Novel Biological Insights and Therapeutic Approaches in High-Risk 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
Houda Alachkar, PharmD
Graduate Program in Integrated Biomedical Sciences
The Ohio State University
2012

Dissertation Committee:
Guido Marcucci, MD Advisor
Michael Caligiuri, MD
Danillo Perrotti, PhD
Lai-Chu Wu, PhD
Abstract

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy characterized by clonal proliferation of myeloid progenitors with a reduced ability to differentiate into more mature functional blood cells. Consequently, an accumulation of leukemic cells (blasts) in bone marrow and blood occurs and leads to hematopoietic failure. Even with recent progress in understanding the biologic and genetic changes that underlie the disease, most patients with AML fail to achieve long-term survival. This highlights the need for novel therapeutic strategies that would improve outcome. Cytogenetic aberrations have long been shown to be the most important risk factor for predicting outcome in AML and have therefore been used to guide treatment. However, each cytogenetic risk group presents with molecular heterogeneity, which may explain different outcome among patients with similar chromosome aberrations. Cytogenetically normal AML (CN-AML) is the largest cytogenetic group (45% of AML) and the best molecularly characterized among both younger and older AML patients. Frequent genetic mutations with prognostic significance have been identified in CN-AML. Patients with FLT3 internal tandem duplication (ITD) or IDH2-R172 have been shown to have worse outcome, while patients with CEBPA or NPM1 mutations are commonly reported to have more favorable prognosis. The cytogenetic and molecular aberrations associated with AML influence the expression of downstream target genes that encode proteins involved in complex biologic networks supporting leukemogenesis. Therefore, microarray genome-wide gene-expression profiling and microRNA-expression profiling assays provide molecular insight into the underlying biology of the different disease
subsets, offer diagnostic and prognostic information, and potentially reveal novel therapeutic targets.

The research we are presenting in the following chapters has two major aims. Aim 1: identify novel therapeutic targets and prognostic markers employing genome-wide analyses of gene expression signatures in high risk AML patients. Aim 2: investigate novel therapeutic approaches directed toward previously identified molecular targets in high risk disease. In chapter #2 we identified SPARC as a novel molecular and therapeutic target in AML. We demonstrated a functional, mechanistic and clinical implication of SPARC in AML, revealing that high SPARC expression promoted growth advantage in vitro, aggressive disease in vivo, and worse outcome in CN-AML patients. SPARC acts likely via activation of ILK/AKT/β-catenin pathways. We also identified RUNX1 and IDH2-R172 mutations as an upstream upregulators of SPARC expression likely via NFκb/SP1 complex mediating mechanisms. In chapter #3 we investigated the preclinical and pharmacological activity of the natural initiation of translation inhibitor, silvestrol in FLT3-ITD positive AML denoting a large proportion of high risk AML patients. We demonstrated that silvestrol had a potent in vitro and in vivo anti-leukemia activity in FLT3-ITD and FLT3 overexpressing AML cells. Silvestrol inhibited FLT3 mRNA translation resulting in FLT3 protein down-regulation and in turn inhibition of aberrant tyrosine kinase activity.

In conclusion, we identified an original molecular target with novel biological insights, and investigated innovative therapeutic approaches suitable for high risk subsets of AML. This research will significantly advance understanding of AML and open new avenues of treatment strategies, which will result in optimized patient care, and improved clinical outcome.
This thesis is dedicated to

The great people of Syria who are dying for their freedom,
    you were my inspiration

My loving Mother who devoted her life for our education,
    you were always in my heart

My amazing family
    father
    sisters and brothers
    Bassem, Wafa, Hana, Nada, Amal, Malack, Mustafa, and Murhaf
    You were always with me

My beloved husband
    Irshad
    You were my best friend and companion
Acknowledgments

I am above all deeply grateful to my advisor, Dr. Guido Marcucci, for his support, mentorship, guidance, patience, and encouragement. It is because of his instructions and guidance that I have been able to complete this work.

I would like to thank my committee members, Drs. Michael Caligiuri, Danillo Perrotti and Lai-Chu Wu, for offering insightful comments and kind advice regarding my research and my progress. I would like to acknowledge Dr. Bloomfield for her overall support for this project, as without her help and support much of this work would not have been possible. I would like to thank Dr. Wolfgang Sadee for offering me a research opportunity in his lab. It was his directions and advices that guided me through setting up my professional goals to further my career as a research scientist.

I would like to acknowledge the current and former members of Dr. Marcucci’s lab, specifically, John Curfman for trying his best maintaining all aspects of the lab and keeping everyone happy. I thank Chris Hickey for being a good listener and for his great help with the ChIP experiments. I greatly appreciate Ramasamy Santhanam for his assistance with in vivo experiments and Yue-Zhong Wu for his technical help with cloning and for very interesting political discussions.

I also like to thank all my friends for their help and support particularly Sana and Barraha for being always with me.
Vita

2001 .................................................. B.S. Pharmacy, Aleppo University, Syria

2005 .................................................. M.S. Pharmacology&Toxicology, Wright
State University, OH, US

2011 .................................................. PharmD. Ohio Northern University, OH, US

2008 to present ........................................ Graduate Research Associate, Integrated
Biomedical Graduate Program, The Ohio
State University, OH, US

Publications


3-  Smith RM, Alachkar H, Papp AC, Wang D, Mash DC, Wang JC, Bierut LJ,


5- Frasch HF, Barbero AM, Alachkar H, McDougal JN. Skin penetration and lag times of neat and aqueous diethyl phthalate, 1,2-dichloroethane and naphthalene. Cutan Ocul Toxicol. 2007;26(2):147-60. PMID:17612981

Fields of Study

Major Field: Integrated Biomedical Science Program

Area of Research Emphasis: Molecular Pharmacology, Pharmacogenomics and Therapeutics
# Table of Contents

Chapter 1: Background

1.1 Definition, etiology, diagnosis and classification .............................................. 1

1.2 Clinical Presentation .............................................................................................. 5

1.3 Risk Stratification and prognosis ........................................................................... 5

1.2 AML Treatment ...................................................................................................... 10

1.4 Molecular signatures in AML ............................................................................... 12

Chapter 2: SPARC Contributes to Leukemia Growth and Aggressive Disease in Acute Myeloid Leukemia ........................................................................................................ 15

2.1 Introduction ........................................................................................................... 15

2.1.1 SPARC gene and expression ........................................................................... 15

2.1.2 SPARC protein structure and functions ......................................................... 15

2.1.3 SPARC signaling pathways ............................................................................ 18

2.1.4 SPARC and Cancer ......................................................................................... 20

2.2 Experimental procedure ....................................................................................... 22

2.3 Results ................................................................................................................... 28

2.3.1 SPARC is expressed in hematopoietic stem cells (HSCs) .............................. 28

2.3.2 SPARC promotes leukemia growth .................................................................. 30

2.3.3 SPARC overexpression negatively impacts clinical outcome in CN-AML ... 37
2.3.4 SPARC overexpression is associated with distinct gene and microRNA expression signatures in CN-AML ..........................................................41

2.3.5 SPARC activates ILK/AKT/β-catenin signaling pathways .........................46

2.3.6 Distinct mutations in IDH2 and RUNX1 genes induce SPARC expression likely via activation of NF-κB/SP1 complex in CN-AML.................................61

2.3.7 SPARC is a novel therapeutic target in AML .........................................70

2.4 Discussion ..................................................................................................73

Chapter 3 : Pharmacological Activity of Silvestrol in High-Risk Acute Myeloid Leukemia .........................................................................................77

3.1 Introduction ..............................................................................................77

3.2 Materials and Methods ...........................................................................80

3.3 Results .......................................................................................................85

3.3.1 Silvestrol antileukemia activity in FLT3-ITD and FLT3-wt expressing AML cells ..............................................................85

3.3.2 Silvestrol downregulates FLT3 expression through inhibition of FLT3 translation initiation ..................................88

3.3.3 Silvestrol downregulates miR-155 expression in FLT3-ITD positive AML ...94

3.3.4 Activity of silvestrol in FLT3-ITD positive leukemia grafts ..................98

3.4 Discussion ..................................................................................................101

Chapter 4 : Summary and Future Directions ..................................................104

References ......................................................................................................114
List of Tables

Table 1.1 FAB classification of Acute Myeloid Leukemia .................................................. 4
Table 1.2 WHO classification of Acute Myeloid Leukemia .............................................. 4
Table 1.3 European LeukemiaNet Classification System for Correlation of Cytogenetic and Molecular Genetic Data in AML with Clinical Data .................................................. 8
Table 1.4 Recurrent Molecular Genetic Abnormalities in Adult AML and their Clinical Significance .......................................................................................................................... 9
Table 1.5 New agents directed at molecular and other specific targets in AML..............11
Table 2.1 Comparison of clinical and molecular characteristics of Low vs. High SPARC all patients ..........................................................................................................................38
Table 2.2 miR expression profile associated with SPARC expression in older patients .44
Table 2.3 miR expression profile associated with SPARC expression in younger patients ..........................................................................................................................45
Table 2.4 List of patient samples used in in vitro experiments .......................................60
Table 3.1 Calculated IC$_{50}$ values for AML primary cells and cell lines ......................88
List of Figures

Figure 1.1 The molecular heterogeneity of cytogenetically normal AML (modified from (21))........................................................... 6

Figure 2.1 A schematic of SPARC protein structure and its multi-functional domains (88)........................................................................................................17

Figure 2.2 SPARC is expressed in hematopoietic stem cells/progenitors. ....................29

Figure 2.3 SPARC promotes growth advantage in AML cell lines. ................................31

Figure 2.4 SPARC promotes growth advantage in primary blasts..............................33

Figure 2.5 SPARC promotes aggressive disease in vivo. ...........................................35

Figure 2.6 SPARC promotes aggressive disease in vivo. ...........................................36

Figure 2.7 SPARC overexpression negatively impacts clinical outcome in younger CN-AML patients. ..........................................................................................................................40

Figure 2.8 SPARC overexpression is associated with distinct gene expression signature in CN-AML........................................................................................................................................42

Figure 2.9 SPARC overexpression is associated with distinct microRNA expression signature in CN-AML..................................................................................................................43

Figure 2.10 SPARC activates ILK/AKT/β-catenin signaling pathways in THP-1 cells.....48

Figure 2.11 SPARC enhances β-catenin nuclear translocation.....................................50

Figure 2.12 SPARC activates TCF/LEF transcriptional activity and increase the expression of β-catenin target genes..............................................................52

Figure 2.13 SPARC activates ILK/AKT/β-catenin signaling pathways in MV4-11 cells...53

Figure 2.14 SPARC promotes growth advantage via ILK activation..........................55
Figure 2.15 SPARC activates ILK/AKT/β-catenin signaling pathways in primary blasts. 57
Figure 2.16 SPARC knock-down decreases β-catenin protein level in primary blasts...58
Figure 2.17 SPARC increases P-β-catenin(Ser675) in primary blasts.................................59
Figure 2.18 SPARC expression in CN-AML patients according to their IDH1 and IDH2 mutational status. ........................................................................................................62
Figure 2.19 SPARC expression in CN-AML patients according to their RUNX1 mutational status. ........................................................................................................64
Figure 2.20 Mutations in IDH2 and RUNX1 genes induce SPARC expression. ..........65
Figure 2.21 SPARC expression is regulated by NF-κB, SP1, and RUNX1 transcription factors. ........................................................................................................67
Figure 2.22 IDH2-R172 and RUNX1 contribute individually to SPARC overexpression through NF-κB and SP1 mediating mechanisms. ........................................69
Figure 2.23 Bortezomib inhibits SPARC expression. .....................................................72
Figure 3.1 Antileukemic activity of silvestrol in vitro. .....................................................86
Figure 3.2 Silvestrol induces apoptosis in primary blasts in vitro.................................87
Figure 3.3 Silvestrol down-regulates FLT3 protein expression.................................91
Figure 3.4 Silvestrol affects FLT3 mRNA expression differently in FLT3-wt and FLT3-ITD AML cells..............................................................92
Figure 3.5 Silvestrol down-regulates FLT3 mRNA expression in THP-1 cells in the presence of FLT3 ligand.................................................................93
Figure 3.6 Silvestrol down-regulates miR-155 and upregulates miR-155 target PU1.....96
Figure 3.7 In vivo antileukemic activity of silvestrol in FLT3-ITD positive MV4-11 xenograft leukemia mouse model.........................................................100
Figure 4.1 SPARC upregulates c-Myc and CyclinD1 mRNA expression in CD34+ cells isolated from cord blood cells. .................................................................108

Figure 4.2 In vitro and In vivo antileukemic activity of silvestrol in KIT mutant AML. ....112
Chapter 1: Background

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is one of the most common and deadly form of acute leukemia among adults in the United States (1). According to Surveillance Epidemiology and End Results (SEER), based on cases between 2005 and 2009 the age-adjusted incidence rate and the age-adjusted death rate were 3.6 and 2.8 per 100,000 men and women per year, respectively (2). It is estimated that approximately 13,780 new case of AML will be diagnosed, and 10,200 patients will die of the disease in 2012 (3). The incidence of AML is rising as the population ages. The median age at diagnosis is 67 years, with 54% of patients diagnosed at 65 years or older and at least 33% at 75 years of age (1).

1.1 Definition, etiology, diagnosis and classification

AML is a heterogeneous hematologic malignancy characterized by clonal proliferation and block of differentiation of myeloid precursors, the so-called blasts. Consequently, an accumulation of leukemic cells (blasts) in bone marrow (BM) and
blood occurs and leads to hematopoietic failure (4). If left untreated, patients with AML die of infection, bleeding and organ failure within few weeks.

Although the etiology of AML is still mostly unknown, the risks for developing the disease have been attributed to several environmental and hereditary factors. Prolonged exposure to petrochemicals; solvents such as benzene, petroleum, pesticides, herbicides and tobacco smoking were found to increase risk for developing AML(5, 6). Ionizing radiation from warfare, occupational, or therapeutic exposure was also found to increase disease incidence (1, 7). Additionally, up to 10% of patients receiving cytotoxic therapy for solid tumors or hematologic malignancies such as alkylating agents and topoisomerase II inhibitors develop AML within one to six years after exposure. Recent studies suggest that therapy-related AML may account for 5% to 20% of patients with AML (8-10). Heredity related risk factors for developing AML include constitutional chromosome aneuploidies such as +21 in Down and XXY in Klinefelter syndromes, and inherited diseases with excessive chromatin fragility, e.g., Bloom syndrome, ataxia telangiectasia neurofibromatosis and Schwachman-Diamond syndrome, Faconi anemia and Kostmann syndrome (11). Additional hereditary risk factors are germline mutations of the CEBPA and RUNX1 genes (12).

According to the World Health Organization (WHO) classification, the term “myeloid” includes cells of granulocytic (neutrophil, eosinophil, basophil), monocytic/macrophage, erythroid, megakaryocytic and mast cell lineages (13). In this system, a myeloid neoplasm with 20% or more blasts in the PB or BM is considered to be AML (13). However, in some cases associated with specific cytogenetic aberrations include t(8;21)(q22;q22); inv(16) (p13;1q22) or t(16;16)(p13.1;q22); and t(15;17)
(q22;q12) [APL subtype], the diagnosis of AML may be made regardless of the 20% cut off blast count in the PB or BM. Therefore, the 20% blast threshold is not required to treat the patient as having AML (13).

Historically, AML was classified according to the French-American-British (FAB) system based on morphologic and cytochemical criteria (Table 1.1) (14). More recently, the WHO system based more on cytogenetic and molecular markers with biological and clinical impact has been adopted (Table 1.2). Previously, abnormalities included in the classification were mainly chromosomal translocations involving transcription factors and associated with characteristic clinical, morphologic and immunophenotypic features. However, experimental evidences leading to advance understanding of leukemogenesis suggest that myeloid differentiation resulting from chromosomal rearrangements involving transcription factors require a second genetic abnormality to promote proliferation of the leukemic clone (15). Incorporating this rationale, at least two classes of mutations in AML pathogenesis are recognized. Class I mutations involve mutations that encode proteins that activate signal transduction pathways to promote proliferation and/or survival advantage. Class II mutations involve mutations that impair hematopoietic differentiation and subsequent apoptosis (15). Since the WHO system has been frequently revised to include the most recent discoveries and updates in the field; it provides more meaningful prognostic information than the FAB system and therefore is considered the more clinically useful classification system (16).
Table 1.1 FAB classification of Acute Myeloid Leukemia

<table>
<thead>
<tr>
<th>FAB subtype</th>
<th>Name</th>
<th>% of adult AML patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Undifferentiated acute myeloblastic leukemia</td>
<td>5%</td>
</tr>
<tr>
<td>M1</td>
<td>Acute myeloblastic leukemia with minimal maturation</td>
<td>15%</td>
</tr>
<tr>
<td>M2</td>
<td>Acute myeloblastic leukemia with maturation</td>
<td>25%</td>
</tr>
<tr>
<td>M3</td>
<td>Acute promyelocytic leukemia (APL)</td>
<td>10%</td>
</tr>
<tr>
<td>M4</td>
<td>Acute myelomonocytic leukemia</td>
<td>20%</td>
</tr>
<tr>
<td>M4 eos</td>
<td>Acute myelomonocytic leukemia with eosinophilia</td>
<td>5%</td>
</tr>
<tr>
<td>M5</td>
<td>Acute monocytic leukemia</td>
<td>10%</td>
</tr>
<tr>
<td>M6</td>
<td>Acute erythroid leukemia</td>
<td>5%</td>
</tr>
<tr>
<td>M7</td>
<td>Acute megakaryoblastic leukemia</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 1.2 WHO classification of Acute Myeloid Leukemia

<table>
<thead>
<tr>
<th>Acute myeloid leukemia and related neoplasms</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute myeloid leukemia with recurrent genetic abnormalities</td>
<td></td>
</tr>
<tr>
<td>AML with t(8;21)(q22;q22); RUNX1-RUNXI T1</td>
<td></td>
</tr>
<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
<td></td>
</tr>
<tr>
<td>APL with t(15;17)(q22;q12); PML-RARA</td>
<td></td>
</tr>
<tr>
<td>AML with t(9;11)(p22;q23); MLLT3-MLL</td>
<td></td>
</tr>
<tr>
<td>AML with t(6;9)(p23;q34); DEK-NUP214</td>
<td></td>
</tr>
<tr>
<td>AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1</td>
<td></td>
</tr>
<tr>
<td>AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1</td>
<td></td>
</tr>
<tr>
<td>Provisional entity: AML with mutated NPM1</td>
<td></td>
</tr>
<tr>
<td>Provisional entity: AML with mutated CEBPA</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia with myelodysplasia-related changes</td>
<td></td>
</tr>
<tr>
<td>Therapy-related myeloid neoplasms</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia, not otherwise specified</td>
<td></td>
</tr>
<tr>
<td>AML with minimal differentiation</td>
<td></td>
</tr>
<tr>
<td>AML without maturation</td>
<td></td>
</tr>
<tr>
<td>AML with maturation</td>
<td></td>
</tr>
<tr>
<td>Acute myelomonocytic leukemia</td>
<td></td>
</tr>
<tr>
<td>Acute monoblastic/monocytic leukemia</td>
<td></td>
</tr>
<tr>
<td>Acute erythroid leukemia</td>
<td></td>
</tr>
<tr>
<td>Pure erythroid leukemia</td>
<td></td>
</tr>
<tr>
<td>Erythroleukemia, erythroid/myeloid</td>
<td></td>
</tr>
<tr>
<td>Acute megakaryoblastic leukemia</td>
<td></td>
</tr>
<tr>
<td>Acute basophilic leukemia</td>
<td></td>
</tr>
<tr>
<td>Acute panmyelosis with myelofibrosis</td>
<td></td>
</tr>
</tbody>
</table>
1.2 Clinical Presentation

Nearly 50% of patients report fatigue, weakness, anorexia and weight loss at the time of diagnosis. Other signs and symptoms at diagnosis include: fever with or without an identifiable infection, and abnormal hemostasis (bleeding and bruising) due to neutopenia and thrombocytopenia, respectively. Occasionally, patients complain of bone pain, cough, headache and diaphoresis. Symptomatic mass lesions in soft tissues, cranial or spinal dura may rarely be present at the time of diagnosis (1, 7).

1.3 Risk Stratification and prognosis

Cytogenetic aberrations have long been the most important prognostic factor for predicting remission rates, relapse risks, and overall survival (OS) outcomes (1). AML associated with t(8;21), t(15;17) or inv(16) predicted a relatively favorable outcome (5-year survival rates are 55-65%). Whereas in patients lacking these favorable changes, the presence of a complex karyotype, -5, del(5q), -7, or abnormalities of 3q defined a group with relatively poor prognosis (5-year survival rates are 5-14%). The remaining group of patients including those with 11q23 abnormalities, +8, +21, +11, del(9q), del(7q) or other miscellaneous structural or numerical defects not included in the favorable or adverse risk groups were found to have an intermediate prognosis (5-year survival rates are 24-41%) (17).
The intermediate-risk cytogenetic category is the most heterogeneous group in AML, because it includes both normal karyotype without cytogenetic abnormalities and those with cytogenetic abnormalities that are considered neither poor-risk nor favorable. Cytogenetically normal AML (CN-AML) is the largest cytogenetic group (45% of AML) and the best molecularly characterized among both younger and older AML patients (18, 19, 20). However this group is also very molecularly and clinically heterogeneous (Figure 1.1) [modified from (21), recently discovered mutations in AML are not included].

**Figure 1.1** The molecular heterogeneity of cytogenetically normal AML (modified from (21))
Frequent mutations in genes have been identified to possess important prognostic values in AML (22). Mutations in FLT3 encoding a receptor tyrosine kinase involved in hematopoiesis such as FLT3-ITD are found in approximately 30% to 40% of CN-AML cases, and are associated with a poor outcome. Survival rate for this subset is 20% to 25% at 4 years when only the FLT3-ITD status is considered (22-26).

NPM1, encoding a shuttling protein in the nucleolus, is found to be mutated in 50% to 60% of CN-AML cases, and when only the NPM1 mutation status is considered, it is reported to be associated with better outcome, approximately 50% survival at 4 years (18, 27-29). Mutations of CEBPA gene encodes for CCAAT/enhancer binding protein alpha (C/EBPα), a transcription factor are found in approximately 15% of CN-AML cases and these patients also have favorable outcome with approximately 60% 4-year survival rate (30, 31). The prognostic impacts of mutations in the IDH1 (8%-16% of CN-AML patients) and IDH2 (19% of CN-AML) encoding the isocitrate dehydrogenase 1 and 2 have been inconsistent (32-38). Mutations of DNMT3A, which encode for DNA methyltransferase 3A have been reported in 29% to 34% in CN-AML, with R882 is the most commonly mutated residue. Data concerning the prognostic significance of DNMT3A mutations have also been conflicting (39-42).

From the 2008 revision of the WHO classification of myeloid neoplasms and acute leukemia, an international expert panel working on behalf of the European LeukemiaNet (ELN) constructed a new stratification system for reporting cytogenetic and selected molecular abnormalities in studies correlating genetic findings with treatment outcome in AML. The novel aspect of the ELN classification is that it divides patients
with CN-AML into genetic groups according to molecular alterations recognized in the WHO classification, specifically \textit{NPM1}, \textit{CEBPA}, and \textit{FLT3} mutations (Table 1.3).

<table>
<thead>
<tr>
<th>Genetic Group</th>
<th>Subsets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favorable</td>
<td>\textit{t}(8;21)(q22;q22); RUNX1-RUNX1T1 \textit{inv}(16)(p13.1q22) or \textit{t}(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD (normal karyotype) Mutated CEBPA (normal karyotype)</td>
</tr>
<tr>
<td>Intermediate-I</td>
<td>Mutated NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 without FLT3-ITD (normal karyotype)</td>
</tr>
<tr>
<td>Intermediate-II</td>
<td>\textit{t}(9;11)(p22;q23); MLLT3-MLL Cytogenetic abnormalities not classified as favorable or adverse</td>
</tr>
<tr>
<td>Adverse</td>
<td>\textit{inv}(3)(q21q26.2) or \textit{t}(3;3)(q21;q26.2); RPN1-EVI1 \textit{t}(6;9)(p23;q34); DEK-NUP214 \textit{t}(v;11)(v;q23); MLL rearranged \textit{–}5 or \textit{del}(5q) \textit{–}7 abnl(17p) Complex karyotype</td>
</tr>
</tbody>
</table>

In addition to structural genetic aberrations, changes in expression of specific genes seem to impact prognosis of molecular subsets of patients with AML. Higher \textit{BAALC} (brain and acute leukemia, cytoplasmic) gene expression levels predicted lower CR rates as well as inferior disease-free and overall survival (OS). \textit{MN1} (meningioma 1) overexpression is also associated with poor response to induction chemotherapy and higher relapse rate and worse OS in AML. \textit{ERG} (\textit{v}-\textit{ets} erythroblastosis virus E26 oncogene homolog, avian) overexpression was observed in AML with complex aberrant karyotypes and the adverse prognostic significance of high blood \textit{ERG} levels was established in CN-AML.

Recently, microRNA, short RNAs that bind to their complementary sequences on
target mRNA resulting in their translation inhibition or mRNA degradation, expression was also found to affect clinical outcome in AML. Low expression levels of let7b and miR-9 were detected in patients classified in the favorable-risk group, while higher expression levels of these microRNAs were found in patients with adverse- or intermediate-risk cytogenetic groups. Also, high level of miR-181a was correlated with favorable outcome in CN-AML. A list of molecular genetic abnormalities that impact clinical outcome are presented in Table 1.4.

Table 1.4 Recurrent Molecular Genetic Abnormalities in Adult AML and their Clinical Significance

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Prognostic relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIT</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FLT3-ITD</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FLT3-TKD</td>
<td>Controversial</td>
</tr>
<tr>
<td>CEBPA</td>
<td>Favorable</td>
</tr>
<tr>
<td>NPM1</td>
<td>Favorable</td>
</tr>
<tr>
<td>MLL-PTD</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>RAS</td>
<td>Controversial</td>
</tr>
<tr>
<td>WT1</td>
<td>Controversial</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>IDH1/IDH2</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>ASXL</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>TET2</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>TP53</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>JAK2</td>
<td>Unfavorable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene Overexpression</th>
<th>Prognostic relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAALC</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>ERG</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>MN1</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>EVI1</td>
<td>Unfavorable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRs Overexpression</th>
<th>Prognostic relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-181a</td>
<td>Favorable</td>
</tr>
</tbody>
</table>
1.2 AML Treatment

Treatment of AML includes induction chemotherapy and post-remission (or consolidation) therapy. The induction therapy aims to achieve complete remission (CR), by reducing the leukemic burden and restoring the normal hematopoiesis. It is decided after considering individual patient characteristics such as age, co-morbidities, performance status, and preexisting myelodysplasia and likely initiated before karyotype and molecular markers information become available (1). Standard induction regimens are based on a backbone of cytarabine and an anthracycline. These regimens are appropriate for patients younger than age 60 years and for older patients with intact functional status, minimal co-morbidity, and favorable cytogenetic or molecular mutations. Another option for older patients who are medically fit is the purine nucleoside analogue clofarabine, epigenetic agents, including hypomethylating drugs such as 5-azacytidine and decitabine, alone or in combination with histone deacetylase inhibitors (1).

Consolidation therapy aims to maintain CR, prolong survival and achieve complete cure. Most patients who do not receive post-remission therapy will experience relapse, usually within 6 to 9 months. A bone marrow aspirate and biopsy should be performed 7 to 10 days after completion of induction therapy to determine the efficacy of the induction therapy. Additional therapy with standard-dose cytarabine and anthracycline or escalation to high-dose cytarabine with or without an anthracycline is the most common consolidation treatment (1). Patients who fail the induction therapy
and additional high-dose cytarabine or patients with high risk AML (such as FLT3-ITD) should be considered for allogeneic HSCT with matched donor, a clinical trial, or best supportive care (1).

Recent progress made in understanding the pathophysiology of AML has helped advancing targeted therapy developments, a summary of some of these therapeutic strategies are listed in (Table1.5) (4, 43).

Table 1.5 New agents directed at molecular and other specific targets in AML

<table>
<thead>
<tr>
<th>New agents</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farnesyltransferase inhibitor</td>
<td>Farnesylation of lamin A and HJJ-2</td>
</tr>
<tr>
<td>FLT3 tyrosine kinase inhibitors:</td>
<td>Internal tandem duplications of FLT3</td>
</tr>
<tr>
<td>PKC-412, MLN518, CEP701.</td>
<td></td>
</tr>
<tr>
<td>MDR modulators: cyclosporine, PSC833, zosuquidar</td>
<td>P-glycoprotein and other multidrug resistance proteins</td>
</tr>
<tr>
<td>Protac 838</td>
<td></td>
</tr>
<tr>
<td>Histone deacetylase inhibitors:</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>phenylbutyrate, depsipeptide</td>
<td></td>
</tr>
<tr>
<td>Proteosome inhibitor</td>
<td>Bortezomib</td>
</tr>
<tr>
<td>DNA demethylating agents</td>
<td>DNMTs</td>
</tr>
<tr>
<td>5-Azacytidine, Decitabine</td>
<td></td>
</tr>
<tr>
<td>Clofarabine</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>BCL-2 antisense deoxynucleotide</td>
<td>BCL-2 protein</td>
</tr>
<tr>
<td>Antiangiogenic agents</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1.4 Molecular signatures in AML

The cytogenetic and molecular aberrations associated with AML influence the expression of downstream target genes that encode proteins involved in complex biologic networks supporting leukemogenesis. Therefore, microarray genome-wide gene-expression profiling and microRNA-expression profiling assays may provide molecular insight into the underlying biology of the different disease subsets, offer diagnostic and prognostic information, and potentially reveal novel therapeutic targets. Indeed, several gene and microRNA expression profiles have been reported in AML, and some of which have resulted in revealing new molecular targets in AML (44).

Gene expression signature associated with CEBPA mutations in a high-risk molecular group of CN-AML, (patients with wild-type NPM1 genes with or without FLT3-ITD or those with NPM1 mutation and FLT3-ITD) showed upregulation of genes involved in erythroid differentiation (e.g. GATA1, ZFPM1) (44). In contrast, genes involved in myeloid differentiation (e.g. RUNX1, SPI1, ID1) and genes belong to the homeobox family were downregulated in patients with CEBPA mutations (45).

MicroRNA expression signatures have also been associated with distinct molecular aberrations in AML. For example microRNA expression signature associated with FLT3-ITD revealed the upregulation of miR-155 (46-48). Sustained expression of miR-155 was shown to cause granulocyte/monocyte expansion and result in pathological features of myeloid neoplasia in a mouse model (49). Gene and microRNA expression signatures associated with NPM1 mutations include the upregulation of the HOX genes and miRs that reside in the genomic cluster of HOX genes such as miR-10a, miR-10b and miR-196a (48, 50). In addition, among the downregulated microRNAs in
the NPM1-associated microRNA signature, were miR-204 and miR-128, which are also predicted to target HOX genes (48).

Thus far, it became clear that AML is very heterogeneous disease, and responses of AML patients to chemotherapy range from days to cure. This heterogeneity in patients clinical outcomes is mere manifestation of the underneath diversity of the molecular and cytogenetic aberrations. Unfortunately, and despite our expanded understanding of the clinical and biological heterogeneity of this disease in the last decade, the treatment remained the same and one for all. This suggests that further research is highly needed to understand the molecular and biological pathways involved in leukemogenesis in order to improve the dismal outcome of high risk subset of AML patients.

The research we are presenting in the following chapters is divided into two major aims, our first aim is to identify novel therapeutic targets and prognostic markers employing previously identified genome-wide analyses of gene expression signatures in high risk AML patients. Our second aim is to investigate novel therapeutic approaches directed toward previously identified molecular targets in high risk disease.

In chapter #2 we identified SPARC as a novel molecular and therapeutic target in AML. We demonstrated a functional, mechanistic and clinical implication of SPARC in AML, revealing that high SPARC expression promoted growth advantage in vitro, aggressive disease in vivo, and worse outcome in CN-AML patients. SPARC acts likely via activation of ILK/AKT/β-catenin pathways.

In chapter #3 we investigated the preclinical and pharmacological activity of the natural initiation of translation inhibitor, silvestrol in FLT3-ITD positive AML representing
a large proportion of high risk AML patients. We demonstrated that silvestrol had a potent \textit{in vitro} and \textit{in vivo} anti-leukemia activity in \textit{FLT3}-ITD and \textit{FLT3} overexpressing AML cells. Silvestrol inhibited \textit{FLT3} mRNA translation resulting in \textit{FLT3} protein down-regulation and in turn inhibition of aberrant tyrosine kinase activity.
Chapter 2: SPARC Contributes to Leukemia Growth and Aggressive Disease in Acute Myeloid Leukemia

2.1 Introduction

2.1.1 SPARC gene and expression

The Secreted Protein Acidic and Rich in Cysteine (SPARC) gene is highly conserved in vertebrates, localizes on human chromosome 5q31–q33 (51) and lacks the canonical CAAT and TATA box sequences in its promoter region. The SPARC gene encodes a 32 kDa secreted matricellular calcium binding glycoprotein also known as osteonectin or BM-40 (52-54). SPARC expression is elevated during development and tissue differentiation and declines following maturation (55). Post-development the expression of SPARC is limited to tissues with high extracellular matrix (ECM) turnover, such as bone and gut epithelia (55). SPARC is also induced during wound-healing, angiogenesis, and by the stromal cells during tumorigenesis (56-59).

2.1.2 SPARC protein structure and functions

Human SPARC protein consists of 303 amino acids (54), 17 amino acids that makes a signal peptide is removed during processing leaving a mature protein of SPARC that consists of three individual structural domains (60) (Figure 2.1). the three
domains are: 1- An N-terminal domain, which consists of 52 amino acids (Ala1-Glu52), is highly acidic, binds calcium with low affinity, inhibits cell spreading, enhances plasminogen activator inhibitor-1 (PAI-1) and decreases fibronectin (FN) and thrombospondin-1 (TSP-1). 2- FS-like domain which is 85 amino acids (Asn53-Pro137) located between the N-terminus domain sequence and EC-binding domain, and contains 10 cysteine residues and several internal disulfide bonds. This domain promotes angiogenesis and proliferation and block focal adhesions (53). Cationic region 1 (Amino acids 54–73) of this domain has been shown to inhibit the proliferation of endothelial cells, while cationic region 2 (amino acids 113–130) stimulates DNA synthesis. 3- EC-binding domain (extracellular calcium-binding domain) which comprises 149 amino acid residues (Cys138-Ile286), and consists of two EF-hand motifs that bind to calcium with high affinity. This domain induces matrix metalloproteinases (MMPs), regulates the interactions between cells and matrix, and inhibits cell spreading, proliferation, and adhesion (61, 62). SPARC is post-translationally modified by N-linked glycosylation. Phosphorylation modification has been also reported in bone osteonectin, however the role of this modification in the function of SPARC is unclear (63).
Matrix metalloproteinases (MMP-2, -3, -7 and -13, plasmin and trypsin) cleave SPARC producing KGHK-containing fragment that can stimulate angiogenesis (64). The presence of truncated form of SPARC protein has been recently reported in hepatocellular carcinoma (HCC) samples and esophageal carcinoma (65, 66).

The phenotype of SPARC-null mice shows characteristics of ECM disruption, including early cataract formation, accelerated dermal wound-healing, osteopenia and a
curly tail (55, 67-69). SPARC-null mice also show deficiencies in connective tissue, such as decreased levels of collagen I in skin, adipose, heart and bone (70-72).

### 2.1.3 SPARC signaling pathways

Although cell surface receptors have not been identified for SPARC yet; several reports have suggested that stabilin-1 and integrins may mediate the intracellular effects of SPARC. Stabilin-1 was found using a phage display as a cellular receptor interacts with SPARC through the extracellular epidermal growth factor (EGF)-like domain resulting in internalization and delivery of SPARC to the endocytic pathway and its targeting to lysosomes. SiRNA knock-down of Stabilin-1 reduces SPARC expression, while elevated SPARC level is observed in stabilin-1 over-expressing cells (73).

Integrins connect cells to the ECM, signal in response to ECM ("outside-in" signaling) and regulate the interactions of the ECM in response to intracellular cues ("inside-out" signaling) (74). Integrins contribute to tumor growth and metastasis via interacting and clustering directly with growth factor receptor to control cell survival, proliferation, adhesion and migration. In addition, integrin activation and signaling are required for proper cell response to cytokine (75-79).

Several studies reported that SPARC binds integrins β1, β3, αv, and β5 and regulates integrin interaction with the ECM. In addition, many studies suggest that SPARC influences downstream components of integrin signaling, specifically the activation of integrin linked kinase (ILK) (80). SPARC was recently found to bind integrin β1 with its copper-binding domain; thereby, directly enhancing integrin/ILK signaling (81,
SPARC also promotes cell survival of lens epithelial cells following serum starvation by enhancing ILK activation (82). SPARC-intergin/ILK interaction is also observed in several cancer cell lines. SPARC increased survival and induced an invasive phenotype in human glioma cells (83-85). In addition, targeting SPARC with short-hairpin RNA decreased the activity of ILK, focal adhesion kinase (FAK) and protein kinase B (Akt) resulting in decrease of cell survival and invasion (85). Forced SPARC expression resulted in increase ILK expression in glioma cells (86). In human ovarian cancer cells, SPARC reduced the surface localization and/or clustering of αv, β1, β3 and β5 integrins resulting in inhibition of adhesion, invasion and proliferation (87).

SPARC protein regulates cell–matrix interactions and tissue remodeling through binding directly to collagen and other ECM proteins, and activating MMPs (88). It also interacts with and regulates several growth factors such as transforming growth factor β (TGFβ), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (89-92).

SPARC does not bind bFGF directly, but it inhibits bFGF-induced migration of endothelial cells (90, 92-95). SPARC binds PDGF and results in dose-dependent inhibition of ligand binding and activation of PDGF receptors on human dermal fibroblasts (92). Similarly, SPARC binds VEGF directly and inhibits VEGFR1 activation and VEGF- induced proliferation of microvascular endothelial cells (90, 96). On the other hand, VEGF induces the expression of SPARC in human vascular endothelial cells (97). SPARC is also implicated in the regulation of TGFβ (89, 95). TGFβ is a master regulator of wound-healing and fibrosis by inducing the synthesis of several ECM proteins including collagen and fibronectin (98). Several studies demonstrate that TGFβ induces SPARC expression (99-102). SPARC was also reported to regulate the expression and
activity of TGFβ (103) suggesting that there is a reciprocal regulatory feedback loop between SPARC and TGFβ.

The interaction of SPARC with several signaling pathways and different growth factors that have a dichotomous effect in cancer progression such as TGFβ (104) influences and explains the apparently paradoxical role of SPARC in cancer.

2.1.4 SPARC and Cancer

The functions of SPARC in regulating ECM deposition, cell-ECM interaction and growth factor signaling suggested to researchers a possible contribution of this protein in several hallmarks of cancer including angiogenesis, migration, proliferation and survival (52, 58, 105). In line with this observation, altered SPARC expression was reported in several malignancies. However, inconsistency and often contradiction, even among the same tumor types were reported.

Conflicting reports have classified SPARC as either a tumor suppressor or an oncogene. The difficulty of assigning a specific role to SPARC may be related to the diversified roles that this protein plays in the tumor and microenvironment cells of distinct types of cancer (58). Low expression levels of SPARC have been found in ovarian cancer (106), colorectal (107, 108), pancreatic (109); while high expression levels have been reported in breast cancer (110, 111) melanoma (112-114), and glioblastomas (115). The association of SPARC expression levels with clinical outcome is also controversial. While stromal SPARC expression has been significantly associated with poor prognosis in non-small cell lung cancer (116) and with recurrence of ductal carcinoma in situ (117), absence of stromal SPARC independently predicted poor
prognosis in colon cancer (118). This controversial role of SPARC in cancer is likely related to the diversified functions of the protein in the microenvironment of distinct types of cancer. Suggesting that SPARC activity to promote or inhibit tumor progression is dependent on the initiating cell-type, the tumor stage, and the context of the tumor microenvironment.

In hematological malignancies, SPARC was found to be downregulated at diagnosis in patients with del(5) MDS and became upregulated post-treatment with lenalidomide (119-121). SPARC was also found downregulated in AML with MLL rearrangements and upregulated in t(8;21) and inv(16) acute myeloid leukemia (AML) (122), although no correlation with outcome was reported in these molecular and cytogenetic subsets. Finally in chronic myelogenous leukemia (CML), the accumulation of intracellular SPARC was found to contribute to imatinib resistance (123).

Recently, we have observed that upregulated SPARC was comprised in gene expression profiles (GEPs) associated with distinct gene mutations [i.e., IDH2-R172 ([34]) or aberrantly overexpressed genes [BAALC and ERG levels (124)] predicting poor outcome in cytogenetically normal (CN)-AML). In contrast, SPARC was found among the most downregulated genes in the GEP associated with NPM1 mutations, which predict favorable outcome in CN-AML (50). Therefore, we hypothesized that SPARC deregulation contributes to the level of disease aggressiveness and, when overexpressed, is associated with unfavorable prognosis in AML.
2.2 Experimental procedure

2.2.1 Cell lines and primary blasts

THP-1, MV4-11 and Kasumi-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10-20% calf serum. HEK 293T cells (ATCC, Manassas) were grown in DMEM medium supplemented with 10% calf serum. Patient AML blasts were maintained in RPMI 1640 medium supplemented with 30% fetal bovine serum, 1% HEPES Buffer, and 1x StemSpan CC100 (Stemcell Technologies, Vancouver, Canada) containing IL-3, IL-6, FLT3 ligand and SCF. All cells were incubated at 37ºC with 5% CO₂. The AML blasts utilized in the experiments 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 the tissue for investigational studies was obtained from each patient according to OSU institutional guidelines.

2.2.2 Transient transfection, RNA interference and viral induction

THP-1, MV4-11 and Kasumi-1 cells and patient AML blasts were transfected with 1-2μg of pLenti-SPARC and pLenti-EV, pLenti-IDH1-wt, pLenti-IDH1-R132, pLenti-IDH2-wt, pLenti-IDH2-R172 or pCMV-RUNX1, pCMV-EV, pCMV-P65, pSuper-SP1 and pSuper-EV.. For RNA interference, cells were transfected with 1.0 nmol (10µl) of SPARC–siRNA, ILK-siRNA, RUNX1-siRNA, t(8,21)-siRNA, SP1-siRNA, P65-siRNA or off target-siRNA control (Dharmacon Inc., Lafayette, CO). Transient transfection was done utilizing the Nucleofector Kit (Amaxa Inc, Walkersville, MD) according to the manufacturer’s instructions. For viral inductions, approximately 2X10⁶ cells were infected...
with lentivirus containing the following vectors pLenti6.2/V5-DEST vectors: Empty vector, SPARC, IDH1-WT, IDH2-WT, IDH1-R132 and IDH2-R172. After 48 hours, antibiotic selection with blasticidin was started for 10 days.

2.2.3 RNA extraction and Real-Time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using SuperScript III reagents (Invitrogen) and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative Real-Time RT-PCR for SPARC, c-MYC, CyclinD1, IDH1, IDH2, P65, SP1, RUNX1, and 18S, expression was performed using commercially available TaqMan Gene Expression Assay primers and probes (Applied Biosystems) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). The comparative cycle threshold (CT) method was used to determine the expression levels normalized by the internal control 18S for gene expression.

2.2.4 Immunoprecipitation, Western Blot Analysis, Chromatin Immunoprecipitation and Antibodies

Immunoprecipitation and western blot were performed as previously described (125) utilizing the following antibodies: Actin (l-19) sc-1616 (Santa Cruz Biotechnology, Santa Cruz, CA); SPARC-ab89218 (abcam, Cambridge, MA) and (Cell Signaling Technology, Danvers, MA), GSK3β, AKT, P-GSK3β, P-AKT, p-β-catenin (Cell signaling), c-MYC (santa cruz), IDH1-3997S (Cell Signaling), IDH2-ab55271 (abcam), SP1 (Santa cruz), P65 (upstate), and RUNX1(Calbiochem, San Diego, CA). ChIP assays were performed using the EZ ChIP Assay Kit (Millipore) according to the manufacturer’s
standard protocol. DNA was quantified using qRT-PCR with SYBR green incorporation (Applied Biosystems). The antibodies used were: anti-RUNX1(abcam), SP1 and NF-κB(p65) (Millipore, Billerica, MA). The primers specific for SPARC gene promoter are:

(F1: CGCAAAAAGAGGAGGAAAGA, R1: GAGACAGGCAACAGGAAACC, F2: GGCTATGGGAGAAGGAGGAG, R2: GGGGTCAACACATACCTCAG, F3: TGCCCTTGTTGGCAAATACAA, R3: TTGTTCAGAGGGCAAGTGAG)

2.2.5 Immunofluorescent Staining, Cell Sorting, Flow Cytometry and Confocal Microscopy

Cells were washed and stained with CD34, CD38, CD45RA and CD123 antibodies (eBioscience), and sorted into the following populations: hematopoietic stem cells and multipotent progenitors (HSC&MPP) or CD34+/CD38-, common-myeloid progenitors (CMP) or CD34+/CD45RA-/CD123low and granulocyte/monocyte progenitors (GMP) or CD34+/CD45RA+/CD123low. Analysis was performed using a BD Aria II cell sorter Version 6.1.2 (BD Biosciences).

For intracellular flow and confocal microscopy analysis; cells (1×10^6) were washed twice with cold PBS, and then fixed in Cytofix/cytoperm buffer (500ul per 1 million of cells) (BD Biosciences, Billerica, MA), by incubation for 20min on ice. Cells were then washed twice with 1ml of Perm/Wash buffer and blocked in 10% goat serum (diluted in PBS pH7.4) on shaker for 2hr. After washing twice with PBS, cells were resuspend in 100ul of Perm/Wash buffer (1X) with the 1ul of β-catenin antibody (cell signaling) and c-MYC antibody (Santa-Cruz) and incubated overnight on shaker at 4C. After washing, the samples were incubated with fluorochrome conjugated secondary antibody for 2 hour at room temperature. Samples were analyzed by flow cytometry on a FACSCalibur
instrument. Nuclei were stained with 1ul of Drag5. 10ul of stained cells were pipetted to a slide, air-dried for 3min and coversliped. Samples were analyzed by confocal microscope (The Ohio State University Image facility).

### 2.2.5 Clonogenic, Viability and Growth Inhibition Analysis

Methylcellulose clonogenic assays were carried out by plating $1 \times 10^3$ of the different cell lines, 1-$2 \times 10^4$ cells from primary blasts in 0.9% MethoCult (Stem Cell Technologies). Colonies from cell lines and primary cells were scored 10 and 14 days later, respectively. For cells viability and growth inhibition, we utilized MTS assay. Briefly, $5.0 \times 10^4$ cells were incubated in triplicate in a 96-well plate in the presence or absence of the indicated test samples in a final volume of 100 µl for 24, 48 and 72 hours at 37°C. Thereafter, 20 µl MTS solution (Promega, Madison WI) was added to each well. After 4 hours incubation at 37°C, plates were shaken and the optical density at 490 nm was measured. Percent cell viability was calculated as cell viability of the experimental samples/cell viability of the control samples × 100. At least three independent experiments were performed.

### 2.2.6 Leukemogenesis in NOD/SCID gamma mice

Four to six-week-old NOD/SCID gamma (NSG) (The Jackson Laboratory, Bar Harbor, ME) were intravenously injected through the tail vein with $1-5 \times 10^6$ cells of THP-1 or MV4-11 infected with pLenti-EV or pLenti-SPARC. Six to eight weeks after engraftment, cell-injected mice ($n = 6$) were euthanized, and spleens, livers, kidneys and sternum were isolated. Parts of the spleens were grounded, and the red blood cells were lysed to attain single MNCs utilized for immunoblotting and qRT-PCR assays. For
pathological examination, tissue sections from liver and spleen were fixed on formalin, embedded in paraffin blocks, and H&E stained. All animal studies were performed in accordance with OSU institutional guidelines for animal care and under approved protocols by the OSU Institutional Animal Care and Use Committee.

2.2.7 Patients

We studied pretreatment bone marrow and blood samples with ≥ 20% blasts from 363 patients (age 19 to 83 years) with de novo CN-AML. Cytogenetic analyses at diagnosis were confirmed by central karyotype review. To establish CN-AML, ≥ 20 metaphase cells from diagnostic bone marrow had to be analyzed and the karyotype had to be normal, Institutional review board–approved informed consent for participation in the studies was obtained from all patients. Younger patients (age < 60 years; n = 153) were treated on CALGB 9621 and 19808 protocols and older patients (age ≥ 60 years; n = 210) were enrolled on protocols 8525, 8923, 9420 9720, or 10201. No patient included in our analysis received allogeneic transplantation in first complete remission (CR). The median follow-up for younger and older patients alive and included in this analysis was 7.0 and 3.8 years, respectively.

2.2.8 Cytogenetic and Mutational Analyses

The diagnosis of CN-AML was based on the analysis of ≥20 metaphases in bone marrow specimens subjected to short-term cultures and confirmed by central karyotype review. Patients were also characterized centrally for FLT3 internal tandem duplication (ITD)(126), FLT3 tyrosine kinase domain mutations (24), MLL partial tandem duplication (127) NPM1 (28, 50), WT1 (128), CEBPA (45), IDH1, IDH2 (34) TET2, (129) ASXL1
(130) and DNMT3A (40) mutations as previously reported.

2.2.9 Genome-Wide Expression Analyses

Gene- and microRNA-expression profiling were conducted using the NanoString nCounter System (NanoString Technologies, Seattle, WA) and the Affymetrix U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) and the Ohio State University custom microRNA array (OSU_CCC version 4.0), respectively, as reported previously (131).

2.2.10 Statistical Analysis

Statistical analyses relative to microarray gene and microRNA expression data were performed by the CALGB Statistical Center. Definitions of clinical end points—CR, disease-free survival (DFS), and OS—are as reported previously (34). The differences among patients in baseline clinical and molecular features according to their SPARC expression levels were tested using the Fisher's exact and Wilcoxon rank sum tests for categoric and continuous variables, respectively. Estimated probabilities of DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions.
2.3 Results

2.3.1 SPARC is expressed in hematopoietic stem cells (HSCs)

SPARC has been linked to tissue remodeling, development and stem cells (55, 132); but its role in hematopoiesis has not been fully investigated yet. To understand the role of SPARC in leukemogenesis, we initially characterized its expression in normal bone marrow (NBM) cells obtained from normal donors by sorting four different NBM samples into CD34+ and CD34- populations using magnetic beads. SPARC mRNA level measured by qRT-PCR was found to be significantly higher in the CD34+ cells compared to that in the CD34- cells (Figure 2.2a). This prompted us to examine SPARC expression in the following sorted stem cells/ progenitor populations: hematopoietic stem cells and multipotent progenitors (HSC&MPP) or CD34+/CD38-, common-myeloid progenitors (CMP) or CD34+/CD45RA-/CD123low and granulocyte/monocyte progenitors (GMP) or CD34+/CD45RA+/CD123low of NBM (n=2). We found SPARC protein expression to be the highest in HSC-MPP and this expression has significantly (70-97%) decreased when cells transitioned into CMP (Figure 2.2b,c). This suggests a possible implication of SPARC in hematopoiesis.
Figure 2.2 SPARC is expressed in hematopoietic stem cells/progenitors.

SPARC mRNA level measured in CD34+ and CD34- cells of NBM samples (n=4) (a). SPARC protein expression measured by western blot in sorted stem cells/progenitors populations of NBM samples (b,c)
2.3.2 SPARC promotes leukemia growth

To investigate the impact of SPARC on leukemia growth, gain and loss of function experiments were initially performed. THP-1 cells expressing low level of SPARC were stably infected with pLenti-SPARC (THP-1/SPARC) or a control empty vector pLenti-EV (THP-1/EV); SPARC ectopic expression was confirmed by western blotting (Figure 2.3a). Colony forming assays were then performed and scored 10-14 days later. A significant increase in the ability of colony formation was observed in THP-1/SPARC compared to the THP-1/EV (164 vs 63; P=0.03) (Figure 2.3b). When we knocked-down SPARC in Kasumi-1 cells expressing high endogenous levels of this gene (Figure 2.3c), we observed a decrease in the colony forming ability in SPARC siRNA transfected cells compared with the off-target siRNA transfected controls (84 vs 46; P=0.03) (Figure 2.3d).
Figure 2.3 SPARC promotes growth advantage in AML cell lines.

SPARC ectopic expression in THP-1 cells was confirmed by western blotting (a) resulted in increase in colony formation (b), SPARC knock-down in Kasumi-1 cells confirmed by western blot (c) resulted in decrease colony formation (d).
These results were then confirmed in AML primary blasts from four different patients (Table 2.4). Blasts were infected with either pLenti-SPARC or pLenti-EV vectors; ectopic SPARC expression was confirmed by western blot (Figure 2.4a). Significant increase in colony forming ability was observed in SPARC infected blasts (16 vs 7; $P=0.005$; 125 vs 77; $P=0.013$; 22 vs 16; $P=0.05$ and 357 vs 170; $P=0.062$) compared to their respective controls (Figure 2.4b) for each patients.
Figure 2.4 SPARC promotes growth advantage in primary blasts.

SPARC ectopic expressions in primary blasts from 4 different AML patients were confirmed by western blotting (a) and resulted in increase in colony formation (b).
To examine the activity of SPARC in vivo, NSG mice were injected with THP-1/SPARC cells or THP-1/EV controls via tail vein. We hypothesized that both mice group developed an AML- disease, albeit those transplanted with THP-1/SPARC cells had a more aggressive phenotype. In fact, 8 weeks later the mice injected with THP-1/SPARC cells had significantly larger spleens (P=0.008) (Figure 2.5a,b) and livers (P=0.02) (Figure 2.5c,d) than THP-1/EV cells injected mice. The livers in THP-1/SPARC mice showed increase tumor nodularity with respect to THP-1/EV controls. Similar results were obtained when we used MV4-11/SPARC xenografts (Figure 2.6). Pathology analysis showed that the THP-1/SPARC cells formed coalescing masses that obliterated large expanses of the liver parenchyma. In contrast, THP-1/EV cells occurred as individual or small clusters of tumor cells within some hepatic sinusoids without effacing the hepatic cords (Figure 2.5e). The THP-1/SPARC had a monomorphic phenotype and high proliferative capacity relative to the pleomorphic character (i.e., variable cell appearance) and elevated apoptotic tendency of the THP-1/EV cells. The pattern and degree of tumor infiltration in the bone marrow (from sternum), heart, and kidney were similar for THP-1/SPARC and THP-1/EV cells injected mice (data not shown). Importantly, THP-1/SPARC-injected mice (n=6) had a significantly shorter survival than THP-1/EV-injected mice (n=7) (P=0.006; Figure 2.5f). These data support increase aggressiveness of leukemia cells overexpressing SPARC.
Figure 2.5 SPARC promotes aggressive disease in vivo.

NSG mice were engrafted with THP-1 cells infected with plenti-EV or plenti-SPARC vectors; spleens (a,b), and livers (c,d) collected 8 weeks following engraftment. Histopathology of liver from the THP-1/EV and THP-1/SPARC (e) Survival analysis of THP-1/EV mice (N=7) compared to THP-1/SPARC (N=6) (f).
Figure 2.6 SPARC promotes aggressive disease in vivo.

NSG mice were engrafted with MV4-11 from a previously engrafted NSG mouse (a) with parental MV4-11 cells (b) before injection, cells were infected with pLenti-EV or pLenti-SPARC vectors. Spleens collected 4-8 weeks following engraftment.
2.3.3 SPARC overexpression negatively impacts clinical outcome in CN-AML

Since we found that SPARC contributes to an aggressive leukemia phenotype in preclinical models, next we tested whether increased SPARC expression also associates with aggressive human disease. We analyzed 362 adult patients with primary CN-AML, enrolled and treated on Cancer and Leukemia Group B (CALGB) upfront protocols with cytarabine-daunorubicin-based regimens for SPARC expression by nCounter assays (nanostring). At diagnosis, higher SPARC expressers harbor more frequently RUNX1 mutations ($P < .0001$) and ASXL1 mutations ($P < .0001$), and higher BAALC, ERG and MN1 expression ($P < .001$, each) and less frequently NPM1 mutations ($P < .0001$) and FLT3-TKD ($P = .02$) compared with lower expressers, but we found no increase frequency of extramedullary disease (Table 2.1). Higher SPARC expressers had lower chances for achieving CR ($P < .001$; OR .75) and shorter DFS ($P < .001$; HR 1.17) and OS ($P < .001$; HR 1.16), once adjusted for age. In a multivariable analyses, higher SPARC expression independently predicted worse odds for achieving CR ($P < .001$), once adjusting for RUNX1 mutation ($P = .01$), WBC ($P < .001$) and age group ($P = .01$) and was associated with a trend for shorter DFS ($P = .07$) once adjusting for FLT3-ITD ($P < .001$), RUNX1 mutation ($P = .03$), WBC ($P = .005$), age group ($P < .001$), and with significantly shorter OS ($P < .001$), once adjusting for FLT3-ITD ($P < .001$), WT1 ($P = .003$) and ASXL1 ($P = .04$) mutations and age group ($P < .001$). Although we used SPARC as a continuous variable, for display purpose only, we dichotomized younger patients into higher and lower expresser groups using the median SPARC expression value and present the DFS and OS Kaplan-Maier curves in (Figure 2.7a,b).
Table 2.1 Comparison of clinical and molecular characteristics of Low vs. High SPARC all patients

<table>
<thead>
<tr>
<th>n=363</th>
<th>Low SPARC (n=182)</th>
<th>High SPARC (n=181)</th>
<th>P (Median cut)</th>
<th>P (Continuous)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol, no. (%)</td>
<td>8525</td>
<td>13 (7)</td>
<td>10 (5)</td>
<td>.28</td>
</tr>
<tr>
<td></td>
<td>8923</td>
<td>13 (7)</td>
<td>4 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9420</td>
<td>1 (1)</td>
<td>4 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9621</td>
<td>38 (21)</td>
<td>37 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9720</td>
<td>46 (25)</td>
<td>49 (27)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10201</td>
<td>32 (18)</td>
<td>38 (21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19808</td>
<td>39 (21)</td>
<td>39 (22)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>62</td>
<td>63</td>
<td>.23</td>
<td>.005</td>
</tr>
<tr>
<td>Range</td>
<td>(18, 81)</td>
<td>(20, 83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age group no. (%)</td>
<td>&lt;60</td>
<td>77 (42)</td>
<td>76 (42)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>≥60</td>
<td>105 (58)</td>
<td>105 (58)</td>
<td></td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td>Male</td>
<td>85 (47)</td>
<td>99 (55)</td>
<td>.14</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>97 (53)</td>
<td>82 (45)</td>
<td></td>
</tr>
<tr>
<td>Race, no. (%)</td>
<td>White</td>
<td>2 unknown</td>
<td>1 unknown</td>
<td>.86</td>
</tr>
<tr>
<td></td>
<td>Non-White</td>
<td>163 (91)</td>
<td>161 (89)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>Median</td>
<td>4 unknown</td>
<td>4 unknown</td>
<td>.003</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(4.8, 15.0)</td>
<td>(4.6, 13.4)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>Median</td>
<td>2 unknown</td>
<td>69</td>
<td>.13</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(4, 507)</td>
<td>(8, 850)</td>
<td></td>
</tr>
<tr>
<td>WBC count (x10^9/L)</td>
<td>Median</td>
<td>2 unknown</td>
<td>24.9</td>
<td>.007</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(1.0, 450.0)</td>
<td>(1.0, 434.1)</td>
<td></td>
</tr>
<tr>
<td>%Blood Blasts</td>
<td>Median</td>
<td>2 unknown</td>
<td>51</td>
<td>.009</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0, 97)</td>
<td>(0.99)</td>
<td></td>
</tr>
<tr>
<td>%Bone Marrow Blasts</td>
<td>Median</td>
<td>73</td>
<td>64</td>
<td>.002</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(4.97)</td>
<td>(6.97)</td>
<td></td>
</tr>
<tr>
<td>FAB, no. (%)</td>
<td>M0</td>
<td>1 (1)</td>
<td>5 (4)</td>
<td>.19</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>36 (26)</td>
<td>31 (24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>36 (26)</td>
<td>41 (32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>32 (23)</td>
<td>33 (26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>30 (22)</td>
<td>16 (13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>2 (1)</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Extramedullary Involvement, no. (%)</td>
<td>CNS</td>
<td>0 (0)</td>
<td>2 (1)</td>
<td>.03</td>
</tr>
<tr>
<td></td>
<td>Hepatomegaly</td>
<td>9 (5)</td>
<td>7 (4)</td>
<td>.05</td>
</tr>
<tr>
<td></td>
<td>Spleenomegaly</td>
<td>12 (7)</td>
<td>9 (5)</td>
<td>.62</td>
</tr>
<tr>
<td></td>
<td>Lymphadenopathy</td>
<td>15 (8)</td>
<td>13 (7)</td>
<td>.51</td>
</tr>
<tr>
<td></td>
<td>Skin Infiltrates</td>
<td>13 (7)</td>
<td>11 (6)</td>
<td>.84</td>
</tr>
<tr>
<td></td>
<td>Gum Hypertrophy</td>
<td>26 (14)</td>
<td>12 (7)</td>
<td>.83</td>
</tr>
<tr>
<td></td>
<td>Mediastinal Mass</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>.02</td>
</tr>
<tr>
<td>Induction Treatment, no. (%)</td>
<td>AD</td>
<td>46 (25)</td>
<td>35 (19)</td>
<td>.32</td>
</tr>
<tr>
<td></td>
<td>ADE</td>
<td>93 (51)</td>
<td>93 (51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AD/Ep</td>
<td>32 (18)</td>
<td>36 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNR-CTX-E-PRED</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G3139</td>
<td>10 (5)</td>
<td>17 (9)</td>
<td></td>
</tr>
</tbody>
</table>

Continued
Table 2.1 continued

<table>
<thead>
<tr>
<th></th>
<th>Low $^1$ SPARC (n=182)</th>
<th>High $^1$ SPARC (n=181)</th>
<th>$^P$ $^3$ (Median cut)</th>
<th>$^P$ $^5$ (Continuous)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NPM1</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>3 unknown</td>
<td>4 unknown</td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>153 (85)</td>
<td>73 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26 (15)</td>
<td>104 (59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FLT3-ITD</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITD positive</td>
<td>3 unknown</td>
<td>3 unknown</td>
<td>$.83$</td>
<td>$.76$</td>
</tr>
<tr>
<td>No ITD</td>
<td>66 (37)</td>
<td>68 (38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>113 (63)</td>
<td>110 (62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CEBPA</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>3 unknown</td>
<td>4 unknown</td>
<td>$.02$</td>
<td>$.37$</td>
</tr>
<tr>
<td>Single mutated</td>
<td>20 (11)</td>
<td>35 (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double mutated</td>
<td>6</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-Type</td>
<td>14</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>159 (89)</td>
<td>142 (80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ELN Risk Group</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>4 unknown</td>
<td>4 unknown</td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Intermediate-I</td>
<td>110 (62)</td>
<td>64 (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68 (38)</td>
<td>113 (64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FLT3-TKD</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TKD positive</td>
<td>3 unknown</td>
<td>4 unknown</td>
<td>$.002$</td>
<td>$.02$</td>
</tr>
<tr>
<td>No TKD</td>
<td>28 (16)</td>
<td>9 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>151 (84)</td>
<td>168 (95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WT1</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>3 unknown</td>
<td>4 unknown</td>
<td>$.28$</td>
<td>$.34$</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>14 (8)</td>
<td>20 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>165 (92)</td>
<td>157 (89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TET2</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>5 unknown</td>
<td>7 unknown</td>
<td>$.18$</td>
<td>$.80$</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>50 (28)</td>
<td>38 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>127 (72)</td>
<td>136 (78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MLL-PTD</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>22 unknown</td>
<td>23 unknown</td>
<td>$.12$</td>
<td>$.09$</td>
</tr>
<tr>
<td>Absent</td>
<td>7 (4)</td>
<td>14 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>153 (96)</td>
<td>144 (91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IDH1</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R132</td>
<td>6 unknown</td>
<td>5 unknown</td>
<td>$.13$</td>
<td>$.16$</td>
</tr>
<tr>
<td>V71i</td>
<td>14 (8)</td>
<td>22 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-Type</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>160 (91)</td>
<td>154 (87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IDH2</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 unknown</td>
<td>31 (18)</td>
<td>35 (20)</td>
<td>$.68$</td>
<td>$.19$</td>
</tr>
<tr>
<td>R140</td>
<td>31</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R172</td>
<td>0</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-Type</td>
<td>145 (82)</td>
<td>141 (80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RUNX1</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>19 unknown</td>
<td>17 unknown</td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>6 (4)</td>
<td>36 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>157 (96)</td>
<td>128 (78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ASXL1</strong>, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>7 unknown</td>
<td>6 unknown</td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Wild-Type</td>
<td>4 (2)</td>
<td>30 (17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>171 (98)</td>
<td>145 (83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNMT3A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>12 unknown</td>
<td>9 unknown</td>
<td>$.11$</td>
<td>$.12$</td>
</tr>
<tr>
<td>R882</td>
<td>67 (39)</td>
<td>53 (31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-R882</td>
<td>39</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-Type</td>
<td>28</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>103 (61)</td>
<td>119 (69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ERG</strong> expression group</td>
<td></td>
<td></td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Median Range</td>
<td>-3.34</td>
<td>-2.64</td>
<td>(-6.10, -0.79)</td>
<td>(-5.68, -0.02)</td>
</tr>
<tr>
<td><strong>BAALC</strong> expression group</td>
<td></td>
<td></td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Median Range</td>
<td>-2.78</td>
<td>-0.29</td>
<td>(-7.65, 5.12)</td>
<td>(-5.66, 7.59)</td>
</tr>
<tr>
<td><strong>MNX1</strong> expression group (median cut), no. (%)</td>
<td></td>
<td></td>
<td>$&lt;.001$</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>High</td>
<td>74 unknown</td>
<td>74 unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>36 (33)</td>
<td>75 (70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 (87)</td>
<td>32 (30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.7 SPARC overexpression negatively impacts clinical outcome in younger CN-AML patients.

Younger CN-AML patients were dichotomized into higher and lower expresser groups using the median SPARC expression value and present the DFS and OS Kaplan-Maier curves.
2.3.4 SPARC overexpression is associated with distinct gene and microRNA expression signatures in CN-AML

To gain a biologic insight into SPARC leukemia activity, we derived genome-wide microarray gene and microRNA expression signatures associated with SPARC overexpression in CN-AML patients. We found 650 genes to be significant at \( P \leq 0.001 \) (Figure 2.8). Among the most upregulated genes (\( \geq 2 \) fold increase) were the two human hematopoietic stem cell markers: \( CD34 \) (3.1 folds, \( P < 1e-07 \)), and \( PROM1 \) (\( CD133 \)) (5.2 folds, \( P < 1e-07 \)); other genes that have been associated with negative prognosis in AML were also upregulated such as: \( BAALC \) (2.8 folds, \( P < 1e-07 \)) and \( MN1 \) (2.4 folds, \( P < 1e-07 \)). Among the most downregulated genes in the signature were members of \( HOXA \) and \( HOXB \) clusters and the \( HOX \) cofactor, \( MEIS1 \). In addition, we were able to derive a microRNA expression signature associated with SPARC expression (Figure 2.9) (Table 2.2 and 2.3). The hematopoietic stem cell-specific \( miR-126 \) and \( miR-130a \) were upregulated in both older and younger patients. miRs previously reported to be upregulated in the favorable molecular group of \( NPM1 \) mutated AML patients were among the most downregulated miRs in high SPARC expressers signature including \( miR-10a, miR-10b, miR100 \) and \( miR-196b \) in older and \( miR10a \) and \( let7b \) in younger patients.
Figure 2.8 SPARC overexpression is associated with distinct gene expression signature in CN-AML.
miR expression profile associated with SPARC expression in older patients

miR expression profile associated with SPARC expression in younger patients

Figure 2.9 SPARC overexpression is associated with distinct microRNA expression signature in CN-AML.
Table 2.2 miR expression profile associated with SPARC expression in older patients

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>Parametric p-value</th>
<th>FDR</th>
<th>UniqueID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.369</td>
<td>1.6e-06</td>
<td>0.000143</td>
</tr>
<tr>
<td>2</td>
<td>0.352</td>
<td>5.1e-06</td>
<td>0.000379</td>
</tr>
<tr>
<td>3</td>
<td>0.273</td>
<td>0.0004676</td>
<td>0.0209</td>
</tr>
<tr>
<td>4</td>
<td>-0.268</td>
<td>0.0005936</td>
<td>0.0241</td>
</tr>
<tr>
<td>5</td>
<td>-0.309</td>
<td>6.75e-05</td>
<td>0.00335</td>
</tr>
<tr>
<td>6</td>
<td>-0.31</td>
<td>6.46e-05</td>
<td>0.00335</td>
</tr>
<tr>
<td>7</td>
<td>-0.33</td>
<td>2.04e-05</td>
<td>0.0013</td>
</tr>
<tr>
<td>8</td>
<td>-0.383</td>
<td>6.0e-07</td>
<td>6.69e-05</td>
</tr>
<tr>
<td>9</td>
<td>-0.44</td>
<td>&lt;1e-07</td>
<td>&lt;1e-07</td>
</tr>
<tr>
<td>10</td>
<td>-0.512</td>
<td>&lt;1e-07</td>
<td>&lt;1e-07</td>
</tr>
<tr>
<td>11</td>
<td>-0.52</td>
<td>&lt;1e-07</td>
<td>&lt;1e-07</td>
</tr>
</tbody>
</table>
Table 2.3 miR expression profile associated with SPARC expression in younger patients

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>Parametric p-value</th>
<th>FDR</th>
<th>UniqueID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.392</td>
<td>1.5e-06</td>
<td>0.000524</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_miR_130a</td>
</tr>
<tr>
<td>2</td>
<td>0.378</td>
<td>3.5e-06</td>
<td>0.000611</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_miR_126</td>
</tr>
<tr>
<td>3</td>
<td>0.338</td>
<td>3.77e-05</td>
<td>0.00329</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_miR_126</td>
</tr>
<tr>
<td>4</td>
<td>0.285</td>
<td>0.0005687</td>
<td>0.0397</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_miR_130b</td>
</tr>
<tr>
<td>5</td>
<td>-0.273</td>
<td>0.0009624</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_let_7b</td>
</tr>
<tr>
<td>6</td>
<td>-0.351</td>
<td>1.85e-05</td>
<td>0.00215</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsa_miR_1</td>
</tr>
</tbody>
</table>
2.3.5 SPARC activates ILK/AKT/β-catenin signaling pathways

Having demonstrated that AML cells overexpressing SPARC supported a more aggressive disease phenotype, next we sought to determine the molecular mechanisms of SPARC in leukemogenesis. We started with the notion that secreted SPARC interact with several cell surface receptors including integrins, such as α5β1, αvβ5, and αvβ3 (133, 134). Integrin β1 was found to interact with SPARC and in turn activate Integrin Linked Kinase (ILK), a multifunctional cytoplasmic serine/threonine kinase (80, 82), shown to activate PI3K/AKT phosphorylation and inactivates glycogen synthase kinase 3β (GSK3β) thereby resulting in enhanced β-catenin signaling pathway that support cell survival (84, 135, 136). Thus, we hypothesized that these mechanisms become aberrantly operative in SPARC overexpressing cells and contribute to leukemia growth.

In the media collected from THP-1/SPARC cell culture, we found a significantly higher levels of SPARC protein when compared to that obtained from THP-1/EV cells (Figure 2.10a). Co-immunoprecipitation assay with SPARC and integrin antibodies showed higher binding of the protein to integrins α5, αv, β1, β3, in THP-1/SPARC cells compared with THP-1/EV cells thereby supporting a potential SPARC autocrine integrin-binding mechanism in leukemia cells overexpressing SPARC (Figure 2.10b).

To determine whether the SPARC binding to integrins enhanced ILK activity, we then performed kinase activity assays. Lysates from THP-1/SPARC cells and THP-1/EV cells were immunoprecipitated with anti-ILK antibody and subjected to assays utilizing GSK3 fusion protein as an ILK substrate. THP-1/SPARC cells showed increased P-
GSK3αβ(Ser21/9) reflecting higher ILK activity than THP-1/EV cells (Figure 2.7c). Furthermore, we showed increase in P-AKT(Ser-473) and P-GSK3β(Ser9) in THP-1/SPARC cells compared to THP-1/EV (Figure 2.7d). Knocking down ILK or blocking integrin receptors with αv, β1, β3, antibodies abolished the AKT and GSK3β phosphorylation in THP-1/SPARC cells, thereby supporting the SPARC signaling through this pathway (Figure 2.10d, e).
Figure 2.10 SPARC activates ILK/AKT/β-catenin signaling pathways in THP-1 cells.

THP-1 cells were infected/transfected with pLenti-EV or pLenti-SPARC vectors, secreted SPARC protein levels were measure by western blot in media collected from cell culture (a). Co-immunoprecipitation assay with SPARC showed binding of SPARC to integrins α5, αv, β1, β3 (b). Lysates from THP-1/SPARC cells and THP-1/EV cells were immunoprecipitated with anti-ILK antibody and subjected to assays utilizing GSK3 fusion protein as an ILK substrate (c). P-AKT(Ser-473) and P-GSK3β(Ser9) levels in THP-1/SPARC cells compared to THP-1/EV following ILK Knock-down (d) or blocking integrin receptors with αv, β1, β3, antibodies (e).

GSK3β promotes β-catenin degradation, and when phosphorylated, this activity is suppressed, and β-catenin stabilization and nuclear translocation are enhanced (137). Similarly P-AKT(Ser473) induces β-catenin stabilization and nuclear translocation through phosphorylation of β-catenin (Ser552) residue (138). Both GSK3β and AKT are
downstream targets of ILK. Indeed we observed higher levels of p-β-catenin(Ser552) in THP-1/SPARC cells compared to THP-1/EV controls (Figure 2.12c). By confocal microscopy, we also showed an increase nuclear translocation of β-catenin in THP-1/SPARC compared to THP-1/EV cells (Figure 2.11).
Figure 2.11 SPARC enhances β-catenin nuclear translocation.

THP-1 cells were transfected with pLenti-EV or pLenti-SPARC vectors. Cells were stained with β-catenin antibody and Draq5 antibody (nuclear staining). Immunofluorescence confocal images showing β-catenin and cell nuclei as green and blue respectively.
Once in the nucleus, β-catenin interacts with TCF/LEF transcription factors and promotes expression of genes supporting cell growth and proliferation. Ectopic SPARC expression resulted in >4 fold increase of TCF/LEF reporter activity measured by luciferase assay in 293T cells transfected with SPARC compared with EV-controls (P<.001; Figure 2.12a). The SPARC-induced TCF/LEF activity was decreased by siRNA knock-down of ILK (Figure 2.12b). The SPARC-induced β-catenin-TCF/LEF activity was also supported by increased expression of the target genes c-MYC in THP-1 cells (P=0.04) (Figure 2.12c,d). Consistent with these results, we observed significant increase in target genes c-MYC and cyclin D1 expression in MV4-11 cells transfected with pLenti-SPARC compared to pLenti-EV transfected MV4-11 cells; also 1.8 fold increase in c-MYC expression in spleens from mice xenografted with MV4-11/SPARC compared to those xenografted with MV4-11/EV (P=0.002) (Figure 2.13).
Figure 2.12 SPARC activates TCF/LEF transcriptional activity and increase the expression of β-catenin target genes.

Luciferase activity were measured in 293T cells 24 hours following co-transfection with pBAR (TCF/LEF reporter vector) or pfuBAR (TCF/LEF reporter vector with mutated site) and pLenti-EV or pLenti-SPARC (a) and off-target siRNA or ILK siRNA (b). P-β-catenin (ser552) and c-MYC protein (c) and c-MYC mRNA (d) expression in THP-1 cells transfected with pLenti-EV and pLenti-SPARC.
Figure 2.13 SPARC activates ILK/AKT/β-catenin signaling pathways in MV4-11 cells.

MV4-11 cells were transfected with pLenti-EV or pLenti-SPARC vectors, P-AKT(Ser-473), P-GSK3β(Ser9), P-β-catenin (ser552) and c-MYC protein levels were measured (a) and (b) and cyclinD1 mRNA expression (c). c-MYC mRNA expression levels were measured in spleen tissues collected from MV4-11/EV and MV4-11/SPARC mice (d).
In order to confirm that SPARC activity in leukemia was mediated by ILK activation, THP-1/EV and THP-1/SPARC cells were treated with 1, 2 and 10 μM of T315, an ILK inhibitor, and MTS assays were performed and scored on days 1, 2, 4 and 7. We observed ~30% SPARC-induced growth advantage on day 7 in the vehicle-treated cells but no significant growth advantage in T315-treated cells (P= 0.04; Figure 2.14).
Figure 2.14 SPARC promotes growth advantage via ILK activation.

THP-1/EV and THP-1/SPARC cells were treated with 2 \( \mu \)M of T315, an ILK inhibitor, and MTS assays were performed and scored on day 7. Cells viability values on day 7 were normalized to that on day 1 and the growth advantage of SPARC was determined by calculating the cell growth ratio of THP-1/SPARC to that of THP-1/EV.
Finally, we validated our findings in primary blasts from AML patients (Table 2.4). Forced expression of SPARC in primary blasts resulted in increase of P-GSK3β(Ser9), P-β-catenin(Ser552) and P-AKT(Ser473) (Figure 2.15.a,b) and increase of c-MYC level measured by intracellular flow (Figure 2.15.d). In addition SPARC knock-out by siRNA decreased total β-catenin, as appeared by confocal microscopy (Figure 2.16) and decreased P-GSK3β(Ser9) and P-β-catenin(Ser552) protein levels (Figure 2.15.c), and c-MYC mRNA expression (Figure 2.15.e). SPARC overexpression and knockdown also resulted in significant increase and decrease of P-β-catenin(Ser675) respectively (Figure 2.17.a,b), a PKA mediated phosphorylation known to stabilize β-catenin (139).
Figure 2.15 SPARC activates ILK/AKT/β-catenin signaling pathways in primary blasts.

Primary blasts were infected/transfected with pLenti-EV or pLenti-SPARC, off target siRNA or SPARC siRNA; immunoblot analysis were performed on P-GSK3β(Ser9) (a,c), P-AKT(Ser473) (b), P-β-catenin (ser552) (a,c) and intracellular flow cytometry were performed to measure c-MYC protein levels (d) and qRT-PCR was used to measure c-MYC mRNA expression (e).
Figure 2.16 SPARC knock-down decreases β-catenin protein level in primary blasts. Primary blasts were transfected with pLenti-EV or pLenti-SPARC. Cells were stained with β-catenin antibody and Draq5 antibody (nuclear staining). Immunofluorescence confocal images showing β-catenin and cell nuclei as green and blue respectively.
Figure 2.17 SPARC increases P-β-catenin(Ser675) in primary blasts.

Primary blasts were infected/transfected with pLenti-EV or pLenti-SPARC, off target siRNA or SPARC siRNA; immunoblot analyses were performed on P-β-catenin (Ser675).
Table 2.4 List of patient samples used in *in vitro* experiments

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytogenetics</th>
<th>Mutation analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt #1</td>
<td>Inv(16)</td>
<td></td>
</tr>
<tr>
<td>Pt #2</td>
<td>Normal</td>
<td><em>FLT3-ITD/ NPM1-mut</em></td>
</tr>
<tr>
<td>Pt #3</td>
<td>Normal</td>
<td><em>FLT3-ITD</em></td>
</tr>
<tr>
<td>Pt #4</td>
<td>Normal</td>
<td><em>FLT3-ITD/ NPM1-mut</em></td>
</tr>
<tr>
<td>Pt #5</td>
<td>Normal</td>
<td><em>NPM1-mut</em></td>
</tr>
<tr>
<td>Pt #6</td>
<td>Normal</td>
<td><em>FLT3-ITD</em></td>
</tr>
<tr>
<td>Pt #7</td>
<td>Normal</td>
<td><em>FLT3-ITD/ NPM1-mut</em></td>
</tr>
<tr>
<td>Pt #8</td>
<td>Inv(16)</td>
<td></td>
</tr>
<tr>
<td>Pt #9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pt #10</td>
<td>Inv(16)</td>
<td></td>
</tr>
<tr>
<td>Pt #11</td>
<td>Normal</td>
<td><em>NPM1-mut</em></td>
</tr>
</tbody>
</table>
2.3.6 Distinct mutations in IDH2 and RUNX1 genes induce SPARC expression likely via activation of NF-κB/SP1 complex in CN-AML

Having dissected the downstream mechanisms that potentially mediate the contribution of SPARC to aggressive AML, next we asked the question of how SPARC expression becomes deregulated in AML. We previously reported that SPARC was among the most upregulated genes in the GEP associated with IDH2-R172 mutation that predicted unfavorable outcome in CN-AML patients (34). Here we showed that SPARC upregulation was indeed specifically associated with IDH2-R172 mutants (P<0.001) and not with other IDH1 or IDH2 mutations (Figure 2.18).
Box-plots of nanostring SPARC by IDH1/2 mutation status

Figure 2.18 SPARC expression in CN-AML patients according to their IDH1 and IDH2 mutational status.
In order to validate the association between \textit{IDH2-R172} and SPARC overexpression, we tested the expression of SPARC in THP-1 cells infected with \textit{IDH2-R172}. A significant increase in SPARC mRNA (3 fold increase) and protein levels were detected in THP-1 infected with \textit{IDH2-R172} compared with empty vector or \textit{IDH2-wt} infected cells (Figure 2.20.a,b). We also verified SPARC upregulation in Kasumi-1 and MV4-11 cells and primary blasts from AML patient (Figure 2.20c,d,e) transiently transfected with \textit{IDH2-R172} and found a significant increase in SPARC expression in \textit{IDH2-R172}-cells compared to \textit{IDH2-wt}-cells. THP-1 and Kasumi-1 cells transfected with \textit{IDH1-R132} and MV4-11 cells transfected with \textit{IDH2-R140Q} mutation constructs did not show significant increase in SPARC expression compared to their respective controls (Figure 2.20a,b,c,f).

Higher SPARC expression was also observed in the unfavorable RUNX1-mutated CN-AML subset compared with the RUNX1-wt subsets (Figure 2.19). Interestingly, SPARC was reported to be overexpressed in patients with t(8,21) and inv(16) AML (122). Because of the heterogeneity of RUNX1 mutations, we utilized a loss of function approach to examine the effect of RUNX1 on SPARC expression. Knockdown of RUNX1-RUNX1T1 translocation by siRNAs in Kasumi-1 cells resulted in significant decrease in SPARC expression (Figure 2.20g); while knock-down of \textit{RUNX1}wt in MV4-11 cells resulted in SPARC overexpression (Figure 2.20h).
Figure 2.19 SPARC expression in CN-AML patients according to their RUNX1 mutational status.
Figure 2.20 Mutations in IDH2 and RUNX1 genes induce SPARC expression.

SPARC protein levels in THP-1 (a) and Kasumi-1 (c) cells and mRNA levels in THP-1 cells (b) were measured in infected/transfected cells with pLenti-EV, pLenti-IDH1-WT, pLenti-IDH1-R132, pLenti-IDH2-WT pLenti-IDH2-R172 by immunoblot and qRT-PCR respectively. SPARC protein levels were measured in Primary blasts infected with pLenti-IDH2-WT and pLenti-IDH2-R172 (e), and in MV4-11 cells transfected with pLenti-EV, pLenti-IDH2-WT, pLenti-IDH2-R172 (d) pLenti-IDH2-R140Q (f). Kasumi-1 cells transfected with AML-ETO siRNA and SPARC protein level was measured 72 hours after (g). MV4-11 cells were transfected with RUNX1 siRNA and SPARC protein expression was measured 48hours after.
To understand how IDH2-R172 and RUNX1 mutations induce SPARC expression, we searched SPARC promoter for transcription factors that might be involved. Utilizing TFSEARCH program, SPARC promoter sequence revealed several binding sites for SP1, RUNX1 and NF-κB among other transcription factors. To examine the effect of these transcription factors on SPARC promoter activity, we cloned SPARC promoter region in PGL4.11 luciferase vector and used it along with expressing vectors of each of the different transcription factor (SP1, RUNX1 and P65 (NF-κB subunit)) to co-transfect 293T cells. Luciferase activities measured 24 hours later, showed significant increase (P<.001, each) in SPARC promoter activity in cells transfected with SP1 or P65 respectively (Figure 2.21). Furthermore, a significant decrease in SPARC promoter activity was observed when cells were transfected with RUNX1 expression vector (80%, P<.001) or SP1 siRNA (50%, P<.001) (Figure 2.21a). In addition, ectopic expression of SP1 and P65 resulted in significant increase in SPARC protein level compared to empty vector transfected MV4-11 and THP-1 cells (Figure 2.21b,e), while SP1 and P65 knock-down by siRNAs resulted in significant decrease in SPARC protein level in Kasumi-1 cells (Figure 2.21c,d).
Figure 2.21 SPARC expression is regulated by NF-κB, SP1, and RUNX1 transcription factors.

293T cells were co-transfect with PGL4.11 luciferase vector contain the SPARC promoter region (PGL4.11/SPARC-promoter) and expressing vectors of each of the different transcription factor (SP1, RUNX1 and P65 (NF-κB subunit)), Luciferase activities measured 24 hours later, and normalized to that in 293T cells co-transfect with PGL4.11/SPARC-promoter and EV vector (a). SPARC protein expression levels were measure following SP1 ectopic expression in MV4-11 cells (b), SP1 or P65 knock-down in Kasumi-1 cells (c,d), and p65 expressing vector in THP-1 cells (e).
IDH2-R172 mutation was found to result in accumulation of 2-Hydroxyglutarate (2HG) in both glioblastoma and AML (140). This accumulation resulted in inhibition of 2-oxoglutarate dependent oxygenases including the Jmjc lysine demethylases such as FBXL11 (140, 141). FBXL11 was found to negatively regulate transcriptional NF-κB activity through lysine demethylation (142). On the other hand, RUNX1 inhibits NF-κB signaling through interaction with IκB kinase complex in the cytoplasm (143). RUNX1 mutants lacked the ability to inhibit NF-κB signaling; importantly patients with RUNX1-related leukemia exhibits distinctly activated NF-κB signaling (143). These reports led us to speculate that IDH2-R172 and RUNX1 mutations contribute individually to SPARC overexpression through NF-κB activating mechanisms.

To test this possibility we utilized NF-κB luciferase reporter assay and examined the effect of IDH2-R172 and RUNX1 ectopic expression on NF-κB activity. 293T cells cotransfected with IDH2-R172 and NF-κB luciferase vectors exhibit higher luciferase activity when compared with IDH2-wt transfected cells (Figure 2.22a), on the other hand RUNX1 ectopic expression in 293T cells resulted in significant decrease of NF-κB luciferase reporter activity compared to the empty vector transfected cells (Figure 2.22a). Importantly, 293T cells cotransfected with SPARC promoter luciferase vector and IDH2-R172 showed also increase of luciferase promoter activity (Figure 2.22b). Furthermore, chromatin immunoprecipitation (ChIP) showed P65 enrichment on SPARC promoter in IDH2-R172 transfected THP-1 cells compared with IDH2-wt transfected cells (Figure 2.22d). Although no significant change in P65 enrichment in RUNX1 transfected cells was observed, we found significant decrease in SP1 enrichment on SPARC promoter in RUNX1 transfected cells compared to EV-transfected cells (Figure 2.22e). These results
suggest that *IDH2*-R172 and *RUNX1* contribute individually to *SPARC* overexpression through NF-κB/SP1 activating mechanisms.

Figure 2.22 *IDH2*-R172 and *RUNX1* contribute individually to *SPARC* overexpression through NF-κB and SP1 mediating mechanisms.

293T cells cotransfected with pLenti-*IDH2*-R172 or pCMV-*RUNX1* and *NF-κB* luciferase vectors (a). 293T cells cotransfected with pLenti-*IDH2*-R172 and PGL4.11/SPARC-promoter vectors (b) and luciferase activities were measured 24 hours later, values were normalized to that in respective controls. Chromatin immunoprecipitation (CHIP) showed P65 enrichment on *SPARC* promoter in *IDH2*-R172 transfected THP-1 cells compared with *IDH2*-wt transfected cells (c). Decrease in SP1 enrichment on *SPARC* promoter in *RUNX1* transfected cells compared to EV-transfected cells (d).
2.3.7 SPARC is a novel therapeutic target in AML

Thus far, we demonstrated that SPARC overexpression resulted in more aggressive AML in vitro and in vivo, correlated with worse outcome in CN-AML patients and resulted in activation of signaling pathways associated with tumor progression. Therefore, SPARC may represent a novel therapeutic target in AML patients exhibiting high expression level of this gene. We found SPARC to be upregulated though mechanisms that activate NF-κB/SP1 complex. In addition, SPARC was previously found to be a target for miR-29a and miR-29c (144) and SPARC 3’UTR region was predicted to have binding sites for miR-29b (145, 146). To confirm these reports we cloned SPARC 3’UTR region in luciferase reporter vector (pGL3) and used the generated vector to co-transfect 293T cells with the following synthetic miRNA (miR-29b, miR-203, and miR-9*) or scramble oligos. We found 60% decrease of SPARC 3’UTR-luciferase activity in the presence of miR-29b compared to that in the presence of the scramble or the other miRs (P<0.0001) (Figure 2.20a).

Additionally we have previously identified SP1 as a direct target for miR-29b (147). In sum, these observations suggest that modulating NF-κB/SP1 and/or miR-29b may provide a therapeutic advantage for AML characterized by SPARC upregulation. Bortezomib, a proteasome inhibitor with antitumor activity was found to exhibit antileukemic activity through miR-29b-dependent SP1 downregulation (125). Therefore we hypothesized that bortezomib, may provide a promising therapeutic approach to disrupt SPARC expression and efficiently override aberrant SPARC activity in AML.
Thus, Kasumi-1 cells were treated with 0, 20, 60 and 100 nM of Bortezomib; immunoblot analysis at 24 hours after Bortezomib treatment showed significant dose dependent increase of \( \text{miR-29b} \) (Figure 2.23a) decrease in SPARC RNA and protein levels (Figure 2.23 b,c). Consistent with these results, we found SPARC levels to be decreased on day 26 in samples of patients treated on a phase 1 dose escalation trial of decitabine in combination with bortezomib (Figure 2.23e).
Figure 2.23 Bortezomib inhibits SPARC expression.

SPARC 3'UTR-luciferase activity in the presence of miR-29b was measured by co-transfecting 293T cells with SPARC-3'UTR reporter and a synthetic miRNA, values were normalized to that of cells transfected with scramble(a). Kasumi-1 cells were treated with 20, 60, and 100 nM of Bortezomib; miR-29b (b) and mRNA levels of SPARC and SP1 (c) were measure 24 hours later by qRT-PCR. SPARC, SP1 and c-MYC protein levels were measured by western blot (d). SPARC mRNA levels in bone marrow samples from patients treated on phase 1 dose escalation trial of decitabine in combination with bortezomib (e).
2.4 Discussion

We demonstrated here the functional, mechanistic and clinical implication of SPARC in AML. Our study reveals that high SPARC expression in AML cells promoted growth advantage in vitro and aggressive disease in vivo, likely via activation of ILK/AKT/β-catenin pathways. Furthermore, SPARC overexpression independently associated with worse outcome in CN-AML patients.

Several studies have reported elevated SPARC expression in invasive malignant tumors (115, 148). High SPARC was associated with malignant melanomas progression (112, 113, 149) and meningiomas and gliomas invasiveness (86, 115, 150-152). On the contrary, in other types of cancer (colon cancer, ovarian cancer), SPARC has also been found to be associated with tumor suppression (153, 154). In MDS, 5q deletion results in the loss of one copy of SPARC among other genes and significant downregulation of SPARC expression in the progenitor compartment (119, 120). The involvement of SPARC in MDS was supported by the dramatic upregulation of SPARC in response to lenalidomide, a drug approved for treatment of MDS patients (119). Although this suggested a possible tumor suppressor role of SPARC in del(5q) MDS, no biological evidence was found to support this notion. Indeed SPARC was found to be dispensable for murine hematopoiesis suggesting that SPARC deficiency does not alter the hematopoietic potential of normal bone marrow (155). Tripodo and colleagues have later reported that increased SPARC expression in the bone marrow stroma contributes to the detrimental fibrotic changes associated with myeloproliferation, while loss of SPARC expression induces a defective stromal niche that supports myeloid expansion (156). Being expressed by both stromal and hematopoietic cells, with different functions
depending on the cellular source, SPARC contributions to myeloid neoplasms seem at least controversial and remain to be fully elucidated.

Here we investigated SPARC overexpression in AML by functional approaches in both mouse models and human AML. We demonstrated that SPARC promotes aggressive leukemia growth, thereby implicating SPARC as a potential oncogene. This seems in disagreement with a previous report by DiMartino et al, where exogenous SPARC protein was found to induce growth inhibition in AML with MLL rearrangements (122). The discrepancy between the two studies may be related to difference in experimental conditions. In fact, while we forced ectopic SPARC expression in leukemia cells, Di Martino et al. exposed the leukemia cells directly to recombinant exogeneous SPARC protein. This raises the question of, whether post-translational modifications occur in the endogenously produced protein and may be required for the leukemogenesis. Furthermore, while we showed a likely autocrine SPARC function that allows for interaction of SPARC with membrane proteins such as the integrins, it cannot be excluded the involvement of SPARC in intracellular signaling pathways different from those activated by the extracellular protein. Indeed, the intracellular retention of SPARC was seemingly required for its protective effect in imatinib resistant CML (123). In support of the negative impact of SPARC in human AML, we showed that expression of this gene predicted a lower CR rate and shorter DFS and OS in younger and older patients with primary CN-AML and treated similarly with cytrabine-anthracyline regimens on CALGB upfront protocols. Indeed, SPARC expression was found higher in patients with concurrent unfavorable prognostic mutations (i.e., IDH2-R172 and RUNX1) or aberrantly upregulated genes (i.e., BAALC and MN1) and lower in patients with
concurrent favorable prognostic mutations (*NPM1*). It should be underscored, however, that multivariable analyses in our study demonstrated that *SPARC* levels are prognostically independent from the aforementioned mutations, suggesting that while *IDH2-R172* and *RUNX1* mutants may activate pathways leading to *SPARC* overexpression, these mechanisms are not private of these mutations. Indeed, analysis of the *SPARC* promoter revealed several binding sites for NF-κB and SP1 in addition to *RUNX1*. These transcription factors have been found to participating in transactivating complexes leading to upregulation of target genes in AML cells (157).

Our initial observation supports the notion that *SPARC* overexpression enhanced β-catenin signaling pathway, known to be required for leukemia growth and stemness (158, 159). Indeed, in GEP and MEP associated with high *SPARC* expression, we observed upregulation of hematopoietic stem cell gene and microRNA markers such as *CD34, CD133, TGFβ* genes and *miR-126* and *miR130a* that have been associated with leukemia stemness. The *SPARC*-dependent activation of β-catenin, resulted from upstream binding of *SPARC* with integrin and activation of ILK, which indeed was found activated in leukemic cells (160). ILK a kinase that links the cell adhesion receptors, integrins, and growth factors to the downstream signaling pathways that involve AKT and GSK3β resulting in the nuclear localization of β-catenin and activated transcription, cell cycle progression, and cell proliferation (161). ILK inhibitors in combination with FLT3 inhibitors were found to result in selective targeting of AML rather than normal hematopoietic progenitors (160, 162). While *SPARC/ILK/AKT* signal transduction pathways were found involved in glioma cell invasion and survival (85), to our knowledge this mechanism has not been previously reported in AML.
In conclusion, high SPARC expression contributes to aggressive AML growth via β-catenin activation-mediated mechanisms. SPARC overexpression independently predicted worse outcome in CN-AML patients. Furthermore, given the involvement of SPARC in leukemia growth and aggressive disease, mapping out the SPARC-related leukemogenic pathways may open new avenues for therapeutic intervention. Indeed, it is equitable to speculate that SPARC may represent a novel therapeutic target in AML for compounds inhibiting NF-κB activity or directly interfering with β-catenin transactivation.
Chapter 3: Pharmacological Activity of Silvestrol in High-Risk Acute Myeloid Leukemia

3.1 Introduction

The outcome for high-risk patients who are treated with allogeneic stem cell transplantation in first complete remission are encouraging, but lack of suitable donors, presence of co-morbidities and treatment-related toxicity and mortality has often limited the application of this approach. Therefore, novel therapeutic strategies that improve the currently poor outcome in AML patients while demonstrating an optimal toxicity index are highly needed.

As we have shown in chapter 1, recurrent cytogenetics and molecular aberrations are known to impact the prognosis of AML. Importantly, several of these genomic aberrations may also comprise novel therapeutic targets. Gain-of-function mutations of the tyrosine kinase (TK) receptor encoding gene FLT3 occur in approximately 30% of AML patients, and result in constitutive TK activity and, in turn, increasing growth and survival of leukemia blasts. Of the FLT3 mutations, the internal tandem duplication (FLT3-ITD) is associated with poorer outcome (23, 163-167). In addition, overexpression of the FLT3-wt receptor and its ligand (FL) occur in a high
percentage of AML and the subsequent autocrine stimulatory loop may contribute to the pathogenesis and aggressiveness of the disease (168, 169). Therefore, investigating compounds that can inhibit both mutant and overexpressed wild type FLT3 in AML leukemia is warranted.

Smith et al. demonstrated that FLT3-ITD likely constitutes a driver mutation in AML and therefore it may represent not only as a prognosticator but also a potential therapeutic target (170). Emerging small molecule inhibitor compounds have been shown to interfere with the aberrant FLT3 TK activity and lead to arrest of leukemia growth (165). Unfortunately the clinical impact of these compounds as single agents or in combination with chemotherapy has not yet fulfilled the promise, likely because of their relatively low potency, lack of specificity and the early onset of mechanisms of resistance (171-173). This underscores the need for additional strategies that effectively target aberrant FLT3 activity in AML blasts and improve the currently poor survival rate in high-risk patients with FLT3-driven AML.

In addition to the activation of FLT3 kinase activity, another hallmark of FLT3-ITD positive AML is the upregulation of miR-155 (46-48). Importantly, studies have shown that sustained expression of miR-155 caused granulocyte/monocyte expansion and resulted in pathological features of myeloid neoplasia in a mouse model (49). Also, miR-155 has a known oncogenic activity in hematologic malignancies and may serve as clinical biomarker for disease progression, and therefore a novel therapeutic target (174, 175).

Silvestrol is a cyclopenta[b]benzofuran rocaglate with a unique dioxanyl ring-containing side chain (176, 177). It was isolated from the Indonesian plant Aglaia foveolata, structurally characterized, and tested for anti-tumor efficacy (176). Silvestrol
showed activity against several solid tumor cell lines (178-181) as well as primary chronic lymphocytic leukemia cells at nanomolar concentrations, and prolonged survival in a murine model of B cell acute lymphoblastic leukemia (182). More recently, silvestrol was reported to have synergistic activity against AML cell lines when combined with chemotherapy (183). Pelletier et al, have demonstrated that silvestrol interferes with assembly of the eIF4F translation complex by promoting an aberrant interaction between capped mRNA and eIF4A, thus blocking protein synthesis at the initiation step (181, 184). This inhibition of protein synthesis results in a preferential depletion of proteins with short half-lives, such as MCL1, to which leukemia and cancer cells may be addicted and thrive on (182, 184). Therefore, we hypothesize that silvestrol could also inhibit translation of FLT3 mRNA and in turn downregulate the expression of FLT3 and decrease aberrant tyrosine kinase in FLT3-driven AML. Thus, we sought to examine the in vitro and in vivo anti-leukemic and biological activity of silvestrol in FLT3-ITD or FLT3-wt overexpressing AML cell lines and primary blasts.
3.2 Materials and Methods

3.2.1 Reagents

Silvestrol was kindly provided by Dr. A. Douglas Kinghorn. PKC412 was purchased from LC Laboratories (Woburn, MA, USA). Human FLT3 ligand (FL) was purchased from PeproTech (Rocky Hill, NJ, USA).

3.2.2 Cell lines and primary blasts

MV4-11 and THP-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% calf serum. Blasts from AML patients were maintained in RPMI 1640 medium supplemented with 30% fetal bovine serum, 1% HEPES buffer, and 1x StemSpan CC100 (StemCell Technologies, Vancouver, BC, Canada) containing IL-3, IL-6, FLT3 ligand and SCF. All cells were incubated at 37°C with 5% CO₂. Patient AML blasts 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. Authenticating tests of these cell lines was done using monoclonal antibodies and FLT3 mutational analysis.

3.2.3 Immunoblot analyses

Cells were suspended 30 min in 1x lysis buffer (20 mM Hepes, 150 mM NaCl, 0.1% NP40) containing protease inhibitor cocktail III (Calbiochem, Darmstadt, Germany) and lysate was recovered by centrifugation. Lysates were separated using 4-20% SDS-PAGE and transferred to PVDF membrane (GE Healthcare, Piscataway, NJ).
Membranes were blocked using 5% milk or BSA in 1x TBS with 0.1% Tween 20 (1xTBS-T) for 1 hour at room temperature with shaking, then incubated overnight at 4°C in the following primary antibodies diluted in 1xTBS-T with 5% milk or BSA: actin (Santa Cruz Biotechnology, Santa Cruz CA), FLT3 (Cell Signaling, Danvers, MA), phosphorylated and total STAT5 (Cell Signaling), P65 antibody (Billerica, MA). Membranes were washed using 1xTBS-T, incubated with HRP-conjugated secondary antibodies diluted in 1xTBS-T with 5% milk or BSA, washed, and developed using ECL Western Blotting Detection reagents (GE Heathcare).

3.2.4 RNA immunoprecipitation (RIP), RNA extraction, Real-Time RT-PCR

MV4-11 cells were treated with 50 nM silvestrol for 3 hour, lysed (5 min) in 100 mM KCl, 5 mM MgCl₂, 10 mM HEPES [pH 7.0], 0.5% NP-40, 1 mM dithiothreitol (DTT), 100 units/ml RNase OUT (Invitrogen), 400 mM vanadyl-ribonucleoside complex and protease inhibitors (Roche, Mannheim, Germany). Extracts were clarified and stored at -80°C. Anti-eIF4E antibody (cell signaling) and goat IgG (Sigma, St. Louis, MO) were incubated with protein sepharose A/agarose G-coupled beads overnight. Beads were subsequently washed four times with 50 mM TRIS/HCl, pH 7.0, 150 mM NaCl, 1 mM MgCl₂, and 0.05% NP-40, and twice after addition of 1M urea. Precipitates were digested with proteinase K (55°C), and eIF4E-associated mRNAs were isolated using Trizol reagent (Invitrogen, Grand Island, NY). cDNA was synthesized using SuperScript III reagents (Invitrogen) and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative Real-Time RT-PCR for FLT3 and PU1 genes and miR-155 and miR-34a expression was performed using commercially available TaqMan Gene Expression Assay primers and
probes and the 7900HT Fast Real-Time PCR System (Applied Biosystems). The comparative cycle threshold (C_{T}) method was used to determine the expression levels normalized by the internal control 18S for gene expression.

### 3.2.5 Clonogenic and Viability Analysis

Methylcellulose clonogenic assays were carried out by plating 2X10^4 primary blasts in 0.9% MethoCult (Stem Cell Technologies). Colonies (>100 mm) from cell lines and primary cells were scored 14 days later.

### 3.2.6 Flow cytometry

For FLT3 detection, cells (5×10^5) were washed with phosphate-buffered saline (PBS) and resuspended in 50 µl binding buffer containing 5 µL FLT3 antibody (BD Biosciences, Billerica, MA). After 15 min incubation, cells were washed with PBS, resuspended in 400 µL flow buffer and analyzed on a FACSCalibur cytometer (BD Biosciences). To assess apoptosis, AML cells were incubated with 10, 30 and 50 nM silvestrol for 24 hours. Cells (5×10^5) were then washed with PBS and resuspended in 50 µl binding buffer containing 2 µL of annexin V-FITC stock (BioWhittaker, Inc, Walkersville, MD) and 5 µL propidium iodide (PI) (BD Biosciences). After 20 min incubation, fluorescence was quantified by flow cytometry on a FACSCalibur instrument.

### 3.2.7 MV4-11 xenograft murine model

This model was developed recently in our laboratory. Briefly, 4~6 week-old non-obese diabetic severe combined immunodeficient gamma (NSG) mice (NOD.Cg-Prkdcsid Ii2rgtm1Wjl/SzJ, The Jackson Laboratory, Bar Harbor, ME) were
intravenously (i.v) injected via tail vein with 2x10^7 MV4-11 cells. Two months later, the spleen mononuclear cells (MNCs) were isolated from MV4-11 cells injected mice (1st-adapted MV4-11 cells in NSG mice). The adapted spleen MNCs were injected into the new NSG mice via tail vein. The loading dose of cells was reduced to 50% in second and third transplantation and only 1X10^7 cells/mouse were used for second injection. About a month later, the spleen MNC were isolated from 2nd-adapted MV4-11 cells in NSG mice. The 3rd transplantation was performed using 0.5 x10^7 cells/mouse spleen MNC from 2nd adapted NSG mice. For the subsequent transplantation model, 0.5X10^6 spleen MNC cells were used. The complete blood count and FACS analysis of CD45 and FLT3 expression and cytospin were examined weekly to monitor the progression of disease. Generally, these mice develop AML-like disease in two weeks after the injection of spleen MNC from 3rd adapted MV4-11 cells of NSG mice and their lifespan is around 3~4 weeks. All the experiments were conducted in accordance with the institutional guidelines for animal care and use. Spleen cells (0.5x10^6) from MV4-11 transplanted NSG mice were intravenously (iv) injected into NSG mice via tail vein, and divided into groups for vehicle (hydroxypropyl beta-cyclodextrin in 30% sterile water; N=6) or silvestrol (1.5 mg/kg in vehicle; N=10) treatment. One control mouse (no leukemia/no treatment) was also included. Two to three weeks after engraftment, white blood count (WBC) and FLT3 expression by flow cytometry were assessed to confirm transplantation. Treatments with silvestrol or vehicle were initiated 21 days after engraftment (based on disease signs documented by WBC count and FLT3 expression). Administration was by intraperitoneal injection every 48 hours for up to three weeks or until euthanasia criteria were met. Expected median survival of untreated animals in this model is 28 days. Mice were weighed daily and
checked for signs of dehydration, discomfort or toxicity. On day of administration, doses were recalculated for each animal after weighing to maintain 1.5 mg/kg. For the pharmacodynamic study, 6 mice were used (3 per group, vehicle and silvestrol). These mice were given 3 doses of either vehicle or silvestrol; and 48 hours following the third dose, spleens were isolated and mononuclear cells obtained for immunoblotting assay. For pathological examination, tissue sections from the liver and spleen were fixed on formalin, embedded in paraffin blocks, and H&E stained.

### 3.2.8 Wright-Giemsa staining

Morphological signs of apoptosis were detected by Wright-Giemsa staining. Smears of control and treated cells were stained with Wright-Giemsa solution for 25 min, rinsed with distilled water and air dried. Cell morphology was studied by light microscopy.

### 3.2.9 MTS assay for growth inhibition

Briefly, 5.0×10^4 cells were incubated in triplicate in a 96-well plate in the presence or absence of the indicated test samples in a final volume of 100 µl for 24, 48 and 72 hours at 37°C. Thereafter, 20 µl MTS solution (Promega, Madison WI) was added to each well. After 4 hours incubation at 37°C, plates were shaken and the optical density at 490 nm was measured. Percent cell viability was calculated as cell viability of the experimental samples/cell viability of the control samples × 100. At least three independent experiments were performed.
3.3 Results

3.3.1 Silvestrol antileukemia activity in \textit{FLT3}-ITD and \textit{FLT3}-wt expressing AML cells

We first examined the antileukemia activity of silvestrol in AML cell lines. MV4-11 cells are \textit{FLT3}-ITD positive, whereas THP-1 cells are negative for \textit{FLT3}-ITD but express robust levels of \textit{FLT3}-wt. measured by MTS assay we found that leukemia growth decreased in a dose- (5 to 160 nM) and time-dependent manner following silvestrol treatment (Figure 3.1a). At 48 hours, ICs\textsubscript{50} (concentration required to inhibit growth to 50\% of control) were 2.6 and 3.8 nM in MV4-11 and THP-1, respectively (Figure 3.1b and Table 3.1). Primary blasts from three AML patients with \textit{FLT3}-ITD and two AML patients with \textit{FLT3}-wt were treated with 5 to 320 nM silvestrol. A dose-dependent decrease in proliferation was observed, with ICs\textsubscript{50} values at 48 hours of 4.0, 6.4 and 3.6 nM for the blasts from the \textit{FLT3}-ITD patients and 6.6 and 16.9 nM for the blasts from the \textit{FLT3}-wt patients (Figure 3.1c and Table 3.1). We also performed a colony forming assay of silvestrol in primary samples from three different patients including two \textit{FLT3}-ITD positive cases. We observed no colony formation for any of the tested samples when blasts were treated with 25 nM of silvestrol (Figure 3.1d).

To determine whether the antileukemic activity of silvestrol was mediated by enhanced apoptosis, we utilized annexin and PI staining of cells treated with 10-50 nM silvestrol for 48 hours. A dose-dependent increase in apoptosis was observed in silvestrol treated compared to vehicle-treated primary blasts from \textit{FLT3}-ITD (n=3) as well as \textit{FLT3}-wt (n=2) AML patients (Figure 3.2). In AML primary cells, silvestrol induced 1 to 7.5 fold increase in apoptosis in both \textit{FLT3}-wt and \textit{FLT3}-ITD blasts (P=0.0001 for every
tested case) treated with 10, 30 and 50 nM of silvestrol compared with vehicle treated controls.

Figure 3.1 Antileukemic activity of silvestrol in vitro.
(a) MV4-11 cells were incubated with 5 to 160 nM silvestrol. Cell viability was evaluated by a MTS assay at 24, 48 and 72 hours. (b) AML cell lines were incubated with 5 to 160 nM silvestrol, and viability was evaluated by a MTS assay 48 hours later. (c) AML primary cells unmutated FLT3 or with FLT3-ITD were incubated with 5 to 320 nM silvestrol. Cell viability was evaluated by MTS assay 48 hours following treatment. (d) Colony forming ability in primary blasts from FLT3-ITD positive and negative AML patients treated with 25nM of silvestrol and scored 14 days later.
Figure 3.2 Silvestrol induces apoptosis in primary blasts \textit{in vitro}.

Primary blasts from \textit{FLT3}-ITD positive and negative AML patients treated with 10, 30 and 50 nM of silvestrol for 48 hours and analyzed by annexin/PI flow cytometry.
Table 3.1 Calculated IC$_{50}$ values for AML primary cells and cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FLT3 Status</th>
<th>IC$_{50}$ (95% Confidence Intervals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV4-11</td>
<td>FLT3-ITD</td>
<td>2.65 nM (1.49 to 4.72)</td>
</tr>
<tr>
<td>THP-1</td>
<td>FLT3-wt</td>
<td>3.81 nM (2.46 to 5.09)</td>
</tr>
<tr>
<td><strong>Primary cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt #1</td>
<td>FLT3-wt</td>
<td>16.9 nM (13.6 to 21.1)</td>
</tr>
<tr>
<td>Pt #2</td>
<td>FLT3-wt</td>
<td>6.61 nM (5.67 to 7.70)</td>
</tr>
<tr>
<td>Pt #3</td>
<td>FLT3-ITD</td>
<td>3.65 nM (2.21 to 6.04)</td>
</tr>
<tr>
<td>Pt #4</td>
<td>FLT3-ITD</td>
<td>4.89 nM (3.90 to 6.12)</td>
</tr>
</tbody>
</table>

3.3.2 Silvestrol downregulates FLT3 expression through inhibition of FLT3 translation initiation

Silvestrol interferes with assembly of the eIF4F translation complex by promoting an aberrant interaction between capped mRNA and eIF4A, thus inhibiting mRNA translation at the initiation step and therefore blocking protein synthesis (181, 184). This inhibition of protein synthesis results in a preferential depletion of proteins with short half-lives to which leukemia and cancer cells may be addicted (182, 184). While several proteins have been shown to be targeted by silvestrol, whether this compound also inhibits FLT3 initiation of translation resulting in inhibition of FLT3 protein synthesis has
not been reported. Thus, using an RNA immunoprecipitation assay, we tested this possibility.

Because of the difficulty immunoprecipitating eIF4A protein for the RNA immunoprecipitation assay using commercially available antibodies, we utilized an antibody against eIF4E, a subunit of the initiation of translation complex that binds the mRNA cap structure. Cells were exposed to silvestrol at concentrations that were higher than the calculated in vitro IC_{50} but that were also achievable in vivo, and harvested at 24 hours or earlier time points, to avoid interfering with cell death observed with more prolonged drug exposure but still ensure adequate pharmacologic activity (185). MV4-11 cells were treated with 50 nM silvestrol for 3 hours and eIF4E was immunoprecipitated in three different experiments. Associated RNA was then assessed for depletion of FLT3 RNA by quantitative RT-PCR. While no change in FLT3 mRNA levels was observed in total RNA from MV4-11 cells treated with silvestrol compared to vehicle-treated cells, a five-fold depletion of FLT3 mRNA was detected in eIF4E immunoprecipitates from silvestrol-treated cells compared to vehicle-treated cells at 3 hours (Figure 3.3a). This resulted in 54% and 46% reduction in FLT3 receptor expression (by flow cytometry) (Figure 3.3b) and 92% and 84% reduction in FLT3-ITD or FLT3-wt protein levels (by western blot) respectively in MV4-11 and THP-1 cells, exposed to 50 nM silvestrol for 24 hours, compared to vehicle-treated controls (Figure 3.3c). Since total FLT3 protein levels were almost undetected following silvestrol treatment, we expected phosphorylated FLT3 to be depleted as well. Therefore, the effect of silvestrol on FLT3 kinase activity was determined by measuring phosphorylation of the FLT3 target protein STAT5. We found a significant decrease in P-STAT5 in MV4-11 cells 24 hours following silvestrol treatment (50nM) (Figure 3.3d). Similarly, we confirmed the effect of silvestrol
on FLT3 expression in primary blasts from FLT3-wt and FLT3-ITD patients. We also found down-regulation of FLT3 protein in silvestrol-treated primary blasts regardless of their mutational status (Figure 3.3e). On the mRNA level, and consistent with a sole effect on mRNA translation, silvestrol treatment resulted in increase in FLT3 mRNA levels in FLT3-wt AML cell line and primary blasts (Figure 3.4). However we observed a significant decrease in FLT3 mRNA expression in FLT3-ITD positive cell lines and primary cells and FLT3-wt cell lines treated with 25ng/ml of FLT3 ligand (FL), suggesting that once the receptors are activated by gain-of-function mutations or ligand binding, FLT3 downregulation may interfere with an autoregulation mechanisms of gene expression (Figure 3.5) (157).
Figure 3.3 Silvestrol down-regulates FLT3 protein expression.

(a) FLT3 mRNA expression in eIF4E immunoprecipitates from 50 nM silvestrol treated MV4-11 cells compared to that from untreated cells. (b) Expression of FLT3 examined by flow cytometry in MV4-11 and THP-1 cells after 24 hours exposure to 50 nM silvestrol. Blue = silvestrol-treated samples; Red = vehicle-treated control. (c) AML cells treated with 50 nM silvestrol and examined for FLT3 expression by immunoblotting. (d) Primary AML cells incubated with increasing concentrations of silvestrol for 24 hours, and assessed for FLT3 protein expression was by immunoblot. (e) MV4-11 cells treated with silvestrol or PKC-412 (50 nM each) for 6 hours, and assessed for STAT5 phosphorylation by immunoblot.
Figure 3.4 Silvestrol affects *FLT3* mRNA expression differently in *FLT3*-wt and *FLT3*-ITD AML cells.

THP-1 and MV4-11 cells and *FLT3*-wt and *FLT3*-ITD primary blasts were treated with 50nM of silvestrol for 24 hours, and *FLT3* mRNA expression was measured thereafter, mRNA expression is normalized to *18S*. 
Figure 3.5 Silvestrol down-regulates *FLT3* mRNA expression in THP-1 cells in the presence of FLT3 ligand.

THP-1 cells treated stimulated with 25ng/ml FL ligand and treated with 50nM of silvestrol for 24 hours, then *FLT3* mRNA expression was measure thereafter.
3.3.3 Silvestrol downregulates miR-155 expression in FLT3-ITD positive AML

We reported that miR-155 is up-regulated in FLT3-ITD positive AML compared to FLT3-wt AML although whether this microRNA directly contributes to the leukemogeneic activity of FLT3-ITD is unknown (46). Having shown that silvestrol downregulated FLT3-ITD protein, next we tested whether silvestrol also altered the expression of miR-155 that appears co-regulated with FLT3. Thus, miR-155 expression was measured in MV4-11 cells treated with 50 nM of silvestrol for up to 24 by qRT-PCR. We found approximately a 40-60% decrease in miR-155 expression in silvestrol treated compared to untreated cells as early as 6 hours following the treatment (P=0.05), while no significant change was observed in the expression of an unrelated miRs (i.e., miR-34) (Figure 3.6a, b and c). We also examined the effect of silvestrol on miR-155 function, we treated MV4-11 cells with 50nM silvestrol and assessed the mRNA expression of the miR-155 target gene PU1 (186). Twenty-four hours following treatment with silvestrol, we observed more than 2 folds increase of PU1 mRNA expression level in treated compared to untreated MV4-11 (P = 0.04) (Figure 3.6d). Similar results were obtained in FLT3-ITD positive primary blasts; these cells exhibited 80% decrease in miR-155 expression (P = 0.058) and a 2-fold increase in PU1 expression (P= 0.005), 24 hours following treatment with 50 nM of silvestrol compared with untreated cells (Figure 3.6e and 3.6f).

Although, the mechanisms through which constitutively activated FLT3 associates with increased miR-155 expression are unknown. miR-155 has been reported to be regulated by NF-κB, which is also potentially activated in AML blasts with constitutively activated FLT3 (187, 188). Importantly, silvestrol belongs to the rocaglate
derivatives, which have been reported to inhibit the activity of NF-κB (178, 189). Therefore we speculated that silvestrol may influence NF-κB activity and/or protein level. When we treated MV4-11 and THP-1 cells with 50 nM of silvestrol for 24 hours, we observed decrease in NF-κB (P65) protein expression in silvestrol-treated cells compared with vehicle-treated cells (Figure 3.6g). This suggests that the effect of silvestrol on miR-155 may be independent or only partially dependent of the silvestrol mediated FLT3 inhibition. Nevertheless, the compound effectively targets both the microRNA and the mutant protein that may concurrently contribute to the aggressiveness of FLT3-ITD AML.
Figure 3.6 Silvestrol down-regulates miR-155 and upregulates miR-155 target PU1.

(a) MV4-11 cells were treated with 50nM of silvestrol and miR-155 expression was measured and normalized to U44 expression at 6, 12 and 24 hours following silvestrol treatment. (b, c) MV4-11 cells were treated with 50nM of silvestrol for 24 hours, and miR-155 (b) and miR-34a (c) expression was measured thereafter. (d) MV4-11 cells were treated with 50 nM of silvestrol for 24 hours, and PU1 expression was measured thereafter. (e) FLT3-ITD positive primary cells were treated with 50nM of silvestrol for 24 hours, and miR-155 expression was measured thereafter. (f) FLT3-ITD positive primary cells were treated with 50nM of silvestrol for 24 hours, and PU1 expression was measured thereafter. (g) MV4-11 and THP-1 cells were treated with 50nM of silvestrol for 24 hours and assessed for P65 protein expression by immunoblot.
Figure 3.7 Continued

e  miR-155 expression in FLT3-ITD primary cells treated with 50nM of silvestrol for 24h

f  PU1 mRNA expression in FLT3-ITD primary cells treated with 50nM of silvestrol for 24h

MV4-11 (FLT3-ITD)  THP-1 (FLT3-wt)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>0.59</th>
<th></th>
<th>1</th>
<th>0.43</th>
</tr>
</thead>
<tbody>
<tr>
<td>P65</td>
<td>-</td>
<td>+</td>
<td>P65</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>-</td>
<td>+</td>
<td>Actin</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
3.3.4 Activity of silvestrol in FLT3-ITD positive leukemia grafts

To investigate the *in vivo* efficacy of silvestrol in AML we employed MV4-11 leukemia graft murine model. NSG mice were subjected to secondary transplant with MV4-11 cells harvested from the spleen of previous MV4-11 engrafted mice that developed an aggressive AML-like disease. Twenty-one days post-engraftment, mice were treated intraperitoneally with vehicle (Group 1) or 1.5 mg/kg silvestrol (Group 2) every other day for 3 weeks. The silvestrol dose and schedule used here were previously reported for lymphoid leukemia models (182, 190). Forty-eight hours following the first two doses, blood samples with circulating MV4-11 cells were taken from 3 mice from each group, and FLT3 expression was assessed by flow cytometry. A two- and three-fold increase in FLT3 expression was observed in vehicle-treated mice 48 hours after the first (P=0.07) and second (P=0.024) treatment doses compared with pretreatment baseline, while no significant change was found in the silvestrol treatment group (Figure 3.7a). After 3 doses (day 6 of treatment), spleens from three mice from each group were examined. Spleens from silvestrol treated mice were 60% smaller (P=0.016) and showed an 80% reduction in FLT3 protein (P=0.002) compared to vehicle-treated controls (Figure 3.7b, c). Cytospins of bone marrow cells and histopathology of bone marrow (sternum), spleen, and liver sections from MV4-11-engrafted mice treated with vehicle showed extensive infiltration of blast cells. In contrast, cytospins from silvestrol-treated leukemic mice were similar to those of the age-matched control mice with some differentiated blasts (Figure 3.7d, e). Silvestrol-treated leukemic mice (n=10) survived significantly longer than the vehicle-treated controls (n=6) (median survival: 63 days from engraftment vs. 29 days, respectively; P<0.0001). All mice in the vehicle group died by day 31 from engraftment (day 11 from
treatment start), while 50% of silvestrol-treated mice were still alive on day 74 from engraftment (day 54 from treatment start) (Figure 3.7f). Of the silvestrol-treated mice, 30% were still alive at 6 months from experiment start day. When sacrificed, these survived mice showed no signs of leukemia.
Figure 3.8 *In vivo* antileukemic activity of silvestrol in FLT3-ITD positive MV4-11 xenograft leukemia mouse model.

NSG mice were engrafted with MV4-11 cells from a previously engrafted NSG mouse. (a) FLT3 expression by flow cytometry evaluated 48 hours after 1st and 2nd doses. (b) spleens size after 3 silvestrol doses (day 6 of treatment) (c) Immunoblot of FLT3 protein expression in spleens from silvestrol-treated mice and vehicle-treated controls. (d) Cytospins of bone marrow cells and (e) histopathology of bone marrow (sternum), spleen, and liver from the silvestrol-treated leukemic mice and age-matched (non-leukemic) control mice. (f) Survival analysis of silvestrol-treated leukemic mice (N=10) compared to the vehicle-treated controls (N=6).
3.4 Discussion

The natural product silvestrol has been shown to inhibit translation initiation by modulating the interaction of capped mRNA with the RNA helicase eIF4A (184). Proto-oncogenes such as \textit{CCND1}, \textit{MCL1}, and \textit{MYC} tend to encode proteins with short half-lives, and are therefore more dependent on active translation to maintain protein levels that support malignant cell growth and survival. Such factors and in turn cancer cells addicted to them, are thus expected to be more sensitive to translation inhibition by silvestrol, as was recently reported (181-183, 190). To our knowledge, the effect of silvestrol on FLT3 expression has not been previously reported. Here we report that silvestrol also inhibits \textit{FLT3} mRNA translation thereby causing depletion of the encoded oncoprotein, inhibition of the protein’s aberrant tyrosine kinase activity. Silvestrol caused growth arrest and apoptosis in AML cell lines and primary blasts treated at nanomolar concentrations. These results therefore extend the list of the oncoproteins targeted by silvestrol and support the antileukemia activity of this compound.

It is expected that interference with translational initiation complex would results in depletion of target proteins, without substantial changes in the encoding mRNAs. Indeed, we noted this in THP-1 cells. In contrast, in MV4-11, THP-1 stimulated with FL and primary blast expressing \textit{FLT3-ITD}, we noted a significant decrease in \textit{FLT3} mRNA following silvestrol treatment. This suggested that activated FLT3 autoregulates its transcription in these cells. The mechanism of this phenomenon is not completely understood. However, STAT and /or NFkB proteins may contribute to this mechanism,
both proteins are constitutively active in FLT3-ITD cells, and their ectopic expression have been reported to upregulate FLT3 mRNA (191) (192)(Onai 2006) (blum 2012).

Most importantly, we showed a remarkable in vivo activity of silvestrol in a FLT3-ITD leukemia engraftment model. Mice engrafted with adapted MV4-11 cells developed leukemia and had a median survival of only 4 weeks. Treatment with silvestrol had no obvious toxicity and lowered the FLT3 expression to undetectable levels after administration of only three doses. Silvestrol treatment significantly prolonged survival and even cured approximately 30% of the animals, further reinforcing the concept that pursuing suppression of the FLT3-ITD protein expression may be a valid therapeutic approach in FLT3-driven AML. Recently, there has been an increased interest in utilizing small molecule kinase inhibitors to target aberrant TK activity of FLT3 mutants that contribute to leukemia growth and poor outcome in AML patients (193). Unfortunately, the lack of selectivity, inadequate pharmacokinetics, and early onset of resistance resulted in relatively disappointing outcomes with these agents (194, 195). Nevertheless, FLT3 down-regulation via siRNA has been shown to result in growth inhibition and apoptosis in FLT3-ITD-positive AML cells (187, 196). This implicates that interfering not only with aberrant activity of this kinase but also with FLT3 expression might be a useful therapeutic strategy in AML. Thus drugs, like silvestrol, that have the ability to decrease the expression of FLT3 may be clinically useful in this distinct subset of AML addicted to the FLT3 aberrant TK activity.

Although activity of silvestrol on the FLT3 expression was impressive, it is likely that silvestrol as a natural product and an inhibitor of eIF4A has the ability to alter the
expression and activity of several other targets that may contribute to the impairment of leukemia growth. Nevertheless, the antileukemia activity of this natural product may result specific for those FLT3-driven molecular subsets of AML that are resistant to chemotherapy and/or enzymatic kinase inhibitors. To this end, we not only showed silvestrol-related FLT3 suppression, but also downregulation of miR-155, an oncomiR whose expression is likely regulated by NFκB often found constitutively activated in FLT3-driven AML (197, 198). This microRNA has been shown to be upregulated in FLT3-ITD positive AML, and is thought to contribute to leukemia growth and aggressiveness in these molecular subsets of AML (23, 48). Thus the dual activity of silvestrol on FLT3 and miR-155 expression in FLT3-ITD positive cells suggests that this compound may represent a potentially valuable therapeutic approach to this high-risk AML.

In conclusion, we showed that silvestrol is a compound with a potent antileukemia activity in FLT3-driven AML. These results thereby provide a novel therapeutic strategy in FLT3-driven AML by a novel mechanism that is not based on the disruption of the aberrant tyrosine kinase activity through enzymatic TK inhibition, but rather it is effective via translation inhibition of FLT3 mRNA. Given these and other previously published findings (182, 190), silvestrol is now under preclinical development in the National Cancer Institute’s NExT Program for a rapid translation into the clinic.
Chapter 4 : Summary and Future Directions

AML is the most common and deadly type of leukemia in adults. The lifetime risk of this disease is 1 in 254 (men and women) (2) and only 10-50% of AML patients achieve long-term survival (4). AML is a heterogeneous clonal disorder characterized by the aberrant proliferation and block of differentiation and maturation of leukemic progenitors and precursor cells called blast (199). Accumulation of somatically acquired genetic alterations in hematopoietic progenitor and stem cells transform these cells into leukemia initiating cells with hallmark properties that define their malignant status such as: self-renewal, limitless proliferation potential and block of differentiation and apoptosis (200, 201). In recent years, identification of gene mutations and deregulated expression of genes and noncoding RNAs (ie, microRNAs) provided enormous insights into the mechanisms of leukemogenesis and unraveled the molecular genetic heterogeneity within distinct cytogenetically defined subsets of AML, in particular the large group of cytogenetically normal (CN) AML (202). Several signaling pathways have been also identified in AML and found implicated not only in the pathophysiology of the disease, but also in the prognosis and response to treatment.

Despite this progress, AML research has gone through in the last decade, this progress was not correlated with rewarding corresponding improvement with regard to patient’s outcome. In fact, the major progress made in AML therapeutic have not been
due to the introduction of new therapeutic agents but rather the more optimal use of previously used chemotherapy (e.g., high-dose cytarabine therapy) as well as to better supportive therapy (control of bacterial and fungal infections and hemorrhagic events). The improvement of survival rates related to allogeneic and autologous transplantation has also accounted for the improvement of overall survival of AML patients (203). This lack of direct cause-effect relationship between the progress in basic research and the improvement of patient's outcome observed in AML is likely due to several disease characteristics.

AML is very heterogeneous disease with regard to clinical and morphological features, and cytogenetic abnormalities and gene mutations and expression. Therefore, it is projected that patients respond differently to treatment and thus have extremely variable survival rates, ranging from several days to complete recovery. Furthermore, representing a medical emergency in most cases, requiring immediate medical intervention; is a critical challenge that faces introducing new individualized therapy based on genetic or molecular findings at diagnosis. Therefore, the application of molecularly or cytogenetic analysis at induction is currently considered to address post-remission therapy only, specifically to identify patients with need for allogeneic stem cell transplantation (1).

An ideal targeted therapy as a replacement of the induction therapy is expected to inhibit key pathways of leukemogenesis, eradicate the leukemic clone and restore normal hematopoiesis (203). However, the large number of different cytogenetic, genetic, and molecular abnormalities makes the individualized treatment of AML a
challenging objective. In addition, genetic aberrations associated with AML are not mutually exclusive and often coexist in the leukemic cells of the single patient. More importantly, we are still far from identifying and distinguishing the somatic alterations imperative in tumor initiation and progression from passenger mutations resulting from genomic instability of leukemic cell. Therefore, it may not be a realistic on the short term to aim for identifying a new single agent that ultimately eliminates the leukemic clone and efficiently restores the normal hematopoiesis. Instead a combination of several new drugs or conventional chemotherapy could represent a more effective and potentially curative strategy.

The work we presented here focused on two objectives, first: identification of novel molecular targets (i.e. SPARC) in AML (chapter 2). Second: investigating novel approaches (i.e. Silvestrol) to target previously identified molecular targets (i.e. FLT3) in AML (chapter 3).

In summary of the first objective, we utilized recently published GEP data to identify new molecular targets that have not been studied in AML before. We found SPARC to be upregulated in several GEPs associated with worse outcome in CN-AML patients. SPARC was among the most upregulated genes associated with IDH2-R172 mutation and among the most downregulated genes associated with NPM1 mutations. These observations indicated a previously unidentified proleukemic contribution of SPARC in AML. Altered SPARC expression has been reported in different types of cancer, but its role in AML remained unknown. In chapter 2 we showed that SPARC promoted AML growth in vitro, aggressive disease in vivo, and worse outcome in CN-
AML patients. SPARC activity was mediated by activation of ILK-AKT-GSK3β pathways and in turn, β-catenin, a protein required for leukemia stemness. Consistently, SPARC overexpression was associated with gene and microRNA profiles comprising upregulated stem cell markers. Analyzing the function of RUNX1 and IDH2-R172 mutations associated with SPARC overexpression and poor outcome in AML, revealed NF-κB/SP1 transactivation as the likely mechanism for SPARC upregulation. These data suggest that SPARC is a novel prognosticator and a potential therapeutic target in AML.

We expect this work to have significant diagnostic, prognostic, and therapeutic implications in AML. SPARC upregulation identifies a novel subset of CN-AML that are more likely to harbor IDH2-R172, RUNX1, or deregulation of the NF-κB/SP1 and miR-29b molecular network. Furthermore, SPARC association with negative clinical outcome makes measuring this protein levels at diagnosis prognostically informative. Being a secreted protein permits feasible and fast quantification techniques (i.e, ELISA based assays) to be utilized for SPARC levels assessments at diagnosis. Further research will be needed in order to obtain meaningful guidelines for serum SPARC levels.

Additionally, we demonstrated that SPARC links the upstream NF-κB/SP1/miR-29b deregulation with its downstream targets: ILK/AKT/β-catenin. Therefore several therapeutic approaches can be investigated in patients with high SPARC expression. Some of these approaches include: 1- Intervention with SPARC upstream network utilizing pharmacological disruption of NF-κB/SP1 with Bortizomib or synthetic miR-29b. 2- Direct interference with SPARC expression and/or activity via SPARC antibodies based treatment, or proteases based therapy that results in SPARC digestion and therefore inhibition of its activity. 3- Intervention with SPARC downstream targets utilizing integrins antibodies, ILK inhibitors, AKT or β-catenin inhibitors.
Supported by *in vitro* and *in vivo* experimental evidence, we demonstrated that SPARC promoted growth advantage and aggressive disease in AML cells and AML mouse model respectively. This work revealed SPARC as a novel molecular contributor to AML aggressiveness and progression. Although, ectopic SPARC expression in normal CD34+ cells resulted in c-Myc and *CyclinD1* mRNA upregulation (Figure 4.1), it was not sufficient to transform these cells; suggesting that SPARC requires other hits or activated pathways in order to enhance the leukemogenic potential of AML cells. Further research will be needed to identify the different molecular targets that interact with SPARC and account for its role in AML. Introducing mutations such as *RAS*, *FLT3-ITD* or *KIT* along with ectopic expression of SPARC will be an initial approach to answer some of these questions.

Figure 4.1 SPARC upregulates *c-Myc* and *CyclinD1* mRNA expression in CD34+ cells isolated from cord blood cells.
In addition, we demonstrated that SPARC upregulation in AML results from activation of NF-κB and SP1 complex and acts through activating ILK, AKT and β-catenin signaling pathways. As a result SPARC represent a cross-link between the NF-κB and AKT/β-catenin pathways, both are crucial for leukemic stem cell survival and self-renewal. In melanoma, ILK also altered the activity and subcellular localization of nuclear NF-κB subunit p65; whether similar effect occurs in AML is still unknown (204). However, it is possible that SPARC is key protein in the regulatory loop of NF-κB/ILK found to be activated in several cancer types, and therefore disruption of this loop can be achieved by targeting SPARC expression or activity.

We speculate that several other matricellular protein might play similar role to the one we reported for SPARC. Osteopontin (OPN) is also a matricellular protein found to be upregulated in AML patients compared to healthy donors and also associated with worse outcome in AML patients (205). Therefore expanding this work to other matricellular protein may provide better understanding to this new class of proto-oncogenes and their clinical and biological impacts.

While the first objective of our work focused on identification of previously unknown prognostic markers and molecular targets in AML, the second objective was directed toward investigating novel therapeutic approaches that target previously known ones. As we mentioned in previous chapter, overexpression and/or activating mutations [i.e., ITD] of the FLT3 are among cytogenetic and molecular aberrations that have an unfavorable prognostic significance for both younger and older AML patients treated with conventional chemotherapy. This substantiates the need for novel therapeutic approaches in this molecular subset of patients. However, the use of emerging small
molecule inhibitors of the enzymatic kinase activity of the FLT3 protein, albeit promising, is limited by early onset of mechanisms of resistance. We showed in chapter 3 that silvestrol, a natural product from *Aglaia foveolata* had a potent anti-leukemia activity in AML cells including those with FLT3-ITD and FLT3 overexpression. Silvestrol induced down-regulation of the FLT3 gene expression and in turn inhibition of tyrosine kinase activity. FLT3 protein downregulation occurred via inhibition of mRNA translation, a mechanism that has also been reported to mediate downregulation of other proteins that contribute to malignant growth and survival (i.e., MYC and MCL1). Remarkably, silvestrol significantly prolonged survival and even cured approximately 30% of mice engrafted with FLT3-ITD positive compared to vehicle-treated controls.

Because of the remarkable antileukemic activity of silvestrol in FLT3-ITD positive AML, we asked whether this activity is valid in other mutant or upregulated RTKs in AML. Similar to FLT3, KIT is frequently mutated and/or upregulated in AML. To examine the activity of silvestrol in KIT mutant AML, we tested silvestrol cytotoxicity in Kasumi-1 cells (*KIT* mutant AML cells) by MTS assay. We showed that silvestrol reduced the viability of Kasumi-1 cells in a time and dose dependent manner with IC$_{50}$ of 7.53 nM at 48 hours. A dose dependent increase in Annexin staining in the treated Kasumi-1 cells when cells were treated with 0, 10, 30, and 50 nM silvestrol for 48 hours was observed suggesting an apoptosis mediated cytotoxicity of silvestrol (Figure 4.2a). To determine whether KIT expression is affected by silvestrol treatment, we treated Kasumi-1 cells with 50nM of silvestrol for 24 hours; KIT protein level then was evaluated by western blot. We found a significant decrease in KIT expression in Kasumi-1 cells (46%) in silvestrol treated cells when compared to the untreated (Figure 4.2b).
To evaluate the *in vivo* efficacy of silvestrol in *KIT* mutant AML, *KIT* mut FCD-P1(D816V) xenograft mouse model was selected to evaluate the *in vivo* efficacy of silvestrol in AML with *KIT* mutation. NOD-SCID Gamma (NSG) mice were engrafted with *KIT* mut FCD-P1(D816V). Twenty-one days post-engraftment, mice were also treated intraperitoneally with vehicle or silvestrol (1.5 mg/kg every 48 hr for 3 weeks). After 3 doses (day 6 of treatment), spleens were obtained from three mice from each group. Spleens from silvestrol treated mice were 53% decreased in size (P=0.02) (Figure 4.2c). Interestingly, cytospins of bone marrow cells from silvestrol treated mice looked similar to cells from NSG-control mice and showed signs of differentiation compared to bone marrow cells obtained from vehicle treated mice (Figure 4.2d). Silvestrol treated leukemic mice (n=8) survived significantly longer than vehicle treated control (n=6) (median survival: 35.5 vs. 29 days from engraftment day respectively; P=0.002). All vehicle-treated mice died by day 31 from engraftment (day 11 from treatment start), while 50% of silvestrol-treated mice are still alive on day 35 from engraftment (day 15 from treatment start) (Figure 4.2e).
Figure 4.2 *In vitro* and *in vivo* antileukemic activity of silvestrol in *KIT* mutant AML.

Kasumi-1 cells treated with 10, 30 and 50 nM of silvestrol for 48 hours and analyzed by annexin/PI flow cytometry (a). Kasumi-1 cells treated with 50 nM silvestrol and examined for KIT protein expression by immunoblotting (b), NSG mice were engrafted with *KIT* mut FCD-P1(D816V) cells from a previously engrafted NSG mouse. Spleens size after 3 silvestrol doses (day 6 of treatment) (c) Cytospins of bone marrow cells and (d) Survival analysis of silvestrol-treated leukemic mice (N=8) compared to the vehicle-treated controls (N=6) (e).
We conclude that silvestrol has a potent *in vitro* and *in vivo* antileukemic activity in AML through downregulation of the expression and activity of FLT3 and other TKs driving leukemia. Given the excellent antileukemic activity of silvestrol via a novel mechanism of TK inhibition that is potentially complementary to that of TKIs (i.e., silvestrol-induced suppression of TK protein expression vs. TKI-induced protein enzymatic inhibition), a rapid translation of this natural product to the clinic as a single agent or in combination with other TKIs ± chemotherapy seems warranted.

To sum up, we identified an original molecular target with novel biological insights, and investigated innovative therapeutic approaches suitable for high risk subsets of AML. This research will significantly advance understanding of AML and open new avenues of treatment strategies, which will result in optimized patient care, and improved clinical outcome for high risk patients.
References


