The Role of PU.1 in Lipid Metabolism and Cell Cycle Regulation in Myeloid Progenitor Cells

Jess Rhee
The University of Western Ontario

Supervisor
DeKoter, Rodney P.
The University of Western Ontario

Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
© Jess Rhee 2018

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Cell Biology Commons, Immunity Commons, and the Other Cell and Developmental Biology Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/5558

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.
Abstract

PU.1 is a transcription factor essential for myeloid development. High PU.1 levels promote cell cycle arrest and differentiation. Low levels promote proliferation and have been associated with leukemia. BN mice are homozygous for a hypomorphic allele of Spi1 that results in expression of PU.1 at 20% of normal levels. Induction of PU.1 expression in BN myeloid progenitor cells causes cell cycle arrest, differentiation, and the upregulation of microRNAs targeting lipid metabolic genes. Acly encoding ATP citrate lyase (ACL) was one of these targets. ACL produces acetyl-CoA which is essential for fatty acid synthesis. We hypothesized that inhibiting ACL would cause cell cycle arrest. BN cells treated with an ACL inhibitor were cell cycle arrested. Acetyl-CoA supplementation rescued cell cycle. The results suggest fatty acid metabolism plays a crucial role in cell cycle progression. Elucidating the mechanism of PU.1’s role in lipid metabolism and cell cycle regulation has implications for disease.

Keywords

PU.1, ATP citrate lyase, ACL, acetyl-CoA, cell cycle, lipid metabolism
I thank my supervisor Dr. Rodney DeKoter, for these two years that have provided many learning opportunities both in and out of academia. I thank you for your mentorship, guidance, and all your patience.

I also thank my advisory committee members, Dr. Bryan Heit and Dr. Murray Huff for all their support and guidance throughout the past two years as well.

Last but not least, I thank all my friends and lab members for their constant support and guidance as well all the memorable moments that I shall cherish.

Thank you to everyone, from the bottom of my heart, who have been involved in this wonderful journey!
Dedication

I dedicate this monograph to both my mother and father. Thank you for your never-ending and unwavering support and love. I appreciate everything that you both have provided and sacrificed for me and I hope the culmination of these two years towards this monograph will make you both proud.
# Table of Contents

Abstract ........................................................................................................................................... i

Acknowledgments .......................................................................................................................... ii

Dedication ........................................................................................................................................ iii

Table of Contents .......................................................................................................................... iv

List of Tables ................................................................................................................................ vi

List of Figures ............................................................................................................................... vii

List of Abbreviations .................................................................................................................... ix

1 Chapter 1: Introduction .............................................................................................................. 1

1.1 Hematopoiesis ....................................................................................................................... 1

1.2 Transcriptional Regulation of Myeloid Development .......................................................... 7

1.3 Dysregulation of Myeloid Development and Acute Myeloid Leukemia.......................... 10

1.4 Purine-Rich Box Binding-1 (PU.1) ...................................................................................... 13

1.5 PU.1 and Myeloid Development .......................................................................................... 15

1.6 Targets of PU.1 .................................................................................................................... 19

1.7 ATP Citrate Lyase’s Role in Metabolism and Cell Cycle ................................................... 20

1.8 Lipid Metabolism, Cell Cycle, and Differentiation ............................................................. 24

1.9 Hypothesis ............................................................................................................................ 30

2 Chapter 2: Material and Methods ............................................................................................ 34

2.1 Inducible BN Cell System ..................................................................................................... 34

2.2 Cell Culture ........................................................................................................................... 34

2.3 Cell Cycle Analysis ............................................................................................................... 37

2.4 Retrovirus Production .......................................................................................................... 37

2.5 Bone Marrow Cell Isolation and Culture ............................................................................ 37

2.6 Tritium Culture and Scintillation Counting ...................................................................... 38
### Chapter 2: Methods

- **2.7 Reverse Transcriptase Quantitative PCR** .......................................................... 39
- **2.8 Flow Cytometry** .................................................................................................. 39
- **2.9 Statistical Analysis** ............................................................................................ 39

### Chapter 3: Results

- **3.1 Acly transcript levels decrease following differentiation of progenitor cells in M-CSF** .................................................................................................................. 41
- **3.2 Acetate and acetyl-CoA rescues cell cycle arrest from impaired ACL function** .......................................................... 48
- **3.3 Acetate and acetyl-CoA supplementation rescues iBN induced cell cycle arrest** .......................................................................................................................... 51
- **3.4 Acetyl-CoA rescues cell cycle arrest from impaired FASN function** ................. 60
- **3.5 Extracellular [³H]-Acetyl-CoA is incorporated into cells** ...................................... 63
- **3.6 [³H]-Acetyl-CoA uptake in iBN cells increases with ACL inhibition** ................... 66
- **3.7 [³H]-Acetyl-CoA uptake in iBN cells decreases with acetate supplementation** ..... 66
- **3.8 [³H]-Acetyl-CoA is incorporated in both lipids and histones** ............................... 69
- **3.9 [³H]-Acetyl-CoA incorporation in lipids increases with ACL inhibition** ............. 72

### Chapter 4: Discussion

- **4.1 Inhibiting ACL results in decreased proliferation** ............................................. 78
- **4.2 Acetate and Acetyl-CoA can rescue the cell cycle** ............................................. 80
- **4.3 The role of lipid metabolism and histone acetylation in cell cycle regulation** .... 83
- **4.4 The mechanism by which acetyl-CoA regulates the cell cycle** ........................ 84
- **4.5 Future directions** ............................................................................................... 85
- **4.6 Summary and conclusions** ............................................................................... 91

References ....................................................................................................................... 92

Curriculum Vitae ............................................................................................................. 110
List of Tables

Table 1 The regulation of myeloid development by transcription factors ........................................... 9

Table 2 Mutated transcription factors in AML ....................................................................................... 11

Table 3 ETS-family transcription factors .............................................................................................. 14

Table 4 RT-qPCR Primer Sequences .................................................................................................. 40
List of Figures

Figure 1 Weissman and Jacobsen models of hematopoiesis .................................................. 5

Figure 2 PU.1 domains and protein-protein interactions ..................................................... 16

Figure 3 Glycolysis pathway ................................................................................................. 28

Figure 4 Acetyl-CoA pathways to promote cell cycle and proliferation in cells .................. 32

Figure 5 iBN cell system model ........................................................................................... 35

Figure 6 Bone marrow cell isolation procedure ................................................................. 42

Figure 7 CD11b and C-kit expression after column separation ........................................... 44

Figure 8 Column separated bone marrow cells grown in M-CSF and GM-CSF undergo proliferation and differentiation as marked by morphological changes .............................. 46

Figure 9 Transcript levels of Acly decrease in bone marrow cells grown in M-CSF .......... 49

Figure 10 ACL inhibition causes cell cycle arrest and acetate supplementation can rescue the cell cycle ......................................................................................................................... 52

Figure 11 ACL inhibition causes cell cycle arrest and acetyl-CoA can rescue the cell cycle 54

Figure 12 Induction of PU.1 iBN cells causes cell cycle arrest and acetate supplementation can rescue the cell cycle .............................................................................................................. 56

Figure 13 Induction of PU.1 iBN cells causes cell cycle arrest and acetyl-CoA supplementation can rescue the cell cycle .................................................................................................. 58

Figure 14 FASN inhibition causes cell cycle arrest and acetyl-CoA supplementation can rescue the cell cycle .................................................................................................................... 61

Figure 15 [3H]-acetyl-CoA is able to enter myeloid progenitor cells ....................................... 64

Figure 16 ACL inhibition increases acetyl-CoA uptake .......................................................... 67
Figure 17 Acetate supplementation decreases the uptake of $[^3\text{H}]$-acetyl-CoA.......................... 70
Figure 18 $[^3\text{H}]$-acetyl-CoA is incorporated in both lipids and histones................................. 73
Figure 19 ACL inhibition increases $[^3\text{H}]$-acetyl-CoA incorporation in lipids......................... 76
Figure 20 Modified iBN cell system to induce miR141 ................................................................. 87
Figure 21 Luciferase assay to detect miR141 binding................................................................. 89
List of Abbreviations

Acute myeloid leukemia (AML)

Common myeloid progenitor (CMP)

Hematopoietic stem cell (HSC)

Common lymphoid progenitor (CLP)

Purine-rich box binding-1 (PU.1)

ATP citrate lyase (ACL)

Acetyl-CoA synthetase 2 (ACSS2)

Granulocyte colony-stimulating factor (G-CSF)

Granulocyte macrophage colony-stimulating factor (GM-CSF)

Macrophage colony-stimulating factor (M-CSF)

microRNA (miR)

cyclin-dependent kinase (CDK)

BMS303141 (BMS)

Fatty acid synthase (FASN)
1 Chapter 1: Introduction

1.1 Hematopoiesis

Hematopoiesis is the process by which all blood cell lineages, including red blood cells and white blood cells (leukocytes), are produced. This developmental process begins with hematopoietic stem cells (HSCs) that are responsible for the development of all blood cell lineages (Galloway & Zon, 2003). These HSCs, like all other stem cells, are able to self-renew to produce new HSCs, and differentiate into the different blood lineages (Gunsilius, Gastl, & Petzer, 2001; Seita & Weissman, 2010). Hematopoiesis in vertebrates is characterized by two waves, first the primitive wave, a transient stage responsible for the production of red blood cells for the oxygenation of tissue, and then the definitive wave, responsible for the production of HSCs. The physical locations of these waves differ in this sequential process (Galloway & Zon, 2003; Orkin & Zon, 2008). These locations include the yolk sac, a specific area surrounding the dorsal aorta called the aorta-gonad mesonephros (AGM) region, the fetal liver, and lastly the bone marrow (Orkin & Zon, 2008). There are differences and nuances in hematopoiesis between species, for example between mouse and human (Jagannathan-Bogdan & Zon, 2013).

Focusing on a mouse model of hematopoiesis, the production of blood in the primitive wave initiates in the mammalian yolk sac by embryonic day 7.25-9 (E7.25-9) (Jagannathan-Bogdan & Zon, 2013; McGrath et al., 2011; Orkin & Zon, 2008). The primary function of hematopoiesis in the yolk sac is to produce erythroid progenitor cells/red blood cells for the oxygenation of tissue as the embryo develops (Jagannathan-Bogdan & Zon, 2013; Orkin & Zon, 2008). These erythroid progenitors are not pluripotent and cannot self-renew (Jagannathan-Bogdan & Zon, 2013). This primitive wave is very transient and is eventually replaced by the definitive wave at around E10 (Jagannathan-Bogdan & Zon, 2013; Orkin & Zon, 2008). The next site of hematopoiesis occurs in the AGM region which produces hematopoietic progenitor cells, regarded as the beginning of definitive hematopoiesis (Orkin & Zon, 2008). Traditionally, the primitive wave was thought to sequentially begin in the yolk sac followed by rapid
transition into the definitive wave beginning in the AGM and migrating to the fetal liver and eventually the bone marrow (McGrath et al., 2011). However, recent discoveries have shown that there is also a transient wave of definitive hematopoiesis that occurs in the blood islands of the yolk sac that produces erythroid-myeloid progenitors (Bertrand et al., 2007; Jagannathan-Bogdan & Zon, 2013; McGrath et al., 2011). Definitive hematopoiesis progresses in the AGM region where multipotent HSCs are formed between E9-10.5 (Bertrand et al., 2007; Jagannathan-Bogdan & Zon, 2013; McGrath et al., 2011; Orkin & Zon, 2008). These HSCs from the AGM region then migrate to the fetal liver at around E12.5-16.5 and then eventually to the bone marrow, where they will reside for the rest of the mature and adult life (Cumano & Godin, 2007; Galloway & Zon, 2003; Jagannathan-Bogdan & Zon, 2013). Additional sites of HSCs migration include the spleen and thymus (Orkin & Zon, 2008).

For humans, hematopoiesis similarly begins in the yolk sac but there is a temporary transition into the liver (first hepatic colonization) before progressing into the AGM and the definitive hematopoiesis establishes in the liver again (second hepatic colonization) and then the bone marrow and thymus (Jagannathan-Bogdan & Zon, 2013).

The process of hematopoiesis depends on two crucial and defining aspects of stem cells: multipotency and self-renewal (Seita & Weissman, 2010). These HSCs are able to generate the entire blood system by differentiating specifically into all the cell subsets in the myeloid and lymphoid lineages (Seita & Weissman, 2010). Specifically, HSCs differentiate into an intermediate precursor, including common myeloid progenitor cells (CMPs) and common lymphoid progenitor cells (CLPs) that are oligopotent (differentiating capacity of several but not all lineages of a tissue). CMPs derive myeloid subsets which includes granulocytes, macrophages, dendritic cells, erythrocytes (red blood cells), and megakaryocytes that give rise to platelets (Seita & Weissman, 2010). CLPs generate lymphoid subsets which includes B cells, T cells, natural killer cells (NKs), and an overlap of dendritic cell formation from the lymphoid lineage as well (Seita & Weissman, 2010). Hematopoietic stem cells are able to undergo differentiation and form all these different subsets due to coordinated, regulated, and cell-specific gene expression (Shivdasani & Orkin, 1996). Included in these regulatory pathways are
cytokine receptors and cell specific transcription factors that control the differentiation into the different blood cell lineages (Metcalf, 1993; Shivdasani & Orkin, 1996; Zhu & Emerson, 2002). Although hematopoietic differentiation is thought to be predominantly determined by transcriptional regulation, evidence suggests that cytokines also have an important role in determining stem cell fate (Sarrazin & Sieweke, 2011; Zhu & Emerson, 2002). Therefore, the intrinsic transcription factors and extrinsic cytokine signaling both play a role in stem cell fate (Sarrazin & Sieweke, 2011; Zhu & Emerson, 2002).

Hematopoietic stem cells are phenotypically characterized as a LIN^− IL-7Rα^− SCA1^+ KIT^+ FLT3^− Thy1\textsuperscript{low} CD34^− population in the Weissman model (Fig. 1A) (Weissman, Anderson, & Gage, 2001; Zhu & Emerson, 2002). The Lin phenotype includes surface antigens B220, CD4, CD8, Gr-1, Mac-1, and Ter-119, indicating mature hematopoietic cell lineages (Challen, Boles, Lin, & Goodell, 2009). These cells have lifelong self-renewal and multilineage potential (Rosenbauer & Tenen, 2007; Weissman et al., 2001). These long-term HSCs give rise to short-term HSCs (LIN^− IL-7Rα^− SCA1^+ KIT^+ FLT3^low Thy1\textsuperscript{low} CD34^+ ) that have multilineage differentiation potential but reduced self-renewal capabilities (Kondo et al., 2003; Weissman et al., 2001; Zhu & Emerson, 2002). These short-term HSCs then give rise to multipotential progenitors (MPPs) and are phenotypically characterized as LIN^− IL-7Rα^− SCA1^+ KIT^+ FLT3\textsuperscript{low−hi} Thy1\textsuperscript{low} CD34^+ (Rosenbauer & Tenen, 2007). MPPs completely lose their self-renewal capacity but can differentiate into all blood-cell lineages (Weissman et al., 2001). The MPPs then branch to give rise to either the complete lymphoid or myeloid lineage via common lymphoid (CLPs) or myeloid progenitors (CMPs), respectively (Rosenbauer & Tenen, 2007). CLPs are characterized as LIN^− IL-7Rα^+ SCA1\textsuperscript{low} KIT\textsuperscript{low} and lose all myeloid potential (Kondo, 2010). CMPs are characterized as LIN^− SCA1^− KIT^+ CD34^+ FcγRII^− FcγRIII^− and lose all lymphoid potential (Kondo, 2010; Rosenbauer & Tenen, 2007). CMPs branch out even further to give rise to more specialized progenitor cells, including granulocyte/monocyte progenitors (GMPs; LIN^− SCA1^− KIT^+ CD34^+ FcγRII^+ FcγRIII^−), megakaryocyte/erythroid progenitors (MEPs; LIN^− SCA1^− KIT^+ CD34^+ FcγRII^+ FcγRIII^−), basophil progenitors, and shared
macrophage and dendritic progenitors (MDPs) (Arinobu et al., 2005; Fogg et al., 2006; Rosenbauer & Tenen, 2007).

However, the classical Weissman model of hematopoiesis has been challenged by many colleagues where the erythroid lineage diverges at a much earlier stage, bypassing the CMP stage (Adolfsson et al., 2005; Chi et al., 2009; Yamamoto et al., 2013). In particular, the Jacobsen group (Fig. 1B) has suggested that at an early stage, a HSCs loses megakaryocyte and erythroid potential to develop into a lymphoid primed multipotent progenitor (LMPP) (Adolfsson et al., 2005). This LMPP can then either differentiate into a GMP or a CLP (Adolfsson et al., 2005). One group has even suggested that an earlier state of hematopoiesis myeloid-restricted progenitors (MyRPs) that have long-term self-renewal capacities are the major suppliers of myeloid cells rather than MPPs or LMPPs (Yamamoto et al., 2013).

Despite all the progress to map out the process of hematopoiesis and all the regulatory pathways involved in managing this process, there is still a lot more to be discovered and established to provide a complete picture of hematopoiesis.
**Figure 1 Weissman and Jacobsen models of hematopoiesis**

This figure is adapted from Rosenbauer & Tenen 2007 (Frank Rosenbauer & Tenen, 2007), Reya et al. 2001 (Reya, Morrison, Clarke, & Weissman, 2001), and Weissman et al. 2001 (Weissman et al., 2001)

**A.** The classical/Weissman model of hematopoiesis. CLPs are responsible for the generation of T, B, and NK cells. CMPs give rise to GMPs, MEPs, and MDPs, and mast cell/basophil progenitors. **B.** The Jacobsen model of hematopoiesis. MEPs are derived directly from ST-HSCs, while myeloid and lymphoid lineages are derived from LMPPs

Abbreviation: 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).
1.2 Transcriptional Regulation of Myeloid Development

Transcription factors play a major role in hematopoietic development and differentiation. There are many transcription factors that play an essential role in the development of the myeloid lineage (Table 1) (Rosenbauer & Tenen, 2007; Tenen, Hromas, Licht, & Zhang, 1997). The myeloid lineage is composed of granulocytes (neutrophils, eosinophils, and basophils), monocytes, macrophages, mast cells, as well as erythrocytes and megakaryocytes, although this has been contested by findings from the Jacobsen group (Adolfsson et al., 2005; Iwasaki & Akashi, 2007). Dendritic cells have also been characterized as having unique developmental programs that can stem from myeloid or lymphoid origins (Manz, Traver, Miyamoto, Weissman, & Akashi, 2001). Myeloid cells have a central role in innate immunity and one of their main functions is to phagocytose foreign antigens, while some myeloid cells have the additional capability to present these antigens (Kawamoto & Minato, 2004).

In general, commitment to the myeloid lineage begins with transcriptional control followed by terminal differentiation in response to specific colony-stimulating factors and cytokines that are released into the circulation (Kawamoto & Minato, 2004). These colony-stimulating factors include macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and multi colony-stimulating factor (multi-CSF, or better known as IL-3) (Barreda, Hanington, & Belosevic, 2004).

The transcription factors involved in controlling and orchestrating myeloid development include PU.1, CCAAT/enhancer binding proteins (C/EBPα, β, and ε), growth-factor independent 1 (GF1), interferon-regulatory factor 8 (IRF8), runt-related transcription factor (RUNX1), stem-cell leukemia factor (SCL), JUNB, Ikaros and MYC (Rosenbauer & Tenen, 2007; Tenen et al., 1997; Zhu & Emerson, 2002). RUNX1 and SCL are crucial at the level of the embryo and deficiency in any of the two are embryonic lethal and hematopoiesis is undetectable (Okuda, van Deursen, Hiebert, Grosveld, & Downing, 1996; Rosenbauer & Tenen, 2007; Shivdasani, Mayer, & Orkin, 1995). Conditional deletion of these two genes in adult HSCs demonstrated that they are not necessary for maintenance of hematopoiesis in the bone marrow but effect lineage-specific
Aside from embryo and hematopoietic dependent transcription factors, PU.1 is the earliest and most essential transcription factor at work for myeloid development (Dakic et al., 2005; Klemsz, McKercher, Celada, Van Beveren, & Maki, 1990; Rosenbauer & Tenen, 2007). PU.1 belongs to the E26 transformation-specific (ETS) family of transcription factors and it is encoded by Spi1 (DeKoter & Singh, 2000; Klemsz et al., 1990). Spi1 deletion in mice is embryonic lethal and there is no B cell or macrophage development (Rosenbauer & Tenen, 2007; Scott, Simon, Anastasi, & Singh, 1994). PU.1’s role as a master transcriptional regulator is made evident by the presence of PU.1-binding motifs in the regulatory sequences of almost all myeloid and many lymphoid-specific genes (Rosenbauer & Tenen, 2007; Tenen et al., 1997). Overall, PU.1 expression is necessary to generate CMPs from HSCs (Frank Rosenbauer & Tenen, 2007a).

C/EBPα is the transcription factor most essential for the generation of GMPs from CMPs (Rosenbauer & Tenen, 2007). C/EBPα is a basic region leucine zipper transcription factor, with expression found in HSCs, myeloid and granulocyte progenitors, but not in macrophages (Radomska et al., 1998). C/EPBα deficiency in mice leads to normal numbers of CMPs but these mice lack GMPs and all granulocyte lineages downstream (Zhang et al., 1997). C/EBPα regulates the expression of many myeloid-specific genes (Tenen et al., 1997) but also plays a role in negatively regulating stem-cell self-renewal properties (Zhang et al., 2004) and coordinating cell cycle exit (Johnson, 2005).

Macrophage or granulocyte differentiation from GMPs is determined by PU.1 and its protein binding partner, IRF8 (Rosenbauer & Tenen, 2007). IRF8 expression is limited to blood cells, and in the myeloid lineage, IRF8 expression is found in myeloid progenitors and macrophages but not granulocytes (Tamura, Nagamura-Inoue, Shmeltzer, Kuwata, & Ozato, 2000). Irf8<sup>−/−</sup> mice compared to wild-type mice have increased numbers of granulocytes and granulocyte precursors, while having fewer macrophages (Scheller et al., 1999). Restoring IRF8 expression in Irf8<sup>−/−</sup> myeloid progenitors is able to shift differentiation back to macrophages (Tamura et al., 2000). Irf8<sup>−/−</sup> myeloid progenitors have normal mRNA levels encoding the M-CSFR, but their
### Table 1 The regulation of myeloid development by transcription factors

Table 1.1 is adapted from Rosenbauer and Tenen 2007

Abbreviation: CCAAT/enhancer binding protein (C/EBP,); common lymphoid progenitor (CLP); chronic myeloid leukemia (CML); common myeloid progenitor (CMP); growth-factor independent 1 (GFI1); granulocyte/monocyte progenitor (GMP); interferon-regulatory factor 8 (IRF8); long-term hematopoietic stem cell (LT-HSC); short-term hematopoietic stem cell (ST-HSC); transcription factor encoded by *SPI1* (PU.1); runt-related transcription factor 1 (RUNX1); stem-cell leukemia factor (SCL)

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Phenotype in KO or conditional KO mice</th>
</tr>
</thead>
</table>
| **RUNX1**            | KO: no definitive hematopoiesis (Growney et al., 2005; Okuda et al., 1996)  
**Conditional KO**: (in HSCs) impaired megakaryocytic maturation, defective B and T cell development, impaired myeloid proliferation (Ichikawa et al., 2004) |
| **SCL**              | KO: complete absence of yolk sac hematopoiesis, lack of angiogenesis (Mikkola et al., 2003; Robb et al., 1995)  
**Conditional KO**: (in HSCs) decreases erythrocytes and megakaryocytes, impaired ST-HSCs (Hall et al., 2003; Verbeek et al., 1999) |
| **PU.1**             | KO: lack of mature myeloid and B cell (McKercher et al., 1996; Scott et al., 1994)  
**Conditional KO**: (in HSCs) blocked in the stage prior to CMP and CLP differentiation, increased granulopoiesis, defective HSCs (Dakic et al., 2005; Iwasaki et al., 2005) |
| **C/EBPα**           | KO: lack of GMPs and granulocytes, impaired monocytes, increased immature myeloid cells (D. E. Zhang et al., 1997)  
**Conditional KO**: (in HSCs) the same as KO mice with increased HSC self-renewal (Zhang et al., 2004) |
| **IRF8**             | KO: increased viral infection susceptibility, increased granulocytic cells, CML-like disease (Holtschke et al., 1996) |
| **GFI1**             | KO: reduction in earliest lymphoid progenitors, complete block in late neutrophil maturation, defective HSCs (Hock et al., 2004, 2003; Karsunky et al., 2002; Zeng, Yücel, Kosan, Klein-Hitpass, & Möröy, 2004) |
| **C/EBPε**           | KO: abnormal late neutrophil maturation, block in eosinophil development, defective macrophage function (Verbeek et al., 1999; Yamanaka et al., 1997) |
response to M-CSF is impaired, suggesting protein degradation (Kallies, Rosenbauer, Scheller, Knobeloch, & Horak, 2002).

Granulocyte differentiation from the GMP stage requires GFI1 and C/EBPε (Rosenbauer & Tenen, 2007). GFI1 expression is found in HSCs, neutrophils, early B and T cells, but not erythroid cells (Hock & Orkin, 2006). Both GFI1 and C/EBPε knockouts lead to an absence in neutrophil production and abnormal granulocyte development (Hock et al., 2003; Hock & Orkin, 2006; Yamanaka et al., 1997).

Overall, for lineage-specific differentiation, the temporal aspect of lineage-specific transcription factors is crucial in determining the fate of HSCs into differentiated myeloid cells. In addition these transcription factors cause cell cycle arrest, which is required for cells to undergo terminal differentiation (Friedman, 2002; Myster & Duronio, 2000).

1.3 Dysregulation of Myeloid Development and Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a cancer of myeloid cells in the bone marrow characterized by an impairment in their maturation or differentiation causing hematopoietic insufficiency (such as granulocytopenia, thrombocytopenia, or anemia), with or without an increase in white blood cell count (Lowenberg, Downing, & Burnett, 1999).

As mentioned in section 1.2, transcriptional regulation plays a crucial role in the differentiation and development of blood cell lineages, especially myeloid lineages. However, when aberrations in differentiation occur, this seems to be a causative event for the development of cancer (Tenen, 2003). Increasing evidence suggests that cancers are sustained by “stem-cell-like” tumor-cell subpopulations that share properties of normal stem cells (Passegue, Jamieson, Ailles, & Weissman, 2003). Mutations in defined genomic regions of transcription factors have been identified in patients with acute myeloid leukemia (AML) (Table 2) (Look, 1997; Rosenbauer & Tenen, 2007).
### Table 2 Mutated transcription factors in AML

Table 2 is adapted from Rosenbauer and Tenen 2007

Abbreviation: *Japanese cohort only. Acute myeloid leukemia (AML); acute
megakaryoblastic leukemia (AMKL); core-binding factor-β (CBFβ); GATA-binding
protein 1 (GATA1); homeobox (HOX); mixed lineage leukemia (MLL); myosin heavy
chain 11 (MYH11); promyelocytic leukemia (PML); retinoic acid receptor-α (RARα)

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Mutation and effects</th>
<th>Frequency in AML</th>
</tr>
</thead>
</table>
| RUNX1-ETO (t(8;21))  | Mutation: RUNX1 DNA binding domain fused to the transcriptional corepressor ETO  
Effects: downregulated expression or activity of PU.1, C/EBPα and RUNX1 | 12-15% |
| CBFβ-MYH11 (inv16)   | Mutation: inversion of breaks in chromosome 16  
Effects: joins CBFβ with the myosin gene MYH11 | 8-10% |
| PML-RARα (t(15;17))  | Mutation: PML gene fused to RARA  
Effects: blocks myeloid transcription factors (such as C/EBPα and PU.1) | 6-7% |
| MLL fusions (t11q23) | Mutation: MLL gene fused with one of 30 distinct genes encoding partner proteins  
Effects: believed to dysregulate HOX genes | 4-7% |
| C/EBPα               | Mutation: amino terminal dominant negative (gene product adversely affects WT gene)  
Effects: carboxy-terminal loss of DNA binding | 7-9% |
| GATA1                | Mutation: Amino-terminal dominant negative | Almost 100% in AMKL (Down’s syndrome) |
| PU.1                 | Effects: decreased heterodimer formation and DNA binding*; PU.1 activity downregulated by RUNX1-ETO and PML-RARα | <7% |
| RUNX1                | Mutation: missense, nonsense, or frame shift mutations clustered within runt domain | 9% |
In a specific subset of AML, characterized as the granulocytic French-American-British (FAB) system M2 subtype AML, C/EBPα is the most frequently mutated transcription factor (Leroy et al., 2005; Pabst et al., 2001). The main mutation in the N-terminus causes a disruption in translation by preventing the formation of the normal and longer 42-kDa isoform, into a shorter 30-kDa variant (Pabst et al., 2001). This causes a block in granulocyte differentiation by reducing C/EBPα function through a dominant-negative effect by the shorter, 30-kDa variant on the normal 42-kDa form (Pabst et al., 2001). Similarly, patients with trisomy 21 (Down’s syndrome) who development acute megakaryoblastic leukemia or myeloproliferative disorders have a mutation in the N-terminus of GATA1 (Rosenbauer & Tenen, 2007). Similarly to C/EBPα, this causes a truncated isoform of GATA1 which is still able to bind to DNA, but has reduced activation potential (Calligaris, Bottardi, Cogoi, Apezteguia, & Santoro, 1995; Gurbuxani, Vyas, & Crispino, 2004). Taken together, mutation in these two transcription factors plays a role in the development of AML, specifically in the granulocyte lineage for C/EBPα mutations.

In addition, PU.1, RUNX1, and IRF8 mutations have also been shown to be contributing causes of AML. RUNX1 is found mutated in 9% of cases of AML, mostly in the immature FAB system M0 subtype of AML (Osato et al., 1999). Runx1−/− mice also develop myeloproliferative syndrome (Growney et al., 2005). There have not been any reported IRF8 mutations, however, it is frequently downregulated in patients with both AML and CML (Schmidt et al., 1998). Furthermore, Irf8 knockout lead to a CML-like disease state in mice (Holtschke et al., 1996) and downregulation of IRF8 was essential for the development of myeloid leukemia in a mouse model (Hao & Ren, 2000). IRF8 has also been shown to cooperate with a RUNX1-ETO fusion construct to transform myeloid cells into malignant cells (Schwieger et al., 2002), and a point mutation in Irf8 led to CML-like disease in a BXH-2 mouse model (Turcotte et al., 2005).

Mutations in PU.1, which is encoded by the Spi1 gene, have been identified in 7% of patients with AML, predominantly in patients with FAB systemic monocytic M4 and M5 subtypes of AML (Mueller et al., 2002). The high leukemic potential due to reduced PU.1 activity has been shown by several mouse models (Rosenbauer et al., 2004; Will et
Rosenbauer and his group were able to show that mice with a deletion of the upstream regulatory element (URE) of Spi1 had decreased PU.1 expression in HSCs and myeloid progenitors, which lead to aggressive forms of AML (Rosenbauer et al., 2004). Findings have suggested that low, but not completely abolished, levels of PU.1 favour malignancy (Rosenbauer, Koschmieder, Steidl, & Tenen, 2005). However, Metcalf and his group have demonstrated that complete loss of PU.1 also leads to AML in adult mice (Metcalf et al., 2006). Although the effects of aberrant PU.1 are evident, the mechanisms leading to AML by PU.1 disfunction are still unclear and require elucidation.

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

PU.1 is a transcription factor that is part of the ETS-family of transcription factors (Sharrocks, 2001) (Table 3.). The first ETS-family transcription factor, Ets-1, was discovered as a fusion oncogene with v-myb in the avian retrovirus E26 (Hromas et al., 1993; Moreau-Gachelin, Tavitian, & Tambourin, 1988; Nunn, Seeburg, Moscovici, & Duesberg, 1983). Members of this family are related based on the homology of their DNA binding domains, also called the ETS-domain (Sharrocks, 2001). This ETS-domain is a variant of the winged helix-turn helix motif (Liang et al., 1994), with structural conservation among the family members, including three α-helices and four β-sheets (Sharrocks, 2001). All ETS-domain proteins bind to DNA sequences with a central GGAA or GGA motif (Sharrocks, 2001). PU.1 was discovered as a putative oncogene isolated from murine Friend virus induced erythroleukemia which resulted from insertional activation at the Spi1 gene locus promoter region by spleen-focus-forming virus (Moreau-Gachelin et al., 1988). PU.1 was first shown to be a transcriptional activator through its ability to trans-activate a reporter gene when PU.1 binding sites (purine rich sequence, 5’-GAGGAA-3’, PU box) were placed next to a thymidine kinase (TK) promoter linked to a chloramphenicol acetyltransferase (CAT) gene (Klemsz et al., 1990).

In addition to the ETS-domain at the C-terminus of PU.1 being responsible for DNA binding, it also binds and interacts with other proteins, including C/EBPα and β as well as GATA-1 (Nishiyama et al., 2004; Reddy et al., 2002). The N-terminus acts as a
Table 3 ETS-family transcription factors

Table 3 is adapted from Hollenhorst, McIntosh, & Graves, 2011.

<table>
<thead>
<tr>
<th>Human ETS-domain protein</th>
<th>Defects in mouse deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPI family</strong></td>
<td></td>
</tr>
<tr>
<td>PU.1</td>
<td>Lethal, myeloid and lymphoid differentiation</td>
</tr>
<tr>
<td>SpiB</td>
<td>B-cell</td>
</tr>
<tr>
<td>SpiC</td>
<td>Red pulp macrophages</td>
</tr>
<tr>
<td><strong>TEL family</strong></td>
<td></td>
</tr>
<tr>
<td>ETV6</td>
<td>Yolk sac, hematopoietic stem cells</td>
</tr>
<tr>
<td>ETV7</td>
<td>N/A (no mouse homolog)</td>
</tr>
<tr>
<td><strong>ESE family</strong></td>
<td></td>
</tr>
<tr>
<td>ELF3</td>
<td>Intestine</td>
</tr>
<tr>
<td>EHF</td>
<td>N/A</td>
</tr>
<tr>
<td>ELF5</td>
<td>Embryo patterning, mammary gland</td>
</tr>
<tr>
<td><strong>ELF family</strong></td>
<td></td>
</tr>
<tr>
<td>ELF4</td>
<td>NK cells, NK-T cells</td>
</tr>
<tr>
<td>ELF2</td>
<td>N/A</td>
</tr>
<tr>
<td>ELF1</td>
<td>No defect</td>
</tr>
<tr>
<td>SPDEF</td>
<td>Intestine</td>
</tr>
<tr>
<td><strong>PEA3 family</strong></td>
<td></td>
</tr>
<tr>
<td>ETV1</td>
<td>Neuronal connections</td>
</tr>
<tr>
<td>ETV5</td>
<td>Male fertility</td>
</tr>
<tr>
<td>ETV4</td>
<td>Male fertility, motor neurons, mammary glands</td>
</tr>
<tr>
<td><strong>TCF family</strong></td>
<td></td>
</tr>
<tr>
<td>ELK3</td>
<td>Vasculature</td>
</tr>
<tr>
<td>ELK4</td>
<td>Thymocytes, peripheral T cells</td>
</tr>
<tr>
<td>ELK1</td>
<td>No defect</td>
</tr>
<tr>
<td><strong>ETS family</strong></td>
<td></td>
</tr>
<tr>
<td>ETS1</td>
<td>T cells, NK cells, NK-T cells</td>
</tr>
<tr>
<td>ETS2</td>
<td>Placenta, hair follicles</td>
</tr>
<tr>
<td>ETV2</td>
<td>Blood, vasculature</td>
</tr>
<tr>
<td><strong>ERF family</strong></td>
<td></td>
</tr>
<tr>
<td>ETV3</td>
<td>N/A</td>
</tr>
<tr>
<td>ETV3L</td>
<td>N/A</td>
</tr>
<tr>
<td>ERF</td>
<td>Placenta</td>
</tr>
<tr>
<td><strong>ERG family</strong></td>
<td></td>
</tr>
<tr>
<td>ERG</td>
<td>Definitive hematopoiesis</td>
</tr>
<tr>
<td>FLI1</td>
<td>Vasculature, megakaryocytes, B cells</td>
</tr>
<tr>
<td>FEV</td>
<td>5-HT neurons</td>
</tr>
<tr>
<td>GABPA</td>
<td>Lethal, T cells</td>
</tr>
</tbody>
</table>
transactivation domain, enriched with acidic and glutamine residues (Nishiyama et al., 2004). The N-terminal domain activates transcription, but also interacts with other proteins such as GATA-1, TATA box binding protein (TBP) and retinoblastoma protein (pRb) (Moreau-Gachelin, 1994; Moreau-Gachelin et al., 1988; Rekhtman, Radparvar, Evans, & Skoultchi, 1999). TBP is a subunit for the basal transcriptional complex TFIID, which increases RNA polymerase II activity and pRb is a cell cycle regulator (Moreau-Gachelin, 1994). The last domain PU.1 is the PEST domain, located between the N-terminus and the ETS domain (Moreau-Gachelin, 1994). This domain is characterized by its high proline (P), glutamic acid (E), serine (S), and threonine (T) content and involved in protein stability and degradation (Fig. 2) (Moreau-Gachelin, 1994).

1.5 PU.1 and Myeloid Development

PU.1 plays an essential role in myeloid development and is considered a master regulator of myeloid gene expression (Will et al., 2015). In addition to its essential role in myeloid development, homozygous mutations in PU.1 have shown to be embryonically lethal in mice by day 18, whereas heterozygous PU.1 mutations lead to an impairment in B and T cell, monocyte, and granulocyte development (Scott et al., 1994). However, PU.1 is expressed at the highest levels in myeloid and B cells (Klemsz et al., 1990; Moreau-Gachelin et al., 1988). PU.1 mRNA is expressed at low levels in murine embryonic stem cells and HSCs but upregulated with myeloid differentiation (Voso et al., 1994). Inhibition of PU.1 function in HSCs blocks subsequent myeloid progenitor cell formation (Cheng et al., 1996; Voso et al., 1994). One prominent study demonstrated that murine fetal liver progenitor (LIN⁻ KIT⁺ CD27⁺) cells cultured with macrophage and B-cell stimulating cytokines (SCF, IL-3, IL-7, Flt3L, M-CSF) had increased protein levels of PU.1 in developing macrophages (Kueh, Champhekar, Nutt, Elowitz, & Rothenberg, 2013). These mice had a PU.1-GFP knock-in reporter and through time-lapse imaging and flow cytometry, they were able to monitor the increasing PU.1-GFP accumulation in the progenitor cells differentiating into macrophages as determined by the macrophage cell surface marker, F4/80 (Kueh et al., 2013). The suggested mechanism behind this was
**Figure 2 PU.1 domains and protein-protein interactions.**

The transcription factor PU.1 is shown with its three domains: N-terminal transactivation domain (in red), PEST domain (in purple), and C-terminal ETS binding domain (in blue). TPB, Rb, and GATA-1 interact with the N-terminal transactivation domain and C/EBPα/β and GATA-1 interact with the C-terminal ETS domain.
H C  terminal
transactivation domain T  T  domain C terminal
 T  domain
 ATA 1
Rb
T  
 ATA 1
C     
C
an induction of cell-cycle lengthening, allowing for stable PU.1 accumulation (Kueh et al., 2013).

Inhibition of PU.1 has been utilized by many studies to investigate its role in myeloid development. Scott et al. first demonstrated that a homozygous PU.1 knockout is embryonic lethal at E18 (Scott et al., 1994). Normal megakaryocytes and erythroid progenitors were developed but the generation of progenitor B and T cell, monocytes, and granulocytes were defective (Scott et al., 1994). Another group also developed a homozygous PU.1 knockout but were able to sustain their survival through the use of antibiotics (McKercher et al., 1996). These mice lacked monocytes and mature B cells, but were able to produce progenitor B-cells. Several days after birth, they were able to produce T cells and neutrophil-like cells as well, however, monocytes and mature B cells were still absent. This demonstrates the requirement for PU.1 in B cells and myeloid development.

The Tenen group was able to demonstrate that AML could be induced through decreased PU.1 expression (Rosenbauer et al., 2004). Using a mouse with a hypomorphic Spi1 allele which expressed PU.1 at 20% of normal levels, resulting in an accumulation of abnormal and immature myeloid precursors in the bone marrow and spleen (Rosenbauer et al., 2004). Myeloid differentiation was blocked in these mice and they developed AML (Rosenbauer et al., 2004).

Similarly, our laboratory generated a hypomorphic allele of Spi1, termed BN, which expresses PU.1 at 20% of wild-type levels (Houston, Kamath, Schweitzer, Chlon, & DeKoter, 2007). This allele was generated by mutating the first coding exon and replacing two ATG start sites with a neomycin resistance cassette (Houston et al., 2007). A third translational start codon is present within the Spi1 gene, enabling Spi1BN alleles to be transcribed but as a truncated protein. As a result of altered transcriptional regulation, functional PU.1 is expressed at reduced levels (this differs from the Tenen group’s hypomorph (Frank Rosenbauer et al., 2004) as their model utilized a cre-loxP-based homologous recombination strategy to replace the upstream regulatory region (URE) of the Spi1 locus with a neomycin resistance cassette). Mice homozygous for the mutated
*Spi*1 allele (*Spi1*<sup>BN/BN</sup> mice), have hyperproliferation of immature myeloid cells in the bone marrow and spleen, and a complete absence of B-cells (Houston et al., 2007). These mice have a short survival period of 1-3 weeks following birth and are characterized by osteopetrosis, failed bone remodeling, which combined result in reduced physical size (Houston et al., 2007). The failure of bone remodeling is a result of failed terminal differentiation of myeloid progenitor cells into osteoclasts (Raggatt & Partridge, 2010). Thus, these findings all suggest the importance of PU.1 and proper PU.1 expression levels and regulation for myeloid development.

### 1.6 Targets of PU.1

In accordance with PU.1’s essential role in myeloid and B-cell development, PU.1 regulates the expression of the *c-fms* and *IL-7Ra* genes, which encode receptors for M-CSF and IL-7, respectively (DeKoter, Lee, & Singh, 2002; DeKoter, Walsh, & Singh, 1998). Genes expressed early in myeloid development such as GM-CSF receptor, G-CSF receptor, and myeloperoxidase, are expressed in PU.1 knockout embryos. However, genes associated with terminal myeloid differentiation such as, CD11b (Mac-1 integrin α-chain), CD64 (phagocytic Fc receptor), and the M-CSF receptor, are not expressed (Olson et al., 1995). Growth factors that bind to M-CSF, GM-CSF, and G-CSF receptors are required for the proliferation and differentiation of myeloid cells, which have actually been shown to activate PU.1 (Mossadegh-Keller et al., 2013; Olson et al., 1995). PU.1 also induces expression of genes required for myeloid phenotypes including CD18, Fc gamma receptor I/IIIA (FcγRI/IIIA), and scavenger receptors type I and II (SR1 and SRII) (Feinman et al., 1994; Moulton, Semple, Wu, & Glass, 1994; Paul et al., 1993; Perez, Coeffier, Moreau-Gachelin, Wietzerbin, & Benech, 1994; Rosmarin, Caprio, Levy, & Simkevich, 1995). CD11b is a cell surface marker expressed on mature monocytes, macrophages, and granulocytes (Paul et al., 1993), and when paired with CD18 forms the macrophage-1 antigen complex (Mac-1), or complement receptor 3 (CR3), which plays important roles in phagocytosis and inflammatory responses (Rosmarin et al., 1995). High affinity FcγRI and low affinity FcγRIIIA are exclusively expressed in the myeloid lineage and are receptors for the Fc domain of immunoglobulin G (IgG) (Feinman et al., 1994; Perez et al., 1994). These receptors play an essential role
in innate immunity by their ability to induce phagocytosis and antibody-dependent cellular cytotoxicity (Feinman et al., 1994; Perez et al., 1994). SRI and SRII expression is highly restricted to monocytes and macrophages and are maximally expressed during differentiation from monocytes to macrophages (Moulton et al., 1994).

In addition to activating genes involved in myeloid differentiation and maturation, PU.1 has also been shown to target genes involved in lipid metabolism and cell cycle regulation (Solomon et al., 2017). Using a PU.1 inducible BN cell system (Methods 2.1) with doxycycline, our laboratory was able to demonstrate that genes encoding cell cycle regulators and lipid anabolism were directly and inducibly bound by PU.1. However, steady-state mRNA levels were reduced (Solomon et al., 2017). Induction of PU.1 in iBN cells via doxycycline reduced expression of *E2f1*, which is an activator of genes involved in cell cycle as well as lipid metabolism, indirectly through microRNA (miR) 223 (Solomon et al., 2017). MicroRNAs have a negative regulatory role by either cleaving mRNA targets or repressing the translation of mRNA (Cai, Yu, Hu, & Yu, 2009). MicroRNA sequencing of PU.1 induced iBN cells identified validated miR targets for cell cycle and lipid metabolism downregulation.

Taken together, these results suggest that PU.1 may regulate the processes of cell cycle progression and differentiation through the induction of microRNAs by targeting cell cycle regulators and lipid metabolism to reduce proliferation and allow for differentiation.

1.7 ATP Citrate Lyase’s Role in Metabolism and Cell Cycle

MicroRNA targets of PU.1 induced iBN cells were genes involved in lipid metabolism and of these genes, *Acly*, the gene encoding ATP citrate lyase (ACL) was a target of interest. ACL’s function is to produce acetyl-CoA from mitochondrial-derived citrate within the cell. ACL has crucial role as the major source of acetyl-CoA for cells, specifically for histone acetylation (Wellen et al., 2009). *Acly* has been shown to be mainly regulated by the transcription factor sterol regulatory element binding protein-1 (SREBP-1) (Bauer, Hatzivassiliou, Zhao, Andreadis, & Thompson, 2005). SREBP-1 upregulates *Acly* at the mRNA level through Akt signaling (a pro-growth and survival
transduction pathway in response to extracellular signals) (Sato et al., 2000). However, ACL protein levels are independent of SREBP-1 (Migita et al., 2008). One study has suggested that the PI3K/Akt pathway stimulates ACL activity through phosphorylation of the ACL protein itself, which stabilizes the protein, instead of transcriptional upregulation of mRNA (Migita et al., 2008). Treatment with PI3K inhibitors has no effect on dephosphorylation or inactivation of ACL in lung cancer cells, suggesting that ACL activity may also be regulated by other pathways (Migita et al., 2008).

As a general overview, acetyl-CoA is a central metabolic intermediate that occupies a critical position in multiple cellular processes, including anabolic and lipogenic pathways, as well as being a key determinant of histone acetylation (Pietrocola, Galluzzi, Bravo-San Pedro, Madeo, & Kroemer, 2015). As a central metabolite, there are many pathways that lead to the catabolic production of acetyl-CoA. These pathways can be located inside or outside the mitochondria (Pietrocola et al., 2015). Within the mitochondria, acetyl-CoA is generated in the matrix by glycolysis, β-oxidation of fatty acids, and the catabolic metabolism of branched amino acids (Pietrocola et al., 2015). Starting with glycolysis, the final metabolic process ends with the generation of cytosolic pyruvate which gets transported into the mitochondria by mitochondrial pyruvate carrier (MPC) (Herzig et al., 2012). While in the mitochondria, pyruvate is decarboxylated to produce acetyl-CoA, CO₂, and NADH via the pyruvate dehydrogenase complex (PDC) (Pietrocola et al., 2015).

Acetyl-CoA can also be produced from β-oxidation from cytosolic free fatty acids or fatty acids within the mitochondria (Pietrocola et al., 2015). This process utilizes a member of the acyl-CoA synthetase family to catalyze CoA and the ATP-dependent conversion of the cytosolic fatty acid into acyl-CoA (Pietrocola et al., 2015). Acyl-CoA is then condensed with L-carnitine to form acylcarnitine and free CoA via carnitine. Acylcarnitine is transported into the mitochondria and in the mitochondria, the acylcarnitine is converted back into L-carnitine, and acyl-CoA. The acyl-CoA is then able to generate NADH and acetyl-CoA in the mitochondria (Pietrocola et al., 2015). Fatty acids within the mitochondria can simply undergo this last step of acyl-CoA β-oxidation.
into acetyl-CoA and NADH without the need to be converted into acylcarnitine (Schulz, 1991).

Branched-chain amino acids, including valine, leucine, and isoleucine, can also be metabolized to produce acetyl-CoA (Harris, Joshi, Jeoung, & Obayashi, 2005; Pietrocola et al., 2015). These branched amino acids are first transaminated to branched-chain α-ketoacids and imported into the mitochondrial matrix (Pietrocola et al., 2015). The α-ketoacids are decarboxylated to produce NADH, acetyl-CoA, and acyl-CoA, which can be further metabolized into acetyl-CoA (Pietrocola et al., 2015).

Despite the many metabolic pathways within the mitochondria that can produce acetyl-CoA, there are cytosolic/extramitochondrial pathways that can also contribute to acetyl-CoA production and are essential for histone acetylation (Wellen et al., 2009) and lipogenesis (Bauer et al., 2005; Chypre, Zaidi, & Smans, 2012). The first of these pathways utilizes acetyl-CoA synthetase 2 (ACSS2) to convert cytosolic acetate into acetyl-CoA (Schug, Voorde, & Gottlieb, 2016). Exogenous acetate sources can be transported into the cell through various monocarboxylate transporter protein family members (Halestrap & Price, 1999). Acetyl-CoA can also be synthesized from ethanol through the conversion of ethanol into acetaldehyde by aldehyde dehydrogenase 1 family member A1 (ALDH1A1) (Cederbaum, 2012). Physiologically, circulating acetate levels in humans are low compared to other species (Frost et al., 2014), however, the ACSS2-dependent conversion of acetate into acetyl-CoA has been shown to be elevated in human primary and metastatic malignant cells of various origins and types (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2016). The second pathway involves the transport of mitochondrial derived citrate into the cytosol via the dicarboxylate antiporter solute carrier family 25 member 1 (SLC25A1, citrate transporter), in which citrate is converted into acetyl-CoA by ATP citrate lyase (Bauer et al., 2005; Pietrocola et al., 2015).

The acetyl-CoA produced within the mitochondria is generally metabolized further in the citric acid cycle to produce NADH for ATP via oxidative phosphorylation (Pietrocola et al., 2015). This highlights the fact that although glycolysis, β-oxidation,
and branched-chain amino acid metabolism can lead to large amounts of acetyl-CoA, much of it is diverted into energy/ATP production via the final pathway of oxidative phosphorylation (Pietrocola et al., 2015). Cytosolic acetyl-CoA on the other hand, presents a unique function as the precursor for many anabolic pathways and reactions for fatty acid, steroid, and even amino acid synthesis (Pietrocola et al., 2015). This is where ACL’s function becomes evident as a crucial cross-link between glucose metabolism and fatty acid synthesis (Chypre et al., 2012).

Acetyl-CoA is the essential substrate for de novo fatty acid synthesis and the mevalonate pathway. In de novo fatty acid synthesis pathway, acetyl-CoA is carboxylated into malonyl-CoA by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce the 16 carbon fatty acid chain, palmitate/palmitic acid (Chypre et al., 2012; Mashima, Seimiya, & Tsuruo, 2009). In the mevalonate pathway, acetyl-CoA is used to synthesize farnesyl-pyrophosphate (FPP), which is involved in cholesterol biosynthesis but can also be synthesized into geranylgeranyl-pyrophosphate (GG-PP) (Chypre et al., 2012). FPP and GG-PP are involved in farnesylation and geranylgeranylation, respectively, which together are referred to as prenylation (Kassai & Fukada, 2011). Briefly, the process of prenylation involves post-transcriptional lipid modification to increase membrane interactions of proteins (Zhang & Casey, 1996). Acetyl-CoA is also heavily and directly involved in acetylation, particularly, histone acetylation for regulating global chromatin structure and gene expression (Wellen et al., 2009).

In regards to histone acetylation, Wellen was able to demonstrate that ACL is found not only in the cytoplasm, but also in the nucleus, and because citrate and acetate are small molecules, they are able to freely diffuse through nuclear pores (Wellen et al., 2009). Therefore, their findings suggest that acetyl-CoA may be produced both in the cytoplasm and nucleus (Wellen et al., 2009). This group also demonstrated that silencing of ACL via small interfering RNA (siRNA) significantly decreases the amount of histone acetylation, but supraphysiological levels of acetate was able to rescue histone acetylation via the mouse homolog of ACSS2 (Wellen et al., 2009). Histone acetylation occurs through the action of histone acetyltransferases (HATs) and acetyl-CoA is the obligate
substrate for this process (Pietrocola et al., 2015). Therefore, the amount of acetyl-CoA present in the nucleus or cytoplasm has a direct link to the epigenetic control of gene expression. High levels of histone acetylation is required for global transcription (Takahashi, McCaffery, Irizarry, & Boeke, 2006) but also for the preferential transactivation of genes involved in cell growth and replication (Cai, Sutter, Li, & Tu, 2011; Donohoe et al., 2012; Lee et al., 2014).

Thus, ACL has an essential position in cellular processes, particularly lipid biosynthesis and histone acetylation, both of which influence the progression of cell cycle and proliferation in cells (Cai et al., 2011; Shi & Tu, 2015; Wellen et al., 2009).

1.8 Lipid Metabolism, Cell Cycle, and Differentiation

The metabolic requirements of cells change drastically as they progress through the cell cycle. Cells must double their DNA, membranes, organelles, and other biomass components of the cell (Kaplon, van Dam, & Peeper, 2015). In order to sustain the energy required for proliferation, cells increase glucose uptake while shutting down oxidative metabolism (Kaplon et al., 2015). In this way, metabolic intermediates, such as acetyl-CoA, are used for the biosynthesis of macromolecules required for cell division, instead of entering the Kreb’s cycle for oxidative phosphorylation (Kaplon et al., 2015). Warburg’s observation showed that cancer cells exhibit a higher rate of glycolysis, and that this was a distinguishing characteristic between normal and cancer cells, and also a primary cause of malignancy (Lunt & Vander Heiden, 2011; Warburg, 1956). However, normal proliferating cells also exhibit a preference for glycolysis as although this pathway is not as efficient as oxidative phosphorylation, it is a much faster pathway for ATP production and the intermediate metabolites are readily available for biosynthetic pathways including nucleotide, amino acid, and fatty acid synthesis (Lunt & Vander Heiden, 2011). It is well established and accepted that signal transduction pathways including cyclins regulate metabolism (Kaplon et al., 2015; Wellen & Thompson, 2012). However, it is also emerging that signaling pathways and cyclins may be regulated by metabolism and the availability of nutrients (Kaplon et al., 2015; Wellen & Thompson, 2012).
Cyclin-dependent kinases (CDKs) contain a serine/threonine-specific catalytic core and bind with regulatory subunits, known as cyclins, to control the kinase activity and substrate specificity (Lim & Kaldis, 2013). These CDK/cyclin complexes were first implicated in cell cycle control in pioneering work done in yeast, where a single CDK was promoting transition between the different cell cycles with its interaction with various cyclins (Lim & Kaldis, 2013; Reed, Ferguson, & Groppe, 1982). The cell cycle is divided into different phases, G0, G1, S, G2, and M phase (Kaplon et al., 2015). G0 is the phase where most differentiated and quiescent cells reside. G1 marks the beginning of proliferation in which the cells prepare for DNA replication by growing in size through metabolic activity. S phase is marked by a doubling of DNA content, followed by the G2 phase where cells prepare for mitosis which then occurs during mitosis/M phase (Kaplon et al., 2015). Lim and Kaldis gave a fitting analogy to the CDK-cyclin complex, with CDKs being the engine that drives cell cycle progression and cyclins as the various gears that are changed to aid this transition between the cell cycle phases (Lim & Kaldis, 2013). These CDK/cyclin complexes are regulated by CDK inhibitors (CKIs) which can impede cell cycle progression (Lim & Kaldis, 2013). Unlike yeast, mammalian cells utilize a variety of CDKs and cyclins for more elaborate and precise control over proliferation of different cell types (Malumbres & Barbacid, 2009).

In regards to cell cycle signaling pathways and their control on metabolism, D-type cyclins play a crucial role in regulating metabolism. This was first demonstrated in cyclin-D deficient mice that displayed marked metabolic phenotypes (Kaplon et al., 2015). Cyclin D2 deficient mice displayed a diabetic phenotype as a result of impaired β-cell expansion and function in the pancreas, and this was further enhanced by co-depletion of cyclin D1 (Kushner et al., 2005). Cyclin D3 deficient mice displayed reduced adipocyte size and increased sensitivity to insulin, a consequence of the inactivation of peroxisome proliferator-activated receptor γ (PPARγ), which is the master regulator of adipogenesis (Sarruf et al., 2005). Cyclin D1 depletion in both oncogenic and normal breast tissue lead to an increase in the glycolytic enzyme, pyruvate kinase (PK) and lipogenic enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) levels (Sakamaki et al., 2006). In hepatocytes, cyclin D1 is responsible for repressing carbohydrate response element binding protein (ChREBP) which is an
important regulator for glucose sensing and lipid metabolism (Hanse et al., 2012). Taken together, these results seem to suggest that cyclin D1 inhibits both glycolysis and lipogenesis (Kaplon et al., 2015). Looking into this further, cyclin D activity accumulates in G1 phase, therefore it seems that D-type cyclins restrain the conversion of glucose for lipids allowing for glucose-derived metabolites to be focused on doubling cell content at the early stages of the G1/S transition (Kaplon et al., 2015).

The interaction of Cyclin D-CDK4/6 complexes regulate the phosphorylation of retinoblastoma protein (pRb) in the G1 phase (Kaplon et al., 2015). Phosphorylation of pRb converts E2F/DP transcription factors from a repressor into an activator, thus allowing for the expression of genes required for DNA synthesis and promoting S-phase entry (Dimova & Dyson, 2005; Kaplon et al., 2015). E2F transcription factors are involved in cell cycle progression and survival by activating genes involved in cell cycle regulation and DNA synthesis (Dimova & Dyson, 2005). However, E2F, particularly E2F1, has a role in regulating metabolism by positively regulating PPARγ, thus regulating adipogenesis (Fajas et al., 2002). The E2F-pRb pathway also regulates glucose metabolism as E2F1-depleted mice displayed a diabetic phenotype (Fajas et al., 2004). E2F1 induces expression of pyruvate dehydrogenase kinase (PDK4) which inhibits pyruvate dehydrogenase and therefore oxidative phosphorylation (Roche & Hiromasa, 2007). E2F1 also stimulates glycolysis by upregulating phosphofructokinase 2 (PFK2) (Darville, Antoine, Mertens-Strijthagen, Dupriez, & Rousseau, 1995). Taken together, the E2F-pRb pathway promotes a metabolic switch from oxidative to glycolytic metabolism which supports the metabolic phenotype of proliferating cells (Kaplon et al., 2015).

Tumor suppressor p53 is also a major regulator of cellular metabolism. The activation of p53 from a variety of signals sets the cell up for two options: inhibition of the cell cycle at the G1/S transition or inducing pro-apoptotic signals (Vousden & Prives, 2009). This gives cells the opportunity to repair damage before cellular division or undergo apoptosis if the damage is beyond repair (Kaplon et al., 2015). However, p53 has also been demonstrated to silence glycolysis and promote oxidative metabolism (Kaplon et al., 2015). For instance, p53 induces TIGAR, an enzyme that lowers the level of the glycolysis activator, fructose-2,6-bisphosphate, causing an inhibition of glycolysis.
(Bensaad et al., 2006). In addition to inhibiting glycolysis, p53 also promotes oxidative phosphorylation by upregulating cytochrome c oxidase, an essential component of the mitochondrial electron transport chain (Matoba et al., 2006). p53 has also been shown to negatively regulate lipogenesis by inhibiting fatty acid synthesis by suppressing expression levels of the transcription factor sterol regulatory element-binding protein (SREBP1c), FASN, and ACL (Yahagi et al., 2003). Overall, p53 is able to promote cell cycle arrest by acting on cell cycle machinery, but also counteracts metabolic profiles that favour proliferation by promoting oxidative metabolism over glycolytic pathways (Kaplon et al., 2015). Cyclin and CDK complexes, the E2F-pRb, and p53 are just a few examples of signaling pathways involved in cell cycle regulation that also regulate metabolism. However, on the other side of this spectrum is the emerging evidence of metabolic pathways regulating cell cycle.

One of the earliest pieces of evidence to suggest that metabolism could regulate the cell cycle was demonstrated in 1974 when cells in the absence of glucose were arrested at the G1/S stage (Pardee, 1974). More recent discoveries have shown that one of the enzymes involved in the glycolytic pathway, 6-phosphofructo-2-kinase (PFKFB3), stimulated proliferation (Kaplon et al., 2015; Yalcin et al., 2009). More specifically, PFKFB3 is responsible for converting fructose-6-phosphate to fructose-2,6-biphosphate (F2,6BP), and F2,6BP is a potent allosteric activator of 6-phosphofructokinase-1 (PFK-1) which stimulates glycolysis (Yalcin et al., 2009) (Fig. 3). PFKFB3 can also localize in the nucleus where its overexpression increases the expression of G1-promoting cyclin D3, mitotic kinase CDK1, M phase-promoting phosphatase Cdc25C, while decreasing the expression of the CDK1 inhibitor (p27Kip1) (Yalcin et al., 2009).

Another glycolytic enzyme that regulates cell cycle progression is pyruvate kinase isoform M2 (PKM2), which catalyzes the final step of phosphoenolpyruvate to pyruvate and ATP (Kaplon et al., 2015). However, PKM2 can also translocate to the nucleus where it binds to β-catenin and localizes to the cyclin D1 and c-Myc promoters to enhance their expression (Yang et al., 2012, 2011). By inducing cyclin D1 expression, PKM2 regulates the G1-S transition and progresses proliferation (Kaplon et al., 2015).
Figure 3 Glycolysis pathway

The metabolic pathway of glycolysis is illustrated in this diagram. Metabolic substrates and products are labelled under representative diagrams and enzymes responsible for metabolism are above pathway arrows.
In general, it is accepted that cell cycle progression and differentiation are distinct and mutually exclusive pathways (Myster & Duronio, 2000). When cells terminally differentiate they no longer proliferate or enter the cell cycle, and vice versa (Myster & Duronio, 2000). This also applies to the differentiation of myeloid progenitors into macrophages. Our laboratory has been able to demonstrate that induction of PU.1 expression in our inducible BN (iBN) cell system (methods 2.1) induces cell cycle arrest and allows for the terminal differentiation of these myeloid progenitor cells into macrophages (Solomon et al., 2017). In addition, following this induction of PU.1 expression, microRNAs were also upregulated. With microRNA sequencing, it was determined that the targets were involved in cell cycle, metabolic pathways and metabolic genes (Solomon et al., 2017). Included in these metabolic genes was Acly, the gene encoding ACL. The increasing evidence of metabolic pathways and enzymes having an obvious role in metabolism, but also as direct regulators of cell cycle, warrants the need to further explore and elucidate the mechanisms of how metabolic pathways can control cell cycle progression. Notably, acetyl-CoA seems to be a central point for this cross over of metabolism and cell cycle regulation. As mentioned before, acetyl-CoA is the essential precursor for de novo fatty-acid synthesis (Mashima et al., 2009), but histone acetylation is also directly dependent on acetyl-CoA (Wellen et al., 2009). Both fatty acid synthesis and histone acetylation are crucial for the progression of cell cycle progression.

1.9 Hypothesis

PU.1 induction in our iBN cell system induces cell cycle arrest and differentiation, while also inducing microRNAs that have been validated to target genes involved in lipid metabolism. Included in these target genes of lipid metabolism was ACL, which is essential for the metabolism of mitochondrial citrate into acetyl-CoA. This acetyl-CoA can then be utilized as the first and essential precursor molecule for fatty acid synthesis via the FASN pathway which contributes to cell cycle progression by providing cells with necessary biological components for proliferation. Acetyl-CoA can also be transported into the nucleus, or localized nuclear ACL can produce acetyl-CoA in the nucleus, to be utilized for histone acetylation that activates genes involved in cell cycle
progression and proliferation (Fig. 4) (Wellen et al., 2009). Therefore, this suggests that PU.1 induced myeloid differentiation and the cell cycle arrest coupled with this differentiation may be a result of inhibited ACL function, which impairs lipogenesis and lipid components available for proliferation. As a result, I intend to investigate the mechanism by which lipid metabolism may regulate myeloid proliferation by determining whether ACL inhibition can impair cell cycle progression in myeloid progenitor cells and if acetyl-CoA supplementation can rescue this effect. In order to conduct this investigation, I have formed the following hypothesis:

**Acetyl-CoA is critical for cell cycle progression and blocking acetyl-CoA production by inhibiting ATP citrate lyase (ACL) function will lead to a decrease in cell cycle progression and proliferation in myeloid progenitor cells.**

In order to assess my hypothesis, I completed these three main aims:

**Aim 1:** Determine whether *Acl* levels decrease following myeloid differentiation

**Aim 2:** Determine if inhibiting acetyl-CoA production is sufficient to block cell cycle progression

**Aim 3:** Explore the mechanisms by which acetyl-CoA inhibition blocks cell cycle progression
Acetyl-CoA is metabolized in the cell from mitochondrial citrate by ACL. Acetyl-CoA can then be utilized by one of two pathways to promote cell cycle and proliferation. The acetyl-CoA can be utilized as the first and essential precursor substrate for fatty acid and lipid biosynthesis or be used in the nucleus to acetylate histones to activate genes involved in cell cycle and proliferation. In addition to acetyl-CoA production by ACL, acetyl-CoA can be produced by many other pathways including β-oxidation of fatty acids in the cytosol or mitochondria, and branched-chain amino acids, including valine, leucine, and isoleucine, can also be metabolized to produce acetyl-CoA as mentioned in section 1.7. However, acetyl-CoA produced by ACL provides an essential pool of cytosolic acetyl-CoA for lipid biosynthesis and histone acetylation.
Acetate

\( \text{mitochondria} \)

\( \text{nucleus} \)

\( \text{reb} \)

\( \text{AC} \)

\( \text{ACL} \)

\( \text{fatty acids and lipids} \)

\( \text{cell cycle progression and proliferation} \)

\( \text{histone acetylation} \)

\( \text{PU.1} \)

\( \text{ACSS2} \)

\( \text{FASN} \)

\( \text{miR141} \)

\( \text{Kreb's cycle} \)

\( \text{acetate} \)

\( \text{acet-CoA} \)

\( \text{citrate} \)
Chapter 2: Material and Methods

2.1 Inducible BN Cell System

Our laboratory has previously generated a novel hypomorphic allele of Spi1 termed BN, which produces PU.1 at 20% of wild-type levels (Houston et al., 2007). These BN mice express a non-truncated and functional PU.1 protein, however, the expression levels are decreased to 20% of wild-type levels due to a neomycin resistance insert in the first exon sequence. The myeloid progenitor cells from the liver of fetal BN mice were infected with a two-vector doxycycline inducible system for an inducible restoration of PU.1 expression (response and regulator vectors). The regulator vector encoded the Tet-On 3G transactivator protein and GFP while the response vector contained PU.1 cDNA under the control of the TRE3G promotor (PTRE3GV) and a puromycin resistance gene. With the administration of doxycycline, Tet-On 3G binds specifically to PTRE3GV and activates transcription of PU.1, hence the term inducible BN cells (iBN cells) (Fig. 5). These iBN cells can be grown indefinitely in culture with GM-CSF but are kept as frozen stocks in liquid nitrogen.

2.2 Cell Culture

iBN cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Wisent, St-Bruno, QC), with 1ng/ml GM-CSF (Peprotech, QC), and additionally supplemented with 10% fetal bovine serum (FBS) (Wisent, St-Bruno, QC), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Mediatech, Manassas, VA), L-glutamine (2mmol/L) (Mediatech), 2-mercaptoethanol (5 x 10⁻⁵ M) (Sigma-Aldrich, St. Louis, MO). PU.1 induction experiments were performed by culture of inducible BN (iBN) cells in 1.0ng/ml GM-CSF in the presence of absence of 1,000ng/ml doxycycline for 48 hours. Additionally, iBN cells were grown with 100µM of acetyl-CoA (Sigma Aldrich, Oakville, ON), 25mM of acetate (Sigma Aldrich, Oakville, ON), 55µM BMS303141 (Cedar Lane, Burlington, ON), or 10µg/ml of C75 (Sigma Aldrich, Oakville, ON) depending on experimental conditions. Platinum-E (Plat-E) retroviral packaging cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wisent) with the same supplements described above. Progenitor B (Pro-B) cells derived from our own
Figure 5 iBN cell system model

BN cells are myeloid progenitor cells derived from the liver of fetal mice that are $Spi1^{BN/BN}$ and express PU.1 at 20% of normal levels. BN cells are transfected with the regulator and response vector to allow them to inducible for PU.1 expression following doxycycline treatment, hence the term inducible BN (iBN) cells. In the regulator vector is the long terminal repeats (LTR), the Tet-On 3G transactivator protein (Tet3G), the internal ribosome entry site (IRES), and a green fluorescent protein (GFP) reporter. In the response vector is the LTR, the puromycin resistance gene (Puro) as a selection marker, the TRE3G promoter ($P_{TRE3G}$) upstream of the PU.1 gene (PU.1).
laboratory were grown in IMDM also with the same supplements as described above, with the addition of recombinant murine interleukin-7 (IL-7) (100ng/ml; R & D Systems, Minneapolis, MN) or 25ml of supernatant from IL-7 producing J558-IL7 cell line (Winkler, Melchers, & Rolink, 1995).

2.3 Cell Cycle Analysis

Cell cycle was analyzed by flow cytometry with an allophycocyanin (APC) BrdU Flow Kit according to the manufacturer’s protocol (BD Biosciences, Mississauga, ON). Cells were labeled with bromodeoxyuridine (BrdU) for 2 hours at 37ºC. Cells were then incubated with the APC-conjugated anti-BrdU antibody using a 1:100 fold dilution. Cells were additionally stained with 7-amino-actinomycin D (7-AAD; BD Pharmingen) to determine cell cycle position. To stain cells with 7-AAD, cells were suspended in PBS containing 0.05mol/L ethylenediaminetetraacetic acid (EDTA; pH 8.0) and 0.5% BSA and then incubated with 7-AAD.

2.4 Retrovirus Production

Plat-E retroviral packaging cells (Morita, Kojima, & Kitamura, 2000) were used to generate retroviral supernatants using polyethylenimine (PEI) transfection (Godbey, Wu, & Mikos, 1999) at a 3:1 PEI/DNA ratio. Supernatant containing virus was collected 48 hours after transfection. Cells were infected by spinoculation by centrifugation at 3000 rpm for 3 hours at 32ºC with polybrene at a final concentration of 8µg/ml. After centrifugation, cells were washed and cultured for 48 hours to allow for retroviral integration and gene expression. Infection frequency was determined by flow cytometric analysis of green fluorescent protein (GFP).

2.5 Bone Marrow Cell Isolation and Culture

Bone marrow cells were extracted from the femurs and tibias of wild-type mice. The bone marrow was flushed with a syringe using PBS and collected in a tube. The bone marrow cells were washed three times with FACS buffer (2.5g BSA Fraction V, 5mM EDTA in 500 ml of PBS). The bone marrow cells were then re-suspended with 100µl of FACS buffer and 1µl each of biotinylated anti-CD11b, GR1, B220, and TER119.
antibodies (BD Pharmingen, Mississauga, ON). The cells were then washed for unbound primary antibody and resuspended with 90µl of labelling buffer as per manufacturer’s protocol (Miltenyi Biotec, Auburn, CA) and 10µl of magnetic streptavidin microbeads (Miltenyi Biotec, Auburn, CA) were added per 10^7 cells. The cells were washed again and resuspended with 500µl of separation buffer. LD columns (Miltenyi Biotec, Auburn, CA) were placed in a magnet and unlabeled cells were collected in a tube (negative selection fraction). As the biotinylated antibodies bind to streptavidin beads, the magnet holds the cells bound by the antibodies in the column and cells without any of the mature cell surface markers flow down into the tube. A fraction of these cells were labelled with phycoerythrin (PE) conjugated antibodies for CD11b and C-kit (BD Pharmingen, Mississauga, ON), and analyzed for their lineage negative characteristics based on CD11b-PE and C-kit-PE expression assessed through flow cytometry. These cells were grown in culture with IMDM and 10ng/ml and 2ng/ml of M-CSF or GM-CSF, respectively (Peprotech, QC).

2.6 Tritium Culture and Scintillation Counting

iBN cells were cultured with 0.5µCi of [³H]-Acetyl-CoA (Perkin Elmer, Waltham, MA) in 1mL of complete media for 24 hours. The media was collected in separate tubes and the pellets were washed three times with PBS. The cell pellets were then solubilized with 200µl of a 3% solution of potassium hydroxide (KOH) (Puchalski & Jasper, 1985). These solubilized cell pellets were then extracted for their lipid using 1ml of a 2:1 (v/v) chloroform:methanol solution, similar to the Folch method (without the neutralization step with acid/chloride salt) (Folch, Lees, & Stanley, 1957). The cell pellets after their washes (skipping the KOH solubilization step) were used directly for histone extraction according to the manufacturer’s protocol (Abcam, Toronto, ON). Scintillation counts were performed using a LS 6500 scintillation counter (Beckman Coulter, Ramsey, MN) using 10µl from either solubilized cell pellets, lipid extracts and histone extracts.
2.7 Reverse Transcriptase Quantitative PCR

RNA was isolated from cells using TRIzol Reagent (Life Technologies, Carlsbad, CA) and reverse-transcribed into cDNA using the iScript kit (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) was performed using iQ SYBR Green Supermix Kit (Bio-Rad) and a QuantStudio5 (Applied Biosystems, Foster City, CA). Relative messenger RNA (mRNA) levels of Acly were normalized to B2m as a reference gene and compared between samples using the comparative threshold cycle method. Acly mRNA levels were also normalized to F4/80, a mature macrophage cell surface marker. Results are presented as the mean and standard deviation (SD) of triplicate samples. Primer sequences are listed in Table 4.

2.8 Flow Cytometry

Flow cytometry analysis was performed on single-cell suspensions of cells washed in flow cytometry buffer consisting of: D-PBS, 0.05mol/L EDTA, and 0.5% BSA. Antibodies directly conjugated to phycoerythrin (PE) against CD11b and C-kit were utilized to determine lineage-negative characteristics of extracted bone marrow cells. APC-BrdU was utilized for cell cycle analysis. Flow cytometric analysis and sorting was performed using a FACSCanto and FACSaria III, respectively (BD Immunocytometry Systems, San Jose, CA) at the London Regional Flow Cytometry Facility. Data was then analyzed using FlowJo, version 10 (Tree Star, Ashland, OR).

2.9 Statistical Analysis

Statistical significance was determined with ratio-paired t test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison using Prism 5 (GraphPad Software, La Jolla, CA, USA).
### Table 4 RT-qPCR Primer Sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acly forward</td>
<td>5' - CCA GTG AAC AAC AGA CCT ATG A - 3'</td>
</tr>
<tr>
<td>Acly reverse</td>
<td>5' - AAT GCT GCC TCC AAT GAT G - 3'</td>
</tr>
<tr>
<td>B2m forward</td>
<td>5’ - TGG CTC ACA CTG AAT TCA CCC CCA – 3’</td>
</tr>
<tr>
<td>B2m reverse</td>
<td>5'- TCT CGA TCC CAG TAG ACG GTC TTG G - 3'</td>
</tr>
<tr>
<td>F4/80 (Adgre1) forward</td>
<td>5’ - GTG GTC ATA ATC TCT GCT TCT GT - 3’</td>
</tr>
<tr>
<td>F4/80 (Adrge1) reverse</td>
<td>5’ - AAA CTC CAG ATA AAC CCC GTC - 3’</td>
</tr>
</tbody>
</table>
3 Chapter 3: Results

3.1 Acly transcript levels decrease following differentiation of progenitor cells in M-CSF

PU.1 levels increase as myeloid progenitor cells undergo differentiation into macrophages (Kueh et al., 2013). Our laboratory was able to demonstrate that inducing PU.1 expression in our iBN cells with doxycycline causes cell cycle arrest and differentiation, as indicated by increased expression of the macrophage cell surface marker, F4/80 (Solomon et al., 2017). In addition to cell cycle arrest and differentiation, increased expression of PU.1 in iBN cells also upregulated microRNAs that targeted and downregulated genes involved in lipid metabolism. Included in these genes was Acly, that encodes ACL. Therefore, I wanted to determine whether Acly levels actually decrease following differentiation of early progenitor cells into myeloid lineages. This differentiation would also indicate cell cycle arrest, as the two processes are coupled. This would help establish the idea that as cells undergo differentiation, they couple this process with cell cycle arrest and a reduction in ACL’s function may be central to regulating these two coupled processes. To determine this, bone marrow cells from WT mice were extracted and labelled with biotinylated antibodies for CD11b, GR-1, B220, and TER119 to bind mature macrophages, granulocytes, B cells, and erythrocytes, respectively, and magnetic streptavidin beads were added and cells were passed through a magnetic column (LD column) (Fig. 6). The cells that pass through the magnetic column and into the collection tube were regarded as lineage negative (lin⁻) cells with early progenitor markers as indicated by a decrease in CD11b expression (Fig. 7D) and an increase in C-kit expression (Fig. 7B) after passing through the column. These lineage-negative cells were then cultured in either M-CSF or GM-CSF (10ng/ml and 2ng/ml, respectively) for up to 6 days and RNA was extracted at day 2, 4, and 6. I expected that these lin⁻ cells, after being grown in M-CSF or GM-CSF for up to 6 days would undergo differentiation as a result of decreased levels of Acly transcripts.

By day 6 there was evidence of proliferation and differentiation marked by morphological changes, especially in lin⁻ cells grown in M-CSF (Fig. 8). RNA was reverse transcribed into cDNA and RT-qPCR analysis was done with Acly as the gene
**Figure 6 Bone marrow cell isolation procedure**

Bone marrow cells from wildtype mice were extracted and labelled with biotinylated antibodies for CD11b, GR-1, B220, and TER119 and magnetic streptavidin beads. They were placed in a magnetic column and all unlabeled bone marrow cells were collected and referred to as lineage negative (lin⁻), due to a lack of mature marker expression.
Antibodies:
CD11b
GR1
B220
TER119

BMCs

Negative Selection

Lin− Cells
Figure 7 CD11b and C-kit expression after column separation

Following magnetic column separation of biotinylated antibody and magnetic streptavidin labelled bone marrow cells, small fractions of cells were labelled with either PE labelled anti-CD11b or anti-C-kit antibodies to determine the effectiveness of the column separation. A. The frequency of C-kit positive bone marrow cells without column separation was low with 12.6% of the bone marrow cells being C-kit+. B. The frequency of C-kit+ cells increases following column separation and increased to 34.1%. C. The frequency of CD11b positive bone marrow cells without column separation was high with 68.2% of cells being CD11b+. D. The amount of CD11b+ cells decreases following column separation to 4.33%. Data is representative of 3 independent experiments.
Control

C-Kit enrichment

12.6

Count

0

10^3

C-Kit PE Fluorescence

10^5

Control

CD11b Depletion

68.2

Count

0

10^3

C-Kit PE Fluorescence

10^5

Depleted

C-Kit enrichment

34.1

Count

0

10^3

C-Kit PE Fluorescence

10^5

Depleted

CD11b Depletion

4.33

Count

0

10^3

C-Kit PE Fluorescence

10^5
Figure 8 Column separated bone marrow cells grown in M-CSF and GM-CSF undergo proliferation and differentiation as marked by morphological changes

Extracted bone marrow cells that were separated from the magnetic column were grown in IMDM with additional 10ng/ml or 2ng/ml M-CSF or GM-CSF, respectively. Pictures and RNA were taken at day 2, 4, and 6 time points. By day 6, especially with BMCs grown in M-CSF, there is noticeable proliferation and differentiation marked by the changes of the cells into a more macrophage-like morphology. Scale bar, 20µm for all images. Data is representative of 3 independent experiments.
target with β2m and F4/80 as reference genes to determine relative fold change through
the threshold cycle method. For cells grown in M-CSF (but not GM-CSF), there was a
significant decrease in the level of transcripts for Acly in reference to both β2m and F4/80
(Fig. 9), suggesting that as these lin⁻ cells undergo differentiation with M-CSF, the
transcript levels for Acly decrease.

3.2 Acetate and acetyl-CoA rescues cell cycle arrest from impaired ACL function

After demonstrating that Acly levels decrease following differentiation of lin⁻ cells
grown in M-CSF, I wanted to determine whether inhibiting ACL function, using the ACL
inhibitor, BMS303141 (BMS), would be able to cause an arrest in cell cycle.

The rationale behind this was that the previous experiment showed that Acly
transcript levels decreased following myeloid differentiation, therefore, in order to
establish that cell cycle arrest was also occurring as a result of decreased ACL function,
we wanted to utilize a direct ACL inhibitor (BMS). This would determine whether cell
cycle could be arrested in myeloid progenitor cells through ACL inhibition. I also wanted
to determine if acetate and acetyl-CoA supplementation could rescue any cell cycle
impairment due to BMS. Acetate was used as a supplement because exogenous sources
of acetate are able to transported into the cell by monocarboxylate transporters
(Comerford et al., 2014) and ACSS2 can metabolize acetate into acetyl-CoA (Wellen &
Thompson, 2012). Acetyl-CoA was used to supplement against ACL inhibition as ACL’s
function is to produce acetyl-CoA from mitochondrial derived citrate. Showing that the
cell cycle could be rescued by these two metabolic substrates would help suggest that
acetyl-CoA and ACL function are important in regulating cell cycle as acetate could
compensate for a lack of ACL function and acetyl-CoA can act as a direct substitute for
impaired ACL function. Therefore, in order to determine this, iBN cells were grown with
or without BMS (55µM) and BMS treated iBN cells were supplemented with acetate
(25mM) or acetyl-CoA (100µM) in 2mL of their normal growth media. These
concentrations were determined by optimization experiments for BMS, acetyl-CoA, and
acetate.
Using RT-qPCR, mRNA levels of *Acl* in the column separated BMCs were determined for day 2, 4, and 6 time points. *Acl* transcript levels were normalized to β2m and F4/80 (*Adgre1*). *Acl* transcript levels decreased by day 6 in reference to both β2m and F4/80 in BMCs grown in M-CSF. **A.** BMCs grown in M-CSF at day 2, 4, and 6 time points with *Acl* target in reference to β2m transcript levels. Two-way ANOVA, p<0.05, and N=3. **B.** BMCs grown in M-CSF at day 2, 4, and 6 time points with *Acl* target in reference to F4/80 transcript levels. Two-way ANOVA, p<0.05, and N=3.
The expectation was that ACL inhibition through BMS treatment would be sufficient to cause cell cycle arrest in iBN cells and that both acetate and acetyl-CoA supplementation would be able to rescue the cell cycle by compensating (acetate) or providing acetyl-CoA directly.

The experiment showed that ACL inhibition via BMS was sufficient to cause cell cycle arrest (Fig. 10B and 11B) which was significantly decreased compared to control iBN cells not given BMS. In addition, both acetate (Fig. 10C) and acetyl-CoA (Fig. 11C) supplementation were able to partially but significantly rescue cell cycle progression in these BMS treated iBN cells as marked by an increase in the percentage of cells in the S-phase. Acetate and acetyl-CoA treatment in iBN cells that were not treated with BMS had no significant impact on cell cycle, suggesting a rescue effect of acetate and acetyl-CoA.

3.3 Acetate and acetyl-CoA supplementation rescues iBN induced cell cycle arrest

After establishing that ACL inhibition causes cell cycle arrest, and that acetate and acetyl-CoA supplementation partially rescues cell cycle, the next step was to demonstrate if acetate and acetyl-CoA supplementation could rescue PU.1 induced cell cycle arrest in iBN cells. Our laboratory has previously demonstrated that PU.1 induced iBN cells undergo cell cycle arrest (Solomon et al., 2017). As mentioned before, PU.1 induction causes cell cycle arrest in iBN cells and we proposed that the upregulation of microRNAs that are targeting and downregulating genes involved in lipid metabolism are responsible for cell cycle arrest. Therefore, I wanted to determine if supplementing these PU.1 induced iBN cells with the direct metabolic product of ACL (acetyl-CoA), and an indirect source of acetyl-CoA (acetate via the ACSS2 pathway), could rescue the cell cycle by providing these iBN cells with the necessary substrates for lipid biosynthesis. We expected that supplementation with both acetate and acetyl-CoA would be able to rescue cell cycle of PU.1 induced iBN cells.

Following induction of PU.1 in iBN cells with doxycycline, the cell cycle was arrested as expected, furthermore, acetate (Fig. 12C) and acetyl-CoA (Fig. 13C)
Figure 10 ACL inhibition causes cell cycle arrest and acetate supplementation can rescue the cell cycle

iBN cells were grown with the ACL inhibitor, BMS at 55µM, and cell cycle was arrested. Supplementation of the BMS treated iBN cells with 25mM of acetate was able to significantly rescue the cell cycle. **A.** iBN cells grown in with BN media (IMDM + 1ng/µl GM-CSF). **B.** iBN cells grown with BN media and 55µM BMS **C.** iBN cells grown with BN Media, 55 µM BMS, and 25mM of acetate. **D.** Ratio of cells in S-phase. **A-C** are representative of, and **D** qualifies as mean ± SEM, 4 independent experiments, *p < 0.05*, ratio paired t-test (data points are connected by lines to indicate paired trials).
A

Control

B

BMS

C

BMS + Acetate

D

Percentage of cells in S-phase

**

Control

Acetate

55uM BMS

55uM BMS + 25mM Acetate
Figure 11 ACL inhibition causes cell cycle arrest and acetyl-CoA can rescue the cell cycle

iBN cells were grown with the ACL inhibitor, BMS at 55µM, and cell cycle was arrested. Supplementation of the BMS treated iBN cells with 100µM of acetyl-CoA was able to significantly rescue the cell cycle. **A.** iBN cells grown in with BN media (IMDM + 1ng/μl GM-CSF). **B.** iBN cells grown with BN media and 55 µM BMS **C.** iBN cells grown with BN Media, 55 µM BMS, and 100 µM of acetyl-CoA. **D.** Ratio of cells in S-phase. **A-C** are representative of, and **D** quantifies as mean ± SEM, 4 independent experiments. * p < 0.05, ratio paired t-test (data points are connected by lines to indicate paired trials).
% of cells in phase.

**Figure 1.**

**A.** Control

**B.** BMS

**C.** BMS + Acetyl-CoA

**D.** Percentage of cells in S-phase

- Control
- Acetyl-CoA
- 55μM BMS
- 55μM BMS + 100μM Acetyl-CoA

* Indicates statistical significance.
Figure 12 Induction of PU.1 iBN cells causes cell cycle arrest and acetate supplementation can rescue the cell cycle

Acetate supplementation was able to rescue proliferation in PU.1 induced iBN cells. Cell cycle was analyzed using a BrdU Flow Kit (BD Pharmingen). A. iBN cells grown in with BN media (IMDM + 1ng/μl GM-CSF). B. iBN cells grown with BN media and 1000ng/ml doxycycline C. iBN cells grown with BN Media, 1000ng/ml doxycycline, and 25mM of acetate. D. Ratio of cells in S-phase. A-C are representative of, and D quantifies as mean ± SEM, 4 independent experiments. * p < 0.05, ratio paired t-test (data points are connected by lines to indicate paired trials).
Percentage of cells in S-phase.

A | Control
---|---

B | Dox

C | Dox + Acetate

D | Percentage of cells in S-phase

- Control: S 60.8
- Dox: S 38.1
- Dox + Acetate: S 40.4
Figure 13 Induction of PU.1 iBN cells causes cell cycle arrest and acetyl-CoA supplementation can rescue the cell cycle

Acetyl-CoA supplementation was able to rescue proliferation in PU.1 induced iBN cells. Cell cycle was analyzed using a BrdU Flow Kit (BD Pharmingen). **A.** iBN cells grown in with BN media (IMDM + 1ng/μl GM-CSF). **B.** iBN cells grown with BN media and 1000ng/ml doxycycline. **C.** iBN cells grown with BN Media, 1000ng/ml doxycycline, and 100 μM of acetyl-CoA. **D.** Ratio of cells in S-phase. **A-C** are representative of, and **D** quantifies as mean ± SEM, 4 independent experiments. * p < 0.05, ratio paired t-test (data points are connected by lines to indicate paired trials).
Percentage of cells in phase

Control

Dox

Dox + Acetyl-CoA

D

Percentage of cells in S-phase

Control Dox

Dox Acetyl-CoA
supplementation were both able to partially but significantly rescue the cell cycle and increase the percentage of cells in the S-phase.

3.4 Acetyl-CoA rescues cell cycle arrest from impaired FASN function

Given that acetate and acetyl-CoA supplementation was able to rescue cell cycle arrest from both ACL inhibition via BMS and PU.1 induction via doxycycline, the next experiment was to determine whether a downstream pathway of fatty acid synthesis could also arrest cell cycle. Specifically, I wanted to determine if inhibiting FASN, with C75 (Landree et al., 2004), could also arrest cell cycle as this would indicate more strongly an important role for fatty acids in cell cycle progression. As a substrate, acetyl-CoA can be utilized for fatty acid and lipid biosynthesis, or it can be used for histone acetylation. However, based on the previous experiments, it cannot be stated with certainty that the acetyl-CoA used to rescue the cell cycle is being utilized by the cells for lipid biosynthesis. Therefore, inhibiting FASN, an essential but more downstream pathway for fatty acid/lipid biosynthesis would provide stronger evidence that inhibition of lipid biosynthesis is a regulatory mechanism that controls cell cycle. If inhibiting FASN causes cell cycle arrest, then rescuing this pathway with acetyl-CoA and acetate supplementation would also help to suggest that these substrates are being utilized for lipid biosynthesis over histone acetylation. The expectation was that FASN inhibition would cause cell cycle arrest and that both acetate and acetyl-CoA supplementation would be able to rescue the cell cycle.

I was able to show that FASN inhibition with 10µg/ml of C75 (determined through optimization) arrested cell cycle (Fig. 14B). Additionally, acetyl-CoA was able to partially, but significantly, rescue cell cycle (Fig. 14D). This rescue was not observed with acetate supplementation. Therefore, taking the results together, FASN inhibition was able to arrest the cell cycle, and supplementing with acetyl-CoA, a competitor of C75, was able to rescue the cell cycle, suggesting that the metabolic pathway for fatty acid and lipid biosynthesis may be responsible for regulating cell cycle progression.
Figure 14 FASN inhibition causes cell cycle arrest and acetyl-CoA supplementation can rescue the cell cycle

Acetyl-CoA supplementation was able to rescue proliferation in FASN inhibited iBN cells. Cell cycle was analyzed using a BrdU Flow Kit (BD Pharmingen). A. iBN cells grown in with BN media (IMDM + 1ng/μl GM-CSF). B. iBN cells grown with BN media and 10μg/ml C75 C. iBN cells grown with BN Media, 10μg/ml C75, and 100 μM of acetyl-CoA. D. Ratio of cells in S-phase. A-C are representative of, and D quantifies as mean ± SEM, 3 independent experiments. * p < 0.05, ratio paired t-test (data points are connected by lines to indicate paired trials).
The image shows a flow cytometry analysis comparing the percentage of cells in different phases of the cell cycle under different conditions.

**A. Control**
- Anti-BrdU APC vs. 7-AAD
- S phase: 47.5%
- G1: 51.2%
- G2: 1.26%

**B. C75**
- Anti-BrdU APC vs. 7-AAD
- S phase: 16.4%
- G1: 78.9%
- G2: 4.79%

**C. C75 + Acetyl-CoA**
- Anti-BrdU APC vs. 7-AAD
- S phase: 26.8%
- G1: 68.3%
- G2: 4.94%

**D. Percentage of cells in S phase**
- Different lines represent different treatments:
  - 10ug C75
  - 10ug C75 + Acetyl-CoA
- Asterisk indicates a significant difference.
3.5 Extracellular $[^3H]$-Acetyl-CoA is incorporated into cells

After establishing that inhibiting FASN could result in cell cycle arrest, the next matter to address was the mechanism by which extracellular acetyl-CoA regulates cell cycle. The first step to uncovering the mechanism by which acetyl-CoA regulates the cell cycle was to confirm that acetyl-CoA is able to enter the cells. This is important as it is well established that the CoA group is too large to allow for passive diffusion into cells and there are no reported transporters that allow for import of extracellular acetyl-CoA (Bhagavan & Ha, 2011). The possibility that extracellular acetyl-CoA was being imported into cells was therefore assessed by the addition of extracellular $[^3H]$-acetyl-CoA, followed by quantification of $[^3H]$-acetyl-CoA in the cells. The tritium is located on the acetyl portion of acetyl-CoA.

Using a liquid scintillator counter, I counted tritium levels in iBN cells as disintegrations per minute (DPM) counts. One million iBN cells were incubated in 1ml of their normal media supplemented with 0.5µCi of $[^3H]$-acetyl-CoA for 24 hours. After 24 hours, the cells were spun down into a pellet and the media was collected in a separate Eppendorf tube. The cell pellets were washed three times with PBS and after the washes, the pellets were solubilized using a 200µl of a 3% solution of KOH to solubilize the cells (Puchalski & Jasper, 1985). If acetyl-CoA was imported into the cells, I would expect to detect tritium levels in the solubilized cell pellets as all the previous experiments with acetyl-CoA supplementation indicated that acetyl-CoA was able to rescue the cell cycle, which would not be possible if acetyl-CoA was not entering the cells.

The results showed that there were significantly higher counts in the supernatant compared to the solubilized cell pellets, however, solubilized cell pellets of iBN cells incubated with $[^3H]$-acetyl-CoA had a significant amount of tritium counts compared to the negative control (iBN cells grown in just their normal media) (Fig. 15). Therefore, these results indicate that iBN cells are able to uptake exogenous acetyl-CoA into their cells.
Figure 15 $[^3\text{H}]$-acetyl-CoA is able to enter myeloid progenitor cells

Tritium DPM counts were higher in both supernatant and BN cells when given $[^3\text{H}]$-acetyl-CoA. iBN cells were incubated for 24 hours with 0.5µCi $[^3\text{H}]$-acetyl-CoA. The supernatant was collected and the cell pellet was solubilized and then counted using scintillation vials to compare the DPM values compared to the negative control (BN). Data is presented as mean ± SEM, one-way ANOVA with Tukey’s multiple comparisons test, p < 0.05, n=5.
3.6 \( [^3\text{H}]\)-Acetyl-CoA uptake in iBN cells increases with ACL inhibition

After establishing that acetyl-CoA is indeed able to enter the cell, the next step was to demonstrate if ACL inhibition with BMS administration could increase \( [^3\text{H}]\)-acetyl-CoA uptake. Knowing that BMS treatment targets ACL and causes cell cycle arrest that is able to be rescued with acetyl-CoA supplementation, we proposed that BMS treatment would starve these iBN cells of acetyl-CoA and as a consequence, they would increase their uptake of \( [^3\text{H}]\)-acetyl-CoA from their environment. Therefore, iBN cells were grown in 1ml of normal media with 0.5µCi of \( [^3\text{H}]\)-acetyl-CoA and 55µM of BMS.

Consistent with my prediction, there was a significant increase in the uptake of \( [^3\text{H}]\)-acetyl-CoA in BMS treated iBN cells (Fig. 16). These results suggest that inhibition of ACL and consequently, impaired acetyl-CoA production in iBN cells, causes these cells to increase their uptake of exogenous acetyl-CoA. These results could also suggest that there may be some sort of sensing mechanism in iBN cells for acetyl-CoA levels within the cell, that could upregulate mechanisms to increase acetyl-CoA uptake from the environment.

3.7 \( [^3\text{H}]\)-Acetyl-CoA uptake in iBN cells decreases with acetate supplementation

Knowing that treatment with BMS increases uptake of \( [^3\text{H}]\)-acetyl-CoA in iBN cells, the next experiment to address was whether or not supplementation with unlabeled acetate or acetyl-CoA could reduce and compete with the uptake of \( [^3\text{H}]\)-acetyl-CoA in BMS treated iBN cells. The rationale for this experiment was to suggest the metabolic pathway that acetate and acetyl-CoA were entering to rescue proliferation in ACL inhibited cells. If acetate was being transported into the cell and then metabolized into acetyl-CoA via ACSS2, then I would expect acetate supplementation to be able to compensate for the lack of ACL function in producing acetyl-CoA, therefore, I would
**Figure 16 ACL inhibition increases acetyl-CoA uptake**

BMS treatment increases [³H]-acetyl-CoA uptake in BN cells. BN cells were incubated with [³H]-acetyl-CoA and BMS for 24 hours. Cell pellets of negative control (BN), BN cells and [³H]-acetyl-CoA, and BN cells given [³H]-acetyl-CoA and BMS were solubilized and DPM values were counted through a liquid scintillator. Data is presented as mean ± SEM, one-way ANOVA with Tukey’s multiple comparisons test, p < 0.05, n=7.
also expect less $[^3]H$-acetyl-CoA uptake in the cells. Similarly, if acetyl-CoA is being taken up from the extracellular environment, and utilized similarly to $[^3]H$-acetyl-CoA, I would expect that unlabeled acetyl-CoA should compete with $[^3]H$-acetyl-CoA for uptake, reducing the amount of tritium present in the cell. Therefore, iBN cells were treated with 55µM of BMS and 0.5µCi of $[^3]H$-acetyl-CoA and additionally supplemented with either 25mM of acetate of 100µM of unlabeled acetyl-CoA.

Unexpectedly, supplementation with unlabeled acetate at 25mM, but not with 100µM unlabeled acetyl-CoA reduced the amount of $[^3]H$-acetyl-CoA uptake by iBN cells (Fig. 17). These results suggest that acetate is sufficient to compensate for ACL function, as shown in the previous experiment where acetate was able to partially rescue the cell cycle in ACL treated iBN cells.

3.8 $[^3]H$-Acetyl-CoA is incorporated in both lipids and histones

One of the most important and crucial experiment of these series of experiments was attempting to identify the mechanism by which acetyl-CoA rescues cell cycle. Acetyl-CoA may be able to regulate cell cycle through fatty acid and lipid synthesis and thereby providing the biological substrates to sustain proliferation or through histone acetylation and promoting gene activation of genes involved in cell cycle. Therefore, to determine which of these two pathways that acetyl-CoA is incorporated into, iBN cells were incubated for 24 hours with 0.5µCi of $[^3]H$-acetyl-CoA and extracted for their lipids (using Folch’s extraction method (Folch et al., 1957) without the acidification/chloride salt neutralization step) and histones (using a kit from Abcam). By comparing the amount of $[^3]H$-acetyl-CoA incorporation into lipid versus histone extracts, the distribution of acetyl-CoA into these two pathways can be elucidated. Based on the previous ACL and FASN inhibition experiments with acetyl-CoA supplementation, the expectation was that $[^3]H$-acetyl-CoA would be more incorporated in lipid extracts than histone extracts.
Figure 17 Acetate supplementation decreases the uptake of $[^3\text{H}]-\text{acetyl-CoA}$

Addition of acetate reduced $[^3\text{H}]-\text{acetyl-CoA}$ DPM counts but acetyl-CoA addition had no significant difference on DPM counts. BN cells were treated with $[^3\text{H}]-\text{acetyl-CoA}$, BMS and acetate and $[^3\text{H}]-\text{acetyl-CoA}$, or BMS and acetyl-CoA and $[^3\text{H}]-\text{acetyl-CoA}$ for 24 hours and counted using a liquid scintillator. Data is presented as mean ± SEM, one-way ANOVA with Tukey’s multiple comparisons test, $p < 0.05$, $n=5$. 
The results showed that $[^3]$H-acetyl-CoA was incorporated into both lipids and histone extracts (Fig. 18A). In each experimental condition, 500,000 iBN cells were used. However, the extraction methods produce extracts of different volumes (histone extracts were ~150ml, lipid extracts were ~750ml), whereas scintillation counts were always performed using 10µl of extracts. Therefore, when normalized to the number of cells and the 5 fold dilution of the lipid extract, relative to the histone extract, it was determined that exogenous acetyl-CoA was preferentially incorporated into lipids over histones (Fig. 18B).

3.9 $[^3]$H-Acetyl-CoA incorporation in lipids increases with ACL inhibition

Lastly, in order to confirm that ACL inhibition causes cell cycle arrest through impaired lipid and fatty acid synthesis, and that acetyl-CoA supplementation is able to rescue the cell cycle by providing the necessary essential substrates for fatty acid synthesis, iBN cells were treated with BMS and given $[^3]$H-acetyl-CoA. The lipids were then extracted from these BMS and $[^3]$H-acetyl-CoA treated iBN cells to determine whether BMS treatment increased the amount of $[^3]$H-acetyl-CoA incorporation in lipids.

Previous rescue experiments have already shown that ACL inhibition is sufficient to cause cell cycle arrest, and that inhibition of lipid synthesis downstream of ACL, via FASN inhibition, is also sufficient to cause cell cycle arrest. Therefore, taken together we know that the cell cycle can be arrested due to impaired lipid biosynthesis. However, showing that BMS treatment in iBN cells can increase the amount of $[^3]$H-acetyl-CoA incorporation in lipids would provide further evidence that the recovery of cell cycle following acetyl-CoA supplementation was a result of acetyl-CoA feeding into the lipid biosynthesis pathway. Therefore, we expected that BMS treatment in iBN cells would cause an increase of $[^3]$H-acetyl-CoA incorporation in lipids.
Figure 18 $[^3$H$]$-acetyl-CoA is incorporated in both lipids and histones

iBN cells were incubated with $[^3$H$]$-acetyl-CoA for 24 hours and extracted for their lipids or histones. A. $[^3$H$]$-acetyl-CoA was incorporated equally into lipid and histone extracts compared to negative control. B. When lipid and histone extracts are normalized to cell concentrations, greater $[^3$H$]$-acetyl-CoA incorporation in lipid extracts was observed. Data is presented as mean ± SEM, one-way ANOVA with Tukey’s multiple comparisons test, $p < 0.05$, $n=5$. 
Normalized DPM

(normalized to cell concentrations)
The results showed that BMS treatment increased the amount of $[^3H]$-acetyl-CoA incorporation in lipids compared to iBN cells just given $[^3H]$-acetyl-CoA (Fig. 19). Therefore, taking these results and previous results together, ACL inhibition impairs cell cycle/proliferation through decreased lipid biosynthesis, and inhibition can be partially reversed by bypassing ACL by the exogenous addition of acetyl-CoA.
Figure 19 ACL inhibition increases [³H]-acetyl-CoA incorporation in lipids

iBN cells were treated with BMS and given [³H]-acetyl-CoA. The cells were extracted for their lipids and there was greater [³H]-acetyl-CoA incorporation in lipids with cells treated with BMS compared to iBN cells that were not treated with BMS. Data is presented as mean ± SEM, one-way ANOVA with Tukey’s multiple comparisons test, p < 0.05, n=6.
4 Chapter 4: Discussion

4.1 Inhibiting ACL results in decreased proliferation

Based on the results, we have been able to demonstrate that early progenitor cells from the bone marrow of WT mice are able to undergo differentiation into myeloid linages when grown in M-CSF and this is marked by a decrease in the transcript levels of Acly. Inhibiting ACL via BMS is sufficient to cause cell cycle arrest in myeloid progenitor cells (iBN cells) but acetate and acetyl-CoA supplementation can rescue the cell cycle. Similarly, PU.1 induced cell cycle arrest in iBN cells can also be rescued through acetate and acetyl-CoA supplementation. We have also been able to show that inhibiting the FASN pathway via C75 is also sufficient to cause cell cycle arrest and that acetyl-CoA supplementation can rescue the cell cycle. Using radiolabeled acetyl-CoA, I was able to confirm that acetyl-CoA is able to enter into myeloid progenitor cells and that BMS treatment increases the uptake of acetyl-CoA. Finally, exogenously added acetyl-CoA was incorporated into both histones and lipids, with ACL inhibition increasing the amount of acetyl-CoA incorporated into lipids. Based on these experiments, my hypothesis that acetyl-CoA is critical for cell cycle progression and blocking acetyl-CoA production by inhibiting ACL function leads to a decrease in cell cycle progression and proliferation in myeloid progenitor cells is supported.

This discovery has many implications in regards to cancers, where altered metabolism is a hallmark of malignant cells, and where ACL has been shown to be overexpressed in many cancer types and thus represents a possible therapeutic target (Hanai, Doro, Seth, & Sukhatme, 2013; Khwairakpam et al., 2015; Pietrocola et al., 2015; Wang, Yin, Wei, Yang, & Jiang, 2017; Zaidi, Swinnen, & Smans, 2012). As mentioned before, the metabolic requirements of cells change drastically as they progress through cell cycle and proliferation, with proliferating cells requiring the sufficient building blocks to double their cellular contents (Kaplon et al., 2015). Highly proliferative cells, including cancers, preferentially use glycolysis over oxidative phosphorylation because, although it is less efficient at generating ATP, glycolysis offers a quicker production of ATP and provides intermediate metabolites for several biosynthetic pathways (nucleotide, amino acid, and fatty acid synthesis) (Lunt & Vander
This preferential use of glycolysis by cancer cells is known as the Warburg effect (Lunt & Vander Heiden, 2011; Warburg, 1956).

Many cancer types also display increased endogenous fatty acid biosynthesis, regardless of the levels of extracellular lipids available (Menendez & Lupu, 2007). However, most normal cells, even highly proliferative cells, preferentially use dietary or exogenous lipids for the synthesis of new structural lipids (Menendez & Lupu, 2007). Upregulated *de novo* fatty acid synthesis in cancer cells provides the necessary lipid structural components for membranes in cancer cells (Rysman et al., 2010). This increase in fatty acid synthesis in cancers are a result of an upregulation in the expression and activity of various enzymes involved in fatty acid synthesis (Menendez & Lupu, 2007). ACL-produced cytosolic acetyl-CoA is central to fatty acid synthesis. Consistent with this, ACL is known to be upregulated in many cancers and inhibition of ACL suppresses proliferation of many cancerous cells (Hanai et al., 2013; Khwairakpam et al., 2015; Pietrocola et al., 2015; Wang et al., 2017; Zaidi, Swinnen, et al., 2012). Thus, my results in BMS treated iBN cells agree with the findings of previous literature (Dufort et al., 2014; Pietrocola et al., 2015). In fact, BMS was designed in the hopes of identifying a more cell-permeable ACL inhibitor (Li et al., 2007).

BMS was first designed as a cell-permeable ACL inhibitor, with the main structure identified as 2-hydroxy-N-arylbenzenesulfonamide (Li et al., 2007). BMS303141 or compound 9 as identified by Li et al., structurally deviates from citrate but is considered a citrate analog and ACL inhibitor (J. J. Li et al., 2007; Ma, Chu, & Cheng, 2009). Similarly, a study that utilized BMS showed that B cells that were treated with BMS blocked glucose incorporation in *de novo* lipid biosynthesis, cholesterol, fatty acids, phospholipids and inhibited proliferation (Dufort et al., 2014). Additionally, it has been established that ACL inhibition can suppress tumor growth (Hatzivassiliou et al., 2005; Pietrocola et al., 2015) and re-emerging as a therapeutic drug target for lowering LDL cholesterol (Burke & Huff, 2017).
4.2 Acetate and Acetyl-CoA can rescue the cell cycle

The biosynthesis of lipids and fatty acids is one pathway that is crucial for proliferation. The first step of lipid biosynthesis is the production of fatty acids from the essential and primary substrate acetyl-CoA. Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) and with FASN, through multiple repeated condensations of the acetyl groups, metabolizes a 16-carbon saturated fatty acid, palmitic acid (Baenke, Peck, Miess, & Schulze, 2013). Fatty acids can then be used to generate many types of lipids including triacylglycerides for energy storage, or converted into phosphoglycerides as structural components of membranes (Baenke et al., 2013). As mentioned above, many cancers upregulate de novo lipogenesis (Menendez & Lupu, 2007; Rysman et al., 2010) and inhibiting FASN and other metabolic enzymes (including ACL) in the fatty acid biosynthesis pathway are effective in limiting the growth and proliferation of cancer cells (Flavin, Peluso, Nguyen, & Loda, 2010; Hatzivassiliou et al., 2005; Khwairakpam et al., 2015). This indicates a clear role for lipid metabolism in regards to the proliferation of both normal and malignant cells, with lipid biosynthesis providing the cell with components for biological membranes which can also be utilized for energy (Baenke et al., 2013).

In addition to lipid biosynthesis, histone acetylation is also an important pathway in proliferation. Acetyl-CoA links lipid metabolism and histone acetylation to proliferation by being the midpoint in these two processes, with ACL produced Acetyl-CoA diverted into both the fatty acid biosynthesis and histone acetylation pathways (Wellen et al., 2009). Wellen was able to demonstrate that ACL is found in both the nucleus and cytoplasm and that using an siRNA to silence ACL significantly reduced global histone acetylation (Wellen et al., 2009). Through supplementing cells with acetate and through the conversion of acetyl-CoA, Cai et al. was able to demonstrate that acetyl-CoA promotes the acetylation of histones bound to cell proliferation and growth genes, including ribosome biogenesis, amino acid metabolism, and even lipid, fatty acid, and isoprenoid metabolism (Cai et al., 2011).

Supplementing iBN cells with acetate or acetyl-CoA following arrest by either BMS or PU.1 induction partially but significantly rescues cell cycle progression. Many
studies and reviews have highlighted the uptake of acetate as a hallmark and distinguishing aberrant metabolic feature of cancer cells (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2016). The plasma membrane is impermeable to acetate anions (CH$_3$COO$^-$) due to the charge, however, the non-ionic form acetic acid (CH$_3$COOH), is modestly permeable (Schug et al., 2016). However, acetic acid is a weak acid (pK$_a$=4.75) and as a result is typically deprotonated—i.e. in a charged and membrane-importable state at physiological pH 5.5 (in the large intestine) to pH 7 (in tissues) (Schug et al., 2016). Therefore, most of the acetate transported into cells is transported via monocarboxylate transporters (MCTs) (Halestrap & Price, 1999; Schug et al., 2016). The acetate is then metabolized into acetyl-CoA by ACSS2 in the cytosol where acetyl-CoA can promote proliferation through fatty acid synthesis or histone acetylation (Schug et al., 2016). High expression of ACSS2 is found in breast cancers, glioblastomas, ovarian cancers, lung cancers, and is often correlated with higher-grade tumors and poorer survival compared to tumors with low ACSS2 expression (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2015). Depletion of ACSS2 in tumor xenografts was able to inhibit growth, suggesting that acetate and the metabolism of acetate into acetyl-CoA are critical for sustaining tumor growth (Comerford et al., 2014; Schug et al., 2015). One study supplemented melanoma cell lines with acetate and demonstrated that there was a high dependence on acetate for cell viability and proliferation in glucose-deprived melanoma cell lines (Lakhter et al., 2016). Although iBN cells are not considered a cell line, their inherent reduced PU.1 expression levels can be characterized as a “pre-leukemic” state (Solomon et al., 2017), which may also explain how acetate supplementation was able to promote proliferation in these iBN cells.

A limitation of my study design is that the physiological concentrations of acetate in human plasma is between 50-200µM (Schug et al., 2016), much lower than the concentration used in my rescue experiments (25mM acetate). This caveat also applies to my radiolabeled experiments where I demonstrated that acetate supplementation (25mM) reduces the uptake of [$^3$H]-acetyl-CoA, but not acetyl-CoA supplementation (100µM). These results could suggest that iBN cells utilize acetyl-CoA present in the environment to a high degree when ACL function is inhibited. Alternatively, the concentrations of acetate supplementation (being 250 fold greater than acetyl-CoA supplementation) could
have simply diluted the solute concentration of $[^3\text{H}]$-acetyl-CoA, therefore reducing the possibility of $[^3\text{H}]$-acetyl-CoA uptake. However, there seemed to be a general trend of acetyl-CoA being a more effective supplement to rescue the cell cycle over acetate. This was evident by the higher percentage of iBN cells in the S-phase when induced for PU.1 (Fig. 11) and supplemented with acetyl-CoA and only acetyl-CoA being able to rescue the cell cycle of FASN inhibited iBN cells (Fig. 14). Although with the FASN inhibition experiment utilizing C75, there is another caveat to explain.

The relevance of acetyl-CoA being able to rescue the cell cycle from C75 administration and not acetate becomes apparent when investigating the mechanism by which C75 inhibits FASN. C75 is regarded as a competitive irreversible inhibitor in that acetyl-CoA is able to compete with C75, until C75 binds to FASN and undergoes irreversible enzyme modification (Rendina & Cheng, 2005). This may explain why acetate was not able to rescue the cell cycle in C75 treated iBN cells as the additional metabolic step of converting acetate into acetyl-CoA may have coincided with irreversible modification of FASN by C75.

However, acetyl-CoA supplementation experiments and studies are almost non-existent in the literature as it is commonly accepted that acetyl-CoA cannot passively diffuse through cell membranes due to the large CoA group, and due to the absence of any identified CoA transporters on the cell surface (Bhagavan & Ha, 2011). Acetyl-CoA transporters have been discovered in other subcellular compartments, notably acetyl-CoA transporters-1 (AT-1), which is expressed on the endoplasmic reticulum (ER) and mediated acetyl-CoA transport into the ER lumen (Hirabayashi, Kanamori, Nomura, & Nomura, 2004; Jonas, Pehar, & Puglielli, 2010; Kanamori et al., 1997). Given that the AT-1 transporter is found to be localized on the ER, although there has been no evidence to suggest this, it could be possible to speculate through the secretory pathway that AT-1 could be exported to the Golgi apparatus, and from the Golgi to the cell membrane (Borgese, 2016).

Despite the uncertainty behind the mechanisms by which acetyl-CoA enters iBN cells, the evidence that solubilized cell pellets of iBN cells treated with $[^3\text{H}]$-acetyl-CoA
had significantly higher DPM counts compared to untreated iBN cells indicates that acetyl-CoA must be imported into the cell cytosol (Fig. 15). This is a novel and exciting finding, as to our knowledge this pathway has not been previously described – perhaps, in part, due to an absence of studies exploring the possibility of exogenous acetyl-CoA import into cells. One possible alternative mechanism by which acetyl-CoA may be entering these iBN cells is through pinocytosis or another means of phagocytic activity in these myeloid progenitor cells.

Therefore, taken together, acetyl-CoA is able to enter iBN cells through an uncharacterized mechanism, and then acts as an essential precursor molecule for both lipid biosynthesis and histone acetylation, with these activities rescuing cell cycle in iBN cells. Acetate supplementation is also able to rescue the cell cycle through the ability of cells to metabolize acetate into acetyl-CoA through ACSS2 function.

4.3 The role of lipid metabolism and histone acetylation in cell cycle regulation

It is clear that lipid metabolism and histone acetylation both have a substantial role in promoting cell cycle progression and proliferation. In accordance with this notion, both histone and lipid extracts of [3H]-acetyl-CoA treated cells had significant DPM counts (Fig. 16A) suggesting that acetyl-CoA was being incorporated in both lipids and for histone acetylation. There is a caveat to the results found with these lipid extraction experiments as the general protocol followed was the Folch method of lipid extraction (Folch et al., 1957), but with the modification that I did not include a chloride salt during cell lysis. Although the cell pellets were solubilized in KOH, which as a strong base completely dissociates into K⁺ and OH⁻, the solubilization solution may have lacked sufficient cations to neutralize all of the negatively charged acidic lipids in the sample. Therefore, it is possible that only a portion of lipids were recovered, thus the DPM values for my lipid extractions may have underestimated the portion of acetyl-CoA incorporated into lipids. Similarly, additional variation in the DPM measurements may have been incurred through the quantification of only a portion of the lipid and histone lysate rather than the total lysate volume. The however, the initial reason for taking only 10µl was because we wanted to utilize a 96 well plate and only use 200µl of scintillation fluid.
Even with the possible under-representation of radiolabeled lipids, normalizing for cell concentrations, it is clear that acetyl-CoA was being incorporated more into lipids than into histones (Fig. 16B).

4.4 The mechanism by which acetyl-CoA regulates the cell cycle

Based on the results of iBN cells treated with $[^3]$H-acetyl-CoA with or without BMS, it suggests that acetyl-CoA is preferentially being incorporated into lipids over histone acetylation. When lipid and histone extracts were normalized to their extraction volumes, there was a significantly higher amount of $[^3]$H-acetyl-CoA incorporation in lipids over histones (Fig. 18B). In addition, when iBN cells were treated with BMS at 55µM, these BMS treated iBN cells had more $[^3]$H-acetyl-CoA incorporation into their lipids compared to iBN cells just supplemented with $[^3]$H-acetyl-CoA (Fig. 19). Therefore, given that BMS is an ACL inhibitor, and that ACL produces acetyl-CoA for either lipid biosynthesis or histone acetylation, the finding that $[^3]$H-acetyl-CoA is incorporated more in lipids when treated with BMS suggests that lipid biosynthesis may indeed be the preferential pathway by which extracellular acetyl-CoA rescues iBN cells from cell cycle arrest. It is possible that both lipid and histone acetyl-CoA incorporation increased following BMS treatment. However, due to limited time this analysis was not performed on histones extracted from BMS and $[^3]$H-acetyl-CoA treated iBN cells. Therefore, while these results demonstrate that $[^3]$H-acetyl-CoA incorporation into lipids increased following BMS treatment – strong evidence that exogenously added acetyl-CoA enters the lipid biosynthesis pathway – it remains possible that histone acetylation increases as well, and that one, or both of these pathways is responsible for the recovery of cell cycle.

Consistent with lipid synthesis being the predominant mechanism by which ACL inhibition blocks cell cycle progression, one study utilized shRNA to silence Acly expression and showed reduced proliferation in these Acly silenced cells when grown in reduced lipid conditions (Zaidi, Royaux, Swinnen, & Smans, 2012). Consistently, this study determined that supplementation with fatty acid, oleic acid, and acetate was sufficient to rescue cell cycle (Zaidi, Royaux, et al., 2012). Although this study did not
look into acetyl-CoA supplementation, their reasoning was very similar to ours (Zaidi, Swinnen, et al., 2012). They proposed that if Acly suppression caused a reduction in lipid biosynthesis, then supplementation with fatty acid could rescue the cell cycle. The group was able to demonstrate these findings, further supporting our model (Zaidi, Royaux, et al., 2012). Therefore, taken together, the results suggest that acetyl-CoA as a supplement is able to restore cell cycle progression in BMS treated iBN cells by restoring the acetyl-CoA available in these cells for lipid biosynthesis to allow for cell cycle progression.

4.5 Future directions

Although the results of this project seem to strongly suggest that acetyl-CoA is able to rescue BMS induced cell cycle arrest in iBN cells by restoring lipid biosynthesis, there are still other experiments and findings that would help provide stronger evidence and provide a better explanation behind the mechanisms. As mentioned, PU.1 induction in iBN cells upregulated microRNAs that target and downregulate genes involved in lipid metabolism. Therefore, providing direct evidence that miR141 downregulated Acly would more definitively establish the link and regulatory mechanism of PU.1 in lipid metabolism. In addition to linking PU.1 to lipid metabolism, elucidating the mechanism of how downregulated lipid biosynthesis and histone acetylation, as a result of downregulated Acly, can be sensed and cause cell cycle arrest is crucial. These two future experiments would help explain and elucidate PU.1’s exact role in regulating lipid biosynthesis, and how myeloid progenitors can sense downregulated lipid biosynthesis and histone acetylation to cause an impairment in cell cycle progression.

Therefore, in order to determine if miR141 is actually binding to Acly and causing downregulation, we propose to establish a modified iBN cell line that upregulates miR141 instead of PU.1 when induced with doxycycline. The rationale for this is because our laboratory was able to show that following PU.1 induction, microRNAs targeting lipid biosynthesis and cell cycle regulators were upregulated (Solomon et al., 2017). Of these microRNAs, miR141 was shown to target Acly. Therefore, the goal would be to demonstrate that miR141 induction is sufficient to block cell cycle.
Instead of the TRE3GV promoter being upstream of PU.1, these cell lines would instead have the miR141 gene (Fig. 20). These miR141 iBN cells would be induced with doxycycline and the transcript levels of and Acly would be determined via quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Their cell cycle would also be assessed via the BrdU assay and flow cytometry. If miR141 is indeed targeting Acly for downregulation, it would be expected that following induction of these miR141 iBN cells with doxycycline, their cell cycle would be arrested and that acetyl-CoA supplementation should be able to directly rescue their cell cycle.

The next step of this aim would be to establish that miR141 actually binds to Acly to exert its effects. In order to demonstrate this, a luciferase reporter system would be utilized, where the 3’-untranslated region (UTR) of Acly would be downstream of Renilla luciferase to measure miR141 binding to its target Acly mRNA. Renilla luciferase would be normalized to Firefly luciferase with a constitutive thymidine kinase promoter upstream of the Firefly luciferase (Fig. 21). If miR141 is actually binding to Acly, it would be expected that the Renilla luciferase bioluminescence would be decreased while Firefly luciferase would remain constitutively active. These experiments would help provide further evidence to suggest that the induction of miR141 is responsible for the expected downregulated mRNA levels of ACL, and a possible mechanism behind how PU.1 expression can lead to cell cycle arrest.

The next aim would be to determine possible mechanisms of how downregulation of lipid biosynthesis and histone acetylation could be sensed in the cell to cause impaired cell cycle progression. One possible avenue to explore is the AMP-activated kinase (AMPK) pathway, as AMPK is a master energy sensor that modulates cellular activities in response to energy stress (Yuan, Xiong, & Guan, 2013). Specifically, AMPK has been shown to decrease fatty acid synthesis by phosphorylating acetyl-CoA carboxylate (ACC, the enzyme responsible for converting malonyl-CoA to acetyl-CoA) and thus inhibiting ACC (Munday, Carling, & Hardie, 1988).
Figure 20 Modified iBN cell system to induce miR141

The modified microRNA iBN cell system. Instead of PU.1 being downstream of the TRE3GV Promoter, the gene for microRNA 141 will be inserted. Following doxycycline administration, microRNA 141 will be induced and expressed. In the regulator vector is the long terminal repeats (LTR), the Tet-On 3G transactivator protein (Tet3G), the internal ribosome entry site (IRES), and a green fluorescent protein (GFP) reporter. In the response vector is the LTR, the puromycin resistance gene (Puro) as a selection marker, the TRE3G promoter ($P_{TRE3GV}$) upstream of the gene encoding microRNA 141 (miR141).
Figure 21 Luciferase assay to detect miR141 binding

Luciferase assay to detect miRNA binding to target gene Acly. Following microRNA binding to the target 3’ UTR of Acly, Renilla luciferase transcript will degrade resulting in less Renilla luciferase bioluminescence but Firefly Luciferase bioluminescence will be constitutively active via the thymidine kinase promoter.
Renilla Luciferase

UTR of Acl promoter

Firefly Luciferase

Transcript 1

pMSCV backbone

Transcript 2

From psiCheck-2 Vector
AMPK may also be responsible for sensing and regulating lipid biosynthesis at the transcriptional level by phosphorylating and inhibiting the lipogenic transcription factor, sterol regulatory element-binding protein 1C (SREBP-1C) (Li et al., 2011). AMPK just highlights one possible pathway used as a sensor for lipid biosynthesis that could contribute to cell cycle regulation as well. As the direct substrate required for acetylation, acetyl-CoA itself is used as a means to sense and regulate histone acetylation (Cai & Tu, 2011). It has been shown that histone acetylation is directly corresponded with increasing acetyl-CoA levels (Cai & Tu, 2011).

Therefore, exploring these two future aims would provide an even stronger foundation behind understanding the mechanisms behind how PU.1 is exerting its role on lipid biosynthesis, and how cells are able to determine their lipid metabolic and histone statuses to undergo cell cycle arrest.

4.6 Summary and conclusions

This project sought to explore the mechanism of how ACL and its metabolic product, acetyl-CoA, can regulate the cell cycle of myeloid progenitor cells. We hypothesized that inhibiting ACL function with the drug, BMS303141, would cause cell cycle arrest in myeloid progenitor cells (iBN cells), and that acetyl-CoA supplementation would be able to rescue the cell cycle. The evidence presented in this project supports our hypothesis. We have been able to demonstrate that ACL inhibition via BMS treatment causes cell cycle arrest and that acetyl-CoA supplementation is able to restore the cell cycle. We have also demonstrated novel evidence that acetyl-CoA can enter cells and that acetyl-CoA is incorporated into both lipids and histones for acetylation. Following BMS treatment, we have demonstrated that acetyl-CoA incorporation in lipids increases suggesting that lipid biosynthesis is the pathway responsible for rescuing cell cycle arrest following BMS treatment. Whether histone acetylation also increases remains to be confirmed. In conclusion, we have shown that ACL inhibition is sufficient to cause cell cycle arrest, but acetyl-CoA supplementation is able to rescue the cell cycle through both lipid biosynthesis and histone acetylation pathways.
References


Li, J. J., Wang, H., Tino, J. A., Robl, J. A., Herpin, T. F., Lawrence, R. M., Biller, S.,


Mashimo, T., Pichumani, K., Vemireddy, V., Hatanpaa, K. J. J., Singh, D. K. K.,


for terminal myeloid differentiation. *Immunity*, 3(6), 703–714. https://doi.org/10.1016/1074-7613(95)90060-8


Rosenbauer, F., & Tenen, D. G. (2007). Transcription factors in myeloid development:


Schmidt, M., Nagel, S., Proba, J., Thiede, C., Ritter, M., Waring, J. F., Rosenbauer, F.,


Curriculum Vitae

Name: Jess Rhee

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2012-2016 BMSc.

The University of Western Ontario
London, Ontario, Canada
2016-2018 MSc.

Honours and Awards:
Canada Graduate Scholarships-Master’s (NSERC)
2017-2018

Related Work Experience
Teaching Assistant
The University of Western Ontario
2017-2017

Publications:

https://doi.org/10.1017/CBO9781107415324.004