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MOLECULAR ALTERATIONS AND CLONAL EVOLUTION IN ACUTE MYELOID LEUKEMIA: PROGNOSTIC AND THERAPEUTIC IMPLICATIONS

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Stockholm 2014

To my family

ABSTRACT

Acute myeloid leukemia (AML) is a clonal disorder of hematopoietic stem cells characterized by inhibition of differentiation, subsequent accumulation of incomplete matured cells at various stages and reduced production of healthy hematopoietic elements. Despite significant progress, the outcome of AML is variable and often suboptimal. Many patients will ultimately relapse and approximately 60% of patients will succumb to their disease. Deep understanding of the leukemogenesis and progression of AML and searching for new prognosis markers, risk stratification as well as therapeutic targets are of great importance to scientists, clinicians and of course future patients.

In the first paper, we conducted a study on 48 *de novo* AML patients and found down-regulation of miR-370 expression as a frequent event. Ectopic expression of miR-370 in two AML cell lines led to cell growth arrest and senescence, while depletion of miR-370 expression enhanced the proliferation of those leukemic cells. Mechanistically, miR-370 targeted the transcription factor FoxM1, a well-established oncogenic factor promoting cell cycle progression. The treatment of AML cells with 5-aza-2'-deoxycytidine (5-aza-CdR), a DNA methylation inhibitor, led to the up-regulation of miR-370 expression, which indicates epigenetic silencing of miR-370 in leukemogenesis. In conclusion, miR-370 acts as a tumor suppressor in AML by targeting FoxM1.

Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by the balanced reciprocal translocation t(15;17)(q22;q12-21) that encodes a fusion protein PML-RARα. In study II, we determined the clonal evolution scenario in an APL patient who presented the same disease after 17 years to distinguish between a very late relapse and newly developed *de novo* APL. The patient APL cells carried the identical *PML-RARa* fusion gene between two occasions, however, exhibited significant other genetic alterations. *FLT3ITD* and *FLT3D835* mutations were observed in the first and second APL cells, respectively. Thus, the patient experienced a very true late relapse of the disease. The data also suggest that *PML-RARa* fusion-mediated APL development needs a second oncogenic event (*FLT3* mutations in the present case).

DNA methyltransferase inhibitors such as 5-azacytidine (5-AZA) have been used for the treatment of AML and other malignancies. In study III, we identified that 5-AZA induced DNA damage, telomere dysfunction and telomerase inhibition in AML cells, which was coupled with cellular apoptosis. Telomerase over-expression significantly attenuated 5-AZA-mediated DNA damage, telomere dysfunction and apoptosis of AML cells. Collectively, 5-AZA-mediated telomere dysfunction contributes to its anti-cancer activity.

Somatic mutation of FMS-like tyrosine kinase 3 (*FLT3*) occurs in 30% of AML, with the majority of mutations exhibiting internal tandem duplication (*ITD*) in the juxtamembrane domain to drive leukemogenesis. In study IV, we observed that, in FLT3ITD-harboring primary cells from AML patients and AML cell lines, FLT3 inhibitor PKC412 down-regulated telomerase (*TERT*) gene expression and telomerase activity in a MYC-dependent manner. This effect was required for its optimal anti-AML efficacy. Ectopic expression of TERT significantly attenuated the apoptotic effect of PKC412 on AML cells. Mechanistically, TERT enhanced the activity of FLT3 downstream effectors or alternative tyrosine kinase receptor signaling pathways through which PKC412 effect was attenuated. In conclusion, FLT3ITD regulates *TERT* expression via a MYC-dependent manner, and TERT plays an important role in FLT3 inhibitor-mediated anti-AML efficacy.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Zhang X***, Zeng J*, Zhou M, Li B, Zhang Y, Huang T, Wang L, Jia J, Chen C. The tumor suppressive role of miRNA-370 by targeting FoxM1 in acute myeloid leukemia. *Mol Cancer*. 2012; 11:56. doi: 10. 1186/1476-4598-11-56.

II. **Zhang X**, Zhang Q, Dahlström J, Tran AN, Yang B, Gu Z, Ghaderi M, Porwit A, Jia J, Derolf Å, Xu D[1], Björkholm M[1]. Genomic analysis of the clonal origin and evolution of acute promyelocytic leukemia in a unique patient with a very late (17 years) relapse. *Leukemia*. 2014; 28, 1751-1754; doi:10.1038/leu.2014.113.

III. **Zhang X***, Li B*, Jonge N, Björkholm M and Xu D. The DNA methylation inhibitor induces telomere dysfunction and apoptosis of leukemia cells that is attenuated by telomerase over-expression. (Manuscript)

IV. **Zhang X**, Li B, Dahlström J, Tran AN, Jia J, Björkholm M, Xu D. Functional interplay between FLT3-ITD and telomerase reverse transcriptase (hTERT) in acute myeloid leukemia (AML). (Manuscript)

*Contributed equally

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LIST OF ABBREVIATIONS

5-AZA	5-azacytidine
5-aza-CdR	5-aza-2'-deoxycytidine
ABL1	Abelson murine leukemia viral oncogene homolog 1
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All-*trans* retinoic acid
BCR	Breakpoint cluster region
BM	Bone marrow
BWA	Burrows-Wheeler Aligner
CBFB	Core-binding factor subunit beta
CEBPA	CCAT/enhancer binding protein alpha
CGH	Comparative genomic hybridization
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CR	Complete remission
CSF	Colony-stimulating factor
DA	Daunorubicin, cytosine arabinoside (ara-C)
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMTI	DNA methyltransferase inhibitor
dNTP	Deoxy-ribonucleoside triphosphate
ECL	Enhanced chemiluminescence
FAB	French-American-British
FBS	Fetal bovine serum
FC	Flow cytometry
FL	FLT3 ligand
FLT3	Fms-like tyrosine receptor 3

FISH	Fluorescence in situ hybridization
FoxM1	Forkhead box M1
HER2	Human epidermal growth factor 2
HSCT	Hematopoietic stem cell transplantation
ITD	Internal tandem duplication
JM	Juxtamembrane
KL	Kit ligand
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic Syndrome
MKL1	Megakaryoblastic leukemia 1
miRNA	microRNA
MMLV	Moloney murine leukemia virus
MPN	Myeloproliferative neoplasm
NK	Natural killer
NPM1	Nucleophosmin1
OGT	Oxford Gene Technology
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PML	Promyelocytic leukemia
qRT-PCR	Quantitative Real-time PCR
RARα	Retinoic acid receptor alpha
RBM15	Putative RNA-binding protein 15
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
RTK	Receptor tyrosine kinase
RUNX1	Runt-related transcription factor 1
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
Sp1	Specificity protein 1
TERC	Telomerase RNA component

TERT	Telomerase reverse transcriptase
TK	Tyrosine kinase
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitors
TIF	Telomere dysfunction-induced foci
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
UTR	Untranslated region
WGS	Whole genome sequencing
WHO	World Health Organization

1 INTRODUCTION

1.1 ACUTE MYELOID LEUKEMIA

1.1.1 Definition

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. It is a malignant disorder of the hematological system characterized by clonal expansion of immature myeloblasts in bone marrow, blood and/or other tissue and reduced production of healthy hematopoietic elements[1]. Cytopenias cause clinical manifestations, with symptoms of anemia (fatigue and dyspnea), neutropenia (infections), and thrombocytopenia (hemorrhage), which are usually present at the time of diagnosis and are dominant throughout treatment[2]. When at least 20% of nucleated cells in a bone marrow sample are myeloblasts, the diagnosis of AML can be established. In AML with certain specific genetic abnormalities the diagnosis is established irrespective of the blast cell count[3].

1.1.2 Classifications

The name of 'leukemia' was given by Rudolf Virchow in 1845, which was derived from Greek meaning 'white blood'[4]. However, it took a long time before a uniform classification system was generally accepted for acute leukemia. In 1976 the French-American-British (FAB) co-operative group published 'Proposals for the classification of the acute leukemias[4]' in which AML was classified into six main subtypes based on morphological characteristics of the leukemic blasts and the direction of differentiation along one or more cell lineages as well as the degree of maturation. Modifications were made in 1985[5]. In 2001, World Health Organization (WHO) introduced a new classification which highlighted the biological and prognostic relevance of the cytogenetic abnormalities. It categorizes AML based on genetic findings, relation to cytotoxic therapy, and presence of myelodysplasia-related changes[6]. Cases that do not fulfill criteria for inclusion in one of these groups are assigned to the group of 'acute myeloid leukemia, not otherwise specified' and classified according to the major lineages involved and the degree of maturation. The WHO classification modified in 2008 is described in Table 1.

Table 1. Classification of acute myeloid leukemia according to WHO[3]

Acute myeloid leukemia with recurrent genetic abnormalities
t(8;21)(q22;q22); *RUNX1-RUNX1TI*
inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
t(15;17)(q22;q12); *PML-RARA* (acute promyelocytic leukemia)
t(9;11)(p22;q23); *MLLT3-MLL*
t(6;9)(p23;q34); *DEK-NUP214*
inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EV11*
t(1;22)(p13;q13); *RBM15-MKL1* (acute megakaryoblastic leukemia)
Provisional entity: acute myeloid leukemia with mutated *NPM1*
Provisional entity: acute myeloid leukemia with mutated *CEBPA*
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Myeloid sarcoma
Acute myeloid leukemia, not otherwise specified
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Acute leukemia of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*
Mixed phenotype acute leukemia with t(v;11q23); *MLL*-rearranged
Mixed phenotype acute leukemia, B/myeloid
Mixed phenotype acute leukemia, T/myeloid
Provisional entity: Natural killer (NK) cell lymphoblastic leukemia/lymphoma

1.1.3 Epidemiology

AML is a relatively rare cancer, but is the most frequent form of acute leukemia[7]. In Sweden, every year approximately 350-400 patients are diagnosed with AML, which corresponds to an annual incidence of 3-4/100,000 inhabitants. The incidence of AML is highest in the U.S., Australia, and Western Europe world wide[8]. AML is diagnosed at all ages, but the incidence increases with increasing age. The median age at presentation is about 70 years. Males are slightly more affected than females[9].

1.1.4 Etiology, Pathogenesis, Clinical Signs and Treatment

The etiology of AML is unknown in most patients. Risk factors include exposure to ionising radiation[3], benzene[10], and cytotoxic chemotherapy[11]. Some have a preceding diagnosis of another hematologic disease such as myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN). Cigarette smoking is also a risk factor which could increase the incidence of AML[12-14]. There is no overall increased risk among first-degree relatives to AML patients[15].

Some AML patients develop the disorder after treatment with cytotoxic chemotherapy (usually for a solid cancer), which is named as therapy-related AML[16]. It can be seen 5–10 years after exposure to alkylating agents or 1–5 years after treatment with drugs such as doxorubicin and etoposide, which interact with DNA topoisomerase II. Both therapy-related AML and AML progressed from MDS or MPN are considered as secondary AML.

Development of AML in a patient is considered to be a process of multiple genetic steps in a hematopoietic stem cell[17]. Mutations can either affect cell proliferation or cell survival (class I mutations: *RAS*, *FLT3* and *c-KIT*)[18,19] or interfere with differentiation and maturation of the hematopoietic cells (class II mutations: *RUNX1/ETO*, *CBFB/MYH11* and *PML/RARα*)[20,21]. The development of the disease can most probably only be possible in the presence of combination of these two mutation types, and either one is not enough to induce leukemia.

AML is an aggressive disease and if left untreated, patients will die within weeks. Normal hematopoiesis is impaired by expansion of myeloid blasts in bone marrow and by other mechanisms, leading to the main symptoms of anemia, bleeding and infection[10]. Expansion of myeloblasts in bone marrow and leukemic infiltration may cause skeletal pain and organ specific signs respectively in some patients.

Intensive chemotherapy is necessary for achievement of complete remission (CR) and the latter is a requiste for long-term survival in AML. CR is defined as that leukemic blasts in bone

marrow is less than 5% as well as a neutrophil count more than 1.0×10^9/L and a platelet count more than 100×10^9/L[22]. The initial phase of treatment is referred to as remission induction or 'induction therapy'. The combined use of daunorubicin (D) and cytosine arabinoside (A; DA) from late 1960's made it possible to induce CR in AML patients[23]. In a small subpopulation, there was even a potential of long-term survival. Several anti-leukemic drugs have been introduced since then, including addition of etoposide[24] and the substitution of daunorubicin for idarubicin or mitoxantrone. Although those combinations may improve overall survival in certain groups of patients[25], DA remains the cornerstone in AML treatment[26]. This phase of treatment takes approximately four to six weeks and usually consists of one or two cycles. Induction therapy frequently results in a CR of AML, but such remissions are usually short-lived unless additional, post-remission therapy is given.

Post-remission therapy is given with the intention of killing leukemic cells remaining in the bone marrow or blood, being undetectable under microscope. There are three main basic choices for post-remission therapy: additional chemotherapy (mainly several cycles of high-dose cytarabin), stem cell transplantation from a donor (allogeneic hematopoietic stem cell transplantation, HSCT), or high-dose chemotherapy with autologous stem cell support.

Advances in the genetic and molecular characterizations of leukemia have enhanced the capabilities to develop targeted therapies[27]. The most dramatic example of targeted therapy to date is the use of targeted BCR-ABL protein tyrosine kinase inhibitors (TKI) in chronic myeloid leukemia (CML)[27]. However, AML is an extremely heterogeneous disease with outcomes that vary widely according to the disease subtypes and patient characteristics. Targeted therapy with monoclonal antibodies and small molecule kinase inhibitors is promising strategy to help improve the cure rates in AML[27,28].

1.1.5 Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a well-recognized entity, characterized as the M3 subtype of AML within the FAB morphologic classification, where the cancer cells stop maturing at the promyelocyte or progranulocyte stage[21,29,30]. APL accounts for approximately 10-15% of all cases of AML in Italy but only 3-4% in Sweden[21,31]. The incidence of APL in China is higher where APL accounts for 20-30% of all AML cases[32]. Secondary APL is also recognized following topoisomerase II inhibitor therapy[33]. APL is characterized by a balanced reciprocal translocation involving the *promyelocytic leukemia* (*PML*) gene on chromosome 15 and the *retinoic acid receptor alpha* (*RARa*) gene on chromosome 17, resulting in a generation

of a fusion gene *PML-RARa* encoding for a onco-protein. PML-RAR*a* fusion protein induces cell renewal and block differentiation[34].

APL differs clinically from other AML subtypes in that patients carry a high risk of bleeding and thromboembolic events[35], even in the absence of leukocytosis, before diagnosis and during early treatment[36].

APL is treated differently from all other subtypes of AML. The vitamin A derivative, all-*trans* retinoic acid (ATRA)[37,38], introduced in the late 1980's[39], has the ability to induce differentiation[40,41] of leukemic promyelocytes in patients with APL and can induce CR as a single drug[42]. Prior to the incorporation of ATRA into the treatment of APL, patients with APL were generally treated according to standard AML chemotherapy protocols, but early death rate was a major problem[42]. Single ATRA could induce CR, however, the duration of remission was relatively short[43]. In combination with an anthracycline-based thermotherapy the efficacy has been further improved[36]. Arsenic trioxide (ATO) was first used to treat APL in 1973, well before the benefits of ATRA were appreciated. It is now recognized that ATO has potent and relatively selective activity against APL-initiating cells[44,45] via its ability to induce PML-RARα fusion protein degradation[46,47]. In addition, ATO has the ability to induce CR in patients with refractory and relapsed APL and is successfully used in combination with chemotherapy in this group of patients[36,48].

1.2 MICRORNAS (MIRNAS)

1.2.1 MiRNA Regulations

MiRNAs, small (~22 nucleotide), single-stranded noncoding RNAs, are a novel class of biological molecules. Genes coding miRNAs may either give rise to single miRNAs, or contain several miRNAs in one transcriptional unit as miRNA clusters[49]. MiRNAs post-transcriptionally repress gene expression by recognizing complementary target sites in the 3'untranslated region (UTR) of target mRNAs[50]. A single miRNA can regulate several mRNA targets and conversely multiple miRNAs can cooperatively regulate a single mRNA target. miRNAs have been implicated in a large variety of biological processes, including cell cycle progression, apoptosis, differentiation and hematopoiesis[51-55], and thereby play important roles in multiple pathological processes, including malignant transformation[56,57]. MiRNAs are most frequently located within intergenic regions (distant from annotated genes) or introns of

protein-coding genes, and less commonly within exons or antisense transcripts.

1.2.2 Role of MiRNAs in Human Cancer

The first evidence of a role for miRNAs in human cancer came from studies of chronic lymphocytic leukemia (CLL)[58]. The expression of miR-15a and miR-16-1 was found to be down-regulated in 50-60% of CLL cases due to a deletion of chromosomal region 13q14[59]. Subsequently, the majority of miRNA genes were found to be located at chromosomal regions that are genetically altered in human cancer[56]. The observed miRNA deregulation in cancers can be attributed to genomic alterations/mutations, defects in miRNA biogenesis, transcriptional deregulations and epigenetic regulations. Epigenetic alterations, such as DNA methylation and histone modifications can regulate miRNA expression. Numerous studies have demonstrated that miRNAs are deregulated by epigenetic modifications in a variety of cancer types. For example, miR-203 is hyper-methylated in several hematological malignancies[60].

MiRNAs can suppress the expression of oncogenes or tumor suppressors, and function as tumor suppressors or oncogenes that regulate tumor growth by coordinating multiple signaling pathways.

1.3 FMS-LIKE TYROSINE KINASE3 (FLT3)

1.3.1 FLT3 Mutations in AML

FLT3, structurally related to the receptors for the platelet-derived growth factor (PDGF), colony-stimulating factor (CSF), and Kit ligand (KL) is a type III receptor tyrosine kinase (RTK)[61] (Figure 1). It is expressed on hematopoietic stem cells within bone marrow and activated by its ligand (FL), thereby supporting survival, proliferation, and differentiation of primitive hematopoietic progenitor cells[62]. Somatic mutation of the *FLT3* gene is an internal tandem duplication (ITD) in the juxtamembrane (JM) domain-coding sequence region, being one of the most frequently mutated genes and occurs in approximately 23% of AML[63-65]. The JM domain of RTKs often has an autoinhibitory function[66]. Disruption of its secondary structure by mutation frequently results in constitutive activation of the tyrosine kinase[67]. FLT3ITD is believed as a leukemogenesis driver and predictor of a poor prognosis in AML patients[68]. The other mutation type of FLT3 is a point mutation in the tyrosine kinase domain

(TKD), comprising approximately 7% of cases. Most commonly, the mutation occurs at codon 835 (FLT3D835) that leads to constitutive activation of FLT3. Several variants at nearby residues have also been reported.

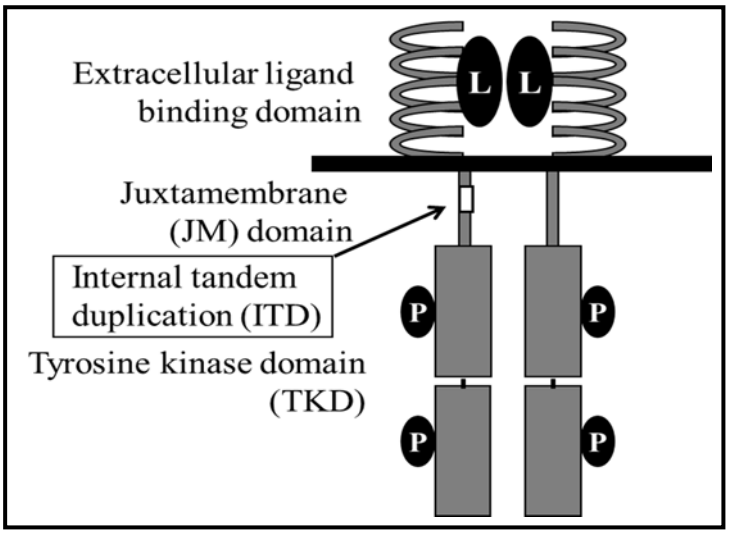

Figure 1. **Structural schematic diagram of FLT3ITD.** FLT3 receptor monomer is composed of an extracellular ligand binding domain, a trans-membrane domain, a juxtamembrane (JM) domain, and a tyrosine kinase domain (TKD). Binding to FLT3 ligand (L) leads to receptor dimerization and activation of the intracellular kinase, which then leads to phosphorylation (P) of multiple sites in the intracellular kinase moiety.

1.3.2 FLT3 Inhibitors

FLT3 has now been well validated as a therapeutic target in AML[69,70]. In the last years, a panel of small-molecule tyrosine kinase inhibitors (TKI) targeting FLT3 have been developed, and these agents are being tested in clinical trials in various contexts—in combination with induction chemotherapy, as single agents in the relapsed/refractory setting, combined with hypo-methylating agents in the relapsed/refractory population, and as maintenance therapy before or after HSCT. Those TKIs include PKC412, tandutinib, sorafenib, sunitinib and others[69,70].

The N-indolocarbazole PKC412 (midostaurin; N-benzoyl-staurosporine; Novartis Pharma AG) is one of several FLT3 inhibitors that is currently in late-stage clinical trials[71]. In preclinical studies, PKC412 caused cell cycle arrest and induced apoptosis in mutant FLT3-positive cells by directly inhibiting FLT3 kinase activity. In a phase II clinical trial, PKC412 was generally well-tolerated, with a decrease in peripheral blast counts observed in roughly a third of PKC412-treated relapsed/refractory AML patients, and a median response duration of 13

weeks[72]. PKC412 is currently in phase III clinical trials in combination with daunorubicin and cytarabine[73,74].

Resistance to therapy is a major obstacle in FLT3-mutated AML[70]. One of the well characterized mechanisms contributing to resistance is the occurrence of new mutations in the FLT3 genome. However, such new mutations were only found in a fraction of patients who had developed FLT3TKI resistance[75].

1.4 TELOMERE, TELOMERASE AND TELOMERASE REVERSE TRANSCRIPTASE (TERT)

1.4.1 Human Telomeres and Telomerase

Human telomeres at the termini of chromosomes are the nucleoprotein complex consisting of up to 20 kb tandemly repeated TTAGGG sequences and associated proteins[76-78]. Six key proteins binding to telomeric DNA include TRF1, TRF2, TIN2, POT1, TPP1 and RAP1, known as shelterin[77,78]. The telomere structure forms protective caps on human chromosome ends, and is essential to maintenance of genomic stability and integrity[76-78]. Telomere length is affected by multiple elements, but the major player in controlling telomere length is telomerase, an RNA-dependent DNA polymerase that adds TTAGGG onto chromosome ends. Telomerase is composed of two core components, the rate-limiting catalytic unit TERT, and the ubiquitously expressed telomerase RNA component (TERC) (Figure 2).

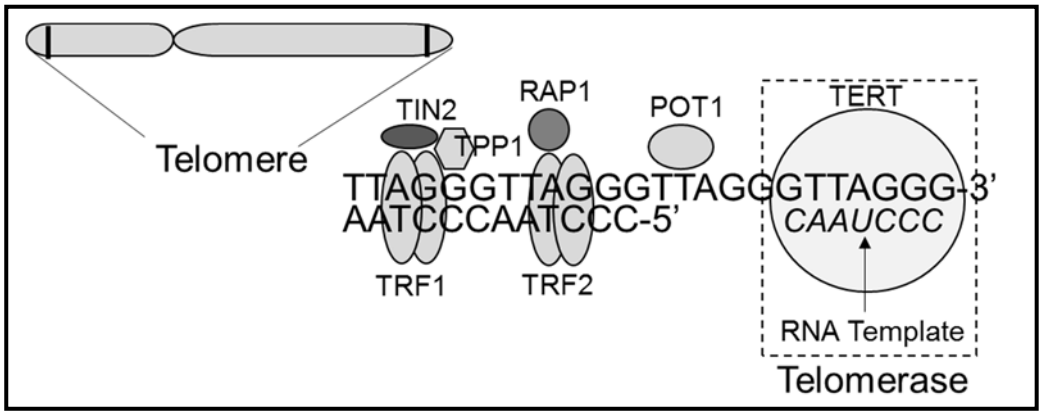

Figure 2. Structural schematic diagram of telomere and telomerase. Human telomeres locate at the termini of chromosomes, which contain tandemly repeated TTAGGG sequences. Six key proteins bind to telomeric DNA, including TRF1, TRF2, TIN2, POT1, TPP1 and RAP1. Telomerase is composed of two core components, the catalytic unit TERT and RNA template.

1.4.2 Regulation of TERT

Transcription, alternative mRNA splicing, phosphorylation, and the maturation and modification of TERT have all been shown to play vital roles in the regulation of telomerase activity. However, the transcriptional controlling of the *TERT* gene is the most important regulatory element of telomerase expression[79]. Sequence analysis revealed that the *TERT* promoter lacks the TATA and CAAT boxes, but contains binding sites for several transcription factors, including *SP1* and *c-Myc*[80].

1.4.3 Telomerase and Cell Proliferation Potential

Telomerase is silent in most human differentiated cells due to the tight repression of the *TERT* gene, which results in progressive telomere shortening with each round of divisions. Cells are triggered to enter a permanent growth arrest stage named "senescence" when their telomeres reach a critical size. Senescence mediated by telomere attrition confers a limited lifespan to normal cells and has been proposed as a potent tumor-suppression mechanism[81].

The relationship of cellular life-span with telomere length and telomerase expression has been well established. Stem/progenitor cells with great proliferation potential exhibit longer telomere coupled with telomerase expression[77]. Telomere dysfunction[82,83] or telomerase deficiency may lead to defective hematopoietic cell proliferation and bone marrow failure while aberrant activation of telomerase is essential for immortalization and transformation of human cells including hematopoietic cells[77,78].

TERT has little or no expression in normal differentiated somatic cells, however, the presence of TERT is obligatory for aberrant cell proliferation and immortalization in most tumors (up to 90%) and recent studies have revealed that cancer stem cells are also TERT-positive. Targeting telomerase/TERT is thus suggested as a novel anti-cancer strategy.

1.4.4 Extra-Telomeric Roles for Telomerase

Besides the canonical telomere elongation function, telomerase can also act as a transcriptional modulator mediating the Wnt–β-catenin signaling pathway. TERT forms a complex with the Wnt transcription factor BRG1 and binds to the promoters of Wnt-target genes following stimulation of Wnt receptors at the plasma membrane, thus regulating the expression of target genes[84]. Moreover, TERT RNA-dependent RNA polymerase activity has been found to contribute to the genesis of double-stranded RNAs that act as precursor of silencing RNAs. In

the mitochondria, TERT has also been shown to play a role in regulating oxidative damage-induced apoptosis. Oxidative stress triggers nuclear export of TERT to the mitochondria[85].

1.5 DNA METHYLATION

1.5.1 DNA Methylation and Gene Transcription

DNA methylation is a process adding a methyl group to the cytosine DNA nucleotide located in CpG sites, which is mediated by DNA methyltransferases (DNMTs). CpG sites are most frequently clustered in regulatory or promoter regions of genes[86]. DNA methylation is an important regulator of gene transcription and a large body of evidence has demonstrated that genes with high levels of 5-methylcytosine in their promoter region are transcriptionally silent, and that DNA methylation gradually accumulates upon long-term gene silencing[87]. Dysregulation of DNMT expression and aberrant DNA methylation widely occurs in human malignancies including AML[86,87].

1.5.2 DNMT Inhibitors (DNMTIS)

Strategies to combat cancer by inhibiting DNA methylation have been developed[88], and DNMTIs including 5-AZA have been applied to the treatment of AML, MDS, and other malignancies[89,90]. It is generally believed that DNMTIs result in global and gene-specific hypo-methylation through which growth arrest and/or apoptosis of malignant cells are induced. A typical example is the tumor suppressor $p16^{INK4}$, a gene that is frequently silent due to its promoter methylation in oncogenesis while re-activated by DNMTI treatment[91]. However, besides DNA demethylation function, DNMTIs may activate or repress many other downstream effectors to achieve their anti-cancer efficacy, for example, DNMTI-mediated growth arrest and apoptosis of leukemic cells has recently been shown to result from the generation of reactive oxygen species (ROS)[92]. In addition, activation-induced cytidine deaminase was down-regulated by DNMTIs via proteasomal degradation rather than a transcriptional regulation, which was believed to play an important role in DNMTI-mediated cytotoxic activities[93].

2 AIMS OF THE STUDY

The overall objective of this translational study is to define the clinical implications of molecular alterations in AML, trying to improve risk stratification and find new therapeutic targets in AML. More specifically, the study aims are:

1. To define the tumor suppressive role of miR-370 in AML, and to explore the underlying mechanism for dysregulation of miR-370 in AML and its relationship with oncogene FoxM1 (Paper I);

2. To define the clonal evolution in a unique APL patient who presented with the same disease 17 years later, and to distinguish between the late relapse of the original disease and new *de novo* or secondary APL, thereby helping select the best therapeutic protocol (Paper II);

3. To probe whether DNMTIs affect telomere function and whether TERT/telomerase interferes with their anti-AML efficacy (Paper III);

4. To determine whether FLT3-ITD regulates TERT expression in AML cells and whether TERT expression affects FLT3 inhibitors' therapeutic efficacy on AML, trying to uncover TERT-mediated drug resistance in FLT3-ITD inhibitor targeted therapy (Paper IV).

3 METHODS

3.1 PATIENT SAMPLES (PAPER I-IV)

Diagnosis of AML was established according to clinical presentation and morphologic criteria of the FAB Classification. Patient's bone marrow (BM) or peripheral blood samples were collected at the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China (**paper I**) and at the Department of Hematology, Karolinska University Hospital, Stockholm, Sweden (**paper II-IV**). Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation[94].

3.2 MUTATION ANALYSIS OF *FLT3-ITD* AND *FLT3-D835* (PAPER II, III & IV)

AML patients' DNA was obtained using QIAamp Blood & Cell Culture DNA Kit (QIAGEN GmbH Hilden, Germany). *FLT3ITD* and *FLT3D835* mutations were studied qualitatively using the fragment length analysis method. PCR primers were fluorescently labeled with 6-FAM, NED or HEX. Amplified fragments were detected using Applied Biosystems 3130 XL and interpretation of the length of each dye-labeled fragment was calculated by comparing it to a size standard using the GeneMapper software[95].

3.3 ARRAY-COMPARATIVE GENOMIC HYBRIDIZATION (CGH) (PAPER II)

Array-CGH[96] of genomic DNA isolated from APL patient BM samples was performed using the Oxford Gene Technology (OGT) platform with four arrays of 180K oligonucleotide probes (60-mer). This platform allowed a complete genome-wide survey with an average resolution of 20-50 Kb. Sample labelling and hybridization were performed following the manufacturer's protocol. The arrays were scanned on an Agilent Microarray Scanner and data was analyzed using the CytoSur Interpret Software (OGT).

3.4 WHOLE GENOME SEQUENCING (WGS) (PAPER II)

Libraries of qualified genomic DNA were prepared for paired-end analysis by the Illumina HiSeq 2000. After the generation of clusters of template DNA, they were sequenced by the Illumina HiSeq 2000 platform. Each sample was at least 30-fold haploid coverage. After a strict QC test, the sequencing data were subjected to bioinformatics analysis. The raw sequencing data were filtered and then aligned by Burrows-Wheeler Aligner (BWA). For the mapping the

human genome build 37 (Hg19) was used as the reference genome. Then, the generated BWA files were processed by the SOAPsnp, SAMtools, BreakDancer and ANNOVAR to analyze and annotate the variants. In order to find the precise translocation sites of *PML-RARα*, the intrachromosomal translocation analysis of paired-end sequence data of initial and second APL samples was performed using BreakDancer[97]. Firstly, we extracted all Maq-mapped reads within 3000bp of chromosomal locations of *PML* and *RARα* by SAMtools[98]. Then the data were subjected to BreakDancer and the analysis parameters were set according to reference.

3.5 CELL LINES AND CULTURE CONDITIONS (PAPER I, III & IV)

Human AML cell lines HL60, K562, HEL, KG1A, MV4; 11 and MOLM-13 were cultured in 10% fetal calf serum-containing RPMI-1640 with addition of 2mM L-glutamine and antibiotics (50 mg/mL penicillin, and 50 mg/mL streptomycin) in a humid atmosphere at 37°C/5% CO2. The DNA methylation inhibitors 5-AZA was bought from Sigma-Aldrich (St. Louis, USA). To assess DNMTIs' effects, exponentially growing cells (HL60 and K562 cells for **paper I**; HEL and KG1A cells for **paper III**) were treated with inhibitors (0, 0.5, 1.0, 2.0, 5.0μM). Culture medium was replaced with freshly prepared DNMTI-containing medium every two days. The specific FLT3 inhibitor PKC412[99] (Sigma-Aldrich, Buchs, Switzerland) was diluted in DMSO and cells were incubated with different concentrations of PKC412 (0.01, 0.025, 0.05, 0.1μM) for various time periods (**paper IV**). Cells were counted for numbers and viability determined by using Trypan-Blue exclusion test or harvested for isolation of miRNA, total RNA or protein.

3.6 PRIMARY AML CELL SEPARATION AND CULTURE (PAPER III & IV)

Primary AML cells were derived from AML patients and their clinical/molecular characteristics are listed in Table 2. Peripheral blood was drawn into heparinized glass tubes and leukemic cells were isolated by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway), and subsequently incubated in complete medium in the absence or presence of 5-AZA or PKC412 as described above.

Table 2. Clinical, cytogenetic and molecular characteristics of AML patients

ID	Gender	Age (years) at diagnosis	Diagnosis(FAB)	Cytogenetics	Molecular Abnormalities
1	Female	22	AML-M3	t(15;17)	*FLT3-ITD* mutation
2	Male	20	AML-M4E0	inv16(p13q22)	*c-KIT* mutation
3	Male	60	AML-M5	Normal	No above mutations
4	Male	78	AML	del(20)	*FLT3-ITD* mutation
5	Male	68	AML-M1	Normal	No above mutations

3.7 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (PAPER I, III & IV)

Total cellular RNA was extracted using the Trizol (Life Technology, Paisley, Scotland, UK) according to the manufacturer's protocol. cDNA was synthesized using random primers (N6) (Amersham, Buckinghamshire, UK) and MMLV reverse transcriptase. The PCR primers used in the study are listed in Table 3. The above primer pairs cross intron/exon boundaries; thus, the resultant PCR products do not represent genomic DNA contamination. β-actin expression was used as a control for RNA loading and RT efficiency and amplified. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in an ABI7900 sequence detector (Applied Biosystems, Foster City, CA, USA) using SYBR Green kit (Applied Biosystems, Foster City, CA, USA).

Table 3. Primers used for qRT-PCR determination of gene expression

Target	Description	Sequence
FoxM1	Forward	5'-TGCAGCTAGGATGTGAATCTTC-3'
	Reverse	5'-GGAGCCCAGTCCATCAGAACT-3'
Skp2	Forward	5'-GGACCTATCGAACTCAGTTAT-3'
	Reverse	5'-CAGCCACCTGTACATGCTTT-3'
p27kip	Forward	5'-ATGTCAAACGTGCGAGTGTCTAA-3'
	Reverse	5'-TTACGTTTGACGTCTTCTGAGG-3'
TERT	Forward	5'-CGGAAGAGTGTCTGGAGCAA-3'
	Reverse	5'-GGATGAAGCGGAGTCTGGA-3'

c-MYC	Forward	5'- TACCCTCTCAACGACAGCAGCTCGCCCAACT-3'
	Reverse	5'-TCTTGACATTCTCCTCGGTGTCCGAGGACCT-3'
c-KIT	Forward	5'-TCATGGTCGGATCACAAAGA-3'
	Reverse	5'-AGGGGCTGCTTCCTAAAGAG-3'
DOK3	Forward	5'-TCCAAAAAGGGGCTTTGTTCC -3'
	Reverse	5'-GGAGGTAGGGTCCTTTCAGC -3'
SULF2	Forward	5'-CCGCCCAGCCCCGAAACC-3'
	Reverse	5'-CTCCCGCAACAGCCACACCTT-3'

3.8 WESTERN BLOT (PAPER I & IV)

Total cellular proteins were extracted from cultured cells or AML BM samples. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with the specific antibodies against FoxM1, p27, c-MYC (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), total FLT3-ITD, phosph-FLT3-ITD, total AKT and phosph-AKT (Cell Signalling Technology, Boston, MA, USA) followed by anti-mouse or rabbit horseradish peroxidase–conjugated IgG and developed with the enhanced chemiluminescence method (ECL). β-actin served as a loading control.

3.9 TAQMAN QRT-PCR MIRNA ANALYSIS (PAPER I)

Quantification of mature miRNAs was performed using qRT-PCR with the TaqMan miRNA assay kit[100] (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. Briefly, 10 ng of total RNA was reverse-transcribed (RT) with specific primers, subsequently 1.5 μL of RT product was used as template for real-time PCR. All real-time experiments were performed in triplicate. Data was normalized by the expression of small nuclear RNA (snRNA) U6 and expressed either as relative expression ($2^{-\Delta Ct}$) or as fold change relative to control ($2^{-\Delta\Delta Ct}$).

3.10 VECTOR CONSTRUCTION (PAPER I, III & IV)

To generate the miR-370-expressing pSilencer3.1-H1 neo vector, a fragment of 212 base pairs (bp) corresponding to the desired miRNA and the surrounding sequences was amplified from human genomic sequence, adding a BamHI site and a HindIII site to the 5' and 3' ends

respectively. MiR-370 inhibitor sequences were synthesized as DNA oligonucleotides; after annealing, were sticking ended and subcloned into a pSuper vector.

The h3.4k-GFP plasmid containing 3.4kb *TERT* promoter (+1 to -3405, ATG as +1) was obtained from Dr. Pei-Rong Huang (National Taiwan University), and the 3.4kb *TERT* promoter fragment was inserted into a pPuro. Cre empty vector (Addgene) just upstream of GFP gene.

For pLenti-III-HA-GFP-TERT vector construction, plenti-III-HA empty vector was bought from Applied Biological Materials Inc. (BC, Canada), a 4.5kb GFP-TERT fragment was cut from pBabe-hygro-GFP-TERT (Addgene) and inserted into pLenti-III-HA. A control plasmid (pLenti-BMN-GFP) encoding a GFP protein was a gift from Rudbeck Laboratory, Uppsala University. The vectors were then packaged[101] in 293FT cells and supernatant containing virual particles collected to infect AML cells to make TERT promoter-driven GFP cells and TERT-over-expressed cells.

3.11 TRANSFECTION AND INFECTION (PAPER I, III & IV)

Cells were incubated in 6-well plates and then transfected with plasmid or siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or infected with lentivirus[101]. Chemical modified Stealth small interfering RNA (siRNA) targeting *FoxM1* and control siRNA were bought from Invitrogen. The sequence for the *FoxM1* siRNA was 5'-GACAACUGUCAAGUGUACCACUCUU-3'. The cells were selected using puromycin (2 μg/ml) after lentivirus infection.

3.12 LUCIFERASE REPORTER VECTOR (PAPER I)

The precursor to miR-370 was synthesized and cloned in pSilencer. Firefly luciferase reporter vectors with the intact putative miR-370 recognition sequence from the 3'-UTR of *FoxM1* (pGL3-FoxM1-wt-3'-UTR) or with random mutations (pGL3-FoxM1-mut-3'-UTR) cloned downstream of the *firefly luciferase* gene were constructed. Wild-type and mutant inserts were confirmed by sequencing. For the 3'UTR-luciferase assays, cells were co-transfected with 0.5 μg pGL3-FoxM1-wt or mut-3'-UTR construct, 4 μg of pSilencer or pSilencer-miR370 and 0.05 μg pRL-TK Renilla luciferase expression construct using Lipofectamine 2000. Luciferase assays were performed 24 hrs after transfection using the Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA).

3.13 SOFT AGAR COLONY FORMATION ASSAY (PAPER I)

HL60 and K562 cells were re-suspended in DMEM (Gibco, Carlsbad, CA, USA) containing 20% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) with equal amount of either 0.3% agar (HL60) or 0.5% agar (K562), and plated in 6-well plates at 5,000 per well on top of a 2.0 mL precast semisolid 1% agar under-layer as described previously[102]. The number of colonies with more than 50 cells was counted after two weeks.

3.14 SENESCENCE-ASSOCIATED BETA-GALACTOSIDASE (PAPER I)

Senescence-associated beta-galactosidase (SA -β-Gal) staining was done as described[103,104]. Briefly, Cells grown in 6-well plates were transfected with pSilencer or pSilencer-miR-370. After 7 days, the cells were rinsed with PBS once, fixed in 3% of formaldehyde for 15 min, and incubated with freshly prepared SA -β-Gal staining[105] solution at 37°C overnight.

3.15 ASSESSMENT OF TELOMERASE ACTIVITY (PAPER III & IV)

Telomerase activity was determined using a commercial Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) according to the manufacturer's instruction. Total cellular proteins were extracted using CHAPS lysis buffer. For each assay, 0.5 μg of protein was used, and 26 PCR cycles were performed after the telomerase-primer elongation reaction. The PCR products were detected with an ELISA colour reaction. The measure telomerase activity was expressed as absorbance [optimal density (OD) in arbitrary units].

3.16 IMMUNO-FISH (PAPER III)

Immuno-FISH was performed as described[106]. Cells were harvested and cytospined onto Superfrost plus slides (Thermo Scientific), fixed with 4% paraformadehyde and permeabilized with Triton PBS for 20 min and blocked with serum free Block (DAKO, Glostrup, Denmark). The slides were then incubated with 53BP1 antibody (Bethyl Inc., Montgomery, TX, USA) followed by incubation with Alexa 594 secondary antibody (Jackson Labs Technologies Inc., Los Gatos, CA, USA). The slides were treated with frozen and thawed cycle in liquid nitrogen, and incubated in 0.1N HCL for 10 min. The PNA telomere probe (PANAGENE Inc., Daejeon, Korea) was finally added.

3.17 FLOW-FISH FOR TELOMERE LENGTH ASSAY (PAPER III)

Flow FISH of AML cells was performed according to a previous protocol by Baerlocher etc[107-109] with minor modifications. Cells from calf thymus were kindly donated from the butchery Ö-slakt AB (Värmdö, Stockholm). All experiments were made with a Gallios flow cytometer (Beckman Coulter) and analysed using the Kaluza software (Beckman Coulter, Caguas, PR, USA).

3.18 SUBTELOMERIC DNA METHYLATION AT CHROMOSOME 4P (PAPER III)

Genomic DNA, extracted from control and TERT-over-expressed HEL cells with or without 5-AZA, was subjected to bisulfite conversion using an EZ DNA Methylation-Gold Kit (ZYMO RESEARCH, Irvine, CA, USA). PCR primers specific to the sub-telomere region of chromosome 4p were used to amplify the target region[110]. The obtained PCR products were then sequenced at both directions. Two independent experiments were performed.

3.19 FLOW CYTOMETRY ANALYSIS OF CELL CYCLE AND APOPTOSIS (PAPER III & IV)

AML cells with different treatment were harvested for ethanol fixation and PI staining as described[111]. The PI fluorescence was measured with a Flow Cytometer (Beckman Coulter). For each sample 1 $\times 10^6$ cells were measured. Data analysis was performed with Kaluza®Flow Analysis Software[112]. The control gate was set based on the negative control.

3.20 TERT PROMOTER ACTIVITY ASSAY (PAPER IV)

The *TERT* promoter reporter plasmid p181wt harboring the core promoter sequence of the *TERT* 5'-flanking region and its mutant variant (p181MYC-) lacking the functional c-MYC motifs (E-boxes) were described previously[113,114]. Cells cultured in 24-well plates at 0.5×10^6 were transfected with p181wt and p181MYC- plasmids using Lipofectamine2000 (Life Technology) according to the manufacturer's protocol, followed by the treatment with PKC412. Luciferase activity in the cell lysates was determined by using a dual luciferase reporter assay system (Promega, Madison, WI, USA) 24 hrs post-transfection of the promoter reporter.

3.21 CDNA ARRAY (PAPER IV)

MOLM-13 cells infected with control pBMN or TERT-expressing lentiviral vectors were treated with 0.1 μM PKC412 for 12 hrs and total RNA extracted. The affymetrix Human Gene 1.0 ST Array was performed[115]. The fold change in gene expression between DMSO- and PKC412-treated cells was then calculated.

3.22 STATISTICAL ANALYSES (PAPER I-IV)

The comparison of mRNA expression, promoter activity, telomerase activity, telomere length, cell cycle analysis and co-localization between control and experimental groups was made using a Student's t-test or One-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. All the tests were two tailed and computed using SPSS18.0 software. Results are depicted as the mean ± standard deviation (SD). P values < 0.05 were defined as statistically significant.

4 RESULTS & DISCUSSION

4.1 TUMOR SUPPRESSIVE ROLE OF MIR-370 BY TARGETING FOXM1 IN AML (PAPER I)

4.1.1 Dysregulation of miR-370 and FoxM1 in AML

We determined the expression level of miR-370 in *de novo* AML patient samples, AML patient in 1st CR samples and healthy control materials, and found that miR-370 was down-regulated in BM blasts from *de novo* AML patients. Compared to that from healthy controls, miR-370 level in patients' samples was significantly reduced ($P < 0.01$, t test), while following acquisition of CR after induction chemotherapy, miR-370 expression level was restored to 0.82 fold of controls. There was no association between the presence of mature miR-370 and age, gender, blast percentage or FAB subtypes.

FoxM1, a master regulator of mitotic gene expression, is required for cell proliferation and its inhibition leads to reduction in anchor-independent growth and tumorigenesis of cancer cells[116]. We determined the expression level of *FoxM1* in all of the clinical samples described above. The results revealed that the *FoxM1* transcript level in AML patients was 21.47-fold higher than that in controls, while following acquisition of CR after induction chemotherapy, *FoxM1* expression level decreased to 1.75 fold of controls, which was negatively correlated with miR-370 levels.

4.1.2 Identification of FoxM1 as a Target of miR-370

Four target prediction programs with different algorithms: DIANA-MicroT[117], TargetScan[118], Miranda[119] and PicTar[120] predicted that *FoxM1* might be a target of miR-370 due to a potential 7-mer binding site for miR-370 in the 3'UTR region of *FoxM1*. The prediction was further confirmed by the following experiments.

First, transfection with miR-370 precursor decreased reporter activity in K562 cells, which containing the miR-370 recognition sequence from the 3'-UTR of *FoxM1* inserted downstream of the *luciferase* gene. Second, random mutations in the recognition sequence resulted in abolition of the reporter activation by miR-370 precursor. Finally, transfection of HL60 and K562 cells with miR-370 precursor resulted in lower expression of FoxM1. However, there was a >2-fold increase in expression of *FoxM1* in HL60 and K562 cells after transfection of miR370 inhibitor. All of the above results confirmed that *FoxM1* is a direct target of miR-370.

4.1.3 Changes in Proliferation and Cellular Senescence of Leukemic Cells Mediated by Altered miR-370 or FoxM1 Expression

We transfected AML cell lines with miR-370-expressing vector as well as its inhibitor to explore the effect of miR-370 on cell proliferation ability. Overexpression of miR-370 decreased cell proliferation, as revealed by colony formation assay (pSilencer vs pSilencer-miR: HL60: 88 ± 15 vs 11 ± 4, $P < 0.01$; K562: 49 ± 5 vs 18 ± 5, $P < 0.01$), while the decline in miR-370 expression was coupled with enhanced cell proliferation (pSuper vs pSuper-miR-inhibitor: HL60: 56 ± 7 vs 72 ± 6, $P < 0.05$; K562: 66 ± 12 vs 93 ± 7, $P < 0.05$). SA β-Gal staining, a specific marker for senescent cells revealed a positive β-Gal staining in the two cell lines transfected with miR-370 precursors [pSilencer vs pSilencer-miR (% of β-Gal-positive cells): HL60: 3 ± 1 vs 28 ± 3, $P < 0.01$; K562: 8 ± 3 vs 40 ± 1, $P < 0.01$;], indicating that cell senescence was at least one of the underlying mechanisms for decreased cell proliferation ability.

In the two AML cell lines, we knocked down the expression of *FoxM1*. Compared to control ones, *FoxM1*-knocked down cells exhibited significantly diminished foci formation in both (Controls vs *FoxM1* siRNA: HL60: 19 ± 3 vs 11 ± 2, $P < 0.05$; K562: 33 ± 5 vs 5 ± 2, $P = 0.001$).

4.1.4 Epigenetic Silencing of miR-370 in AML

The chromosomal location of miR-370 on chromosome 14q32.31 has been shown to be regulated by DNA methylation, or deleted by loss of heterozygosity[121,122] or by hyper-methylation of a CpG island 200 bp upstream in the mother allele[123]. Treatment with 5.0 μM 5-aza-CdR, a DNA methylation inhibitor, for 72 hrs, substantially (>2.0-fold) and significantly ($P < 0.05$) increased the expression of miR-370 in both HL60 and K562 cells and decreased cell proliferation, as revealed by clonal formation assay (control vs CdR: HL60: 24 ± 4 vs 7 ± 2, $P < 0.01$; K562: 152 ± 5 vs 78 ± 5, $P < 0.001$). This result demonstrated that the dysregulation of miR-370 in AML was at least partially due to abnormal hyper-methylation mechanism. Demethylation by DNMTI led to restoration of miR-370 expression and decline in expansion of leukemic cells.

MiR-370 has been noted to be down-regulated in papillary thyroid carcinoma, colorectal cancer [124]and malignant cholangiocytes[125], but evidence of a biological role for this miRNA in AML has not been reported. A link between microRNAs dysregulation and hematological malignancies has been reported dating back to a decade ago, and recent documents further reveal that miRNA expression profiles are AML subtype-specific[126,127].

Collectively, we demonstrate that miR-370 is a tumor suppressive factor by targeting multiple oncogenic pathways, and miR-370 is down-regulated in primary cells from *de novo* AML patients as well as AML cell lines. FoxM1 is a direct downstream target of miR-370, and restoring miR-370 expression down-modulates FoxM1, induces senescence, and dampens cell proliferation in AML cells, suggesting miRNA-based therapy as a novel anti-AML approach (Figure 3).

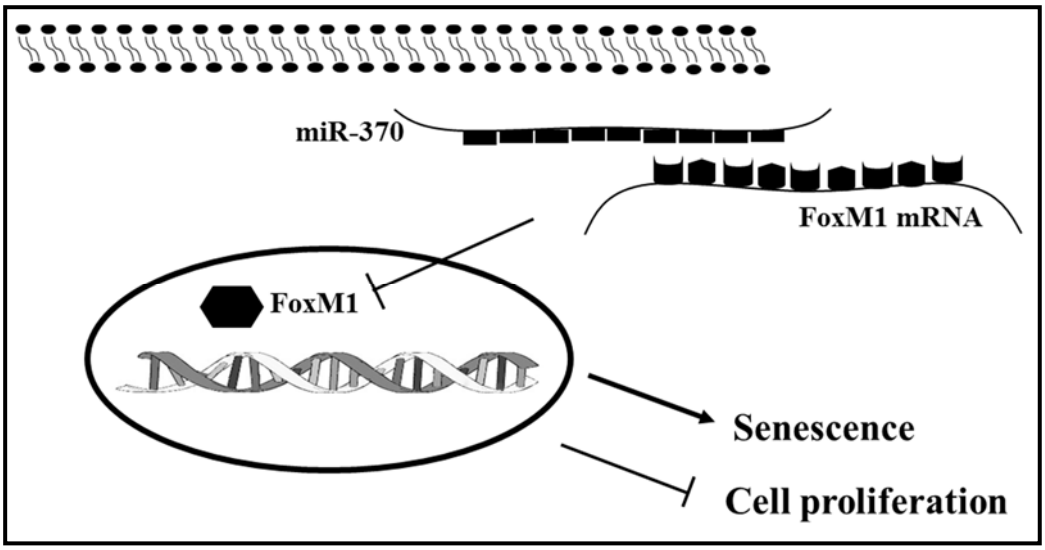

Figure 3. Regulation of FoxM1 by miR-370 in AML cells. MiR-370 acts as a tumor suppressive factor by targeting FoxM1. Restoring miR-370 expression could down-modulate FoxM1, induce senescence, and dampen cell proliferation.

4.2 CLONAL ORIGIN AND EVOLUTION OF APL (PAPER II)

4.2.1 A Very Late (17 Years) Relapse in a Unique Patient

A 42-year-old woman was admitted to hospital in April 1994 and diagnosed with APL and the typical t(15;17)(q22;q12) as revealed by cytogenetic analysis. The patient achieved hematological and cytogenetic CR following induction treatment and consolidation courses. In February 2011, almost 17 years after the initial APL diagnosis, she presented at the hospital and an APL diagnosis was subsequently made. The patient acquired CR following the induction treatment, two consolidation courses followed by two additional consolidation courses with ATRA and ATO. The disease history is summarized in Figure 4. A routine diagnostic work-up did not discriminate between a relapse of her previous APL, the appearance of a secondary APL or a new *de novo* APL.

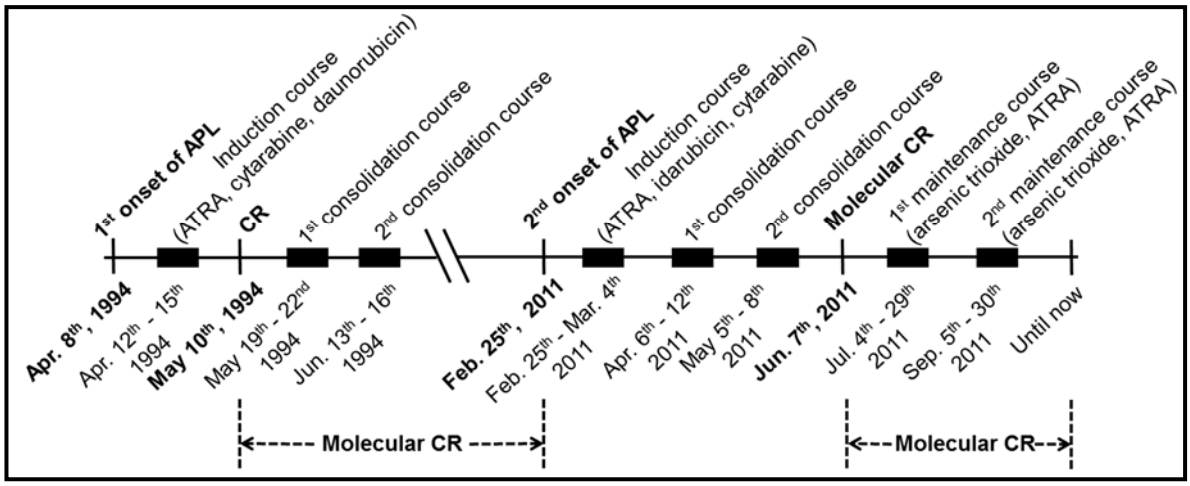

Figure 4. The summary of the clinical history of the APL patient.

4.2.2 Potential APL Clonal Evolution in the Patient

An array-CGH assay was conducted using genomic DNA material from both the two manifestations of APL. Data analysis showed the initial APL sample in 1994 harboring a main cluster of discontinuous losses in chromosome 19 (p13.2–p13.11 and q13.11–q13.43), nearly up to 24 Mb, for the second diagnostic sample in 2011, most of the chromosome 9 (p12-p11.2 and q12-q31.1) was lost.

Whole genome sequencing (WGS) was implemented with the same material mentioned above which revealed two different *PML/RARa* gene fusions (Chromosome17:38489469-Chromosome15:74316176 and Chromosome15:74316160-Chromosome17:38489139) in APL cells from both samples, with the first fusion being predominant in both. Although the fusion genes/breakpoints were identical, significant differences in genetic aberrations were shown by WGS. Fragment length analysis and WGS both demonstrated *FLT3ITD* and *FLT3D835* point mutations in the first and second APL samples, respectively.

Likely, the patient's hematopoietic cells underwent *PML-RARa* gene fusion following a genetic attack, leading to the generation of abnormal ancestral or pre-leukemic clones. These ancestral clones did not transform into APL until the acquisition of *FLT3ITD* by another genetic attack. The patient obtained a CR following ATRA treatment plus chemotherapy in 1994. APL blast clones were eradicated after treatment, but the ancestral clones carrying the *PML-RARa* fusion gene were persistent and acquired a *FLT3D835* point mutation later. The *PML-RARa/FLT3D835* clones then contributed to the second onset of APL in 2011 (Figure 5).

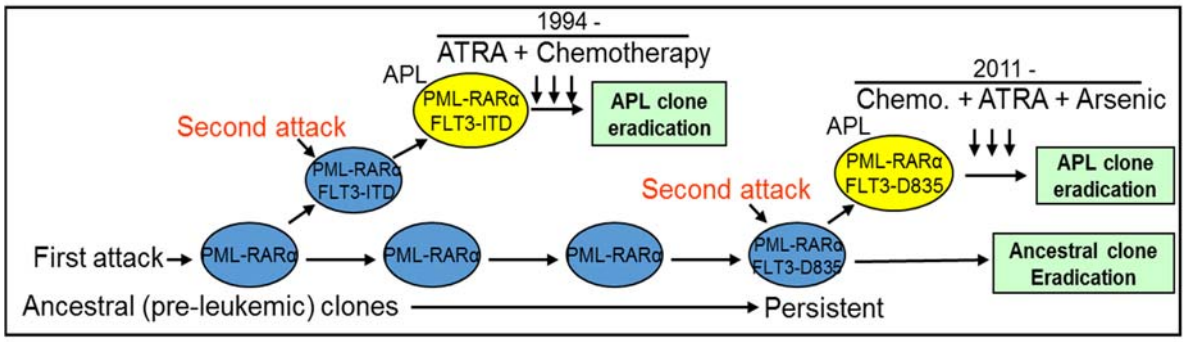

Figure 5. Potential APL clonal evolution in the APL patient. ATRA: all-*trans*-retinoic acid.

The relapse rate of APL is approximately 5-20%[31], which mostly occurs within 2-3 years from CR achievement and late relapse is very rare beyond 7 years[128-130]. Late relapse up to 111,135 and 155 months, respectively, have been reported[128,131,132]. The approach for establishing the diagnosis of relapse in patients with APL relies on the presumption that recurrent disease will be similar to the neoplasm before therapy. However, the characteristics of APL at the time of relapse are not well described in the literature.

In summary, we documented here an APL patient who presented the longest known interval between diagnosis and relapse in the literature to date. Genomic analyses showed how APL clones evolved between the two manifestations of APL in this patient. It seems that the presence of *PML-RARa* fusion gene alone is not sufficient to cause APL and ancestral *PML-RARa*-positive clones may be resistant to anti-APL therapy, causing future relapses when a new genetic attack occurs. Very late relapses in APL, as seen in this unique patient, are more likely caused by a new genetic attack on existing pre-APL clones, which differs from early relapses resulting from the re-growth of original residual APL blasts. ATO, a drug with potent and relatively selective activity against APL-initiating cells was also used here, hopefully preventing APL recurrence.

4.3 ROLE OF DNA METHYLATION INHIBITOR RELATED TO TELOMERE AND TELOMERASE IN AML (PAPER III)

4.3.1 Telomere Dysfunction, Telomere Length Shortening and TERT Down-Regulation Induced by 5-AZA

DNMTIs have been used for the treatment of AML and other malignancies, and inhibition of global/gene-specific DNA methylation is widely accepted as a key mechanism behind their anti-tumor activity[133]. In the present study, we wanted to know whether DNMTIs affect telomere function and whether TERT/telomerase interferes with their anti-cancer efficacy.

Here, telomere dysfunction-induced foci (TIF): co-localization of 53BP1 foci with telomere signals using immuno-fluorescence in situ hybridization (Immuno- FISH) showed: telomeres, revealed as green signals, were readily detectable in both control and 5-AZA-treated KG1A and HEL cells, whereas red 53BP1 foci only occurred in the treated cells. The merged image demonstrated that parts of 53BP1 foci were localized at telomeres in cells exposed to 5-AZA. Thus, 5-AZA induces telomere dysfunction (Figure 6).

To probe potential mechanisms behind 5-AZA-mediated telomere dysfunction, we determined telomere length in these AML cells. FLOW FISH analysis showed that compared to the control cells, both KG1A and HEL cells in the presence of 5-AZA at 2.0μM only exhibited slight telomere shortening, however, significant telomere attrition was observed at 5.0μM.

To further explore the mechanism behind telomere shortening in 5-AZA treated cells, we determined whether 5-AZA inhibited TERT expression and telomerase activity in KG1A and HEL cells. TERT mRNA expression was significantly down-regulated by 5-AZA treatment in both cells in a dose-dependent manner. Consistent with the down-regulation of TERT expression, telomerase activity was diminished in 5-AZA-treated cells.

Telomere 53BP1 Merge

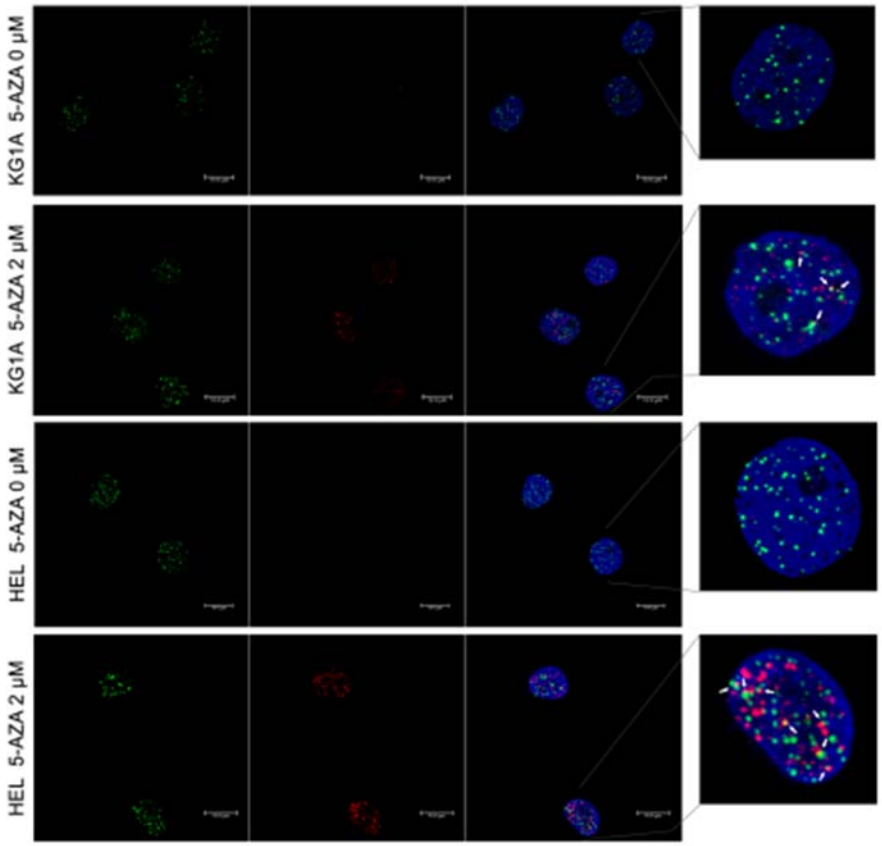

Figure 6. DNA damage and telomere dysfunction mediated by 5-AZA in AML cells. Red and Green: 53-BP1 foci and telomere signals, respectively. Yellow: Co-localization of 53-BP1 foci and telomere signals.

4.3.2 TERT Over-Expression Attenuates Telomere Shortening and Telomere Dysfunction in 5-AZA-Treated AML Cells

We introduced a lentiviral TERT expression vector into HEL cells, making a TERT-over-expressing HEL cell subline (HEL-TERT). This subline expressed two-fold higher telomerase activity than its parent one and TERT expression/telomerase activity was not inhibited by 5-AZA. The parent control HEL cells with an empty pBMN vector (HEL-pBMN) and HEL-TERT cells were first treated with 5-AZA and potential differences in cell numbers, viability or apoptosis, telomere length, telomere dysfunction, DNA damage response were then compared between these two sublines. First, more HEL-TERT cells survived than HEL-pBMN cells in the presence of 5-AZA, especially at a high concentration (mean ± SD, 69.7 ± 7.9% vs 30.5 ± 16.2%, $P = 0.021$). Consistently, apoptotic death of HEL-TERT cells was 10% less than that of HEL-pBMN cells ($P < 0.05$). Second, there were no detectable decline in telomere length in HEL-TERT cells treated with 5-AZA at 5.0μM; Finally, 5-AZA-induced TIFs and p53-BP1 foci were significantly fewer in HEL-TERT cells than in HEL-pBMN cells.

In summary, we show that the DNMT inhibitor 5-AZA down-regulates TERT expression in both AML cell lines and primary leukemic cells, and shortens telomere length coupled with telomere dysfunction, DNA damage response and apoptosis. Ectopic TERT expression partially attenuated telomere dysfunction and DNA damage, thereby protecting AML cells from apoptosis. Conceivably, TERT down-regulation and telomere dysfunction mediated by 5-AZA may contribute to the anti-tumor activity of DNMTIs. Thus, it may be worthwhile to evaluate the therapeutic efficacy of DNMTIs on hematological malignancies based on their induction of TERT inhibition/telomere dysfunction in future clinical trials.

4.4 ROLE OF FLT3ITD INHIBITOR RELATED TO TELOMERASE AND TERT IN AML (PAPER IV)

4.4.1 Down-Regulation of TERT Expression and Inhibition of TERT Promoter Activity by PKC412 in FLT3ITD-Carrying AML Cells

PKC412 is a TKI specifically targeting FLT3ITD, and as expected, the PKC412 treatment of FLT3-ITD-carrying MV4, 11 and MOLM-13 cells inhibited FLT3 phosphorylation and activity. qRT-PCR analysis displayed a time and dose-dependent down-regulation of *TERT* mRNA expression in the presence of PKC412 in both cells. Consistent with diminished *TERT* mRNA expression, telomerase activity was significantly repressed in MV4, 11 and MOLM-13 cells treated with PKC412.

To assess whether PKC412 regulated *TERT* transcription, we infected MV4, 11 cells with a GFP expression vector driven by a full-length *TERT* promoter and then treated the cells with PKC412. There were more than 50% of GFP-positive cells in DMSO-containing culture while the presence of PKC412 led to the disappearance of most GFP+ cells. We further transfected the same cells with a core *TERT* promoter reporter construct (p181), and then incubated them with DMSO or PKC412. The *TERT* promoter activity, reflected as the level of luciferase activity, was significantly inhibited in the cells exposed to PKC412 compared to the DMSO-treated ones. Those data revealed that PKC412 inhibited *TERT* transcription.

4.4.2 MYC-Dependent Inhibition of the TERT Transcription Activity by PKC412

It is well established that c-MYC is the core transcription factor in trans-activating the *TERT* gene, and we thus examined the link between FLT3ITD and c-MYC in regulating *TERT*

transcription. The treatment of FLT3ITD-carrying cells with PKC412 led to a fast and robust inhibition of *c-MYC* mRNA and protein expression, which preceded a decline in *TERT* expression. Then we transfected both cells with wt *TERT* core promoter-harboring vectors and its MYC binding site-deleted counterparts, respectively. Those cells were then exposed to PKC412. Wt *TERT* promoter activity declined significantly in PKC412-treated cells compared to that in control cells (DMSO-treated). However, PKC412 did not affect the *TERT* promoter activity any longer once two MYC binding sites on the promoter were disrupted. Taken together, PKC412-mediated repression of the *TERT* transcription is MYC-dependent.

4.4.3 Attenuation of PKC412-Mediated AML Cell Apoptosis by Ectopic Expression of TERT through Alternative Tyrosine Kinase (TK) Signaling Pathways

Compared to control cells (MOLM-13-pBMN), significantly more TERT-overexpressed MOLM-13 cells were left in the presence of PKC412. FACS analysis also revealed that PKC412 induced apoptosis in 35% of control cells, and the ectopic *TERT* expression significantly attenuated apoptosis of MOLM-13 cells mediated by PKC412 (18%).

Then we compared differences in gene expression profiles between MOLM-13-pBMN and MOLM-13-TERT cells in the presence or absence of PKC412. Intriguingly, we identified that the ectopic *TERT* expression significantly affected *FLT3* and other receptor tyrosine kinase (RTK) signaling pathways (Figure 7). First, the expression of *c-KIT*, another RTK structurally similar to *FLT3*, was up-regulated by the ectopic *TERT* expression, and its mRNA level was even much higher upon the exposure of MOLM-13-TERT cells to PKC412. In contrast, *c-KIT* expression did not change in MOLM13-pBMN cells with and without PKC412. Second, *DOC3*, an endogenous inhibitor of the RAS-MAPK signaling, was down-regulated in MOLM-13-TERT cells and the PKC412 treatment led to further dramatic decline in *DOC3* levels. Finally, *SULF2* that activates the PDGF signaling pathway exhibited enhanced expression in MOLM13-TERT cells and its robust increase was observed following PKC412 treatment of these cells, whereas there was no detectable alteration in its expression in MOLM-13-pBMN cells with and without PKC412.

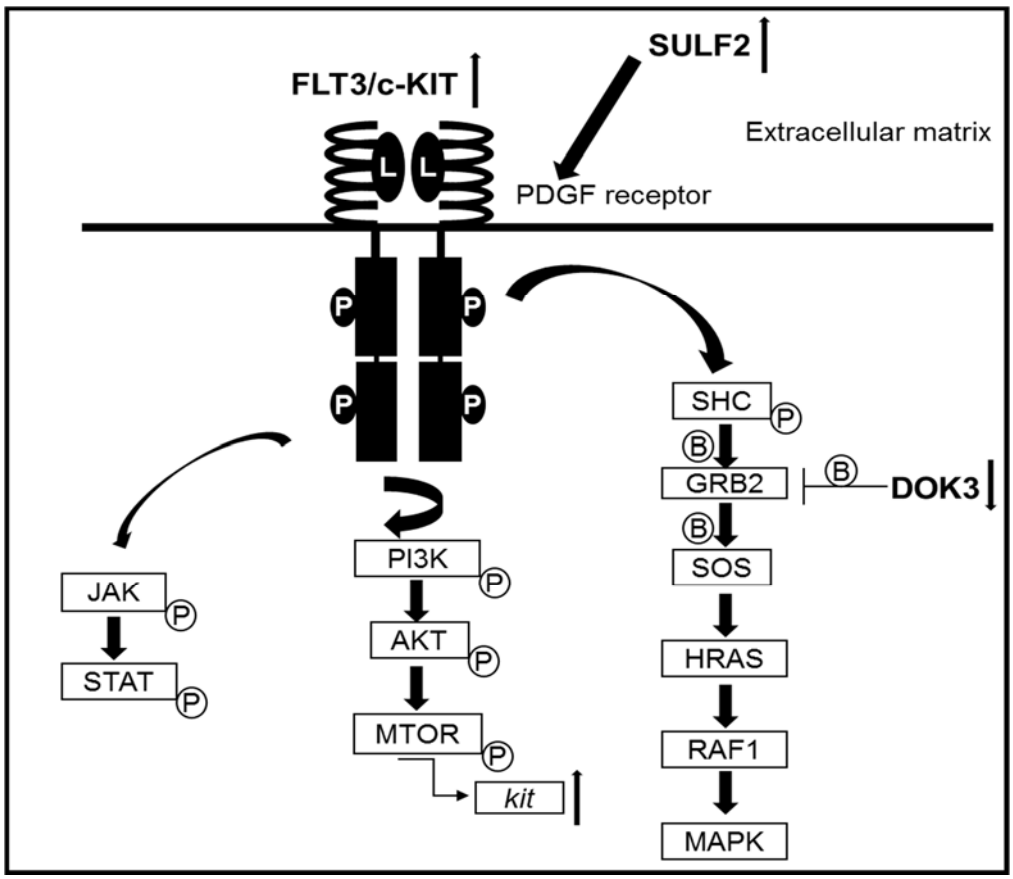

Figure 7. TERT stimulation of the FLT3 downstream effectors and alternative TK pathways in the presence of PKC412. PI3K, phosphatidylinositol 3-kinase; GRB2, growth-factor-receptor-bound protein 2; SHC, SH2-containing sequence protein; mTOR, mammalian target of rapamycin.

Here we identified that PKC412, a TKI specifically targeting FLT3ITD, repressed *TERT* transcription and telomerase activity in FLT3ITD-carrying AML cells in a MYC-dependent manner. This effect of PKC412 is likely associated with its therapeutic efficacy on AML. Importantly, we demonstrate that TERT significantly attenuates the apoptotic cell death mediated by PKC412, which strongly indicates that TERT is capable of mediating resistance to cancer targeted therapy. Collectively, the present findings reveal a functional link between FLT3ITD and telomerase, and may have an important implication in AML pathogenesis and targeted therapy.

5 SUMMARY&CONCLUSIONS

AML is a heterogeneous disease. Degulation of multiple oncogenes and tumor suppressors contributes to the process of leukemogenesis. At the genetic level, depletion of tumor suppressor expression or gain of oncogene copy number plays a great role in this process. In addition, at the epigenetic level, abnormal hyper-methylation in promoter region or global hypo-methylation leads to silence of tumor suppressor genes, thus inducing uncontrolled expansion of leukemic cells.

In this project, several factors which play potential roles in leukemogenesis are studied both from experimental and clinical aspects. Specially, the conclusions are:

1. MiR-370 is a tumor suppressive factor by targeting FoxM1, and its down-regulation results from DNA hyper-methylation. Targeting miR-370 may have a therapeutic implication in AML (Paper I).

2. We reported an extremely late relapse of APL and the leukemic clonal evolution. Very late relapses in APL, as seen in our unique patient, are more likely caused by a new genetic attack on existing pre-APL clones, which differs from early relapses resulting from the re-growth of original residual APL blasts. Genomic characterization of late relapses may have therapeutic implications (Paper II).

3. DNMT inhibitor 5-AZA down-regulates TERT expression, and shortens telomere length coupled with telomere dysfunction and apoptosis in AML. Ectopic TERT expression partially attenuates telomere dysfunction and DNA damage, thereby protecting AML cells from apoptosis. TERT down-regulation and telomere dysfunction mediated by 5-AZA may contribute to the anti-tumor activity of DNMTIs. Thus, it may be worthwhile to evaluate the therapeutic efficacy of DNMTIs on hematological malignancies based on their induction of TERT inhibitor/telomere dysfunction in future clinical trials (Paper III).

4. The FLT3 inhibitor PKC412 repressed TERT transcription and telomerase activity in FLT3ITD-carrying AML cells in a MYC-dependent manner. This effect of PKC412 is likely associated with its therapeutic efficacy on AML. TERT significantly attenuates the apoptotic cell death mediated by PKC412, which strongly indicates that TERT is capable of mediating resistance to cancer targeted therapy. The present findings reveal a functional link between FLT3ITD and telomerase, and may have an important implication in AML pathogenesis and targeted therapy (Paper IV).

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7 REFERENCES

1. Jabbour E, Estey E, Kantarjian H. Adult acute myeloid leukemia. *Mayo Clin Proc.* 2006;81(2):247-260.
2. Ferrara F, Schiffer CA. Acute myeloid leukaemia in adults. *Lancet.* 2013;381:484-495.
3. Sewerdlow S, XCampo E, Harris N, et al. WHO classification of Tumours Haematopoetic and Lymphoid Tissues. *Lyon: IARC Press.* 2008.
4. Seufert W, Seufert WD. The Recognition of Leukemia as a Systemic-Disease. *J Hist Med All Sci.* 1982;37(1):34-50.
5. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Annals of internal medicine.* Oct 1985;103(4):620-625.
6. Vardiman J, Harris N, Brunning R. The World Health Organization(WHO) classification of the myeloid neoplasms. *Blood.* 2002;100:2292-2302.
7. Greenlee R, Hill-Harmon M, Murray T, Thun M. Cancer statistics. *CA Cancer J Clin.* 2001;51:15-36.
8. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics. *CA Cancer J Clin.* 2002;52:23-47.
9. Phekoo KJ, Richards MA, Moller H, Schey SA. The incidence and outcome of myeloid malignancies in 2,112 adult patients in south East-England. *Haematol-Hematol J.* Oct 2006;91(10):1400-1404.
10. Estey E, Dohner H. Acute myeloid leukaemia. *Lancet.* Nov 25 2006;368(9550):1894-1907.
11. Pedersen-Bjergaard J, Andersen MK, Andersen MT, Christiansen DH. Genetics of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia.* Feb 2008;22(2):240-248.
12. Speer SA, Semenza JC, Kurosaki T, Anton-Culver H. Risk factors for acute myeloid leukemia and multiple myeloma: a combination of GIS and case-control studies. *Journal of environmental health.* Mar 2002;64(7):9-16; quiz 35-16.
13. Kane EV, Roman E, Cartwright R, Parker J, Morgan G. Tobacco and the risk of acute leukaemia in adults. *British journal of cancer.* Dec 1999;81(7):1228-1233.
14. Fernberg P, Odenbro A, Bellocco R, et al. Tobacco use, body mass index, and the risk of leukemia and multiple myeloma: A nationwide cohort study in Sweden. *Cancer Res.* Jun 15 2007;67(12):5983-5986.

15. Goldin LR, Kristinsson SY, Liang XS, Derolf AR, Landgren O, Bjorkholm M. Familial aggregation of acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol.* Jan 10 2012;30(2):179-183.
16. Smith SM, Beau MML, Huo D, et al. Clinical-cytogentic associations in 306 patients with therapy-related myelodysplasia or myeloid leukemia: the University of Chicago series. *Blood.* 2003;102:43-52.
17. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia.* May 2008;22(5):915-931.
18. Daver N, Cortes J. Molecular targeted therapy in acute myeloid leukemia. *Hematology.* 2012;17(s1):s59-s62.
19. Scholl C, Gilliland DG, Frohling S. Deregulation of signaling pathways in acute myeloid leukemia. *Seminars in oncology.* Aug 2008;35(4):336-345.
20. Gilliland D, Jordan C, Felix C. The molecular basis of leukemia. *Hematology Am Soc Hematol Educ Program.* 2004:80-97.
21. Lo-Coco F, Hasan SK. Understanding the molecular pathogenesis of acute promyelocytic leukemia. *Best practice & research. Clinical haematology.* Mar 2014;27(1):3-9.
22. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *Journal of Clinical Oncology.* 2003;21(24):4642-4649.
23. Boiron M, Weil M, Jacquillat C, et al. Daunorubicin in the treatment of acute myelocytic leukaemia. *Lancet.* Feb 15 1969;1(7590):330-333.
24. Bishop J, Lowenthal R, Joshua D. Etoposide in acute nonlymphocytic leukemia. Australian Leukemia Study Group. *Blood.* 1990;75:27-32.
25. Mandelli F, Vignetti M, Suciu S. Daunorubicin Versus Mitoxantrone Versus Idarubicin as Induction and Consolidation Chemotherapy for Adults with Acute Myeloid Leukemia: The EORTC and GIMEMA Groups Study AML-10. *J Clin Oncol.* 2009;27:5397-5403.
26. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood.* Jan 21 2010;115(3):453-474.
27. Jabbour E, Cortes J, Ravandi F, O'Brien S, Kantarjian H. Targeted Therapies in Hematology and Their Impact on Patient Care: Chronic and Acute Myeloid Leukemia. *Semin Hematol.* Oct 2013;50(4):271-283.

28. Tettamanti. S, Magnani. CF, Biondi. A, Biagi. E. Acute myeloid leukemia and novel biological treatments: monoclonal antibodies and cell-based gene-modified immune effectors. *Immunology letters.* Sep-Oct 2013;155(1-2):43-46.
29. Mi J. How to manage acute promyelocytic leukemia. *Leukemia.* Aug 2012;26(8):1743-1751.
30. Grimwade D, Enver T. Acute promyelocytic leukemia: where does it stem from? *Leukemia.* 2004;18(3):375-384.
31. Lehmann S, Ravn A, Carlsson L, et al. Continuing high early death rate in acute promyelocytic leukemia: a population-based report from the Swedish Adult Acute Leukemia Registry. *Leukemia.* Jul 2011;25(7):1128-1134.
32. Zhang L, Zhu X. Epidemiology, diagnosis and treatment of acute promyelocytic leukemia in children: the experience in china. *Mediterranean journal of hematology and infectious diseases.* 2012;4(1):e2012012.
33. Mistry AR, Felix CA, Whitmarsh RJ, et al. DNA topoisomerase II in therapy-related acute promyelocytic leukemia. *N Engl J Med.* 2005;352:1529-1538.
34. D The H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. *Nat Rev Cancer.* Nov 2010;10(11):775-783.
35. Breen KA, Grimwade D, Hunt BJ. The pathogenesis and management of the coagulopathy of acute promyelocytic leukaemia. *British journal of haematology.* Jan 2012;156(1):24-36.
36. Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood.* Feb 26 2009;113(9):1875-1891.
37. Degos L. All-trans retinoic acid (ATRA) therapeutical effect in acute promyelocytic leukemia. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie.* 1992;46(5-7):201-209.
38. Chen Z, Tao R, Xie X. The Present Status in All-Trans-Retinoic Acid (Atra) Treatment for Acute Promyelocytic Leukemia Patients - Further Understanding and Comprehensive Strategy Are Required in the Future. *Leukemia Lymphoma.* Nov 1992;8(4-5):247-252.
39. Huang M, Ye Y, Chen S, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood.* 1988;72(2):567-572.
40. Degos L. All-trans-retinoic acid treatment and retinoic acid receptor alpha gene rearrangement in acute promyelocytic leukemia: a model for differentiation therapy. *International journal of cell cloning.* Mar 1992;10(2):63-69.

41. Hofmann SL. Southwestern Internal-Medicine Conference - Retinoids - Differentiation Agents for Cancer-Treatment and Prevention. *Am J Med Sci.* Sep 1992;304(3):202-213.
42. Iland HJ, Wei A, Seymour JF. Have all-trans retinoic acid and arsenic trioxide replaced all-trans retinoic acid and anthracyclines in APL as standard of care. *Best Pract Res Cl Ha.* Mar 2014;27(1):39-52.
43. Fenaux P, Castaigne S, Dombret H, et al. All-trans retinoic acid followed by intensive chemotherapy gives a high complete remission rate and may prolong remissions in newly diagnosed acute promyelocytic leukemia: a pilot study on 26 cases. *Blood.* 1992;80(9).
44. Zheng X, Seshire A, Rüster B, et al. Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARalpha-positive leukemic stem cells. *Haematologica.* 2007;92:323-331.
45. Nasr R, Guillemin M-C, Ferhi O, et al. Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nature Medicine.* 2008;14:1333 - 1342.
46. Chen G-Q, Shi X-G, Tang W, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. *Blood.* 1997;89(9).
47. Ablain J, Leiva M, Peres L, Fonsart J, Anthony E, de The H. Uncoupling RARA transcriptional activation and degradation clarifies the bases for APL response to therapies. *The Journal of experimental medicine.* Apr 8 2013;210(4):647-653.
48. Lengfelder E, Hofmann WK, Nowak D. Treatment of acute promyelocytic leukemia with arsenic trioxide: clinical results and open questions. *Expert review of anticancer therapy.* Sep 2013;13(9):1035-1043.
49. Altuvia Y, Landgraf P, Lithwick G, et al. Clustering and conservation patterns of human microRNAs. *Nucleic acids research.* 2005;33(8):2697-2706.
50. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* Jan 23 2004;116(2):281-297.
51. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic acids research.* 2005;33(4):1290-1297.
52. Karp X, Ambros V. Developmental biology. Encountering microRNAs in cell fate signaling. *Science.* Nov 25 2005;310(5752):1288-1289.

53. Xu P, Guo M, Hay BA. MicroRNAs and the regulation of cell death. *Trends Genet.* Dec 2004;20(12):617-624.
54. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science.* Jan 2 2004;303(5654):83-86.
55. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature.* Nov 11 2004;432(7014):226-230.
56. Calin GA, Sevignani C, Dan Dumitru C, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *P Natl Acad Sci USA.* Mar 2 2004;101(9):2999-3004.
57. Rooij EV, Sutherland LB, Liu N, et al. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *P Natl Acad Sci USA.* Nov 28 2006;103(48):18255-18260.
58. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *P Natl Acad Sci USA.* Nov 26 2002;99(24):15524-15529.
59. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A.* Sep 27 2005;102(39):13944-13949.
60. Bueno MJ, Perez de Castro I, Gomez de Cedron M, et al. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. *Cancer cell.* Jun 2008;13(6):496-506.
61. Mathews W, Jordan C, Wiegand G, Pardoll D, Lemischka I. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell.* 1991;65:1143-1152.
62. McKenna H, Smith F, Brasel K, et al. Effects of FLT3 ligand on acute myeloid and lymphocytic leukemic blasts from children. *Exp Hematol.* 1996;24:378-385.
63. Kiyoi H, Naoe T, Nakano Y, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood.* May 1 1999;93(9):3074-3080.
64. Kiyoi H, Towatari M, Yokota S, et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia.* Sep 1998;12(9):1333-1337.
65. Rombouts W, Blokland I, Lowenberg B, Plemacher R. Biological characteristic and prognosis of adult acute myeloid leukemia with internal tandem duplications in the FLT3 gene. *Leukemia.* 2000;14:675-683.
66. Hubbard SR. Juxtamembrane autoinhibition in receptor tyrosine kinases. *Nature reviews. Molecular cell biology.* Jun 2004;5(6):464-471.

67. Griffith J, Black J, Faerman C, et al. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Molecular cell.* Jan 30 2004;13(2):169-178.
68. Jilani I, Estey E, Manshuri T, et al. Better detection of FLT3 internal tandem duplication using peripheral blood plasma DNA. *Leukemia.* Jan 2003;17(1):114-119.
69. Swords R, Freeman C, Giles F. Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. *Leukemia.* Oct 2012;26(10):2176-2185.
70. Leung AY, Man CH, Kwong YL. FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia. *Leukemia.* Feb 2013;27(2):260-268.
71. Williams CB, Kambhampati S, Fiskus W, et al. Preclinical and phase I results of decitabine in combination with midostaurin (PKC412) for newly diagnosed elderly or relapsed/refractory adult patients with acute myeloid leukemia. *Pharmacotherapy.* Dec 2013;33(12):1341-1352.
72. Fischer T, Stone R, Deangelo D, et al. Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. *J Clin Oncol.* 2010;28(28):4339-4345.
73. ClinicalTrials.gov. NCT 00651261 Daunorubicin, Cytarabine, and Midostaurin in Treating Patients With Newly Diagnosed Acute Myeloid Leukemia.
74. Wander SA, Levis MJ, Fathi AT. The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond. *Therapeutic advances in hematology.* Jun 2014;5(3):65-77.
75. Grunwald MR, Levis MJ. FLT3 inhibitors for acute myeloid leukemia: a review of their efficacy and mechanisms of resistance. *Int J Hematol.* 2013;97:683-694.
76. Blackburn EH. Switching and signaling at the telomere. *Cell.* Sep 21 2001;106(6):661-673.
77. Calado R, Young N. Telomere diseases. *N Engl J Med.* 2009;361:2353-2365.
78. Warner JK, Wang JC, Takenaka K, et al. Direct evidence for cooperating genetic events in the leukemic transformation of normal human hematopoietic cells. *Leukemia.* Oct 2005;19(10):1794-1805.
79. Cukusic A, Vidacek NS, Sopta M, Rubelj I. Telomerase regulation at the crossroads of cell fate. *Cytogenet Genome Res.* 2008;122(3-4):263-272.
80. Kanzawa T, Komata T, Kyo S, Germano IM, Kondo Y, Kondo S. Down-regulation of telomerase activity in malignant glioma cells by p27KIP1. *International journal of oncology.* Dec 2003;23(6):1703-1708.

81. Aubert G. Telomere dynamics and aging. *Prog Mol Biol Transl Sci.* 2014;125:89-111.
82. Martine P, Blasco MA. Role of shelterin in cancer and aging. *Aging cell.* 2010;9:653-666.
83. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis.* 2010;31:9-18.
84. Choi JK, Southworth LK, Sarin KY, et al. TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. *Plos Genet.* Jan 2008;4(1).
85. Cong Y, Shay JW. Actions of human telomerase beyond telomeres. *Cell research.* Jul 2008;18(7):725-732.
86. Mizuno S, Chijiwa T, Okamura T, et al. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood.* Mar 1 2001;97(5):1172-1179.
87. Schoofs T, Berdel WE, Muller-Tidow C. Origins of aberrant DNA methylation in acute myeloid leukemia. *Leukemia.* Jan 2014;28(1):1-14.
88. Subramaniam D, Thombre R, Dhar A, Anant S. DNA methyltransferases: a novel target for prevention and therapy. *Frontiers in oncology.* 2014;4:80.
89. Flotho C, Claus R, Batz C, et al. The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. *Leukemia.* Jun 2009;23(6):1019-1028.
90. Raj K, John A, Ho A, et al. CDKN2B methylation status and isolated chromosome 7 abnormalities predict responses to treatment with 5-azacytidine. *Leukemia.* Sep 2007;21(9):1937-1944.
91. Claus R, Lubbert M. Epigenetic targets in hematopoietic malignancies. *Oncogene.* 2003;22:6489-6496.
92. Fandy TE, Jiemjit A, Thakar M, Rhoden P, Suarez L, Gore SD. Decitabine induces delayed reactive oxygen species (ROS) accumulation in leukemia cells and induces the expression of ROS generating enzymes. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Mar 1 2014;20(5):1249-1258.
93. Tsai CT, Yang PM, Chern TR, et al. AID downregulation is a novel function of the DNMT inhibitor 5-aza-deoxycytidine. *Oncotarget.* Jan 2014;5(1):211-223.
94. Paoliello-Paschoalato AB, Azzolini AE, Cruz MF, et al. Isolation of healthy individuals' and rheumatoid arthritis patients' peripheral blood neutrophils by the

gelatin and Ficoll-Hypaque methods: Comparative efficiency and impact on the neutrophil oxidative metabolism and Fcgamma receptor expression. *Journal of immunological methods.* Oct 2014;412:70-77.

95. Rinehart T. AFLP analysis using GeneMapper software and an Excel macro that aligns and converts output to binary. *Biotechniques.* 2004;37(2):186-188.
96. Arico A, Ferraresso S, Bresolin S, et al. Array-based comparative genomic hybridization analysis reveals chromosomal copy number aberrations associated with clinical outcome in canine diffuse large B-cell lymphoma. *PloS one.* 2014;9(11):e111817.
97. Chen K, Wallis J, McLellan M, Larson D, Kalicki J, Pohl C. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nature Methods.* 2009;6(9):677-681.
98. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* Aug 15 2009;25(16):2078-2079.
99. Odgerel T, Kikuchi J, Wada T, et al. The FLT3 inhibitor PKC412 exerts differential cell cycle effects on leukemic cells depending on the presence of FLT3 mutations. *Oncogene.* May 15 2008;27(22):3102-3110.
100. Wang S, Xue S, Dai Y, et al. Reduced expression of microRNA-100 confers unfavorable prognosis in patients with bladder cancer. *Diagnostic pathology.* 2012;7:159.
101. Nasri M, Karimi A, Allahbakhshian Farsani M. Production, purification and titration of a lentivirus-based vector for gene delivery purposes. *Cytotechnology.* Dec 2014;66(6):1031-1038.
102. Li XN, Parikh S, Shu Q, et al. Phenylbutyrate and phenylacetate induce differentiation and inhibit proliferation of human medulloblastoma cells. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Feb 1 2004;10(3):1150-1159.
103. Dimri G, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA.* 1995;92:9363-9367.
104. Lindvall C, Hou M, Komurasaki T, et al. Molecular characterization of human telomerase reverse transcriptase-immortalized human fibroblasts by gene expression profiling: activation of the epiregulin gene. *Cancer Res.* Apr 15 2003;63(8):1743-1747.
105. Cristofalo VJ. SA beta Gal staining: biomarker or delusion. *Experimental gerontology.* Oct 2005;40(10):836-838.

106. Cesare AJ, Kaul Z, Cohen SB, et al. Spontaneous occurrence of telomeric DNA damage response in the absence of chromosome fusions. *Nature structural & molecular biology.* Dec 2009;16(12):1244-1251.
107. Baerlocher GM, Vulto I, Jong Gd, Lansdorp PM. Flow cytometry and FISH to measure the average length of telomeres (flow FISH). *NATURE PROTOCOLS.* 2006;1(5):2365-2376.
108. Roos G, Hultdin M. Flow cytometric determination of telomere length. *Cytometry.* Sep 1 2001;45(1):79-80.
109. Baerlocher GM, Mak J, Tien T, Lansdorp PM. Telomere length measurement by fluorescence in situ hybridization and flow cytometry: tips and pitfalls. *Cytometry.* Feb 1 2002;47(2):89-99.
110. Ng L, Cropley J, Pickett H, Reddel R, Suter C. Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. *Nucleic Acides Res.* 2009;37:1152-1159.
111. Moore A, Donahue CJ, Bauer KD, Mather JP. Simultaneous measurement of cell cycle and apoptotic cell death. *Methods in cell biology.* 1998;57:265-278.
112. Granada JF, Alviar CL, Wallace-Bradley D, et al. Patterns of activation and deposition of platelets exposed to the polymeric surface of the paclitaxel eluting stent. *Journal of thrombosis and thrombolysis.* Jan 2010;29(1):60-69.
113. Takakura M, Kyo S, Kanaya T, et al. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res.* Feb 1 1999;59(3):551-557.
114. Strååt K, Liu C, Rahbar A, et al. Activation of telomerase by human cytomegalovirus. *J Natl Cancer Inst.* 2009;101(7):488-497.
115. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science.* Oct 20 1995;270(5235):467-470.
116. Laoukili J, Stahl M, Medema RH. FoxM1: At the crossroads of ageing and cancer. *Bba-Rev Cancer.* Jan 2007;1775(1):92-102.
117. Kiriakidou M, Nelson PT, Kouranov A, et al. A combined computational-experimental approach predicts human microRNA targets. *Genes & development.* May 15 2004;18(10):1165-1178.

118. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* Jan 14 2005;120(1):15-20.
119. John B, Enright A, Aravin A, Tuschl T, Sander C, Marks D. Human microRNA targets. *PLoS Biol.* 2004;2:1862–1879.
120. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nature genetics.* May 2005;37(5):495-500.
121. Starczynowski DT, Morin R, McPherson A, et al. Genome-wide identification of human microRNAs located in leukemia-associated genomic alterations. *Blood.* Jan 13 2011;117(2):595-607.
122. Calin GA, Croce CM. MicroRNAs and chromosomal abnormalities in cancer cells. *Oncogene.* Oct 9 2006;25(46):6202-6210.
123. Royo H, Cavaille J. Non-coding RNAs in imprinted gene clusters. *Biology of the cell / under the auspices of the European Cell Biology Organization.* Mar 2008;100(3):149-166.
124. Bandres E, Cubedo E, Agirre X, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Molecular cancer.* 2006;5:29.
125. Meng F, Wehbe-Janek H, Henson R, Smith H, Patel T. Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. *Oncogene.* Jan 10 2008;27(3):378-386.
126. Mi S, Lu J, Sun M, et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *Proc Natl Acad Sci U S A.* Dec 11 2007;104(50):19971-19976.
127. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood.* Mar 15 2008;111(6):3183-3189.
128. Ferrara F, Selleri C, Mele G, et al. Late relapse of acute promyelocytic leukemia treated with all-trans retinoic acid and chemotherapy: report of two cases. *Ann Hematol.* Jul 2004;83(7):484-486.
129. Fenaux P, Chastang C, Chevret S, et al. A randomized comparison of all transretinoic acid (ATRA) followed by chemotherapy and ATRA plus chemotherapy and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. *Blood.* Aug 15 1999;94(4):1192-1200.

130. Tallman MS, Andersen JW, Schiffer CA, et al. All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood.* Dec 15 2002;100(13):4298-4302.
131. Latagliata R, Carmosino I, Breccia M, et al. Late relapses in acute promyelocytic leukaemia. *Acta haematologica.* 2007;117(2):106-108.
132. Zhan H, Rajasree R, Russo L, Patel D. Late relapse of acute promyelocytic leukemia in a patient with no maintenance therapy. *Am J Hematol.* Mar 2007;82(3):248.
133. Issa J, Kantarjian H. Azacitidine. *Nat Rev Drug Discov.* 2005;Suppl:S6-7.