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B Cell Lymphoma Malignancy is Associated with a Long non-coding RNA, MIAT, Expression.

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ABSTRACT

Hematopoietic malignancies are highly dysregulated processes in which many molecular aspects are involved. Long non-coding RNAs (lncRNAs) are increasingly recognized as important regulators of gene expression. They are functional RNAs longer than 200 nucleotides in length, with low coding potential. MIAT was originally identified as a long non-coding RNA expressed in neuronal cells and constitutes a component of the nuclear scaffold. Moreover it affects the kinetics of the splicing process. Recently, MIAT is genetically associated with heart disease. However the molecular basis of MIAT function as well as its role in human disease is still in the beginning.

In the present thesis, Expression of MIAT was studied in leukemic cell lines as well as a large cohort of CLL patients. Quantitative analysis of MIAT, revealed extremely MIAT expression in B cell lymphoma, while other types of leukemia like AML, ALL and CML did not show considerable expression. Feasible prognosis of outcome in CLL sample groups was related to MIAT expression. Abundant expression of MIAT achieved in unfavorable outcomes group of CLL patient samples (trisomy 12, 17p13 deletion, 11q22 deletion) compared to favorable (13q deletion) cytogenetic group. Intriguingly, MIAT expression might be associate to aggressiveness and poor outcome in CLL which also empower a role for MIAT in leukemia. Since, genetic networks controlling MIAT expression was the focus of our intense interest, an association beyond the transcription factors, Oct4, and human MIAT transcript was also studied. We showed that mRNA and protein level of Oct4 is in direct modulation of MIAT expression in leukemic cell lines as well as CLL patient samples. RNAi mediated knockdown of MIAT transcript lead to robust changes in Oct4 mRNA level. We further characterized a regulatory feedback loop between Oct4 and MIAT by developing lentivirus shRNA to downregulate Oct4 level. We showed that a reciprocal correlation of MIAT and Oct4 regulates their expression. Most importantly, We demonstrated that suppression of either Oct4 or MIAT induced apoptosis and reduced viability in lymphoma derived cell line.

In haematological malignancies, More studies on lncRNAs MIAT, may help to identify patient populations at risk of leukemia, may classify patients into aggressive or mild cancer groups and may also promisingly facilitate the derivation of conventional therapeutic interventions by transfering lncRNA research to clinical oncology.

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DEDICATION

I would like to dedicate my dissertation

To:

My Father & My Mother

For teaching me to be diligent and hopeful

My Devoted and Kindly Wife

Who was with me in each step of the way

My Great Son

Whom I would love him forever

CHAPTER 1 : INTRODUCTION

1.1. Leukemia

Leukemia is part of a broader group of neoplasms which affect the blood, bone marrow, and lymphoid system, known as tumors of the hematopoietic and lymphoid tissues. Leukemia usually begins in the bone marrow and results in high numbers of abnormal white blood cells. These white blood cells are not fully developed and are called *blasts* or *leukemia cells*. WBCs can be formed from different cell lineages, lymphoid or myeloid. In 2012 leukemia developed in 352,000 people globally and caused 265,000 deaths. Both inherited and environmental (non-inherited) factors are believed to be involved (health september 2013). Risk factors include smoking, ionizing radiation, some chemicals (such as benzene), prior chemotherapy and Down syndrome as well as a family history of leukemia. Outcomes have improved in the developed world. The average five-year survival rate is 57% in the United States. In children under 15, the five-year survival is greater than 60 to 85%, depending on the type of leukemia. The cell lineage affected by the cancer determines the kind of leukemia and the affect can be sudden or "acute" or can be developing slowly or "chronic". This results in 4 subtypes: acute lymphocytic leukemia (ALL) – most common in children, acute myelogenous leukemia(AML, #1) – most common in adults, chronic lymphocytic leukemia (CLL) – most an adult disorder, chronic myelogenous leukemia (CML) – most common in adults.

1.1.1. Acute lymphoblastic leukemia (ALL)

(Incidence, Causes and Risk Factors).

Acute lymphoblastic leukemia (ALL), results from an acquired or a genetic injury to the DNA of a single cell in the marrow. The effects of ALL include uncontrolled and exaggerated growth and accumulation of cells called "lymphoblasts" or "leukemic blasts," which fail to function as normal blood cells. The presence of the leukemic blasts blocks the production of

normal cells. As a result, when ALL is diagnosed, the number of healthy blood cells (red blood cells, white blood cells and platelets) is usually lower than normal(Raetz 2014). (ALL) is the most common type of leukemia in young children. ALL occurs most often in the first decade of life but increases in frequency again in older individuals. A few factors have been associated with an increased risk of developing the disease. Exposure to high doses of radiation is one such factor. Also reducing children's exposure to bacterial infections during the first year of life and multiple diagnostic x-rays during childhood may have increased the risk of childhood. Previous chemotherapy and radiation treatment may be a cause of ALL in adults(Raetz 2014). Some cases of ALL relate to a mutation in a lymphocyte that occurs during the prenatal period (in utero).

ALL Subtypes:

ALL Subtypes include precursor B acute lymphoblastic leukemia, precursor T acute lymphoblastic leukemia, Burkitt's leukemia, and acute biphenotypic leukemia.

Immunophenotyping, a process used to identify cells based on the types of proteins (antigens) on the cell surface, is necessary to establish the diagnosis of either B-cell ALL, T-cell ALL or acute myeloid leukemia (AML, #2). Mature B-cell leukemia is also known as "Burkitt leukemia/lymphoma." It accounts for 2%-3% of ALL patients. In some studies, ALL has been subdivided into CD10 (the common acute lymphoblastic leukemia antigen, abbreviated cALLa) positive and CD10 negative. Genetic classification of ALL cells is summarized in table 1.1. Translocations are the most common type of DNA change that is associated with ALL. In a translocation, the DNA from one chromosome breaks off and becomes attached to a different chromosome. Other chromosome changes such as deletions and inversions can also lead to the development of ALL, but these changes are less common. In many cases of ALL, the genetic changes are not known(Raetz 2014).

Table 1.1: Genetic classification of ALL.

	Abnormalities	Involved genes	Incidence	Molecular Techniques
B-ALL	t(9;22)(q34;q11)	BCR ABL	Adults: 30% Childrens: 3%	RT-PCR
	t(12;21)(p13;q22)	TEL AML1 (ETV6 RUNX1)	Adults: <1% Childrens: 20%	RT-PCR
	t(4;11)(q21;q23)	MLL AF4 (ALL1 AF4)	Adults: 5% Childrens: 60%	RT-PCR
	t(1;19)(q23;p13)	E2A PBX1 (TCF_3 PBX1)	5%	RT-PCR
	t(8;14)(q24;q32)	c-MYC IgH	1%	FISH
	t(17;19)(q22;p13)	E2A HLF	<1%	RT-PCR
	t(11;19)(q23;p13)	MLL ENL	<1%	RT-PCR
		JAK1/2/3 mutations	10%	Sequencing
T-ALL	t(10;14)(q24;q11) t(7;10)(q34;q24)	HOX11 TCRα/δ HOX11 TCRβ	Adults: 31% Childrens: 7%	RT-PCR
	t(5;14)(q35;q32)	HOX11L2 TCRα/δ	Adults: 13% Childrens: 20%	RT-PCR, FISH
	t(1;14)(p32;q11)	TAL1 TCRα/δ	1-3%	RT-PCR
	Normal 1p32	SIL TAL1	9-30%	RT-PCR
	inv(7)(p15q34), t(7;7)	HOXA genes TCRβ	5%	FISH, RT-PCR
	t(10;11)(p13;q14-21)	CALM AF10	10%	FISH
	t(9;9)(q34;q34)	NUP214 ABL1	6%	FISH
	t(9;14)(q34;q34)	EML1 ABL1	<1%	FISH
	NOTCH1 mutations	NOTCH1	50%	Sequencing
	JAK1 mutations	JAK1	18%	Sequencing

1.1.2. Acute Myeloid Leukemia (AML)

AML results from acquired changes in the DNA (genetic material) of a developing marrow cell. Once the marrow cell becomes a leukemic cell, it multiplies into 11 billion or more cells. These cells, called “leukemic blasts,” do not function normally. However, they grow and survive better than normal cells. The presence of the leukemic blasts blocks the production of normal cells (Karp 2011).

(Incidence, Causes and Risk Factors).

Most patients diagnosed with AML have no clear-cut triggering event. Repeated exposure to the chemical benzene can be a factor in AML development. Benzene damages the DNA of normal marrow cells. A small but increasing percentage of AML cases arise following treatment with chemotherapy (especially with alkylating agents or topoisomerase II inhibitors)

or radiation therapy for other cancers, such as lymphoma, myeloma and breast cancer. But only a small proportion of people exposed to chemotherapy, radiation therapy and/or benzene develop AML. A theory about why AML develops in some people is that they have inherited genes that limit their ability to detoxify the causative agents. (Karp 2011). Genetic disorders, such as Fanconi's anemia, Shwachman syndrome, Diamond-Blackfan syndrome and Down syndrome, are also associated with an increased risk of AML. AML may develop from the progression of other blood cancers, including polycythemia vera, primary myelofibrosis, essential thrombocythemia and myelodysplastic syndromes (MDS) (Karp 2011). AML is the most common acute leukemia affecting adults. The risk for developing AML increases about 10-fold from ages 30 to 34 years (about 1 case per 100,000 people) to ages 65 to 69 years (about 10 cases per 100,000 people). For people over 70, the incidence rate continues to increase, peaking between the ages of 80 and 84.(Karp 2011).

AML Subtypes:

Most people who are diagnosed with AML have one of the eight AML subtypes shown in Table 1.2. This table is based on the French, American, British (FAB) classification system . The World Health Organization (WHO) classification system for AML which is based on the expected outcomes include :

AML with recurrent genetic abnormalities , AML with myelodysplasia-related changes ,Therapy-related AML , AML not otherwise specified, AML with a translocation between chromosomes 8 and 21, AML with a translocation or inversion in chromosome 16, AML with changes in chromosome 11,Acute promyelocytic leukemia (APL, M3), which usually has a translocation between chromosomes 15 and 17. (Karp 2011).

Table 1.2: Subtypes of AML

Cell Subtype	Description
Myeloblastic	M0 – minimally differentiated AML
Myeloblastic, with minimal maturation	M1 – myeloblasts are the dominant leukemic cells in the marrow at the time of diagnosis.
Myeloblastic, with maturation	M2 – many myeloblasts, but some cells are developing toward fully formed blood cells.
Promyelocytic	M3 – leukemic cells have a translocation between chromosomes 15 and 17.
Myelomonocytic	M4 – leukemic cells often have a translocation or an inversion of chromosome 16.
Monocytic	M5 – leukemic cells have features of developing monocytes (white cells).
Erythroleukemic	M6 – leukemic cells have features of developing red cells.
Megakaryocytic	M7 – leukemic cells have features of developing platelets.

1.1.3. Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) results from an acquired mutation to the DNA of a single marrow cell that develops into a lymphocyte. In 95 percent of people with CLL, the change occurs in a B lymphocyte. In the other 5 percent of people with CLL, the cell that transforms from normal to leukemic has the features of a T lymphocyte or a natural killer (NK) cell. Thus, any of the three major types of lymphocytes (T cells, B cells or NK cells) can undergo a malignant transformation that causes diseases related to B-cell CLL. The leukemic cells that accumulate in the marrow in people with CLL do not prevent normal blood cell production as extensively as is the case with acute lymphoblastic leukemia. This is an important distinction: It is the reason for the generally less severe early course of CLL comparing to ALL (Byrd 2014).

Incidence, Causes and Risk Factors.

CLL has generally not been associated with any environmental or external factors. First-degree relatives of patients with CLL are three to four times more likely to develop CLL than people who do not have first-degree relatives with the disease. However, the risk is still small. The incidence of the disease increases from less than one per 100,000 in individuals aged 40 to 44 years to more than 30 per 100,000 in individuals aged 80 and older. Older patients tend to have a worse outcome due to being diagnosed with a more aggressive CLL and the inability to tolerate treatment and symptoms of the disease (Byrd 2014).

Chromosomal Changes:

About half of CLL patients who are tested with "G-banding karyotyping" are found to have CLL cells with chromosomal abnormalities. About 80 percent of CLL patients who are tested with "fluorescence in situ hybridization (FISH)" are found to have chromosomal abnormalities. The following examples are some of the more common chromosomal abnormalities:

Del(13q) Deletions on the long arm of chromosome 13, del(13q), are the most common. Del(13q) with no other chromosomal abnormalities is associated with a relatively more favorable outcome.

Trisomy 12 About 10 to 20 percent of patients have CLL cells with three copies of chromosome 12 (trisomy 12) instead of the expected two chromosomes. Trisomy 12 is associated with intermediate-risk CLL. Trisomy 12 with other chromosomal abnormalities is associated with a higher risk than trisomy 12 alone.

Del(11q) Up to 20 percent of people with CLL have deletions in CLL cells in the long arm of chromosome 11, del(11q). The proportion of CLL patients with del 11q tend to be younger with large lymph nodes and have high-risk disease.

Chromosome 14 or Chromosome 6 Structural abnormalities of chromosome 14 or chromosome 6 in CLL cells indicate higher-risk disease.

Del(17p) About 5 percent of people with CLL at diagnosis have deletions in the short arm of chromosome 17, del(17p). The critical TP53 gene in this region is typically deleted. People who have CLL with del(17p) tend to have higher-risk disease and usually do not respond as well to standard initial therapy. Their CLL treatment needs to be approached in a different

manner. Other factors may be signs of faster-growing disease (higher-risk CLL) and indicate the need for closer follow-up with the doctor. For example:

Blood lymphocyte doubling People with CLL whose lymphocyte number doubles in one year have higher-risk CLL and need closer follow up; a lymphocyte number that remains stable indicates a relatively lower risk.

CD38 The expression of CD38 on CLL cells may be an indicator of higher-risk CLL.

B2M A higher level of serum beta2-microglobulin (B2M), a protein that is shed from CLL cells, is associated with a greater extent of disease. Several studies have found that B2M and other serum markers, such as CD23, may help predict survival or progression-free survival.

Un mutated IgHv The un mutated immunoglobulin heavy chain variable region gene (IgHv) suggests the likelihood of higher-risk disease. Forty percent of CLL patients at diagnosis will have this whereas 60 percent will have the more favorable IgHv-mutated disease.

ZAP-70 (zeta-associated protein 70), when increased, may be associated with higher-risk disease. It should be noted that further study in clinical trials is needed to standardize the assessment of ZAP-70. The National Comprehensive Cancer Network (NCCN) guidelines state that the evaluation of ZAP-70 expression by flow cytometry can be challenging and is not recommended outside of a clinical trial. There are new tests such as ZAP-70 methylation which may represent a better way to measure this. Additionally, other prognostic markers such as **CD49d** expression have also been suggested as a better biomarker than ZAP-70 (Byrd 2014).

NOTCH1 gene Notch1 is a gene involved in the development of different type of blood cells. In CLL, approximately 10 to 15 percent of patients have mutations of this gene causing it to be more active than it should be.

SF3B1 gene This gene is involved in the forming of select proteins in CLL and other blood cancers. It is mutated in several blood cancers including CLL, AML, and MDS. In CLL, approximately 10 to 15 percent of patients have mutations of this gene, resulting in dysfunctional protein processing. Several studies have suggested that CLL patients who have SF3B1 gene mutations may progress more quickly, requiring therapy and have a shorter remission and overall survival .

TP53 gene mutations The TP53 gene is viewed as the gatekeeper to protecting the DNA of cells from damage. Mutated DNA of cancer cells lead to increased cancer growth and

resistance to chemotherapy cancer treatments. Mutation of the TP53 gene is very commonly seen in patients who also have del(17p) findings on their interphase cytogenetics. Some patients just have mutation of the TP53 gene and, in general, these patients have a higher likelihood of progressing more quickly, requiring therapy, not responding well to traditional therapies and having an overall shorter survival. Select newer therapies work better for patients who have del(17p) or the TP53 gene mutations (Byrd 2014).

1.1.4. Chronic myeloid leukemia (CML)

CML is a type of cancer that starts in the blood-forming cells of the bone marrow and invades the blood. The National Cancer Institute estimates 33,990 people in the United States are living with CML, with another 5,980 new cases expected in 2014. Chronic myeloid leukemia (CML) is called by several other names, including, Chronic myelogenous leukemia, Chronic granulocytic leukemia, Chronic myelocytic leukemia. CML results from an acquired or a genetic injury to the DNA of a single bone marrow cell. The mutated cell multiplies into many cells (CML cells). CML does not completely interfere with the development of mature red cells, white cells and platelets. As a result, chronic phase myeloid leukemia is generally less severe than acute leukemia, and often patients do not have any symptoms when diagnosed.

Incidence, Causes and Risk factors:

Most cases of CML occur in adults. The frequency of CML increases with age, from about less than 1.2 in 100,000 people until about 40 years, to about 2.4 in 100,000 people at 55 years, to about 9.6 in 100,000 people at 80 years and older. No one is born with CML. Scientists do not yet understand why the BCR-ABL gene that leads to CML is formed in some people and not in others. However, in a small number of patients, CML is caused by exposure to very high doses of radiation. This effect has been most carefully studied in the survivors of the atomic-bomb blast in Japan. A slight increase in risk also occurs in some individuals treated with high-dose radiation therapy for other cancers, such as lymphoma. Most people treated for cancer with radiation do not go on to develop CML, and most people who have CML have not been exposed to high-dose radiation. Exposures to diagnostic dental or medical x-rays have not been associated with an increased risk of CML(Neil P. Shah 2014).

The Philadelphia Chromosome.

CML was initially distinguished from other types of leukemia by the presence of a genetic abnormality of chromosome 22 found in the blood and marrow cells of patients with CML.

In 1960, doctors from the University of Pennsylvania School of Medicine in Philadelphia discovered the 22nd chromosome in people with CML was shorter than it was in healthy people. This shortened 22nd chromosome was later named the "Philadelphia chromosome" or "Ph chromosome."Figure 1.1.

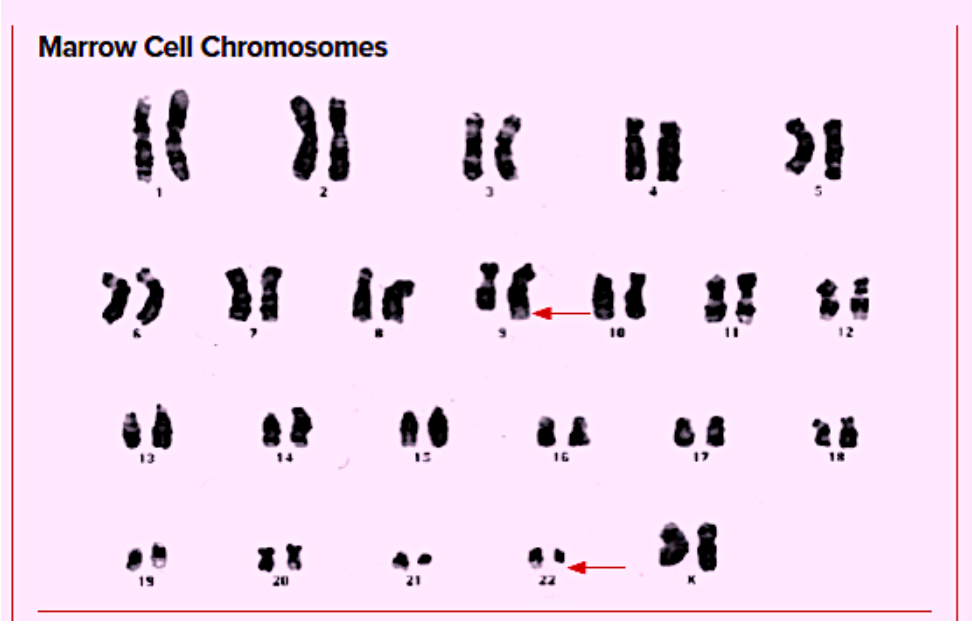


Figure1.1 Shown here is the set of chromosomes from a marrow cell of a female patient with CML.The arrow in the second row indicates chromosome 9,which is elongated.The arrow in the forth row indicates shortened arm of chromosome 22 (the Ph chromosome).

The BCR-ABL Cancer-Causing Gene Further studies of CML cells stablished that translocation of two chromosomes take place. The translocation of chromosome 9 and chromosome 22 is found only in the leukemia cells of CML patients and in some patients with acute lymphoblastic leukemia (ALL). One theory that scientists propose about why this switch occurs is that when the cells are dividing, chromosomes 9 and 22 are very close to each other, making this error more likely. The break on chromosome 9 leads to a mutation of a gene called “ABL” (for Herbert Abelson, the scientist who discovered this gene). The break on chromosome 22 involves a gene called “BCR” (for breakpoint cluster region). The mutated ABL gene moves to chromosome 22 and fuses with the remaining portion of the BCR gene. The result of this fusion is the leukemia-causing fusion gene BCR-ABL . Genes provide cells with instructions for making proteins. The BCR-ABL gene produces a dysfunctional protein called“BCR-ABL tyrosine kinase”. The BCR-ABL tyrosine kinase leads to the abnormal regulation of cell growth and survival and is responsible for the development of CML. For that reason, the BCR-ABL tyrosine kinase is a target for specific drug therapies that block its effects in many people with CML (Neil P. Shah 2014).

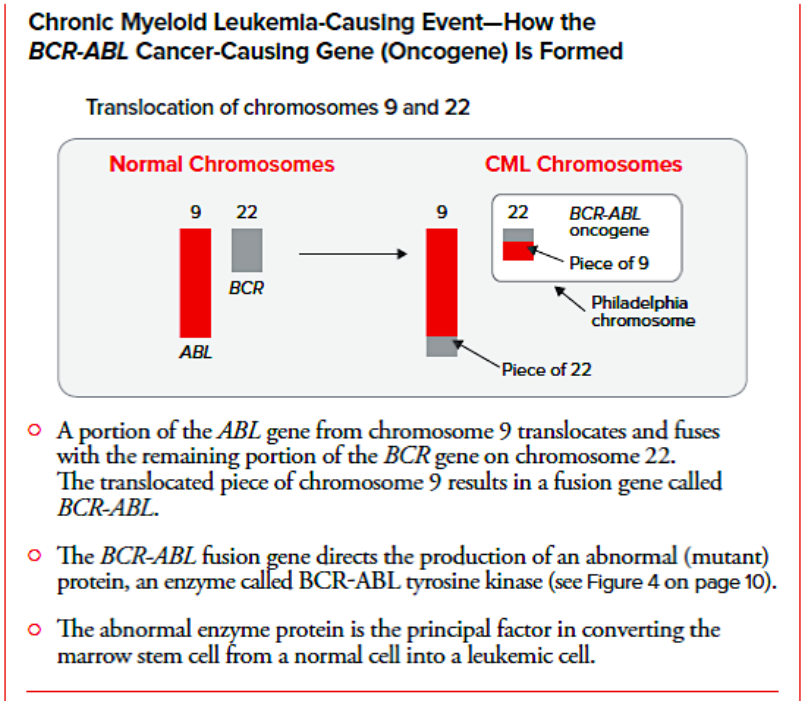


Figure 1.2. The process of translocation between the genes on chromosomes 9 and 22

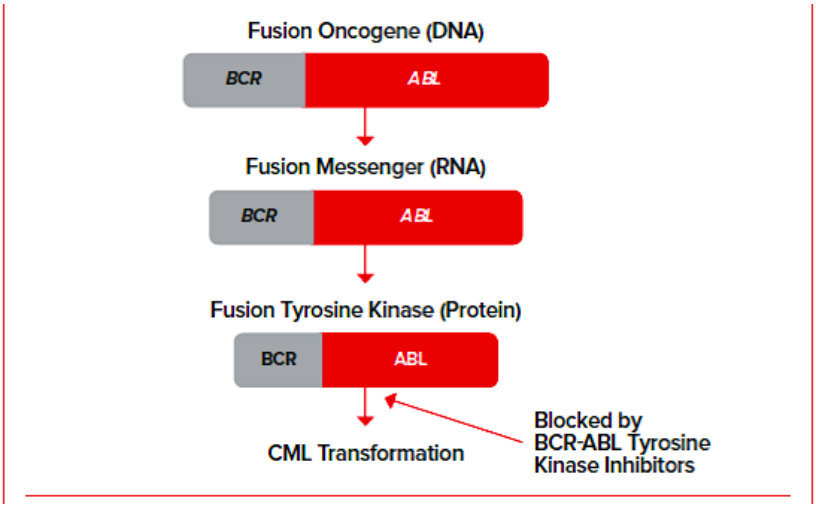


Figure 1.3. Leukemia causing process in a Marrow stem cell

Diagnosis

Complete Blood Count (CBC). People with CML often have Decreased hemoglobin concentration, Increased white blood cell count, often to very high levels Possible increase or decrease in the number of platelets depending on the severity of the person's CML, Specific pattern of white blood cells Small proportion of immature cells (leukemic blast cells and promyelocytes) Larger proportion of maturing and fully matured white blood cells (myelocytes and neutrophils).

Bone Marrow Aspiration and Biopsy. These tests are used to examine marrow cells to find abnormalities and are generally done at the same time. The sample is usually taken from the patient's hip bone after medicine has been given to numb the skin. Both samples are examined under a microscope to look for chromosomal and other cell changes.

Cytogenetic Analysis. This test measures the number and structure of the chromosomes. Samples from the bone marrow are examined to confirm the blood test findings and to see if there are chromosomal changes or abnormalities, such as the Philadelphia (Ph) chromosome. The presence of the Ph chromosome (the shortened chromosome 22) in the marrow cells, along with a high white blood cell count and other characteristic blood and marrow test findings, confirms the diagnosis of CML. A small percentage of people with clinical signs of CML do not have cytogenetically detectable Ph chromosome, but they almost always test positive for the *BCR-ABL* fusion gene on chromosome 22 with other types of tests.

FISH (Fluorescence In Situ Hybridization). FISH is a more sensitive method for detecting CML than the standard cytogenetic tests that identify the Ph chromosome. FISH is a quantitative test that can identify the presence of the *BCR-ABL* gene . FISH uses color probes that bind to DNA to locate the BCR and ABL genes in chromosomes. Both BCR and ABL genes are labeled with chemicals each of which releases a different color of light. The color shows up on the chromosome that contains the gene— normally chromosome 9 for ABL and chromosome 22 for BCR—so FISH can detect the piece of chromosome 9 that has moved to chromosome 22 in CML cells. Since this test can detect BCR-ABL in cells found in the blood, it can be used to determine if there is a significant decrease in the number of circulating CML cells as a result of treatment.

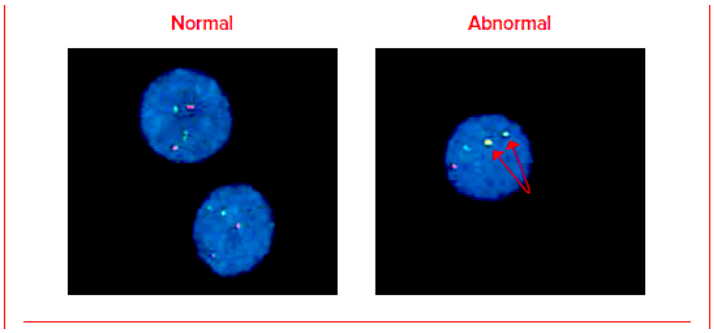


Figure 1.4. Identifying the BCR-ABL gene using FISH

Polymerase Chain Reaction (PCR) The *BCR-ABL* gene is also detectable by molecular analysis. Quantitative PCR is used to determine the relative number of cells with the abnormal *BCR-ABL* gene in the blood. This has become the most used and relevant type of PCR test because it can measure small amounts of disease, and the test is performed on blood samples, so there is no need for a bone marrow biopsy procedure.

Blood cell counts, bone marrow examinations, FISH and PCR may also be used to track a person's response to therapy once treatment has begun (Neil P. Shah 2014).

CML-Related Disorders

There are other subtypes of myeloid leukemia that have a chronic course and have some of the signs and symptoms of CML. These include

Chronic myelomonocytic leukemia (CMML)

Juvenile myelomonocytic leukemia (JMML)

Chronic neutrophilic leukemia (CNL).

These diseases are less common "myeloproliferative neoplasms." People with these diseases do not have the BCR-ABL gene; the absence of the BCR-ABL gene is one of several distinguishing features used to make the correct diagnosis. In general, CMML, JMML and CNL create more severe changes in blood cell counts early in the course of the disease; these changes are not as well controlled with current drug treatments (Neil P. Shah 2014).

Other subtypes of Leukemia:

Hairy cell leukemia (HCL) is sometimes considered a subset of chronic lymphocytic leukemia, but does not fit neatly into this pattern. About 80% of affected people are adult men. No cases in children have been reported. HCL is incurable, but easily treatable. Survival is 96% to 100% at ten years.(Else, Ruchlemer et al. 2005)

T-cell prolymphocytic leukemia (T-PLL) is a very rare and aggressive leukemia affecting adults; somewhat more men than women are diagnosed with this disease.(Matutes 1998) Despite its overall rarity, it is also the most common type of mature T cell leukemia;(Valbuena, Herling et al. 2005) nearly all other leukemias involve B cells.

Large granular lymphocytic leukemia may involve either T-cells or NK cells; like hairy cell leukemia, which involves solely B cells, it is a rare and indolent leukemia.

Adult T-cell leukemia is caused by human T-lymphotropic virus (HTLV), Like HIV, HTLV infects CD4+ T-cells and replicates within them; however, unlike HIV, it does not destroy them. Instead, HTLV "immortalizes" the infected T-cells, giving them the ability to proliferate abnormally. Human T cell lymphotropic virus types I and II (HTLV-I/II) are endemic in certain areas of the world.

1.1.5. Lymphoma

Lymphoma is the name for a group of blood cancers that develop in the lymphatic system. Hodgkin lymphoma and non-Hodgkin lymphoma are the two main types. About 90 percent of people with lymphoma have non-Hodgkin lymphoma (NHL). The rest have Hodgkin lymphoma. The lymphoma cells pile up and form lymphoma cell masses. These masses gather in the lymph nodes or other parts of the body. Lymphadenopathy or swelling of lymph nodes is the primary presentation in lymphoma. B symptoms (systemic symptoms) can be associated with both Hodgkin lymphoma and non-Hodgkin lymphoma. They consist of Fever, Night sweats, Weight loss, Loss of appetite or anorexia, Fatigue, Respiratory distress or dyspnea, Itching(Walter 2013).

WHO classification

The current accepted definition, the WHO classification published in 2001 and updated in 2008 is the latest classification of lymphoma and is based upon the foundations laid within the "Revised European-American Lymphoma classification" (Djebali, Davis et al.). This system attempts to group lymphomas by cell type (i.e. the normal cell type that most resembles the tumor) and defining phenotypic, molecular, or cytogenetic characteristics.

Hodgkin Lymphoma

Hodgkin lymphoma (HL), one of the most curable forms of cancer, was named for Thomas Hodgkin, a British pathologist, In 1832. It was officially renamed “Hodgkin lymphoma” in the late 20th century when it became evident that the disease results from an injury to the DNA of a lymphocyte . The damage to the DNA is acquired rather than inherited. The altered DNA in the lymphocyte produces a cancerous change that—if untreated—results in the uncontrolled growth of the cancerous lymphocytes. The accumulation of the cancerous lymphocytes results in the tumor masses that are found in the lymph nodes and other sites in the body(Carla Casulo 2013). HL is distinguished from other types of lymphoma by the presence of “Reed-Sternberg cells”. Reed-Sternberg cells are usually B cells and have differences and variations to them.

Incidence, Causes and Risk Factors

HL is most likely to be diagnosed in people in their 20s or early 30s. It is less common in middle age but becomes more common again after age 65. The following are examples of risk factors.

Patients who have a history of a blood test confirming mononucleosis have a 3-fold increased risk of HL compared to the general population. People infected with human T-cell lymphocytotropic virus (HTLV) or human immunodeficiency virus (HIV) also have increased probability of developing HL.

There are occasional cases of familial clustering, as with many cancers, and there is an increase in the incidence of HL in siblings of patients with the disease. These cases are uncommon, but the concept of genetic predisposition is under study to determine its role in the

sporadic occurrence of HL in otherwise healthy individuals. Epstein-Barr virus has been associated with nearly half of all cases. However, this virus has not been conclusively established as a cause of HL(Carla Casulo 2013).

Diagnosis

Imaging: The imaging test(s) may show enlarged lymph nodes in the chest or abdomen or both. Tumor masses can also occur outside the lymph nodes in lung, bone or other body tissue.

Lymph node biopsy: HL can be confused with various types of non-Hodgkin lymphoma—since the treatment is different, a precise diagnosis is needed. A biopsy of an involved lymph node or other tumor site is needed to confirm the diagnosis of HL. A needle biopsy of the lymph node is usually not sufficient to make a firm diagnosis.

Immunophenotyping:"immunophenotyping" is sometimes used to distinguish HL from other types of lymphoma or other noncancerous conditions. The presence of Reed-Sternberg and Hodgkin cells can confirm a diagnosis of HL.(Carla Casulo 2013).

Subtypes of Hodgkin Lymphoma

There are two main HL subtypes:

Classical Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma.

About 95 percent of HL patients have the classical subtype.

Classical Hodgkin Lymphoma: Classical HL can be further subdivided. Four major subtypes have been identified.

Nodular Sclerosis.

Mixed Cellularity.

Lymphocyte-Depleted.

Lymphocyte-Rich Classical.

Nodular Lymphocyte-Predominant Hodgkin Lymphoma. The cells in NLPHL, known as "lymphocytic" and "histolytic" cells, are different from classic Reed-Sternberg B cells. Patients with this subtype may have no symptoms and are usually diagnosed with very limited disease. It is most common in young men. The NLPHL subtype is indolent and is associated with long-term survival (Carla Casulo 2013).

Non-Hodgkin Lymphoma:

Non-Hodgkin lymphoma (NHL) is the term used for a diverse group of blood cancers that share a single characteristic. They arise from an injury to the DNA of a lymphocyte parent cell. NHL generally develops in the lymph nodes or in lymphatic tissue found in organs such as the stomach, intestines or skin. The REAL/WHO (Revised European-American Lymphoma/World Health Organization) classification categorizes subtypes by the appearance of the lymphoma cells, the presence of proteins on the surface of the cells and genetic features. **Follicular lymphoma** and **diffuse large B-cell lymphoma** are the two most common types and together account for about 53 percent of cases. Lymphocytic or lymphoblastic leukemias and lymphomas are closely related. A cancer that originates in the lymphatic tissue in the marrow is designated “lymphocytic leukemia” or “lymphoblastic leukemia”; the acute and chronic forms of lymphocytic or lymphoblastic leukemia are the two major examples of this type of blood cancer. A cancer that begins in a lymph node or other lymphatic structure in the skin, the gastrointestinal tract or another site in the body is called a “lymphoma”(Carla Casulo 2013) .

Table 1.3. Diagnostic Designations for Non Hodgkin Lymphoma

NHL Subtypes and Frequency

B-Cell Lymphoma

1. Diffuse Large B-Cell Lymphoma (31%)
2. Follicular Lymphoma (22%)
3. Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma (7.5%)
4. Small Cell Lymphocytic Lymphoma–Chronic Lymphocytic Leukemia (7%)
5. Mantle Cell Lymphoma (6%)
6. Mediastinal (Thymic) Large B-Cell Lymphoma (2.4%)
7. Lymphoplasmacytic Lymphoma–Waldenström Macroglobulinemia (less than 2%)
8. Nodal Marginal Zone B-Cell Lymphoma (less than 2%)
9. Splenic Marginal Zone Lymphoma (less than 1%)
10. Extranodal Marginal Zone B-Cell Lymphoma (less than 1%)
11. Intravascular Large B-Cell Lymphoma (less than 1%)
12. Primary Effusion Lymphoma (less than 1%)
13. Burkitt Lymphoma-Burkitt Leukemia (2.5%)
14. Lymphomatoid Granulomatosis (less than 1%)

T-Cell and Natural Killer (NK)-Cell Lymphoma (about 12%)

1. Peripheral T-Cell Lymphoma, not otherwise specified
2. Cutaneous T-Cell Lymphoma (Sézary Syndrome and Mycosis Fungoides)
3. Anaplastic Large Cell Lymphoma
4. Angioimmunoblastic T-Cell Lymphoma
5. NK-Cell Lymphoma

Immunodeficiency-Associated Lymphoproliferative Disorders

The percentages above are approximate; they are provided to give a sense of the relative distribution of NHL subtypes. Immunodeficiency-associated lymphoproliferative disorders account for a very small percentage of total NHL cases.

Incidence, Causes and Risk Factors

About 69,740 cases of non-Hodgkin lymphoma are expected to be diagnosed in the United States in 2013 (Surveillance, Epidemiology, and End Results [SEER] Program; National Cancer Institute, 2013). The incidence of NHL increases with age. In the 20- to 24-year age-group, 2.5 cases occur per 100,000 persons. The rate increases almost 20-fold to 44.6 cases per 100,000 individuals by age 60 to 64 years, and over 40-fold to more than 100 cases per 100,000 persons after age 75. While NHL is between 50 and 100 times more prevalent among people with HIV/AIDS than among uninfected individuals, newer therapies for HIV infection have lowered the incidence of AIDS-related lymphoma.There is a higher incidence of NHL in farming communities. Studies suggest that specific ingredients in herbicides and pesticides such as organochlorine, organophosphate and phenoxy acid compounds are linked to lymphoma (Carla Casulo 2013). Exposure to certain viruses and bacteria is associated with NHL. It is thought that infection with a virus or bacterium can lead to intense lymphoid cell proliferation, increasing the probability of a cancer-causing event in a cell. for examples, Epstein-Barr virus (EBV) infection—in patients from specific geographic regions—is strongly associated with African Burkitt lymphoma. The role of the virus is unclear, since African Burkitt lymphoma also occurs among people who have not been infected with EBV. Epstein-Barr virus infection may play a role in the increased risk of NHL in persons whose immune systems are suppressed as a result of organ transplantation and its associated therapy. Human T-lymphotropic virus (HTLV) is associated with a type of T-cell lymphoma in patients from certain geographic regions in southern Japan, the Caribbean, South America and Africa. The bacterium Helicobacter pylori causes ulcers in the stomach and is associated with the development of mucosa-associated lymphoid tissue (MALT) lymphoma in the stomach wall. About a dozen inherited syndromes can predispose individuals to later development of NHL. Having autoimmune diseases such as Sjögren's syndrome, lupus, or rheumatoid arthritis, may also increase a person's risk of developing lymphoma (Carla Casulo 2013).

Diagnosis

A diagnosis of NHL is usually made by examining a lymph node biopsy specimen. the examination includes tests called "immunophenotyping" and "cytogenetic analysis."

Lymph Node Biopsy: Non-Hodgkin lymphoma can involve parts of the body that do not involve lymph nodes. When lymphoma is detected exclusively outside of the lymph nodes, it is called "primary extranodal lymphoma," and the biopsy specimen is taken from the involved tissue, such as the lung or bone (Carla Casulo 2013).

Staging

A physical examination, and the findings from imaging tests, computed tomography (CT) scans, tissue biopsies and blood tests are used to determine the extent of the patient's NHL. This process is called "staging," and the information is used to determine appropriate treatments (chemotherapy, radiation). FDG-PET scanning differs from x-rays, CT, MRI and ultrasonography, which only provide anatomical images; FDG-PET also measures altered tissue metabolism (activity). This imaging technique relies on a radioactive tracer called "FDG ([18F]-fluorodeoxyglucose)." FDG is a special form of glucose. The radioactive tracer is given intravenously to the patient and enters the cells. Cancer cells have a greater attraction to this glucose than normal cells, so cancer cells trap more of the radioactive tracer; then the local tracer concentration is measured. This technique allows the cancer cells to be separated from normal cells. Using FDG-PET to measure increased FDG uptake in lymphoma cells may provide a very sensitive and relatively rapid assessment of the lymphoma cells' response to therapy.

Blood and Marrow Tests. Blood tests determine whether lymphoma cells are present in the blood and if the immunoglobulins made by lymphocytes are deficient or abnormal. Check indicators of disease severity such as blood protein levels, uric acid levels and erythrocyte sedimentation rate (ESR). Assess kidney and liver functions and hepatitis A, hepatitis B and hepatitis C status. Measure two important biological markers, lactate dehydrogenase (LDH) and beta2-microglobulin, which are helpful prognostic indicators for several NHL subtypes.

Most patients diagnosed with NHL will have a bone marrow biopsy to make sure there is no spread of the disease to the bone marrow and to evaluate the use of specific therapies including radioimmunotherapy (Carla Casulo 2013).

1.2. Non coding RNA

Transcriptome analysis by tiling arrays and RNA sequencing has led to the amazing conclusion that while 70%–90% of the genome is transcribed, only 2% is dedicated to the transcription of protein coding sequences(Mattick 2001). This result has caused a great impression in a scientific community that is deeply proteocentric, *i.e.*, is dedicated to the study of proteins and generally does not pay much attention to other molecules such as lipids or RNAs.

Most cellular RNA is composed of highly expressed non-coding RNAs whose relevance in cell functionality has been well-known for years. However, their transcription requires a relatively small proportion of the genome. These housekeeping non-coding RNAs include transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), required for mRNA translation; small nuclear RNAs (snRNAs), essential for splicing; and small nucleolar RNAs (snoRNAs), involved in RNA modification. More recently, several small RNAs have been described as playing essential roles in gene expression and transposon silencing. These include microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi interacting RNAs (piRNAs). Less clear is the role and the molecular mechanisms involved in the function of other small RNAs derived from retrotransposons or 3' untranslated regions or associated with transcription start sites, promoters, termini or repeats. All these non-coding RNAs, with the exception of some of the housekeeping RNAs (some rRNAs and a few snRNAs and snoRNAs), share the common characteristic of being smaller than 200 nts. Therefore the remaining non-coding RNAs, longer than 200 nts, have been grouped under the name of long non-coding RNAs (lncRNAs).

LncRNAs are not really long, just longer than the limit of 200 nts imposed by small RNAs. In fact, the average size of coding mRNAs is near 2500 nts while the average length of all the lncRNAs recently described by the Encode project is less than 600 nts(Dunham and Frietze 2012). Thus, most of the long non-coding RNAs are shorter than the coding mRNAs, even if some of the lncRNAs may be longer than 100 kbs. Apart from not being really long, it is difficult to determine whether lncRNAs are indeed non-coding. Traditionally, lncRNAs have been characterized by what they do not have: they lack open reading frames (ORFs) longer than 100 amino acids, conserved codons and homology to protein databases (Lin, Carlson et al. 2007),(Lin, Deoras et al. 2008). Therefore, they have poor coding potential, although they could still code for small open reading frames or non-conserved peptides. Some authors have also analyzed coding capacities of specific

lncRNAs by matching their sequences with ribosome footprints or peptide fragments from mass spectrometry analysis. Hits would indicate translation(Pueyo and Couso 2011),(Ingolia, Lareau et al. 2011),(Banfai, Jia et al. 2012),(Derrien, Johnson et al. 2012). In spite of these efforts, it should be borne in mind that what makes lncRNAs interesting for most scientists is not whether they can encode for proteins or not but the fact that they are functional as RNA molecules. The demonstration of function as an RNA should be required for annotation as an lncRNA, as a functional long RNA is the best definition for lncRNAs. To complicate things further, there are several cases of coding mRNAs that contain regulatory RNA elements and act as bifunctional RNAs; on one hand they code for a protein (p53, for instance) and on the other hand they have a function asRNAs(Kloc, Wilk et al. 2005),(Wadler and Vanderpool 2007),(Dinger, Pang et al. 2008),(Leygue 2007),(Jenny, Hachet et al. 2006),(Candeias, Malbert-Colas et al. 2008). Furthermore, several coding genes are transcribed to non-coding alternative splicing variants. Functional or lncRNA genes are very similar to coding genes at the DNA and chromatin level as they share the same epigenetic marks. Similar to mRNAs, most lncRNAs are transcribed from RNA polymerase II, are capped at the 5' end, contain introns and approximately 40% are polyadenylated at the 3' end(Guttman, Amit et al. 2009). The lncRNAs recently described by Encode show a bias for having just one intron and a trend for less-efficient cotranscriptional splicing(Derrien, Johnson et al. 2012),(Kloc, Wilk et al. 2005),(Wadler and Vanderpool 2007),(Dinger, Pang et al. 2008),(Leygue 2007),(Jenny, Hachet et al. 2006),(Candeias, Malbert-Colas et al. 2008),(Guttman, Amit et al. 2009),(Tilgner, Knowles et al. 2012). It has been estimated that there could be as many lncRNA genes as coding genes, but the number of lncRNAs is still growing and some authors consider that it could increase from ~20,000 to ~200,000(Gibb, Vucic et al. 2011),(Mercer, Dinger et al. 2008). Compared to mRNAs, most lncRNAs localize preferentially to the nucleus, are more cell type specific and are expressed at lower levels(Djebali, Davis et al. 2012). In fact, there is less than one copy per cell of many lncRNAs. The low expression levels and the fact that the sequence of lncRNAs is poorly conserved have convinced many scientists that they are not relevant for cell functionality. However, although lncRNAs are under lower selective pressure than protein-coding genes, sequence analysis shows that lncRNAs are under higher selective pressure than ancestral repeat sequences with neutral selection. Moreover, promoters of lncRNAs have similar selection levels than promoters of protein coding genes(Derrien, Johnson et al. 2012). Even in the absence of strong sequence

conservation, the genomic location and structure of many lncRNAs is conserved together with short stretches of sequences, suggesting that lncRNAs could be under selective pressure to maintain a functional RNA structure rather than a linear sequence(Derrien, Johnson et al. 2012). Recent publications in the field have led to the hypothesis that many lncRNAs may be key regulators of development and may play relevant roles in cell homeostasis and proliferation. In fact, several lncRNAs have been described that function as oncogenes or tumor suppressors(Hauptman and Glavac 2013). It is expected that for cell biology the role of lncRNAs could be as revolutionary as the role of small non-coding RNAs such as miRNAs. miRNA studies have highlighted the relevance of gene regulation in cell homeostasis, differentiation and proliferation and may impact the clinic with new therapies and new diagnostic and prognostic tools for many diseases. The relevance of miRNAs has been clearly established for haematological malignancies(Agirre, Jimenez-Velasco et al. 2008),(Agirre, Vilas-Zornoza et al. 2009).

1.2.1. Classes of lncRNAs and lncRNA Functionality

Classification by Genomic Location

Under the name of lncRNAs there are RNAs with many different characteristics, which complicates classification. Therefore a well accepted method is based on genomic location rather than on functionality, conservation or origin. From a genetic point of view lncRNAs can be classified into one or more of the following categories:

(a) sense, when overlapping with one or more exons of another transcript in the same strand;

(b) antisense, when overlapping with one or more exons of another transcript in the opposite strand;

(c) intronic, when derived from an intron of another transcript;

(d) divergent or bidirectional, when they share a promoter with another transcript in the opposite strand and therefore are coregulated;

(e) intergenic, when they are independent, located in between two other genes. Long intergenic non coding RNAs (lincRNAs) are a special class of intergenic lncRNAs whose genes have histone mark signatures of active transcription (trimethylation in lysine 4 and lysine 36 of histone 3: H3K4m3, H3K36m3)(Huarte, Guttman et al. 2010).

In the case of antisense transcripts, classification based on genomic location helps to predict functionality. 50%–70% of sense transcripts have natural antisense partners (Panzitt, Tschernatsch et al.) (Katayama, Tomaru et al. 2005),(Carninci, Kasukawa et al. 2005),(Galante, Vidal et al. 2007). NATs are generally involved in the regulation in *cis* of the corresponding sense RNA by mechanisms that act at the transcriptional and post transcriptional level. NATs can induce transcriptional interference or recruit chromatin modifiers and remodelers to establish a local transcriptionally active or inactive chromatin conformation (Yap, Li et al. 2010). NATs can modify processing and induce or reduce the expression or the translation of their sense counterpart. Some intronic lncRNAs also regulate the expression of their genomic partners. Intronic lncRNAs may be generated by stabilization of the intron after splicing of the host gene but, more commonly, they are produced from independent transcription (Guil and Esteller 2012).

Classification by Specific Characteristics

Most lncRNAs with special characteristics cannot be easily classified into a single group according to genomic location. These include enhancer RNAs (eRNAs), lncRNA-activating (lncRNA-a) genes, transcribed ultraconserved regions (T-UCRs), pseudogenes, telomere-associated ncRNAs (TERRAs), circular RNAs, *etc.* eRNAs are transcribed by RNA polymerase II at active enhancer regions, characterized by H3 Lys4 mono methylation or Lys27 acetylation and binding of the regulatory protein p300 (Heintzman, Stuart et al. 2007), (Heintzman, Hon et al. 2009), (Visel, Blow et al. 2009), (Kim, Hemberg et al. 2010). eRNAs are not polyadenylated. Many are bidirectional and poorly expressed (Kim, Hemberg et al. 2010),(Wang, Garcia-Bassets et al. 2011),(De Santa, Barozzi et al. 2010), but expression of several eRNAs seems to be tightly regulated (Kim, Hemberg et al. 2010), (Wang, Garcia-Bassets et al. 2011). Although many eRNAs were thought to be by-products of the presence of RNA pol II in enhancers, recent evidence suggests that some may function to control the expression of neighbouring genes (Orom, Derrien et al. 2010). LncRNA-a genes generally transcribe intergenic RNAs which are involved in the expression of neighbouring genes (Orom, Derrien et al. 2010). Thus, downregulation of the lncRNA-a results in downregulation of the neighbour gene. This effect requires expression of the Mediator complex and it has been shown that interaction of the lncRNA-a with Mediator is required for the upregulation of nearby genes (Lai, Orom et al. 2013). T-UCRs and pseudogenes are lncRNAs that share sequence similarity to other mammalian genomes or other regions of the same genome, respectively. There are 481 UCRs longer than 200 bp

that are absolutely conserved between human, rat, and mouse genomes (Bejerano, Pheasant et al. 2004). Pseudogenes originated from duplication of ancestor or parental coding genes (duplicated pseudogenes) or through retrotransposition of processed RNAs transcribed from ancestor genes (processed pseudogenes). Subsequently, they have lost their coding capacity as a result of the accumulation of mutations. When pseudogenes are expressed, they may regulate the expression and function of their parental gene by several mechanisms (Hirotsune, Yoshida et al. 2003), (Hawkins and Morris 2010). For instance, pseudogenes may act as miRNA decoys that lead to increased stability and translation of their parental gene (Harrison, Zheng et al. 2005),(Pink, Wicks et al. 2011), (He 2010), (Salmena, Poliseno et al. 2011). Circular RNAs, newcomers to the RNA list, can also function as RNA decoys (Ledford 2013), (Kosik 2013),(Memczak, Jens et al. 2013).

Classification as* cis *or* trans-*Acting Molecules

LncRNAs can also be classified according to their functionality as cis and/or trans acting molecules (Figure 5). Trans-acting lncRNAs function away from the site of synthesis while cis-acting lncRNAs function at the site of transcription to affect the expression of neighbouring genes. Several cis-acting lncRNAs guide epigenetic regulators to their site of transcription while they are being transcribed. Thus, lncRNA transcription is critical and rapidly creates an anchor to recruit proteins involved in chromatin re modeling (Chu, Qu et al. 2011), (Gabory, Jammes et al. 2010), (Mancini-Dinardo, Steele et al. 2006),(Pauler, Koerner et al. 2007). This molecular mechanism has tremendous advantages: (i) it responds very fast, as it only requires transcription of an RNA and a proper accumulation of nuclear chromatin remodelers; (ii) it is very specific, as the targeting does not involve RNA-DNA interactions other than those required for lncRNA transcription and (iii) it may function with just a single molecule of lncRNA per locus. This may explain the low abundance of cis-acting lncRNAs and the relatively high concentration of lncRNAs close to developmental genes whose expression is strictly controlled(Engstrom, Suzuki et al. 2006). Thus, cis-acting lncRNAs control the epigenetic regulation of some imprinted genes. Imprinting depends on the parental origin of the imprinted genes, which play critical roles in mammalian development and therefore, their expression must be tightly regulated (Li and Sasaki 2011). Many imprinted gene loci express lncRNAs that appear to regulate the expression of neighbouring imprinted protein-coding genes in cis, allele specifically (Mohammad, Mondal et al. 2009).

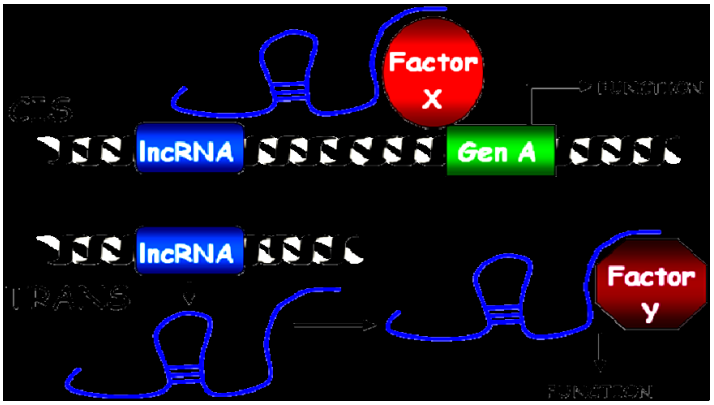


Figure 1.5. Schematic representation of cis and trans-acting lncRNAs. cis-acting lncRNAs function at the site of transcription and affect the expression of neighbouring genes.Trans-acting lncRNAs function away from the site of synthesis.

Trans-acting lncRNAs regulate gene expression on a genome-wide scale. A good example is HOTAIR, which binds the chromatin-modifying complexes PRC2, LSD1 and CoREST/REST (Khalil, Guttman et al. 2009), (Rinn, Kertesz et al. 2007), (Gupta, Shah et al. 2010), (Tsai, Manor et al. 2010).

1.2.2. lncRNA Functionality

Guiding chromatin remodeling factors seems to be the predominant function exerted by lncRNAs. In fact, it has been estimated that 20% of all lncRNAs may bind PRC2 (Khalil, Guttman et al. 2009). Several lncRNAs have also been shown to bind to PRC1, the CoREST/REST repressor complex (Khalil, Guttman et al. 2009), the histone ethyl transferase associated with the activating trithorax complex, MLL1 (Bertani, Sauer et al. 2011), (Dinger, Amaral et al. 2008) and H3-K9 methyltransferase, G9a (Nagano, Mitchell et al. 2008), (Pandey, Mondal et al. 2008). However, lncRNAs have also been shown to exert several other functions in the cell nucleus and cytoplasm, including regulation of DNA bending and insulation, RNA transcription, splicing, translation and stability, organization of subnuclear structures and protein localization, among others.

DNA looping CTCF can induce chromosomal bending and protect specific genes from the effects of distal enhancers and regulatory elements. The lncRNA *SRA* can interact with and enhance the function of CTCF (Yao, Brick et al. 2010). Also, endogenous but not exogenous nascent *HOTTIP* lncRNA, binds target genes via chromosomal looping (Wang, Yang et al. 2011).

Transcription LncRNAs may activate or inhibit transcription of specific targets. Some lncRNAs act as co activators that bind transcription factors and enhance their transcriptional activity (Feng, Bi et al. 2006),(Lanz, McKenna et al. 1999), (Caretti, Schiltz et al. 2006).This is the function of *SRA* lncRNA in the progestin steroid hormone receptor (Watanabe, Yanagisawa et al. 2001), (Lanz, Chua et al. 2003). However, some lncRNAs act as decoys of transcription factors (Kino, Hurt et al. 2010) and may move them to the cytoplasm to keep them away from their nuclear targets (Willingham, Orth et al. 2005). Thus, p53-induced lncRNA *PANDA* binds transcription factor NF-YA and prevents NF-YA activation of cell death genes (Hung, Wang et al. 2011). Finally, the act of lncRNA transcription may interfere with transcription initiation, elongation or termination of another sense or antisense gene (Mazo, Hodgson et al. 2007). Transcriptional interference can also lead to activation of gene expression by inhibiting the action of repressor elements.

Organization of subnuclear structures LncRNAs can recruit protein factors to nuclear structures. This is the case of lncRNA *MALAT1* and *NEAT-1*. *MALAT1* recruits serine/arginine–rich splicing factors to nuclear speckles (Tripathi, Ellis et al. 2010). More importantly, *NEAT-1* is an essential structural component of paraspeckles, subnuclear structure implicated in RNA splicing and editing (Mao, Sunwoo et al. 2011), (Bond and Fox 2009). Depletion of *NEAT-1* leads to loss of paraspeckles while overexpression of *NEAT-1* causes an increase in the number of paraspeckles (Clemson, Hutchinson et al. 2009), (Chen and Carmichael 2009), (Sunwoo, Dinger et al. 2009). *MALAT1* and *NEAT-1* are genomic neighbors over expressed in several tumors compared to healthy tissues. Surprisingly the mouse knockouts of either *NEAT-1* or *MALAT1* had no detectable phenotype, suggesting that there could be redundant or compensatory molecules (Eissmann, Gutschner et al. 2012), (Ip and Nakagawa 2012), (Nakagawa, Naganuma et al. 2011), (Zhang, Arun et al. 2012).

Splicing Splicing can be inhibited by lncRNAs antisense to intron sequences that impede spliceosome binding causing intron retention (Annilo, Kepp et al. 2009), (Allo, Buggiano

et al. 2009),(Hastings, Milcarek et al. 1997), (Krystal, Armstrong et al. 1990), (Yan, Hong et al. 2005). Furthermore, alternative splicing can be altered by lncRNA-mediated sequestration or modification of splicing factors. Thus, *MALAT1* binds splicing factors present in nuclear speckles and modulates the activity of SR proteins, involved in the selection of splice sites, and therefore regulates the splicing of many pre-mRNAs (Tripathi, Ellis et al. 2010). Some snoRNA-containing lncRNAs (sno-lncRNAs) are retained close to their sites of transcription where the splicing factor Fox2 is enriched. Changes in the level of the sno-lncRNA lead to a nuclear redistribution of Fox2 and to changes in alternative splicing. Thus, the sno-lncRNAs could function as a regulator of splicing in specific subnuclear domains (Yin, Yang et al. 2012).

Translation LncRNAs have been described that increase or inhibit translation of specific targets (Carrieri, Cimatti et al. 2012), (Yoon, Abdelmohsen et al. 2012).

Stability LncRNAs have been described that increase or decrease stability of specific targets(Kretz, Siprashvili et al. 2013), (Gong and Maquat 2011). Binding of lncRNAs containing ancestral Alu repeats to complementary Alu sequences in the 3′UTR of coding mRNAs forms a dsRNA recognized by the dsRNA binding protein Stau1, which induces Stau-mediated RNA decay (Gong and Maquat 2011). Instead, lncRNA *TINCR* localizes to the cytoplasm, where it interacts with Stau1 and promotes the stability of mRNAs containing the TINCR box motif (Kretz, Siprashvili et al. 2013).

miRNA binding LncRNAs can regulate mRNA stability and translation by binding to miRNAs and preventing their action. Besides the already described role of some pseudogenes and circular lncRNAs in miRNA sequestration, other lncRNAs such as *linc-MD1*, have been shown to serve as “sponge” for miRNAs. *Linc-MD*1 binds two miRNAs, which downregulate transcription factors involved in muscle differentiation and therefore muscle differentiation is induced upon *Linc-MD*1 expression (Cesana, Cacchiarelli et al. 2011). To date few lncRNAs have been described to have catalytic properties. The high number of lncRNAs and their heterogeneity helps them to exert such a myriad of functions. In fact, all lncRNA functions respond to just three different mechanisms: decoys, scaffolds and guides (Ma, Hao et al. 2012). Decoy-acting lncRNAs impede the access of proteins such as transcription factors and RNAs such as miRNAs to their targets. LncRNAs *MD-1* and *PANDA* act as decoys for miRNAs and transcription factors, respectively (Hung, Wang et al. 2011), (Cesana, Cacchiarelli et al. 2011). Scaffold-acting lncRNAs serve as adaptors to bring two or more factors into discrete ribonucleoproteins

(RNPs) (Spitale, Tsai et al. 2011). LncRNA *TERC*, *HOTAIR* or *NEAT-1* act as scaffolds to form the telomerase complex (Zappulla and Cech 2006), a silencing complex (Tsai, Manor et al. 2010) or the paraspeckle, respectively (Mao, Sunwoo et al. 2011), (Bond and Fox 2009). Guide-acting lncRNAs are required to localize protein complexes at specific positions. *XIST* or *AIR* lncRNAs act as guides to target gene silencing activity in an allele-specific manner. In many cases though, the secondary and tertiary structure of lncRNAs dictates their function. Thus, lncRNAs generally have complex structures with higher folding energies than those observed in mRNAs (Kertesz, Wan et al. 2010). Proteins are expected to be the major partners of lncRNAs to form functional RNP particles. RNA binding proteins represent more than 15% of the total amount of proteins (Castello, Fischer et al. 2012). In several cases studied to date, interaction between proteins and RNAs results in conformational changes to the protein, the RNA or both, which could endow the complex with a novel ability. LncRNA function impacts cell behaviour. LncRNAs have specially emerged as regulators of development. Some transcription factors involved in pluripotency bind promoter regions of more than 100 mouse lncRNAs (Guttman, Amit et al. 2009). 26 lincRNAs have already been described as being required for the maintenance of pluripotency in mouse (Guttman, Donaghey et al. 2011). Two lncRNAs regulated by pluripotency transcription factors such as Oct4 and Nanog are essential for pluripotency maintenance, as they, in turn, control the expression of Oct4 and Nanog (Sheik Mohamed, Gaughwin et al. 2010). Therefore, these lncRNAs participate in positive regulatory loops. Similarly, several lncRNAs have been implicated in human disease, including several cancers (Taft, Pang et al. 2010). Dysregulated lncRNAs have been described in heart disease, Alzheimer disease, psoriasis, spinocerebellar ataxia and fragile X syndrome (Faghihi, Modarresi et al. 2008), (Ishii, Ozaki et al. 2006), (Pasmant, Laurendeau et al. 2007), (Sonkoly, Bata-Csorgo et al. 2005), (Daughters, Tuttle et al. 2009), (Khalil, Faghihi et al. 2008), and in several tumors including breast, brain, lung, colorectal, prostate and liver cancers, melanoma, leukaemia and others (Calin, Liu et al. 2007), (Gupta, Shah et al. 2010), (Taft, Pang et al. 2010), (Kogo, Shimamura et al. 2011), (Chung, Nakagawa et al. 2011), (Yang, Zhou et al. 2011), (Lai, Yang et al. 2012), (Calin, Pekarsky et al. 2007), (Khaitan, Dinger et al. 2011), (Huarte and Rinn 2010). LncRNAs have been described that function as oncogenes (Li, Feng et al. 2009), tumor suppressors (Huarte, Guttman et al. 2010), (Yu, Gius et al. 2008) or drivers of metastatic transformation, such as *HOTAIR* in

breast cancer (Gupta, Shah et al. 2010). In this study we will concentrate on those lncRNAs whose expression is altered in haematological malignancies.

1.2.3. LncRNAs Deregulated in Haematological Malignancies

The impact of non-coding RNAs on haematological malignancies has been well described for microRNAs (Lawrie 2013), (Agirre, Martinez-Climent et al. 2012). The list of lncRNAs involved in the initiation and progression of blood tumors is still very short and expected to grow exponentially in the near future. Some of the lncRNAs that play a role in haematological malignancies are in fact host genes of miRNAs with oncogenic or tumor suppressor properties. Others endow oncogenic or tumor suppressor properties in the long non-coding RNA molecule. The mechanism of action of few of them has been studied in some detail.

Host Genes of Small RNAs

BIC and C13ORF25

Some lncRNAs were described to have oncogenic properties in blood cells before the discovery of miRNAs. This is the case of the B cell Integration cluster (Banfai, Jia et al.) or host gene *mir-155* (*MIR155HG*. *BIC* and *miR-155* expression is increased in Hodgkin lymphoma, Acute Myeloid Leukemia (AML) and Chronic Lymphocytic Leukemia (CLL) but it is not detected in healthy samples (Elton, Selemon et al. 2013). Similarly, *C13ORF25* or host gene *mir-17* (*MIR17HG*) encodes the *miR-17-92* cluster and its expression is increased in B-cell lymphoma(Ji, Rao et al. 2011), Mantle Cell Lymphoma (MCL) (Rinaldi, Poretti et al. 2007) and other tumors (Hayashita, Osada et al. 2005), (Humphreys, Cobiac et al. 2013).

nc886 or *vtRNA2-1*

vtRNA2-1, previously known as *pre-miR-886*, is a short ncRNA suppressed in a wide range of cancer cells that inhibits activation of protein kinase R (PKR) (Kunkeaw, Jeon et al. 2013). *vtRNA2-1* could be a tumour suppressor for AML and its role could be mediated by PKR.

Table 1.4. lncRNAs in hematopoiesis and hematological malignancies.

LncRNAs	LOCATION	HEMATOLOGIC DISEASE/SYSTEM	FUNCTION	MOLECULAR MECHANISM	MECHANISMS INVOLVED IN DYSREGULATION	CITATIONS
MIR155HG BIC	21q21.3	Burkitt, Hodgkin lymphoma, AML, CLL	Host of miRNAs	miR-155	Target MYB and NFKB	[134]
MIR17HG	13q31.3	B-cell lymphoma, MCL	Host of miRNAs	miR-17-92	Target MYC	[136,137,142]
vtRNA2-1	5q31.1	AML (poor prognosis)		PKR inhibition	DNA methylation Deletion 5q	[140,141]
PVT1	8q24.21	MM, Burkitt Lymphoma, T-cell Leukemia, CLL	Oncogene and host of miRNAs	miR-1204 MYC activation	Translocation t(8;14)(q24;q11) t(2;8)(p11;q24) t(8;22)(q24;q11)	[143–148]
CDKN2B-AS1/ANRIL	9p21.3	AML, ALL	Oncogene	PRC1 and PRC2 targeting	rs3731217-G SNP Deletion, hypermethylation	[26,128,149–151]
MEG3	14q32.2	AML,MM	Tumor suppressor	PRC2 binding to control DLK1 imprinting. p53 activation.	DNA methylation	[149,150,152–155]
DLEU1/DLEU2	13q14.2	CLL, MM, Lymphoma	Tumor suppressor	*hsa-miR-16-1* and *15a* BCL2 targeting. NFKB activation	Histone modification, DNA methylation, deletion	[156]
GAS5	1q25.1	B-cell Lymphoma, Leukemia	Tumor suppressor	Glucorticoid receptor repression. Regulated by mTOR pathway.	Translocation (1;3)(q25;q27)	[87]
H19	11p15.5	AML, CML, MPN, T-cell Leukemia, Lymphoma	Oncogene/tumor suppressor	Activated by Myc and down-regulated by p53. miR-675 targeting Rb	Loss of imprinting	[157]
T-UCRs		CLL (prognosis marker)	Oncogene/tumor suppressor	miR control		[46]
lincRNA-p21	Not annotated in human	ALL, CML	Tumor suppressor	Activated by p53 binds hnRNP K to induce apoptosis	Not known	[158]
TCL-6	14q32.13	T cell leukemia	Poorly characterized	Not described	Translocation and inversions with TCR	[151]
WT1-AS	11p13	AML, ALL	Poorly characterized	WT-1 control	Not known	[159]
CRNDE	16q12.2	AML, MM, T-cell leukemia	Oncogene	PRC2 and COREST binding	Not known	[160]
RMRP	9p13.3	Non-Hodgkin lymphoma	Poorly characterized	Not described	Mutation	[161]
SNHG5	6q14.3	B-cell Lymphoma	Poorly characterized	snoRNA host	Translocation (1;3)(q25;q27)	[162]
HOXA-AS2	7p15.2	APL	Poorly characterized		Not known	[163]
HOTAIRM1	7p15.2	Hematopoietic regulator	Regulator of myelopoiesis	HOX A genes.		[164]
EGOT	3p26.1	Hematopoietic regulator	Regulator of eosinophil development			[165]
PU.1-AS	11p11.2 Non annotated	Hematopoietic regulator	*PU.1-AS* regulate the hematopoiesis regulator PU.1	PU.1 control		[166]
EPS	Mouse 4qC7	Hematopoietic regulator	Regulator of erytropoyesis	Pycard repression		[167]
ThyncR1	1q23.1	Hematopoietic regulator	Regulator of T cell selection and maduration.	Riboregulator		[168]

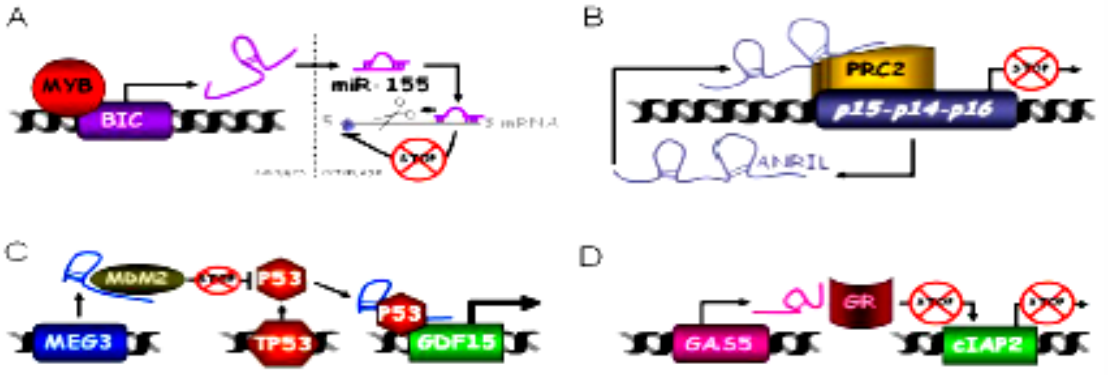


Figure 1.6. Schematic representation of the function of lncRNAs deregulated in haematological malignancies. (A) BIC. Myb transcription factor increases the expression of BIC in several leukemias and lymphomas. This results in increased levels of miR-155 and miR-155-mediated downregulation of several tumor suppressor genes; (B) ANRIL. The INK4 p15INK4b-p14ARF-p16INK4a cluster transcribes for an antisense transcript named ANRIL; PcG complex (PRC2) is targeted to the INK4 locus by ANRIL, and locus expression is inhibited; (C) MEG3. MEG3, among other functions, stimulates p53-dependent tumor suppressor pathways by several mechanisms. MEG3 down-regulates MDM2 expression, therefore decreasing p53 MDM2-mediated degradation. MEG3 increases p53 protein levels and stimulates p53-dependent transcription. MEG3 enhances p53 binding to some target promoters such as GDF15; (D) GAS5. GAS5 binds the DNA binding domain of glucocorticoid receptors (GR) and impedes GR binding to DNA and induction of GR-dependent genes such as cIAP2.

PVT1

It is not clear whether the role of Plasmacytoma variant translocation 1 (*PVT1*) lncRNA in haematological malignancies depends exclusively on being a miRNA host gene. *PVT1* is overexpressed, compared to healthy tissues, in breast and ovarian cancer, pediatric malignant astrocytomas, AML and Hodgkin lymphoma (Guan, Kuo et al. 2007), suggesting that *PVT1* could be an oncogene. In fact, upregulation of *PVT1* contributes to tumor survival and chemoresistance (Guan, Kuo et al. 2007), (Palumbo, Boccadoro et al. 1990), (You, Chang et al. 2011) while its downregulation inhibits cell proliferation and induces a strong apoptotic response (Guan, Kuo et al. 2007).

1.2.3.1. LncRNAs with Oncogenic Properties

ANRIL* or *CDKN2B-AS1

Antisense Non-coding RNA in the INK4 Locus (*ANRIL*) or *CDKN2B-AS1* is transcribed antisense to the *p15INK4b-p14ARF-p16INK4a* cluster, whose members are key effectors of oncogene-induced senescence. *ANRIL* is located in a genetic susceptibility locus (9p21) associated with several diseases, including coronary artery disease (Palumbo, Boccadoro et al.), atherosclerosis, intracranial aneurysm, type 2 diabetes, and several cancers, such as glioma, basal cell carcinoma, nasopharyngeal carcinoma, and breast cancer (Pasmant, Sabbagh et al. 2011). Several single nucleotide polymorphisms (SNP) in this locus alter *ANRIL* structure(Burd, Jeck et al. 2010) and *ANRIL* gene expression (Liu, Sanoff et al. 2009), (Cunnington, Santibanez Koref et al. 2010), mediating susceptibility to disease. There is a statistically significant association between an *ANRIL* polymorphism and Philadelphia positive Acute Lymphoblastic Leukemia (Ph+ ALL) (Iacobucci, Sazzini et al. 2011). Furthermore, 69% of samples (n = 16) from patients with ALL and AML showed relatively increased expression of *ANRIL* and downregulated p15 compared to controls(Yu, Gius et al. 2008). The expression of *ANRIL*, CBX7, and EZH2 is coordinated and elevated in preneoplastic and neoplastic tissues, leading to decreased p16INK4a expression and decreased senescence (Yap, Li et al. 2010).

1.2.3.2. LncRNAs with Tumor Suppressor Properties

MEG3

The maternally expressed gene 3 (*MEG3*) was the first lncRNA proposed to function as a tumor suppressor. *MEG3* is a paternally imprinted polyadenylated RNA, expressed in many normal human tissues as several alternative splicing variants (Miyoshi, Wagatsuma et al. 2000), (Zhang, Zhou et al. 2003). *MEG3* expression was decreased compared to healthy tissues in various brain cancers (pituitary adenomas, glioma and the majority of meningiomas and meningioma cell lines) (Zhang, Gejman et al. 2010), (Gejman, Batista et al. 2008), bladder, lung, renal, breast, cervix, colon and prostate cancers and haematological malignancies such as MM, AML or myelodysplastic syndromes. Surprisingly *MEG3* is overexpressed in Wilms tumor and may be increased or decreased in

different hepatocellular carcinomas *versus* healthy livers (Braconi, Kogure et al. 2011). *MEG3* activates the tumor suppressor protein p53 at different levels. On one hand *MEG3* down-regulates MDM2 expression, therefore decreasing p53 MDM2-mediated degradation(Zhou, Zhang et al. 2012). On the other hand, *MEG3* significantly increases p53 protein levels and stimulates p53-dependent transcription(Benetatos, Vartholomatos et al. 2011). Finally, *MEG3* enhances p53 binding to some target promoters such as *GDF15(Zhang, Rice et al. 2010), (Zhou, Zhong et al. 2007; Zhou, Zhang et al. 2012)*. In bladder cancer a negative correlation has been shown between *MEG3* expression and autophagy (Ying, Huang et al. 2013).

DLEU1* and *DLEU2

Deleted in leukemia 1 (*DLEU1*) and 2 (*DLEU2*) are two genes transcribed head to head in a 30-kb region located in the long arm of chrormosome 13 (13q14), which is lost in more than 50% of patients with CLL and that predicts a poor prognosis(Dal Bo, Rossi et al. 2011). Recent studies show that *DLEU1* and *DLEU2* control transcription of their neighbouring candidate tumor suppressor genes, which may act as positive regulators of NF-kB activity (Garding, Bhattacharya et al. 2013). Increased expression of *DLEU2* leads to reduced proliferation and clonogenicity (Lerner, Harada et al. 2009).

GAS5

Growth arrest specific 5 (*GAS5*) is induced under starvation conditions and is highly expressed in cells that have arrested growth(Coccia, Cicala et al. 1992), (Mourtada-Maarabouni, Pickard et al. 2009). *GAS5* modulates cell survival and metabolism by antagonizing the glucocorticoid receptor (GR)(Kino, Hurt et al. 2010). *GAS5* binds the DNA binding domain of GRs directly, preventing GRs from binding to DNA, from functioning as transcription activators and from reducing cell metabolism(Kino, Hurt et al. 2010). Expression of *GAS5* is sufficient to repress GR-induced genes, such as the cellular inhibitor of apoptosis 2 (*cIAP2*) and sensitizes cells to apoptosis(Kino, Hurt et al. 2010). Thus, *GAS5* behaves as a tumor suppressor. *GAS5* expression is decreased in breast cancer and is almost undetectable in growing leukemia cells and increases after density-induced cell cycle arrest(Kino, Hurt et al. 2010),(Coccia, Cicala et al. 1992), (Mourtada-Maarabouni, Pickard et al. 2009). Downregulation of *GAS5* by RNA interference protects

leukemic and primary human T cells from the anti-proliferative effect of rapamycin(Mourtada-Maarabouni, Hasan et al. 2010).

1.2.3.3. LncRNAs with Dual Functions

H19

H19 is an imprinted lncRNA located close to the *IGF2* gene. *H19* is expressed from the maternal allele and *IGF2* from the paternal allele (Gabory, Jammes et al. 2010), (Barsyte-Lovejoy, Lau et al. 2006). A key feature of cancer is the loss of this imprinting, which results in the well documented overexpression of *H19* in cancers of the colon, liver, breast and bladder and in hepatic metastases, compared to healthy tissues (Barsyte-Lovejoy, Lau et al. 2006), (Hibi, Nakamura et al. 1996), (Fellig, Ariel et al. 2005), (Matouk, DeGroot et al. 2007), (Berteaux, Lottin et al. 2005). Loss of *H19* imprinting has been described in adult T-cell leukaemia/lymphoma (ATL) (Takeuchi, Hofmann et al. 2007) and decreased *H19* expression was found in the bone marrow of patients with clinically untreated chronic myeloproliferative disorders, including chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and chronic myelomonocytic leukaemia (CMML)(Nunez, Bashein et al. 2000), (Bock, Schlue et al. 2003) and AML (Tessema, Langer et al. 2005). *H19* can behave as an oncogene or as a tumour suppressor(Gabory, Jammes et al. 2010). *H19* expression can be activated by the oncogene c-Myc(Barsyte-Lovejoy, Lau et al. 2006) and downregulated by the tumor suppressor p53(Dugimont, Montpellier et al. 1998), (Farnebo, Bykov et al. 2010).

1.2.4. LncRNAs and Hematopoietic Malignancies

1.2.4.1. LncRNAs Poorly Characterized in Haematological Malignancies

LincRNA-p21: is a p53 activated lncRNA identified in mouse that binds to and guides hnRNP K to target genes. *LincRNA-p21* bound hnRNP K acts as a transcriptional repressor that leads to the induction of apoptosis(Huarte, Guttman et al. 2010). As BCR-ABL1 stimulates nRNP-K expression and stability and promotes tumor progression, it has been

suggested that *lincRNA-p21* could play a relevant role in acute or chronic leukemia (Notari, Neviani et al. 2006), (Du, Wang et al. 2010).

TCL6: T cell Leukemia/Lymphoma 6 (*TCL6*) is transcribed from a locus involved in translocations and inversions with T cell receptor (*TCR*) (Bhagwat and Ramachandran 1975). These rearrangements in TCR commonly lead to activation of *TCL6* lncRNA and other oncogenes related to T cell leukemogenesis (Saitou, Sugimoto et al. 2000).

***WT1-AS*:** is an antisense lncRNA to WT-1, a well-characterized developmental gene that is mutated in Wilms' tumor (WT) and AML. *WT1-AS* has been shown to regulate WT1 protein levels. *WT1-AS* binds the exon 1 of WT1 mRNA in the cytoplasm. It has been suggested that the abnormal splicing of *WT1-AS* in AML could play a role in the development of this malignancy(Dallosso, Hancock et al. 2007).

***CRNDE*:** is overexpressed, compared to healthy tissue, in more than 90% of colorectal adenomas tested, but also in hepatocellular, prostate, brain, kidney and pancreas carcinomas and different haematological neoplasia such as AML, MM and T cell leukemia(Ellis, Molloy et al. 2012).

RMRP: Ribonuclease mitochondrial RNA processing (*RMRP)* is a lncRNA mutated in Cartilage-Hair Hypoplasia (CHH), an autosomal recessive chondrodysplasia with short stature, which entails a high risk of developing Non-Hodgkin lymphoma disease (Taskinen, Ranki et al. 2008), (Ridanpaa, van Eenennaam et al. 2001).

SNHG5: is a precurssor of snoRNAs, involved in diffuse large B-cell lymphoma(Tanaka, Satoh et al. 2000).

HOXA-AS2: In an acute promyelocytic leukemia (APL) cell line, *HOXA-AS2* upregulation correlated with inhibition of apoptosis. Treatment with all-*trans* retinoic acid (ATRA) blocked the expression of *HOXA-AS2* and increased apoptosis of the APL cell line (Zhao, Zhang et al. 2013).

1.2.4.2. LncRNAs Involved in Hematopoiesis

The best studied lncRNA in hematopoiesis is ***HOTAIRM1*** (HOX antisense intergenic RNA myeloid 1). *HOTAIRM1* is as an essential regulator of myeloid cell differentiation that locates at the 3' end of the *HOXA* cluster and controls *HOXA1* expression(Zhang, Lian et al. 2009). HOXA genes are important transcriptional regulators in normal and malignant hematopoiesis and are known to be important for many cancers including leukemias

harbouring MLL rearrangements. *HOTAIRM1* is expressed specifically in the myeloid lineage and is induced during the retinoic acid-driven granulocytic differentiation of the NB4 promyelocytic leukaemia cell line and normal human hematopoietic cells. Knockdown of *HOTAIRM1* affects retinoic acid-induced expression of *HOXA1* and *HOXA4* (but not distal *HOXA* genes) and attenuates induction of myeloid differentiation genes (Zhang, Lian et al. 2009).

Other lncRNAs involved in hematopoiesis have also been described. ***EGO*** (or *EGOT* in human) lncRNA was identified in mouse eosinophil differentiation of CD34+HSCs where it stimulated major basic protein and eosinophil-derived neurotoxin mRNA expression (Wagner, Christensen et al. 2007).

The lncRNA ***PU.1-AS*** is an antisense transcript of *PU.1* that negatively regulates *PU.1* mRNA translation by a mechanism similar to miRNAs (Ebralidze, Guibal et al. 2008). *PU.1* is a master hematopoietic transcriptional regulator essential for normal hematopoietic development and suppression of leukaemia development.

LincRNA erythroid prosurvival **(*EPS*)** is one of the about 400 lncRNAs whose expression is modulated during red blood cell formation and is required for differentiation during hematopoiesis in mouse(Zhang, Lian et al. 2009), (Wagner, Christensen et al. 2007), (Hu, Yuan et al. 2011). *EPS* is an erythroid-specific lncRNA that represses expression of *PYCARD*, a proapoptotic gene, and therefore inhibits apoptosis(Hu, Yuan et al. 2011), (Paralkar and Weiss 2011). EPS is not well conserved among mammals. It is presently unclear whether a human version of EPS exists.

Finally, ***THY-ncR1*** is a thymus-specific lncRNA expressed in cell lines derived from stage III immature T cells in which the neighbouring *CD1* gene cluster is also specifically activated(Aoki, Harashima et al. 2010).

1.2.5. Regulation of the Expression of lncRNAs Involved in Haematological Malignancies

Altered expression of lncRNAs, similar to that of coding genes, can be the result of genomic alterations, epigenetic regulation or a change in response to transcription factors or stability effectors such as miRNAs. The presence of mutations in the lncRNA primary sequence correlates highly with human diseases. In fact, most mutations in the genome occur in noncoding regions (Halvorsen, Martin et al. 2010). Mutations can be large or small. Large-scale mutations are deletions and amplifications of hundreds of nucleotides

and chromosomal translocations occurring at fragile sites. Genome-wide analyses looking for fragile sites in lncRNA genes have not yet been performed. However, it is expected that lncRNAs will have a clear association with common chromosomal aberrations similar to that found for miRNAs in human haematological malignancies and carcinomas (Calin, Liu et al. 2007). In fact, several studies have described lncRNAs affected by large scale mutations. One of the best examples is *ANRIL*, affected by a large germline deletion that includes the complete INK4/ARF locus. This deletion is associated with hereditary cutaneous malignant melanoma and neural system tumors syndrome (Pasmant, Sabbagh et al. 2011). *DLEU1* and *DLEU2* lncRNAs also locate in a region commonly deleted in CLL.

Small scale mutations are deletions or insertions of a few nucleotides. The relevance of small scale mutations for lncRNAs is obscured by the fact that little is known about the relevance of the primary sequence in lncRNA functionality and expression. It is expected that small mutations can lead to disease if they affect relevant linear sequences or they alter the structure of domains important in lncRNA functionality or accumulation. In fact, several disease-associated SNPs have been described as affecting the structure of the 5' and 3' non-translated regions of coding genes(Halvorsen, Martin et al. 2010). Furthermore, GWAS studies have shown that SNPs in noncoding regions are associated with higher susceptibility to diverse diseases. Germline and somatic mutations in lncRNA genes have been identified in haematological malignancies and colorectal cancers (Wojcik, Rossi et al. 2010). Genetic aberrations of the *GAS5* locus have been found in melanoma, breast and prostate cancers (Morrison, Jewell et al. 2007), (Nupponen and Carpten 2001), (Smedley, Sidhar et al. 2000). Several lncRNAs are regulated at the transcriptional level. Thus, lncRNAs, such as *lincRNA-P21*, are activated in response to DNA damage by the direct binding of the tumour-suppressor protein p53 to the promoter (Huarte, Guttman et al. 2010). Similarly, the expression of several lincRNAs responds to pluripotency factors or oncogenes. Epigenetic modifications are key regulators of lncRNA expression. This has been well described for *MEG3* and *DLEU1/DLEU2*. Expression of the *MEG3* locus is regulated by two regions, which are hypermethylated in several solid tumors leading to downregulation of *MEG3* expression (Zhang, Zhou et al. 2003), (Kagami, O'Sullivan et al. 2010), (Astuti, Latif et al. 2005). AML patients with aberrant hypermethylation of the *MEG3* promoter showed decreased overall survival (Benetatos, Hatzimichael et al. 2010), (Khoury, Suarez-Saiz et al. 2010). Thus, *MEG3* methylation status may serve as a useful biomarker in this leukemia. A similar *MEG3* hypermethylation was observed in 35% of the

patients with myelodysplastic syndrome, but in this case there was no statistically significant correlation between *MEG3* hypermethylation and prognosis (Benetatos, Hatzimichael et al. 2010). Demethylation correlated with transcriptional deregulation of the neighbouring candidate tumour suppressor genes. Finally, the expression of lncRNAs can be regulated by miRNAs. Several miRNAs have been described as regulating T-URC expression. This has been best described for *miR-155*, which is overexpressed in CLL compared to healthy cells. *miR-155* targets T-UCRs both *in vitro* and in CLL patient samples (Calin, Liu et al. 2007). Interestingly, *miR-29a* has also been shown to regulate *MEG3* expression in hepatocarcinoma cell lines(Braconi, Kogure et al. 2011).

1.2.6. MIAT (myocardial infarction associated transcript)

MIAT (myocardial infarction associated transcript), also known as RNCR2 (retinal non-coding RNA 2) or Gomafu, is a long non-coding RNA. Single nucleotide polymorphisms (SNPs) in MIAT are associated with a risk of myocardial infarction.(Ishii, Ozaki et al. 2006). It is expressed in neurons, and located in the nucleus (Sone, Hayashi et al. 2007). It plays a role in the regulation of retinal cell fate specification(Rapicavoli, Poth et al. 2010). This gene encodes a spliced long non-coding RNA that may constitute a component of the nuclear matrix. Altered expression of this locus has been reported to be associated with a susceptibility to myocardial infarction. It has also been proposed that pathways involving this transcript may contribute to the pathophysiology of schizophrenia. A similar gene in mouse has been associated with retinal cell fate determination. Alternatively spliced transcript variants have been identified. [provided by RefSeq, Dec 2014].

MIAT transcript escapes from nuclear export, thereby constituting a component of the nuclear matrix. Recent studies have revealed that nuclear lncRNAs are essential for the maintenance of nuclear structures and can affect pre-mRNA processing and export, thereby regulating gene expression(Ip and Nakagawa 2012). It was suggested that the MIAT RNA regulates splicing efficiency by changing the local concentration of splicing factors within the nucleus. MIAT RNA binds to the SF1 splicing factor through a tandom repeats of UACUAAC sequence with a high affinity, which affects the kinetics of the splicing process(Ishii, Ozaki et al. 2006). Interestingly, single nucleotide polymorphisms in the human homologue of MIAT are associated with an increased risk of myocardial infarction,

and thus the gene has been named myocardial infarction associated transcript (MIAT)(Tsuiji, Yoshimoto et al. 2011). Recent studies characterized MIAT as a co-activator of Oct4 in a regulatory feedback loop. Oct4 is considered a master transcription factor for pluripotent cell self-renewal. A defined Oct4 level controls the establishment of naive pluripotency as well as commitment to all embryonic lineages (Rapicavoli, Poth et al. 2010; Sheik Mohamed, Gaughwin et al. 2010; Radzisheuskaya, Chia Gle et al. 2013).

Cloning and expression

By screening a human fetal brain cDNA library, followed by 5-prime and 3-prime RACE, (Ishii, Ozaki et al. 2006) obtained 4 MIAT splice variants. Northern blot analysis detected a 10-kb transcript that was highly expressed in fetal brain and in adult brain and spleen. Weaker expression was detected in adult peripheral blood leukocytes, lung, liver, thymus, colon, and small intestine. In vitro translation resulted in no protein products, suggesting the MIAT variants may function as RNAs.

Mapping

By genomic sequence analysis, (Ishii, Ozaki et al. 2006) mapped the MIAT gene to chromosome 22q12.1. The MIAT gene is located on the long (q) arm of chromosome 22 at position 12.1. More precisely, the MIAT gene is located from base pair 26,657,481 to base pair 26,676,477 on chromosome 22.

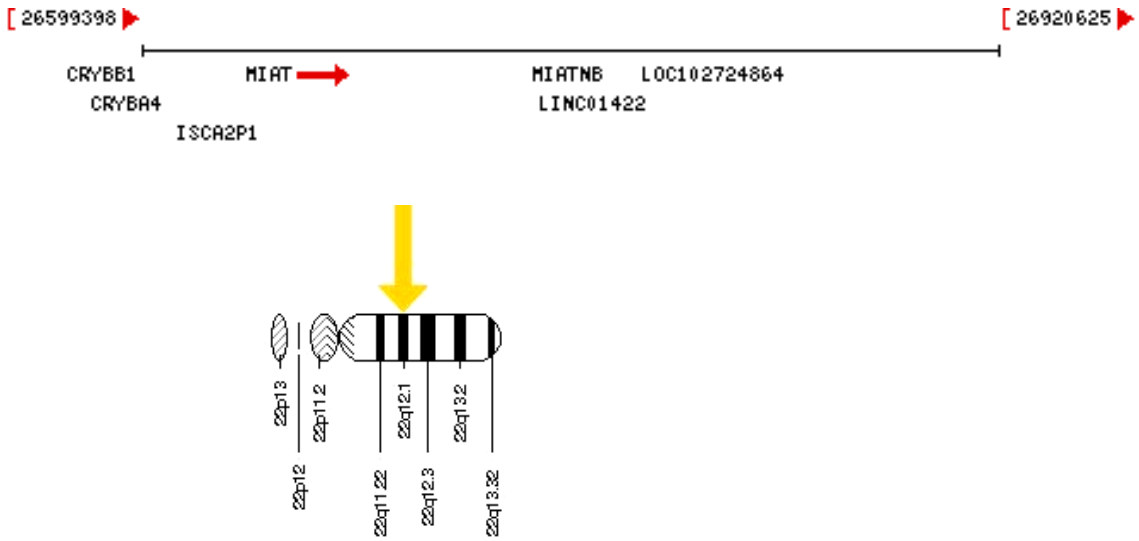


Figure 1.7. MIAT gene location on long arm of chromosome 22

Molecular Genetics

(Ishii, Ozaki et al. 2006) performed a case-control association study in 188 Japanese myocardial infarction (MI; 608446) patients and 752 controls using 52,608 haplotype-based SNPs and identified a SNP (rs2301523) on chromosome 22q12.1 that was significantly associated with MI (p = 0.0006). Further analysis using a total of 3,464 MI patients and 3,819 controls confirmed the association with MI (chi square = 22.71; p = 0.0000019; odds ratio, 1.36). Following linkage disequilibrium (LD) mapping, haplotype analyses revealed that 6 SNPs in this locus, all of which were in complete LD, showed markedly significant association with MI.

In a case-control association study involving 3,464 Japanese myocardial infarction (608446) patients and 3,186 controls, (Ishii, Ozaki et al. 2006) identified a 6-SNP haplotype block, defined by the SNPs 5338C-T in intron 1, 8813G-A and 9186G-A in exon 3, and 11093G-A, 11741G-A, and 12311C-T in exon 5 of the MIAT gene, that was significantly associated with myocardial infarction (p less than 0.0000030 for each SNP).

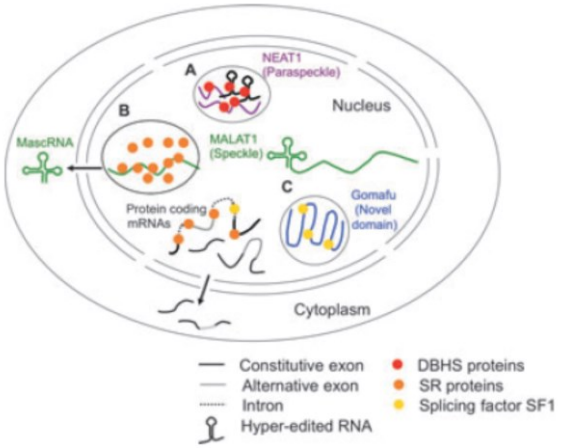


Figure 1.8a. MIAT is located within novel subnuclear domains. It interacts with the splicing factor,SF1, and can affect the efficiency of splicing *in vitro;* therefore it may also have the ability to affect splicing *in vivo*

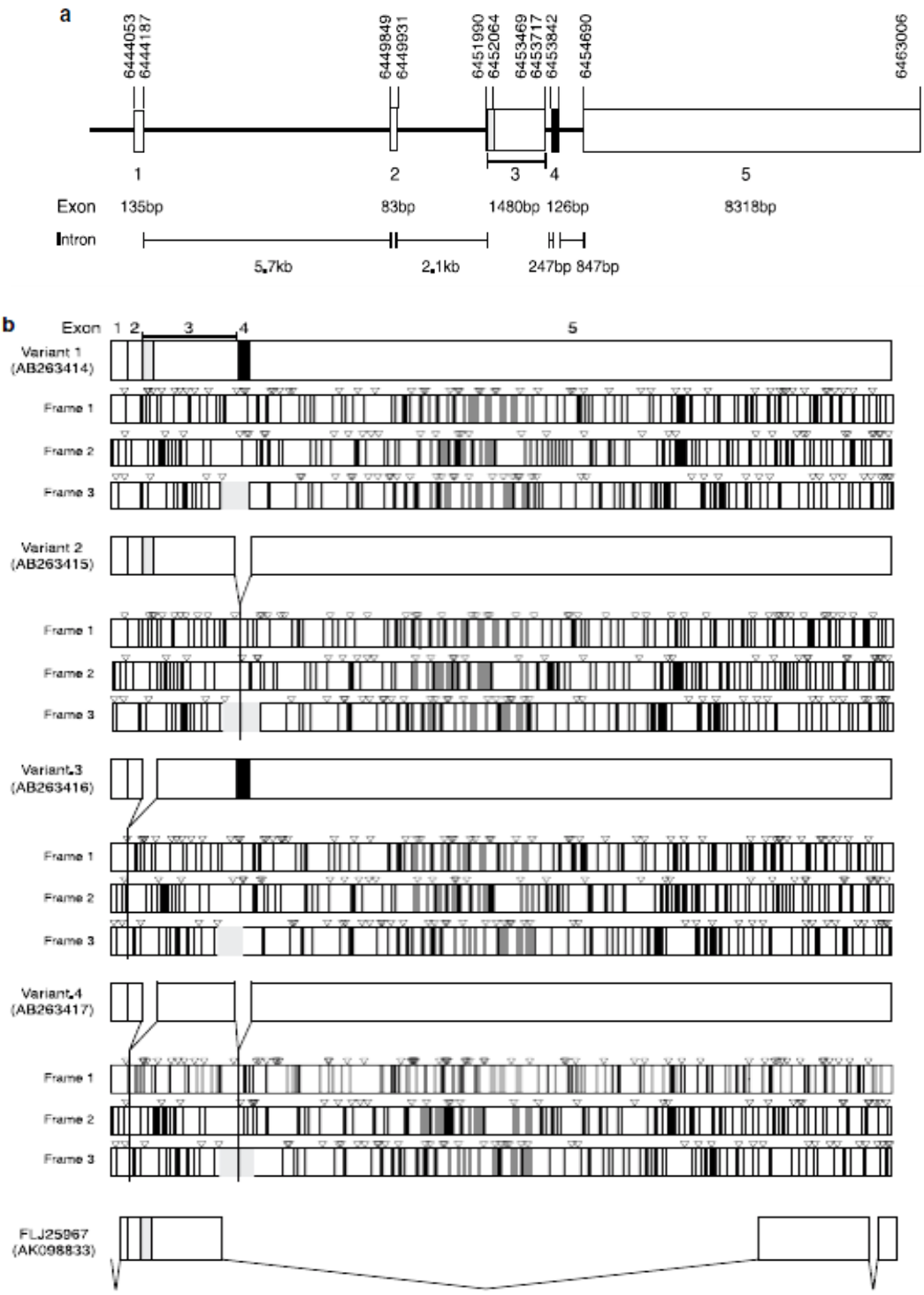


Figure1. 8b. Genomic organization and splicing variant of MIAT. a, Genomic structure of MIAT. Base pair numbering at the top was based on an entry from Gene Bank DNA database NT011520,10.b, Four splicing variants of MIAT and their possible open reading frames. The longest ORF was shown in grey. inverted triangle and vertical line indicate ATG initiation codon and stop codon, respectively

1.3. Oct4 transcription factor

Oct-4 (octamer-binding transcription factor 4) also known as **POU5F1** (POU domain, class 5, transcription factor 1) is a protein that in humans is encoded by the POU5F1 gene.(Takeda, Seino et al. 1992) Oct-4 is a homeodomain transcription factor of the POU family. This protein is critically involved in the self-renewal of undifferentiated embryonic stem cells. As such, it is frequently used as a marker for undifferentiated cells. Oct-4 expression must be closely regulated; too much or too little will cause differentiation of the cells.(Niwa, Miyazaki et al. 2000). The octamer (made of eight units) in this family of transcription factors is the DNA nucleotide sequence "ATTTGCAT", the etymology for the naming of the octamer transcription factor(Petryniak, Staudt et al. 1990).

During embryonic development, Oct4 is expressed initially in all blastomeres. Subsequently, its expression becomes restricted to the ICM and downregulated in the TE and the primitive endoderm. At maturity, Oct4 expression becomes confined exclusively to the developing germ cells (Pesce and Scholer 2001),(Pesce and Scholer 2000). Targeted disruption of Oct4 in mice has produced embryos devoid of a pluripotent ICM(Nichols, Zevnik et al. 1998), suggesting that Oct4 is required for maintaining pluripotency. Furthermore, quantitative analysis of Oct4 expression revealed that a high level of Oct4 expression drives ES cells towards the extra-embryonic mesoderm or endoderm lineages, while those with a low level of Oct4 become trophectodermal cells; ES cells with a normal level of Oct4 remain pluripotent(Niwa 2001)],(Niwa, Miyazaki et al. 2000). Thus, it has been proposed that Oct4 is a key regulator of stem cell pluripotency and differentiation(Pesce and Scholer 2001),(Pesce and Scholer 2000). Further investigation of Oct4 may help unravel the molecular and cellular mechanisms of stem cell pluripotency.

Expression and Function

Oct-4 transcription factor is initially active as a maternal factor in the oocyte but remains active in embryos throughout the preimplantation period. Oct-4 expression is associated with an undifferentiated phenotype and tumors.(Looijenga, Stoop et al. 2003) Gene knockdown of Oct-4 promotes differentiation, thereby demonstrating a role for these factors in human embryonic stem cell self-renewal.(Zaehres, Lensch et al. 2005). Oct-4 can form a heterodimer with Sox2, so that these two proteins bind DNA together(Rodda, Chew et al. 2005). Mouse embryos that are Oct-4-deficient or have low expression levels of Oct-4 fail to form the inner cell mass, lose pluripotency

and differentiate into trophectoderm. Therefore, the level of Oct-4 expression in mice is vital for regulating pluripotency and early cell differentiation since one of its main functions is to keep the embryo from differentiating.

Implications in disease

Oct-4 has been implicated in tumorigenesis of adult germ cells. Ectopic expression of the factor in adult mice has been found to cause the formation of dysplastic lesions of the skin and intestine. The intestinal dysplasia resulted from an increase in progenitor cell population and the upregulation of β-catenin transcription through the inhibition of cellular differentiation.(Hochedlinger, Yamada et al. 2005).

Pluripotency in embryo development

Animal model

In 2000, Niwa et al. used conditional expression and repression in murine embryonic stem (ES) cells to determine requirements for Oct-4 in the maintenance of developmental potency.(Niwa, Miyazaki et al. 2000) Although transcriptional determination has usually been considered as a binary on-off control system, they found that the precise level of Oct-4 governs 3 distinct fates of ES cells. A less-than-2-fold increase in expression causes differentiation into primitive endoderm and mesoderm. In contrast, repression of Oct-4 induces loss of pluripotency and dedifferentiation to trophectoderm. Thus, a critical amount of Oct-4 is required to sustain stem cell self-renewal, and up- or down regulation induces divergent developmental programs. Niwa et al. suggested that their findings established a role for Oct-4 as a master regulator of pluripotency that controls lineage commitment and illustrated the sophistication of critical transcriptional regulators and the consequent importance of quantitative analyzes. The transcription factors Oct-4, Sox2 and Nanog are capable of inducing the expression of each other, and are essential for maintaining the self-renewing undifferentiated state of the inner cell mass of the blastocyst, as well as in embryonic stem cells (which are cell lines derived from the inner cell mass).(Rodda, Chew et al. 2005). Oct-4 is one of the transcription factors used to create induced pluripotent stem cells, together with Sox2, Klf4 and often c-Myc in mouse (Okita, Ichisaka et al. 2007),(Wernig, Meissner et al. 2007),(Maherali, Sridharan et al. 2007) demonstrating its capacity to induce an embryonic stem cell-like state. It was later deterimined that only two of these four factors, Oct4 and Klf4 were sufficient to reprogram mouse adult neural stem cells. Finally it was shown that a single factor, Oct-4 was sufficient for this transformation.(Kim, Sebastiano et al. 2009).

In adult stem cells

Several studies suggest a role for Oct-4 in sustaining self-renewal capacity of adult somatic stem cells (i.e. stem cells from epithelium, bone marrow, liver, etc.)(Tai, Chang et al. 2005) Other scientists have produced evidence to the contrary,(Lengner, Camargo et al. 2007) and dismiss those studies as artifacts of in vitro culture, or interpreting background noise as signal,(Lengner, Welstead et al. 2008) and warn about Oct-4 pseudogenes giving false detection of Oct-4 expression.(Zangrossi, Marabese et al. 2007). Oct-4 has also been implicated as a marker of cancer stem cells.(Kim and Nam 2011), (Atlasi, Mowla et al. 2007).

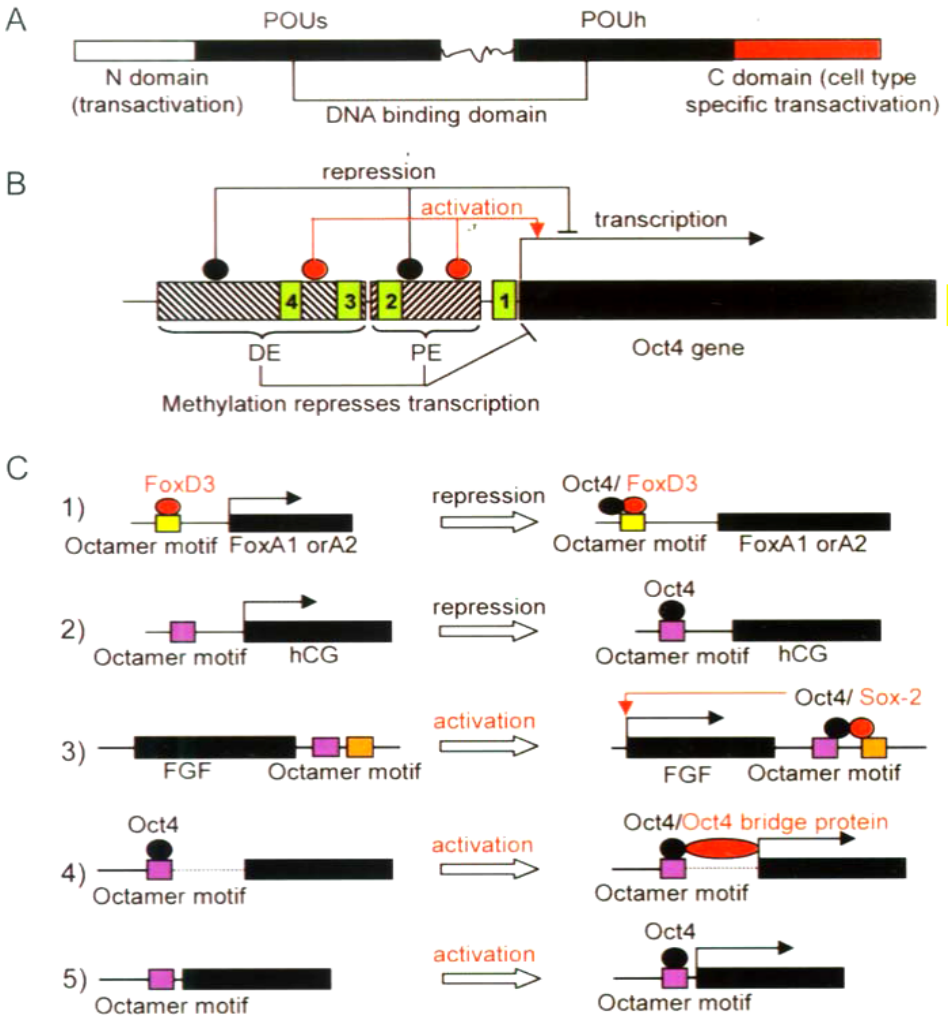


Fig 1.9. Structure and Function of Oct4 A. a schematic illustration of Oct4 domains. Note the C domain behaves differently from the N domain with respect to cell type-specific transactivation. B. The upstream regulatory elements of the Oct4 gene. DE, distal enhancer, and PE, proximal enahncer, are important for regulating Oct4 expression. There are 4 regions that are highly conserved among human, bovine and mouse Oct4 promoter/enhancer elements, shown as green box 1 through 4 relative to DE and PE. Conserved region 1 (CR1) is downstream of PE and immediately upstream of exon 1. Each enhancer contains multiple potential binding sites for transcription factors that can either activate (red) or repress Oct4 expression. In addition, methylation in these regions represses Oct4 expression in differentiated cells. C.Modes of action of Oct4 on different target genes. Oct4 represses gene expression either indirectly by neutralizing activators such as FOXD3 (example 1), or directly by binding to promoters (example 2). Oct4 also acts as an activator of gene transcription by binding to octamer sites located upstream (example 4 and 5) or downstream (example 3) of target genes. In the simplest mode, Oct4 binds to octamer sites immediately upstream of the promoter to activate gene expression directly (example 5). Alternatively, Oct4 can synergize with other factors like Sox2 to activate gene transcription (example 3). When located at a considerable distance, as in example 4, adaptor proteins must be involved to bridge Oct4 to the basic transcription machinery for transcriptional activation.

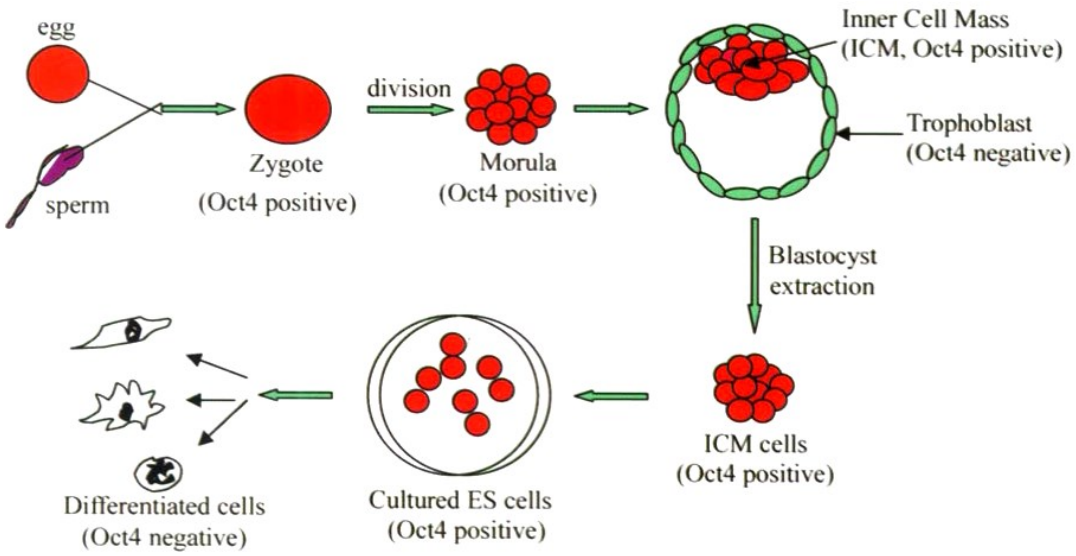


Fig 1.10. ES cells and Oct4 expression The isolation and differentiation of ES cells in vitro are illustrated schematically starting with the fertilization of an egg by a sperm to form a zygote. At the blastocyst stage, inner cell mass (ICM) becomes visible and can be extracted and cultured in vitro to form embryonic stem (ES) cells. Cultured ES cells can be induced to differentiate into various cell types that are negative for Oct4. The stages of Oct4 expression are noted and the cells with Oct4 expression are marked in red colour. There is a general correlation between Oct4 expression and totipotency.(Pan, Chang et al. 2002).

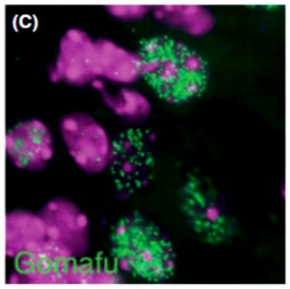


Figure 1.11. Long non- coding RNA(lncRNA) MIAT localized in pyramidal neurons in the cerebral cortex. Green: lncRNA

CHAPTER 2: SPECIFIC AIMS OF STUDY

Long non-coding RNAs (lncRNAs) constitute a significant fraction of the transcriptional output from the mammalian genome; lacking considerable protein-coding abilities which mostly involved in transcriptional and post-transcriptional gene regulation (Ponting, Oliver et al. 2009; Lipovich, Johnson et al. 2010; Hu, Alvarez-Dominguez et al. 2012). MIAT (also referred to as RNCR2/Gomafu) was originally identified as a noncoding RNA which is widely and abundantly expressed in the nervous system throughout development, and its expression continues into adulthood(Sone, Hayashi et al. 2007). Recent studies have revealed that nuclear lncRNAs are essential for the maintenance of nuclear structures and can affect pre-mRNA processing and export, thereby regulating gene expression(Ip and Nakagawa 2012). However the molecular basis of MIAT function as well as its role in human disease is still in the beginning.

We set two major specific aims:

(i) Studying the differential expression of lncRNA MIAT in Leukemic malignancies.

Quantitative Real Time PCR assay employ to established the differential expression of MIAT in available number of B and T cell lines derived from all types of leukemia . As well, analyzing the expression level of MIAT transcript in a large cohort of patient samples, will be done to establish the clinical significance of the dysregulation of MIAT non coding RNA in an aggressive form of Leukemia, CLL. Careful analysis of MIAT expression in different cytogenetically groups of CLL patient, also provide a guidelines to associate the *lncRNAs* expression levels with malignancy prognosis as well as aggressiveness or even response to treatment. The present study also try to assess the effect of the dysregulation of MIAT long non coding RNA in abnormal behavior of cancerous cells, as proliferation and apoptosis programmed death. This would be the first study, to our knowledge, where expression of *lncRNA* MIAT is evaluated in a hematopoietic malignancy.

(ii) **Studying the regulatory correlation of the *lncRNA* MIAT and the master pluripotency and differentiation transcription factor , Oct4 .**

Oct4 is considered as a master transcription factor for pluripotent cell self-renewal. A defined Oct4 level controls the establishment of naive pluripotency as well as commitment to all embryonic lineages (Rapicavoli, Poth et al. 2010; Sheik Mohamed, Gaughwin et al. 2010; Radzisheuskaya, Chia Gle et al. 2013). Recent studies characterized mouse Gomfu/MIAT as a co-activator of Oct4 in a regulatory feedback loop. In this study we evaluate the relationships between human MIAT and Oct4 expression. Towards this end, RNAi-mediated MIAT-silencing as well as shRNA Oct4 knockdown will be performed in leukemic cell lines. Development of recombinant lentiviruses for delivery of shRNA guarantee the high and consistent suppression of targets. Affected cells will be analyzed for modulation in expression of MIAT and Oct4 targets through real time analysis. Western blotting analysis following loss of function of either MIAT or Oct4 targets provide additional confirmation. Complementary study of correlation between MIAT and Oct4 will attain through gain of function studies. Thus, we will try to over-express MIAT by developing an expression construction contains MIAT ORF. In conclusion, The expected results will be used to explain the molecular network in which MIAT regulated. It also might open a window to understanding the role of MIAT in differentiation of Leukemic cell.

CHAPTER 3. MATERIALS AND METHODS

3.1. Plasmids

pGIPZ lentiviral vector for Oct4 shRNA (GIPZ POU5F1 shRNA Transfection Starter Kit GE healthcare, dharmacon, USA, Cat# RHS4287-EG5460).

This plasmid is designed to express short hairpin RNA (shRNA) constructs which designed based on microRNA-30 Hairpin. pGIPZ lentiviral vector adds a Drosha processing site to the hairpin construct and has been shown to greatly increase gene silencing efficiency(Boden, Pusch et al. 2004). The hairpin stem consists of 22 nucleotides of dsRNA and a 19 nucleotides loop from human miR-30. Adding the miR-30 loop and 125 nucleotides of miR-30 flanking sequence on either side of the hairpin results in greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs (Silva, Li et al. 2005). Increased Drosha and Dicer processing translates into greater shRNA production and greater potency for expressed hairpins. Use of the miR-30 design also allows the use of 'rules-based' designs for target sequence selection. One such rule is the destabilizing of the 5' end of the antisense strand, which results in strand specific incorporation of microRNA/siRNAs into RISC. pGIPZ lentiviral vector map shown in Figure 3.1. To assure the highest possibility of modulating the gene expression level, Oct4 gene is represented by four shRNA constructs, each covering a unique region of the target gene. Features of the vector depicted in Figure 3.2.

The features that make this vector a versatile tool for RNAi studies include:

• Ability to perform transfections or transductions using the replication incompetent lentivirus .

• TurboGFP (Evrogen, Moscow, Russia) and shRNA are part of a bicistronic transcript allowing the visual marking of shRNA expressing cells.

• Amenable to *in vitro* and *in vivo* applications.

• Puromycin drug resistance marker for selecting stable cell lines.

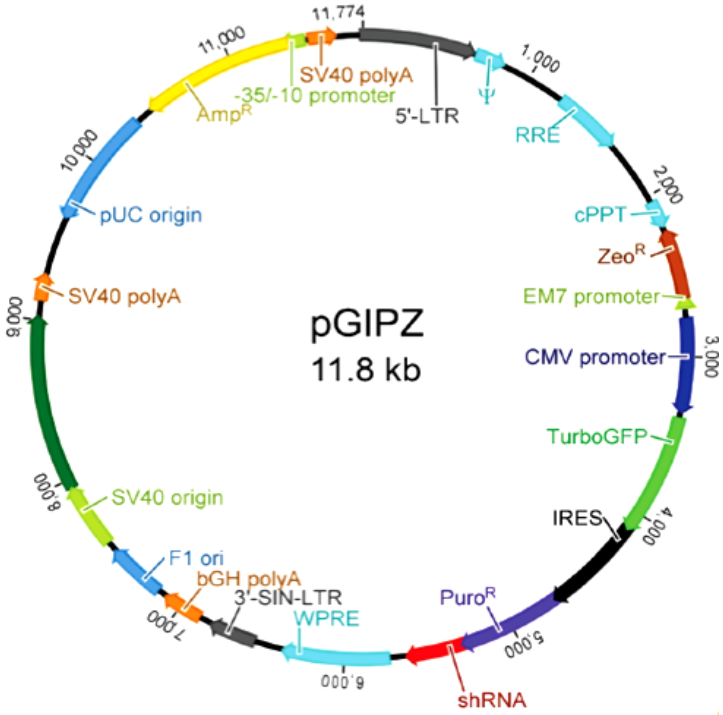
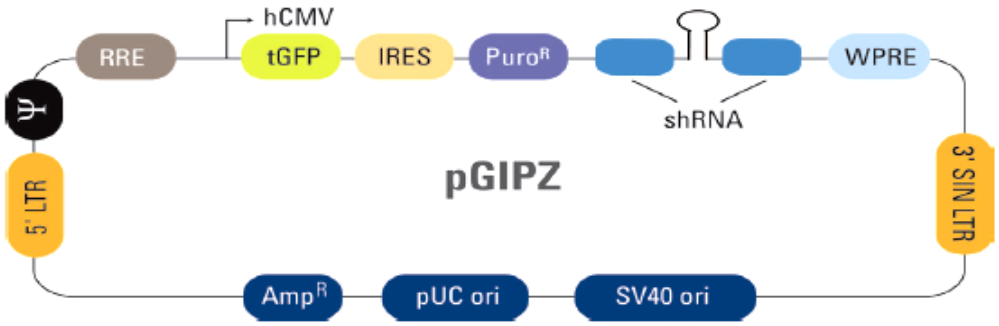


Figure 3.1: Map of pGIPZ Vector

Antibiotic resistance conveyed by pGIPZ include:

Antibiotic	**concentration**	**Utility**
Ampicillin(carbencillin)	100µg/ml	Bacterial selection marker (outside LTRs)
Zeocin	25µg/ml	Bacterial selection marker (inside LTRs)
Puromycin	variable	Mammalian selection marker



Vector Element	**Utility**
hCMV	Human cytomegalovirus promoter drives strong transgene expression
tGFP	TurboGFP reporter for visual tracking of transduction and expression
Puro	Puromycin resistance permits antibiotic selecting pressure and propagation of stable integrants
IRES	Internal ribosomal entry site allows expression of TurboGFP and Puromycin resistance genes in a single transcript
ShRNA	micrRNA-adapted shRNA(based on miR-30) for gene knockdown
5′LTR	5′ long terminal repeat
3′SINLTR	3′self-inactivating long terminal repeat for increased lentivirus safety
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev response element enhances titer by increasing packaging efficiency of full-length viral genomes
WPRE	Woodchuck hepatitis post transcriptional regulatory element enhances trans gene expression in target cells

Figure 3.2: Feature of pGIPZ Vector

3.2. Primers and Probes

The Primers and probes that used in this study are listed in Table 3.1.

Table 3.1: List of primers and probes

Name	Sequence	Application
MIAT Forward	5' tactcgAGCTACAAAGACGACGCCGGCTG3' (XhoI equipped)	Cloning steps of MIAT
MIAT Reverse	5' agacGCGTGAATTGATTTTTAATAGCAAA3' (MulI equipped)	
35MIAT Forward	5' ctcgAGCTACAAAGACGACGCCGGCTGCGCTCGCG 3'	Cloning steps of MIAT
2126MIAT Forward	5' ctcGAGGACAGCTCCAGGGGTATGAGGGAGGCCTG 3'	
6274MIAT Forward	5' ctcGAGGCCTGTTGGTCTAGACTCTAGACTGTGGAG 3'	Cloning steps of MIAT
10148MIAT Reverse	5' acGCGTGAATTGATTTTTAATAGCAAAATGGCATT 3'	
MIAT prob	TaqMan® Gene Expression Assays Cat no:4331182	Real Time PCR
OCT4 prob	TaqMan® Gene Expression Assays POU5F1 Cat no:4331182	
Human TBP (TATA-box binding Protein)	Endogenous Control (FAM™/MGB probe, non-primer limited), Cat no:433376	

3.3. DNA and Protein Marker/Ladder

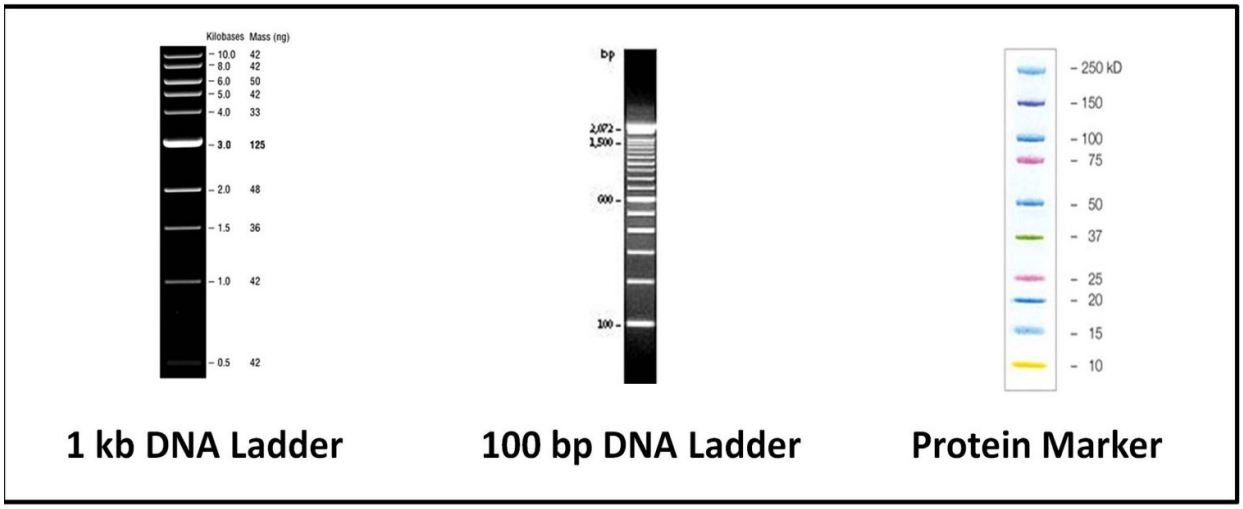
- **DNA Ladder(Gene Ruler)**

100 bp DNA Ladder, (Invitrogen, Cat#15628-019) which is suitable for sizing double-stranded DNA from 100 to 1,500 bp.

1 kb DNA Ladder(New England Biolabs,USA, Cat# N3232S) with effective size range between 500bp to 10,002bp.

- **Protein Marker**

Precision Plus Protein™ Kaleidoscope™ Standards #161-0375 which are a mixture of ten multicolor recombinant proteins (10–250 kD).



3.4. Cell lines and Cultures

3.4.1. Cell lines

The leukemic cell lines Were obtained from the American Type Culture Collection (ATCC, Manassas, VA,USA) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) (Table 3.2). The human embryonic kidney cells HEK293FT transformed with the SV40 large T antigen were obtained from Invitrogen (Carlsbad, CA, USA).

Table 3.2. leukemic cell lines.

No	Cell line	Cell type	Morphology	Disease
1	GM1500	lymphoblast B lymphocyte; Epstein-Barr virus (EBV) transforme	lymphoblast	Lymphoma
2	B1	Lymphoblastoid B cells	Lymphoblastoid	CLL
3	DB	B lymphoblast	lymphoblast	large cell lymphoma
4	SU-DHL-6	B lymphocyte	lymphoblast-like	large cell lymphoma; diffuse mixed histiocytic and lymphocytic lymphoma; follicular B cell lymphoma
5	ML1	Myeloid leukemia lines	Lymphoblastoid	Human acute myeloblastic leukaemia
6	MOLM13	acute myeloid leukemia	most cells are round growing in suspension	Acute myeloid leukemia (Yuan, Loya et al.)
7	SEM	B cell precursor	Round to polygonal single	Acute lymphoblastic leukemia (ALL)
8	CA-46	B lymphocyte	lymphoblast	Burkitt's lymphoma

9	DOHH-2	B cell lymphoma	cells grow as single cells or in clumps in suspension	immunoblastic B cell lymphoma progressed from follicular centroblastic/centrocytic lymphoma
10	MOLT4	T cell leukemia	round cells growing in suspension, singly or in clusters	acute lymphoblastic leukemia (ALL)
11	NU-DHL-1	B lymphocyte	early B-cell	undifferentiated B lymphoma, non-Burkitt's type
12	SU-DHL-10	B Lymphocyte	lymphoblast-like	Large B Cell Lymphoma
13	SUPT11	T-ALL	Small, polymorph cells	T cell leukemia
14	Raji	B lymphocyte	Lymphoblast-like	Burkitt lymphoma
15	JURKAT	T lymphocyte	lymphoblast	acute T cell leukemia
16	Nalm-6			B cell precursor leukemia
17	SU-DHL-8	B Lymphocyte	lymphoblast-like	Large B Cell Lymphoma
18	MV4;11	macrophage	lymphoblast	biphenotypic B myelomonocytic leukemia
19	BL-41			Burkitt lymphoma
20	HL60			acute myeloid leukemia
21	WSU-DLCL-2	B Lymphocyte	Round to oval	B cell lymphoma
22	380	B Cell Precursor	Round small cells	B cell precursor leukemia
23	BJAB			Burkitt lymphoma
24	U937	Monocyte-like characteristics	Lymphoblast-like / Lymphocyte-like, round to polygonal, single cells	histiocytic lymphoma

25	THP1	Monocyte	Monocyte-like	acute monocytic leukemia
26	Namalwa			Burkitt lymphoma
27	697	B cell leukemia	rounded morphology	B cell precursor leukemia
28	MOLM14	acute myeloid leukemia	most cells are round growing in suspension	AML-M5a
29	KARPAS-422	B cell	Round to polygonal cells, growing singly or in small clusters	Pleural effusion, B cell non-Hodgkin lymphoma (intra-abdominal, diffuse large cell lymphoma, refractory, terminal)
30	CESS			
31	OCI-LY-19	B cell lymphoma	single round cells growing in suspension	Diffuse large B-cell lymphoma (DLBCL)
32	RS4;11	B cell leukemia	Lymphoblast-like / single, relatively small, round cells	B cell precursor leukemia (ALL L2)
33	KG1	Macrophage	myeloblast	acute myelogenous leukemia
34	PER377	mature B-cell		ALL
35	MEC1	Mature B cell	round to polymorphic cells growing in suspension, singly or partly in small aggregates, a few cells are slightly adherent	chronic B cell leukemia
36	MonoMac6			acute monocytic leukemia
37	CCRF-SB			T cell leukemia
38	DAUDI			Burkitt lymphoma

39	REH	B cell	Lymphoblast-like / small, round, single cells	Peripheral blood, Acute lymphoblastic leukemia, non-T; non-B, (ALL at first relapse)
40	CI-1			
41	K562	NK cells	lymphoblast	chronic myelogenous leukemia (CML)
42	NB4			acute promyelocytic leukemia (M3)

3.4.2. Cell cultures

All leukemic cell lines were cultured in RPMI 1640, 20% FBS and 1% Pen/strep. Medium for OCI-LY-19 cell line was MEM α, GlutaMAX™ Supplement, no nucleosides. HEK293FT were Cultured in Dulbecco's Modified Eagle's Medium(DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/ml 0.1% (v/v) penicillin/streptomycin (Sigma, St Louis, MO). Specification of media used in this study listed in Table 3.3. Cells were maintained in a humidified incubator at 37°C and 5% CO2 and sub-cultured every 2-3 days .Early passages (passage 4–7) were used in each steps of the study. Freezing Media was prepared as 90%FBS+10% DMSO and was used while keeping on ice.

Table 3.3. Cell culture media and reagents

Medium	**Catalog Number**	**Company**
Dulbecco's Modified Eagle's Medium	D5796	Sigma Aldrich
MEM-α glutamax no nucleosidase medium	32561102	life technologies
RPMI-1640 Medium R8758	R8758	Sigma Aldrich
Penicillin-Streptomycin	P4333	Sigma Aldrich
Fetal Bovine Serum	F2442	Sigma Aldrich

3.4.3. Counting the Cell Number

At the indicated times, cells were trypsinized, (exclude suspended cells) and the total number of cells per ml of media were counted using Scepter 2.0 Handheld Automated Cell Counter (Millipore Corporation, Billerica, MA, USA).

3.5. Development of Lentivirus for shRNA Against Oct4

3.5.1. Plasmid Preparation

For plasmid preparation, all GIPZ shRNA clones were grown at 37 °C in 2x LB broth (low salt) medium plus 100 μg/mL carbenicillin only. Plasmid DNA was isolated using Endo Free Plasmid Maxi Kit cat# 12362, Qiagen as follows:

1. Overnight LB culture was harvested by centrifuging at 6000 x g for 15 min at 4°C.

2. The bacterial pellet was completely re-suspend in 10 ml Buffer P1.

3. 10 ml Buffer P2 was added and mixed thoroughly by inverting 4–6 times, and incubated at room temperature (15–25°C) for 5 min. Because of using LyseBlue reagent, the solution turned blue.

4. 10 ml chilled Buffer P3 was added, mixed thoroughly by inverting 4–6 times. The solution was mixed until it got completely colorless.

5.Tube was centrifuged at max speed(4.7K at 4°C for 30 min) and supernatant recovered (27 to 30 ml) into 50 ml tube.

6. 2.5 ml Buffer ER was added to the collected lysate, mixed by inverting the tube approximately 10 times, and incubated on ice for 30 min.

7. Equilibrated a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and the column allowed to empty by gravity flow.

8. The lysate was applied from step 6 to the QIAGEN-tip and allowed to enter the tip.

9. The QIAGEN-tip was washed with 2 x 30 ml Buffer QC.

10. DNA was eluted with 15 ml Buffer QN into a 50 ml endotoxin-free or pyrogen-free tube.

11. DNA was precipitated by adding 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA and mix. Centrifuged at 4300 x g for 60 min at 4°C. The supernatant carefully was decanted.

12. DNA pellet was washed with 1 ml of endotoxin-free room-temperature 70% ethanol and centrifuged at ≥15,000 x g for 10 min. The supernatant carefully was decanted without disturbing the pellet.

13. In sterile condition the pellet was air-dried for 5–10 min and the DNA re-dissolved in a 250µl volume of endotoxin-free sterilized water.

3.5.2. Production and Packaging of Lentiviral Particles

Trans-Lentiviral Packaging Kits (Thermo Scientific, Cat# TLP5912) was used to generate lentiviral particles. The production of lentiviral particles was begun with co-transfection of the Thermo Scientific Trans-Lentiviral packaging mix with an shRNA vector into HEK293T packaging cells, using the calcium phosphate reagent. Following co-transfection, replication-incompetent virions are released into the medium for collection and downstream use.

3.5.3. Cell Plating

HEK293T cells seeded in a 6-well plate for each transfer vector to be packaged into lentiviral particles.

1. The day before transfection, cells was diluted in normal growth medium (DMEM High Glucose, Sodium Pyruvate, 10% FBS, 1% Pen/Strep) to achieve the optimal cell density of about 85-95% confluence at time of transfection. For each well of a 6-well plate, 1×10^6 cells were prepared in 2mL medium.
2. Cells was incubated at 37 °C with 5% CO_2 overnight.

3.5.4. Co-Transfection with Calcium Phosphate Transfection Reagent

The CaCl2 and 2x HBSS were thawed briefly in a 37 °C water bath. After thawing, both reagents may be stored for several weeks at 4 °C without detectable loss of function. Reagents should be brought to ambient temperature prior to proceeding with transfection.

1. For each well of a 6-well plate, the indicated quantity of transfer vector DNA and Trans-Lentiviral Packaging Mix were prepared in a 5 mL (Fisher Scientific Cat #14-959-1A) or 50 mL (Fisher Scientific Cat #14-432-23) polystyrene tube. Sterile water used to bring DNA mix to the indicated total volume.

	Lentiviral Transfer Vector DNA (shRNA or ORF)	Trans-Lentiviral Packaging Mix	Total Volume (with sterile water)
One well of a 6-well plate	6 µg	4.3 µL	135 µL

2. The indicated volume of $CaCl_2$ was added to the diluted DNA above:

	Cacl2
One well of a 6-well plate	15

3. The tube was vortexed at a speed sufficient to thoroughly mix reagents without spillover. While vortexing, the indicated volume of 2x HBSS was added drop – wise:

	2X HBSS
One well of a 6-well plate	150 µL

4.Incubated at room temperature for 3 minutes. A light chalky precipitate was appeared during this incubation (the precipitate may not always be obvious).

5. The total volume (300 µL) of transfection mix was added drop-wise to the cells.

Note: The exact volume may be slightly less due to pipetting loss, but this will not negatively impact transfection efficiency.

6. The cells were incubated at 37 °C with 5% CO2 for **10-16** hours (do not extend this time).

7. After 16 hours of incubation, the cells were examined microscopically for the presence of a fluorescent reporter protein, TurboGFP as an indicator of transfection efficacy.

Note: The color of the medium may be orange or orange/yellow; this does not affect viral production.

8. Reduced serum medium was prepared as follows:

a. High Glucose DMEM (Fisher Scientific Cat #SH30243.LS)

b. 5% Fetal Bovine Serum (Fisher Scientific Cat #SH30070.03)

c. 2 mM L-glutamine (Fisher Scientific Cat #SH30034.01)

d. 1x Penicillin/Streptomycin (Fisher Scientific Cat #SV30010)

9. Calcium phosphate-containing medium was removed from cells and replaced with the indicated volume of reduced serum medium:

	Reduced serum medium
One well of a 6-well plate	2ml

10. The cells were incubated at 37 °C with 5% CO2 for an additional 48 hours.

Note: Transfection efficacy can be determined by Fluorescent microscopy of GFP ,before harvesting 48 hours supernatant.

3.5.5. Viral Particle Collection and concentration

1. Viral particle-containing supernatants was harvested 48 hours after the medium change by removing medium to a 15mL sterile, capped, conical tube and stored at 4^{o}C.

2. 2ml reduced serum medium was added to each well again and the cells incubated at 37°C with 5% CO2 for an additional 24 hours.

3. Viral particle-containing supernatants harvested after 24 hours by removing medium to the last 15 mL sterile, capped, conical tube contains 48 hours supernatant.

4. non-adherent cells were pelleted by centrifugation at 1600 × g at 4 °C for 10 minutes to pellet cell debris.

5. After the low-speed centrifugation step, to remove any remaining cellular debris, filtration step was done in which supernatant was passed through a sterile 0.45 μM low protein binding filter (Millipore Millex-HV 0.45 μM PVDF filters).

6. Lenti-X™ Concentrator (Clontech Cat. Nos. 631231 & 631232) was used for concentrating of virus particles.

7. 1 volumes of clarified Viral supernatant from step 6 was mixed with 3 volume of the Lenti-X Concentrator and incubated overnight at 4°C.

8. Sample was centrifuged at 1,500 x g for 45 minutes at 4°C. After centrifugation, an off-white pellet will be visible.

9. Supernatant was carefully removed. Residual supernatant was removed with either a pipette tip or by brief centrifugation at 1,500 x g.

10. The pellet gently was re-suspend in 1/100th of the original volume using RPMI1640 without serum and antibiotics. The pellet can be somewhat sticky at first, but will go into suspension quickly.(when add RPMI to the pellet, don’t shake or agitate it).

11. The mixture was incubated at 4°C for overnight.

12. The mixture was centrifuged at max speed ,4°C for 15 minutes.

13. Supernatant was recovered and aliquoted into small volumes.

14. single-use aliquots were immediately stored at –80°C.

3.5.6. Viral Iteration

1. The day before transduction, a 24-well tissue culture plate was seeded with HEK293T Cells at 5×10^4 cells per well in DMEM (10% FBS, 1% pen-strep).

Note: The following day, each well should be no more than 40-50% confluent.

2. Dilutions of the viral stock were made in a round bottom 96-well plate using serum-free media(Figure 3.3 A). The goal was to produce a series of 5-fold dilutions to reach a final dilution of 390,625-fold.
3. 80 µL of serum-free media was added to each well.
4. 20 µL of thawed virus stock was added to each corresponding well in column 1 (five-fold dilution).

Note: Pipette contents of well up and down 10-15 times. Discard pipette tip.

5. With new pipette tips, 20 µL from each well of column 1 was transferred to the corresponding well in column 2.

Note: Pipette up and down 10-15 times and discard pipette tip.

6. With new pipette tips, 20 µL from each well of column 2 was transferred to the corresponding well in column 3.

Note: Pipette up and down 10-15 times and discard pipette tip.

7. Repeated transfers of 20 µL from columns 3 through 8, pipetted up and down 10-15 times and changing pipette tips between each dilution.
8. the dilutions of the virus stock was incubated for 5 minutes at room temperature.
9. The 24-well plate was labeled as shown in (Figure3.3 B) using one row for each virus stock to be tested.
10. Culture media was removed from the cells in the 24-well plate.
11. 225 µL of serum-free media was added to each well.
12. Cells were transduced by adding 25 µL of diluted virus from the original 96-well plate (Figure 3.3 a.) to a well on the 24-well destination plate (Figure 3.3 B.) containing the cells.
13. Transduced cultures were incubate at 37 °C for 4 hours.
14. Remove transduction mix from cultures and add 1 mL of DMEM (10% FBS, 1% Pen-Strep).
15. Culture cells for 48 hours.
16. Count the TurboGFP expressing cells or colonies of cells.

Note: Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour cultureperiod.

17. Transducing units per mL (TU/mL) can be determined using the following formula: # of TurboGFP positive colonies counted × dilution factor × 40 = # TU/mL.

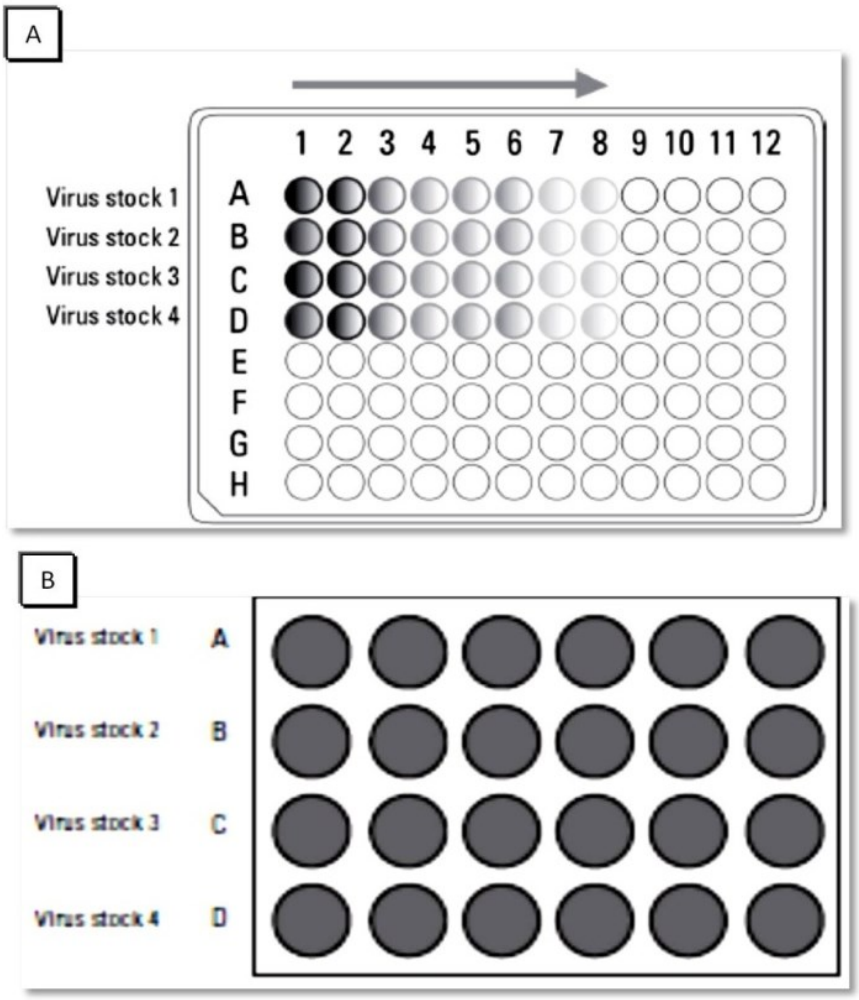


Figure 3.3. (A). Five-fold serial dilutions of virus stock (B). Twenty-four well tissue culture plate, seeded with HEK293T cells, used to titer the virus.

3.5.7. Transduction of Recombinant Lentiviral Particle

The day before transduction, 1×10^5 HEK293T cells were seeded in a 12-well tissue culture plate in 1ml DMEM (10% FBS, 1% Pen/Strep). The following day, the wells should be no more than 40-50% confluent. Transduction mix was prepared by adding 1µl Polybrene (Hexadimethrine bromide – Sigma #H9268) to desired amount of Viral particle and scaled up to 1ml with DMEM (without serum, 1% Pen/Strep). The media was aspirated and transduction mix was added gently onto the cells. plate was incubated for 4 to 8 hours at 37^{o}C. Transduction mix was removed from cultures and 1mL of DMEM (10% FBS, 1% Pen/Strep) added gently to each well. Cells kept in culture for 72 hours and GFP efficacy was monitored using fluorescent microscope.

3.5.8. Infection of Target Cells with Recombinant Lentiviral Particle

$1*10^5$ target cells was mixed with 0.5µl Polybrene (Hexadimethrine bromide – Sigma #H9268) and 500µl of RPMI in a capped cuture tube .Appropriate amount of recombinant viral particle or control virus was added to the mixture. Centrifuged in 2000g, Room Temperature (24^{o}C) for 180 min. Then incubated overnight at 37 ^{o}C. After completing the incubation time, the mixture was centrifuged at 1000 rpm 10 min RT. The supernatant aspirated and the pellet was re-suspended in 0.5 ml RPMI with 10 % FBS and 1% antibiotics and seeded in 24 well for 48-72 hours.

3.6. Cloning of MIAT ORF

3.6.1. Restriction Enzyme Digestion

A) Materials and Reagents

• DNA sample in water or TE buffer
• 10x digestion buffer
• Restriction enzyme

B) Procedure

1. The following reagents were pipetted into a microfuge tube:

Material	**Final concentration**
Vector	0.1 to 4µg
TE Buffer (10X)	1X
RE enzyme	1-5u/µg DNA
ddH2O	Rest of volume
Final volume	30µl

2. Incubated for 2 hours at 37◦C.
3. 2 to 5 µl of the digested sample along with the uncut DNA and a DNA marker were run on agarose gel and checked for the exact size of resulted fragments.

Tips:

• For checking DNA, 0.1 µg DNA was used

• For cloning, 4 µg DNA was used.

3.6.2. Ligation

Ligation was done with 50 ng of cut and purified vector in combination with 3-fold molar excess of insert in the presence of 1µl T4 ligase (Fermentase,USA) and 1µL of 10X T4 ligase buffer. Total volume was adjusted at 10µl with ddH2O. Ligation control was included with substituting the amount of insert with water. It allows evaluating the relegation efficiency. The ligation reactions were incubated at 15°C in a thermocycler for overnight.

3.6.3. Competent Cells

Transformation was done in DH5α-T1 bacterial competent cells. invitrogen cat# K4530-20.

3.6.4. Transformation

Transformation was done according to manual of One Shot® TOPO®TA Cloning® Kit for sequencing, invitrogen cat# K4530-20.

A) Materials and Reagents

- The TOPO® Cloning reaction from Set up the TOPO® Cloning reaction
- LB plates containing 50 µg/mL ampicillin or 50 µg/mL kanamycin
- 15-mL snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g., plates, spreaders)

Components supplied with the kit:

- S.O.C. medium

Prepare for transformation

- A water bath to 42°C was equilibrated (for chemical transformation)
- The vial of S.O.C. medium warmed to room temperature.
- Selective plates warmed at 37°C for 30 minutes .
- 1 vial of One Shot® cells for each transformation thawed on ice.

B) One Shot® chemical transformation procedure

1. 2 µL of the TOPO® Cloning reaction was added into a vial of One Shot® chemically competent E. coli and mix gently. Do not mix by pipetting up and down.
2. Incubated on ice for 5–30 minutes. Note: Longer incubations on ice do not seem to affect transformation efficiency.
3. The cells were heat-shocked for 30 seconds at 42°C without shaking.
4. The tubes immediately transferred to ice.
5. 250 µL of room temperature S.O.C. medium was added.
6. The tube was capped tightly and shaked horizontally (200 rpm) at 37°C for 1 hour.

7. 10, 25, 50 and 100µL from each transformation (and 50 µL from negative) were spreaded on a pre-warmed selective plate and incubated overnight at 37°C.

3.6.5. Glycerol Stocks Preparation

Every plasmid construct, whether it was created or purchased, maintained as a glycerol stock.

A) Materials and Reagents

- Appropriate LB media, liquid & solid.
- 80% Glycerol (80ml autoclaved glycerol was mixed with 20ml autoclaved ddH2O).

B) Procedure

1. A single colony of the clone picked off of a plate and grown in the appropriate selectable liquid medium for overnight (e.g., LB with desired antibiotic).
2. The construct was labelled (clone ID # and date). Placed this label onto a sterile screw cap of cryo-vial and then placed on ice.
3. 0.5ml of the o/n culture was added to 0.5ml of 80% sterile glycerol in the sterile screw cap micro centrifuge tube (on ice) and vortexed.
4. the glycerol stock was freezed at –80°C
5. All pertinent information (host strain, vector, cloning site(s), selection criteria, date prepared, origin/source and/or reference, and any other important information) regarding this accession were entered into the lab stock collection book. Also include a map or sequence if possible.

To streak out from a glycerol stock

1. The location of the construct was determined.
2. the tube was took and placed onto dry-iced box
3. A portion from the top of the frozen glycerol stock scraped off using a plastic inoculating loop and streaked onto plate.

3.6.6. Manipulation of DNA

3.6.1.1. Plasmid Mini prep

In cloning and sub-cloning steps of plasmid DNA, QIAGEN Plasmid Mini kit (cat≠12125) (Qiagen, Hilden, Germany) was used according the manufacturer's instruction .

3.6.1.2. Recovery of DNA fragments from the gel

Recovery and of DNA fragments from the agarose gel was performed by dialysis method as follows:

1. The gel was put into the dialysis membranes containing 0.5 ml buffer TBE
2. Two sides of membrane were closed by clips
3. The membrane was put in the electrophoresis tank contains 0.5 % TBE as electrical flows go through negative to positive for 30 min at 100 V.
4. Buffer included DNA was collected from bag(250µl) and transferred to a tube and DNA extracted as follows:

3.6.1.3. Extraction of DNA by Phenol-Chloroform

A) Materials and Reagents

- phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform isoamyl alchohol
- TE buffer

B) Procedure

1. 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample and vortexed or shakeed by hand thoroughly for approximately 20 seconds.
2. Centrifuged at room temperature for 5 minutes at 16,000 × g. The upper aqueous phase was carefully aspirated and transferred to a fresh tube. Be sure not to carry over any phenol during pipetting. 100µl TE buffer was added to precipitate and mixed well.

3. Centrifuged at room temperature for 5 minutes at 16,000 × g. The upper aqueous phase was carefully aspirated and transferred to the previous separated aqueous phase.
4. 300µl chloroform isoamyl alchohol was added to the tube and centrifuged at room temperature for 5 minutes at 16,000 × g. The upper aqueous phase was carefully aspirated and transferred to a fresh tube.
5. Proceeded to Ethanol Precipitation.

3.6.1.4. Ethanol Precipitation

A) Material and Reagents

Reagent	**Volume**
Glycogen (20 µg/µL)	1 µL
7.5 M NH_4OAc	25µl
100% ethanol	

B) Procedure

1. The reagents in the listed order in above table, were added to the aqueous phase of extraction procedure (see above).
2. The tube was placed at –20°C overnight to precipitate the DNA from the sample.
3. The sample was centrifuged at 4°C for 30 minutes at 16,000 × g to pellet the DNA.
4. The supernatant was carefully removed without disturbing the DNA pellet.
5. 500 µL of 70% ethanol was added and centrifuged at 4°C for 5 minutes at 16,000 × g. The supernatant was carefully removed.
6. The pellet was dried at room temperature for 5–10 minutes.
7. The pellet was resuspended in adequate volume of H2O .
8. Centrifuged briefly to collect the sample, and was placed on ice.

3.6.1.5. Quantification of DNA

The amount of DNA was measured by a Nanodrop® spectrophotometer (ND-1000)(Thermo scientific).

3.7. Quantitative Molecular Assays

3.7.1. RNA Assay

3.7.2.1. RNA Extraction

RNA of cell lines were extracted using TRIzol method. In the case of cells infected with lentivirus, we used RNA Clean Up and Concentration Kit (Norgen Cat# 23600)

- **RNA Extraction using TRIzol®**

A) Material and Reagents

- TRIzol®Reagent(Ambion®) (Invitrogen, Cat#: 15596-018)
- Chloroform (Sigma Aldrich, Cat#: C2432-500ML)
- Isopropanol 100%
- Ethanol 75%

B) Procedure

Cultured cells were harvested, washed with PBS and pelleted by centrifuging at 200×g for 5 min. The cell pellet was dissolved in 1mL of TRIzol®Reagent. The homogenized samples were incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. 0.2 mL of chloroform per 1mL of TRIzol was added and the tubes were shaked vigorously by hand for 15 seconds. After 2–3 minutes incubation at

room temperature, the samples were centrifuged at 12,000×g for 15 minutes at 4°C. The aqueous phase of the samples were aspirated and placed into new tubes. 0.5mL of 100% isopropanol was added to the aqueous phase, for 1mL of TRIzol® Reagent used for homogenization. The samples were incubated at room temperature for 10 minutes followed by centrifuge at 12,000×g for 10 minutes at 4°C. The RNA pellets were washed with 500µL of 75% ethanol. The samples were vortexes briefly, then centrifuged at 7500 × g for 5 minutes at 4°C. The wash was discarded. the RNA pellets were air dried for 5–10 minutes and re-suspended in 30µl of in RNase-free water. Absorbance of RNA at 260 nm and 280 nm was used to determine concentration. Extracted RNA were qualified on the 0.8% agarose gel.

- **RNA clean up and concentration kit(Norgen Cat# 23600)**

The Protocol for RNA Clean-up and Concentration from Phenol/Guanidine Based RNA (Trizol or Tri Reagent) Isolation Methods was followed as below:

1. Sample Preparation

a. RNA was Isolated using a phenol/guanidine-based reagent such as Trizol or Tri Reagent, according to manufacturer's instruction. After the separation of the aqueous and organic phases, the upper (aqueous) fraction containing the RNA was collected into a new RNase-free microcentrifuge tube . Note the volume.

b. Added one volume of 70% ethanol (provided by the user) to the fraction from step 1a. Mixed by vortexing for 10 seconds.

2. Binding to Column

a. Assembled a column with one of the provided collection tubes.

b. Applied up to 600 µL of the RNA mixed with the ethanol (from Step 1b) onto the column and centrifuged for 1 minute at ≥ 3,500 x g (~6,000 RPM).

Note: Ensure the entire sample volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute at 14,000 x g (~14,000 RPM).

c. Discarded the flow through. Reassembled the spin column with its collection tube.

d. If the volume of RNA mix is greater than 600 µL, repeat Steps 2b and 2c until all the remaining RNA mix has passed through the column.

3. Column Wash

a. Applied 400 µL of Wash Solution A to the column and centrifuged for 1 minute.

Note: Ensure the entire Wash Solution A has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

c. Discarded the flow through and reassemble the spin column with its collection tube.

d. Repeated steps 3a and 3b to wash the column a second time.

e. Washed column a third time by adding another 400 µL of Wash Solution A and centrifuging for 1 minute.

f. Discarded the flow through and reassembled the spin column with its collection tube.

g. Spined the column for 2 minutes in order to thoroughly dry the resin. Discarded the collection tube.

4. RNA Elution

a. Placed the column into a fresh 1.7 mL Elution tube provided with the kit.

b. Added 20 µL of water to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended.

c. Centrifuged for 2 minutes at 200 x g (~2,000 RPM), followed by 1 minute at 14,000 x g (~14,000 RPM) Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for 1 additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate micro centrifuge tube (Repeat Steps 4b and 4c).

5. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

3.7.2.2. Quantification of the extracted RNA

Quantified the RNA in Nanodrop and measured absorbance at 260 nm and 280 nm. (A260/280 of >1.8 was considered for assessment of quality of RNA. Partially dissolved RNA samples have an A260/280 ratio <1.6).

3.7.2.3. DNaseI treatment of extracted RNA

A) Materials and Reagents

DNase I, Amplification Grade, manufactured by life technology, Unite size,100U

Catalog #s 18068−015, Lot No. 1465877

B) Procedure

The following materials were added to an RNase-free, 0.5-ml microcentrifuge tube on ice:

1 µg RNA sample

1 µl 10X DNase I Reaction Buffer

1 µl DNase I, Amp Grade, 1 U/µl

DEPC-treated water to 10 µl

Tube was incubated for 15 min at room temperature. The DNase I was inactivated by the addition of 1 µl of 25mM EDTA solution to the reaction mixture. Heated for 10 min at 65°C.

NOTE: It is important not to exceed the 15-minute incubation time or the room temperature incubation. Higher temperatures and longer time could lead to Mg++-dependent hydrolysis of the RNA. Additionally, it is vital that the EDTA be added to at least 2mM prior to heat-inactivation to avoid this problem.

3.7.2.4. cDNA synthesis

A) Materials and Reagents

Super Script VILO DNA synthesis Kit (Invitrogen™ Cat #s 11754-050)

B) Procedure

For a single reaction, the following components were combined in a tube on ice. For multiple reactions, a master mix was prepared without RNA.

component	concentration
5X VILO™ Reaction Mix	1X
10X SuperScript® Enzyme Mix	1X
RNA	100ng
DEPC-treated water	--
Total volume	10µl

2. Tube contents was gently mix and incubated at 25°C for 10 minutes.

3. Tube was incubated at 42°C for 120 minutes.

4. The reaction was terminated at 85°C at 5 minutes.

5. Stored at –20°C until use.

3.7.2.5. Real Time PCR

A) Materials and Reagents

1. Human TBP (TATA-box binding Protein) Endogenous Control (FAM™/MGB probe, non-primer limited), Life Technologies**,** Cat# 433376
2. TaqMan Gene Expression Assays , Life Technologies**,** Cat# Hs00402814_m1
3. TaqMan® Gene Expression Assays POU5F1, Life Technologies**,** Cat no:4331182
4. Optical 96-Well Fast Thermal Cycling Plate with Barcode (code 128), part No. 4346906.

5. Fast advantage 2X real time master mix

B) Protocol

Components	**Final concentration**
2X TaqMan Universal Fast PCR Master Mix	**1X**
20X Taq Man Gene Expression Assay for **MIAT,TBP,OCT4**	**1X**
cDNA Sample	**1:4 diluted**
RNase-free Water	**Up to 20µl**

Each sample was loaded in triplicate. PCR was done in Biorad-Chromo4 thermal cycler real
time PCR instrument as follows:

Sample Volume: 20		**No. of Cycles: 40**		
Step	**UNG incubation**	**Ampli Taq Gold Active.**	**PCR 40 Cycle**	
Action	Hold	Hold	Denature	Anneal/Extend
Temperature	50 ^{o}C	95 ^{o}C	95^{o} C	60^{o} C
Fast chemistry time	2 min	20 min	1 min	20 min

3.7.2.6. polyA SpinTM mRNA Isolation kit(New England Biolabs, Cat#S1560S)

polyA SpinTM mRNA Isolation was used for isolation of mRNA from other RNA (like rRNA, tRNA and snRNA) in a RNA samples. Isolation procedure was done according to the manual of instructor as follows:

• Allow oligo (dT)25-cellulose, column(s) and buffers to come to room temperature.

• Prepare a 65–70°C bath and an ice bath.

• Spin tube containing oligo (dT)25-cellulose in a micro centrifuge for 10 seconds. Using a micropipette remove storage buffer. Be careful to avoid drawing of cellulose beads into pipette tip.

• Equilibrate cellulose by adding 200 µl of Wash Buffer to cellulose beads, mix thoroughly then micro centrifuge for 10 seconds. Using a micropipette decant supernant.

• Pre warm Elution Buffer in 70°C bath.

Isolation Procedure

1.100µl of Cl1 nuclear RNA(891 ng/µl) and 60µl of Cl1 cytoplasmic RNA(485 ng/µl) were diluted with Elution Buffer to final volume of 450 µl.

2 . Add 50 µl of 5M NaCl per 450 µl total RNA solution.

3 . Heat at 65°C for 5 minutes and quickly cool in an ice bath for 3 minutes.

4 . Apply total RNA solution to equilibrated oligo $(dT)_{25}$ -cellulose, seal cap and mix thoroughly . Let stand at room temperature for 5 minutes agitating by hand or place horizontally on rotary shaker 5 . Micro centrifuge for 10 seconds . Note: It is important to agitate beads during binding, washing and elution steps .

6 . Pipet supernatant back into original micro centrifuge tube . Repeat steps 3 thru .

7 . Pipet supernatant back into original micro centrifuge tube for storage . It is recommended that no spin-column elutes be discarded until entire isolation procedure is completed and the results are evaluated . Elutes can be stored in sterile test tubes on ice .

8 . Add 400 µl of Wash Buffer to oligo (dT)25 -cellulose beads . Agitate by hand to resuspend the cellulose beads . Using a 1 ml micropipette with sterile pipette tip, transfer Wash Buffer and beads to the column reservoir of a clean micro centrifuge spin column unit (provided with kit) .

9 . Let stand at room temperature for 2 minutes agitating by hand or place horizontally on rotary shaker . Micro centrifuge for 10 seconds . Remove column reservoir and transfer column eluent to a clean 13 x 100 mm test tube .

10 . Add 400 µl of Wash Buffer to column reservoir and wash as in Step 9 three times .

11 . Using the same method wash column once with 400 µl of Low Salt Buffer .

*Note: This wash step with 0 .1 M NaCl removes residual poly(A)– RNA which is bound to the cellulose column . This step can be omitted during a second round purification .

12 . Remove spin-column reservoir and place in a clean micro centrifuge tube (provided with kit) .

13 . Add 200 µl of pre warmed Elution Buffer to column reservoir . Agitate by hand resuspending the cellulose beads . Let stand for 2 minutes agitating by hand or place on rotary shaker . Micro centrifuge for 10 seconds .(we add 50 and 100µl in two independent rounds)

14 . Repeat Step 12 using fresh pre warmed Elution Buffer. (we add 50µl in two independent rounds only for nuclear sample)

15 . Place Elution Buffer eluate on ice.

3.7.2. Protein Assay

3.7.2.7. Protein Isolation

Total protein from mammalian cell lines were prepared using RIPA lysis buffer (150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) (Sigma, St Louis, MO) with

complete protease and phosphatase inhibitor cocktails (Sigma, St Louis, MO,USA). Homogenates were then centrifuged at 13000 rpm for 15 min at 4°C and supernatants collected. Protein concentration was measured using Bradford Reagent and the BSA Protein for tracing a standard curve.

3.7.2.8. Western Blotting

A) Reagents and Solutions

- **SDS-PAGE Running Buffer**

Tris-base 3.0 g

Glycine 14.4 g

20% SDS 5 ml

Add ddH2O to 1 L

- **Western Blot Transfer Buffer**

Tris-base 3.0 g

Glycine 14.4 g

Methanol 200 ml

Add ddH2O to 1 L

- **TBS Buffer(pH 7.4)**

Tris-base 2.420 g

NaCl 8.78 g

Add ddH2O to 1 L

Adjust the pH to 7.4

- **TBST Buffer**

0.05 % tween 20

Add to TBS Buffer

- **Western Blot Blocking Buffer**

5% milk

Add to TBS Buffer

Mix well and filter

- **Ponceau Red Staining Solution**

Ponceau S 0.5g

Acetic acid 25 ml

Add ddH2O to 500 ml

B) Procedure

Thirty micrograms of total extracted protein were loaded onto 4-15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories,Hercules,CA94547). The separated proteins were transferred to PVDF membranes (BioRad) for 2h at 100mA. After blocking with 5% non-fat milk ,the membrane was incubated with Oct4 primary antibody (Anti- OCT4 antibody adcam cat# 19857) (concentration: 1µl/ ml), dilution 1:1000). GAPDH (mouse) (concentration dilution 1:15000) was used for normalization purposes at 4°C overnight. The membrane was washed three times in TBST buffer, each for 10min. Then followed by incubation with Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit (Sigma Aldrich,A9044 dilution 1:10000) to recognize the primary antibodies for 1hr. at room temperature. Signals were developed with Precision Plus Protein Dual Color Standard (BIO-RAD) and Protein bands were visualized using the ChemiDoc™MP Imaging System and quantified using Image lab 4.0 software (Bio-Rad Laboratories,Hercules,CA94547).

3.8. Quantitative Cell Analysis

3.8.1. CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. 20000 Leukemic Cells transferred to 96-white-walled microtiter plates. Cell viability was determined by adding 100µl of the CellTiter-Glo luminescent cell viability kit (catalog# G7571) from Promega Corporation (Madison, WI, USA) directly to cells suspended in serum-supplemented mediumand The plate was incubated for 90min at room temperature in a dark room to stabilize luminescent signal. For each sample, triplicate wells were considered. Also we considered wells without cells as a blank control as negative background controls. The contents were mixed for 2 minutes on an orbital shaker to induce cell lysis. Luminescence was recorded in Infinite F200 PRO multimode microplate reader(Tecan Group Ltd, Seestrasse 1038708 Männedorf ,Switzerland). Calculation of results was done by subtracting the average of luminescence value of the culture medium background from all Luminescence value of experimental wells. Data were confirmed in at least two independent experiments.

3.8.2. Caspase-Glo® 3/7 Apoptosis Assay

The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in purified enzyme preparations or cultures of adherent or suspension cells. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The Caspase-Glo® 3/7 Reagent is optimized for caspase activity, luciferase activity and cell lysis. Addition of the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal. For preparation of the reagent the Caspase-Glo® 3/7 Buffer and lyophilized Caspase-Glo® 3/7 Substrate were equilibrated to room temperature before use. Then the contents of the Caspase-Glo® 3/7 Buffer bottle was transferred into the amber bottle containing Caspase-Glo® 3/7 Substrate. The contents was mixed by swirling or inverting, until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 Reagent.

Caspase-3/7 activity was measured immediately after the detection of CellTiter-Blue® Cell Viability Assay (described above) on the same wells, by adding 100µl of the homogeneous Caspase-Glo® 3/7 assay Reagent (catalog#G8091)(Promega Corporation ,Madison, WI , USA) at the established time after transfection, to each well of a 96-well plate containing 100µl of blank or negative control cells. Because of the sensitivity of this assay, the plate was covered with a plate lid. contents of wells gently was mixed using a plate shaker at 300–500rpm for 30 seconds, depending upon the cell culture system. The optimal incubation period was determined empirically between 60, 90 and 120min. the luminescence of each sample was measured in a plate-reading illuminometer (infinite F200 PRO) (Tecan Group Ltd, Seestrasse 1038708 Männedorf ,Switzerland), as directed by the luminometer manufacturer. The value for the blank reaction which show the background luminescence from experimental values was subtracted. Negative control reactions were important for determining the basal caspase activity of the cell culture system and vehicles. Caspase-3/7 activity is expressed as luminescence of treated sample / mock control×100. Data were confirmed in at least two independent experiments.

3.8.3. MTS assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay(a) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliu m, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability. The experiment was established by adding 20µl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium. We used multichannel pipettes for convenient delivery of uniform volumes of CellTiter 96® AQueous One Solution Reagent to the 96-well plate. The plate was incubated at 37°C for 15 min in a humidified, 5% CO2 atmosphere while covering to avoid light 96-well plate reader. Absorbance was measured at 490nm using a plate-reading illuminometer (infinite F200 PRO) (Tecan Group Ltd, Seestrasse 1038708 Männedorf ,Switzerland), as directed by the luminometer manufacturer.

3.9. Statistical Analysis

Significance was determined by the two-tailed Student's test. A p-value threshold < 0.05 was considered significant. All real time PCR (assayed in triplicate), Western blotting, and transfection experiments were repeated twice, and reproducible results were obtained. Values were presented as the mean ± standard deviation (Carninci, Kasukawa et al.).

CHAPTER 4. RESULTS

4.1. LncRNA MIAT is Differentially Expressed Among Leukemic Cell Lines

A total of 42 leukemic cell lines were analyzed for MIAT expression including 7 ALL, 2 CLL, 10 AML, one CML, 19 B cell lymphoma and 4 T cell leukemia cell lines.

The 7 ALL cell lines included 380,697, RS4;11, SEM, Nalma6 with the characteristic of B precursor leukemic cells, PER377 which originated from mature B cell and REH which derived from the peripheral blood of a non B, non T acute lymphoblastic leukemia (ALL at first relapse) patient.

In the group of CLL , MECI derived from the peripheral blood of a patient with B-chronic lymphocytic leukemia (B-CLL) in prolymphocytoid transformation to B-PLL. B1 cell characteristics established from a chronic lymphocytic leukemia clone by *in vitro* EBV infection.

The 10 AML cell lines included NB4, HL-60 as promyeloblast leukemic cell type, Monomac6, U937,THP-1, MOLM13, MOLM14 that derived from peripheral blood of a patient with acute monocytic leukemia, CESS with lymphoblast cell type from myelomonocytic leukemia and KG1, MV4;11 with cell type of macrophage and ML-1 which originated from lymphoblast.

The only CML cell line, K562, derived from a CML patient in blast crisis. Analysis of properties of B and T lymphocytes showed that K562 is not a B cell line, while it has some T cell properties.

The 19 B cell lymphoma included BJAB,BL41,Raji,Daudi,CA46,Nalmava, CI-1 belong to Burkitt lymphoma, DoHH2,SU-DHL-6 from follicular B cell lymphoma, NU-DHL1 early B cell from non-differentiated B lymphoma, GM1500, WSU-DLCL-2 with lymphoblast morphology and DB, SU-DHL-8, SU-DHL-10, OCI-LY-19,Karapas form large B cell lymphoma.

The Four T Leukemia cell lines included CCRF-SB, SUP11, MOLT4 and JURKAT.

Total RNAs of cell lines were extracted and treated by *DNase I, Amplification Grade Kit* in order to eliminate genomic DNA contamination. 80 ng of purified total RNAs was used in cDNA synthesis reaction using Super Script VILO DNA synthesis Kit (Invitrogen™ Cat #s 11754-050) (see material and methods for details). Real Time PCR was done providing TaqMan Gene Expression Assays probe for MIAT (Life Technologies**,** Cat# Hs00402814_m1), in three replicates for each samples. Human TBP (TATA-box binding Protein)(FAM™/MGB probe, non-primer limited (Life Technologies**,** Cat# 433376)) was selected as endogenous Control. The results were depicted in Figure 4.1. The lowest level of MIAT was detected in ALL and AML as well as CML cell lines. MECI cell line belonging to CLL type, showed moderate level of MIAT. In B cell lymphoma variable expression of MIAT from low to high was detected. B lymphoma cell lines with Low expressed MIAT mostly had Lymophoblast-like morphology. Two cell lines, SU-DHL-6 and DOHH2 originated from follicular B cell lymphoma fell down in category of moderate MIAT expression. Other member of B lymphoma cell lines with moderate level of MIAT expression originated from Burkitt lymphoma or Large B cell lymphoma. In the group of high MIAT expression which included CI1,DB and Nalmava, the highest level belonged to CI1 cell lines. CI1 cells are at intermediate stages of B-cell differentiation with abnormalities involving chromosomes 2, 8 and 22 and its karyotype was 46, XX, t(2;8) t(14;22). The t(2;8) had the same breakpoints as those reported in some cases of Burkitt's lymphoma. DB cell line which established from ascites of a 45-year-old caucasian man with diffuse large cell lymphoma; assigned to GCB-like lymphoma subtype (germinal center B-cell) was stand for the second high MIAT expression B Lymphoma. Nalmava belonged to Burkitt lymphoma ,too. It also did not seem a correlation between MIAT expression and differentiation stage of B cells. T cell leukemia cell lines showed also a variable level of MIAT expression from low to moderate.

In conclusion, CLL and B cell lymophoma cell lines express significant high level of MIAT whereas AML,ALL and CML cell lines did not show a considerable MIAT expression. T cell leukemia cell lines are variable in MIAT expression (Figure 4.2.).

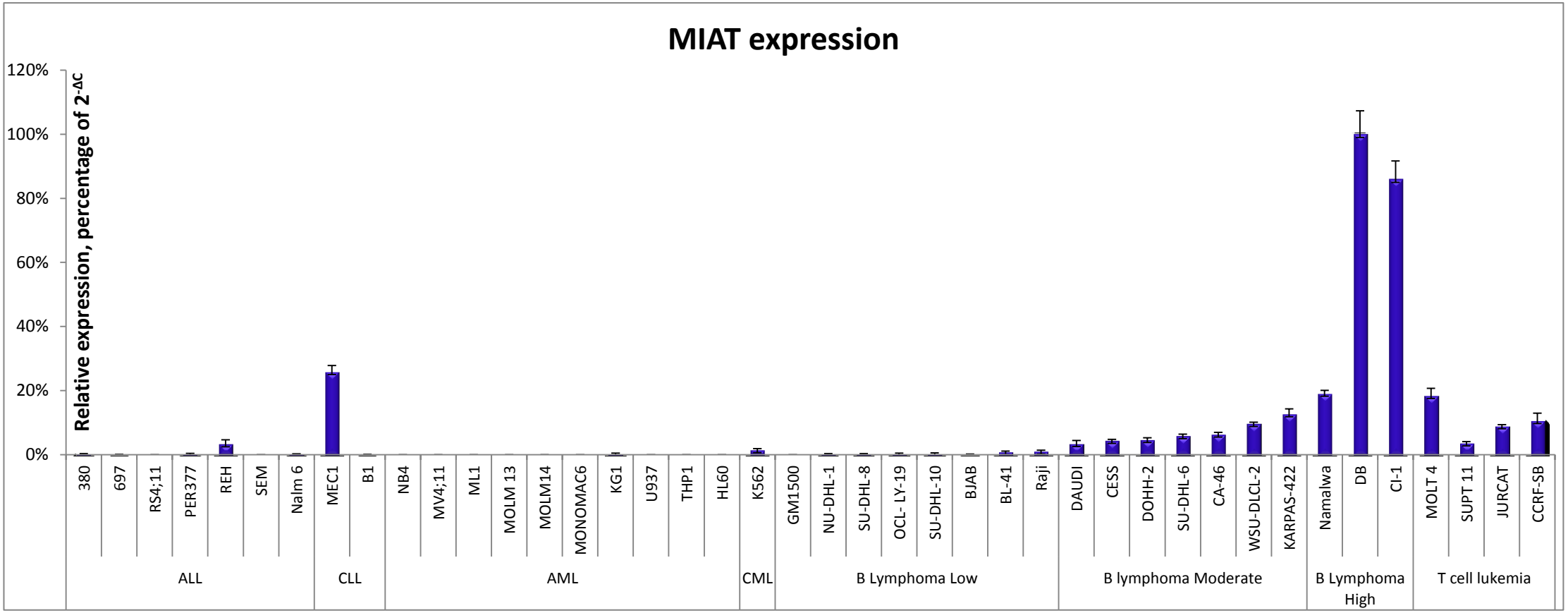


Figure 4.1: MIAT expression in Leukemic cell lines. Differential expression of MIAT in leukemic cell lines was examined through Real time PCR using specific taqman probe for MIAT and normalized according to TBP (TATA binding protein) expression. Data are represented as mean values +/- SD from three replicates. Percentage of of $2^{-\Delta ct}$ was used to explain the MIAT expression.

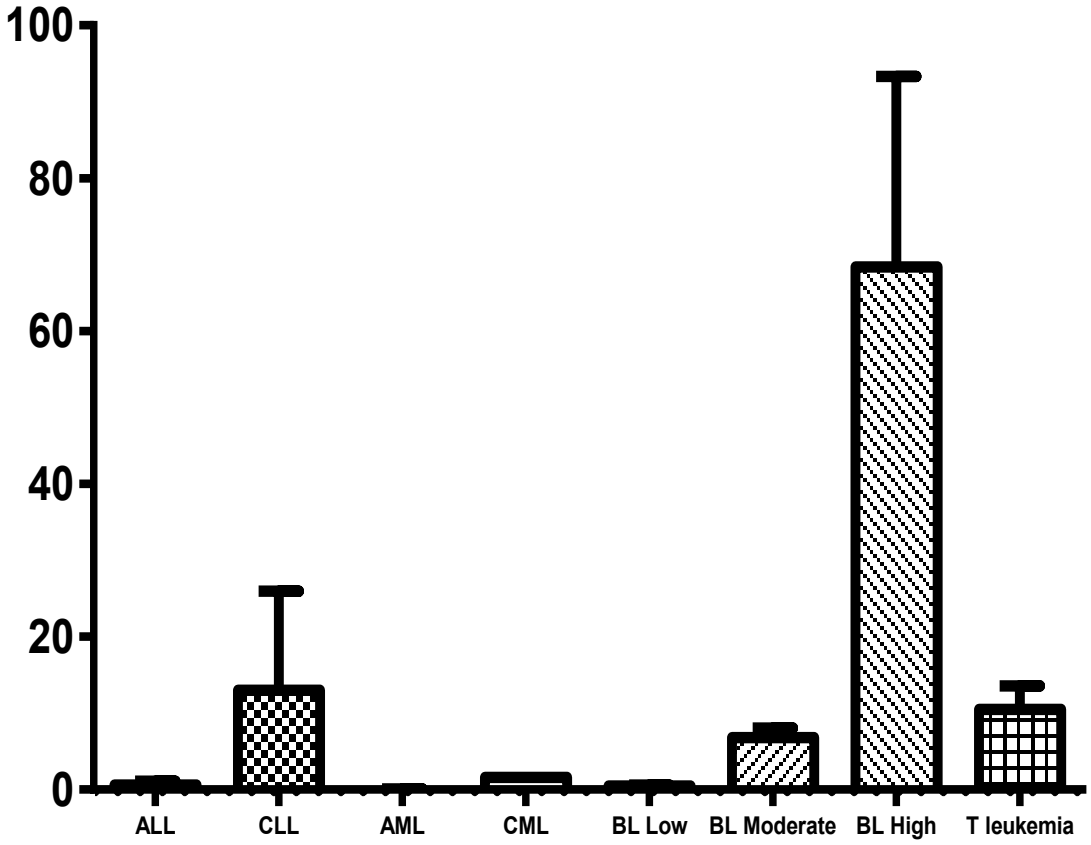


Figure 4.2: MIAT expression in different type of Leukemia cell lines. Data are represented as mean values +/- SM from each group.

4.2. CLL Patients Differentially Express MIAT Transcript

80 CLL patient's RNA were subjected for survey of MIAT expression. The study was approved by the Ethics Committee of the Ohio State University of Medical Center (OSUMC), and written informed consent was obtained from all patients and subjects. The expression level of MIIAT was evaluated by Real Time PCR. The experiment was done in triplicates for each samples. The percentage of $2^{-\Delta ct}$ of MIAT target gene and TBP as normalizer gene was used to compare the differential expression of MIAT in representative CLL patients (Figure 4.3A). CLL patient's samples were classified according to four main cytogenetic changes to 11q del, 12 trisomy, 13q del and 17p del. As depicted in Figure 4.3B. Evaluation of mean of MIAT expression in each group revealed that mean of MIAT expression in group of 17p del is significantly higher than Del(13q) by 61% (p value 0.001). Predicting features of CLL patients with 17p del showed more aggressiveness, poor prognosis and outcome and these patients tend to have higher-risk disease. As well CLL patient with cytogenetic abnormality of 17p del usually do not respond to standard initial therapy whereas CLL patients with 13q del with no other chromosomal abnormalities are associated with a relatively more favorable outcome(1). MIAT expression in 11q del and 12 trisomy, were relatively high comparing to 13q del by 37% (p value: 0.014) and 57% (p value: 0.002) respectively. The proportion of CLL patients with del 11q tend to be younger with large lymph nodes and have high-risk disease. Regarding to these information, Suggested that there should be a relation between MIAT expression in CLL patients and prognosis features of the disease.

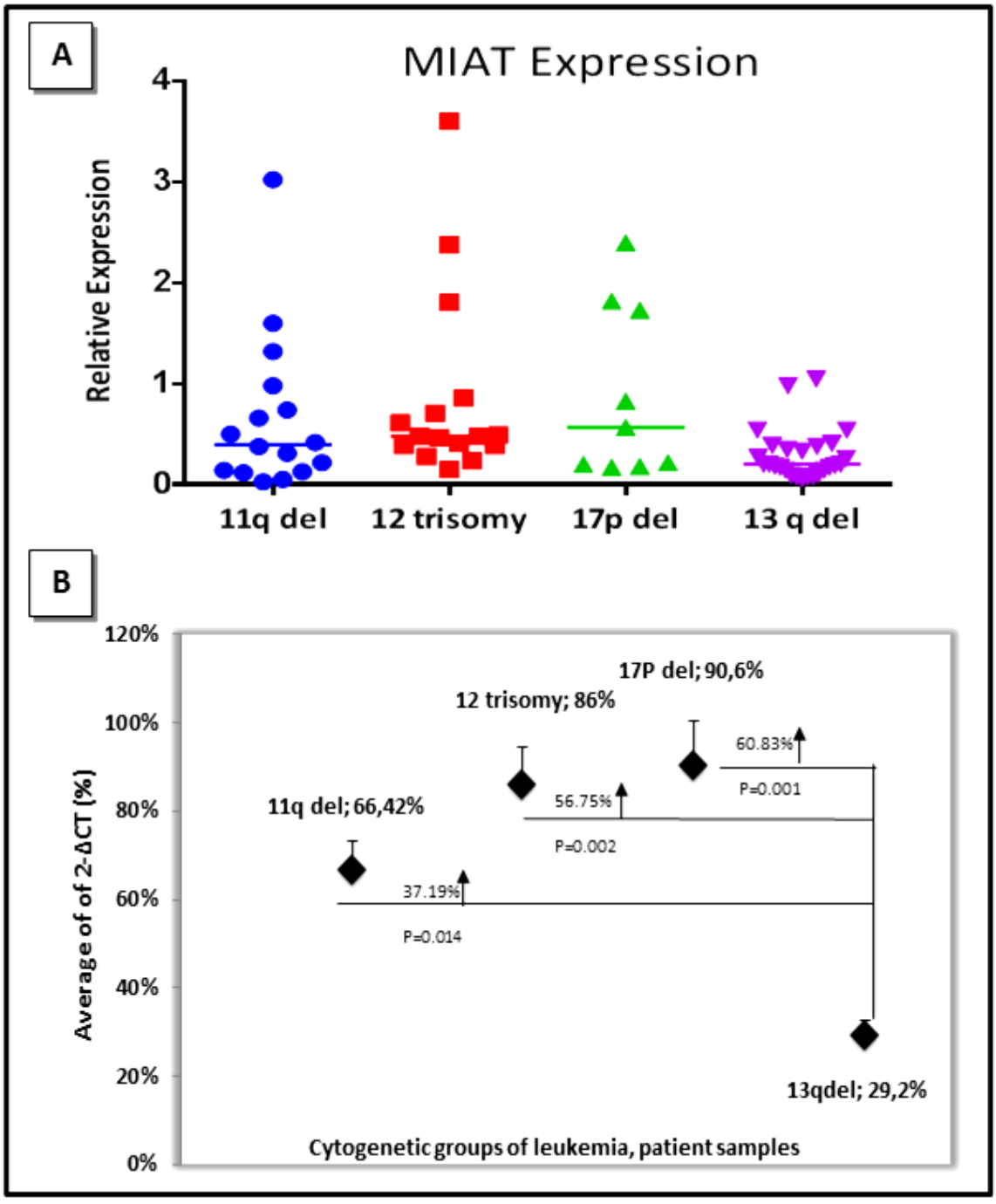


Figure 4.3. MIAT expression in CLL patient. A: relative expression of MIAT in four of cytogentic different group of CLL patients.**B:** mean of lncRNA MIAT expression in each cytogenetic groups of CLL patients. Results were shown as mean+/- SD . Expression of MIAT were significantly higher in 11q del (p value 0.014), 12 trisomy (p value 0.002) and 17pdel (p value: 0.001) respect to 13q del**.**

4.3. Transcription Factor Oct4 Modulates MIAT Expression

For an in-depth understanding the molecular pathway of MIAT function the expression of transcription factor Oct4 was studied which is suggested as regulator of long non coding RNA of Gomafu in mouse Esc(2). Providing cDNAs which synthesized in step 3.1 and 3.2, expression level of Oct4 were analyzed in CLL patients as well as Leukemic cell lines by using specific probe for human Oct4 (TaqMan® Gene Expression Assays POU5F1, *Life Technologies*, Cat no:4331182) in a real time PCR reaction. TBP (*Life Technologies*, Cat# 433376) was used as reference gene. The mean expression of MIAT and Oct4 in each four cytogenetic types of CLL was compared. Interestingly we found that the expression of Oct4 was highest in 17p del group and gradually decreased in 12 trisomy and 11q del toward 13q del (Figure 4.4 A). This results was in accordance with expression of MIAT in different cytogenetic groups of CLL samples that were analyzed in this study, suggesting a possible transcriptional regulation of MIAT by Oct4. Analyzing the Oct4 expression in leukemic cell lines revealed the same results, as in CLL and B lymphoma were detected the highest expression of Oct4 as well as MIAT(Figure 4.4 B). Next, western blot analysis was used to compare protein levels of Oct4 in leukemic cell lines. leukemic cell lines CI1,MEC1 and DB with high expression of MIAT and leukemic cell lines NU-DHL-1, BJAB and BL-41 with low expression of MIAT were cultured and proceed to protein extraction as described in material and method (Figure 4.5.). We observed higher expression of Oct4 protein level in CI1 and DB cells that also had been shown higher expression of MIAT transcribed during Real time PCR (Step 3.1). The lowest Oct4 protein was observed in BL-41 and NU-DHL-1 with very low expression of MIAT expression. While BJAB with low expression of MIAT transcript still showed moderate Oct4 expression in protein level. These results validated that Oct4 and MIAT transcript could participate in a feedback regulation.

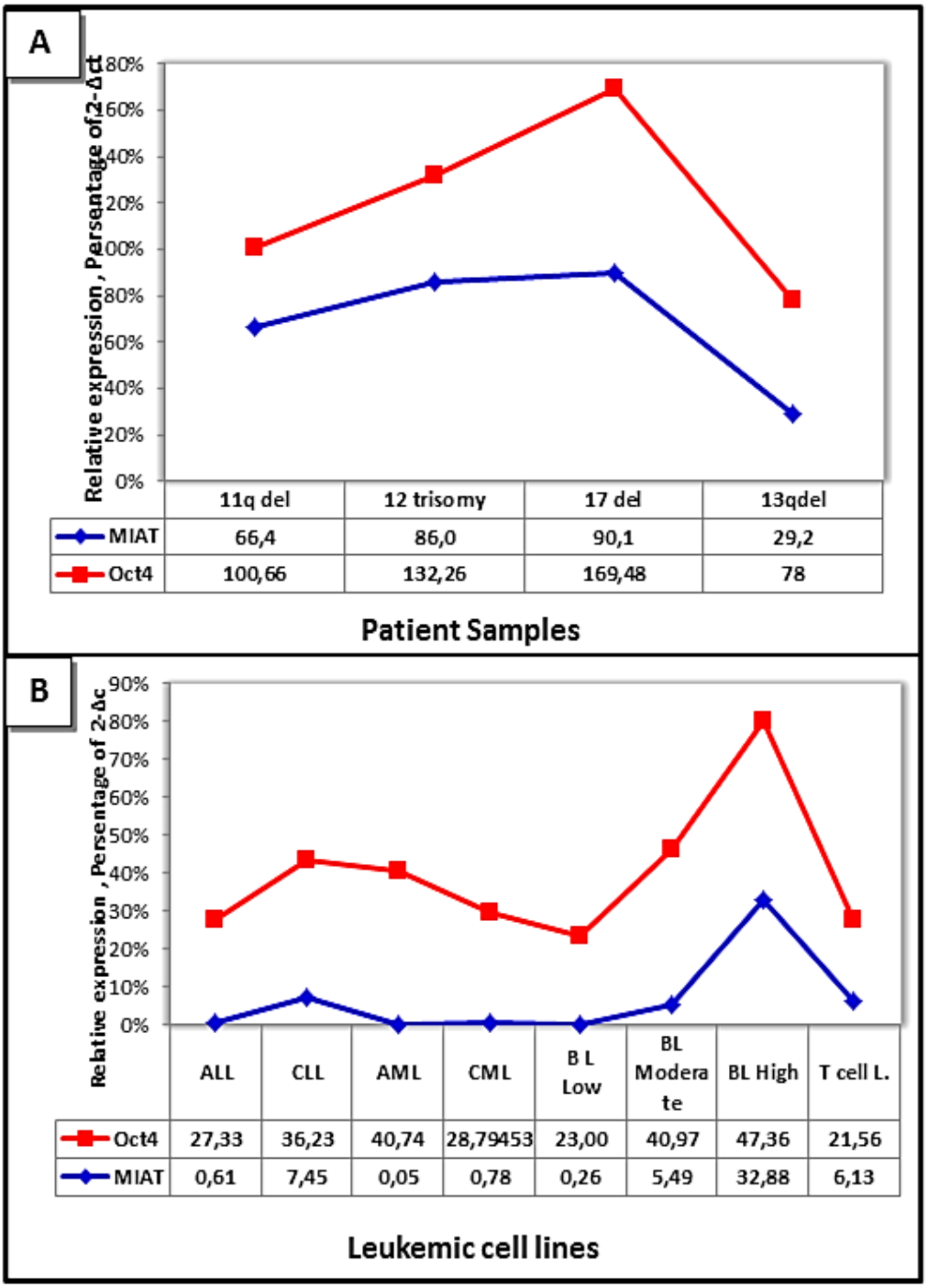


Figure 4.4: Quantities real time PCR for Oct4 transcript and MIAT. Result were established through as mean values +/- SD from three replicate (p value<0.05). **A: Oct4 transcript expression in CLL patients**. Oct4 as well as MIAT express highly in 17pdel CLL samples. The least expression of Oct4 was achieved in 13q del CLL patients that was in accordance with MIAT expression. **B: Oct4 expression in Leukemic cell lines.** The mean of Oct4 expression in each category of leukemic cell lines represent the same pattern of lncRNA MIAT expression.

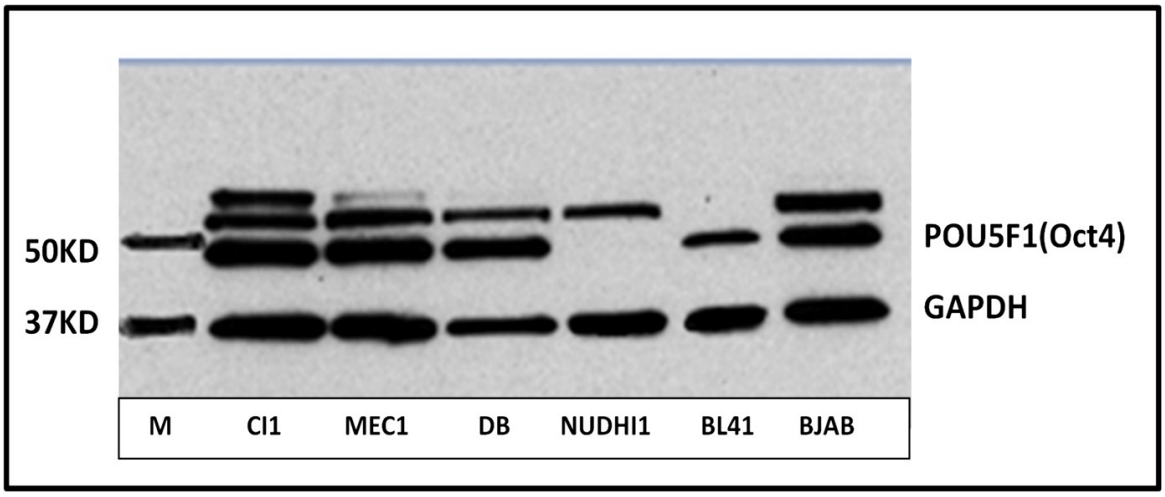


Figure 4.5. Western Blot analysis of POU5F1(Oct4) protein in leukemic cells. CI1 , MECI and DB which express lncRNA MIAT at highest level showed higher expression of Oct4 at protein level. NUDHI1 did not show any expression of Oct4 protein. Oct4 expression in BL41 was low. BJAB cell line showed medium level of Oct4 protein. The latest three cell lines distinguished as low expressing MIAT leukemic cell lines. GAPDH was employed as reference normalize

4.4. Knockdown of lncRNA MIAT in leukemic cell lines

4.4.1. Figuring out the most Effective Leukemic Cell Line for Chemical Transfection of Synthetic Oligos like siRNAs

As the transfection in suspend cell lines like leukemic cell lines is not feasible as adherent cell lines, Leukemic cell lines that showed high expression of MIAT were selected as DB, CI1 and MEC1 to be checked for transfection efficacy with different chemical reagents for transfection. Transfection of BLOCK-iT™ Fluorescent Oligo(invitrogen) using lipofectamine 2000(invitrogen) and Dharmafect (GE) and reverse transfection using Neo FX Rt reagent(invitrogen) were compared in DB,CI1,MEC1. $4.5*10^4$ DB, CI1 and MEC1 cells were seeded into 96 well plates on the day of transfection for transient transfection. HEK-293 cell line which known as easily transfect among adherent cell lines, transfected with BLOCK-iT™ Fluorescent Oligo in parallel, to compare the efficiency of different chemical transfection reagents and stability of BLOCK-iT oligo during times (Data not shown). For HEK-293, $0,8*10^4$ cells were seeded into 96 well plate on the day before transfection for transient transfection, however for reverse transfection using Neo FX Rt reagent (invitrogen), $2.5*10^4$ cells were used on the day of transfection. The cells were monitored during 5 days using florescent microscope. After 24,48,72,96 and 120 hours post-transfection, transfection efficiency was determined under florescent microscope. Among leukemic cell lines, DB and CI1 were detected as the best transfectable cell lines in comparison with MEC1 (Figure 4.6.). On the base of transfection efficacy, DB and CI1 cell lines were selected for the next steps. Also we checked three different concentrations of BLOCK-iT™ Fluorescent Oligo(invitrogen) (10nM,50nM, and100nM), and different volume (0.2µl,0.5µl and 1µl) of lipofection reagents of Lipofectamine 2000(invitrogen) and Dharmafect (GE) in DB and CI1 cell lines. Our goal was set up the best effective transfection situation with the least cytotoxicity. According to Figure 4.7 the results showed the best transfection efficacy with 100nM concentration of BLOCK-iT™ Fluorescent Oligo trasfected using 1µl of Dharmafect (GE) reagent.

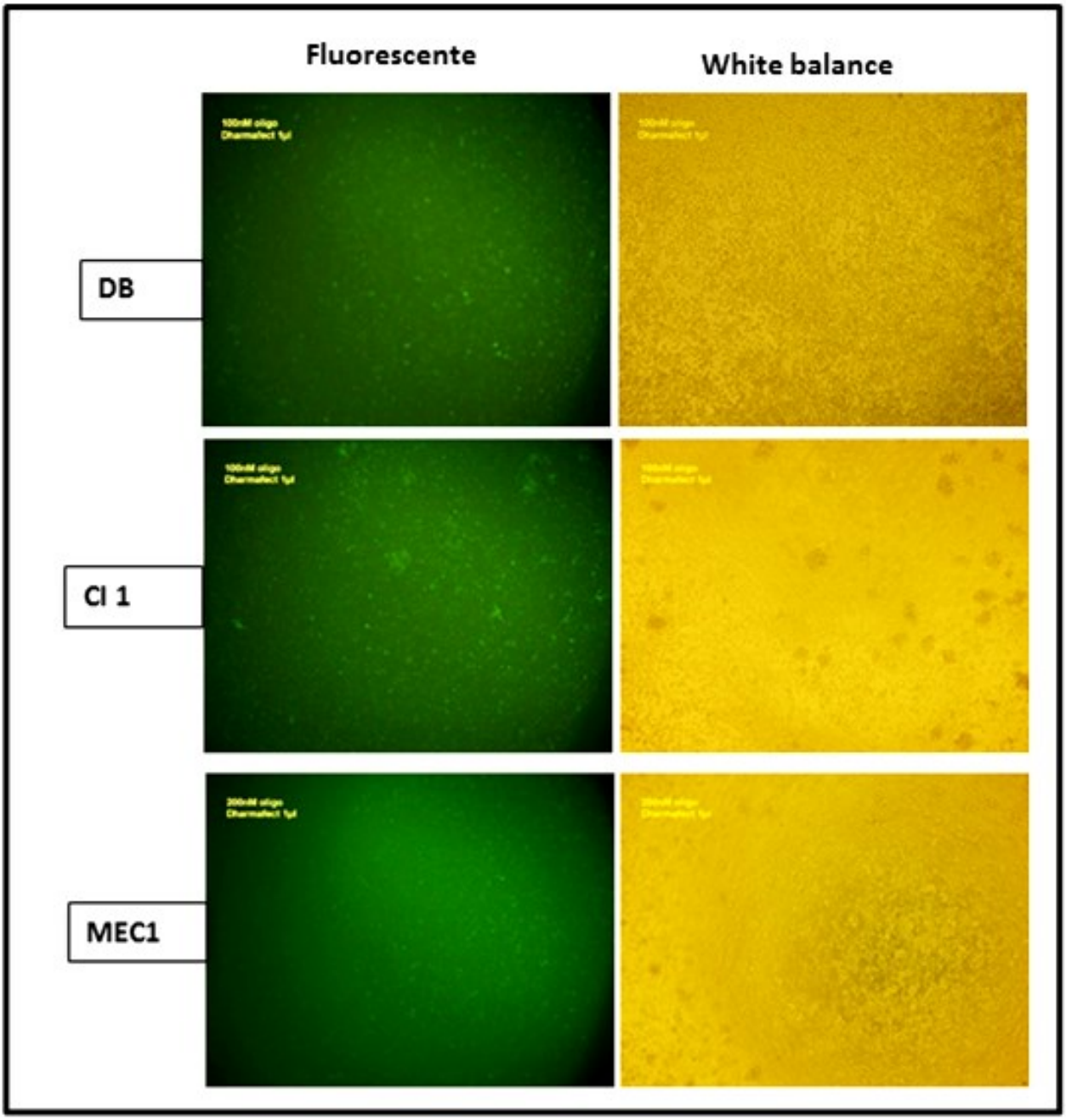


Figure 4.6. Transfection effiency of leukemic cell lines. DB, CI1 and MEC1transfected with BLOCK-iT florescent oligos to compare the efficiency of transfection. Features of transfected cells after 48 hours visualized under light microscope(10X) . DB and CI1 were detected as most efficient during lipofection

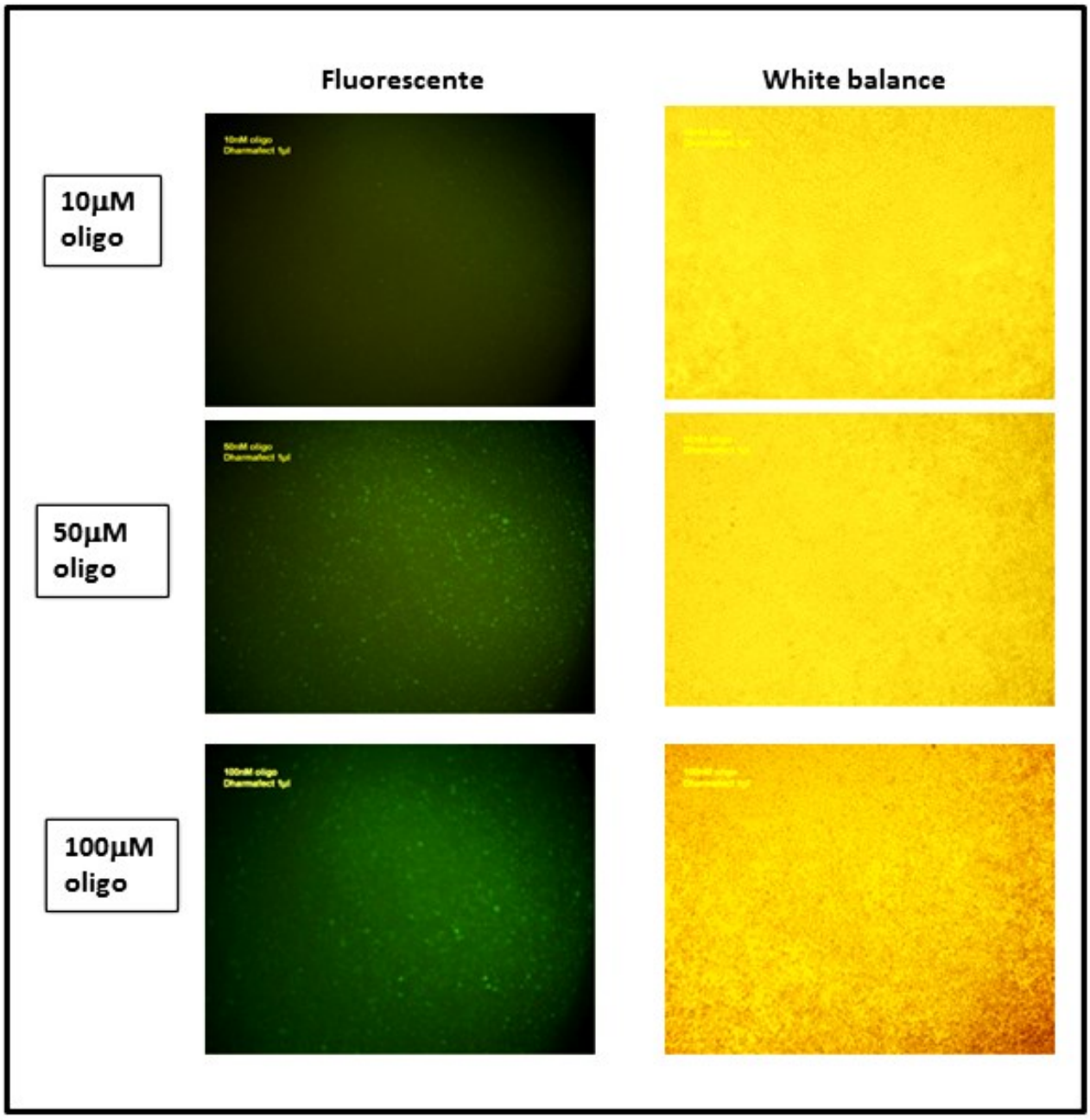


Figure 4.7. Set up analysis for lipofection of synthetic oligos. 10µM ,50µM and 100µM Block-iT oligos were transfected with different concentration as well as reagents available for lipofection. Efficiency of transfection was revealed 48h after transfection under florescent microscope (10x). 100µM oligo trasfected using 1µl of Dharmafect reagent showed to be the most efficient .

4.4.2. MIAT Knockdown in CI1 and DB Cell Lines

100nM of Human MIAT siRNA was transfected in DB and CI1 cell lines ($5*10^5$ DB and CI1 cells in 24 well plates) using Dharmafect (GE) Transfection reagent. We used a pool of 4 siRNAs reaction to achieve the most effective knockdown of MIAT. In this experiment Negative controls was established for verification of experiment. Negative control cells were transfected by ON-TARGETplus Non-targeting siRNA in the same concentration and condition of target siRNA. Cell lines harvested in different time points 24h,48h,72 and 96 hours and subjected for analysis of MIAT expression level through Real Time PCR. The experiment was done in biological triplicates. Technical replicate for Real Time PCR reaction was also considered. The results are shown in Figure 4.8 for DB and CI1 cell lines. According to the real time PCR results single transfection of 100nM siRNA against MIAT was able to inhibit the expression of MIAT by 26% (p value: 0.01) in DB cells and 14% (p value: 0.04) in CI1 cells, for 24 hours. However at longer time their effect diminished, suggesting degradation of synthetic siRNA by RISC complex. To reach the most efficient knockdown of MIAT, we increased the amount of siMIAT and transfected it in DB for 24 hours. ON-TARGETplus Non-targeting siRNA as negative controls were considered for verification of experiment. This result showed that the best rate of knocking down of MIAT was seen in 150nM of siRNA against MIAT respect to negative control. The value of down regulation was achieved by 32.7% (p value=0.01) in time point 24 hours (Figure 4.9).

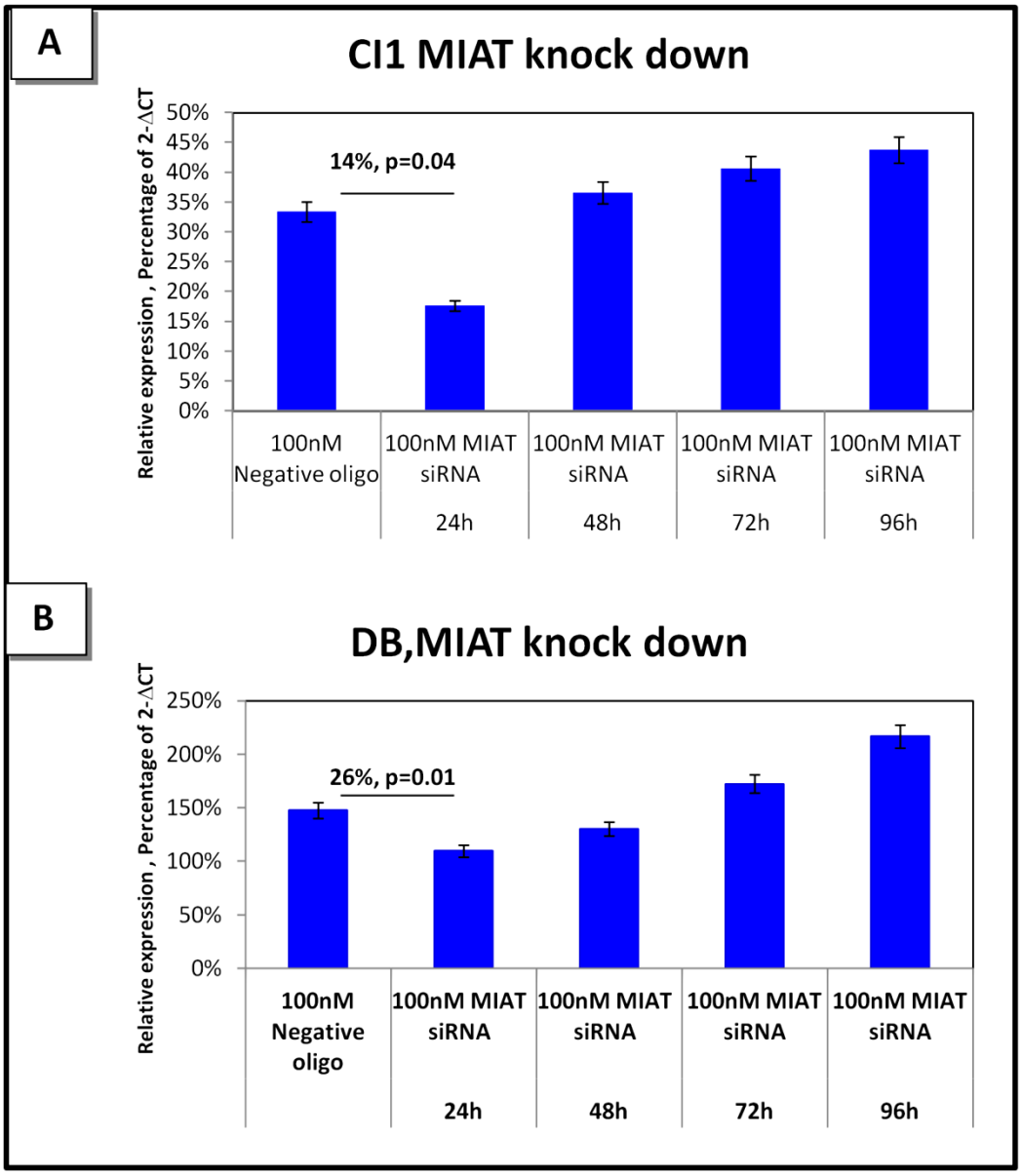


Figure 4.8.Directected know down of MIAT in DB and CI1 cells. DB and CI1 cell lines were transfected with 100nM MIAT siRNA and negative oligo as control. Real Time PCR of transfected cells for 24,48,72 and 96 hours showed that MIAT down regulation was achieved for 24h post transfection. The value of down regulation was higher in DB by26% respect to 14% in CI1(P value<0.05).

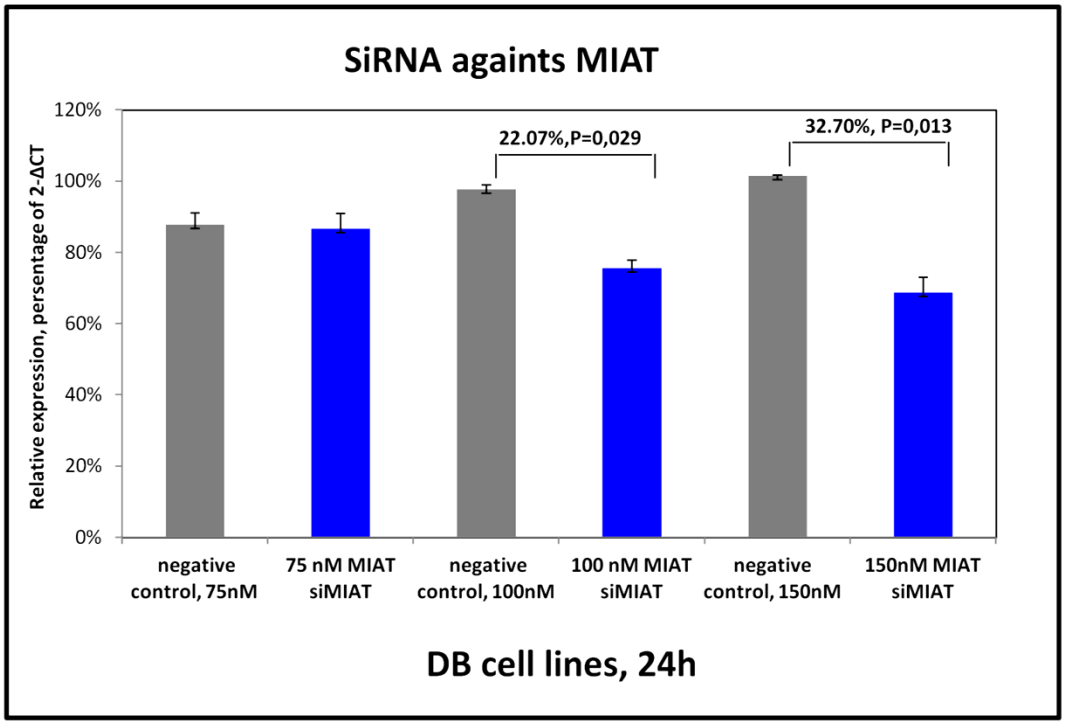


Figure 4.9. Different concentration of siRNA for know down of MIAT in DB cells. DB cell line was transfected with 75nM, 100nM and 150nM of MIAT siRNA and negative oligo as control. Real Time PCR of transfected cells after 24 hours post transfection revealed using 150nM of siRNA, we can down regulate MIAT expression by 32.70% (P value=0.013).

4.4.3. Directed Knockdown of the lncRNA MIAT Promotes Down Degulation of Oct4

To check whether the lncRNA MIAT might participate in a feedback loop affecting the level of Oct4 transcription factor, we examined if RNAi against MIAT could change Oct4 mRNA levels in leukemic cell lines. siRNA transfection was done in DB leukemic cell lines. $5*10^5$ DB cells were seeded into 24 well plates on the day of transfection. Transfection was done using 100nM of Human MIAT siRNA and transferred to the cell by Dharmafect (GE) Transfection reagent. The same concentration of ON-TARGETplus Non-targeting siRNA #1

were transfected in negative control cells. Cells harvested after 24 hours and level of lncRNA MIAT and mRNA of Oct4 were analyzed through Real Time PCR providing specific probes for MIAT and Oct4 and TBP as normalizer. The results showed that siRNA of MIAT resulted in reduced mRNA level of Oct4 (Figure 4.10.). Interestingly, increasing concentration of RNAi against MIAT, affected the RNA level of Oct4 as well as MIAT down regulation (Figure 4.10.). That means transcription of MIAT and Oct4 are regulated in a potential synergistic feedback mechanism.

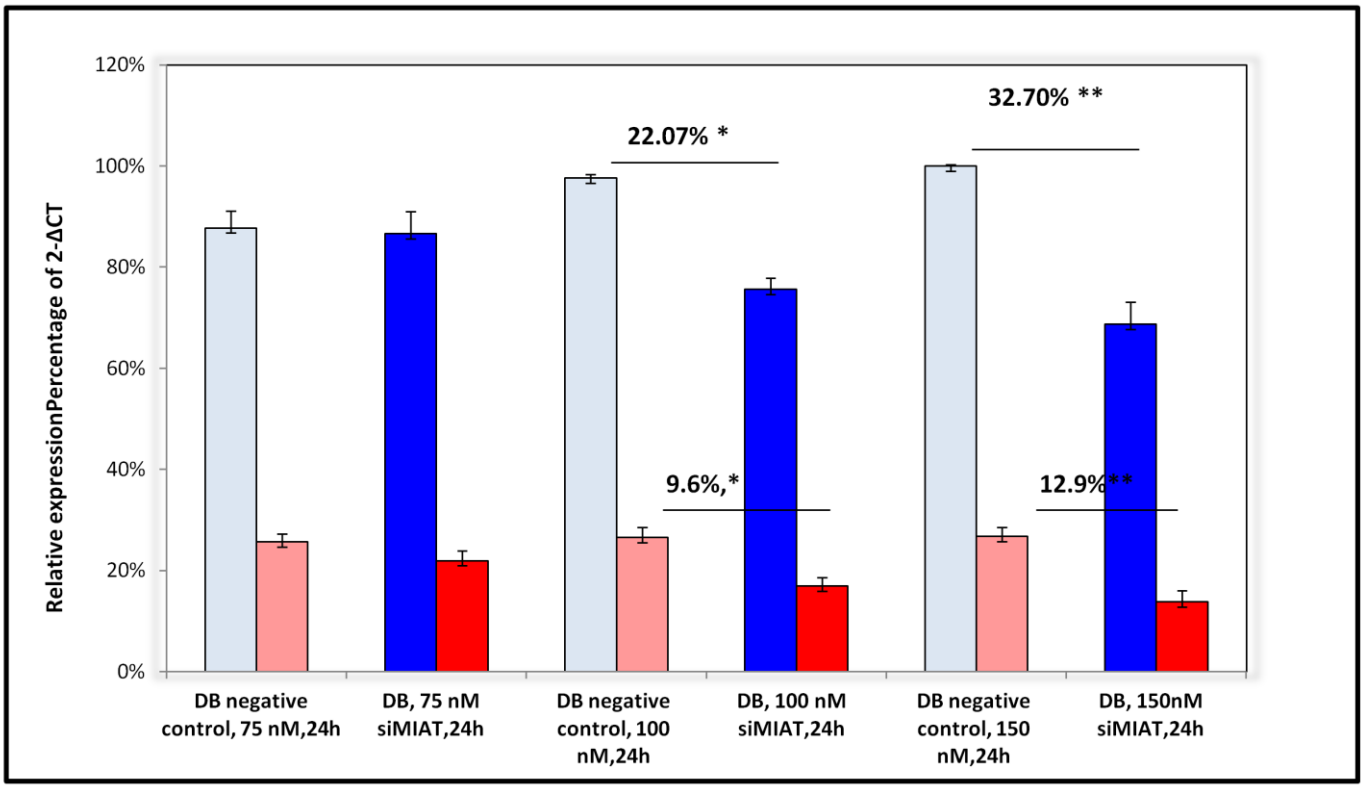


Figure 4.10. RNAi mediated know down of MIAT, down regulate the expression of Oct4. DB cell line was transfected with 75nM, 100nM and 150nM of MIAT siRNA and negative oligo as control. Real Time PCR was done for Oct4 and MIAt as well. 12.9% and 9.6% down regulation of Oct4 expression were observed using 100nM and 150nM of siRNA against MIAT respectively. Knockdown efficiency of MIAT was validated by showing 32.7% and 22.07% reduction of MIAT expression level in samples transfected by 100nM and 150nM of siRNA against MIAT respectively. *P value<0.01, **P value<0.05.

4.4.4. Directed Knockdown of the lncRNA MIAT Decreased cell Proliferation and Induced Apoptosis in Leukemic Cell Lines

To check whether lncRNA MIAT transcript level reduction is correlated with alteration in cell viability and programmed cell death levels, three sequential rounds of transfection of Human MIAT siRNA in DB cell lines was employed. We proposed that continuous transfection ensure the highest and continuous inhibition over the 72-hour time course ,that is needed for cell function studies. 5*105 DB cells were seeded into 24 well plates on the day of transfection and 150nM of Human MIAT siRNA was transfected by Dharmafect (GE) Transfection reagent. In this experiment, Negative scrambled oligo was used as control (ON-TARGETplus Non-targeting siRNA # Dharmacon). Three sequential rounds of transfection of Human MIAT siRNA (GE company) were done at 0, 24h and 48h. To be sure that repeating transfection by lipofection reagent has no considerable toxic effect, the viability of cells were analyzed by MTS Assay during the time points (Data not shown). Before each rounds of transfection, cells washed gently with complete media and seeded in wells again. After 72 hours cells harvested and proceed to RNA extraction step by NORGEN RNA Clean Up and Concentration Kit. Real Time PCR reaction was done in significant statistical replicates for each samples. The results showed that triple transfection of siMIAT, significantly depleted endogenous level of MIAT by 51% relative to nonsilencing control siRNA-transfected (p value=0.001), confirming that these lncRNAs are susceptible to Dicer-mediated suppression (Figure 4.11A). Furthermore, we showed the co-downregulation of MIAT and Oct4 RNA level is still detected (Figure 4.11A), which powering the hypothesis of existence of a potential synergistic feedback mechanism between lncRNA MIAT and transcription factor, Oct4.

Also we showed that lncRNA MIAT RNAi induced apoptosis by 40%(P value0.007) corresponding to negative control treated cells(Figure 4.11BI) after 96 hours from starting point of experiment. Consistently the number of viable cells decreased by 10% (P value 0.05) (Figure 4.11 BI). The lesser effects was observed in 72 time point (Figure 4.11 BII).

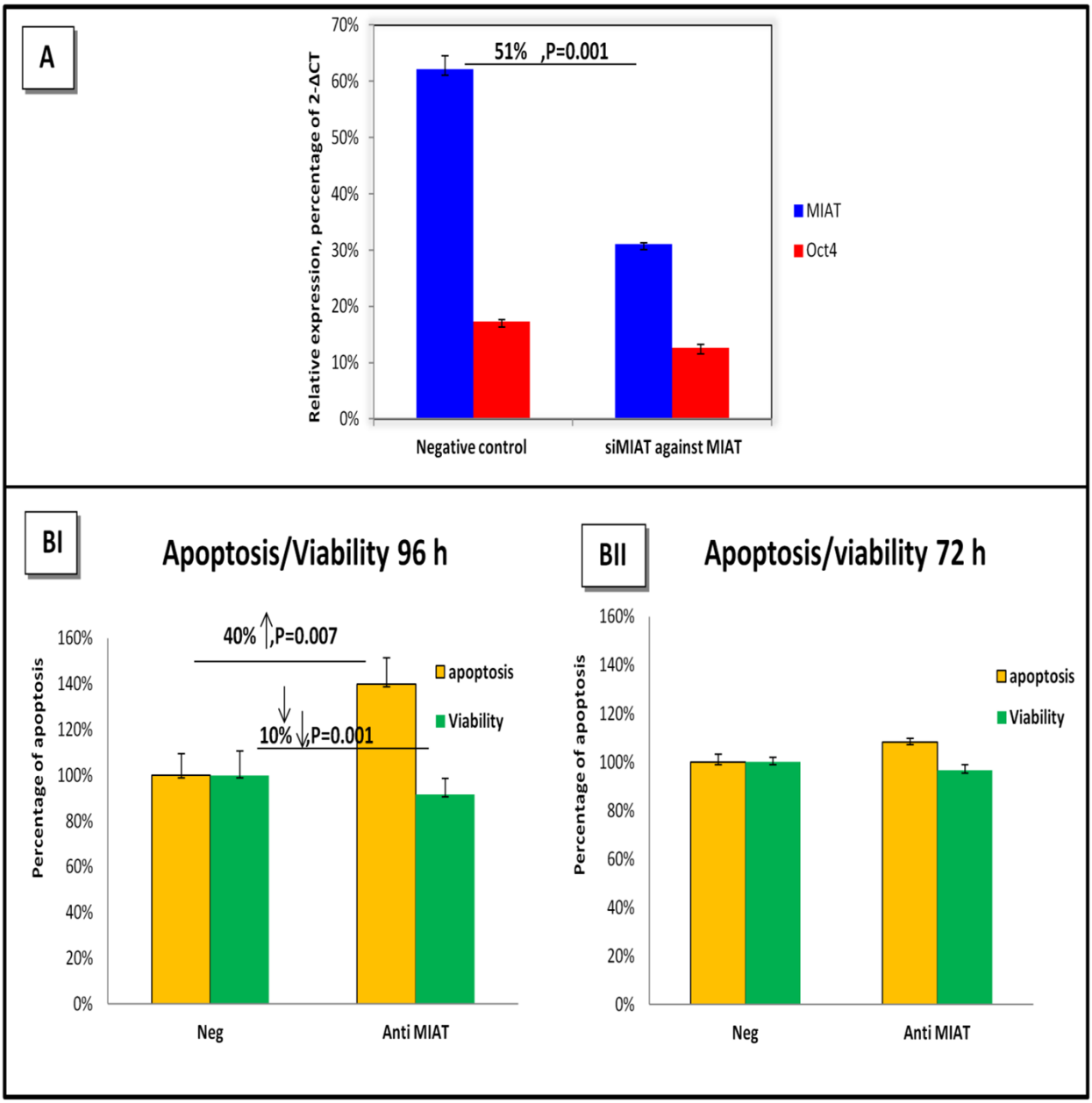


Figure 4.11. Cell based assay mediated knockdown of MIAT by siRNA. A: Down regulation of MIAT as well as Oct4 were detected following triple transfection of DB cells with siRNA against MIAT. **B: Apoptosis and cell proliferation assay following directed knockdown of MIAT by siRNA. BI .** After 96 hours from the start point of experiment, 40% (P value 0.007) induction in apoptosis and 10%(P value 0.05) decrease in viability were observed. **BII.** Apoptosis and viability of cells transfected with siRNA through three days did not show any significant chang related to the control. Control was considered as not transfected DB cells

4.5. Oct4 Knockdown by shRNA Mediated Lentiviral delivery: in vitro Study

4.5.1. Development of Recombinant Lentivirus for shRNA against Oct4

ShRNA against Oct4 were delivered as 4 individual constructs of : V3LHS-311240, V3LHS-311241, V3LHS-311243 and V2LHS-222507. A set of four expression constructs were used to guarantee that at least one of the four, offered an efficient knockdown for Oct4 with 50% or more. Lentivius for shRNA clone collection against Oct4 as well as control viral particles were produced in HEK-293T Cells, providing Thermo Scientific Trans-Lentiviral shRNA Packaging Kit including helper plasmids which co-transfected with expression constructs using Calcium Phosphate Transfection Reagent (see details in material and methods). Transfection efficacy of four individual constructs were monitored 48h post-transfection for GFP reporter under fluorescent microscopy (Figure 4.12.). Transient transduction of four recombinant lentiviral particles were done in HEK293 cells to determine the most efficient viral particle for transduction of target cells. For each individual viral particle, transduction efficiency of $1*10^5$,$2*10^5$ and $4*10^5$ IU were compared. The efficacy of transduction monitored under fluorescent microscopy, 72 hours post transduction. The results showed that the most efficient transduction of recombinant lentiviral particle achieved using $4*10^5$ IU compare to IU $2*10^5$ and $1*10^5$ (Figure 4.13.A). Infected cells with recombinant lentiviral particle at IU $4*10^5$ were trypsinized and harvested 72 hours post infection and proceed to protein extraction. Western blot analysis of Oct4 was done by using Oct4 antibody (Anti- OCT4 antibody adcam cat# 19857) (concentration: 1µl/ ml), dilution 1:1000). GAPDH (mouse) (concentration dilution 1:15000) was used for normalization (Figure 4.13.B). The results showed that lentivirus V3LHS-311241 knockdown Oct4 effectively compared to other viral particles.

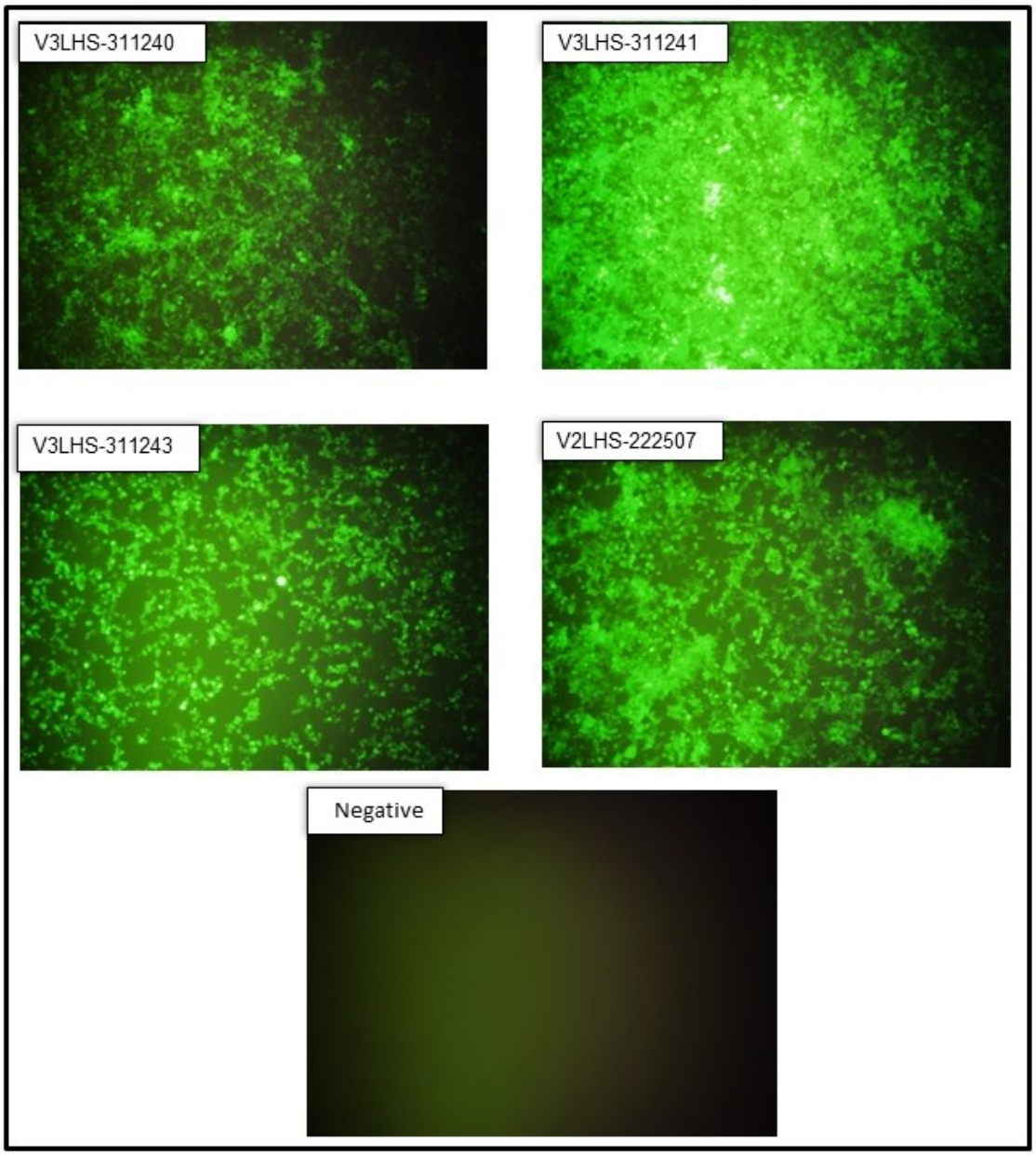


Figure 4.12. Transfection efficiency of four Oct4 shRNA expression constructs. Untransfected sample considered as a negative control for GFP expression.

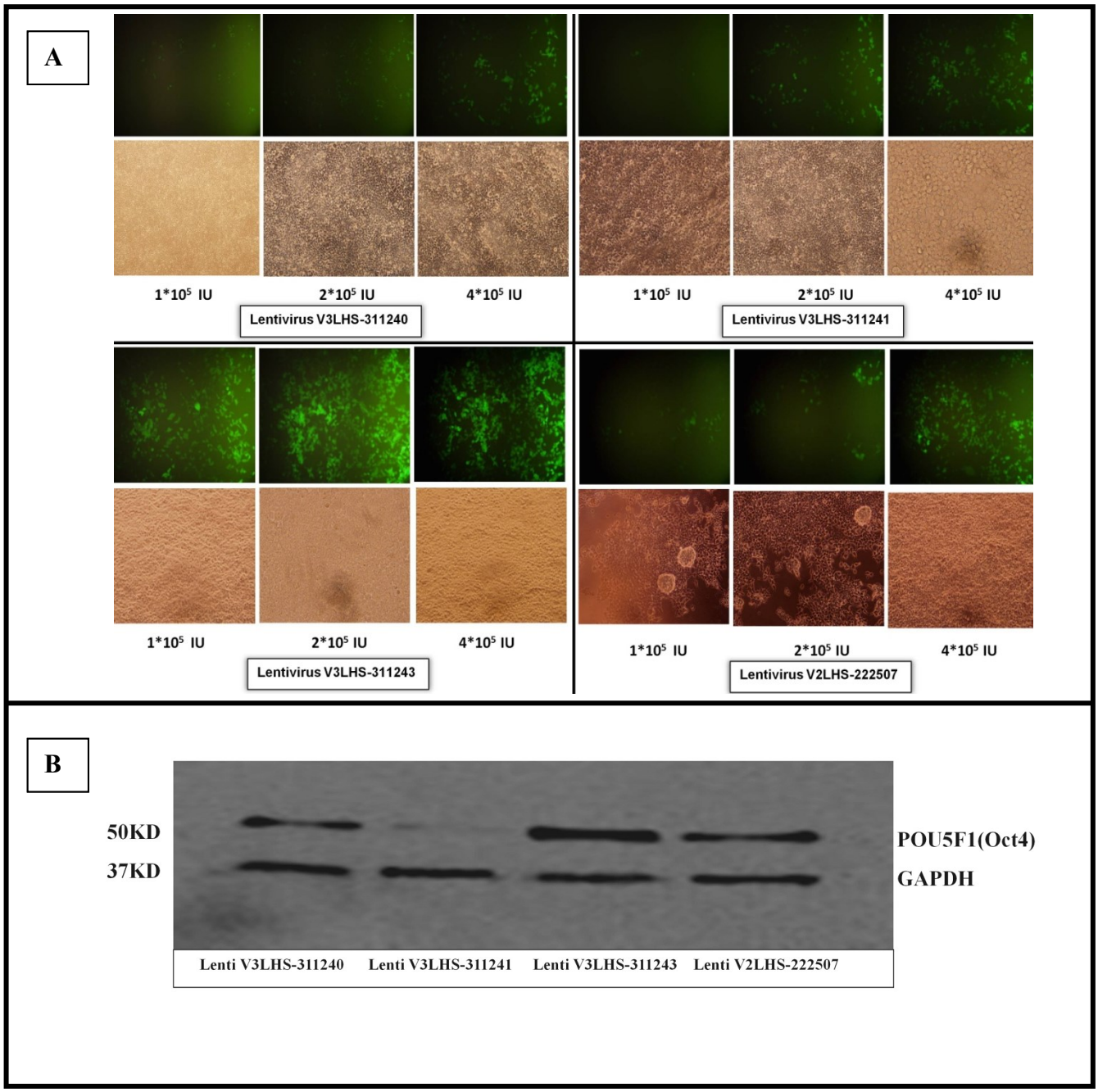


Figure 4.13. A.Transduction efficiency of four Oct4 shRNA lentivirus. DB cells were infected with recombinant Lentivirus at $1*10^5$,$2*10^5$ and $4*10^5$ IU. Infection efficiency and concentration of each particle were compared by florescent features of GFP reporter expression. **B. Western blot analysis for Oct4**. Functionality of four recombinant particles for Oct4 knockdown was determined at protein level, 72h after infection. Mouse GAPDH was used for normalization. The most efficient viral paricle was determined as LentiV3LHS-311241.

4.5.2. Lentiviral Delivery of shRNA Suppress the Expression of Oct4 in Leukemic cell line

The ability of shRNA mediated lentiviral delivery to knock-down endogenous Oct4 was assessed in DB cells. toward this end, Virus no V3LHS_311241 was selected for transduction because of its higher efficacy in transduction of HEK293 cells (see section 3.5.1.). pGIPZ non-silencing shRNA lentiviral control virus RHS 4346 was used as Negative control in our experiment. 100000 cells were mixed with 0.5ml RPMI and 0.5 µl of Polybrene in one tube for each well of 24 well plate. Recombinant virus was added to the mentioned mixture with different IU $1*10^5$, $2*10^5$, $4*10^5$ and $10*10^5$. Centrifuged in 2000g, Room Temperature for 180 min followed by Incubation over night at 37. The day after, Centrifuged at 1000 rpm 10 min RT. Aspirated supernatant and resuspended pellet in 0.5 ml RPMI with 10 % FBS and 1% antibiotics and seeded in 24 well plate. Cytotoxicity of recombinant virus was assessed by using MTS viability method which did not show any considerable cytotoxicity effect. After 48 hours GFP expression studied in florescent microscope and DB cell line with more than $4*10^5$ IU of virus was selected for proceeding the experiment (data not shown).

$10*10^5$ DB cell lines were infected by $5*10^6$ virus expressing shRNA of Oct4 and control virus as well. Experiment was done in triplicate in 12 well plate . Figure 4.14A shows the efficacy of transduction which is monitored by GFP expression under fluorescent microscopy. Infected cells peletted 72 hours post infection and followed for RNA and protein extractions. The significant reduction of Oct4 mRNA by shRNA resulted in Real Time PCR (-18,64 ± 5,634 difference between means shRNA oct4 v. control, P value= 0.02 relative to control virus infected cells, N = 3 replicates). Lentivirus delivery of shRNA against Oct4 also significantly reduced protein level of Oct4 ($P < 0.001$ relative to control virus infected cells). These data demonstrated that shRNA against Oct4 provides an effective knockdown.

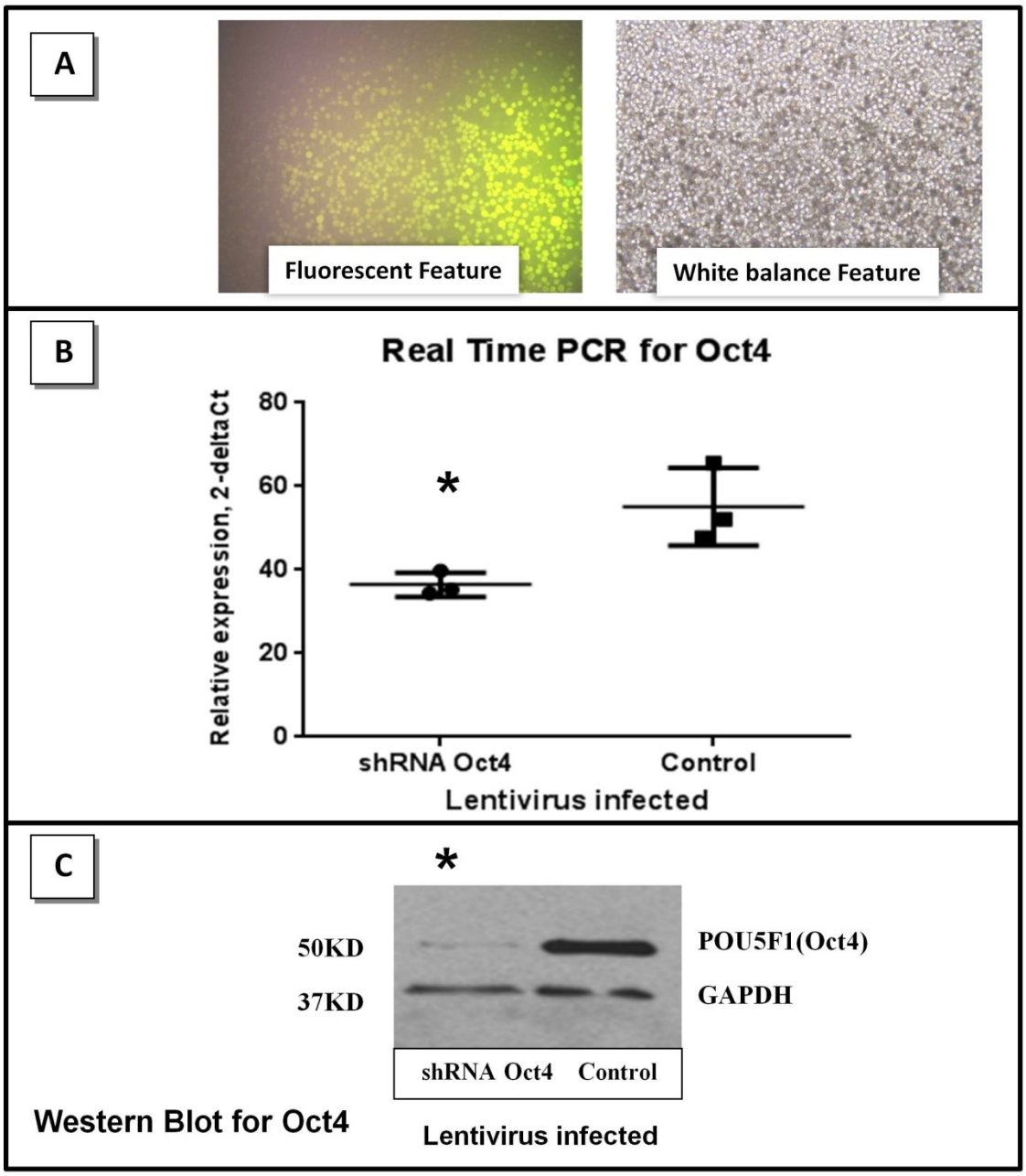


Figure 4.14. shRNA-Oct4 knockdown mediated lentivirus delivery. A. Fluorescent features of $10*10^5$ DB cell lines which infected by $5*10^6$ virus(10X) represent the percentage of infected cells. **B. Real time PCR analysis for Oct4 mRNA.** shRNA directed Oct4 knockdown resulted in around 18,64 ± 5,634 fold reduction of Oct4 mRNA, compared with the control virus infected samples, 3 d post-infection (p value <0.01). **C. Western blot analysis of POU5F1/Oct4 protein**, confirmed an robust downregulation of protein level of Oct4 in comparison with control virus in DB cells. GAPDH protein was employed as reference normalize.

4.5.3. Oct-4 knockdown Modulates MIAT Expression

To further explore whether MIAT modulation following Oct4 knockdown was concordant with Oct4 mRNA silencing using RNAi MIAT (see section 3.4.3.), we examined whether shRNA against Oct4 mediated lentiviral delivery could change lncRNA MIAT transcript level in leukemic cells. Indeed, shRNA against Oct4 (Fig. 4.15) resulted in reduced levels of MIAT as shown by real time PCR($P < 0.01$). In conjunction with RNAi MIAT-mediated suppression of Oct4, the result suggests the potential for an auto feedback loop between lncRNA MIAT and Oct4.

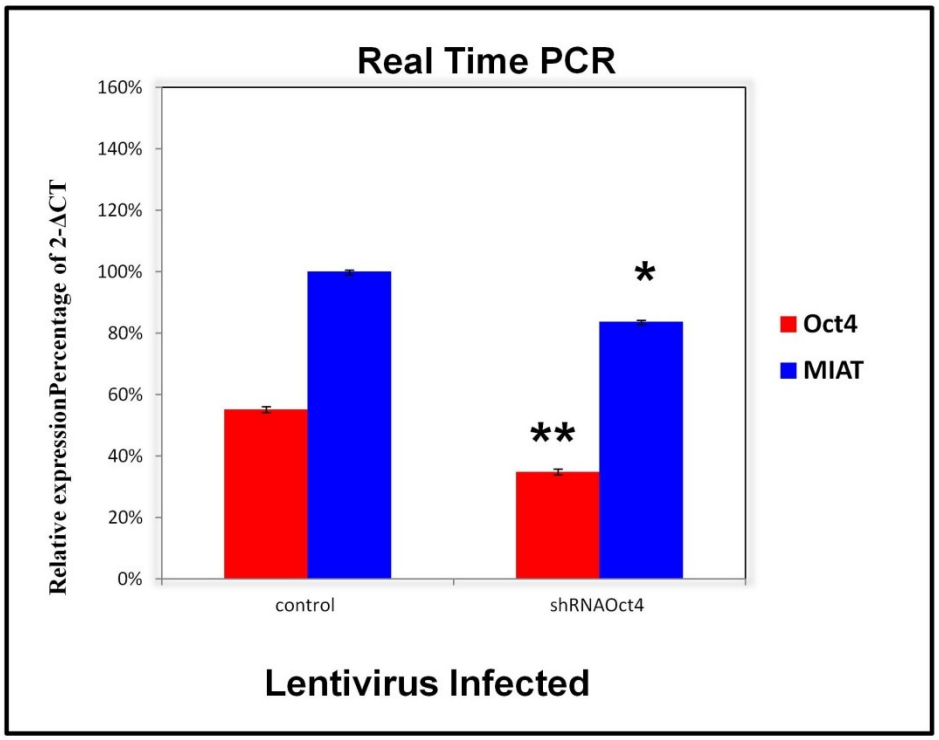


Figure 4.15. Differential expression of lncRNA MIAT upon robust Oct4 shRNA knockdown. shRNA-directed Oct4 knockdown resulted in down-regulation of MIAT compared with the non target control virus infected, 3 d post-infection. (Asterisks) Significant difference from control samples(*, $P < 0.05$; **, $P < 0.01$).

4.5.4. Induced Apoptosis and Depleted Cell Viability Following Oct4 Knockdown

To establish whether Oct-4 knockdown induced changes in biological properties of leukemic cell lines, DB cells were infected with lentivirus express shRNA against Oct4(IU=$10*10^5$). Cell apoptosis and viability were analyzed after 24,48,72 and 96 hours post infection. 20000 infected cells in each time points were used for caspase3/7 apoptosis as well as cell viability. At 24h time point the effect of Oct4 shRNA was not enough to show significant difference in apoptosis and viability, means that knockdown of the target OCT4 transcripts was also time dependent. However after 48h, Oct4 knockdown leads significant specific effects on number of viable cells and cells went to programmed death(Figure 4.16A). Higher effect also observed at 72 h after infection (Figure 4.16A). Real Time PCR was done on harvested cells at 72 h to show that the existence differences in apoptosis and viability are consequences of Oct4 knockdown(Figure4.16B).

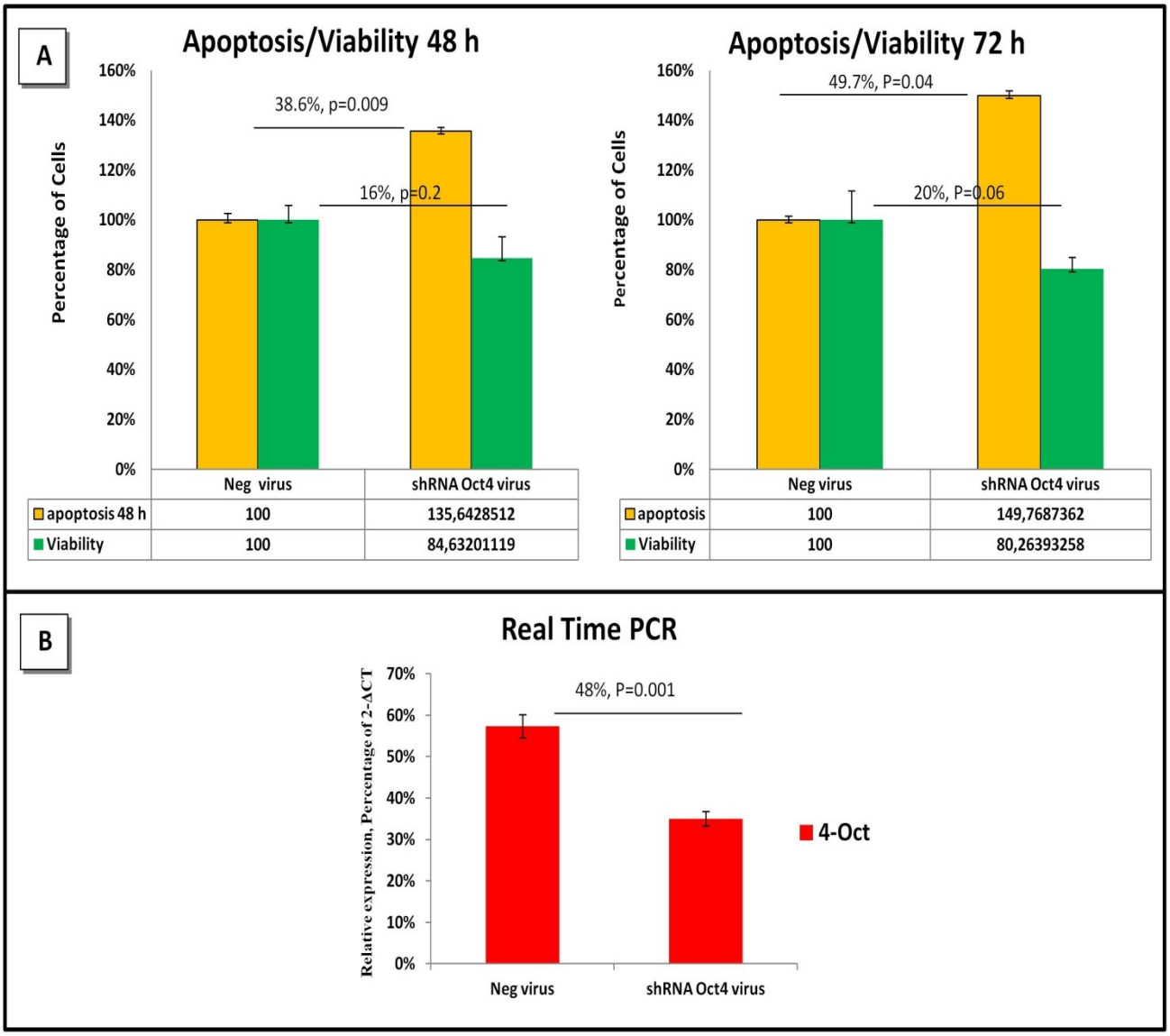


Figure 4.16. Apoptosis induction and proliferation inhibition following Oct4 knockdown. A. Apoptosis and viability of DB cells infected with shRNA against Oct4 mediated lentivirus at IU $10*10^5$ significantly changes regards to control virus infected cells. Greater induction in apoptosis and inhibition of proliferation observed after 72 h post infection. **B.** Increase in apoptosis and Decrease in viability was a consequence of Oct4 as confirmed by real time PCR.

4.6. Development of Vectors for Over-expression of lncRNA MIAT

We next asked whether over-expression of lncRNA MIAT coding sequence could modulate tumorogenesity-associated transcript levels. Moreover, over-expression of MIAT introduce a new possibility for breakthrough in understanding the correlation between MIAT and Oct4 expressions. In this step leukemic cell lines which express MIAT at high level were used as a source of MIAT template.

4.6.1. LncRNA MIAT is Enriched in the Nucleus

There is growing evidence that lncRNAs are recruited to chromatin and epigenetically regulate gene expression(4). Thus, lncRNAs expected preferentially localized in the chromatin and nuclear RNA fractions, in contrast to protein-coding mRNAs that are trafficked to the cytoplasm for translation(5). There are also evidences that show spliced mature Gomafu RNA is localized to the nucleus despite its mRNA-like characteristics(6). We checked the localization of MIAT transcript in leukemic cell lines in which express high level of MIAT as DB, CI1 and MECI. The cultured cells were dissolved in hypotonic solution and homogenized in homogenizer tubes. Centrifuging of homogenized solution separated the cytoplasmic and Nuclear compartments. Both cytoplasmic and nuclear fractions were followed for extraction of RNA using Trizol and Trizol LS (Invitrogen) reagents, respectively(see material and methods for details). 40ng of extracted RNAs were reverse transcripted to cDNA by Super Script VILO DNA synthesis Kit (Invitrogen™ Cat #s 11754-050). Synthesized cDNAs were amplified in Real time PCR procedure using TaqMan specific probes for MIAT (Life Technologies, Cat# Hs00402814_m1) and TBP (Life Technologies**,** Cat# 433376) as reference control. Analysis of the Real time PCR data for nucleus and cytoplasm from three different B cell lines indicates that indeed the MIAT transcript is abundant in the nucleus over the cytoplasm. In Figure 7.17 we displayed the mean of percentage of $2^{-\Delta ct}$ for three technical replicates of each samples. In all cell lines we observed a robust and highly statistically significant enrichment of lncRNAs in the nucleus (p value<0.05). Highest differential ratio was belonged to DB by 50.7% MIAT enrichment in nuclear over cytoplasmic compartment(p value= 1,24E-05).

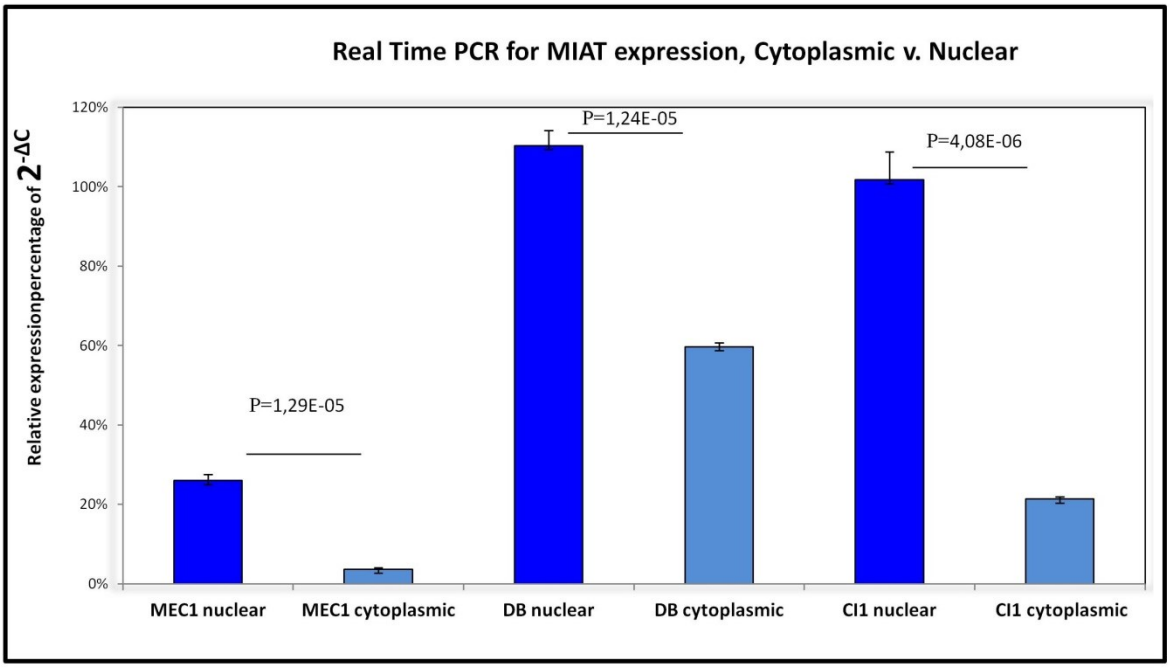


Figure4.17. LncRNAs MIAT are enriched in the cell nucleus. the chromatin/cytoplasm expression ratios of lncRNAs in MECI, DB and CI1 cell lines. Data are represented as percentage of 2-Δct ratios of MIAT and TBP expression.

4.6.2. Cloning of MIAT ORF

4.6.2.1. Isolation of polyA+ RNA from RNA Component of Nucleolus

LncRNAs possess mRNA-like features, as they generally undergo splicing, are transcribed by RNA polymerase II, and are polyadenylated. Most RNA component of nucleolus included ploy(A)- RNAs like ribosomal RNA (rRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA). Thus, we employed polyA Spin™ mRNA Isolation Kit to isolate poly(A)+ RNA, like lncRNA MIAT from total RNA of nuclear component. Poly(A)+ RNA selection kit (New England Biolabs, Cat#S1560S) functions through affinity chromatography using pre-packed oligo (dT)25-cellulose beads. PolyA Spin™ mRNA Isolation Kit was used according to manufacturer's instruction. Because of their poly(A) tails and other mRNA-like features, lncRNAs are represented in typical cDNA cloning. We utilized poly(A) tails of lncRNA MIAT to made cDNA. cDNA Synthesis was done by providing 0.5 µg/µl oligo(dT) 12–18 as well as random Primers in the SuperScript™ First-Strand cDNA synthesizing protocol. PolyA$^+$ RNA content of both

nuclear and cytoplasmic compartment were applied as template for reverse transcription. Then, the products of cDNA synthesis step were subjected for real time PCR using specific probes for MIAT (Life Technologies, Cat# Hs00402814_m1) and TBP (Life Technologies**,** Cat# 433376) as a reference gene. As expected the load of MIAT in cytoplasmic compartment was lower than nucleus. Either Oligo(dT) or Random primers in PCR reaction did not effect on the amount of final product of polymerase reaction.

4.6.2.2. Amplification of MIAT ORF

We designed Forward primers from start point of MIAT transcript (NCBI Reference Sequence: NR_003491.2) and equipped it to *Xho*I restriction site and reverse primers from a few nucleotide upstream of polyA tail and equipped it to *Mlu*I restriction site. cDNA synthesized with oligo(dT) primer in step3.6.2.1. was used as template in a PCR reaction. As the size of our amplicon(MIAT) estimated to be between 9 to 10kb, polymerization was done providing high fidelity, advantage HD polymerase(2.5U/µl) (clontech , cat# 639241). PCR was done in three-Step as 98°C for 10 sec, 55°C for 5 sec and 72°C for 10min(1 min/kb) for 30 cycles. At the end 1µl of Taq polymerase and dATPs was added and incubation was continued by a single extension at 72°C for 20min to add 3´A overhang to amplicons, which will facilitate cloning steps of PCR products in commonly use TOPO®TA Cloning vectors. Amplification of a 10kb PCR product was illustrated on agarose gel 0.8% in TBE 0.5x along with 1kb DNA ladder(Figure 4.18A). The 10kb and heavier bands were cut from the gel and kept in separate tubes (Figure 4.18B). Gel pieces transferred into the dialysis membrane containing 0.5 ml buffer TBE and the two sides of membrane closed thoroughly by clips. The membrane lay down in the electrophoresis tank contains TBE 0.5x as electrical flows go through negative to positive for 45 min 100 V Tank. the Buffer included DNA collected from bag and transferred to a tube.

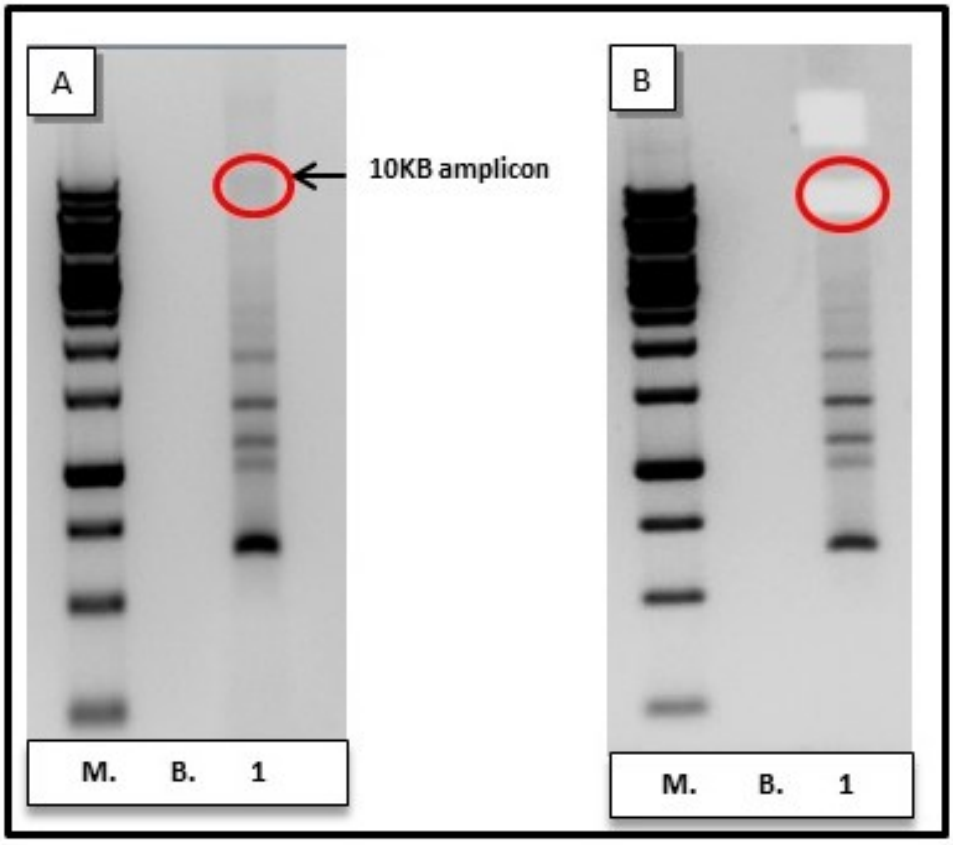


Figure4.18. PCR Amplification of MIAT ORF with around 10kb length, using MIAT forward and reverse primers digned in this study. Ploy A+RNA reverse transcript to cDNA using oligodt primers and subjected for amplification as a template. **A.** Eletophoresis of PCR product revealed a 10kb band. **B.** 10kb amplicon was cut from the gel for further cloning steps.

4.6.2.3. Engineering the clones, contain MIAT ORF

DNA from previous step ,proceeded to Ethanol Precipitation (see material and methods for details). Cloning steps was done into TOPO®TA Cloning vector. The Ligated products were then transformed into DH10β cells and allowed to grow overnight on ampicillin plates to select for cells containing plasmids. Screening of appropriate recombinant clones were done by PCR and restriction digestion analysis. Colony PCR screening was performed with primers flanked full sequence of MIAT transcript. polymerization was done providing high fidelity, advantage HD polymerase(2.5U/µl) (clontech , cat# 639241) in three-Step as 98°C for 10 sec, 55°C for 5 sec and 72°C for 10min(1 min/kb) for 30 cycles. The PCR product visualized on the agarose gel 0.8% after electrophoresis at 80v for 45 min. As Figure 4.19A show, it seemed three over four clone contains our interest fragment. Restriction digestion screening of all four clones was done using *EcoR*I (New England) according to it's manual. Two of four clones confirmed as correct clone after illustrating the digested PCR on gel agarose 0.8% along with 1kb DNA marker(Figure 4.19B).

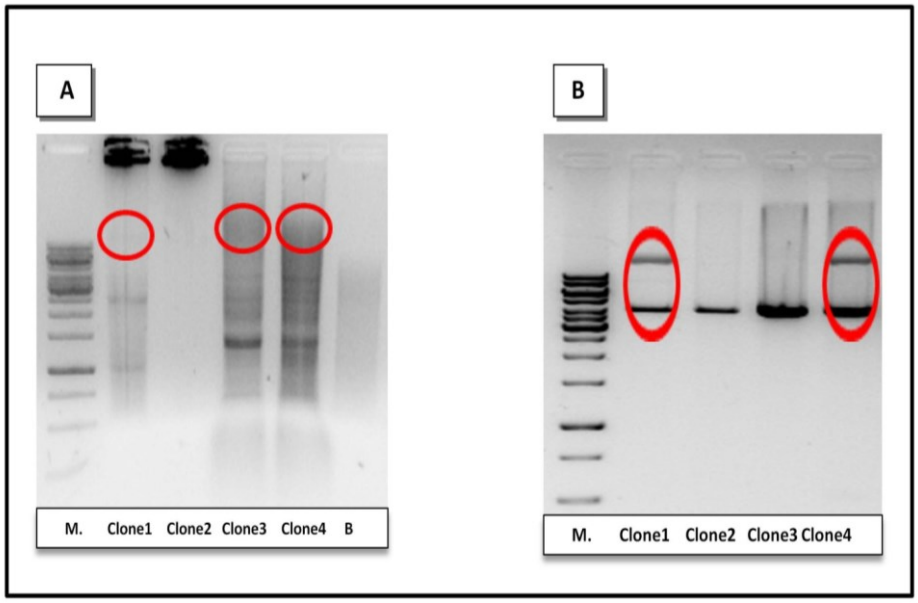


Figure 4.19. Screening of recombinant construct contains MIAT. A. PCR screening, clone 1, 3 and 4 contain our interest fragment with the size of 10kb**. B. Restriction digestion screening. Red circle** show the expected yielding fragments after cut with *EcoR*I. M: 1kb DNA marker.

CHAPTER 5. DISCUSSION

Leukemia is cancer of the body's blood-forming tissues, including the bone marrow and the lymphatic system. The disease develops when blood cells produced in the bone marrow, grow out of control. Approximately every 3 minutes one person in the United States (US) is diagnosed with a blood cancer. 71% of men survive leukemia (all subtypes combined) for at least one year, and this is predicted to fall to 54% surviving for five years or more. Survival for women is slightly lower, with 66% surviving for one year or more and 58% predicted to survive for at least five years. Traditionally biologists have concentrated their efforts on understanding the functions of coding genes. It may therefore be a little surprising that only a tiny fraction of the human genome encodes proteins, yet in contrast recent studies showed that the majority of our genome is transcribed into non-coding RNAs (ncRNAs). NcRNAs include highly abundant and functionally important RNAs, such as ribosomal RNAs (rRNAs), transfer (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs). However, two classes of recently discovered ncRNAs, microRNAs (miRNAs) and long ncRNAs (lncRNAs), appear to play a significant role in the regulation of gene expression programs that occur in higher eukaryotes. LncRNAs, which are abundantly encoded in mammalian genomes, numbering in the tens of thousands, in contrast to microRNA-encoding genes, which are an order of magnitude less numerous. However, functionalizing the rich repertoire of long non-protein-coding transcripts remains a challenge. LncRNAs may also be involved in all levels of gene expression regulation within the cell and, eventually, they have also been implicated in many diseases, including cancers. EGO was one of the first lncRNA to be identified in the hematopoietic system [63]. This lncRNA was identified in eosinophil differentiation of CD34+ HSCs where it stimulated differentiation and mature cell function by transcriptionally regulating eosinophil granule protein expression [62]. However, its mode of action has not yet been described.

Nowadays, Long non-coding RNAs (lncRNAs) are increasingly recognized as important regulators of gene expression, chromatin structure and nuclear architecture. Although many thousand lncRNAs have been identified over the last years, little is known about their biological relevance in normal and cancerous development.

LncRNA MIAT/AK028326 has been previously described as retinal noncoding RNA 2 (RNCR2), strongly expressed in the developing retina(Blackshaw, Harpavat et al. 2004). Subsequent studies have demonstrated that RNCR2/AK028326 is a 3′ terminal fragment of a ∽9-kb lncRNA, *Gomafu*/*Miat*, widely expressed in central nervous system neurons(Sone, Hayashi et al. 2007).

Another study showed that RNCR2/AK028326 is also expressed in mESCs and may be regulated by ES-associated transcription factors. (Sheik Mohamed, J., P. M. Gaughwin, et al. (2010). Their original annotations described AK028326 as the nearest target to the Oct4 ChIP-PET-supported binding site in the region; in addition, during the early stages of their work, EST evidence was lacking to conclusively link AK028326 to the Gomafu/Miat transcriptional unit (Sone et al. 2007). Both AK028326 and the Gomafu/Miat cDNAs were derived from a 5 cap-trapped, dT-primed cDNA library, suggesting that multiple transcriptional initiation and termination events, generating a variety of mature transcripts, including possibly the original AK028326 isoform, may take place at this locus.

Although their results explore the synergy of Oct4 and AK028326 expression, they portray an apparently contradictory increase in mRNA levels of several differentiation markers when AK028326 is over expressed. While this particular finding may be due to over expression far beyond maximum endogenous levels that saturates the regulatory network and leads to unanticipated cell differentiation triggers, their results also do not exclude functional outcomes associated with the longer transcripts from this locus, whose expression did not investigate.

Of potential relevance to their AK028326 work is the finding that MIAT, an lncRNA encoded at the human chromosome 22 locus orthologous to AK028326/Gomafu, is genetically associated with heart disease (Ishii et al. 2006) and, similarly to AK028326/Gomafu, exhibits multiple alternative transcription initiation and termination sites, which are supported by cDNA evidence and are accompanied in EST data by different expression specificities of the different transcripts encoded at the locus. It is therefore remarkable that RNAi and over expression of even a partial fragment of this particular lncRNA are sufficient to promote mESC differentiation under self-renewing conditions, and to promote meso- and ectodermal gene transcription, respectively.

The mechanisms by which AK028326 exert its impact, remain unknown but worthy of future studies.

Our study provides the first evidence that MIAT/AK028326 is also expressed during hematopoetic malignancy, and may be regulated by ES-associated transcription factors (Oct4), expanding our understanding of this particular lncRNA by implicating it in regulatory networks outside of the central nervous system.

In our quantitative analysis of the expression patterns of MIAT transcript in leukemic cell lines, the highest expression were shown to belong to B cell lymphoma. AML and ALL cell lines revealed the lowest level of MIAT expression.

Gomafu is evolutionarily conserved from mammals to birds and amphibians (Rapicavoli et al. 2010; Tsuiji et al. 2011). The previous studies revealed that In contrast to NEAT1 and MALAT1, which are expressed in a wide range of tissues, Gomafu is only expressed in subsets of neurons (Blackshaw et al. 2004; Rapicavoli et al. 2010; Tsuiji et al. 2011). But in this study we showed that MIAT is expressed in lymphoma cell lines as well.

We also associated the aberrant expression of MIAT with clinical significance by analyzing 80 Chronic lymphocytic leukemia (CLL) patient samples from four different cytogenetic abnormalities. CLL is the most prevalent type of leukemia which is characterized by a extremely variable clinical course: while some patients present quick progressive evolutions, others have an indolent course with more than 30 years of survival. 51.7% of the CLL patients present chromosome abnormalities. The most important numerical and structural abnormalities found in CLL include trisomy 12 and deletions in several chromosome regions, such as 13q14, 11q, and 17p13, as well as other less frequently occurring aberrations. The most frequent observed aberration is del 13q14 observed in 34.5% of cases and It is associated to other alterations in 17.2%. 17p13 deletions are found in 17.2% and trisomy 12 in 13.8% (in isolation in 6.9% and associated to del 13q14, in 6.9% of the cases). An 11q22 deletion was found in one case associated to a 13q14 deletion.

Nascimento et al evaluated the relationship between chromosome aberrations and other prognostic factors in CLL. They considered two cytogenetics groups: in isolation and no alteration) and unfavorable outcomes (trisomy 12, 17p13 deletion, 11q22 deletion and two

simultaneous alterations).The unfavorable alterations were more frequently seen among young individuals (<60y).

We identified abundant expression of MIAT in unfavorable outcomes (trisomy 12, 17p13 deletion, 11q22 deletion) compared to favorable (13q deletion) cytogenetic group. By this mean, MIAT expression might be associated to aggressiveness and poor outcome in CLL which also empower a proposed role for lncRNA MIAT in development and regulation of leukemiasis. Expanding this experiments may also issue MIAT to identify patient populations at risk of development of cancer and may classify patients into aggressive or mild cancer groups.

Thus, analysis of the function of lncRNAs is expected to have a tremendous impact on the management of human disease, we also performed functional studies. Recently, the role of lncRNAs in pluripotency was examined in another study by interpreting the genomic context of the ncRNAs relative to nearby protein-coding genes and expression upon embryoid body (EB) differentiation. Out of the 945 ncRNAs expressed during EB differentiation, 174 were differentially expressed, many correlating with pluripotency and cell fate decisions (Dinger et al. 2008b). AK028326 (synonyms: Gomafu, MIAT) was among the 174 differentially expressed lncRNAs, but it was not singled out for in-depth regulatory or functional analysis in that study.

In CLL patient samples and Leukemic cell lines, we showed the strong correlation between MIAT transcript and mRNA of Oct4. The mean expression of MIAT and Oct4 in each four cytogenetic types of CLL was compared. Interestingly we found that the expression of Oct4 was highest in 17p del group and gradually decreased in 12 trisomy and 11q del toward 13q del. This results was in accordance with expression of MIAT in different cytogenetic groups of CLL samples that were analyzed in this study, suggesting a possible transcriptional regulation of MIAT by Oct4. Analysis of protein was somehow in accordance with quantitative transcript expression. We observed higher expression of Oct4 protein level in CI1 and DB cells that also had been shown higher expression of MIAT transcribed during Real time PCR . The lowest Oct4 protein was observed in BL-41 and NU-DHL-1 with very low expression of MIAT expression. These results validated that Oct4 and MIAT transcript could participate in a feedback regulation.

Given the recent evidence that lncRNAs such as NRON, Evf-2, and MEG3 may directly co-regulate transcription factors, testing for direct interactions between Oct4 and

AK028326/Gomafu in the nucleus is a promising potential avenue for future investigation, especially given that nuclear localization of several other functional lncRNAs, including a regulatory lncRNA that forms a ribonucleoprotein complex with transcription factors in vivo (Bond et al. 2009), has been highlighted in recent studies (Hutchinson et al. 2007; Clemson et al. 2009). In view of these precedents, testing for interactions of the lncRNA Gomafu (MIAT) with the Oct4 protein may be promising and can be accomplished by gain and loss of function studies.

We used RNAi to knockdown MIAT transcript in leukemic cell lines and showed that mRNA of Oct4 affected by this treatment to be significantly downregulated. Providing the timely Oct4 knockdown by developing lentiviruses express shRNA against Oct4 we validated regulation of MIAT and Oct4 in a potential synergistic feedback mechanism.

The relevance to our work is the finding that characterized mouse Gomafu/MIAT should be as a co-activator of Oct4 in a regulatory feedback loop. In one study was demonstrated that these lncRNAs are not merely controlled by mESC transcription factors, but they themselves regulate developmental state(Sheik Mohamed, 2010 #195). We analyzed the similarity of MIAT sequence of human and Gomafu of mouse that supposed to be participated in the same feedback loop with Oct4, and demonstrate only ∽200bp resemblances in these two long non coding RNA with more that 9kb length. However the mechanisms by which MIAT and Oct4 feedback loop exert their impact remain unknown but worthy of future studies. Our study was also demonstrated concordance impression of the cancerous cellular features like apoptosis and cell viability after knockdown of either Oct4 or MIAT, that also presented a unique results to our knowledge. The results showed that lncRNA MIAT RNAi induced apoptosis corresponding to negative control treated cells. Consistently the number of viable cells decreased. In addition, oct4 knockdown leads significant specific effects on number of viable cells and cells went to programmed death. The observation that existence differences in apoptosis and viability are consequences of Oct4 knockdown was proved by Real Time PCR on harvested cells.

Our findings elucidate strong associations between lncRNA MIAT and a life threatening human diseases as leukemia. It also should help to explain the mechanisms by which lncRNAs MIAT can function as a modulator of pluripotency. Such future studies of transcription factors and their lncRNA targets should expand our grasp of regulatory

networks in which MIAT plays a role and potentially facilitates the derivation of rational therapeutic interventions and transfers lncRNA research to clinical oncology.

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