Novel epigenetic role and therapeutic targeting of protein arginine methyltransferase 5 (PRMT5) in acute myeloid leukemia

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Somayeh Samadzadeh Tarighat, M.S.
Graduate Program in Molecular, Cellular and Developmental Biology

The Ohio State University
2014

Dissertation Committee:
Guido Marcucci, MD, Advisor
Robert Baiocchi, MD, PhD
Michael Caligiuri, MD
Qianben Wang, PhD
Copyright by
Somayeh Samadzadeh Tarighat
2014
Abstract

Acute myeloid leukemia (AML) is caused by accumulation of somatic mutations in hematopoietic stem and progenitor cells which in turn result in disruption of normal cell differentiation and proliferation processes in the bone marrow. AML is a highly heterogeneous disease in which numerous recurrent gene mutations as well as deregulated expression of genes and microRNA (miR)s establish enormous variation in response to treatment and clinical outcome. These disease-related variations however have been facilitated in defining distinct clinical entities and serve as prognostic markers for risk-adapted therapies especially in AML patients with normal karyotype. Despite the advances in stratification of AML and improved therapeutic approaches for younger (<60 years) patients, the long-term survival rate for older (>60 years) AML patients remains as low as only 10%. As vast efforts to identify novel therapeutic targets continue, epigenetic changes have emerged as relatively new source of investigation. These modifications which affect gene expression without altering DNA sequence are highly reversible and therefore can serve as ideal therapeutic targets. One of the rather less-studied of epigenetic mechanisms is protein arginine methylation catalyzed by a class of enzymes known as protein arginine methyltransferases (PRMTs). Here we have studied PRMT5, an important member of PRMT family of enzymes which is responsible for
most symmetric arginine dimethylation events on histones and other proteins in mammalian cells. PRMT5 is frequently upregulated in human malignancies and often functions to promote cancer growth by altering the expression of tumor suppressor genes or oncogenes in a cell context-dependent manner. Here, we describe a positive role for PRMT5 in promoting leukemia growth both \textit{in vitro} and \textit{in vivo}. PRMT5 when active in leukemia cells substantially participates in hyperproliferation and maintenance of malignant cells while inhibition of this enzyme results in significant reduced proliferation rate, induction of differentiation and eventual cell death.

The main mechanism by which PRMT5 functions in the cell is by altering gene expression through either methylating histones or as a component of repressor and chromatin remodeling complexes. Our analysis of AML samples led to identification of a novel pathway which becomes effectively targeted and regulated by PRMT5. Tumor suppressor miR-29b is directly targeted for suppression by PRMT5-induced transcription silencer mark: H4R3me2, which transient removal from promoter region of this miR allows for significant re-expression of miR-29b. In this context suppression of miR-29b levels by PRMT5 results in uninterrupted activity of its target pro-tumor transcription factor SP1. Enhanced transcriptional activities of SP1 mediated by PRMT5 in turn can result in increased expression of oncogenic receptor tyrosine kinase known as fms-like tyrosine kinase 3 or FLT3. Therefore, our findings suggest a dual role for PRMT5 in silencing miR-29b and activating FLT3 expression, albeit both give rise to an enhanced pro-leukemic phenotype.
Furthermore, inhibition of PRMT5 using a first in class selective small molecule compound known as C12 (HLCL61) demonstrated significant antileukemic activities through increasing expression of miR-29b and thus reduced activity and expression of SP1 and FLT3 in AML cells. MiR-29b is commonly silenced in AML and efforts to introduce or deliver this miR to older AML patients, which cannot tolerate aggressive standard chemotherapy regimens has led to improvement in clinical response to alternative treatments with DNA hypomethylating agent “decitabine”. Incubation of AML cell lines and primary tumor blasts from patients with C12 stimulated a potent response to PRMT5 inhibition in these cells which include hindered proliferation and colony forming ability in addition to induction of differentiation and apoptosis. These findings strongly support the potentials of PRMT5 as a “druggable” epigenetic target with prospects to overcome the challenges of leukemic cell ablation using conventional chemotherapy, especially by providing an alternative remedy besides FLT3 inhibitors in treating high-risk FLT3-ITD AML.

Moreover, we found that PRMT5 forms a physical association with transcription factor NFkB(p65) and epigenetic corepressors histone deacetylase (HDAC)2 and DNA methyltransferase (DNMT)3A in AML cells which represents an additional means of PRMT5 regulation in need of further deciphering. Together these findings suggest that PRMT5 inhibition alone or in combination with other epigenetic agents may indeed contribute to eradication of leukemic cells and improve clinical outlook in high-risk AML patients. Here we report for the first time, on potentials and effectiveness of recently synthesized PRMT5 inhibitor compound (C12) in targeting leukemic cell growth in
AML. Given the significant antileukemic effects of PRMT5 inhibitor in treating AML cells, studies to optimize and calibrate C12 administration *in vivo* is highly warranted. In addition, further functional assays to evaluate gene and protein expression profiles are required to better understand the factors which function to regulate PRMT5 expression and enzyme activities in cells. Thus, through inhibiting PRMT5 followed by disruption of key oncogenic mechanisms including SP1 and FLT3 activities we have discovered a novel mechanism to eliminate leukemic cells.
This document is dedicated to my parents; Maryam and Bagher,

and to my husband; Mohammad for love, friendship and support.
Acknowledgments

I would like to offer my humble gratitude to my advisor Dr. Guido Marcucci. Not only he has been extremely insightful and approachable throughout my training but more importantly kindly trusted me with the opportunity to develop on my own. I am honored to be among his students as I have learned from him, not only leukemia research and how to present my work but also professional ethics and respect. I believe that the scientific and personal teachings under his supervision will continue to impact my scientific confidence, independence and academic career in the most constructive manner.

I am deeply grateful for support, motivation and guidance I received from my co-advisor Dr. Robert Baiocchi. His input has been indispensible for developing and completing my dissertation project.

I would like to thank my committee members Dr. Michael Caligiuri and Dr. Qianben Wang, for offering their valuable time and expertise in helping me to improve my dissertation.
I would also like to thank my graduate program especially Dr. David Bisaro and Dr. Dawn Chandler for providing me with this opportunity in the first place. I am especially thankful to Dr. Dawn Chandler for additional personal support.

To my friends and colleagues in Marcucci lab, past and present, and all the friends I have made during my studies at OSU; they have made this experience considerably more pleasant.

I would like to sincerely acknowledge my parents, Ms. Maryam and Mr. Bagher Tarighat, which have given me unconditional love and support throughout my life. Words cannot express my gratitude for their fortitude and personal sacrifices. I owe this achievement to constant support and the inspiration for learning and self-improvement I received from them and for this I am forever indebted to them.

Finally, I need to thank my husband, Dr. Mohammad Montazeri, who encouragement, support and love have been the major source of determination and strength in this journey. I have been truthfully blessed with a best friend who has been the most patient, loving and compassionate through this challenging time; thank you for sharing your life with me.
**Vita**

August 2003 .......................................................B.S., Agricultural Engineering and Plant Breeding, Urmia University, Iran

May 2010 ..............................................................M.S., Biological Sciences, University of Cincinnati, Cincinnati, Ohio

June 2010 to present .................................Graduate Research Associate, Department of Biology, The Ohio State University, Columbus, Ohio

**Publications**


x


Fields of Study

Major Field: Molecular, Cellular and Developmental Biology

Research Focus: Cancer Biology
Table of Contents

Abstract........................................................................................................................................... ii

Acknowledgments......................................................................................................................... vii

Vita.................................................................................................................................................. ix

Publications...................................................................................................................................... ix

Fields of Study ............................................................................................................................. xi

Table of Contents ........................................................................................................................... xii

List of Tables .................................................................................................................................... xv

List of Figures ............................................................................................................................... xvi

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

1.1 Acute myeloid leukemia and demand for improved therapies................................. 1

1.2 Search for novel therapeutic targets to better treat AML................................. 4

1.3 Protein arginine methylation ............................................................................. 6

1.4 PRMT5 and cancer ............................................................................................. 12

1.5 PRMT5 as novel therapeutic target in leukemia ........................................ 16

Chapter 2: Protein arginine methyltransferase 5 (PRMT5) contributes to leukemia growth
and PRMT5 enzyme activity can be targeted in acute myeloid leukemia .................. 23
2.1 Introduction ........................................................................................................... 23
2.2 Materials and Methods .......................................................................................... 27
2.3 Results .................................................................................................................... 31
  2.3.1 Upregulated PRMT5 contributes to leukemia growth ..................................... 31
  2.3.2 Development of novel class of inhibitors to selectively target and inhibit 
      PRMT5 activity ..................................................................................................... 33
  2.3.3 Antileukemic activities of PRMT5 inhibition in AML cells ............................. 35
2.4 Discussion .............................................................................................................. 36
2.5 Figures ..................................................................................................................... 39

Chapter 3: Protein arginine methyltransferase 5 (PRMT5) modulates miR-29b and FLT3 
expression and activity in acute myeloid leukemia ................................................... 49
  3.1 Introduction .......................................................................................................... 49
  3.2 Materials and Methods ....................................................................................... 52
  3.3 Results ................................................................................................................... 55
    3.3.1 PRMT5 suppresses miR-29b expression via its methylation mark H4R3-me2 
         ......................................................................................................................... 55
    3.3.2 PRMT5 upregulates FLT3, a critical oncogene involved in AML 
         pathogenesis, through enhancing FLT3 transcription .................................. 56
    3.3.3 PRMT5 regulates transcription of FLT3 by modulating SP1 transcription 
         factor .................................................................................................................. 59
3.4 Discussion ................................................................................................................. 62
3.5 Figures ......................................................................................................................... 69

Chapter 4: Identifying key molecular players involved with protein arginine methyltransferase 5 (PRMT5) expression and regulation in acute myeloid leukemia ..... 82

4.1 Introduction ................................................................................................................ 82
4.2 Materials and methods ............................................................................................. 87
4.3 Results ......................................................................................................................... 89
  4.3.1 PRMT5 and miR-96 are caught in a negative regulatory loop in AML .......... 89
  4.3.2 PRMT5 associates with DNMT3A, HDAC2 and NFκB(p65) in AML ....... 91
4.4 Discussion ................................................................................................................... 92
4.5 Figures ......................................................................................................................... 95

Chapter 5: Conclusions and future directions ............................................................... 101

References ....................................................................................................................... 106

Appendix: Promoter and ChIP primer sequences ......................................................... 121
List of Tables

Table 1.1  List of methylation substrates of PRMT5 ................................................................. 22
List of Figures

Figure 1. 1 Blood stain from normal and AML samples showing myeloblast (blast) infiltration in AML. .............................................................. 3

Figure 1. 2 Protein arginine methyltransferase (PRMT) family of enzymes and illustration of methylation events catalyzed by each type. ........................................ 11

Figure 2. 1 PRMT5 upregulation significantly contributes to leukemia growth in the in vitro and in vivo models of AML. .................................................. 41

Figure 2. 2 PRMT5 inhibitor, C12, selectively and potently targets PRMT5 activities. 45

Figure 2. 3 Preclinical and pharmacological activities of C12 in AML samples. .......... 47

Figure 3. 1 PRMT5 suppresses miR-29b expression in AML cells via its dimethylated histone mark................................................................. 71

Figure 3. 2 PRMT5 actively upregulates FLT3 expression and activity in AML .......... 75

Figure 3. 3 PRMT5 regulates FLT3 levels by modifying activities of SP1 transcription factor in AML. ............................................................. 79

Figure 4. 1 PRMT5 levels are altered by activities of miR-96 in AML .................... 97

Figure 4. 2 PRMT5 associates with other corepressors and chromatin modifiers in AML. .......................................................................... 100
Chapter 1: Introduction

1.1 Acute myeloid leukemia and demand for improved therapies

Acute myeloid leukemia (AML) is a cancer of the bone marrow and is characterized by arrest in differentiation and maturation of hematopoietic cells. AML is recognized by an increase in the number of immature myeloid cells (i.e. myeloblasts or blasts) which often results in hematopoietic inefficiency (granulocytopenia, thrombocytopenia and anemia) (Figure 1.1). The annual incidence of AML in the United States is nearly 3.7 per 100,000; however it continues to have the lowest survival rate of all leukemias (American Cancer Society). AML is less common in children and with the median age of 64 years accounts for 30% of all leukemias diagnosed in adults. Although over the past decades significant advances have been made in improving survival rate in younger age groups, the prognosis in older AML patients remains very poor; <10%. Considering the aging population in the Western world awareness of AML attribution has been increased as it represents one of the leading causes of death due to cancer in adults. Unfortunately, the majority of older AML patients, >55-60 years, die from their disease and despite effort in the last three decades only marginal progress has been made in long-term survival of older adults with AML.
The challenge in successfully treating AML is largely due to the highly heterogeneous genetic, epigenetic and clinical nature of AML. Current strategies for induction and post-remission therapy have been guided by prognostic factors as different subgroups of AML can be considered different entities\textsuperscript{2}. In this regard, the most important indicators of the outcome of therapy are the aberrations acquired in leukemia cells including both chromosomal abnormalities and mutations observed in cytogenetically normal AML cases. Mutations that drive irregular signal transduction especially represent pathways that are ideal for targeted therapy\textsuperscript{2}. Indeed, innovative strategies to target pathways and signaling networks that direct and maintain malignant transformation of hematopoietic cells with promise of providing effective treatment regimens while minimizing cytotoxic chemotherapy are undertaken and necessary to overcome the disappointing outcome of therapy in older AML patients.
Figure 1.1 Blood stain from normal and AML samples showing myeloblast (blast) infiltration in AML.

Adapted from “www.sanger.ac.uk”
1.2 Search for novel therapeutic targets to better treat AML

Hematopoiesis is an intricately regulated cellular process which controls expression of genes that regulate maturation of hematopoietic stem and progenitor cells into differentiated and functional blood cells. These processes which are often disrupted in AML, offer insight into activation of oncogenes and regulators of proliferative pathways which are frequently achieved via chromosomal translocations, recurrent mutations and more recently chromatin remodeling and epigenetic mechanisms. Cancer develops in a multistep molecular process where genetic alterations (e.g. gene mutations and deletions) disrupt activity of tumor suppressor and regulatory genes or result in activation of oncogenes.

Epigenetic mechanisms involve stable and mitotically-maintained changes in gene expression without modification in DNA base pair sequence. Epigenetic mechanisms that regulate gene expression include DNA CpG methylation (often very stable), histone modifications (more dynamic biochemical changes) and small non-coding RNAs (dynamic and targetable). Epigenetic processes play a key role in malignant transformation of cells. Recent studies suggest that more than 40% of de novo AML patients lack a detectable mutation in a signaling gene while more than half of AML patients do not have a mutation in transcription factors or a gene fusion event meaning that there exists additional and unknown causes for malignant transformation in this subset of patients. Chromatin modifier enzymes have been implicated in development of AML, as a recent analysis of de novo AML patients showed that >70% of cases harbored
at least one mutation in an epigenetic modifier gene\textsuperscript{13}. More specifically, these enzymes modify chromatin by inducing cytosine methylation in DNA or modifications in multiple residues (e.g. lysine, arginine, tyrosine and serine) of histones\textsuperscript{6}. Irregularities in chromatin remodelers may result in epigenetic activation of oncogenes or inactivation of tumor suppressor genes and therefore have been regarded as new sources for targeted therapies in AML among other malignancies. Structure of chromatin is largely determined by posttranslational modifications of histones which in turn directly influence gene transcription\textsuperscript{14}. For example, increased acetylation of histones is often associated with increased gene transcription while transcriptional activity diminishes by reduced acetylation\textsuperscript{14}. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) determine the acetylation status of histones\textsuperscript{14} and acute myeloid blasts show lower levels of histone H3 acetylation compared to normal progenitor cells suggesting that many genes are epigenetically silenced in AML\textsuperscript{15}. Epigenetic therapies offer the means to reverse epigenetic silencing and re-express genes with hope to induce a therapeutic effect including differentiation, apoptosis or proliferation arrest\textsuperscript{8}. Application of HDAC inhibitors have shown efficacy in treatment of AML by reversing the acetylation of histone tails and consequently reactivating tumor suppressor genes, for example miR-29b, which in turn leads to re-expression of other anti-tumor genes\textsuperscript{16,17}.

Histone tails undergo further posttranslational modifications including methylation and more interestingly, these modifications can influence each other. Methylation of specific arginine (R) residues on histones H3 and H4 are associated with states of gene
transcription\textsuperscript{18}. Furthermore, symmetrically methylated R3 residue of histone H4 can enable acetylation of histone H4 and therefore enhance gene transcription in this region\textsuperscript{19}. The impact of epigenetic modification on histones, catalyzed by chromatin modifier enzymes, and their role in directing gene transcription and determination of cell fate has further underlined the effectiveness of targeting epigenetic processes in addition or in parallel with genetic networks in cancer therapeutics.

\textbf{1.3 Protein arginine methylation}

Posttranslational modifications of histones play pivotal role in determining the structure of chromatin and therefore direct gene transcription processes in the cell\textsuperscript{20}. These epigenetic changes act by altering chromatin compaction and serve as recognition sites for transcription factors as well as secondary proteins which may function to antagonize or enhance transcription\textsuperscript{21}. Methylation events on arginine or lysine residues which are catalyzed by protein methyltransferases (PMTs) appear to play an especially significant role in controlling gene expression\textsuperscript{22,23}. PMT class of proteins comprise two main groups of enzymes namely protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs)\textsuperscript{22}.

PRMTs have 11 known members and can transfer a methyl group, donated by S-adenosylmethionine (AdoMet) to a terminal guanidino nitrogen atom of arginine\textsuperscript{24}. The product of methyltransferase activity by PRMTs is S-adenosylhomocysteine (AdoHcy).
and methylarginine\textsuperscript{25}. PRMTs are classified into Type I, Type II or Type III and there are three main forms of methylated arginine in mammalian cells which include monomethylarginines (MMA), asymmetric dimethylarginines (ADMA) and symmetric dimethylarginines (SDMA)\textsuperscript{24,25,26}. Type I and Type II PRMTs catalyze MMA intermediate event followed by production of either asymmetric dimethylarginines (by Type I; PRMT1, 2, 3, 4, 6 and 8) or symmetric dimethylarginines (Type II; PRMT5 and 7)\textsuperscript{24,27} (Figure 1.2). Type III PRMTs catalyze only monomethylation (MMA) events and PRMT7 has been described as having this activity.

Although histone tails are major target for these enzymes, PRMTs target arginine substrates on both histones and proteins\textsuperscript{24}. PRMT5 is the main Type II arginine methyltransferase and is often accountable for induction of strong gene suppression\textsuperscript{28}. For instance, PRMT5 was found present and active as part of a repressor element in transcription start site of cyclin E1 promoter region\textsuperscript{28}. This enzyme was first identified as a Jak2-binding protein\textsuperscript{29} and has been shown to methylate arginine residues of both histones and other proteins including H3 (H3R8-me2), H4 (H4R3-me2), H2A (H2AR3-me2)\textsuperscript{30,31}, HOXA9-R57\textsuperscript{32}, SHP-R140\textsuperscript{33}, p65-R30\textsuperscript{34}, CRAF-R563\textsuperscript{35}, p53\textsuperscript{36}, RPS10-R158 and 160\textsuperscript{37}, GM130 N-term\textsuperscript{38}, E2F-1 central motif\textsuperscript{39}, SPT5\textsuperscript{40}, FCP1\textsuperscript{41} (Table 1.1).

PRMT5 associates with many cellular proteins in a cell context-dependent manner but is predominantly found in association with WD-repeat-containing protein MEP50 (methylosome protein 50). MEP50 had been regarded as adaptor protein between
PRMT5 and its methylation substrates. Methyltransferase activity of PRMT5 can be modulated via phosphorylation of MEP50 or PRMT5 itself with both inhibitory (when phosphorylated by Jak2 in myeloproliferative neoplasms) and enhancer (phosphorylation with CDK4) effects on PRMT5/MEP50 enzyme activity.

PRMT5 specificity towards nuclear histones is in part determined by its interaction with nuclear protein COPR5 (cooperator of PRMT5). COPR5 tightly binds to PRMT5 and increases RPMT5 affinity to H4R3 and is required to recruit PRMT5 to reconstituted nucleosomes at promoter of target genes such as cyclin E1. PRMT5 is involved in regulation of several cellular functions including ribosome biogenesis, assembly of the Golgi apparatus, germ cell specification, cell growth and proliferation, cell differentiation and apoptosis.

Methylation of ribosomal protein RPS10 by PRMT5 affects the proper assembly of ribosomes with direct consequences on protein synthesis and regulation of cell proliferation. These PRMT5-induced methylation events stabilize RPS10 and protect it from proteasomal degradation hence significantly improving its efficient assembly into ribosomes. PRMT5 also interacts and methylates Golgi matrix protein GM130 which is involved in maintenance and functionality of Golgi apparatus. Methylation event by PRMT5 appears to be required for proper Golgi assembly following mitosis which depends on Golgi matrix proteins’ interactions and is central for transportation of proteins, sorting and posttranslational modifications.
Studies suggest that PRMT5 methylation marks H4R3me2 and H2AR3me2 result in block of cell differentiation in testicular germ cells. PRMT5 interacts with transcriptional repressor BLIMP1 and co-localization of BLIMP1/PRMT5 results in hypermethylation of H4R3 and H2AR3 in primordial germ cells and thus suppression of BLIMP1 target gene DHX38\textsuperscript{46,47}. Removal of these epigenetic marks leads to somatic differentiation in tumors of testicular germ cells. More importantly, PRMT5 was found to be essential for proliferation while knockdown of this enzyme resulted in G1 arrest in a MCF7 breast cancer cell line\textsuperscript{48}. Suppression of PRMT5 results in reduced levels of p53 and as a result downregulation of p53 target genes; MDM2 (p53 antagonist which targets p53 for proteasomal degradation) and p21 (regulates growth arrest). In fact, depletion of PRMT5 prevents p53 protein synthesis by downregulating expression of eIF4E translation initiation factor. P53 when active inhibits the proliferation of cells that carry DNA damage(s) with potentially oncogenic properties\textsuperscript{52} while in unstressed cells MDM2 constantly monoubiquitinates p53 for proteasomal degradation to limit p53 function in unstressed cells.

Other studies have shown that PRMT5 is involved in muscle development and differentiation by depositing dimethylated H3R8 at promoter of myogenic regulator gene called myogenin in differentiating cells. Cho et al., 2012 provided data which suggest a role for PRMT5 in regulating induction of apoptosis. PRMT5 methylates E2F-1 and regulates its function by altering stability and DNA-binding features of this transcription factor. Progression of cell cycle and apoptosis are two opposing mechanisms regulated by
E2F-1 in a context-dependent manner. Depletion of PRMT5 results in E2F-1-dependent reduction in cell growth which in turn sensitizes the cells to E2F-1-dependent apoptosis. Overall, multiple line of evidence supports a key role for PRMT5 in regulating an array of cellular functions in both normal and malignant mammalian cells.
Figure 1. 2 Protein arginine methyltransferase (PRMT) family of enzymes and illustration of methylation events catalyzed by each type.

Adapted from Yang and Bedford\textsuperscript{53}
We are gaining increasingly mechanistic understanding on the role of PRMTs in regulating cell transformation. Data supporting the idea that PRMT5 might be an oncoprotein has emerged from evidence showing PRMT5 involvement in different stages of tumor progression mechanisms. Overexpression of PRMT5 is detected in the number of malignancies including gastric cancer\textsuperscript{54}, lymphoma and leukemia cells\textsuperscript{44,55,56}, ovarian cancer\textsuperscript{57}, gliomas\textsuperscript{58}, melanoma\textsuperscript{59}, breast\textsuperscript{60}.

PRMT5 but not PRMT1 or PRMT4 was described to be essential for cell proliferation in MCF7 cell lines in efforts to determine PRMT5 impact on cell growth\textsuperscript{48}. Furthermore PRMT5 inhibition triggers apoptosis and cell-cycle arrest in a p53-dependent manner\textsuperscript{36}. Jansson et al., 2008 demonstrated that arginine methylation of p53 can alter target gene specificity of p53 and that PRMT5 depletion may trigger p53-dependent G1 arrest in response to DNA damage. More evidence supporting PRMT5 role in regulating p53 has emerged from the data showing that knockdown of PRMT5 inhibits p53 protein synthesis\textsuperscript{48}. PRMT5 regulates the expression of translation initiation factor eIF4E, a key component of protein synthesis, and doing so can modulate protein translation processes. PRMT5 is identified in complex with many repressor elements. Interaction of PRMT5 with SNAIL is critical for the function of the SNAIL repressor complex in suppressing the expression of E-cadherin\textsuperscript{61}. Loss of E-cadherin is considered as hallmark of epithelial-mesenchymal transition which actively directs the metastatic spread of tumor cells\textsuperscript{62}. Additionally, PRMT5 was shown to be involved in promoting anchorage-
independent cell growth\textsuperscript{63}. Together these data suggest a role for this enzyme in controlling and promoting tumor progression.

Furthermore, PRMT5 overexpression in glioblastoma correlated with higher growth rate in patient’ tumor cells and conferred worse overall survival rate\textsuperscript{64}.

PRMT5 is also considered a potential anti-cancer therapeutic target by regulating splicing in mammalian cells; deletion of PRMT5 in neural stem/progenitor cells results in postnatal death in mice via reduced methylation of Sm proteins and irregular splicing of Mdm4 mRNA which in turn leads to activation of p53 response and defective cell cycle progression\textsuperscript{65}.

PRMT5 was identified as HSP90 binding partner suggesting that methylation events induced by this enzyme can impact HSP90 function\textsuperscript{66}. This is major finding as HSP90 inhibition is highly relevant in cancer therapy because of its essential role in chaperoning and stabilizing specific oncogenes required for cell proliferation, apoptosis, metastasis and cell cycle regulation e.g. mutant p53, ERBB2, B-RAF, C-RAF, and CDK4\textsuperscript{67}.

PRMT5 activity in connection with Hedgehog signaling has offered a potential target for the treatment of multiple endocrine neoplasia tumors. In murine pancreatic cancer, Menin represses oncogenic Hedgehog pathway by directly interacting and recruiting PRMT5 and subsequently facilitating formation of H4R3me2 repressive mark to Gas1 gene promoter. Gas1 expression is imperative for activation of Hedgehog signaling pathway
and studies have demonstrated that it represents a potential PRMT5 target. Study by Girardot et al., 2013, to investigate the specific regulation and genomic distribution of PRMT5-induced H4R3me2 repressive mark in mouse embryonic fibroblasts (differentiated) and stem cells revealed that gene promoters were predominantly marked by H3R4me2, further underlining the importance of PRMT5 function in cell growth. The presence of this epigenetic mark at G+C-rich regions which is regulated independently from other chromatin repressor marks plays a potent role in chromatin configuration in nucleus.

Involvement of PRMT5 in cancer biogenesis has become even more evident by the studies that describe a defining role for this enzyme in pathways that control malignant transformation of cells and normal cell growth. PRMT5 is found in complex with chromatin remodeler hSWI/SNF in which it can hypermethylate H3R8 and H4R3 at promoter of tumor suppressor genes, RBL2, NM23 and ST7, resulting in effectively silencing these genes and enhancing cell growth.

PRMT5 has also been implicated in cell cycle progression. Cyclin D1 which activates CDK4 increases cell growth by phosphorylating and inactivating retinoblastoma (RB) protein. Aggarwal et al., 2010 discovered that CDK4 phosphorylates PRMT5 coregulatory factor; MEP50 and doing so results in increased enzyme activity of PRMT5/MEP50. Identification of MEP50 as CDK4 substrate has introduced a new paradigm for cyclin D1 regulatory cascade where epigenetic regulation of target genes is
achieved through an RB-independent manner. These data highlight the role of PRMT5/MEP50 as key effector of cyclin D1-dependent gene expression during cancer growth\textsuperscript{44}.

An additional important role for PRMT5 during development and differentiation was characterized following discovery that loss of Prmt5 function is embryonic lethal via abrogation of pluripotent cells in blastocysts\textsuperscript{70}. PRMT5 presence and upregulation in cytoplasm was proven to be essential for maintenance of pluripotency in association with Stat3. Prmt5/Mep50 actively suppressed the expression of differentiation genes in embryonic stem cells via methylating cytosolic H2A (H2AR3me2). Furthermore, transcriptional repressor Blimp1 which directs specification of primordial cells during early development of mice, interacts with Prmt5 to mediate Prmt5-induced H4R3me2 and H2AR3me2 methylation events at promoter of specific genes in germ cells\textsuperscript{46}.

A novel role for PRMT5 was postulated in reprogramming of somatic cells into pluripotent stem cells. Introduction of Prmt5 into mouse embryonic fibroblasts lead to remarkable reprogramming of these differentiated cells into pluripotent stem cells with indistinguishable characteristics that are unique to embryonic stem cells including teratoma formation, germline transmission and expression of pluripotent genes\textsuperscript{71}. A specific group of genes including Oct3/4, Sox2, Klf4 and c-Myc, many of which are oncogenes, were identified by Yamanaka and colleagues, 2006 which could induce pluripotent stem cells from mouse embryonic or adult fibroblasts under embryonic stem
cell culture conditions. The process of somatic cell reprogramming directed by Yamanaka factors may offer insight into the mechanisms by which cancer stem cells may originate as they may be the result of dedifferentiation of somatic cells following tumorigenesis. Postulated role for PRMT5 in reprogramming cells to promote induction of pluripotency and self-renewal indeed suggest a novel area of research for PRMT5 in deciphering the mechanisms of resistance and recurrence in cancer with potential to advance the efforts in more efficiently targeting and eradicating cancer.

1.5 PRMT5 as novel therapeutic target in leukemia

Several lines of evidence support the relevance of dysregulated PRMT5 expression in cell transformation in a range of hematologic malignancies. In lymphoid malignancies it was shown that PRMT5 was overexpressed in patient-derived mantle cell lymphoma cell lines and that there was a positive correlation between its expression and symmetrically methylated H3R8 and H4R3 levels. In addition, in chronic lymphocytic leukemia (CLL) PRMT5 is expressed at higher levels in transformed B-cell lines in comparison to normal B-cells. The observation that resting or activated B cells did not show PRMT5 expression suggested that PRMT5 overexpression may occur selectively in transformed cells. Furthermore, PRMT5 when experimentally overexpressed induces hyperproliferation (i.e. cancerous transformation) in lymphoma cells at least in part by targeting RB family of tumor suppressor genes whereas knockdown of PRMT5 correlates with decelerated cell growth.
Efforts for prognostication and treatment response prediction as well as identifying novel therapeutic targets in AML has benefitted from dissecting epigenetic players and mechanisms. Previous studies have shown that dysregulated DNA methylation (induced by irregular activity of DNA methyltransferase (DNMT) enzymes) can drive myeloid leukemogenesis by altering methylation events. Interestingly PRMT5 activity links histone methylation to DNA methylation during mammalian gene silencing as there is evidence for requirement of PRMT5-induced H4R3me2 for DNA methylation. In a human β-globin locus, H4R3me2 induced by PRMT5 serves as a direct binding site for DNMT3A. Depletion of PRMT5 results in loss of DNA methylation and subsequently gene expression in erythroid progenitor cells. Such functional cooperation may offer instances for innovative cancer treatment strategies where dual targeting of epigenetic modifiers DNMTs and PRMT5 can generate synergistic effect in arresting leukemia cell growth.

In myeloproliferative neoplasms, an oncogenic tyrosine kinase (mutated JAK2) was shown to modulate chromatin modification through directly interacting and phosphorylating PRMT5. In this case phosphorylation of PRMT5 impaired the ability of PRMT5/MEP50 to methylate its histone substrates but more interestingly, demonstrated the significance of cell context on PRMT5 function. In essence, knockdown of PRMT5 in terminally differentiated cell lines led to induction of cell death or growth arrest whereas enhanced proliferation was observed following PRMT5
knockdown in normal CD34+ cells from peripheral blood\textsuperscript{43}. This study by Liu et al., 2011, shows that PRMT5 blocks differentiation of blood stem/progenitor cells and although inhibition of PRMT5 in many cell lines induces apoptosis but its knockdown in CD34+ cells promotes progenitor cell expansion and accelerates erythroid cell expansion. In highly chemotherapy-resistant T-cell leukemia/lymphoma (ATL), PRMT5 levels was found upregulated in ATL cell lines compared to normal T-cells and inhibition of PRMT5 resulted in decreased cell proliferation and viral gene expression\textsuperscript{78}. Human T-cell leukemia virus-1 (HTLV-1) is the causative infectious module of ATL and knockdown of PRMT5 in ATL cells results in reduced expression of viral gene expression i.e. p19, recommending that PRMT5 can positively regulate HTLV-1 gene expression\textsuperscript{78}. Taken together, numerous studies suggest that PRMT5 expression and methyltransferase activities can influence different steps of tumor initiation and progression processes and is highly dynamic depending on the context of cell, nature of the malignancy and developmental stages.

In addition, the novelty of histone protein methylation processes as therapeutic targets is further underlined considering that for a long time they were thought to be permanent histone marks\textsuperscript{79,80}. Indeed, recent discovery of a large number of enzymes with ability to demethylate methylated lysine residues of histones by amine oxidation, hydroxylation or deamination\textsuperscript{81} rectified the previously-held notion that reverting histone methylation may only be achieved through histone exchange, DNA duplication or cleavage of the methylated histone tail\textsuperscript{80,81}. So far, two main histone demethylase (lysine) families of
enzymes are identified which include lysine-specific demethylase (LSD also known as KDM) and Jumonji C (JMJC) histone demethylases. Lysine demethylation of non-histone substrates is also reported; tumor suppressor p53 is demethylated at K370me2 by LSD1 which in turn can suppress transcriptional activities and apoptosis mediated by p53 via limiting p53 interaction with its binding proteins. More interestingly, lysine demethylase enzymes poses distinct role in human diseases and are especially implicated in cancer growth thus utility of biochemical agents to alter their enzyme activity have been explored as targets for cancer therapy.

Despite the extensive progress in identification and characterizing many lysine demethylases, our knowledge regarding biological enzymes which can catalyze the reversal of arginine methylation remains particularly sparse. The only postulated histone arginine demethylase was described by Chang et al., 2007; as Jumonji domain-containing 6 protein (JMJD6) which was found capable of removing the methyl group from arginine 2 on histone H3 (H3R2me2) and arginine 3 of histone H4 (H4R3me2) without any activity towards methylated lysine residues. Although, their findings was not really confirmed by additional groups and is even somewhat challenged, important functions for JMJD6 (previously known as PTDSR) is described in differentiation and maturation during embryogenesis, interaction with proteins involved in RNA processing, and a possible role in driving proliferation and adverse outcome in breast cancer cells. Additionally, H4R3 demethylation is necessary during oocyte development and is required for proper maturation in mouse. Demethylation of H3R2 is
especially important in the cases where asymmetric dimethylation of H3R2 (H3R2me2a) (a transcription suppressive mark) acts to antagonize H3K4me3 (transcription activator) and the mutual methylation status of these two marks functions to fine-tune specific epigenetic status and gene transcription. With regard to PRMT5, study to investigate the recruitment of this enzyme to the promoter of target genes facilitated by PRMT5-interacting protein bromodomain protein 7 (BRD7) revealed that JMJD6 was only detectable in BRD7 knockdown cells. This work in particular suggested that JMJD6 may in fact be a functional arginine demethylase and may be involved in transcriptional regulation of PRMT5 target genes as its recruitment to promoter of these genes is naturally diminished.

The other enzyme with a potential role in reverting arginine methylation was reported as peptidyl arginine deiminase (PADI) 4 which can deiminate arginine residues by converting methyl-arginine into citrulline. This mechanism however is not really a demethylation but rather an antagonist of arginine methylation. Citrullination, as a posttranslational modification of histones has immense role in transcription regulation and PADI4 was found to citrullinate linker histone H1 and result in its dissociation from chromatin therefore global decondensation of chromatin. In addition, histone H3 deimination by PADI4 in human was documented as important process during response to inflammation and neutrophil stimulation.
Together these findings underline the significance of developing selective inhibitors for arginine methyltransferase enzymes as promising means to control the maintenance of specific methylation status of histones and proteins while the efforts to acquire a sizable knowledge about the presence and functions of naturally occurring arginine demethylases continues.
Table 1.1 List of methylation substrates of PRMT5

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Arginine (R) residue</th>
<th>Functional relevance</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>R8</td>
<td>Transcriptional silencing e.g. RB tumor suppressor family in leukemia and lymphoma and ST7 tumor suppressor.</td>
<td>30,31,7,4,94</td>
</tr>
<tr>
<td>Histone H4</td>
<td>R3</td>
<td>Peak at G+C rich regions of mouse embryonic fibroblasts. Transcriptional silencing of genes. Proposed activation of IL2 by PRMT5-induced methylation.</td>
<td>30,39,7,7,95,96</td>
</tr>
<tr>
<td>Histone H2A</td>
<td>R3</td>
<td>Appears late in <em>Xenopus</em> oogenesis and is involved in global transcriptional repression.</td>
<td>31</td>
</tr>
<tr>
<td>Npm</td>
<td>R187</td>
<td>An exceedingly abundant maternally deposited protein in <em>Xenopus</em> and important for premid blastula transition events in embryo. Npm directs Prmt5/Mep50 towards histones.</td>
<td>31</td>
</tr>
<tr>
<td>HOXA9</td>
<td>R140</td>
<td>Methylation is required for HOXA9 activation by TNF-α and induction of E-selectin (cytokine and leukocyte adhesion molecules).</td>
<td>32</td>
</tr>
<tr>
<td>SHP</td>
<td>R57</td>
<td>SHP activity is increased by methylation which may reverse metabolic syndromes by increasing glucose tolerance.</td>
<td>33</td>
</tr>
<tr>
<td>P65</td>
<td>R30</td>
<td>Methylated p65 exhibits enhanced DNA binding and transcriptional activities.</td>
<td>34,97</td>
</tr>
<tr>
<td>CRAF</td>
<td>R563</td>
<td>Methylation lead to enhanced degradation and thus reduced catalytic activity of CRAF in phosphorylating ERK1/2 in melanoma.</td>
<td>35</td>
</tr>
<tr>
<td>P53</td>
<td>R333, 337 (proposed)</td>
<td>Methylation affects p53 specificity towards its targets. PRMT5 inhibition triggers p53-dependent apoptosis.</td>
<td>36,48</td>
</tr>
<tr>
<td>RPS10</td>
<td>R158,160</td>
<td>Proper assembly of ribosomes and protein synthesis followed by cell proliferation are affected by methylation.</td>
<td>98</td>
</tr>
<tr>
<td>GM130</td>
<td>N-term</td>
<td>Methylation is required for proper assembly of Golgi apparatus.</td>
<td>38</td>
</tr>
<tr>
<td>E2F-1</td>
<td>Central motif</td>
<td>Methylation impacts E2F-1-dependent growth control by increasing its stability and DNA binding activity.</td>
<td>51</td>
</tr>
<tr>
<td>SPT5</td>
<td>R681, 696, 698</td>
<td>SPT5 regulates transcriptional elongation. Methylation by PRMT5 regulates SPT5 interaction with RNA polymerase II.</td>
<td>40</td>
</tr>
<tr>
<td>FCP1</td>
<td>R913, 916</td>
<td>FCP1 stimulates transcription elongation and is required for cell viability.</td>
<td>41</td>
</tr>
<tr>
<td>MBD2</td>
<td>N-term</td>
<td>PRMT5 and H4R3 are recruited to CpG islands via interacting and methylating MBD2 subunit of MBD2/NuRD complex and help to modify chromatin and suppress gene transcription.</td>
<td>99</td>
</tr>
<tr>
<td>PDCD4</td>
<td>N-term (R110)</td>
<td>Methylation of this tumor suppressor results in accelerated tumor growth and enhanced cell viability. Coexpression of PRMT5-PDCD4 is needed for protumor phenotype and methylation enhances interaction with eIF4A translation initiation RNA helicase.</td>
<td>60,100</td>
</tr>
</tbody>
</table>
Chapter 2: Protein arginine methyltransferase 5 (PRMT5) contributes to leukemia growth and PRMT5 enzyme activity can be targeted in acute myeloid leukemia

2.1 Introduction

Discovery of several recurrent mutations in chromatin modifiers and their prognostic value in clinical outcomes has made these epigenetic regulators subject of recent cancer therapeutics. Examples in acute myeloid leukemia (AML) include mutations in TET2, DNMT3A and ASXL1 [which result in adverse outcome] and IDH1 [conferring favorable outcome]\textsuperscript{101}. In addition, DNA and chromatin epigenetic modifications are largely reversible which contributes to susceptibility of these dynamic biochemical changes to pharmacological interventions\textsuperscript{6}. Targeting DNA and chromatin modifiers which are involved in pathogenesis of leukemia has led to discovery of some potent directed epigenetic therapeutics. There has been promising responses to epigenetic therapy in AML especially cases with adverse clinical outcome. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors to respectively induce DNA hypomethylation and histone acetylation of tumor suppressor genes, have been successfully evaluated for cancer therapy\textsuperscript{6,16,102,103}.
Application of DNA hypomethylating agents such as decitabine and 5-azacitidine in leukemic cells has provided anti-leukemia activity especially in older AML patients where cytotoxic chemotherapy may not be practical. A phase II clinical trial delivering low dose decitabine to older AML patients whom were spared intensive chemotherapy resulted in complete remission induction (47%) and overall response rate of 64% by targeting epigenetic mechanisms rather than cytotoxicity\(^\text{103}\). In a different study, priming of AML cells with an HDAC inhibitor (AR-42) increased the response to decitabine treatment by releasing the expression of silenced tumor suppressor miR-29b which can directly target DNMTs reducing their methyltransferase activity\(^\text{16}\). HDAC enzymes are shown to suppress the expression of miR-29b by localizing to the regulatory region of this miR\(^\text{104}\) whereas treatment with HDAC inhibitor can effectively result in re-expression of this tumor suppressor microRNA. In addition, specific IDH1/2 inhibitors have been utilized in preclinical treatment of AML or glioma patient samples with recurrent IDH mutations which in part function by releasing the expression of differentiation genes and induction of trimethylation event on H3K9\(^\text{105}\).

Among chromatin modifiers are protein methyltransferases (PMT) which have emerged as novel targets for oncology due to their recurrent genetic alteration or aberrant expression in specific human cancers. PMTs comprise an important class of enzymes responsible for site-specific methylation of arginine or lysine substrates on histones and proteins\(^\text{21}\). Few selective single molecule inhibitors of PMT enzymes have been developed and have demonstrated anti-tumor potentials. For instance selective inhibitors of DOT1L and EZH2 have already entered phase 1 clinical trials\(^\text{106}\). DOT1L inhibition
has been utilized in MLL-rearranged leukemia which comprise an aggressive disease with poor prognosis while efforts to improve inefficient current treatments continue\textsuperscript{107}. A hallmark of this disease is chromosomal translocation affecting MLL gene which encoded protein, regulates the expression of developmental genes e.g. HOX genes and also has lysine methyltransferase activity to catalyze methylation of H3K4 to generate a transcriptional activation mark. Rearrangement of MLL however results in an in-frame fusion of MLL gene to one of more than 60 potential fusion partners\textsuperscript{107}. MLL fusion proteins have the ability to transform hematopoietic cells into leukemia stem cells\textsuperscript{108}. MLL-rearrangement also leads to hypermethylation of H3K79 by recruiting methyltransferase DOT1L to MLL target genes (DOT1L catalyzes methylation on lysine 79 of H3). DOT1L is required for MLL fusion leukemia maintenance and growth\textsuperscript{107}. Application of selective DOT1L inhibitor to block H3K79 methylation in leukemia provided a potent and selective induction of cell death in MLL-fusion-harboring leukemia cells in vitro and in vivo xenograft mouse model\textsuperscript{107}.

In other cases, promising results have emerged from selective inhibition of EZH2 in induction of cell death in lymphoid cells\textsuperscript{109,110}. EZH2 is the catalytic subunit of polycomb repressive complex 2 (PRC2), a multi-protein complex that catalyzes mono-, di and tri-methylation of H3K27 which is largely a repressive chromatin mark\textsuperscript{111}. Increased activity of EZH2, mainly caused by recurrent point mutations, results in hypertrimethylation of H3K27 and contributes to tumorigenesis and poor prognosis in subpopulations of lymphoma cells\textsuperscript{109,110}. Studies have successfully demonstrated utility of selective EZH2 inhibitors in diminishing H3K27 repressive marks with direct consequences in induction
of apoptosis in EZH2 mutant cells without considerable effects on viability of wild-type lymphoid cells\textsuperscript{109,110}.

One of the emerging epigenetic mechanisms is methylation events on arginine residues of proteins including histones and transcription factors. Catalytic modules responsible for protein arginine methylation are identified as protein arginine methyltransferase (PRMT) family of enzymes\textsuperscript{24}. The absence of studies on protein arginine methylation has been largely due to lack of pharmacologic inhibitors for PRMT family of enzymes.

PRMT5 as major type II protein arginine methyltransferase represents a viable and exciting therapeutic target in multiple human neoplasms. Overexpression of PRMT5 in multiple solid and hematologic malignancies as well as studies supporting a role for this enzyme in promoting cancer cell survival and growth prompted us to investigate the potential implications of PRMT5 inhibition in targeting or reversing the myeloid leukemogeneic processes. Discovery and development of a first in class selective PRMT5 inhibitor was made possible by efforts of a multidisciplinary team at Ohio State University including biochemists, medicinal/synthetic chemists, translational biomedical scientists and computational chemists. Several promising compounds with selective PRMT5 inhibitory activity have been identified, synthesized, and rigorously evaluated \textit{in vitro} and demonstrated potent anti-tumor activities in several types of solid (glioblastoma\textsuperscript{64} and melanoma\textsuperscript{59}) and hematologic tumors including lymphomas (MCL, DLBCLs, Burkits and PRMT5 transgenic lymphomas)\textsuperscript{56,64} and reported here for the first time, AML. In particular, development of small molecule inhibitors of PRMT5 activity
will expedite rapid translation of a new class of drug capable of targeting a newly
discovered oncogenic factor and lead to improved strategies to treat patients with AML.
We sought to investigate PRMT5 involvement in maintaining or promoting leukemia cell
growth and its preclinical therapeutic values in treating AML samples.

2.2 Materials and Methods

Cell lines and primary blasts: THP-1, MOLM13, MV4-11, OCI-AML3, U937 and KG-1 cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented
with 10% calf serum and Kasumi-1 cells were cultured in 20% RPMI. Blasts from AML
patients were maintained in RPMI 1640 medium supplemented with 20% fetal bovine
serum, 1% HEPES buffer, and 1x StemSpan cytokine cocktail CC100 (StemCell
Technologies, Vancouver, BC, Canada) containing IL-3, IL-6, and SCF. All cells were
incubated at 37°C with 5% CO2.

Blasts from AML patients were obtained from apheresis blood samples collected from
patients treated at the Ohio State University (OSU) and stored in the OSU Leukemia
Tissue Bank. Informed consent to use cells for investigational studies was obtained from
each patient under an OSU Institutional Review Board-approved protocol, according to
the Declaration of Helsinki.

Plasmids, transient transfections and reagents: PRMT5 cDNA and shRNA were
cloned, respectively, into pCDH1-MSCV-green-puro-cDNA and pGreenPuro shRNA
cloning lentivector (SBI system biosciences). On-target plus siRNA-SMART pool specific for PRMT5 and off-target scrambled control were obtained from Dharmacon (Lafayette, CO). Gene knockdown and overexpression was carried out either by electroporation using Nucleofector Kit (Amaxa, Walkersville, MD) or infection by lentivirus (SBI system biosciences reagents and methods). HLCL-61 (C12) compound was prepared/synthesized by Hongshan Lai, College of Pharmacy, Ohio State University. Bortezomib was obtained from Millennium Pharmaceuticals Inc., Cambridge, MA. In vitro histone methylase assays were carried out with HMT Assay Reagent Kit (Upstate Catalog # 17-330) following manufacturer instructions.

**Western immunoblotting:** Whole cell lysate were prepared by suspending cell pellets in RIPA buffer for 20min (1% NP-40, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) supplemented with 1X complete EDTA free protease inhibitor (Roche) 1X PhosStop (Roche). Lysates were separated by 4-20% SDS-PAGE and transferred to PVDF membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% milk or BSA in 1x TBS with 0.1% Tween 20 for 1 hour at room temperature with shaking. Primary antibodies for GAPDH (Santa Cruz Biotechnology, Santa Cruz CA), PRMT5 (Millipore), H4R3me2 and H3R8me2, (Abcam) and Ubiquitin (Cell signaling) were diluted 1:1000 or 1:2000 in 5% milk or BSA and incubated for 1-2hr at room temperature. Membranes were washed using 1x TBS-T, incubated with HRP-conjugated secondary antibodies diluted in 1 × TBS-T with 5% milk or BSA, washed, and developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific).
Colony formation assay and cell viability analysis: Clonogenic assays were set up by plating 1x10^3 cell/mL in semisolid Methylcellulose medium (MethoCult, Stem Cell Technologies). Colonies were counted after 10-14 days using inverted microscope. Growth inhibition assays were measured using colorimetric MTS assay, 5x10^4 cells were plated in 100μL final volume in 96-well plate in presence of the different concentrations of C12 for 24, 48 and 72 hours at 37°C. Afterwards, 20 μL of the CellTiter 96W AQeuous One Solution Reagent which contains tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (Promega, Madison WI) was added to each well. Within 1-4 hours incubation at 37°C, the optical density at 490 and 690 nm was measured. Cell viability was calculated with respect to the control samples and reference background wavelength at 690nm. At least three independent experiments were performed. Growth curve assays were done by counting live cells using Trypan blue exclusion and inverted microscope for >12 days after plating the cells in 1x10^5 concentrations.

Flow cytometry and Fluorescence Activated Cell Sorting (FACS): Cells (1x 10^6) were washed and passed through a 40μM filter (BD), then resuspended in 200 μl binding buffer with or without 5 μl AnnexinV (for apoptosis detection) or CD11b (for maturation detection) antibody (BD Biosciences, Billerica, MA). After 15 min incubation, cells were washed with PBS, resuspended in 500 μL flow buffer and analyzed on a FACSCalibur cytometer (BD Biosciences). For apoptosis detection 5 μL propidium iodide (PI) (BD Biosciences) was added to cell before running flow cytometry. Sorting for GFP-positive
cells was carried out on BD FACS Aria instrument after filtering and washing cells 2x with ice cold PBS supplemented with 2% calf bovine serum and resuspending in FACS buffer.

**RNA isolation and real-time PCR:** Total RNA was extracted using TriZol reagent (Invitrogen) and subjected to Reverse Transcription using SuperScript III reagents (Invitrogen). Quantitative Real-Time PCR was performed on resulting cDNA using commercially available TaqMan Gene Expression Assay primers and probes, and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Mature microRNA levels were quantified using reverse transcription product made with TaqMan MicroRNA Reverse Transcription Kit (Life Technologies).

**THP-1 xenograft murine model:** To generate PRMT5 overexpressing AML xenograft mouse model, THP-1 cells were transduced with PRMT5 overexpressing lentivirus marked with GFP. Positively infected cells (1x10^6) were injected via tail vain into 4 ~ 6 week-old non-obese diabetic severe combined immunodeficient gamma (NSG) mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory, Bar Harbor, ME). After 10 weeks, animals were sacrificed and blood, bone marrow, spleen, liver, sternum were processed for RNA, protein isolation, Wright-Giemsa staining and pathology. Mouse survival assays were carried out similarly but by injecting 2x10^6 THP-1 cells and sick mice were scored and sacrificed upon manifestation of AML signs.

**Wright-Giemsa and histopathology staining:** For blood staining cells were fixed with methanol on slides and stained with Wright-Giemsa solution. Slides were washed with
deionized water and then observed under the light microscope (ZEISS Axio Observer.A1). Histology of bone marrow, spleen and liver was carried out on paraffin sections marked with hematoxylin and eosin (H&E) stain and light microscopy. Sites of infiltration are hypercellular due to increased myeloblast levels.

2.3 Results

2.3.1 Upregulated PRMT5 contributes to leukemia growth

Previous studies have demonstrated a role for PRMT5 in supporting cancer cell growth. Here we investigated PRMT5 impact on leukemia growth in AML. We found that PRMT5 was expressed at relatively higher levels in the AML cells (primary patients’ blasts; n=6 and patient-derived cell lines; n=7) than in normal bone marrow (n=4) (P=0.03) (Figure 2.1A). We then ectopically expressed PRMT5 in three AML cell lines: MV4-11 (FLT3-ITD), Kasumi-1 (KIT^mut) and THP-1 (KIT- and FLT3-WT) using Lentivirus (Lenti-PRMT5) and compared the growth rate of these cells with empty vector-transfected (Lenti-EV) negative controls. Upregulation of PRMT5 was confirmed by Western blotting (Figure 2.1B) and resulted in a significantly enhanced cell growth as measured over time by cell counts and colony forming assays (Figure 2.1B). Similarly, in primary blasts from FLT3-ITD and FLT3-WT AML patients, overexpression of PRMT5 led to significantly higher cell growth rate measured by increased number of colonies (Figure 2.1B).
To examine the impact of PRMT5 on leukemia growth in vivo, we then injected NOD/SCID/Gamma (NSG) mice, through tail vein, with THP-1 (1x10^6) cells which were stably transduced with either Lenti-PRMT5 (THP-1/PRMT5) to overexpress PRMT5 or Lenti-EV (THP-1/EV) as negative control. We selected THP-1 cells as they grew slower than other cells in immunodeficient mice and we reasoned that they would allow us to better identify the postulated pro-leukemic activities of PRMT5 upregulation. Ten weeks post engraftment three mice from each group were sacrificed. Mice with THP-1/PRMT5 had significantly larger spleens (p=0.03) and livers (p=0.031) and higher number of circulating blasts than THP-1/EV controls (Figure 2.1C, D and E). Higher PRMT5 expression was confirmed in RNA from bone marrow (BM), spleen and blood of THP-1/PRMT5 engrafted animals (Figure 2.1F). In addition, PRMT5 protein levels and its specific histone methylation marks, H3R8me2 and H4R3me2, were significantly higher in the THP-1/PRMT5 than control animals (Figure 2.1G). The NSG mice engrafted with THP-1/PRMT5 cells had also a significantly shorter survival rate than the controls engrafted with THP-1/EV cells; (median survival, 60 vs. 88 days; P>0.001) (Figure 2.1H).

PRMT5 involvement in leukemia growth was further supported by in vitro knockdown assays where depletion of PRMT5 significantly hindered AML cell growth evaluated by colony forming assay. To this end, MV4-11 (FLT3-ITD) and THP-1 (FLT3-WT) cell lines and two primary patient blasts (FLT3-ITD and FLT3-WT) were treated with either scrambled control (sc) or RNA interference (small interfering RNA (siRNA) or short hairpin RNA (shRNA)) against PRMT5. A significantly smaller number of colonies were
formed by PRMT5-depleted cells than controls (>1.6 fold, P<0.01) (Figure 2.1I). Long-term growth assay via cell count was made impractical by the fact that prolonged inhibition of PRMT5 in AML samples resulted in significant increase in cell death. Taken together these data highlight the potential role of PRMT5 in enhancing leukemia growth.

2.3.2 Development of novel class of inhibitors to selectively target and inhibit PRMT5 activity

PRMT5 can catalyze mono and symmetric dimethylation of arginine residues of peptides using the methyl group provided by S-Adenosylmethionine (SAM). Given that at the time of initiation of inhibitor detection efforts crystal structure of PRMT5 was yet to be described, an in silico model of human PRMT5 catalytic domain was developed with MODELLER9v1 software using crystal structure of rat PRMT1 co-crystalized with SAM and an arginine peptide in the catalytic site (Figure 2.2A). After validating proper docking of SAM cofactor and conformational arrangement in the catalytic domain, a library of 10,000 compounds from ChemBridge CNS-Set™ library was screened and compounds with lowest binding energy were visually inspected for contacts that mimic PRMT-SAM-peptide(ARG) and eight compounds were selected for biological investigation. Among these compounds namely CMP3, CMP5 CMP6 were further tested. CMP6 had no inhibitory activity on any of the PRMT enzyme conditions so the efforts continued with CMP5 and CMP3.
The specificity of potential PRMT5 inhibitors was demonstrated using enzyme activity experiments with CMP3 and CMP5 in presence of PRMT1 showing that neither of these compounds was capable of diminishing PRMT1 enzyme activity (Figure 2.2B).

Selectivity of PRMT5 inhibitor drugs was further established in experiments utilizing flag-tagged human PRMT4 (type I PRMT), PRMT5 or PRMT7 (type II PRMT) enzymes affinity-purified from HeLa nuclear extracts. Results from in vitro histone methyltransferase assays showed that CMP5 and CMP3 exhibit selective inhibition of affinity purified PRMT5 and no activity on type I PRMT4 or type II PRMT7 enzymes (Figure 2.2C). Furthermore, a new generation of enhanced CMP5 labeled HLCL10, HLCL61 (C12) and HLCL64 were developed that showed higher potency in specifically targeting PRMT5. Among these C12 was proved to be most potent in targeting PRMT5 enzyme activity (Figure 2.2D). Specificity of C12 in targeting PRMT5 was supported by the lack of inhibition of other members of PRMT family including the type I (PRMT1 and PRMT4) as well as type II (PRMT7) PRMTs (Figure 2.2D). We chose C12 from this group for biological investigation in AML samples as C12 proved to be the most potent as shown by its ability to eliminate H3R8me2 and H4R3me2 methylation marks in AML cells (Figure 2.2E). To further rule out the possibility that C12 may function by inducing protein degradation we compared the effects in cells treated with C12 and proteasome inhibitor bortezomib. Detection for ubiquitinated proteins revealed that cells treated with C12 lacked the ubiquitination mark while cells treated with bortezomib were highly ubiquitinated, suggesting that proteasomal degradation was not the mechanism of function for PRMT5 inhibitor (Figure 2.2F).
2.3.3  Antileukemic activities of PRMT5 inhibition in AML cells

To provide further insight into PRMT5 potential therapeutic implications, we sought to investigate the preclinical and pharmacological inhibition of the enzymatic activity of the protein using C12. Treatment of subset of AML cell lines and primary patients’ blasts with C12 (1 to 100 μM) resulted in a dose-dependent inhibition of cell proliferation, measured by MTS assay (Figure 2.3A and B, mutation status is listed in Figure 2.1A). At 24, 48 and 72hrs post treatment; IC50 values were calculated for cell lines and patient samples (Figure 2.3A and B). Colony forming assay also demonstrated the growth inhibition in AML cell lines MV4-11 (FLT3-ITD), Kasumi-1 (KITmut) and THP-1(FLT3-WT) and AML patient blasts (#2 (FLT3-ITD), and #4 (FLT3-WT) in presence of C12. Significantly fewer colonies were formed by the AML cell lines and blasts when cultured in presence of nanomolar doses of C12 (100nM) when compared to vehicle-treated control cells (Figure 2.3C). PRMT5 inhibition by C12 was also effective in promoting apoptosis and differentiation. A dose-dependent increase in apoptosis was observed in AML cell lines (MV4-11, Kasumi-1 and THP-1) treated with 25 and 50μM of C12 for 48 hours (Figure 2.3D). In addition, incubation of AML cells (MV4-11 (FLT3-ITD), Kasumi-1 (KITmut) and THP-1 (FLT3-WT)) with C12 resulted in a dose-dependent upregulation of early differentiation marker CD11b (Figure 2.3E). Similarly, >4 fold increase in expression of CD11b was observed in primary blasts treated with 25μM C12 compared to vehicle-treated controls (Figure 2.3E).
2.4 Discussion

Pharmacological inhibition of chromatin modifiers has emerged as novel target class for oncology since mutations or aberrant enzyme activities of these elements contribute to carcinogenesis and growth of cancer cells\textsuperscript{114,115}. Epigenetic modifiers including, histone modifying enzymes and chromatin remodeling complexes, control a variety of cellular signals by inducing biochemical conversions on histones and DNA\textsuperscript{116}. The relevance of epigenetic modifiers in leukemogenesis of acute myeloid leukemia (AML) was described recently by prevalence of recurrent mutations in chromatin modifying genes and genes related to DNA methylation\textsuperscript{13}. AML accounts for largest number of deaths related to leukemias in the US and successful incorporation of epigenetic targeting agents such as inhibitors of histone deacetylation (HDACs) and DNA methylation (DNMTs) in treatment regimens for AML has offered optimism to advance treatment outcome for high risk AML patients\textsuperscript{16,103,117–119}.

Protein methyltransferases which target arginine or lysine residues on proteins are implicated in directing malignant transformation of cells and represent a relatively new source of epigenetic therapeutics\textsuperscript{106}. While the value of agents to target histone lysine methylators including DOT1L and EZH2 have been documented\textsuperscript{106}, an effort to target protein arginine methylation has been absent. We sought to characterize and study the role and pharmacologic inhibition of protein arginine methyltransferase 5 (PRMT5), the one and most potent enzyme to catalyze symmetric dimethylation of arginine with robust impact on gene expression and cellular reprogramming.
Our data suggest that PRMT5 expression and enzymatic activity is essential for leukemia growth both *in vitro* and *in vivo* AML models. The aggressiveness of leukemia and progression of diseases was significantly exacerbated in xenograft mouse model of AML generated by engraftment of AML cells transformed to overexpress PRMT5 when compared to control engraftment mice. PRMT5 overexpressing animals had developed significantly larger spleens and livers, suffered blast infiltration in blood and bone marrow and were perished significantly faster than control mice. Similarly, overexpression of PRMT5 in both patient-derived cell lines and primary tumor cells from AML patients resulted in significantly enhanced cell proliferation and colony formation ability in cells. Conversely, transient depletion of PRMT5 in AML samples led to significantly reduced cell growth and eventually induced cell death. These observations are in support of previous studies which suggested a role for PRMT5 overexpression in various human cancers by influencing different stages of carcinogenesis and tumor progression. In AML, PRMT5 when overactive supports a proliferative phenotype characteristic of cancer cells and thus represents a viable therapeutic target in treating acute leukemia cases.

We tested this notion by studying the preclinical activities of a first in class single molecule inhibitor of PRMT5 labeled C12 developed by a multidisciplinary research group at Ohio State University. This compound which is designed to inhibit PRMT5 methyltransferase activities has been effective in a set of solid and lymphoid malignancies in which PRMT5 activity is correlated with more aggressive phenotype or hyperproliferation\(^{59,64}\). Treatment of AML cell lines and patient samples with this
compound significantly hindered leukemia cell proliferation and resulted in a time- and dose-dependent induction of apoptosis. Furthermore, incubation of AML samples with C12 affected the maturation state of cells shown by a dose-dependent increase in expression levels of early monocyte differentiation marker (CD11b) in patient-derived cell lines and primary tumor cells from patients. Based on these results, PRMT5 inhibition using C12 can greatly target hyperproliferating cancer cells by either inducing programmed cell death or more remarkably by activating differentiation signals which are often blocked in AML. For instance, inactivation of regulators of cell maturation such as C/EBPα is a hallmark of AML and using indirect means of re-expressing or reactivating these signals can improve efficacy of sub-toxic cancer therapies to induce cell reprogramming rather than cytotoxicity. Therefore, it is of utmost significance to test and optimize this compound for effectiveness in the in vivo setting with ultimate goal to enter this epigenetic therapy into clinical trials and improve the lives and treatment outcome of AML patients. Current work represents the first comprehensive effort to describe oncogenic properties for PRMT5 in promoting leukemia growth and introduce first preclinical evidence for C12, a selective PRMT5 inhibitor compound, in targeting AML cells for epigenetic cancer therapy.
2.5 Figures

Figure 2.1 PRMT5 activity is vital for AML cell survival and growth.

(A) Transcript levels of PRMT5 measured by qRT-PCR analysis. * is p<0.05 and ** is p<0.005. Significance was calculated using t test and error bars indicate SEM. List of cell lines and patient samples and their mutational status is provided. (B) Growth curve and colony formation assay comparing proliferation rate of AML cells overexpressing PRMT5 lentivirus and empty lentivector. Western blotting was used to confirm PRMT5 overexpression in Lenti-PRMT5 treated cells compared to Lenti-EV while GAPDH levels served as loading control. For growth curve assay cells were counted for extended period of time (>10 days). Each treatment was set up in triplicates. Colony formation assay comparing proliferation potential of AML cells transduced with Lenti-PRMT5 or empty vector (EV-Lenti) control. Colonies represented in bar graphs were counted 10-14 days after plating. Each assay was repeated in three independent experiments per treatment. (C) Spleens harvested from mice engrafted with THP-1/PRMT5 to overexpress PRMT5 and THP-1/EV as negative control. 1x10^6 cells were injected per mouse and animals were euthanized after 10 weeks. Enlarged spleens were observed in mice overexpressing PRMT5. The bar graphs represent significant increase in size (depicted as weight) of the spleens and livers and white blood cell count (WBC) taken from PRMT5 overexpressing animals compared to negative control mice. (D) Wright-Giemsa staining of the blood showing blast infiltration in blood of THP-1/PRMT5 engrafted mice. Staining was carried out after red cell lysis and the presence of normal
differentiated white blood cells in normal animals and dominant presence of blasts in PRMT5 overexpressing mice is clearly distinguishable. (E) Pathology for spleen and liver depicting significant infiltration of blasts in THP-1/PRMT5 mouse when compared to THP-1/EV normal mouse. (F) qRT-PCR analysis depicting PRMT5 transcript upregulation in THP-1/PRMT5 mice vs. THP-1/EV animals. The difference was statistically significant in spleen samples but, nevertheless, elevated in PRMT5 overexpressing model. (G) Western blotting confirmed apparent upregulation of PRMT5 and its epigenetic mark, symmetrically dimethylated H3 (H3R8me2) and H4 (H4R3me2), in representative spleen sample from THP-1/PRMT5 mouse than in THP-1/EV normal mouse. Immunostaining was done using anti-PRMT5 antibody followed by stripping and re-staining with anti-H3R8 antibody while GAPDH levels served as internal loading control. (H) Kaplan-meier survival curve comparing survival of THP-1/PRMT5 mice with THP-1/EV control animals. 2x10^6 cells were injected per mouse and PRMT5 overexpressing mice exhibited significantly reduced survival potential and began to die starting 34 days post engraftment. (I) Colony formation assay to measure proliferation rate in AML cell lines and patient primary blasts transfected with either PRMT5 RNA interference (siPRMT5 or shPRMT5) or scrambled control (sc). Transfections were carried out in triplicates and western blotting was used to confirm sufficient downregulation of PRMT5 in presence of siPRMT5 or shPRMT5.
Figure 2. PRMT5 upregulation significantly contributes to leukemia growth in the in vitro and in vivo models of AML.

continued
Figure 2.1: continued

C) Spleen

D) Blood Wright-Giemsa

E) Pathology

F) PRMT5 transcript levels

G) PRMT5 survival curve

continued
Figure 2.1: continued

I)  

MV4-11

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>shPRMT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>shPRMT5</td>
<td>10</td>
<td>*</td>
</tr>
</tbody>
</table>

THP-1

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>shPRMT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>shPRMT5</td>
<td>50</td>
<td>*</td>
</tr>
</tbody>
</table>

FLT3-ITD blast

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>siPRMT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>siPRMT5</td>
<td>80</td>
<td>*</td>
</tr>
</tbody>
</table>

FLT3-WT blast

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>siPRMT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>siPRMT5</td>
<td>60</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 2.2  Development of single molecular inhibitor to selectively target and inhibit PRMT5 enzyme activities.

(A) Modeled crystal structure of PRMT5 co-crystallized with S-adenosylmethionine (SAM) and an arginine peptide in catalytic site used for compound screening. (B) Measurement of in vitro enzyme activity of two PRMT5 inhibitor compounds (CMP3 and 5), neither were capable of diminishing PMRT1 activity. (C) In vitro enzyme assay showing the selectivity of PRMT5 inhibitor drugs while no effectiveness towards PRMT4 and PRMT7 was observed. (D) In vitro enzyme assay measuring methylase activity in presence of PRMT5 inhibitor compounds and different PRMT enzymes including type I PRMT1 and 4 and type II PRMT7. C12 shown by arrow was most potent in inhibiting enzymatic activity of PRMT5. PRMT5 inhibitors were not effective towards PRMT1, PRMT4 and PRMT7 enzymatic activities. to demonstrate C12 efficacy in inhibiting PRMT5 in AML cell lines. Methylation marks of PRMT5 were detected using antibodies specific for symmetrically dimethylated H3R8 and H4R3 before and after PRMT5 inhibition with C12. GAPDH levels served as internal loading control. (F) Western blotting to show the lack of protein ubiquitination for proteasomal degradation induced by C12 treatment. Anti-ubiquitin staining was used to stain ubiquitinated proteins following treatment with either C12 or proteasome inhibitor bortezomib while PRMT5 and GAPDH levels remained unchanged.
Figure 2. PRMT5 inhibitor, C12, selectively and potently targets PRMT5 activities.
Figure 2.3 Preclinical data supports antileukemic activities of PRMT5 inhibition in AML samples.

(A) Colorimetric MTS assay measuring the proliferation rate of AML cell lines after 24h, 48h and 72h incubation with different doses of PRMT5 inhibitor compound (C12). Dose-dependent decrease in absorbance directly correlates with number of metabolically active live cells. IC50 values represent the concentration of the compound at which 50% cell death was achieved. Mutational status of cells is listed. (B) MTS growth assay in primary blasts from patients following incubation with C12. (C) Colony formation assay to measure significantly reduced proliferations potential of AML cell lines and primary tumor cells in presence of sub-lethal doses of C12. Colonies were counted 10-14 days after plating and each treatment was carried out in triplicates. (D) Flow cytometry analysis of AML cell lines treated with PRMT5 inhibitor and stained for cell surface markers AnnexinV and PI showing a dose-dependent increase in percentage of apoptotic and dead cells when compared to DMSO treated cells. (E) Histogram of staining with early differentiation marker CD11b shows induction of differentiation in AML cell lines and patient samples treated with C12 for 48 hours. The increase in CD11b expression is AML cells lines followed a dose-dependent manner.
Figure 2. Preclinical and pharmacological activities of C12 in AML samples.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mutation Status</th>
<th>IC50 μM; 24h</th>
<th>IC50 μM; 48h</th>
<th>IC50 μM; 72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV411</td>
<td>FLT3-ITD</td>
<td>17.9</td>
<td>14.12</td>
<td>10.94</td>
</tr>
<tr>
<td>OCI-AML3</td>
<td>NPM1+, DNMT3A R882C</td>
<td>21.41</td>
<td>19.06</td>
<td>12.14</td>
</tr>
<tr>
<td>Kasumi1</td>
<td>KIT mutation (Asn822lys)</td>
<td>6.987</td>
<td>7.211</td>
<td>5.631</td>
</tr>
<tr>
<td>KG1</td>
<td>P53 mutation</td>
<td>10.12</td>
<td>17.97</td>
<td>14.22</td>
</tr>
<tr>
<td>THP1</td>
<td>FLT3 WT</td>
<td>31.44</td>
<td>16.74</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Patient #1 FLT3-ITD, NPM1+ 8.078 3.986 6.314
Patient #2 FLT3-ITD 7.521 8.729 5.236
Patient #4 - 4.244 5.99 7.403
Patient #6 NPM1+ 7.154 6.302 6.01

Figure 2. Preclinical and pharmacological activities of C12 in AML samples.
Figure 2.3: continued

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV4-11</td>
<td>FLT3-ITD</td>
</tr>
<tr>
<td>Kasumi1</td>
<td>KIT mutant</td>
</tr>
<tr>
<td>THP-1</td>
<td>FLT3-WT</td>
</tr>
<tr>
<td>Patient #2</td>
<td>FLT3-ITD, NPM1-</td>
</tr>
<tr>
<td>Patient #4</td>
<td>FLT3-WT, NPM1-</td>
</tr>
</tbody>
</table>

Cells Mutation status

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV4-11</td>
<td>FLT3-ITD</td>
</tr>
<tr>
<td>Kasumi1</td>
<td>KIT mutant</td>
</tr>
<tr>
<td>THP-1</td>
<td>FLT3-WT</td>
</tr>
<tr>
<td>Patient #2</td>
<td>FLT3-ITD, NPM1-</td>
</tr>
<tr>
<td>Patient #4</td>
<td>FLT3-WT, NPM1-</td>
</tr>
</tbody>
</table>

Figure 2.3: continued
Chapter 3: Protein arginine methyltransferase 5 (PRMT5) modulates miR-29b and FLT3 expression and activity in acute myeloid leukemia

3.1 Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemias in adults. The incidence and mortality rate associated with this disease increase by age and the long-term overall survival achieved by younger (60 years) and older (>60 years) AML patients remains as low as only ~30-40% and <10%, respectively. The major complexity in treating AML is given by the highly heterogeneous nature of this disease cytogenetically, genetically, epigenetically and clinically, which further highlights the significance of using prognostic biomarkers and clinical features to guide the selection of the type and the intensity of the treatment.

Epigenetic modifiers represent a novel class of therapeutic targets by playing a defining role in cellular malignant transformation. Pharmacologic alterations of distinct epigenetic processes such as DNA methylation, histone modifications and non-coding RNA expression have been proven effective in restoring normal gene function, increasing the activity of tumor suppressor proteins and ultimately inducing anti-tumorigenic effects. In AML, for example, abnormal DNA methylation events play key role in
myeloid leukemogenesis\textsuperscript{129} and the use of hypomethylating agent decitabine at lower doses has been successfully explored\textsuperscript{102,16}. More recently, aberrant protein arginine methylation has been linked to cancer and it is being explored as a novel therapeutic target\textsuperscript{53,130}. Protein arginine methyltransferase (PRMT) family of enzymes, with 11 known members, catalyze transfer of one or two methyl groups to the guanidine nitrogen atoms of arginine residues of peptides\textsuperscript{55,27}. Type I PRMTs (i.e. PRMT1, 3, 4, 6 and 8) are responsible for asymmetric dimethylation (aDMA) of arginine residues while type II PRMTs (i.e. PRMT5, 7 and 9) catalyze symmetric dimethylaion (sDMA) event. The intermediate mono-methylation (MMA) event is carried out by both types of PRMTs\textsuperscript{55,27}.

Protein Arginine Methyltransferase 5 (PRMT5), with multiple substrates including both histones (H3, H4 and H2A) and proteins like P53, MBD2\textsuperscript{36,99} has gained attention as emerging regulator of protein function in cancer\textsuperscript{24}. The post-translational changes induced by PRMT5 have a significant impact on cell growth and proliferation\textsuperscript{48}. Overexpression of PRMT5 has been reported in hematologic and solid malignancies (mantle cell lymphoma\textsuperscript{74,94}, lung and bladder cancer\textsuperscript{34}, gastric cancer\textsuperscript{54}, germ cell tumors (type II testicular germ cell tumors (TGCT), i.e. seminomas)\textsuperscript{47} and represents a promising therapeutic target.

Recently, it was shown that mutant constitutively active tyrosine kinase, JAK2V617F in myeloproliferative disorders interacts and phosphorylates PRMT5 leading to reduced enzyme activity and in turn progenitor cell expansion and accelerated erythroid
differentiation in CD34+ cells\textsuperscript{43}. We demonstrate here that PRMT5 contributes to AML cell growth and survival while inhibition of enzymatic function of this protein results in a significant antileukemia activity. We also found that miR-29b is effectively suppressed by PRMT5 enzyme activity. MiR-29b, with known antileukemic activity, targets key proliferation pathways in AML including FLT3 and KIT through disrupting activity of SP1; a pro-growth transcription factor\textsuperscript{104,102}. We further confirmed here that PRMT5 can promote leukemia growth by suppressing miR-29b transcription which in turn results in increased SP1 activity and consequently elevated FLT3 transcription.

These findings are especially important in specific subgroup of high-risk AML cases (~30%) which harbor mutations of the FMS-like tyrosine kinase 3 gene (FLT3) resulting in overactive receptor tyrosine kinase\textsuperscript{131,132}. Targeting of FLT3 kinase activity with small molecules has provided evidence of antileukemia activity, but this approach remains far from being curative\textsuperscript{133}. In AML, SP1 transcription factor is required in dynamically activating the transcription of FLT3\textsuperscript{104,102}. Here we report that PRMT5 also participates to FLT3 regulation and that inhibition of the epigenetic activity of this enzyme leads to suppression of FLT3 transcription. Our data describes a novel role for PRMT5 in promoting leukemia growth by suppressing miR-29b which in turn leads to increased SP1 and FLT3 levels. Therefore targeting PRMT5 may represent a new therapeutic approach in AML.
3.2 Materials and Methods

**Cell lines and primary blasts:** THP-1 and MV4-11 cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% calf serum. Blasts from AML patients were maintained as described in Chapter 2.

**Plasmids, transient transfections and reagents:** PRMT5 cDNA and shRNA were cloned, respectively, into pCDH1-MSCV-green-puro-cDNA and pGreenPuro shRNA cloning lentivector (SBI system biosciences). On-target plus siRNA-SMART pool specific for PRMT5, SP1 and off-target scrambled control were obtained from Dharmacon (Lafayette, CO). A locked nucleic acid (LNA)-antimiR-29b inhibitor (hsa-miR-29b mercury LNA microRNA Power inhibitor, Exiqon, Woburn, MA) was used to knockdown miR-29b and synthetic Pre-miR™ miRNA Precursor (Ambion) was used to overexpress miR-29b. MicroRNA transfections were carried out by siPORT NeoFX transfection reagent (Life technologies) including proper scrambled negative control for each treatment. Gene knockdown and overexpression was carried out either by electroporation using Nucleofector Kit (Amaxa, Walkersville, MD) or infection by lentivirus (SBI system biosciences reagents and methods). Commercially available PKC412 (Sigma-Aldrich, M1323) and FLT3 inhibitor (CALBIOCHEM #343020) was purchased while HLCL-61 (C12) compound was prepared/synthesized by Hongshan Lai, College of Pharmacy, Ohio State University.

**Western immunoblot and Immunoprecipitation (IP) analyses:** Western blotting on whole cell lysates was carried out as described in Chapter 2. Primary antibodies for
GAPDH and FLT3 (Santa Cruz Biotechnology, Santa Cruz CA), PRMT5 and SP1 (Millipore), H4R3me2 and H3R8me2 (Abcam), pFLT3, pSTAT5 and pERK1/2 (Cell Signaling) were diluted 1:1000 or 1:2000 in 5% milk or BSA and incubated for 1-2hr at room temperature. Membranes were washed using 1x TBS-T, incubated with HRP-conjugated secondary antibodies diluted in 1 × TBS-T with 5% milk or BSA, washed, and developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Whole cell lysate (at least 500μg) was used to pull down protein complexes using Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore) and ~4μg antibodies against PRMT5, SP1 (Millipore), pTYR (Santa Cruz) and 1μg normal IgG as negative control (Millipore). Denatured pull down samples were subjected to regular western blotting with exception of using TrueBlot IP secondary antibody (Rockland) for immunodetection.

**Cell viability analysis:** Colorimetric MTS assay for FLT3 rescue experiments were set up by plating 5x10⁴ cells in 100μL final volume in 96-well plate in presence of the different concentrations of C12 in presence or absence of FLT3 ligand (R&D Systems, Minneapolis, MN) for 48 hours at 37°C. Later, 20 μL of the CellTiter 96W AQueous One Solution Reagent was added to each well and the optical density at 490 and 690 nm was measured. Cell viability was calculated with respect to the control samples and reference background wavelength at 690nm. At least three independent experiments were performed.
**Chromatin Immunoprecipitation (ChIP):** Crosslinked chromatin was prepared by incubating cells in 1% formaldehyde for 10min at room temperature and quenching with ice cold 1M Glycine. Cells were sonicated in 1% SDS lysis buffer and sheared chromatin was used to pull down protein/DNA complexes using antibodies against SP1, PRMT5 (ChIP-grade Millipore), H4R3me2, H3R8me2 (Abcam) and RNA Pol II as positive control and normal IgG (Millipore) as negative control. Reverse crosslinked DNA was purified using QIAquick PCR Purification kit (Qiagen) and quantitative real-time PCR was carried out using SYBER green incorporation and primers designed for either FLT3 promoter region or miR-29b enhancer region. DNA signals were calculated relative to input DNA amount and in comparison to expression values of negative control IgG.

**RNA isolation and real-time PCR:** Total RNA was extracted using TriZol reagent (Invitrogen) and subjected to Reverse Transcription using SuperScript III reagents (Invitrogen) for mRNA measurements. For mature miR measurements qRT-PCR was done using TaqMan MicroRNA Assay (Applied Biosystems) following manufacturer’s instructions and normalization was carried out according to U44 (for human) and U6 (for mouse) levels.

**Luciferase reporter assay:** Promoter region of FLT3 (Sequence in supplementary data) was cloned into pGL4.11[luc2P] vector. THP-1 cells were transfected with empty vector or FLT3-promoter luciferase plasmid and *Renilla* in presence or absence of C12. Firefly luciferase and *Renilla* luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega).
3.3 Results

3.3.1 PRMT5 suppresses miR-29b expression via its methylation mark H4R3-me2

Symmetric dimethylation of the PRMT5 substrates H4R3 and H3R8 has been shown to regulate gene transcription both negatively and positively\textsuperscript{96,28}. Our recent ChIP-Seq data collected from mantle cell lymphoma cell line Jeko and Mino probed with PRMT5 and symmetrically dimethylated H3R8 specific antibodies, revealed miR-29b promoter to be >10-fold enriched for the PRMT5-catalyzed epigenetic mark H3R8-me2 (Figure 3.1A) suggesting that this regulatory locus may represent a potential PRMT5 target. As we have previously reported, miR-29b is often silenced in AML resulting in the overexpression of several oncogenes, thus we hypothesized that PRMT5 may be implicated in aberrant expression of this miR in leukemia cells. Indeed, we confirmed miR-29b to be upregulated in FLT3-ITD and FLT3-WT AML cell lines subjected to knockdown of PRMT5 (Figure 3.1B). In addition, suppression of miR-29b levels was clearly detectable in the in vivo model of THP-1/PRMT5 mice when compared to THP-1/EV controls (Figure 3.1B). We found that inhibitory effect of PRMT5 on miR-29b was excreted at gene transcription stages as inhibition of PRMT5 using siRNA (Figure 3.1C) or C12 (Figure 3.1D) against PRMT5 led to substantial and time-dependent increase in pri-miR-29b transcript levels (pri-miR-29b-1 and pri-miR-29b-2) in both AML cell lines and patient blasts. This notion was supported by observations where suppression of pri-miR-29b levels was achieved by ectopic expression of PRMT5 in patient blasts and THP-1/PRMT5 xenograft mice (Figure 3.1E).
When we assessed the regulatory region of pri-miR-29b for possible docking of PRMT5 (indirect binding) or H3R8me2 and/or H4R3me2 marks (direct binding) using Chromatin immunoprecipitation (ChIP) assay in MV4-11 and THP-1 cells, we observed enrichment of PRMT5 and H4R3me2 methylation mark, whereas blocking PRMT5 with C12 resulted in significant decrease of PRMT5 and H3R8me2 and/or H4R3me2 enrichment. Overexpression of PRMT5 on the other hand significantly enhanced binding of H4R3-me2 and localization of PRMT5 onto the regulatory region of pri-miR-29b (Figure 3.1F). Together these data suggest that PRMT5 contributes to miR-29b transcription regulation by facilitating the localization of transcription silencer H4R3-me2 onto the miR enhancer region.

Co-immunoprecipitation (Co-IP) in AML cells using antibodies against PRMT5 and SP1 revealed a physical association between these two proteins (Figure 3.1G). Presence of SP1 transcription factor in miR-29b regulatory region is previously described\textsuperscript{104}. The affinity of PRMT5 for SP1 may in part be responsible for facilitating the recruitment of PRMT5 to miR-29b transcription enhancer region.

3.3.2 PRMT5 upregulates FLT3, a critical oncogene involved in AML pathogenesis, through enhancing FLT3 transcription

PRMT5 has been reported to interact with, and to be regulated via posttranslational phosphorylation by mutant constitutively active tyrosine kinase; JAK2V617F in
myeloproliferative neoplasms \textsuperscript{43}. In this context, phosphorylated PRMT5 is rendered less active and results in enhanced progenitor cell expansion and erythroid differentiation (whereas PRMT5 knockdown triggers cell death in K562 and HEL cell lines). These data have suggested interplay of PRMT5 with tyrosine kinases. Interestingly, in our work, we noted that albeit both FLT3-ITD and FLT3-WT AML cells responded to pharmacologic PRMT5 inhibition, the former were more sensitive than the later (IC\textsubscript{50}=17\mu M vs IC\textsubscript{50}=31\mu M, respectively after 24h treatment with C12). Therefore, we tested for a potential interplay between PRMT5 and activated FLT3. Using Co-Immunoprecipitation (Co-IP) assay, we found however that PRMT5 did not physically interact with either wild-type or mutated FLT3 in THP-1 and MV411 cells, respectively (Figure 3.2A). FLT3 inhibition by either tyrosine kinase inhibitor (TKI) PKC412 or a specific FLT3 inhibitor (CALBIOCHEM #343020) did not change PRMT5 expression and phosphorylation levels (Figure 3.2B). Therefore we concluded that there was not a physical interaction between these two proteins.

However, we noted that inhibition of PRMT5, tested with both C12 (Figure 3.2C) and transient transfection with PRMT5-specific siRNA and shRNA (Figure 3.2D), resulted in significant downregulation of wild-type or mutated FLT3 RNA and protein in MV4-11 (FLT3-ITD) and THP-1 (FLT3-WT) cells and FLT3-ITD blasts. Activated FLT3 can contribute to malignant phenotype in leukemia blasts by signaling through downstream effector STAT\textsubscript{5} \textsuperscript{134}. Thus, to confirm decrease of FLT3 activity following PRMT5 knockdown, we measured the downstream phosphorylated STAT5 and ERK1/2 levels, and found that FLT3 downregulation via PRMT5 inhibition resulted in reduced
phosphorylation of STAT5 (pSTAT5) (Figure 3.2E). While FLT3-ITD cells generally expressed high level of the protein, THP-1 cells carrying wild type FLT3 have low basal levels of endogenous protein expression and activity and we reasoned they represented an appropriate model to investigate the activity of PRMT5 on FLT3 expression. We showed that overexpressed PRMT5 indeed resulted in increase in FLT3 mRNA and protein expression (Figure 3.2F). Similar observations were recapitulated in spleen of mouse engrafted with THP-1/PRMT5 cells where higher FLT3 levels were associated with overexpression of PRMT5 (Figure 3.2F). To confirm FLT3 involvement in driving PRMT5-induced leukemia growth we conducted FLT3 rescue experiment to evaluate if FLT3 activation following PRMT5 depletion would restore cell growth capacity. AML cells (MV4-11 (FLT3-ITD) and THP-1 (FLT3-WT)) were cultured in presence of C12 with or without supplementing FLT3 ligand to activate the FLT3 pathway and cell growth was measured using MTS assay within 48 hours of treatment. As shown in Figure 3.2G, forced activation of FLT3 pathway following PRMT5 inhibition lead to significant reversal of hindered cell growth when compared with cells treated with PRMT5 inhibition without subsequent FLT3 activation. These data provide evidence for PRMT5 role in positively contributing to AML cell growth via upregulating FLT3 expression and activity.

Overexpressed SP1 and FLT3 comprise the major signal transduction pathways which direct the growth and maintenance of leukemia cells in FLT3-ITD AML (Ref). Given the dependence of FLT3-ITD AML cells on SP1 transcription activity and FLT3 kinase
activity in driving proliferation and blocking cell maturation processes, FLT3-ITD AML samples show relatively higher sensitivity to PRMT5 inhibition.

3.3.3 *PRMT5 regulates transcription of FLT3 by modulating SP1 transcription factor*

Previous work by our group has identified transactivation complex composed of transcription factors SP1 and NFκB(p65) as a regulator of FLT3 expression in AML\(^{104,102}\). We also have shown that miR-29b effectively targets SP1 transcription factor\(^{135}\), as enforced expression of miR-29b in AML cells resulted in marked suppression of SP1 while transient depletion of miR-29b led to significant increase in SP1 protein levels (Figure 3.3A). Activity of miR-29b can negatively influence leukemogenesis by targeting apoptosis, cell cycle and proliferation pathways\(^{136}\). So we hypothesized that PRMT5–dependent upregulation of FLT3 may be mediated via its repressive function on miR-29b, which in turn may lead to uninterrupted transcriptional activity of SP1.

We first tested PRMT5 role in recruitment and localization of SP1 to regulatory region of FLT3 using ChIP assay and antibodies specific for SP1 and primers designed to amplify predicted binding sites at the promoter region of FLT3 (Appendix) in presence and absence of functional PRMT5. ChIP results indicated that inhibition of PRMT5 in AML cells following C12 treatment can lead to significant decrease in SP1 enrichment on the FLT3 promoter (Figure 3.3B). Conversely, binding of SP1 to FLT3 promoter was
significantly enhanced following forced upregulation of PRMT5 in AML cells (Figure 3.3C). The positive regulatory effects of SP1 on FLT3 expression was then validated by siRNA-mediated depletion of SP1 which resulted in significant downregulation of FLT3 (>2.6 fold) (Figure 3.3D). Additionally, supporting a functional role for PRMT5 in modulating FLT3 activity, inhibition of PRMT5 following exposure to C12 disrupted the FLT3 response element (i.e. FLT3-promoter-Luciferase reporter) activities in THP-1 cells (Figure 3.3E).

Since we identified a potential interaction between PRMT5 and SP1 proteins in AML cells (Figure), we decided to test for the presence of PRMT5 or its methylated histone marks (H3R8me2 and H4R3me2) at FLT3 promoter. Similar to miR-29b regulatory site, ChIP results suggested a considerable recruitment of PRMT5-induced histone marks H3R8me2 and H4R3me2 to promoter region of FLT3 (Figure 3.3F). This suggested that additional players are at work to induce the opposite transcriptional activities that we observed in FLT3 and miR-29b (i.e., one is activated while the other is suppressed). After enrichment of chromatin using antibody against acetylated lysine we found the promoter of FLT3 to be highly acetylated (Figure 3.3F). The striking trend on miR-29b regulatory site however was a significant enrichment of HDAC2 with marginal levels of acetylated histone (Figure 3.3F). Provided in Figure 3.3G is the landscape of ikb promoter region of FLT3 showing the spread and localization of Acetylated histones, PRMT5-induced methylated histones and SP1. This observation may suggest that if hyperacetylation of histones dominates to direct the active transcription of FLT3 oncogene, it is likely that at
hypoacetylated promoters such as miR-29b, histone arginine methylation can act as an effective transcription suppressor.

We also found that inhibition of PRMT5 in AML cell lines and patient samples (FLT3-ITD and FLT3-WT) using transient transfection and C12 treatment resulted in significant decrease of SP1 protein levels (Figure 3.3H). In contrast, overexpression of PRMT5 in THP-1 cells resulted in significant SP1 upregulation (Figure 3.3I). We documented a similar trend in the in vivo murine model of PRMT5 overexpression generated by THP-1/PRMT5 engraftment, as we measured a significant increase in SP1 levels when compared to THP-1/EV control mice (Figure 3.3I). In addition, according to qRT-PCR analysis, SP1 protein upregulation following PRMT5 overexpression, occurred without meaningful changes in SP1 mRNA levels. These data further recommend that PRMT5 contribution to SP1 transactivation activity is predominantly mediated posttranslationally and via suppressing miR-29b activity.

The efficacy of PRMT5 inhibition in downregulating SP1 is quite specific as no changes in SP1 protein levels was observed in presence of FLT3 inhibitors and tyrosine kinase inhibitors (Figure 3.3J). AML cells were treated with either FLT3 inhibitors or C12 and presence and levels of FLT3, pFLT3, pSTAT5, pERK1/2 was measured to evaluate FLT3 inhibition efficiency. In this context, Inhibition of FLT3 via SP1 downregulation appears to be unique to C12 treatment and this strong response to PRMT5 inhibition further confirms the impact of SP1 and its regulators, i.e., miR-29b in mediating the biological functions of PRMT5 in leukemic cells. Moreover, SP1 transcription factor is
overexpressed in multiple human cancers and several agents and biochemical approaches have been employed to downregulate SP1 and numerous SP1-regulated oncogenes\textsuperscript{137}. Antitumorigenic effects of targeting PRMT5 may actually be amplified through indirect and additive downregulation of protumor factors such as SP1.

Overall, these results provide meaningful insight into PRMT5 function in enhancing FLT3 and SP1 levels via diminishing miR-29b anti-tumorigenic activity. Therefore, inhibition of PRMT5 may provide an additional or alternative approach to stimulate miR-29b expression and consequently target overactive SP1 and FLT3 in AML cells.

### 3.4 Discussion

Increasingly encouraging results has emerged from deciphering the implications of both genetic and epigenetic processes, which can cross-talk and impact problematic oncogenic signaling cascades by altering the function or expression of oncoproteins and thus offering additional therapeutic targets\textsuperscript{102,16,138}. We have demonstrated that in AML, application of agents to target epigenetic modifiers at doses below cytotoxic levels can result in improved clinical response in older patients via upregulating a tumor suppressor microRNA; miR-29b, which is often silenced in AML\textsuperscript{139}. MiR-29b, which effectively targets DNA methyltransferase (DNMT)s and transcription factor SP1\textsuperscript{135}, is suppressed by a repressor complex containing HDAC enzymes\textsuperscript{104}, indeed, miR-29b is re-expressed following treatment with HDAC inhibitors\textsuperscript{16}. 

62
Previous studies have shown that protein arginine methyltransferase 5 (PRMT5) upregulation in number of malignancies is associated with growth of cancer cells and represents a novel target for disease treatment. PRMT5 catalyzes the mono or symmetric di-methylation of arginine residues in histones, and other non-histone substrates, including transcription factors. Symmetric di-methylation of histones is generally associated with gene repression and PRMT5 appears to play important role in hematologic malignancies, as overexpression of PRMT5 in lymphoma cells correlates with hyperproliferative phenotype. However, more studies are required to elucidate the impact of protein arginine methylation in acute leukemia growth to further utilize this chromatin modifier as new druggable targets for leukemia therapy.

Here we sought to demonstrate PRMT5 positive contribution to AML cell growth in vitro and aggressiveness of disease in vivo. Upregulation of PRMT5 resulted in survival and proliferation advantage in panel of patient-derived cell lines and primary blasts cells from AML patients. In contrast, PRMT5 attenuation in AML cells significantly decreased the proliferation potential of AML. We also note that in xenograft model of AML generated by engraftment of AML cells overexpressing PRMT5, significant elevation in immature white blood cell (myeloblast or blast) count in blood and bone marrow, enlargement of spleen and liver and reduced overall survival potential were achieved. Our work indicates that PRMT5 dynamically supports leukemia growth.

One of the more clinically challenging subtypes of AML is patients with mutations in FMS-like tyrosine kinase 3 (FLT3) gene, a membrane-bound receptor tyrosine kinase
(RTK) belonging to class III subfamily of RTK enzymes \(^{140}\). FLT3 upon stimulation by FLT3-ligand regulates a number of cellular functions including cell survival, proliferation and differentiation via phosphorylating and subsequently activating substrate proteins in secondary mediator pathways such as MAP kinase, STAT and PI3 kinase/Akt \(^{140}\). Ligand-independent activation of FLT3 in hematologic malignancies confers a poor clinical outlook by providing a survival and proliferation benefit to AML blasts \(^{141}\). Nearly 35% of all AML patients harbor an activating FLT3 mutation \(^{141}\), while two main groups of these aberrations include the most frequently occurring (~25%) internal-tandem duplications (\(FLT3\)-ITD) and less frequent (~7%) point mutations of tyrosine kinase domain (\(FLT3\)-TKD) \(^{131,132}\). An overview of the clinical application of FLT3 inhibitors (e.g. Sorafenib, Lestaurtinib, Quizartinib and Midostaurin) indicates an incomplete or transient response induction in AML patients with constitutively active FLT3 \(^{133}\). Such relative low activity may be mainly attributed to either acquired drug resistance or inefficient inhibition of FLT3 activity \(^{132,142,143}\) but, nevertheless highlights the requirement to identify and develop novel approaches to improve FLT3 inhibition in clinic.

When assessing the mechanism responsible for proliferative phenotype in PRMT5-overexpressing cells we found that FLT3 pathway represents one of the means by which PRMT5 can mediate its leukemogenic functions. Supporting evidence for possible interplay between a tyrosine kinase oncoprotein and PRMT5 was previously provided by Liu et al., 2011 who demonstrated that constitutively active mutant JAK2 promotes myeloproliferative neoplasm by directly interacting and phosphorylating PRMT5 \(^{43}\). This
interaction results in diminished enzymatic activity of PRMT5 via reduced binding to Mep50 cofactor and in turn release of differentiation block in stem/progenitor cells. Our current work reveals an epigenetic role for PRMT5 to upregulate FLT3 transcription by modulating expression of transcription factor SP1. Using gain- and loss-of function and ChIP assays we documented SP1-dependent changes in expression of FLT3 caused by experimental alterations in PRMT5 levels and enzymatic activity. The similarity of results obtained in both FLT3-ITD and FLT3-WT AML specimens examined, support PRMT5 biological potency in upregulating FLT3 pathway without considerable specificity for FLT3 mutant over wild type.

Furthermore, present results reveal that PRMT5 cellular functions influence SP1 levels via negatively regulating the expression of miR-29b, which is previously shown to target SP1 mRNA and effectively inhibit SP1 activity in AML cells. In support of these findings, we documented the presence of PRMT5-induced histone modification, H4R3me2, at enhancer region of pri-miR-29b with help of ChIP experiment. Symmetrically dimethylated H4R3 is shown to negatively regulate gene transcription and indeed may serve to negatively affect transcription of pri-miR-29b via localizing to the enhancer region of this gene. Moreover, introduction of miR-29b to increase available cellular levels of this miR has been shown to improve clinical outcome in AML cases by inducing suppression of pro-growth factors such as SP1, DNMTs, KIT and FLT3. The observed FLT3 and SP1 regulation in association with PRMT5 status in AML samples is partly explained by ability of this epigenetic factor in modulating miR networks in malignant cells evaluated here. Importantly, herein described novel
biological role for PRMT5 to upregulate FLT3 by altering SP1 levels is a potent and rather specific mechanism which defines the extent of regulatory attributions of epigenetic activities of this enzyme. Further gene expression profiling studies are required to identify additional genes and proteins which may be altered upon experimentally induced changes in PRMT5 levels. Nevertheless, PRMT5 activity in AML cell lines and patient blasts promotes a myeloproliferative phenotype that could be established via activation of FLT3 pathway. Future studies addressing the potentials of PRMT5 in more primitive hematopoietic cells i.e. stem/progenitor cell types will be further informative as previous studies have suggested a definitive role for cell context on PRMT5 function.

We also found that transcriptional complex composed of SP1, PRMT5, H3R8me2 and H4R3me2 elicit gene expression signal in a differentially and a gene-dependent manner which is most likely determined, at least in regard to FLT3 and miR-29b, by acetylation status of the histones. The notion that histone acetylation and deacetylation can impact histone methylation and vice versa has been postulated previously as interactions between regulators of each of these processes may act to target these regulators to the same histones. It was demonstrated that PRMT5 as part of nuclear chromatin remodeling and repressor complexes (i.e., SWI/SNF / mSin3A/HDAC) can efficiently methylate hypoacetylated histone H3 and H4 more so than hyperacetylated histones, suggesting that histone deacetylation is required for efficient histone methylation. In fact, specificity of PRMT5 to target H3 and H4 for methylation may be affected by its association with SWI/SNF and mSin3A/HDAC which exhibit preference to deacetylate
Histone acetylation via promoting a less compact chromatin is generally responsible for gene activation and although PRMT5 is active at FLT3 promoter it appears that histone acetylation and SP1 activity in this site signal transcription activation. Therefore, it is plausible that deficiency of acetylated histones in miR-29b regulatory region induced by predominant presence of HDAC enzymes allows for histone arginine methylation by PRMT5 with subsequently potent gene suppression signals in this region.

Given the ability of PRMT5 to impact AML cell fate, we sought to determine the potential of PRMT5 inhibition as viable therapeutic option in treating AML by investigating the preclinical and pharmacological activities of a first in class small molecule inhibitor of PRMT5 labeled C12. Treatment of AML cell lines and patient tumor samples with this selective and potent inhibitor of PRMT5 significantly reduced cell survival and proliferation potential, in addition to induction of apoptosis and in some cases release of differentiation block in AML blasts. These data are in line with recent observations which link antisense or compound induced knockdown of PRMT5 with slower growth (mouse fibroblasts cells, melanoma cell lines), cell cycle arrest, apoptosis and loss of cell migration. Further pharmacokinetics studies are strongly justified to adapt and evaluate C12 power and potency in vivo which can be later employed to design improved clinical trials. In AML especially, application of PRMT5 inhibition may serve as additional line of defense to improve response to standard chemotherapy via both upregulating miR-29b and consequently suppressing proliferative factors including SP1 and FLT3.
In conclusion, we have established that PRMT5 contributes to leukemia growth through complex epigenetic mechanisms that interfere with the expression of tumor suppressor microRNAs and oncogenic transcription factors and result in enhanced proliferation signals therefore may represent a novel target. Given the substantial oncogenic properties of PRMT5 an assessment of therapeutic implications of targeting this enzyme in improving treatment regimens for AML patients is highly warranted.
3.5 Figures

Figure 3.1 PRMT5 suppresses miR-29b transcription via deposition of H4R3me2

(A) ChIP-seq data from lymphoma cells probed with anti-PRMT5 and anti-dimethylated histone H3 showing a >10 fold enrichment of methylated histones at promoter of miR-29b when compared to negative IgG control. (B) qRT-PCR analysis showing a significantly elevated mature miR-29b in AML cell lines treated with C12. qRT-PCR analysis measuring reduced mature miR-29b levels in THP-1/PRMT5 mouse compared to control THP-1/EV mouse in vivo. Significance was calculated using t test and n=3 samples per group were compared. (C) Time-dependent increase in expression of pri-miR-29b levels following transient knockdown of PRMT5 using siRNA in AML patient blasts. Western blotting shows efficient knockdown of PRMT5 following transfection of cells with siPRMT5 (D) Time and dose-dependent increase in expression levels of pri-miR-29b isoforms following treatment of AML cell lines and patient blasts with C12. (E) Significant suppression of pri-miR-29b transcription following ectopic expression of PRMT5 in AML blasts and THP-1/PRMT5 mouse model of PRMT5 overexpression. (F) ChIP assay demonstrating enrichment of PRMT5 and especially H4R3me2 (methylation mark deposited by PRMT5) onto the enhancer region of miR-29b. Inhibition of PRMT5 with C12 resulted in significant decrease of H4R3me2 localization to miR-29b enhancer site. PRMT5 overexpression significantly enhanced the localization of H4R3me2 methylation mark at miR-29b regulatory region. (G) Immunoprecipitation (IP) in AML cells showing physical association between PRMT5 and SP1. Pull downs were carried
out using antibodies specific to PRMT5 and SP1 and normal IgG as negative control. Immunobloting was done with anti-PRMT5 and anti-SP1 antibodies and was executed in sequential manner including stripping and re-staining following each antibody.
Figure 3. 1 PRMT5 suppresses miR-29b expression in AML cells via its dimethylated histone mark.
Figure 3.1: continued

E) FLT3-ITD

Spleen from PRMT5 xenograft mouse

F) PRMT5

H3R8

H4R3

G) Input IgG IP w/ PRMT5 IP w/ SP1 B: PRMT5 B: SP1
**Figure 3.2** PRMT5 upregulates FLT3 mRNA and protein levels in AML cells.

(A) Immunoprecipitation (IP) assay in AML cell lines where pulling down with anti-PRMT5 and immunostaining with anti-PRMT5 and anti-FLT3 reveals no apparent physical association between PRMT5 and FLT3. (B) Inhibition of FLT3 kinase activity did not influence overall phosphorylated PRMT5 levels. Whole cell lysate was used to pull down phosphorylated tyrosine residues (anti-p-Tyr) in cells treated with FLT3 inhibitor. Phosphorylated ERK1/2 (p-ERK1/2) level was detected as control for p-Tyr IP and effectiveness of kinase inhibitory effects of treatment. (C) Inhibition of PRMT5 in AML cell lines and patient samples carried out with C12 lead to significant downregulation of FLT3 RNA and protein expression. qRT-PCR was used to measure relative PRMT5 mRNA levels and western blotting to detect protein levels of PRMT5, FLT3 and GAPDH respectively. (D) Transient knockdown of PRMT5 using siPRMT5 and shPRMT5 in AML cell lines and FLT3-ITD patient sample resulted in downregulation of FLT3 RNA and protein levels. (E) Western blotting showing significant downregulation of phosphorylated STAT5 (pSTAT5) a kinase target of FLT3 in AML cell lines following C12. C12-induced downregulation of FLT3 negatively impacts its kinase activity as evident by reduced levels of pSTAT5. (F) Ectopic overexpression of PRMT5 in AML cell line THP-1 with low basal levels of FLT3 using Lentivirus, sufficiently upregulated FLT3 transcription and protein expression. qRT-PCR and protein detection in representative spleen samples comparing THP-1/PRMT5 and THP-1/EV mouse model of PRMT5 overexpression indicated a PRMT5-dependent upregulation of FLT3 levels in vivo. (G) Rescue experiment in MV4-11 and THP-1 cells
to conform involvement of FLT3 in mediating PRMT5 pro-growth effects. Cells were treated with different doses of C12 in presence or absence of induced FLT3 activity which was provided by supplementing cells with 50nM FLT3-ligand (FL). Colorimetric MTS assay was used to measure cell viability and growth within 48 hours of treatment.
Figure 3. 2 PRMT5 actively upregulates FLT3 expression and activity in AML.
Figure 3.2: continued

---

**E)**

**F)**

---

**G)**

---

**H)**

---

---
Figure 3.3  PRMT5 regulates FLT3 expression by modulating SP1 protein levels.

(A) Overexpression and knockdown of miR-29b in AML cell lines resulted in respectively significant downregulation and upregulation of SP1 protein levels. Western blotting was used to measure SP1 levels after 48h following transient transfection with either miR-29b/mimic or miR-29b/LNA. (B) Chromatin immunoprecipitation (ChIP) assay showing localization of SP1 to promoter region of FLT3 and significant decrease in binding of this transcription factor to FLT3 promoter following PRMT5 inhibition which was concomitant with significant downregulation of FLT3 mRNA levels. (C) ChIP assay in cells which ectopically overexpressed PRMT5 showed an enhanced localization of SP1 to promoter region of FLT3 which coincided with significant upregulation of FLT3 transcription. (D) Transient transfection of AML cells with siRNA specific to SP1 resulted in sufficient knockdown of SP1 and as a result downregulation of FLT3 demonstrated by qRT-PCR assay and western blotting. (E) Luciferase reporter assay to confirm regulatory functions of promoter region of FLT3 for which ChIP primers were designed. The promoter site was cloned before luciferase gene and THP-1 cells were transiently transfected with Luciferase-negative-control or Luciferase-FLT3-promoter construct in presence or absence of PRMT5 inhibitor. Luciferase activity measured relative to Renilla and as a measure of FLT3 activity was significantly decreased following PRMT5 inhibition. (F and G) ChIP experiment showing the differential recruitment of HDAC2, acetylated histones and methylated histones to the promoter of FLT3 and miR-29b. While acetylation is significantly high in FLT3 promoter miR-29b is clear of acetylated histone marks most likely due to presence of HDAC2 in this site. (H)
Western blotting used to measure the downregulation in protein levels of SP1 following inhibition of PRMT5 in AML cell lines. PRMT5 inhibition was carried out using both small molecule inhibition and siPRMT5 or shPRMT5, and in both cases blockage of PRMT5 resulted in significant knockdown of SP1. Actin or GAPDH staining was used as internal loading control. (I) Western blotting in AML cell lines transduced with Lenti-PRMT5 or Lenti-EV showing an upregulated SP1 in Lenti-PRMT5 treated cells compared to negative control. Significant SP1 upregulation was recapitulated in western blotting of spleen samples taken from THP-1/PRMT5 mouse when compared to normal THP-1/EV animal in vivo. (J) Treatment of AML cells with PKC412, specific FLT3 inhibitor (FLT3i) and C12 for 5 hours. Levels of phosphorylated STAT5 (pSTAT5) and ERK1/2 (pERK1/2) were detected as positive control to confirm the effective FLT3 inhibition. Immunostaining was carried out sequentially after striping the membrane following staining with anti-FLT3, anti-pFLT3, anti-SP1, anti-PRMT5, anti-pSTAT5, anti-pERK1/2 and anti-GAPDH.
Figure 3. PRMT5 regulates FLT3 levels by modifying activities of SP1 transcription factor in AML.
Figure 3.3: continued

F) Modified histone marks at:

G) Sp1, PRMT5 and HDAC2 at:

Folds change

<table>
<thead>
<tr>
<th>Protein</th>
<th>IgG</th>
<th>Acetyl-Lys</th>
<th>H3R8me2</th>
<th>H4R3me2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>1</td>
<td>63</td>
<td>15</td>
<td>8</td>
</tr>
</tbody>
</table>

Folds change

<table>
<thead>
<tr>
<th>Protein</th>
<th>IgG</th>
<th>Sp1</th>
<th>PRMT5</th>
<th>HDAC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>1</td>
<td>30</td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>

Folds change

<table>
<thead>
<tr>
<th>Protein</th>
<th>IgG</th>
<th>Acetyl-Lys</th>
<th>H3R8me2</th>
<th>H4R3me2</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29b</td>
<td>1</td>
<td>0.2</td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>

Folds change

<table>
<thead>
<tr>
<th>Protein</th>
<th>IgG</th>
<th>Sp1</th>
<th>PRMT5</th>
<th>HDAC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29b</td>
<td>1</td>
<td>18</td>
<td>22</td>
<td>83</td>
</tr>
</tbody>
</table>

continued
Figure 3.3: continued
Chapter 4: Identifying key molecular players involved with protein arginine methyltransferase 5 (PRMT5) expression and regulation in acute myeloid leukemia

4.1 Introduction

The data presented and discussed in previous chapters provide compelling proof for PRMT5 participation in leukemia growth by altering the expression and activity of tumor suppressors and oncogenes. In addition, in current work we demonstrated the preclinical potency of a first in class single molecule inhibitor of PRMT5 in targeting growth and maintenance of leukemia cell. However, the remaining question to address is to identify the molecular factors which regulate the expression and enzymatic activities of PRMT5 in AML. In this chapter we describe few of the networks and repressor complexes that interact with and influence PRMT5 levels in leukemic cells.

PRMT5 expression is modulated by microRNA (miR)-96

The mechanisms responsible for control of PRMT5 expression are largely unknown, however regulation mediated by microRNA (miR)s was brought into attention as one of the potential mechanisms. Efforts by Pal et al., 2007 revealed that while expression of
PRMT5 protein was enhanced in number of transformed lymphoid cell lines it was not due to direct increase in transcription of this enzyme. Evaluation of PRMT5 mRNA translation rate in normal and mantle cell lymphoid (MCL) cells using sucrose gradient sedimentation experiments suggested that PRMT5 mRNA translation was enhanced in transformed MCL cell lines when compared to normal cells. These observations implicated PRMT5-specific miRs in regulating PRMT5 translation and therefore a search for potential miR binding sites at 3’ UTR region of PRMT5 was carried out. Among identified candidate miRs, miR-92b and miR-96 expression was differentially represented in normal vs. lymphoma cells. In this regard, miR-96 has been previously presented with potent tumor suppression activities. Oncogenic fusion gene anaplastic lymphoma kinase (ALK) which promotes tumor cell survival in T-cell lymphoma was described as a direct target for miR-96. Furthermore, inhibition of ALK by miR-96 was associated with diminished cell proliferation, colony forming ability and migration capacities in cancer cells. Study by Yu et al., 2010 suggested that increased miR-96 activity directly targets KRAS oncogene in pancreatic cancer cells and therefore induces apoptosis by hindering Akt signaling pathway. MiR-96 as a potent regulator of KRAS also negatively affected cancer cell invasion and migration capabilities serving as tumor suppressor element in pancreatic cancer. We investigated the interplay between miR-96 and PRMT5 in acute myeloid leukemia and found a potential negative regulatory loop between these two factors. As miR-96 can target PRMT5 mRNA and effectively suppress protein levels of this enzyme, PRMT5 appears to target miR-96 expression by localizing and depositing its repressive dimethylated histone marks at promoter region of this miR. This
observation provides a clue into PRMT5 machinery in leukemia cells where a network of epigenetic modifiers including posttranslational modifications and non-coding RNAs function to determine the molecular activities of this enzyme.

**PRMT5 associates with corepressors in AML**

Activities of PRMT5 is essentially connected to the control of gene transcription by methylating histones H3R8 and H4R3 or by associating with number of nuclear chromatin remodeling complexes. Thus, the means by which PRMT5 is recruited to chromatin may provide insight into the molecular specifics of its function. Data in support of this notion was provided by Lacroix et al., 2008 by showing that a PRMT5 binding partner called cooperator of PRMT5 or COPR5 functions as key adaptor for PRMT5 activity on some of its target genes (e.g. Cyclin E1) by binding to nucleosome and histone H4. COPR5 strongly affects substrate specificity of PRMT5 by exhibiting high preference towards H4R3 and therefore can influence the results of PRMT5 enzymatic activities\(^{45}\).

Furthermore, chromatin remodeling in combination with histone modification has been shown to play essential role in regulation of specific genes. In this regard, studies to understand the mechanisms underlying transcription repression executed by corepressor complexes hSWI/SNF and mSin3A/HDAC has revealed PRMT5 involvement as interacting component of these complexes to direct gene transcription\(^{63,146}\). SWI/SNF is a
chromatin remodeling complex with four core subunits including Brg1/hBrm, BAF155, BAF170 and INI1. Brg1 and hBrm are the catalytic ATPase subunits and PRMT5 associates with SWI/SNF by directly interacting and binding to Brg1. Moreover, mSin3A comprises the core component of a corepressor complex, mSin3A/HDAC2, with histone deacetylase enzymatic activities. These two multiprotein repressor complexes i.e. SWI/SNF and mSin3A/HDAC can cross talk as mSin3A interacts with Brg1 subunit of SWI/SNF complex. Importantly, it was demonstrated that PRMT5 as part of SWI/SNF / mSin3A/HDAC can efficiently methylate hypoacetylated histone H3 and H4 more so than hyperacetylated histones, suggesting that histone deacetylation is required for efficient histone methylation. In fact, specificity of PRMT5 to target H3 and H4 for methylation may be affected by its association with SWI/SNF and mSin3A/HDAC which exhibit specificity to deacetylate H3 and H4. In support for this interaction, efficient expression of certain genes (e.g. cad) is shown to be regulated by chromatin remodeling and histone deacetylation specifically via localization of PRMT5, Brg1 and HDAC2 to promoter of the gene. We now know that histone acetylation and deacetylation can impact histone methylation and vice versa and interactions between regulators of each of these processes may assure directing these regulators to target the same histones. Rank et al., 2010 discovered that unimpaired PRMT5 activity leads to dramatic decrease in acetylation of H4K12 while significant increase in multiple repressive histone modifications (in addition to H4R3me2) including phosphorylation of H4S1, tri-methylation of H4K20, tri-methylation of H3K9 and tri-methylation of H3K27. They also showed that binding of PRMT5 to the γ-globin gene promoter and its methylase activity
is essential for assembly of multiprotein complexes on this region including MBD2 and MBD3, HDAC1, DNMT3A as well as CK2α and SUV4-20h1 (enzymes responsible for phospho-H4S1 and H4K20me3 respectively)\textsuperscript{145}.

PRMT5 arginine methylase activity was also determined to be essential for DNMT3A DNA methylation activity in human β-globin locus as studies revealed that PRMT5-mediated H4R3me2 epigenetic mark in this site provides a direct binding target for DNMT3A\textsuperscript{77}. These findings are in support of previous data suggesting that certain trimethylation events on histones such as H3K9\textsuperscript{154}, H3K27\textsuperscript{155} and H4K20\textsuperscript{156} are essential for subsequent DNA methylation events.

In addition, association of PRMT5 with transcription factors has been proven relevant in mediating cell growth signals. PRMT5 was found to upregulate NFκB signaling by directly dimethylating R30 residue on p65 subunit of NFκB which in turn can increase DNA binding and subsequent transcriptional activity of p65\textsuperscript{34}. Furthermore, active transcription of CXCL10 which is regulated by p65 was found to be dependent on PRTM5-induced methylation of p65\textsuperscript{97}. Our protein-protein interaction assays revealed that PRMT5 associates with a number of important transcription factors and corepressors in AML cells including NFκB(p65), HDAC2 and DNMT3A. These results indicate that recruitment of PRMT5 to chromatin and its subsequent epigenetic impact is in part determined by its affinity towards other protein members of chromatin remodeling complexes. These intermediate proteins, which can link or bridge PRMT5 to chromatin
by binding to histones, act as prerequisite for PRMT5 recruitment and serve to promote its association with chromatin.

4.2 Materials and methods

Cell lines and primary blasts: MV4-11 and THP-1 cell lines and primary patient blasts were maintained as described in Chapter 2.

Plasmids, transient transfections and reagents: A locked nucleic acid (LNA)-antimiR-96 inhibitor (hsa-miR-96 mercury LNA microRNA Power inhibitor, Exiqon, Woburn, MA) was used to knockdown miR-96 and synthetic Pre-miR™ miRNA Precursor (Ambion) was used to overexpress miR-96. MicroRNA transfections were carried out by siPORT NeoFX transfection reagent (Life technologies) including proper scrambled negative control for each treatment. Commercially available PKC412, mithramycin A and decitabine (Sigma-Aldrich) were purchased while HLCL-61 (C12) compound was prepared/synthesized by Hongshan Lai at College of Pharmacy, Ohio State University.

Western immunoblot and Immunoprecipitation (IP) analyses: Western blotting was carried out as described in Chapter 2. Primary antibodies for GAPDH and FLT3 (Santa Cruz Biotechnology), PRMT5, p65, HDAC2 and SP1 (Millipore) and DNMT3A (Abiocode), were diluted 1:1000 or 1:2000 in 5% milk or BSA and incubated for 1-2hr at room temperature. Whole cell lysate (at least 500μg) was used to pull down protein
complexes using Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore) and ~4μg antibodies against PRMT5, SP1, p65 and 1μg normal IgG as negative control (Millipore). Denatured pull down samples were subjected to regular western blotting with exception of using TrueBlot IP secondary antibody (Rockland) for immunodetection.

**Chromatin Immunoprecipitation (ChIP):** ChIP assay was carried out as described in Chapter 3 using antibodies against SP1, PRMT5 (ChIP-grade Millipore), H4R3me2, H3R8me2 (Abcam) and normal IgG (Millipore) as negative control. Reverse crosslinked DNA was purified using QIAquick PCR Purification kit (Qiagen) and quantitative real-time PCR was carried out using SYBER green incorporation and primers designed for miR-96 promoter region. DNA signals were calculated relative to input DNA amount and in comparison to expression values of negative control IgG.

**RNA isolation and real-time PCR:** Total RNA was extracted using TriZol reagent (Invitrogen) and subjected to Reverse Transcription using TaqMan MicroRNA Assays (Applied Biosystems). Quantitative Real-Time PCR was performed using commercially available TaqMan miR Expression Assay primers and probes, and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Mature microRNA levels were normalized to U44 levels.

**Luciferase reporter assay:** NFκB(p65) target sequences were cloned into pGL4.11[luc2P] vector. 293T cells were transfected with empty vector or NFkB luciferase plasmid and Renilla in presence or absence of C12. Firefly luciferase and
Renilla luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega).

4.3 Results

4.3.1 PRMT5 and miR-96 are caught in a negative regulatory loop in AML

Using transient transfection to overexpress and knockdown miR-96 we demonstrated that PRMT5 may serve as a direct target for miR-96 in AML cells. PRMT5 protein levels were significantly downregulated upon ectopic expression of miR-96 (Figure 4.1A) whereas depletion of miR-96 resulted in considerable increase in PRMT5 levels (Figure 4.1B).

Tumor suppressor function for miR-96 was described previously. Given that PRMT5 inhibition can result in re-expression of tumor suppressor genes or microRNAs, we also evaluated miR-96 levels following depletion of PRMT5. Inhibition of PRMT5 activity in AML cell lines and patient samples using C12 resulted in a significant and dose/time-dependent increase in expression levels of mature miR-96 levels in AML cell lines and patient samples (Figure 4.1C and D). To investigate a possible role for PRMT5 in suppressing the expression of miR-96, we conducted chromatin immunoprecipitation (ChIP) assay to detect the presence of PRMT5 and its dimethylated histone marks H3R8 and H4R3 at the promoter region of miR-96 (Sequence and primers: Appendix 1). Our ChIP data suggest that PRMT5 is localized to miR-96 promoter and is involved in active
methylation of histones H3 and H4 (Figure 4.1E). It is notable that albeit both H3R8me2 and H4R3me2 are present at miR-96 promoter, H4R3me2 appears to be the more prominent methylation mark deposited by PRMT5 in this specific region. Inhibiting PRMT5 activity negatively affected the enrichment of methylated histone marks created by PRMT5 at miR-96 promoter (Figure 4.1E). Furthermore, miR-96 promoter was tested for candidate transcription factor activities in hope to provide further insight into miR-96 regulatory machinery in AML cells. We tested the predicted SP1 binding sites at miR-96 promoter region in presence or absence of PRMT5 activity. We found SP1 to be strongly present at the promoter of miR-96, while its binding was diminished following treatment with PRMT5 inhibitor (Figure 4.1F). These observations recommend that in AML, SP1 acts to contribute to repression of miR-96 expression while its transcriptional activities in this region are weakened by PRMT5 inhibition. Another interesting observation was that treating cells with inhibitor of tyrosine kinases (PKC412), DNA binding transcriptional inhibitor (mithramycin A) and DNA hypomethylating agent (decitabine) did not have significant effects on PRMT5 levels (Figure 4.1G, H and I). However a combination of C12 (PRMT5 inhibitor) and decitabine (DNMT inhibitor) led to significant downregulation of PRMT5 (Figure 4.1 I). These data may recommend that in AML PRMT5 expression is controlled by coordinated processes of protein (most likely histones) arginine methylation and DNA methylation.
4.3.2 PRMT5 associates with DNMT3A, HDAC2 and NFκB(p65) in AML

We used antibodies specific to PRMT5 to conduct Co-immunoprecipitation (Co-IP) pull-down assay to enrich this protein and its binding partners from whole cell lysates prepared from different AML cell lines (FLT3-ITD: MV4-11 and MOLM13, FLT3-WT: THP-1 and FLT3-null: U937). Western blotting was conducted to detect for presence of HDACs and DNMTs in association with PRMT5. In the AML cell lines tested, we found that PRMT5 can interact with DNMT3A and HDAC2 (Figure 4.2A). We later tested the possible changes in the nature of this interaction following PRMT5 inhibition. Treatment of cells with C12 resulted in loss of DNMT3A levels most likely by increasing miR-29b expression (Chapter 3). HDAC2 protein levels remained unchanged however its binding to PRMT5 was diminished following C12 treatment (Figure 4.2B).

Previous studies has shown that PRMT5 can upregulate NFκB signaling by catalyzing dimethylation of R30 residue on p65 subunit of NFκB and lead to increased transcription of genes by enhancing DNA binding potential of p65. In support of these studies we found that PRMT5 inhibition using C12 can significantly reduce NFκB signaling, measured by reduced NFκB reporter activity suggesting that also in AML PRMT5 may influence gene expression via upregulating NFκB transcriptional activity (Figure 4.2C). Furthermore, testing for protein-protein interactions in AML cells revealed that p65 can tightly bind to PRMT5 (Figure 4.2D).
4.4 Discussion

Protein arginine methylation by PRMT5 has been identified as one of the most important epigenetic modifications that participate in regulation of gene expression. Overexpression of PRMT5 contributes to leukemia growth and inhibition of this enzyme offers anti-leukemic activities in AML (Chapters 2 and 3). Identifying the key regulators of PRMT5 which are responsible for control of the expression and activities of this enzyme is essential to better target its oncogenic properties in number of human malignancies. However, efforts to determine the key modulators of PRMT5 in mammalian cells have been scarce. Studies have revealed that PRMT5 mRNA can be targeted by number of microRNA (miR)s which then greatly alter its downstream activities\textsuperscript{74}. Our observations in AML samples suggest that miR-96 represents a potent suppressor of PRMT5 and that variations in the expression of miR-96 can induce significant changes in PRMT5 levels. Ectopic expression of miR-96 in AML samples resulted in significant downregulation of PRMT5 protein levels while experimental depletion of miR-96 is followed by increased PRMT5 protein levels. A tumor suppressor property for miR-96 has been documented in T-cell lymphoma (by targeting ALK oncogenic fusion gene)\textsuperscript{150} and pancreatic cells (via targeting KRAS oncogene)\textsuperscript{151}. More interestingly, PRMT5 and miR-96 are most likely the components of a negative regulatory loop in AML cells. We found that PRMT5 can negatively affect miR-96 expression via localizing to the promoter region of this miR and by catalysis of H3R8 and H4R3 dimethylation. MicroRNAs has gained increasing value in regulation of various cellular functions and altered miR levels can contribute to
formation and development of multiple human malignancies\textsuperscript{157,158,159}. Distinctive changes in pattern of tumor suppressor or oncogenic miR expression have been associated with different subtypes of AML and as a result response to therapy\textsuperscript{160}. As a result, manipulation of the miRs with functional relevance in leukemogenesis has provided an additional platform in enhancing the care and treatment regimens for AML patients\textsuperscript{161}.

Furthermore, expression of PRMT5 in AML models tested here appears to be controlled by combined participation of histone arginine methylation and DNA methylation. These data are highly preliminary but may point out to the presence of a set of coordinated activities of epigenetic mechanisms including protein arginine methylation and DNA methylation necessary to direct the expression of this enzyme. Previous studies have demonstrated a functional association between histone arginine methylation and DNA methylation in regulating gene expression\textsuperscript{77}. Considering that both histone arginine and DNA methylation marks tend to act as transcription silencers, it almost seems that inhibitors of PRMT5 expression (transcription factors, microRNAs, etc.) may be constantly hypermethylated at both histones and DNA and removing these epigenetic marks using C12 and decitabine may allow for PRMT5 silencers to function and suppress the expression of this enzyme. Nevertheless, more studies to identify genes that control PRMT5 expression in leukemia cells is required to further elucidate the meanings of these observations.

Another level of control for PRMT5 function is provided through protein-protein interactions which essentially facilitate the recruitment and localization of this enzyme to
the promoter site of the target genes. PRMT5 as member of repressor complexes in charge of chromatin remodeling such as SWI/SNF and mSin3A/HDAC actively participates in gene expression machinery\textsuperscript{56,153}. We captured PRMT5 in tight association with repressors HDAC2 and DNMT3A and transcription factor NFκB(p65). Physical association between PRMT5 and these elements translate into important functional signals especially by directing this enzyme towards specific histones or chromatin modifications. Recruitment of PRMT5 highly relies on binding partners which interact with histones or DNA and act as mediators between this enzyme and chromatin.

In addition, development and application of selective inhibitors for key chromatin modifiers has been successfully tested as promising and viable targets in epigenetic therapeutics\textsuperscript{16,117–119}. The interaction between PRMT5 and DNMT3A and HDAC2 may offer opportunities for combination therapies where administration of sub-toxic and low doses of these compounds may provide synergistic or additive effects in eradicating or reprograming malignant cells especially in patients who cannot tolerate aggressive standard cytotoxic chemotherapy.
4.5 **Figures**

4.1 *PRMT5 and miR-96 are caught in a negative regulatory loop in AML*

(A) Transient overexpression of miR-96 in AML cells results in significant suppression of PRMT5 protein levels. pRT-PCR was used to measure mature miR-96 levels in the cells following transfection. (B) Transient depletion of miR-96 in AML cells is followed by significant upregulation of PRMT5 protein levels. Western blotting was carried out on whole cell lysates 48hrs after transfection. (C) Inhibition of PRMT5 using C12 in AML cell line results in time and dose dependent increase in expression levels of mature miR-96 levels measured by qRT-PCR. Treatments were done in triplicates. (D) Treatment of AML patient samples with C12 leads to dose-dependent increase in miR-96 expression. (E) Chromatin immunoprecipitation (ChIP) assay in AML cells showing localization of PRMT5 and its methylation marks H3R8me2 and H4R3me2 onto the promoter region of miR-96 gene. The binding of these factors was significantly reduced after inhibition of PRMT5 using C12. Negative IgG ChIP was used as control and antibodies specific for PRMT5 and symmetrically dimethylated H3R8 and H4R3 were used for chromatin pull down. (F) ChIP assay demonstrating enrichment of transcription factor SP1 onto the promoter site of miR-96. Anti-SP1 antibody was used for ChIP and inhibition of PRMT5 resulted in reduced SP1 binding to miR-96 promoter. (G) Treatment of AML cells with kinase inhibitor PKC412 did not affect PRMT5 levels. Phospho-ERK1/2 (p-ERK1/2) level was measured as positive control for activity of PKC412 in inhibiting kinase activity in cells. (H) Inhibition of SP1 using mithramycin A did not affect PRMT5 levels.
in AML cells. Staining with anti-mono and dimethyl arginine was used to investigate methylation activity of PRMT5. FLT3 levels were measured as positive control for efficiency of SP1 inhibition. (I) Treatment of AML cells with hypomethylation agent decitabine results in downregulation of PRMT5 protein levels only in the presence of C12.
Figure 4. 1 PRMT5 levels are altered by activities of miR-96 in AML.
Figure 4.1: continued
Figure 4.2  PRMT5 associates with corepressors and transcription factors in AML

(A) Co-Immunoprecipitation (Co-IP) assay to pull down PRMT5 and its binding partners using antibody against PRMT5 and whole cell lysate from AML cells. Immunostaining was carried out with antibodies against DNMT3A and HDAC2. Negative IgG control and mock control without antibody was included to ensure specificity of pull-down towards PRMT5.  (B) Co-IP assay in AML cells with and without C12 treatment showing the physical association between PRMT5 and DNMT3A and HDAC2. DNMT3A protein levels was downregulated following PRMT5 inhibition due to miR-29b upregulation. HDAC2 association with PRMT5 was diminished following C12 treatment. (C) Luciferase reporter assay to measure NFκB signaling before and after PRMT5 inhibition. A group of NFκB(p65) target sequences are cloned under luciferase reporter and significantly reduced luciferase activity after C12 treatment correlates with reduced NFκB activity. (D) Co-IP analysis showing interaction between PRMT5 and p65 proteins. IP was carried out separately with both anti-PRMT5 and anti-p65 antibodies.
Figure 4. PRMT5 associates with other corepressors and chromatin modifiers in AML.
Chapter 5: Conclusions and future directions

This work describes the role and mechanism of function of an epigenetic modifier, protein arginine methyltransferase 5 (PRMT5), in promoting leukemia cell growth and validates for the first time, the preclinical impacts of targeting PRMT5 in acute myeloid leukemia (AML) using a first in class selective PRMT5 inhibitor (C12). Mono- and symmetric dimethylation events on arginine residues of peptides catalyzed by PRMT5 retain a critical role in directing gene expression and transformation of mammalian cells\textsuperscript{27,95-97}. Oncogenic properties of PRMT5 are mainly mediated by its methylated histone marks, H3R8me2 and H4R3me2, which often signal suppression of target tumor suppressor genes\textsuperscript{63,94,95}. Yet, in addition to transcription silencing via histone methylation or as part of repressor and chromatin remodeling complexes, PRMT5 enzymatic activities may also lead to elevated gene expression largely by inducing methylation of transcription factors, e.g. NF\kappa B(p65), which in turn increase the DNA binding and transcriptional activities of the modified substrates\textsuperscript{34,97}.

Overexpression of PRMT5 is noted in multiple human cancers\textsuperscript{162,163} and functional studies have presented convincing data to support the oncogenic properties of this enzyme as evident by accompanied hyperproliferative and metastatic phenotype in these malignancies\textsuperscript{59,64,112}. We evaluated the potential contribution of PRMT5 to myeloid
leukemogenesis and found that overexpression of this enzyme is in fact a contributing factor to the aggressiveness and maintenance of acute leukemia both in vitro and in an in vivo mouse model of AML. Our efforts here have led to identification of novel direct and indirect targets for PRMT5 which play defining roles in leukemia growth machinery in AML. Methyltransferase activities of PRMT5 effectively target the expression of tumor suppressor microRNA (miR); miR-29b in AML cells as presence of PRMT5-induced dimethylated histone H4R3 at promoter region of miR-29b was found to be required for silencing this miR. Depletion of PRMT5 in AML cells on the other hand results in significant re-expression of miR-29b. Tumor suppressor properties of miR-29b is largely attributed to its ability in actively targeting a number of oncoproteins implicated in leukemogenesis including transcription factor SP1 and DNA methyltransferases (DNMTs)\textsuperscript{104,135}.

Remarkably, miR-29b is frequently downregulated in AML which is often followed by uninterrupted transcriptional activities of SP1; a potent activator of oncogenic receptor tyrosine kinase FLT3 expression. The interaction of PRMT5 with miR-29b, SP1 and its transcription targets is indeed an important modulator of cell behavior in leukemia as induced inhibition of PRMT5 activity could significantly alter the dynamics of this network. Thus, PRMT5 inhibition (via mRNA depletion or single molecule agents) offers a novel mean to hinder leukemic growth by releasing the block of miR-29b expression and consequently downregulating SP1 and FLT3 levels in the cells which in turn directly translates into a slower cell proliferation rate as well as induction of apoptosis and differentiation. These observations coupled with results supporting the apparent
antileukemic activities of C12 in AML cell lines and patient blasts offers a new class of promising epigenetic therapeutic approaches to treat hematologic malignancies.

AML in fact comprises the leading cause of death due to leukemias since most adults (>60 years) with AML are not cured with standard therapies. Search for novel therapies is accelerated by studies underlying the significance of understanding and targeting epigenetic mechanisms in charge of controlling key cellular functions. Our primary analysis here has revealed a highly complex network of genetic and epigenetic elements that function to regulate PRMT5 expression and activity in leukemia cells. Further studies are exceedingly required to identify the genes and transcription factors that mediate the expression and enzymatic functions of PRMT5. As described in this work, miR-96 can potently target and suppress PRMT5 protein levels.

In addition protein binding partners of PRMT5 play a defining role in directing PRMT5 to the chromatin where scaffolding or methyltransferase activities of the protein becomes crucial in determining the expression patterns of the target genes. Among the important corepressors and transcription factors which physically associate with PRMT5 in leukemic cells are HDAC2, DNMT3A and p65 subunit of NFκB. These interactions while point to a multilayer and complex mechanism of actions which affect PRMT5 function in leukemic cells, offers opportunities for combination epigenetic therapies in lieu of aggressive cytotoxic chemotherapy of which multiple success examples are present. Provided by selective agents to target the activities of each chromatin modifier it is possible to explore the combination of these agents in low doses with
potential synergistic or additive effects in altering epigenetic processes rather than inducing toxicity in leukemic cells. Together, these findings for the first time describe a novel role for PRMT5 as a participant of leukemia growth and demonstrate preclinical antileukemic activities for a novel selective inhibitor of PRMT5 in acute leukemia. Future efforts to optimize and evaluate the potency and safety of C12 application in vivo is required to translate and extrapolate these antileukemic effects into clinical trials which may benefit high-risk AML patients with poor prognosis. The insight from these novel findings is especially valuable by providing a platform to advance epigenetic therapeutics especially in the less-studied protein arginine methylation processes with the ultimate goal of improving the care regimens for AML patient in urgent need of personalized and more effective treatments.

Another novel field of research is commenced by the proposed involvement of PRMT5 in promoting pluripotency and reprogramming of somatic cells into pluripotent stem cells. This is especially promising regarding cancer (and leukemia), as failure to eliminate all cancer cells can be attributed to chemoresistance. Cancer stem cells (CSCs) which escape cytotoxic chemotherapy encompass the primary sites of recurrence and induction failure. Markedly, CSCs exhibit characteristics similar to induced pluripotent stem cells and embryonic stem cells including self-renewal, maintaining pluripotency and quiescence (i.e. immortal cell growth). The development of therapeutic approaches that target quiescent CSCs might therefore be expected to have a profound impact on cancer eradication. Multiple studies suggest that constitutively active FLT3 is involved in reinitiating malignancy via functioning within leukemia initiating
cells (LICs)\textsuperscript{171–177}. We uncovered that PRMT5 is involved in upregulating FLT3 pathway by interacting with key regulators of FLT3 (i.e. SP1 and NFkB(p65)) and consequently modulating the activity of this oncogenic tyrosine kinase. Furthermore, recent studies suggesting a potential role for PRMT5 in promoting pluripotency and reprogramming of somatic cells highlight the requirement for investigating the possible relationship between PRMT5 and FLT3 in blocking differentiation and inducing stem-like properties (i.e. quiescence and pluripotency) in leukemic cells.

Finally, given the immense impact of PRMT5-induced methylation events on gene expression and cell transformation, valuable insight will be gained from comparing the global arginine methylation patterns in normal vs. malignant hematopoietic cells. These efforts particularly may facilitate the identification of more PRMT5 targets and the true depth of effects mediated by these epigenetic modifications on reprogramming and malignant transformation of leukemic cells.
References


137. Safe, S., Imanirad, P., Sreevalsan, S., Nair, V. & Jutooru, I. Transcription factor SP1, also known as specificity protein 1 as a therapeutic target. 11, 1–11 (2014).


149. Chen, H., Tini, M. & Evans, R. M. HATs on and beyond chromatin. 218–224


Appendix: Promoter and ChIP primer sequences

Here the sequences for predicted promoter regions of FLT3, miR-29b and miR-96 are provided respectively. Primers to amplify predicted transcription factor binding sites (~200bp amplification product) are listed.

Following web-based search tools were used to:

Predict transcription start sites (TSS):  [http://www.cbs.dtu.dk/services/Promoter/](http://www.cbs.dtu.dk/services/Promoter/)

Predict transcription binding sites:  [http://www.cbrc.jp/research/db/TFSEARCH.html](http://www.cbrc.jp/research/db/TFSEARCH.html)

FLT3

GeneBank aligned region spanning 4252 to 5337 on NG_007066

SP1 binding site from TSS: -266

**Red**: predicted SP1 binding site

↗: transcription start site

**Underlined**: ChIP PCR forward and reverse primer binding

FLT3-ChIP-F: CAGAGTTCGGGGACTCACAG

FLT3-ChIP-R: GAGAGGTTGGGCAGAGCCGAG

Promoter: CTTATTGCAAGAAGAAATAAATAAACTTTTCAACTCGAAAAATTATGC

GATGAAGAACAAGAACATATTGAGCGGATGATTCGCAACATCCTCTGGGATGT

ATTTTCTGTGTGTTTTTGGTTTTGAAGGCTGGTTTTGTTAAACCTCCTTAATTGCCTTG

GTTGACATTGAAAAACCCTGCCTTCTTTTTCTTGATCCCCGCGCTGACTGGGTCTCT

GAGCATTCTAGGAGATGGAGGCAGAGACAAAGGAAATCAGTCACAATTAA

GAATGGAATGGCTTTATGGAATGCTCGCCTGTGCGCTGGCGGGAGTTGCC

AGCGTGCCGAGCGGATTCAGCGCCTTTCTCAGGGCCTCAAAGATCCCAGCG

ACTAGGAGGTTGGTTTGTGGCTCCCTGCAAGTCCCCACGCCCCGGGATCCAAAC

GACAGAGTTCGGGGACTCACAGGGGAGCAAGGCGGAGAGCCCGCAGCCGAGCC

AGTGCAACTTTTCCTCGGCGGGGAGCCCGCGCAGTCCGGG

AGCCCCGGGGCCGCGAGACACAGGTGCTCGGGAACCAGGCGGGGACGCTGGGC

TCGGCTGCAGGCAGCGCCAGGCACCAGCGCTGCTGGCTCTGGCCAAACCTCTCC

GCTCCCGCCTCGGCTCCCTGCTGCTCGGAGGGGTTCTCCTCCCCCTCTTCACCT
TGCACCAGTCCGAGGGAATTTCGGGTGACGCATCTTAAAGAGAGCC
ACCTGCAGCGCGAGGCAGCCGGCGCT
↗

CCAGGCGCCATCGCAAGCTGGGCCGC
CGCGCGCCCTGGGGACCCCGGCTCCGGAGGCCATGCCGGCGTTGGCGCGGA
CGGCGGCCAGCTGCCCTGCTGCTGGTAAAGGCCCCTGCTGCTGCTGCTGCAAGCCC
CTCGCGGTCCCTCAGCCCCACCCCGCAGTGGACCCCGGCGCGCGCGCCCTCC
TCCGGCCAGCGCTCGCTCCTCCCCCGCCTCGCTCCTCTTTCCATTGCCTCCCCG
CCGCCCTCCCCTCTTTCCGGCGCCCTCAGGACTCGCCCACTACTTGCCTGCGGCC
CCGGCGCTTTCCACCCCGGCTCTTCCCTTCTCTCTCCCTCGAAGTCCTCTCC
miR-29b

GeneBank aligned region spanning 32566 to 34078 on EU154353

↗: Predicted TSS: 35100

Underlined: ChIP PCR forward and reverse primer binding:

miR-29b-ChIP-F: TCCTGAGTAGCTGGGATTGC
miR-29b-ChIP-R: CATGCTGTAATGAGGTCTGA

Promoter: TCTCCCCAAGGACATAAAGCAATGTGGGAAGATATACAAATGAC
CTTGTCAACTGGGCTAATATGAAGAGGCCACAGATGCAAAAATAATGTATCA
TACCTTTATTCTTCTGATTTTTTTTTTTTTTTTTTTTTTTCGGAGACAGTCTC
CTTCTTCAGGGCTGGAATCCAGTGGCGCGATCTTTGGCTACTGCAACCTCG
CCACCCGGGTTCCAGGAAATCTCTCGGCTGCCCCTCCCCGCCCAAGTACTTTTTT
GTATTATTTAGTAGAGGAGAGGGGTTCACCTTCTTGGCCAGGCTAGTCTC
AACTCTGCCACCTCGTGATACACCTCGGCTTGGCCCTCCAAAGTGCTGGGATTAC
AGGCGTGAGCACCAGTGGGCTGGCCTCTCTTTGTGGGCCATGGGCTAGTCTTTC
ATGGAGTCTGGCTGTATTGCGGGAGGCTGGGAATGGCATGTACTTGCTGTC
ACTGCAACCTCTCGCCTCCCCAGGTCCAAGTGATTCTCTGGCCCTGCAGCATCGCT
GTAAGCTGGGGAATTGCAGGCAATCCATCCCGGCTAAATTAAAAAAGGAGG
CTTGTTCCTGCACCCAGGGCTGGGAATGGGCACGATCTCAGCTCAGTGCA
ACCTCCGCTACTGGGCTCAAGGAACTCTCTCGTCCGCTGCTCAGCTC
CAGGCATGTGCCACCCAGCGCTGAGTCTGGTGTTTTTTTTTTTTTTTTTTTTTTT
AGTAGATATGGGGTTTCACCCATGTTGGCCAGGATTGGTCTCAAAACTTTCTGACCT
GAAAAACCTATAAAAAACCTTTTGTTTGGAGAAAGTGGGAGAAGTTGGATGT
AGGCTGGGATTTTGTGGGTAACCAAGGAATTGTTTTTTCTTTAGGTATGGTAA
GATTTTGTTGTTATGTGAAAGAGTGTCTTCTTTTTATAGAAGCACCTCAAAGT
AGTTAGGAGCAGTGTATGTATGGGTTGTTATCAATTACTTTCAAAAAAATGC
GCAAAAAATATGATCTATATTACATATAATTAAAGCAAACATGATAAAAAA
GGAATAGTTATTGAATTTAGATAGTATGGGATTAGGAATTCATTGTATTTGTC
TTCCAATTAAATGTATAAAAAAATTCTATAACAGTTTTATAAAAACAGTCACAC
CCCTTTAAATTAGGTTGGCCTGGGTACATGAAGTATCTTTTTGGATGCTAGAAC
ACAAAAAGGGACGACAACAGGCCACCTAAAATAAGATGATAACAAAGAATATA
CCATGAGGCAGAAAAAGACTGCTGCAAAAAATTATTCCAAAGGAACACT
CAGAGTACAAAAAGCCTTACCTTGGAGATAGGAAGGAGAGAGTTAAGTAA
GCTCACAACAC
AGCGACATTAGCATTCTCATTAGTGCATTATTATGTGACTTTACTTGCCCTGAC
TAGTCTATTCTCTCCTAGACCCCCAATTCTCAGTAATCCTTTGGATGCAAAG
TTATCTCAAAGTGAATAAATAGTTTAGGAAGACGTTTTCTTTCAAACCTCTA
TGGAGCAGTTTGCTTTGTTCTATTTGCTATGTCGACCGAGCTGTTCTGTCAG
CACCAGACGCTAGTGCTCTCCGCTGGCCTGGGTACTTGATACACAGGATGCGCTC
TGACTTCTCTGCTTCTTACCAAGCAAGGAGATTTTTCTTTGCTTCTCCACCAAAAA
GAGTGACGGGGTGACATGTGGCCTTGCTCTCATAATGATGAGGCTGACACCTT
TGTCGAGGGCAGCTTACATTAGAATAGGAAGGAGTGTCACCAGGATGCTCTCCCAT
CAATAACAAATTCAGTGACAT
miR-96

GeneBank aligned region spanning 3572 to 5293 on NG_023385

Underlined: ChIP PCR forward and reverse primer binding:

miR-96-ChIP-F: CTTCCAGGATATTTTCTAGCCAC
miR-96-ChIP-R: GAGGCTCAGGAGCGAGGAG

Region 1

CCCACCCAAGGCTGGGAAGTCTGGTCTCTTCCTTCTAGTAGAGGATTCAGAAAAT
TGTAGTAAGGGAAACTGAGGAGGCTAGGAGGAGTGAACCTTCTGAGATCAC
CACCTCATCAAGCTGGATTGTCTCTCTGGGGAGATAGACCAGCTAGAAGGGCA
GAGCCCTGTAGTGGGGTGTGTGAGGAGCAGGACAAAGAGGTCTCCCGGGAGCA
GGAAGGCTTGGAGCATTAAGCAGTGCTAGGGGAGAGCGGAGGATGGCTTTGGCC
AAGGTACCCAGATGGAGCTCCTGAAGCTCCACGGGAGGTTAGTCTCTGGGGGTAA
CATCGCCCAAGAGGTCTGCAATGCCTTCTGGGCACAGGCACCTGTCTCTGACCCCTT
CCAGGATATTATTCTGACCCACCTGCTTCTTCTGGGCCACCGCTGAGCTGGAGTAG
GCTGAGACTCTTAGTGCCACTCTGCGGAGGCCCCACGTTTGCTGCCCTGGGT
GCCAGCTCCCCAGAGACCCTCCTCCCACCTCCTTCTTCTCCGTCCTTCCC
CAGGGGCTGTGACACACAGAGGCCAGGGCCTCCACAGCAACTTCTCTGAGCA
GCTGCTTCTGGCAACACCACCGATGCAACCTTCCACGCCCCCTCCCTCCTGCTCCTGAG
CCTCCGCTTTCTCCCGAGTACACTCCAGACGCGCCAGGGCCAGCCACTCCCTTGC
TAGAGTGCATCCGGCTGCTCCGGCTCCCTCCAAGGCCACCTGGGTTTGCTGACAGG
ACTTCTCTTTCTCTCCCTGGCTTTCTTCTGCCCTTGATCCTAGGCCGAC
AGCAGAGATGCCCAGCCTGGGCGGTTCACAGTCTGGCCCAATCTGGTCTGGTTTGG
GATGGGAGTGGGGGTGAGCAGCAGATTCGGTTTGTTCCTGGGGCTCTGTTTCTGCCACAGGGCAGGCTGGGGGGTGGAGGATAATGGAGACCAAAGTGCC
TAGGAGCCTGGGCTGCTGGTGTCTGGGCTCAGAGGCTACGAGAGGCATCTTGGATGTCCCACTGGTCACTGCCCCTGTGGCAGGTTGGGTAGAGAGGAGGCTCTGGCCAGCTGCTTGCCTCTCCGAGCCAGAGTTACTCTGGCAAGGAGATGGATGGTCCTGACCCACTCCCTCCCCAGCCTGGAGGCGCAGTCTGGGTGATGTGGAGGGATGTGGGCCTTCAGGTGGAGATAGGAGACACCTTGGTGTGGTCTTCTCCTC
TGCAAGGCCAGAAGGCAGTCAGCTCCTCTCCCGCACTCTGCTCCCTCTTGAAGG
TCATCTTTGGGCTGATGGGGCAGTGGATCTTTGTGAAGAGGATGGGACATCGTGGGCCGCTGGTCT
GGGGGTAGAGACCTGCAGGCTAGGGCTCGTGCAGGGTCGGCAGGCCGCAGAGTGTGACTCCTGTTCTGTATGGCACTGGTAGAATTTCACTGTGAACATGTCTCAGTCAGTCAGTAATTACCAGAGGGCCATAAACAGAGCAGAGACAGATCCACGAGGGCCTCCGGAGCACCTTACCCACTTCTCGGCTAGACGTGGAAACAGGCTGCTTCCAAGGG
TGCAAGGCCCCTCGTCCAGTGTGTCCCCAGAGAGCCCGCACCAGTGCCATCTGTGGCCGATTTTGGCACTAGCACATTTTTGC
ATCATGTGCAGTGCCAATATGGGAAA

→Highlighted in Orange: stem loop sequence and Purple: mature miR-96 sequence
miR-96 promoter further upstream of stem-loop region:

GeneBank aligned region spanning 73018 to 73995 on AC084864

**Underlined**: ChIP PCR forward and reverse primer binding:

miR-96-ChIP-F: GATTGTCATGGAGCTGGGAAG

miR-96-ChIP-R: CACGCTGCCGGAGCTCGTG

Region 2:

```
GGGGTTTATCTAGTTATGCCATGGGGGCAAGGTAGCAGAGGAACCTCACCA
TTCGGAGAGGGGCAATTGGCAGAACCACGGCCCATCAGTGTTGCTGCTGAG
GGGGCTTGTATCCCGGGCACCTTTCCCCCTAGTCTCTGCGAGCGAGGAATGG
GAAGGGAGGGGAGCCAGATGTTACTTCTTTGGCAAGGTCTTCTAGCAGCA
GGGGGACTTGCTGAGGTCAAGGGTGGGTTCTTTGCCCAAGGTTACTGCA
AGAGGGCAAGCCAGAGGACAAGGCGAGACACCATTAAAAAGGACGGAGCG
CAAGGCCAGCTGCTTCTAGGTTGGGCCCCAGAAGGAGACAGCGGGTCTTTTC
CTAAACCTGGCAAGCGTCCAGAACCAACTACCCCTCCCCGGGCGCAGC
GACTCCGGGCAGCGTGGGACGCTGACACACTGTCTGATCTCTCCAGGC
GAGAGGAATGGGGCAATTGGCAGACGGACATTGGGATCTCTGAGAAGGGG
GGTAGGATACACGCGGCTTGGAGTGCAGCTGGACAGCCAGCAAAGGTGAGG
ACCCAGGGTCTCCTGAGGCACACTCTTCTCTCCCAGCTGAGGCTGAGCTAAAG
GGATCCAGGATCATCGTCCCCGGGGGAGGCGCCCCCTCACCCGAGTCCAG
```
CTCCGGTTACCTGCTGGCCTGCTCTTTAAAATATGTATGAGGCCCTTGGG
TGCAGGTTTCGCGGCCCCTCCCGGGAGTGGGGCTGGGGGACTCAGGGTGCAGGG
GGAGCTGAGGGGGAGGAAGCGACGCAGCTCCCCGGCCCGGGGCTTCTCG
ACTTTCAAGCAGCAGCATTTAAAGAAAAAGGATGGAAATCGGATCTGTCTCGCGCCG
GCCGCAGCCCCCAGGGCAGCTGGATGGACCCCGGACCTCAGCGGCCCAGCGGGCTAGCT
TTCTGCTGCGCGGTTGTCGCTTCTCGACAGGTTGGAATGGGCCA