped and sequenced.

The regulator vector consisted of the Tet-On 3G transactivator genomic fragment cloned into the MIGR1 vector. BgI1II and EcoR1 restriction sites were PCR amplified (using primers listed in Table 2.1) into the Tet-On 3G gene, part of the pRetroX-Tet3G vector. The Tet-On 3G gene was then PCR amplified out of the pRetroX-Tet3G vector using TET3Gfwd-ATG-BgI1II and TET3Grev-TAA-EcoRI primers (listed in Table 2.1). The MIGR1 plasmid was digested with EcoR1 and BgI1II and the Tet-On 3G gene was inserted into the MIGR1 plasmid by sticky end ligation. Separate response and regulator retroviral supernatants were produced (see below).

2.4 Retrovirus Production

Platinum-E (Plat-E) retroviral packaging cells were used to generate retroviral su-
pernatants using polyethylenimine (PEI) transfection. Twenty-four hours before transfection, Plat-E cells were plated at a concentration of 5 x 10⁶ cells in a 10 cm Petri dish and incubated at 37°C overnight. The next day, media (DMEM complete) was removed and replaced with 10 ml of fresh, serum-free media. Cells were incubated in the serum-free media for two hours at 37°C prior to transfection. For transfection, 1 ml of serum-free DMEM complete with 150 mM of NaCl, 1 μg of pCL-Eco plasmid, and 9 μg of DNA of interest were mixed and vortexed for 15 seconds. Following vortex, 25 μl of PEI (1 μg / μl) (Polysciences, Warrington, PA) was added to the mixture, the mixture was vortexed and the entire solution was incubated at room temperature for 20 minutes without disturbance. Finally, 1 ml of the DNA/PEI solution was added to Plat-E cells, followed by 1 ml of DMEM complete media to ensure cells were not serum starved overnight. Transfected cells were incubated overnight at 37°C. All media was removed the next morning and replaced with 2 ml of DMEM complete. Retrovirus-containing supernatant was harvested 48 hours post transfection.

2.5 Retroviral Transduction of BN cells

BN cells were infected by resuspension in cell-free retroviral supernatants and centrifugation at 3100 rpm for 3 hours in the presence of polybrene (10 μg /ml). Following centrifugation retroviral supernatant was removed and 1 ml of fresh media was added. Cells were incubated for 48 hours post infection to allow retroviral integration and protein expression.

2.6 Cell Cycle Analysis

The BD Pharmingen BrdU Flow Kit was purchased from BD Biosciences (Mississauga, ON, Canada). Bromodeoxyuridine (BrdU) incorporation was measured by flow cytometry with an allophycocyanin (APC ) BrdU Flow Kit according to the manufacturer’s protocol. Briefly, cells were labeled with BrdU for 6 hours at 37°C. Cells were incubated
with the APC-conjugated anti-BrdU antibody using a 1:800 fold dilution. Staining with 7-amino-actinomycin D (7-AAD) was also conducted to determine cell cycle position. For 7-AAD staining, cells were suspended in PBS containing 0.5 M EDTA (pH 8.0) and 0.5 % BSA and then incubated with 7-AAD (BD Pharmingen).

2.7 Real-time PCR

RNA was extracted with TRIzol Reagent (Life Technologies, Carlsbad, CA) and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) was then performed using the iQ SYBR Green Supermix Kit (Bio-Rad) and Rotor-Gene 6000 (Corbett Life Sciences, Valencia, CA). Relative mRNA transcript levels were normalized to beta-2-microglobulin (B2M) as a reference gene and compared between samples using the comparative threshold (Ct) cycle method (135). Results are presented as the mean and SD of three experiments performed in triplicate. Primer sequences for E2f1 forward and reverse primers are listed in Table 2.1.

2.8 Immunoblotting

Cells were lysed with 6X Laemmli lysis buffer. Proteins from whole cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Thermo Scientific, Waltham, MA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Immunoblotting was performed with polyclonal rabbit anti-PU.1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-E2F1 antibody (Thermo Scientific), polyclonal goat anti-actin antibody (Santa Cruz Biotechnology), HRP-conjugated anti-rabbit secondary antibody (Pierce, Rockford, IL, USA) and HRP-conjugated anti-goat secondary antibody (Pierce). All antibodies were diluted according to the manufacturer’s protocol. Immunoreactive proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).
2.9 Transplantation Studies

Non-obese diabetic/severe combined immunodeficient/IL-2 receptor gamma chain null (NOD/SCID/γc⁻) (NSG) mice received 300 rad of irradiation prior to injection. NSG mice were used as they are the most immunodeficient xenotransplantation model to date (136). NSG mice not only lack T and B cells (like the NOD/SCID mouse), but also have defects in NK cell activity, macrophage function, complement activity, and dendritic cell function (136). The immunodeficiency of NSG mice means they lack the ability to reject foreign tissue (136), making them strong candidates for transplantation (136). Irradiation was given to further deplete recipient bone marrow cells. Each NSG recipient mouse received 1 x 10⁶ cells via tail vain injection. In separate experiments, recipient mice received either spleen cells from Sfpi1BN/BN mice, or cultured BN cells (cell line derived from Sfpi1BN/BN fetal liver progenitors, as previously described (102)).

Spleens were removed from 3-week-old, Sfpi1BN/BN neonates, homogenized, and subject to ACK (Ammonium-Chloride-Potassium) lysing buffer to remove erythrocytes. Spleen cells (splenocytes) were then washed three times in phosphate-buffered saline (PBS) (Lonza, Walkersville, MD). 1 x 10⁶ splenocytes were resuspended in 500-700 μl of PBS and kept on ice in preparation for transplant.

Cultured BN cells were washed three times in PBS. 1 x 10⁶ BN cells were resuspended in 500-700 μl of PBS and kept on ice in preparation for transplant.

Post transplantation, mice were monitored for symptoms of disease, specifically laboured breathing and scruffy appearance. When it appeared that the mouse was likely to die within 1-2 days, the mouse was euthanized via CO₂ overdose. Following euthanasia, recipient spleens were analyzed via flow cytometry.

2.10 Flow Cytometry

Single-cell suspensions from spleens of recipient mice were analyzed by flow cytometry, as well as single-cell suspensions of cultured BN cells. Single-cell suspen-
sions were washed in PBS containing 0.5 M EDTA (pH 8.0) and 0.5 % BSA prior to flow cytometry analysis. Cells were analyzed on a FACSCalibur (BD, Franklin Lakes, NJ). Antibodies directly conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), or peridinin-chlorophyll protein with cyanine dye (PerCP-Cy5.5) against the following markers were used: Mac-1/CD11b (M1/70), c-Kit/CD117 (2B8), FcγRII/III/CD32/CD16 (2.4G2), B4/CD19 (1D3), Ki-67 (M1K67). For Gr-1/Ly-6C (8C5) the primary antibody was conjugated to biotin and detected using streptavidin-PE secondary antibody. Analysis was performed using FlowJo version 9.3.2 (Ashland, OR) according to standard protocols. Gates on viable cells were set according to forward and side light scatter, as well as the exclusion of propidium iodide staining. Fluorescence activated cell sorting (FACS) was performed for transduced BN cells at the London Regional Flow Cytometry Facility (Robarts Research Institute) (London, Ontario, Canada) using a FACSARia III flow cytometer using the blue laser (488 nm) for green fluorescent protein (GFP) positive cells.

**2.11 Statistical Analysis**

Statistical significance was determined by unpaired, two-tailed Student’s t-test or one-way ANOVA and Tukey’s Multiple Comparison Test using GraphPad Prism v.6. Differences were considered significant with a p-value <0.05.
CHAPTER 3: RESULTS

3.1 Sfpi1^{BN/BN} spleen cells do not propagate in culture

We have previously shown that Sfpi1^{BN/BN} fetal liver cells can be cultured indefinitely in GM-CSF, whereas Sfpi1^{BN/+} and Sfpi1^{+/+} fetal liver cells cannot (unpublished data). We therefore wanted to establish whether the same would be true of Sfpi1^{BN/BN} spleen cells, compared to their Sfpi1^{BN/+} or Sfpi1^{+/+} counterparts. In order to determine whether Sfpi1^{BN/BN} spleen cells could be propagated in culture, Sfpi1^{BN/BN} spleen cells were obtained from a neonatal Sfpi1^{BN/BN} mouse (as well as a Sfpi1^{BN/+} littermate) and 10,000,000 cells from each mouse were placed into 10 ml of media containing the growth factor GM-CSF (1 ng/ml). Cells were passaged every 2-3 days and viable cell counts were recorded at each passage. At the first passage, the number of both Sfpi1^{BN/BN} and Sfpi1^{BN/+} spleen cells had dropped dramatically to just over 1,000,000 cells (Figure 3.1). By the second passage, viable cell counts decreased further to ~500,000, and a little over 300,000 at the third passage for both Sfpi1^{BN/BN} and Sfpi1^{BN/+} spleen cells (Figure 3.1). The experiment was repeated three times and two of the three times all spleen cells from both the Sfpi1^{BN/BN} and Sfpi1^{BN/+} were dead by the fourth passage (Figure 3.1). In one of the experiments, the spleen cells from the Sfpi1^{BN/BN} mouse, but not the heterozygote littermate, were able to grow past the fourth passage (up to passage number eight) but cell counts remained extremely low (data not shown). This experiment showed that Sfpi1^{BN/BN} spleen cells cannot be cultured, and thus colony forming assays could not be performed using these cells.

3.2 The effect of G-CSF on BN cell differentiation and growth

As mentioned, our laboratory has created a novel hypomorphic allele of Sfpi1 termed BN, which produces PU.1 at 20% of wild-type levels (102). Sfpi1^{BN/+} mice were mated to breed Sfpi1^{BN/BN} neonates. We have also generated a cultured cell line from
Figure 3.1 *Sfpi1*\(^{BN/BN}\) spleen cells do not propagate in culture. Spleen cells were obtained from a neonatal *Sfpi1*\(^{BN/BN}\) mouse (as well as *Sfpi1*\(^{+/BN}\) littermate) and 10 000 000 cells from each mouse were placed into culture containing GM-CSF (1 ng/ml). Cells were passaged and counted every 2-3 days until all cells were dead and experiment was terminated. Cell counts were recorded at start of experiment and each passage. Black bars represent spleen cells from *Sfpi1*\(^{BN/BN}\) mouse. Results are presented as the mean ± SD of three experiments (n=3).
$Sfpi1^{BN/BN}$ mice. The cell line is derived from $Sfpi1^{BN/BN}$ fetal liver progenitors (102). The cell line is referred to as BN (102). BN cells are cultured in the growth factor, GM-CSF, as they are a GM-CSF dependent cell line (unpublished data). They have also been shown to thrive in interleukin-3 (IL-3) (102).

Previous work by Panopoulos et al. showed that the cytokine G-CSF is capable of stimulating a pathway dependent on Stat3, a major G-CSF responsive signaling protein (137), that may result in the differentiation of myeloid cells. As a result, we attempted to assess the differentiation potential of cultured BN cells as a result of G-CSF by culturing them with or without G-CSF (10 ng/ml). In order to determine whether or not differentiation had occurred as a result of G-CSF treatment, we analyzed expression of the myeloid-specific, terminal differentiation cell surface marker CD11b (107). We also analyzed expression of the cell surface marker, c-Kit, which is a marker of myeloid progenitor cells (12). We hypothesized that if BN cells were able to differentiate as a result of exposure to G-CSF, CD11b would be up-regulated in BN cells grown with G-CSF compared to those grown without G-CSF. Furthermore, BN cells grown in G-CSF that had differentiated would down-regulate c-Kit. We cultured 4,000,000 BN cells in one of the following cytokine conditions: GM-CSF (10 pg/ml) alone, or GM-CSF (10 pg/ml) and G-CSF (10 ng/ml), and analyzed for CD11b and c-Kit expression via flow cytometry after 3 days of culture. Our results showed that there was no difference in either CD11b or c-Kit expression between BN cells grown with or without G-CSF (Figure 3.2). There was no significant difference ($p = 0.5537$) between mean fluorescence for CD11b of BN cells grown without G-CSF (black bar, Figure 3.2 B) versus those grown with G-CSF (grey bar, Figure 3.2 B). There was also no significant difference ($p = 0.9346$) between mean fluorescence for c-Kit of BN cells grown without G-CSF (black bar, Figure 3.2 D) versus those grown with G-CSF (grey bar, Figure 3.2 D). Therefore, our data suggests that G-CSF does not induce differentiation of BN cells based on the expression of the terminal differentiation marker CD11b, and the immature, progenitor marker, c-Kit.
Figure 3.2 BN cells cultured in G-CSF do not up-regulate expression of CD11b or down-regulate expression of c-Kit. Cultured BN cells were grown in media containing either GM-CSF (10 pg/ml) alone, or a combination of GM-CSF (10 pg/ml) and G-CSF (10 ng/ml). Following 3 days of culture, cells were analyzed by flow cytometry for expression of cell surface markers. (A) Histogram shows expression of CD11b, a myeloid-specific cell surface marker, for BN cells grown in GM-CSF alone (black), or a combination of GM-CSF and G-CSF (grey). (B) Bar graph shows the mean fluorescence of CD11b expression for BN cells grown in GM-CSF alone (black bar), or BN cells grown in GM-CSF and G-CSF (grey bar). (C) Histogram shows expression of c-Kit, a cell surface marker of myeloid progenitor cells, for BN cells grown in GM-CSF alone (black), or a combination of GM-CSF and G-CSF (grey). (D) Bar graph shows the mean fluorescence of c-kit expression for BN cells grown in GM-CSF alone (black bar), or BN cells grown in GM-CSF and G-CSF (grey bar). Histograms are representative images from one of three experiments (n=3), while bar graphs are presented as the mean ± SD of the three experiments.
We next investigated whether or not BN cells, a GM-CSF dependent cell line (unpublished data), could also be grown in G-CSF. We cultured 100 000 BN cells in one of the following cytokine conditions: GM-CSF (1 ng/ml) alone, GM-CSF (1 ng/ml) and G-CSF (10 ng/ml), or G-CSF (10 ng/ml) alone, and measured their proliferation after 4 days of culture. Cultured BN cells grown in GM-CSF alone or the combination of GM-CSF and G-CSF grew equally well; both showed over 20-fold expansion (Figure 3.3). Nonetheless, there was no significant difference (p = 0.8142) between BN cells grown in GM-CSF alone or the combination of GM-CSF and G-CSF. In contrast, cells grown in G-CSF only were unable to proliferate (Figure 3.3). BN cells grown in GM-CSF only proliferated significantly more than BN cells grown in G-CSF only (p = <0.0001) (Figure 3.3, ****). BN cells grown in the combination of GM-CSF and G-CSF also proliferated significantly more than BN cells grown in G-CSF only (p = 0.0170) (Figure 3.3, *).

Therefore, our data illustrates that BN cells are not responsive to G-CSF. G-CSF did not permit growth when used exclusively, but could be used in combination with GM-CSF. However, the combination of growth factors did not increase proliferation compared to GM-CSF used independently.

3.3 Co-culture with retroviral producing cells does not allow effective restoration of PU.1 expression

In order to test the hypothesis that restoration of PU.1 expression in cultured BN cells would result in cell cycle exit, reduced proliferation and ultimately differentiation, cultured BN cells were co-cultured with mitomycin C treated GP + E86 packaging cells, a retroviral producing cell line (Figure 3.4A). GP +E86 packaging cells were treated with mitomycin C to prevent proliferation. BN cells were grown in the presence of GP + E86 cells producing a previously constructed retrovirus (termed MIG-PU.1) that expresses a 3X-FLAG tagged PU.1 gene, as well as the marker GFP. As a control, BN cells were grown in the presence of GP + E86 cells producing an empty retroviral vector, (termed
Figure 3.3 The cytokine G-CSF does not enable nor inhibit growth of cultured BN cells. Cultured BN cells were grown in media containing either GM-CSF (1 ng/ml) alone (hatched bar), a combination of GM-CSF (1 ng/ml) and G-CSF (10 ng/ml) (black bar), or G-CSF (10 ng/ml) alone (white bar). 100 000 cells were plated in each cytokine condition and cell counts were taken following 4 days of culture. Results are presented as the mean + SD of one experiment (n=1) performed in triplicate. Significant differences are indicated with asterisk (*).
Cytokine treatment

Cell count (/100000)

GM-CSF
GM-CSF + G-CSF
G-CSF

Cell count (/100000)
MIGR1) where PU.1 was not introduced. The MIGR1 retroviral vector still expresses GFP. BN cells were cultured in direct contact with the retrovirus producing cells for 3 days before they were transferred to a second co-culture with fresh retroviral packaging cells. Following the two rounds of retroviral transduction, we attempted to select for BN cells where PU.1 expression had been restored, by culturing cells in media containing GM-CSF, as well as M-CSF. While BN cells are known to express the GM-CSF receptor, they do not express the M-CSF receptor. However, PU.1 activates expression of the M-CSF receptor (104) and theoretically, BN cells that successfully take up the MIGPU.1 retrovirus should be able to respond to stimulation with M-CSF and grow in the presence of M-CSF while uninfected cells should not (Figure 3.4A). As mentioned, MIG retroviruses are tagged with GFP and retrovirally infected BN cells will be GFP positive. The highest transduction frequencies achieved using the co-culture method are illustrated in Figure 3.4B. While transduction frequencies were able to reach a relatively high level, this did not occur consistently. Furthermore, infected BN cells that were recovered after selection in M-CSF were mostly dead based on trypan blue cell counting.

3.4 Inducible expression system enables restoration of PU.1, up-regulation of PU.1 target genes and differentiation

In order to assess the effect of PU.1 restoration on BN cells before cell death, we developed a technique in which we could systematically induce PU.1 expression. The system exploited two separate retroviral vectors, termed the regulator and the response. The regulator vector encoded the Tet-On 3G transactivator protein, as well as GFP (Figure 3.5A). The response vector contained the gene encoding PU.1 under the control of a TRE3G promoter (PTRE3GV), as well as a puromycin resistance gene (Figure 3.5A). In the presence of doxycycline (Dox), Tet-On 3G binds specifically to PTRE3GV and activates transcription of downstream PU.1. In order to set up the system, cultured BN cells were infected with the regulator and response retroviruses simultaneously (Figure
Figure 3.4 Infection of BN cells via co-culture. (A) Schematic of co-culture method used to restore PU.1 expression in BN cells. BN cells, a GM-CSF dependent cell line, were cultured in the presence of mitomycin C treated, GP + E86 packaging cells producing either MIGR1 or MIGPU.1 retrovirus for 3 days, after which they were transferred into a second culture of fresh GP + E86 packaging cells. Following the second 3-day co-culture, BN cells were transferred to media containing GM-CSF (10 pg/ml) and M-CSF (10 ng/ml) to select for infected cells. (B) Highest infection frequencies reached for BN cells infected with either MIGR1 (left panel) or MIGPU.1 (right panel) retrovirus using the co-culture technique. Open histograms show expression of green fluorescent protein (GFP). Black histograms represent negative (uninfected) cells. Histograms are representative images from one of three experiments (n=3); the percentage of infected BN cells +/- SD of the three experiments is listed in the top right hand corner.
**A**

BN cells

IMDM + GM-CSF

BN cells

GP + E86 retroviral producing packaging cells

BN cells

IMDM + GM-CSF + M-CSF

**B**

**MIGR1**

48.8 +/- 17.0%

**MIG-PU.1**

31.2 +/- 15.4%
Figure 3.5 Construction of inducible system used to restore expression of PU.1. (A) Two separate retroviral vectors were used in the inducible system. The regulator vector contains the Tet-On 3G transactivator protein, which in the presence of doxycycline is able to bind and activate expression of the PTRE3G inducible promoter. The PTRE3G promoter controls expression of PU.1 and is located in the pRetroX-TRE3G response vector. A selectable marker was also part of each vector. The regulator vector contains the gene encoding GFP and the response vector contains a puromycin resistance gene. (B) Cultured BN cells were infected with both regulator and response retroviral vector supernatants simultaneously via spin infection. Following infection, BN cells were cultured in media containing puromycin to select for BN cells that had successfully taken up the response vector containing the puromycin resistant gene. Puromycin resistant cells were then screened for GFP positivity using a combination of cloning and cell sorting to select for cells that also contained the regulator vector. Finally doxycycline was added at a concentration of 1000 ng/ml to BN cells infected with both regulator and response retroviruses, effectively turning on PU.1 expression.
A

LTR

\[ \text{Tet-On 3G} \]

IRESGFP

Regulator vector

\[ \text{Puro} \]

\[ P_{\text{TRE3GV}} \text{PU.1} \]

Response vector

B

Regulator

\[ \text{Response} \]

\[ \text{DOX} \]

\[ \text{Puro} \]

\[ \text{GFP} \]

Response

\[ \text{DOX} \]

\[ \text{PU.1} \]
Infected cells were selected by growth in the presence of puromycin (Figure 3.6A). Puromycin resistant cells were also screened for GFP positivity by flow cytometry. FACS for GFP expression was conducted to enrich a population of purified GFP positive cells (Figure 3.6B,D). The presence of the Tet-On 3G protein was confirmed in selected cells by immunoblot using the TetR monoclonal antibody (Figure 3.6C). GFP positive, puromycin resistant BN cells were then grown in culture with doxycycline (1000 ng/ml) to induce PU.1 expression (Figure 3.5B). BN cells successfully infected with both retroviral vectors expressed high levels of PU.1, when cultured in the presence of doxycycline, as confirmed by immunoblot (Figure 3.7A). Finally, CD11b expression was analyzed by flow cytometry in dox induced versus non-induced cells. CD11b is directly activated by PU.1 (107) and therefore PU.1 reintroduction should result in an up-regulation of CD11b expression. As well, CD11b is a cell surface marker, specific to mature myeloid cells (107) and indicates differentiation. As expected, CD11b was up-regulated in dox induced BN cells compared to non-induced cells (Figure 3.7B). Thus, following PU.1 restoration, BN cells showed signs of differentiation by expressing a terminal differentiation marker, CD11b.

3.5 Restoration of PU.1 expression in cultured BN cells results in reduced proliferation and cell cycle exit at the G0/G1 phase

Once PU.1 restoration was established, we assessed the biological effect of restoration in cultured BN cells. In order to address the hypothesis that PU.1 restoration will allow immature progenitors to exit the cell cycle and differentiate, we looked at the ability of BN cells to grow over 4 days of culture, in the presence of various concentrations of doxycycline. At a concentration as low as 100 ng/ml, induction resulted in a decrease in cell proliferation, compared to untreated control (Figure 3.8). The cell count was significantly lower (p < 0.05) (Figure 3.8, *) for regulator and response infected BN cells grown in the presence of doxycycline (100 ng/ml) than infected BN cells grown in
Figure 3.6 Selection of BN cells infected with both regulator and response retroviral supernatants. (A) Selection of BN cells successfully infected with the response vector. (+) indicates retroviral infected BN cells. (-) indicates uninfected BN cells. Cells able to grow in the presence of puromycin contain the response vector (n=1). (B) Selection of BN cells successfully infected with the regulator vector (n=1). GFP positive cells indicate the presence of the regulator vector. The histogram shows expression of GFP. Red represents negative (uninfected) BN cells. Blue represents regulator and response infected BN cells. (C) An immunoblot was conducted to confirm the presence of the Tet-On 3G transactivator protein in GFP positive cells using the TetR monoclonal antibody (n=1). b-actin served as a control. (D) Using fluorescence microscopy, an image was taken of puromycin resistant, GFP⁺ve BN cells (n=1).
Retrovirus infected BN cells

Cell count

A

Retrovirus infected BN cells

B

GFP

C

Non-induced

Dox-induced

30 kDa

TetR

43 kDa

Actin

D
Figure 3.7 Restoration of PU.1 expression and up-regulation of CD11b following induction. (A) An immunoblot was conducted using anti-PU.1 antibody to demonstrate PU.1 restoration following induction with doxycycline (1000 ng/ml) (n=1). An anti-actin antibody was used as a control. (B) Flow cytometry on induced BN cells, 5 days following doxycycline (1000 ng/ml) induction. The histogram shows expression of CD11b, a direct target of PU.1. Open histogram represents non-induced BN cells while filled black histogram represents CD11b expression after doxycycline treatment of BN cells. Histogram is a representative image from one of three experiments (n=3).
A

Non-induced  Dox-induced

PU.1

Actin

B

# Cells

CD11b
Figure 3.8 Restoration of PU.1 expression following induction by doxycycline results in a block in proliferation. Cell growth of retrovirally infected BN cells at various concentrations of doxycycline (black bars). Uninfected BN cells (white bars) were grown in the presence of doxycycline (1000 ng/ml) as a control for possible growth inhibition by doxycycline. Results are presented as the mean and SD of three experiments (n=3). Cell growth at each different doxycycline concentration of retroviral infected BN cells was compared to cell growth of infected BN cells grown in the absence of doxycycline, following 4 days of culture. Significant differences are indicated with asterisk (*).
Doxycycline concentration (ng/ml)

Cell count (/100000)

0 500000 1000000 1500000 2000000 2500000

5000 1000 1000 100

Uninfected BN cells

Regulator and response infected cells

Cell count (/100000)

0 500000 1000000 1500000 2000000 2500000

0 1000 5000 10000 100

Doxycycline concentration (ng/ml)
the absence of doxycycline (Figure 3.8). There was a gradual reduction in cell counts as the concentration of doxycycline increased (Figure 3.8). To eliminate the possibility that the doxycycline was toxic to the cells, we grew uninfected BN cells in the presence of a high concentration of doxycycline (1000 ng/ml) (Figure 3.8). The cell counts were not significantly different (p > 0.05) between uninfected BN cells grown in the presence of doxycycline (1000 ng/ml) and those grown in the absence of doxycycline (Figure 3.8), which indicated that the concentration of doxycycline being used for induction was not toxic. These results suggest that increased PU.1 expression might be promoting cell cycle exit or apoptosis.

The reduction in proliferation following PU.1 restoration prompted investigation into the cell cycle. Cell cycle analysis was conducted using BrdU and 7-AAD to identify cells actively synthesizing DNA during the different stages of the cell cycle (G0/G1, S, or G2/M). This technique permits quantification of the frequency of cells in each phase of the cell cycle. Doxycycline induced cells were mostly apoptotic (47.5%) (Figure 3.9B). Importantly, more non-induced cells were in S phase (52.5%), compared to doxycycline induced cells (25.2%) (Figure 3.9C,D), implying a block in the G1 to S phase transition following PU.1 restoration. These results suggest that PU.1 restoration results in cell cycle exit, specifically at the G1 to S phase transition.

3.6 PU.1 regulates the cell cycle by repressing E2F1

Previous work in our lab determined an inverse relationship between PU.1 expression and the cell cycle regulator E2F1 (unpublished data). In Sfpi1\(^{BN/BN}\) mouse spleen cells, where PU.1 levels were reduced, E2F1 levels were increased compared to Sfpi1\(^{BN/+}\) mice (unpublished data). Our cell cycle analysis results support this finding as E2F1 functions in the transition from G1 to S phase (126). As a result, we hypothesized that PU.1 regulates the cell cycle via repression of E2F1. To test this hypothesis, we examined whether E2f1 transcript and E2F1 protein was reduced following PU.1 restoration in
Figure 3.9 PU.1 restoration blocks G1 to S phase transition. Cell cycle analysis was conducted to determine the position of cells within the cell cycle. Cycling of non-induced BN cells (white) was compared to doxycycline induced BN cells (black). (A) Gating strategy used (gating of non-induced cells is shown); G1 represents apoptotic cells; G2 represents live cells. (B) Quantification of the percentage of apoptotic (G1) and live (G2) cells under the two conditions (non-induced (white) compared to doxycycline induced (black) BN cells). (C) BrdU incorporation was measured by flow cytometry on G2 cells in non-induced BN cells (white) and doxycycline induced BN cells (black). (D) Quantification of the percentage of cells in S phase as indicated by BrdU incorporation for non-induced BN cells (white) and doxycycline induced BN cells (black).
A

B

C

D

Apoptotic, G1
Non-apoptotic, G2

Percentage of cells

Percentage of cells

BrdU# Cells

S phase

Non-induced
Dox induced

Non-induced
Dox induced

Non-induced
Dox induced

Non-induced
Dox induced

BrdU

# Cells

BrdU
cultured BN cells. Reverse transcriptase qPCR (RT-qPCR) analysis demonstrated a 3.1 fold reduction in E2f1 transcripts in BN cells where PU.1 expression had been restored (Figure 3.10). Western blot analysis confirmed a substantial reduction in E2F1 protein in doxycycline induced BN cells compared to non-induced cells (Figure 3.11). In conclusion, our results establish PU.1 as a repressor of E2f1 gene expression.

3.7 The spleens of Sfpi1^BN/BN mice contain a population of highly proliferative myeloid cells

As described in the introduction, our laboratory has developed a mouse model in which mice homozygous for the Sfpi1 BN allele produce PU.1 at 20% of wild-type levels (102). Mice homozygous for Sfpi1 allele (Sfpi1^BN/BN mice) also demonstrated hyperproliferation of immature myeloid cells in the bone marrow, spleen and other tissues (102). To demonstrate increased proliferation in the spleens of Sfpi1^BN/BN mice, flow cytometric analysis was performed for the nuclear protein Ki-67, as well as the myeloid specific cell surface marker CD11b. Ki-67 is highly expressed in proliferative cells; it is present during active cell cycle but absent from resting cells (138). Flow cytometric analysis was performed using single-cell suspensions from the spleens of 21-day Sfpi1^BN/BN and Sfpi1^+/+ mice. There was an up-regulation of both CD11b and Ki-67 in Sfpi1^BN/BN spleen cells compared to Sfpi1^+/+ mice (Figure 3.12). In the representative images, the mean fluorescence intensity of Ki-67 expression in Sfpi1^BN/BN spleen cells was 752 compared to 459 in Sfpi1^+/+ spleen cells (Figure 3.12). Flow cytometric analysis of Sfpi1^BN/BN spleens demonstrated an increase in the frequency of myeloid, proliferative cells compared to littermate control, as indicated by CD11b and Ki-67 expression, respectively (Figure 3.12). These results suggest that myeloid proliferation is increased in the spleens of Sfpi1^BN/BN mice.

3.8 Transplantation studies
Figure 3.10 *E2f1* is down-regulated upon induction of PU.1 with doxycycline. RT-qPCR was performed to determine relative frequencies of *E2f1* transcripts in doxycycline induced BN cells compared to non-induced BN cells. RNA was prepared from retroviral infected BN cells cultured in the presence of doxycycline (1000 ng/ml) and absence of doxycycline. Fold change is shown in reference to the housekeeping gene beta-2-microglobulin (B2M). Results are presented as the mean ± SD of three experiments (n=3) performed in triplicate. Significant differences are indicated with asterisk (*).
Gene of interest

Relative transcript levels

B2M  E2F1

Gene of interest
Figure 3.11 E2F1 protein level is decreased upon induction of PU.1 with doxycycline.
An immunoblot was conducted using anti-E2F1 antibody to demonstrate E2F1 repression by PU.1 following PU.1 restoration with doxycycline (1000 ng/ml) (n=1). As a control an immunoblot was conducted using anti-actin antibody.
Non-induced  Dox induced

E2F1

Actin
Figure 3.12 *Sfpi1*<sup>BN/BN</sup> myeloid cells are increased in frequency *in vivo*. The spleens of 21-day *Sfpi1*<sup>BN/BN</sup> mice (right panels) contain a proliferative (Ki-67<sup>high</sup>), myeloid (CD11b<sup>+</sup>) population that is increased compared to wild-type (wt) littermates (left panels). Flow-cytometric analysis of CD11b, a myeloid-specific cell surface marker, and Ki-67, a nuclear protein highly expressed in proliferative cells, was performed using single-cell suspensions from the spleens of 21-day *Sfpi1*<sup>BN/BN</sup> or *Sfpi1*<sup>+/+</sup> mice. Flow-cytometric analysis of single-cell suspensions from the spleens of 21-day *Sfpi1*<sup>BN/BN</sup> mice (right panels) demonstrates a larger population of CD11b<sup>+</sup> myeloid cells (top) as well as an upregulation of Ki-67 (bottom), in comparison to *Sfpi1*<sup>+/+</sup> mice (left panels). Histograms are representative images from one of two experiments (n=2); the percentage of CD11b +/- SD cells of the two experiments is listed in the top two plots. The mean fluorescence (MF) of Ki-67 expression of the representative image is listed in the bottom two plots.
$Sfpi1^{+/+}$

**CD11b**

- MF = 459

- 8.27 +/- 2.7%

$Sfpi1^{BN/BN}$

**CD11b**

- MF = 752

- 34.4 +/- 7.6%
In order to determine whether reduced expression of PU.1 in Sfpi1\(^{BN/BN}\) myeloid cells is sufficient to cause the development of AML in mice, transplantation studies were conducted. NSG mice were each injected with either 1 x 10^6 splenocytes from Sfpi1\(^{BN/BN}\) mice, or cultured BN cells. Recipient mice were monitored for symptoms of AML and when a mouse showed laboured breathing and scruffy appearance (likely to die within 1-2 days), the mouse was euthanized and the morphology of the spleen was analyzed, as highly enlarged spleens are characteristic of leukemic mice (Figure 3.13). Single cell suspensions were prepared from the spleens of recipient mice and analyzed for engraftment and myeloid cell composition by flow cytometry. In order to conclude that mice have become sick due to AML, a population of immature, myeloid cells must be present. To verify the composition of cells, CD11b and c-Kit were used. As previously mentioned, c-Kit is expressed at high levels in immature progenitors (12), while CD11b is a cell surface marker of the myeloid lineage (107). Other myeloid cell surface markers, such as Gr-1 and FcγRII/III were also analysed. CD19, a B cell-specific marker (139) was also examined to exclude the possibility of a B cell leukemia. The cell surface marker CD45.2 was used for donor cells from Sfpi1\(^{BN/BN}\) mice to indicate whether or not engraftment took place. CD45.2 is expressed on the surface of donor cells (Figure 3.14A) but not in the recipient NSG mice; whereas NSG mice express the CD45.1 cell surface marker (Figure 3.14A). Because the cultured BN cell line did not express CD45.2 (Figure 3.16A), we generated a line of GFP +ve BN cells (Figure 3.16B), used to determine the origin of the cell population present in recipient mice for transplant work with the cultured BN cells.

NSG mice transplanted with Sfpi1\(^{BN/BN}\) spleen cells (n = 6) had a median survival of 50 days (Figure 3.13A), while NSG mice transplanted with cultured BN cells (n = 8) had a median survival of 90.5 days (Figure 3.13B). A large population of immature (c-Kit\(^+\)), myeloid (CD11b\(^+\), CD19\(^-)\) cells was present in the spleens of NSG mice injected with Sfpi1\(^{BN/BN}\) spleen cells (Figure 3.15 A, B, C, middle panels) or BN cells (Figure 3.15 A, B, C, right panels), compared to wild-type spleens (Figure 3.15 A, B, C, left panels).
Figure 3.13 NOD/SCID/\( \gamma_c \) mice transplanted with \( Sfpi1^{BN/BN} \) splenocytes or cultured BN cells become sick and require euthanasia after transplantation. (A) Survival of NOD/SCID/\( \gamma_c \) (NSG) mice (n=6) injected with splenocytes from \( Sfpi1^{BN/BN} \). The median survival was 50 days. (B) Survival of NSG mice (n=8) injected with cultured BN cells. The median survival was 90.5 days.
A

Survival of NSG(s) injected with $Sfpi^{BN/BN}$ splenocytes

B

Survival of NSG(s) injected with cultured BN cells
Figure 3.14 NOD/SCID/γc mice transplanted with $Sfpi1^{BN/BN}$ splenocytes or cultured BN cells contain a donor-derived population of cells.

Flow cytometric analysis was conducted using single cell suspensions from the spleens of wild type C57/Bl6 mice, wild type NOD/SCID/γc (NSG) mice, NSG recipient mice transplanted with $Sfpi1^{BN/BN}$ splenocytes, or NSG recipient mice transplanted with cultured BN cells. $Sfpi1^{BN/BN}$ splenocytes were isolated from C57/Bl6 $Sfpi1^{BN/BN}$ mice. $Sfpi1^{BN/BN}$ splenocytes and cultured BN cells were isolated and transplanted by tail vein injection into sublethally irradiated NSG recipients. Recipient mice were injected with $1 \times 10^6$ cells/recipient mouse.

(A) Donor mice (C57/Bl6) express CD45.2 (grey histograms) while recipient mice (NSG) express CD45.1 (black histograms). Histograms are representative images from one of three experiments (n=3). (B) The dot plots show expression of the cell surface markers CD45.1 and CD45.2. Cultured BN cells did not express either CD45 marker. Engraftment of $Sfpi1^{BN/BN}$ CD45.2$^+$ donor cells in NSG CD45.1$^+$ recipient mice is shown in the middle dot blot. Engrafted cultured BN cells are CD45.1$^-$ and CD45.2$^-$ (right dot plot).
Wild type (C57Bl/6)

Wild type (NSG)

A

CD45.1 CD45.2

0.15% 99%

67% 9.98%

B

Wild type (C57Bl/6) NSG injected with Sfpi1BN/BN splenocytes NSG injected with cultured BN cells

98.8% 85.4% 4.81%

0.569% 14.5% 92.9%
Figure 3.15 NOD/SCID/γc mice transplanted with $Sfpi1^{BN/BN}$ splenocytes or cultured BN cells contain a large population of immature (c-Kit$^+$), myeloid (CD11b$^+$, FcγRII/III$^+$, Gr-1$^+$, CD19$^-$) cells, indicative of AML. Flow cytometric analysis was conducted using single cell suspensions from the spleens of WT C57/Bl6 mice (left panels), NOD/SCID/γc (NSG) recipient mice transplanted with $Sfpi1^{BN/BN}$ splenocytes (middle panels), or NSG recipient mice transplanted with cultured BN cells (right panels). $Sfpi1^{BN/BN}$ splenocytes were isolated from C57/Bl6 $Sfpi1^{BN/BN}$ mice. $Sfpi1^{BN/BN}$ splenocytes and cultured BN cells were isolated and transplanted by tail vein injection into sublethally irradiated NSG recipients. Recipient mice were injected with 1 x 10$^6$ cells/recipient mouse. Histograms show expression of c-Kit (A), which is a cell surface marker of immaturity, CD11b (B) a myeloid-specific cell surface marker, and CD19, a B cell-specific marker (C). Histograms also show expression of FcγRII/III (D) and Gr-1 (E), both myeloid cell surface markers. Open histograms represent the negative (unstained) control. Histogram gating strategies are shown in Figure 3.14 on the CD45.2$^+$ population (wt C57/Bl6 mice and NSG recipient mice transplanted with $Sfpi1^{BN/BN}$ splenocytes) and CD45.1$^-$ population (NSG recipient mice transplanted with cultured BN cells). All histograms are representative images.
Wild type (C57Bl/6) NSG injected with Sfpi1
BN/BN splenocytes NSG injected with cultured BN cells

A  c-Kit

B  CD11b

C  CD19

D  FcyRII/III

E  Gr-1

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Spleen cells of NSG transplanted with either $Sfpi1^{BN/BN}$ spleen cells or BN cells also expressed high levels of FcγRII/III and Gr-1, compared to wild-type spleens (Figure 3.15 D, E). Engraftment was demonstrated in NSG mice transplanted with $Sfpi1^{BN/BN}$ spleen cells by the CD45.2 expression of cells present in recipient spleens (Figure 3.14 B middle dot plot). Engraftment was suggested in NSG mice transplanted with cultured BN cells by the lack of CD45.1$^+$ cells (Figure 3.14 B right dot plot). Engraftment was confirmed in NSG mice transplanted with GFP $^+$ve BN cells by the population of cells expressing GFP in recipient spleens (Figure 3.16C). Finally, upon euthanasia, recipient mice had significantly enlarged spleens (data not shown), a characteristic of AML. Taken together, these results suggest that NSG transplanted with $Sfpi1^{BN/BN}$ splenocytes or cultured BN cells become sick with disease resembling AML following transplantation.
Figure 3.16 Generation of a GFP⁺ve BN cell line to use in transplantation studies.
(A) Cultured BN cells did not express the CD45.2 cell surface marker (n=1). (B) Cul-
tured BN cells were made GFP⁺ve using a retroviral infection in order to identify donor
cell population in transplantation studies (n=1). (C) Flow-cytometric analysis was con-
ducted using single-cell suspensions from the spleen of a NOD/SCID/γc (NSG) recipi-
ent mouse transplanted with cultured GFP⁺ve BN cells. The right panel shows a GFP⁺ve 
population present in a NSG recipient spleen, demonstrating engraftment (n=1). The 
left panel shows the spleen of a NSG injected with non-GFP⁺ve BN cells (n=1).
A. Cultured BN cells

B. Cultured GFP+ve BN cells

C. NSG injected with cultured BN cells

C. NSG injected with cultured GFP+ve BN cells
CHAPTER 4: DISCUSSION

4.1 Overview

The goal of this project was to determine the role of PU.1 in cell cycle regulation and the development of AML. We have shown that a reduction in PU.1 expression resulted in the development of AML in NOD/SCID/gc mice ~70 days after transplantation. Based on our in vitro results, we propose that the development of AML in these mice was likely due to lack of cell cycle exit and terminal differentiation, which corresponds to insufficient PU.1 levels. Our work demonstrated that restoration of PU.1 expression promotes cell cycle exit and permits terminal myeloid differentiation. Furthermore, we have elucidated the mechanism by which cell cycle exit is able to occur; recovered expression of PU.1 resulted in repression of the potent cell cycle activator, E2F1. Taken together, these results provide strong evidence that PU.1 regulates the cell cycle through repression of E2F1, and insufficient E2F1 repression due to reduced levels of PU.1 expression results in the development of AML.

4.2 Restoration of PU.1 expression results in decreased proliferation, increased differentiation, and cell cycle block in G1 to S phase transition

While the phenotype of mice and cultured cells with reduced expression of PU.1 have been previously characterized (47, 102), we sought to determine if the characteristics of these mice and cells could be reversed upon PU.1 restoration. In order to do this, we used a cell line, derived from the fetal liver cells of Sfpi1BN/BN mice (BN cells). In order to reintroduce PU.1, we employed a retrovirus encoding a PU.1 cDNA that was under the control of an inducible promoter. This strategy allowed us to precisely induce expression of PU.1 in the presence of doxycycline. Upon induction with doxycycline, proliferation of BN cells decreased dramatically. Furthermore, BN cells in which PU.1 expression had been restored showed the progression of differentiation by expressing higher levels of the terminal differentiation marker CD11b. In summary, PU.1 restoration enabled cell
cycle exit, and induced cell differentiation, demonstrating that myeloid cell proliferation/differentiation was strictly related to PU.1 levels, implying a role for PU.1 in cell cycle regulation.

To assess the role of PU.1 in cell cycle progression, we measured 5-bromodeoxyuridine (BrdU) incorporation in combination with 7-amino-actinomycin D (7-AAD) staining in vitro. BrdU permits identification of cells that are actively synthesizing DNA while 7-AAD defines cell cycle stage (G0/1, S, or G2/M). We compared cell cycle stage in PU.1-deficient BN cells versus doxycycline induced BN cells, where PU.1 expression had been restored. BrdU/7-AAD staining revealed that PU.1-deficient BN cells, were actively cycling, as evidenced by an increased number of S stage cells. In contrast, doxycycline induced cells had a large proportion of cells that were apoptotic. This experiment suggests that PU.1 inhibits the G1 to S phase transition, allowing cells to exit the cell cycle and differentiate. This concept is supported by the role PU.1 plays in terminal myelopoiesis. Several groups have shown that PU.1 levels increase during granulocytic and monocytic differentiation (47, 87-88, 140). When PU.1 levels are decreased to only 20% of wild-type levels, as in the BN cell line, cells are deregulated and remain in the cell cycle, unable to differentiate. This supports the phenotype of Sfpi1^{BN/BN} mice that contain a highly proliferative, immature myeloid population (102). In summary, a role for PU.1 in cell cycle regulation is consistent with the necessity of PU.1 in terminal myeloid differentiation. In addition, cells must properly exit the cell cycle for differentiation to occur. Due to the requirement of PU.1 for terminal myeloid differentiation, it makes sense that PU.1 drives cell cycle exit.

Our results are in agreement with the recently published data of Staber et al. that shows that PU.1 regulates proliferation in HSCs and that this effect is directly related to PU.1 levels (141). This group discovered that compared to wild-type mice, the proliferative fraction of HSCs was doubled and there was a substantial increase in S, G2, and M phase cells in mice expressing reduced levels of PU.1 (141). Importantly, restoration of
PU.1 levels reversed the S/G2/M fraction to normal levels (141). Of note, the mouse knock-in model (PU.1 \textit{ki/ki}) used in the work by Staber \textit{et al.} (with targeted disruption within the -14 kb URE of murine PU.1) had unchanged PU.1 levels in the whole bone marrow compartment, but HSCs specifically demonstrated a PU.1 reduction to 40% of wild-type levels (141). Comparing this mouse model to that of Rosenbauer \textit{et al.}, which deleted the entire -14 kb enhancer region of PU.1, shows that the major phenotypic difference between the two strains appeared after the HSC stage, in the myeloid progenitor compartment (47, 141). FACS profile for LIN\textit{-}SCA\text{'}\textit{KIT}+ HSCs was similar between strains; the myeloid progenitor profile was completely disturbed in Rosenbauer \textit{et al.}’s URE-KO mice, whereas it appeared mostly normal in Staber \textit{et al.}’s PU.1\textit{ki/ki} mice (47, 141). Furthermore, HSCs of URE KO mice progressed to leukemia whereas PU.1\textit{ki/ki} mice did not (47, 141). In comparing our study to that of Staber \textit{et al.}, two important differences emerge between their model and our group; in the Staber \textit{et al.} model PU.1 expression was reduced to 40% of wild-type levels (whereas ours was 20%) and reduced PU.1 expression was restricted to HSCs (while our reduction in PU.1 expression was applicable to all blood cell types, in which PU.1 is normally expressed) (102). Furthermore, Staber \textit{et al.} investigated the long-term repopulation potential of HSCs (141), while we looked at the ability of reduced expression of PU.1 in myeloid progenitors to induce AML. As well, our mouse model developed leukemia, whereas theirs does not. This suggests that the reduction in PU.1 expression must be sufficiently low and widespread in order to block terminal differentiation, leading to the development of AML.

\textbf{4.3 E2F1 and its role in myeloid proliferation/differentiation}

The finding that PU.1 restoration inhibited cell cycle progression, specifically at the G1 to S phase supported previous unpublished work in our lab that found an inverse correlation between PU.1 and E2F1 protein levels. E2F1 is a transcription factor that promotes cell cycle progression and S-phase entry (123), by controlling the transcription of
genes required for cell division (126). Our results showed that following PU.1 restoration, both transcript and protein levels of E2F1 were down-regulated, indicating a repressive function for PU.1 on E2F1. In support of a role for E2F1 in the blood cell system, Zhu and colleagues have reported E2F1 as a regulator of hematopoietic cell proliferation and differentiation (142). Also, Amanullah et al. have demonstrated that E2F1 is a negative regulator of myeloid differentiation (143). They showed that deregulated expression of E2F1 blocked terminal myeloid differentiation, and promoted leukemogenicity in interleukin-6 (IL-6) treated M1 cells in vivo (143). This finding nicely supports our evidence that reduced PU.1 levels and deregulated E2F1 levels (due to lack of PU.1 repression), result in the development of AML.

The strength of E2F1 as an activator of genes that are essential for cellular proliferation is highlighted by the fact that E2F1 can override various growth-arrest signals, including TGF-β and the CKIs p16, p21, and p27, in some situations (144-146). This finding agrees with the role of E2F1 in cancer development, as deregulation of E2F1 has been largely implicated in human cancers (125, 130). Not unexpectedly, Rb function is compromised in most, if not all, of human cancers (130). Binding by Rb to E2F1 inhibits its transcriptional activation capacity (130); thus when Rb function is compromised, E2F1 is deregulated.

Other myeloid specific transcription factors have also been associated with cell cycle regulation both positively and negatively. The proto-oncogenes c-myb and c-myc are positive regulators of cell cycle progression (34), while C/EBPα is a negative regulator and promotes cell-growth arrest by coordinating exit from the cell cycle. C/EBPα has been shown to inhibit G1 to S phase transition in myeloid cells (147-148). Perhaps the most important mechanism by which C/EBPα exerts its effect is by direct binding to E2F1 where the interaction between C/EBPα and E2F1 enables growth inhibition in myeloid cells and was required for terminal differentiation (149-151). Due to the necessity of both C/EBPα and PU.1 in myelopoiesis, it would not be surprising if they both
shared the ability to promote cell cycle exit via repression of E2F1.

Lastly, Gibbs et al. have shown that the protein product of the PU.1 target gene Egr-1 is able to abrogate the E2F1 block in terminal myeloid differentiation and consequently suppress leukemia (134). In concordance with the fact that Egr-1 functions as a tumor suppressor when myeloid differentiation is blocked by E2F1, is the discovery that the human EGR-1 gene is frequently deleted or subject to monosomy in patients with myelodysplastic syndromes or AML (152-153). Thus, the tumour suppressor role of Egr-1 may represent a strong candidate for differentiation treatment in certain types of leukemias.

4.4 The mechanism by which PU.1 regulates the cell cycle

While we have successfully demonstrated PU.1 repression of E2f1 gene expression, the detailed mechanism by which PU.1 is regulating the expression remains unclear. PU.1 may directly bind the E2f1 promoter and repress transcription. Alternatively, the mechanism may be indirect, in which case PU.1 might activate another regulator which in turn blocks E2f1 transcription.

Support for an indirect mechanism of repression comes from published evidence that PU.1 activates expression of a microRNA (miRNA), miR223, which then goes on to disrupt translation of E2F1 protein (154-156). MicroRNAs are noncoding RNA molecules that regulate the expression of target genes by binding imperfectly with regions in target messenger RNAs (mRNAs) (154). MicroRNAs control gene expression through both translational repression and degradation of target mRNAs (154). Recently it has been shown that the primary mechanism of action of miRNAs is translational inhibition and that this must occur before mRNA may be targeted for degradation (154). PU.1 has been shown to activate miR223 in the myeloid lineage (155). Pulikkan et al. have demonstrated that miR223 targets and represses E2F1 during granulopoiesis (156). They went on to report that E2F1 protein was up-regulated in miR223 null mice and that miR223
blocks cell cycle progression in myeloid cells (156), providing substantial evidence for
the claim that miR223 targets E2F1. Another group has confirmed the role of miR223 in
terminal myeloid differentiation by showing that miR223 knockout mice display defec-
tive granulopoiesis (157). Furthermore, miR223 is down-regulated in different subtypes
of AML (156) and miR223 is inactivated by the AML1-ETO fusion oncoprotein, which
is present in many cases of AML (158). All this data supports a pathway by which, under
normal conditions, PU.1 is expressed in myeloid progenitors, resulting in miR223 activa-
tion, and subsequent suppression of E2F1, cell cycle arrest at the G1 to S phase bound-
ary, and terminal myeloid differentiation. Conversely, when PU.1 is expressed at reduced
levels, miR223 is not expressed, resulting in deregulated E2F1 protein levels, continual
cell cycle progression of immature myeloid cells, and ultimately AML.

Alternatively, one group has shown, through chromatin immunoprecipitation
(ChIP), that there is a PU.1 binding site within the E2f1 promoter (159), suggesting a
direct mechanism of repression. Staber et al. also went on to elucidate the mechanism
through which PU.1 levels control the cell cycle. Using ChIP sequencing, they found
that PU.1 positively regulated the transcription of cell-cycle inhibitors GFI1 and Cdkn1a
(p21) and negatively regulated the transcription of the cell-cycle activators CDK1, E2F1,
and Cdc25a through direct binding to their promoters and enhancers (141).

In disagreement with the findings of Wontakal et al. and Staber et al., there is
evidence that PU.1 does not function as a repressor (160). While Staber et al. demon-
strated that PU.1 binds cell cycle activator promoters and enhancers, they did not actually
demonstrate repression as indicated by chromatin remodeling. Nevertheless, Staber et al.
demonstrated increased cell cycle inhibitor activity with increasing amounts of PU.1, and
decreased cell cycle activator activity (141). This finding suggests PU.1 is able to repress
cell cycle activators, such as E2F1. As mentioned, PU.1 is normally associated with tran-
scriptional activation (160), and it is not known how PU.1 might act as a repressor.
4.5 Reduced PU.1 levels result in AML

In order to conclusively demonstrate that reduced expression of PU.1 is sufficient to induce AML, transplantation studies were conducted. We injected $1 \times 10^6$ spleen cells from $Sfpi1^{BN/BN}$ mice into NSG recipient mice. All the recipient mice that received transplanted cells developed AML, and required euthanasia within 6-12 weeks. Recipient mice had splenomegaly due to an invasion of myeloblastic cells. We also injected $1 \times 10^6$ cultured BN cells, into NSG recipients and found the same results as those obtained using the $Sfpi1^{BN/BN}$ splenocytes. Our results confirm what Dr. Tenen’s group found in 2004 (Rosenbauer et al. 2004). Like our lab, Dr. Tenen’s group also characterized the phenotype of mice expressing reduced levels of PU.1, and performed transplantations. Importantly, while both mouse models displayed PU.1 expression at 20% of wild-type levels, the strategy used to reduce PU.1 expression was different. Dr. Tenen’s group deleted the entire -14 kb enhancer region of PU.1, while we targeted a coding region. As well, the length of time it took for mice to develop the disease, differed between studies. Mice homozygous for this ΔURE neo allele in Rosenbauer et al.’s work developed leukemia in as early as 3 weeks (47), however it took at least 6 weeks for our mice to become sick. Furthermore, Dr. Tenen’s group did not propose a mechanism behind the development of AML in their mice. In another collaborative paper, it was mentioned that HSCs of URE KO mice progress to leukemia, and it is likely that dysregulated cell cycle regulators might be involved (141). Our work suggests that PU.1 repression of the cell cycle activator E2F1 is involved in cell cycle exit in the myeloid lineage.

Together, these results indicate that reduced expression of PU.1 is directly responsible for the development of AML and suggest that restoration of PU.1 expression in individuals with AML could be a therapeutic approach to restore normal myeloid differentiation. The potency and relevance of E2F1 in cell cycle progression and cancer make it an extremely attractive target in the treatment of AML. The association between PU.1 levels and E2F1 may be manipulated in the future treatment of cancer.
4.6 Future directions

This project provided insight into the role that PU.1 plays in cell cycle regulation and the development of AML. Nonetheless, certain issues still need to be addressed. Primarily, the mechanism by which PU.1 regulates E2F1 must be determined. Several experiments are proposed that could illustrate whether the mechanism is indirect or direct.

In order to demonstrate indirect repression, manipulation of miR223 expression is required. Firstly, it must be established that reintroduction of PU.1 into cultured BN cells induces miR223 expression. Secondly, it needs to be determined whether transduction of cultured BN cells with miR223 using a retroviral vector, blocks, or at the very least, slows proliferation. As well, E2F1 protein levels should be assessed, following transduction with miR223. This is a key experiment because PU.1 expression is not being affected, but the block in proliferation would be attributed to miR223 translational repression of $E2f1$ mRNA, indicating an indirect mechanism of repression. However, miR223 may not be the only form of regulation, or may not play a role at all, and other mechanisms of indirect repression might exist. Exploring the role of miR223 would still be worthwhile, however, given the large amount of data supporting the indirect pathway involving miR223.

In order to demonstrate direct repression, ChIP sequencing is required to compile predicted PU.1 binding sites within the regulatory regions of the $E2f1$ gene. Next, validation of the predicated PU.1 binding sites would be performed using standard ChIP. To confirm a repressive function, mutagenesis of the PU.1 binding site(s) within the $E2f1$ gene would be conducted, in AML cells. Mutagenesis could be accomplished using zinc-finger nucleases, which create double-strand breaks in target DNA sequences (161). If mutagenesis of the PU.1 binding site relieves repression of $E2f1$ transcriptional activity, the mechanism can be definitively stated as direct. As well, ChIP could be used to identify chromatin alterations, such as histone deacetylation or methylation, indicative of
transcriptional inactivation, within the $E2f1$ promoter, following PU.1 restoration.

We will exploit the inducible system to further assess the effect of E2F1 on BN cell proliferation/differentiation. For example, following restoration of PU.1 expression in cultured BN cells, we could overexpress E2F1 to ascertain E2F1’s ability to block cell cycle arrest. Presumably, overexpression of E2F1 should prevent PU.1 from enabling cell cycle exit and differentiation of BN cells. As well, knockdown of E2F1 using short hairpin RNA (shRNA), could be conducted to determine if a dose-dependent reduction in BN cell proliferation occurs. A significant reduction in E2F1 activity should slow BN cell growth.

Finally, further elucidating mechanisms by which PU.1 regulates the cell cycle, aside from E2F1 repression, is necessary. This avenue would permit the discovery of more therapeutic targets in the treatment of AML. For example, verifying the cell cycle targets proposed by Staber et al. is one option.

4.7 Summary and conclusions

In this project we sought to explore the mechanism of how PU.1 works to regulate proliferation and differentiation of myeloid progenitors, and its role in the development of AML. In doing so, we hypothesized that reduced expression of PU.1 in $Sfpi1^{\text{BN/BN}}$ myeloid cells will result in the development of AML in transplanted mice due to deregulation of the cell cycle, as well as reduced repression of E2F1. The evidence presented in this monograph supports our hypothesis. We successfully showed how PU.1 expression at 20% of wild-type levels is sufficient to induce AML in transplanted mice. Furthermore, we demonstrated that the effect of reduced PU.1 expression is deregulation of the cell cycle, due to lack of repression of E2F1. Whether PU.1 repression of E2F1 is direct or indirect remains to be confirmed. In conclusion we have shown that normal PU.1 levels are required to repress E2F1, enabling cell cycle exit and terminal differentiation of myeloid progenitors.
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- My thesis project was to determine the molecular mechanism of transcriptional regulation by the transcription factor PU.1 on myeloid cell development and its role in acute myeloid leukemia

4th Year Project
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Dr. Michelle Belton, Schulich School of Medicine and Dentistry, Western University, London, ON
- Worked closely with rodents investigating the effects of Atorvastatin on lowering serum cholesterol levels
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September 2009-April 2011
Dr. Chris Brandl, Department of Biochemistry, Western University, London, ON
- Assisted Dr. Brandl with his research on mechanisms of transcriptional regulation using yeast as a model system, focusing on the SAGA complex that regulates transcription through modification of chromatin structure
• Worked independently on investigating the effects of different length myc protein tags on signal strength

TEACHING EXPERIENCE

Learning Skills Assistant – The Learning Help Centre
September 2012 – April 2013
Student Development Centre, Western University
• Responded to a wide variety of student questions about learning, and helped students develop skills and strategies to succeed in school. Assisted undergraduate students having difficulties in various courses including but not limited to biology, chemistry, physics, calculus, genetics, biochemistry, cell biology, organic chemistry and statistics.

Teaching Assistant (TA) – Medical Sciences 4900G
January 2012 – April 2012
January 2013 – April 2013
Schulich School of Medicine and Dentistry, Western University
• Instruction and supervision in classes (one 5 hour laboratory class/week) – course introduces students to a variety of techniques used in medical research; major topics include animal models of human disease, real-time PCR, biochemical assays, histology and medical imaging.

Teaching Assistant (TA) - Biology 1001A/1201A
September 2011 – December 2011
September 2012 – December 2012
Department of Biology, Western University
• Instruction and supervision in laboratory component of Biology 1001A/1201A (one 3 hour lab/week) with focus on genetics, evolution and ecology.

Fitness Instructor
September 2010-Present
Campus Recreation, Western University
• Teach group fitness classes including Cardio Kickbox, Sleek and Strong, Rock Bottom, Skip and Sculpt, and Ab Attack.

English Conversation Circle Leader
September 2010-April 2012
Student Development Centre, Western University
• Lead and facilitated an English Conversation Circle, to help non-native speakers of English attending UWO (e.g., graduate and undergraduate students, professors, researchers, etc.) gain confidence, improve their proficiency, and enhance spoken English skills.
Academic/Guidance Tutor
January 2009-April 2013
Learning Disabilities Association (LDA) – London Region
• Volunteered as an Academic Tutor for LDA’s One to One Tutoring Program.
• Assisted students in grades 3 to 8 with learning disabilities and/or AD/HD with homework or a specific skill that the student needs to practice (such as reading, writing or math) in working towards improving their grades and competencies.

Swimming Instructor
September 2005-September 2009
Parks and Recreation, The City of Burlington
• Delivered engaging and enjoyable swimming lessons to participants of various ages.

PUBLICATIONS AND PRESENTATIONS


Rachel Ziliotto, Marek Gruca, Heather Broughton, David Hess, and Rodney DeKoter. Gene repression and cell cycle regulation by PU.1 in acute myeloid leukemia

Rachel Ziliotto, Marek Gruca, Heather Broughton, David Hess, and Rodney DeKoter. Gene repression and cell cycle regulation by PU.1 in acute myeloid leukemia
Oral presentation given at the Canadian Society for Immunology Conference 2013, Whistler, BC. April 2013

Rachel Ziliotto, Marek Gruca, Heather Broughton, David Hess, and Rodney DeKoter. Gene repression and cell cycle regulation by PU.1 in acute myeloid leukemia
Poster presented at the Canadian Society for Immunology Conference 2013, Whistler, BC. April 2013

Rachel Ziliotto, Marek Gruca, Heather Broughton, David Hess, and Rodney DeKoter. Gene repression and cell cycle regulation by PU.1 in acute myeloid leukemia

Rachel Ziliotto, Marek Gruca, Heather Broughton, David Hess, and Rodney DeKoter. Gene repression and cell cycle regulation by PU.1 in acute myeloid leukemia

Rachel Ziliotto and Hoa Vo. Purification of Enolase from Saccharomyces cerevisiae Poster presented at Umeå University, Umeå, Sweden. June 2010

SCHOLARSHIPS AND ACADEMIC HONORS

• Canadian Society for Immunology (CSI) 2013 Graduate Student Travel Award
• Poster Presentation Award – Oncology Research & Education Day
• UWO Division of Experimental Oncology 2012 Graduate Student Travel Award – received $1500 to cover travel expenses to attend scientific meetings
• Developmental Biology Student Award – received $1250 to be used during graduate studies
• Schulich Graduate Scholarship – Scholarship covers graduate tuition for two years
• Ontario International Education Opportunity Scholarship – received $2500 to study abroad on exchange
• Trois-Pistoles French Immersion Explore Bursary – received $2000 to study in Trois-Pistoles, Quebec
• University of Western Ontario Scholarship of Excellence – received $2000 for first year of University
• Q.E. II Aiming for the Top Tuition Scholarship - received $3500/year for four years
• Nelson H.S. Gold Medal Award
• Nelson H.S. J.L. Philips Memorial Scholarship – received $2500 for first year of University
• Nelson H.S. Principal’s Leadership Award – received $1000 for first year of University
• Nelson H.S. Physical Education Award
• Nelson H.S. Academic English Proficiency Award
• Nelson H.S. Academic History Award
• Nelson H.S. MVP for Senior Girls Basketball Team

PROFESSIONAL LICENSES/CERTIFICATIONS

• Successfully completed Advanced and Basic Rat Training through Animal Care and Veterinary Services at UWO - Skills obtained in this course include the ability to competently socialize, hold, inject (IP, IV), euthanize and dissect laboratory rodents for experimental purposes
• Successfully completed Basic Mouse Handling through Animal Care and Veterinary Services at UWO
• Comprehensive WHMIS certification
• Biosafety Training certification
• Can-Fit-Pro Certified Fitness Instructor Specialist
• SMART SERVE Certification
• Certified Lifeguard/ Swim Instructor:
  National Lifeguard Service (NLS) – Lifesaving Society, Red Cross Water Safety (WSI) 
  and LSS Instructors – Canadian Red Cross, PAD Provider – Public Access Defibrillation 
  – Joseph Brant Hospital, Airway Management – Lifesaving Society, Standard First Aid 
  and CPR Level C – Lifesaving Society, Bronze Cross – Lifesaving Society
• Grades 1-5 RAD (Royal Academy of Dance) Classical Ballet Exam Certification

TECHNICAL SKILLS

• Data entry and analysis utilizing SPSS and GraphPad Prism
• Fluorescence Activated Cell Sorting (FACS) - London Regional Flow Cytometry Facility

EMPLOYMENT HISTORY

Teaching Assistant (TA) – Western University, London ON    2011-2013
Fitness Instructor – Western University, London, ON    2010-Present
Lanscapre – Lakeland Gardening, Burlington, ON    2010
Swim Instructor – Private Lessons, Burlington ON    2007-2011
Sales Associate – Boutique Jacob, London, ON    2008-2009
Lifeguard/ Swim Instructor – City of Burlington, Burlington ON   2005-2009
Hostess - Jack Astor’s Bar and Grill, Burlington, ON    2006-2007

RECENT VOLUNTEER EXPERIENCE

2012
• Triage Volunteer - In February 2012 I took part in a life-changing experience 
as part of the Alternative Spring Break (ASB) program. With support from our 
community partner (International Service Learning, ISL) my team travelled to 
San Jose, Costa Rica to provide medical clinics to under-serviced communities in 
Costa Rica.

2011-2013
• Let’s Talk Science leader - In September 2011, I began volunteering with the 
award-winning, national, charitable organization, Let’s Talk Science. Let’s Talk 
Science delivers science learning programs to children and youth with the goal 
of prompting kids’ interest in science and making them aware of their individual, 
unique abilities. As a Let’s Talk Science leader, I worked with a grade 1 class, 
conducting classroom visits, to help the students discover the relevance/import-
tance of science in their daily lives. I lead the kids through fun, exciting, hands-on 
activities that enable them to participate in “real-world” science and learn some-
thing new at the same time.
2009-2011

- Emergency Liaison Volunteer at Joseph Brant Memorial Hospital, working primarily in the ER performing traffic control and miscellaneous jobs for the nurses. I greeted and provided information at the entrance for visitors and patients; I escorted patients to examination rooms in the ER, brought visitors into the ER to see patients, assembled patient’s charts, and obtained medical record files.

EXTRACURRICULAR INTERESTS AND ACTIVITIES/PERSONAL ACCOMPLISHMENTS

- I have participated on at least 2-3 different university intramural teams including basketball, soccer, inner tube water polo, softball and dodgeball each year of my university career, both undergraduate and graduate.
- As a member of the Infection and Immunity Research Forum (IIRF) Committee, I helped organize the IIRF scientific meeting.
- As a member of the Microbiology and Immunology Social Committee, I put on social events within the department such as Pumpkin Carving contests, Candy gram sales, and the Departmental Christmas party.
- As Trip Development Executive of Western’s SOS (Students Offering Support), I organized a student-run trip to Cabbage, Belize where students developed a project aimed to help the community.
- I enjoy travelling and spent a semester in Europe as an exchange student, studying at Umeå University in Sweden. While on exchange, I travelled to 12 different countries in Europe. I also participated in UWO’s Trois-Pistoles Immersion Program and spent 2 months in Quebec, taking a French course.