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Regulation of Myeloid Transcription Factors by Leukemic Fusion Protein AML1-ETO in Leukemic Cell Line U937.

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ABSTRACT

The therapies for acute myeloid leukemia (AML) require the discovery of novel protein pathways in the systems biology of a specific AML- subtype. We have shown that in the AML-subtype with translocation t (8; 21), the leukemic fusion protein AML1-ETO inhibits the function of transcription factors PU.1 and C/EBPalpha via direct protein-protein interaction. AML1-ETO up-regulated the differentiation inhibitory factor NM23 protein expression after 6 hours and the NM23 mRNA expression was also elevated in t (8; 21)-AML patient samples in comparison to normal bone marrow. AML1-ETO inhibited the ability of C/EBP transcription factors to down-regulate the NM23 promoter. These data suggest a model in which AML1-ETO inhibits the C/EBP-induced downregulation of the NM23 promoter, and thereby increases the protein level of differentiation inhibitory factor NM23. Proteomic pathway discovery can identify novel functional pathways in AML, such as the AML1-ETO-C/EBP-NM23 pathway, as main step towards a systems biology and therapy of AML.

Keywords: AML1-ETO,C/EBPalpha,NM23 promoter,leukemic fusion protein.

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INTRODUCTION

Hematopoietic malignancies are of different inclusions like leukemia, lymphoma and multiple myeloma. Diagnosis of such syndromes is on the basis of morphological features of affected cells, which appear in peripheral blood, bone marrow and lymphoid organs. By taking advantage of the repetitive accessibility of the neoplastic cells within the peripheral blood or bone marrow aspirates, morphological tests are conducted not only for diagnosis but also for evaluation of clinical outcomes and prognosis, suggesting that the morphological features are considered as a clinical biomarker in hematopoietic malignancies. However, outstanding progress in molecular targeted therapy and allogeneic hematopoietic stem cell transplantation has improved the long-term prognosis of patients with hematopoietic malignancies, and some patients are curable. (This data was added during verbal discussion with Dr Sheo mohan Singh)

Myeloid leukemias:

Leukemia:

Cancer that affects the White Blood Cells abnormal growth and diffrentiation of the blood and the bone marrow is termed as Leukemia.

Chronic Myeloid Leukemia

In this leukemia, part maturity of cells are seen.Cells do not mature completely. These cells may look fairly normal but they are not. They generally do not fight infection as compared to normal white blood cells also these cells survive longer.Chronic leukemias tend to progress over a longer period of time, and survival rates of most of the patients is many years. But chronic leukemias are generally harder to cure than acute leukemias.

Causes

95% of CML patients report the presence of a specific marker, the Philadelphia (Ph) chromosome . CML is characterized by the presence of a specific marker, a chromosomal abnormality called the Philadelphia (Ph) chromosome. Approximately 95% of those suffering from CML possess this abnormality. The Ph chromosome results from a translocation of genes located on the long arms of chromosomes 9 and 22.

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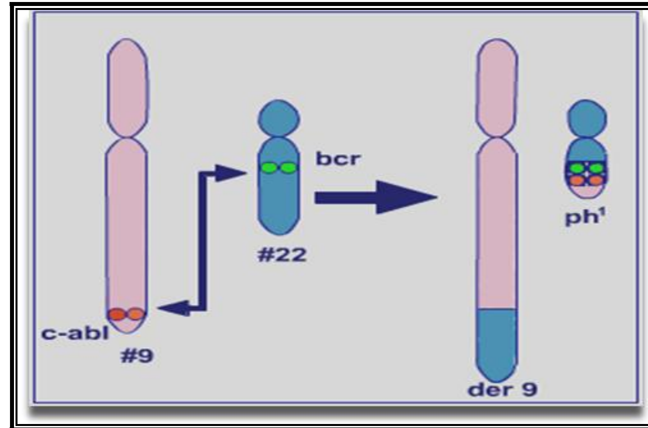

Fig.1.1: CML Translocation

The Fig 1.1 translocation brings two different genes together- the breakpoint cluster region gene (BCR), on chromosome 22 and the Ableson leukemia virus gene (ABL), a proto-oncogene, on chromosome 9. The resulting hybrid gene, the BCR-ABL codes for a protein, endowed with tyrosine kinase activity, which has the power to activate signal transduction pathways. This protein fosters uncontrolled cell proliferation and genomic instability.

In mouse models a CML-like disease has been inculcated on administering bone marrow cells infected with a BCR-ABL gene-containing virus. In other animal models, the fusion proteins have been shown to transform normal progenitor blood cells to malignant cells.

Stages

Chronic myeloid leukemia has three stages depending on the clinical characteristics and laboratory findings.

They are –

1.Chronic phase
2. Accelerated phase
3.Blast crisis phase

As the name indicates the disease may exist for many years with very few signs and symptoms and with marginally increased number of leukemic cells. CML typically begins with the chronic phase, which has very less symptoms. More symptoms begin to appear as the disease progresses. Over a period of time, more chromosomal abnormalities appear in addition to the Ph chromosome. The patient then moves on to an accelerated phase and ultimately progresses to the blast crisis phase when the leukemic macrophage-granulocyte precursor cells begin to proliferate without differentiation.

Acute Myeloid Leukemia

In acute leukemia, the bone marrow cells are unable to mature properly. Immature leukemia cells continue to reproduce and build up. Without treatment, most patients with acute leukemia would live only a few months. Some types of acute leukemia respond well to treatment, and many patients can be cured. Other types of acute leukemia have a less favorable outlook. Acute myeloid leukemia (AML) is a disease that is differentiated by uncontrolled proliferation of clonal neoplastic cells and accumulation in the bone marrow of blasts with an impaired differentiation program[13]. AML accounts for approximately 80% of all adult leukemias and remains the most general cause of leukemia death. Two major types of genetic events that are crucial for leukemic transformation: alterations in myeloid transcription factors governing hematopoietic differentiation and activating mutations of signal transduction intermediates. Transcription factor fusion proteins such as AML1-ETO, PML-RARalpha or PLZF-RARalpha block myeloid cell differentiation by repressing target genes, thus providing one necessary event for leukemogenesis[10]. Disordered cell growth and upregulation of cell survival genes is a proposed necessary second event. Mutations in growth regulatory genes such as FLT3, Ras and c-Kit are common in AML patients.

AML Classification

There are different types of leukemia, depending on the type of WBC they arise from and on the stages that these cells pass through as they mature.

WHO Classification

The World Health Organisation (WHO) classification of AML is clinically important and produces more information that is relevant to prognosis. AML with characteristic genetic abnormalities: such as translocations between chromosome 8 and 21 [t(8;21], translocations between chromosome 15 and 17 [t(15;17)] and inversions involving chromosome 16 [inv(16)]. Patients belonging to this subtype have a high rate of remission and a suitable prognosis compared to the other subtypes. AML with multilineage dysplasia: This category includes patients who suffer from Myelodysplastic Syndrome (MDS) or Myeloproliferative Diseases (MPD) before eventually developing AML. This subtype occurs mostly in older patients and has a unfavourable prognosis. AML and MDS, therapy-related: This category includes patients who have been subjected to chemotherapy and/or radiation and who subsequently develop AML or MDS. These leukemias are often differentiated by specific chromosomal abnormalities, and are associated with poor prognosis. Acute leukemias of ambiguous lineage: Acute leukemias of ambiguous lineage (also known as mixed phenotype acute leukemia) are a subtype of Acute Leukemia where the leukemic cells are difficult to be identified as either myeloid or lymphoid cells, or where both types of cells are present.

AML1 GENE: AML1 humane gene, also named CBFA2 or RUNX1.Located in the 21q22 chromosomal band.

Encodes for one of the two subunits forming a heterodimeric transcription factor, the human core binding factor (CBF).Its protein contain a evolutionary conserved domain of 128 amino acids called runt domain, cause for both heterodimerization with the beta subunit of CBF and for DNA binding.

Function:

This gene is generally expressed in all hematopoietic lineages.The function is to regulate the expression of various genes specific to hematopoiesis playing a pivotal role in myeloid differentiation.

AML1 is one of the genes most commonly deregulated in leukemia through different mechanisms including translocation, mutation and amplification. Translocations lead to the formation of fusion genes(AML1/ETO) encoding for chimerical proteins.Other mechanisms of AML1 deregulation are by point mutations or amplification.

ETO gene:

The Eight-Twenty-One (ETO) nuclear co-repressor gene belongs to the ETO homologue family also containing Myeloid Translocation Gene on chromosome 16 (MTG16) and myeloid translocation Gene-Related protein 1 (MTGR1). By chromosomal translocations ETO and MTG16 become parts of fusion proteins characteristic of morphological variants of acute myeloid leukemia.

Functions:

The common function of ETO homologues have as yet not been examined.

AML1-ETO FUSION PROTIEN:

The pathogenesis of acute myeloid leukemias (AMLs) is linked to oncogenic fusion proteins, generated as a consequence of

A) Primary chromosome translocations or inversions:
B) Many different types of translocations have been described in AMLs, the most frequent being the t(8;21), t(15;17), inv(16), and t(9;11), which, taken together with their variants, account for approximately 40% of AML cases[11].
C) Biological mechanisms in AMLs. In particular, one of the components of each fusion protein is invariably a transcription factor, commonly involved in the regulation of differentiation

It has been suggested, therefore, that AML-associated fusion proteins contribute to the leukemic phenotype by inducing a differentiation block: a biological activity progresses with the

main phenotypic trait of AMLs (i.e., the accumulation of hemopoietic precursors blocked at particular stages of myeloid development).

Translocation-t (8;21):

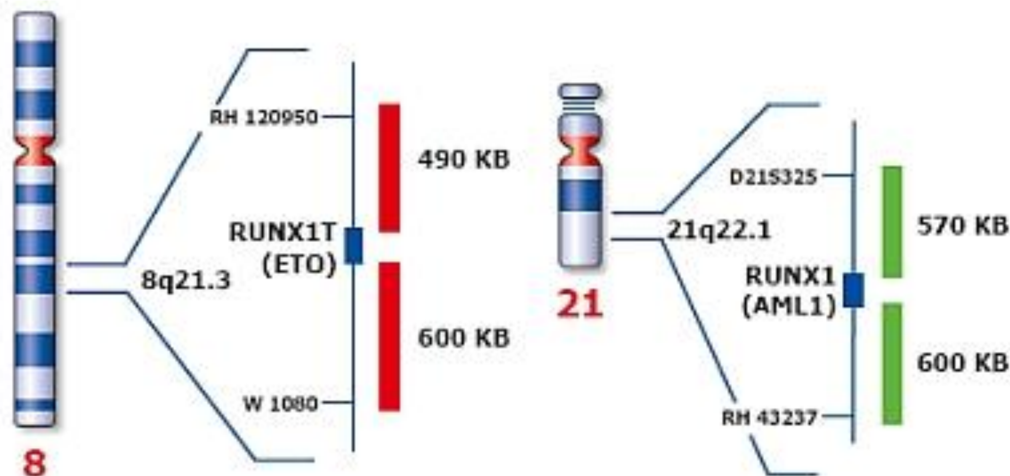

fig 1.3 AML1-ETO Translocation-t(8;21):

This translocation give rise to AML1-ETO fusion coprotein (also known as RUNX1/MTG8). This translocation creates a fusion protein consisting of the acute myeloid leukemia-1 transcription factor and the eight-twenty-one corepressor (AML1 ETO), which represses transcription through AML1 (RUNX1) DNA binding sites and immortalizes hematopoietic progenitor cells.AML1/ETO has the N-terminal DNA-binding domain of AML1,a transcription factor important for definitive hematopoiesis, and almost all of ETO, a protein thought to function as a corepressor for a variety of transcription factors.

AML1-ETO stops myeloid transcription factor C/EBPalpha by downregulating its mRNA, protein and DNA binding activity in t(8;21)-myeloid leukemia and inactivates the myeloid master regulator PU.1 by direct protein-protein interaction in myeloid differentiation.Because AML1-ETO disrupts and stops the normal function of myeloid transcription factors and requires other cooperating factors to induce leukemia, I hypothesized that the systematic identification of AML1-ETO target proteins on a global proteome-wide level might lead to novel insights into the pathogenesis and systems biology of AML1-ETO-induced leukemia on a post-genomic functional level.

U937:

U937 is a human monocytic cell line received from histiocytic lymphoma and typically derived from the bone marrow.As these are monocytic cells,secrete chemokines,cytokines [14].

Histocyte:It is a cell that is part of the mononuclear phagocytic system that takes keen part in the immune responses.

Lymphoma:It is a cancer that starts in the lymphatic cells of the immune system and presents as a solid tumor of lymphoid cells. It is treatable with chemotherapy, and in some cases radiotherapy and/or bone marrow transplantation, and can be curable, depending on the histology, type, and stage of the disease. These malignant cells often originate in lymph nodes, presenting as an enlargement of the node (a tumor)[16].

U937 cells were first isolated from a 37 year old male patient for the cells to be used in properties of differentiation of monocytes. The results obtained showed that the U937 cells were able to differentiate under a variety of stimulus.

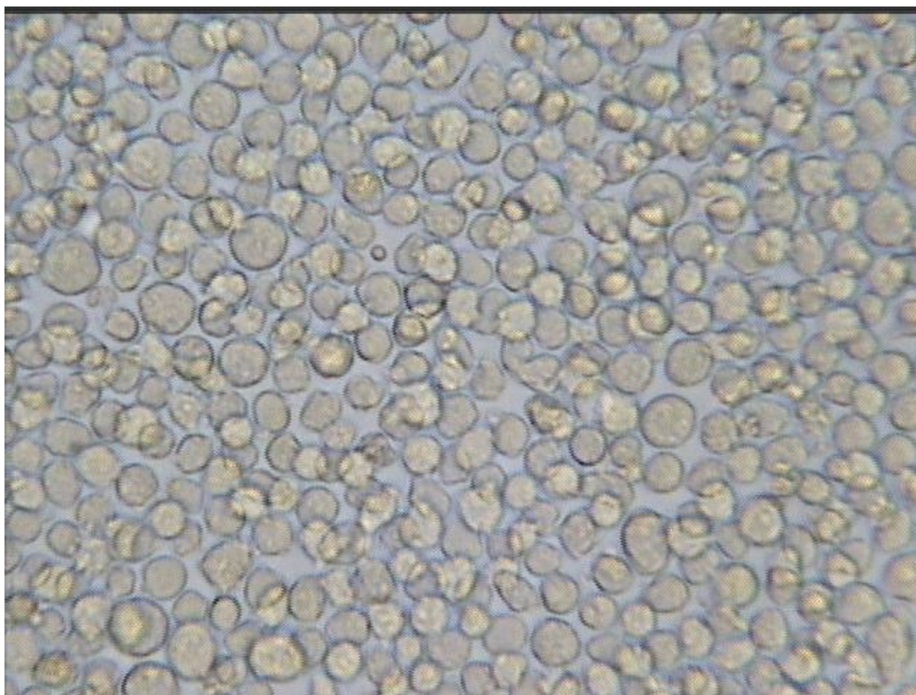

FIG: 1.4 U937 cells under microscope

The U937 cell line is an extensively used cell line in Tissue Culture labs allowing their properties to be used as a monocyte capable of differentiating into macrophages.

GROWTH MEDIUM:

Growing U937 can be done with DMEM and RPMI 1640(Roswell Park Memorial Institute) medium plus 10% Fetal bovine serum.

RPMI 1640: It was developed by Moore et. al. at Roswell Park Memorial Institute.We have use RPMI 1640 with l-glutamine adjusted to contain 1.5g/l Na biocarbonate,4.5g/l glucose ,10% Fetal bovine serum.

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GROWING CONDITIONS:

Temprature 37% and 5% carbon dioxide.

RPMI Storage and Stability

Store the dry powdered medium at 2-8°C under dry conditions and liquid medium at 2-8°C in the dark.

Deterioration of the powdered medium may be recognized by any or all of the following:

Color change from pinkish to yellow, granulation/clumping, insolubility

Deterioration of the Liquid medium may be recognized by any or all of the following:

pH change,

Precipitate or particulates,

Cloudy appearance

Color change

CELL CULTURE:

Culuture can be maintained by the addition of fresh medium or replacement of medium. Alternatively culture can be established by centrifugation with subsequent resuspension at 1 to 2x10^5 to 2x10^6viable cells/ml.

AML1-ETO FUSION PROTEIN AND THEIR REGULATIONS:

AML1/Runx1, originally identified as a gene located at the breakpoint of the t(8;21) translocation, encodes a transcription factor that is widely seen in multiple hematopoietic lineages and that regulates the expression of a variety of hematopoietic genes. Numerous studies have shown that AML1 is a critical regulator of hematopoietic development. In addition, AML1 is a frequent target for chromosomal translocation in human leukemia. The activity of AML1 can be modulated by different types of posttranslational modification, including phosphorylation and acetylation. Phosphorylation by extracellular signal-regulated kinase (ERK) is one of the mechanisms that dictate whether AML1 acts as either a transcriptional repressor or an activator of gene expression. Recently, a physiological role for AML1 in adult hematopoiesis was revealed by conditional gene targeting in mice. Remarkably, adult hematopoietic progenitors are maintained even in the absence of AML1, in stark contrast to the total disruption of definitive hematopoiesis during embryogenesis. AML1 is, however, critical

for megakaryopoiesis and plays an important role in T-cell and B-cell development in adult mice[9]. Recent analyses engineered to recreate hematopoiesis in vitro revealed that the transcriptional activity of AML1 is closely related with the potential of AML1 to generate hematopoietic cells and support thymocyte development.

Transcription factor AML1/Runx1, initially isolated from the t(8;21) chromosomal translocation in human leukemia, is essential for the development of multilineage hematopoiesis in mouse embryos. AML1 negatively regulates the number of immature hematopoietic cells in adult hematopoiesis, whereas it is required for megakaryocytic maturation and lymphocytic development. However, it remains yet to be determined how AML1 contributes to homeostasis of hematopoietic stem cells (HSCs).

The acute myelogenous leukemia 8;21 (AML1-ETO) fusion protein is encoded by a rearranged gene created by the ETO chromosomal translocation. This protein lacks the nuclear matrix-targeting signal that directs the AML1 protein to appropriate gene regulatory sites within the nucleus. Here we report that substitution of the chromosome 8-derived ETO protein for the multifunctional C terminus of AML1 precludes targeting of the factor to AML1 subnuclear domains. Instead, the AML1-ETO fusion protein is redirected by the ETO component to alternate nuclear matrix-associated foci. ETO chromosomal translocation in AML with modifications in the intranuclear trafficking of the key hematopoietic regulatory factor, AML1.Thus misrouting of gene regulatory factors as a consequence of chromosomal translocationsis an dynamic characteristic of acute leukemias.

Approximately 15% of all acute myeloid leukemia (AML) cases and up to 40% of those classified by the French-American-British system as M2 subtype harbor the t (8;21) translocation that gives rise to the AML1/ETO fusion oncoprotein (also known as RUNX1/MTG8)[4]. The t (8;21) is one of the most common chromosomal translocations associated with acute leukemia. This translocation creates a fusion protein consisting of the acute myeloid leukemia-1 transcription factor and the eight-twenty-one corepressor (AML1 ETO), which represses transcription through AML1 (RUNX1) DNA binding sites and immortalizes hematopoietic progenitor cells.AML1/ETO consists of the N-terminal DNA-binding domain of AML1,a transcription factor essential for definitive hematopoiesis, and almost all of ETO, a protein thought to function as a corepressor for a variety of transcription factors.

AML1/ETO impacts multiple processes involved in normal myelomonocytic development where the fusion protein interferes with multiple signal transduction pathways, promoting early myeloid cell self-renewal and interfering with proper hematopoietic differentiation[7]. The targeted therapies for cancer and leukemia requires the discovery of novel target proteins and protein pathways in cancer cells, and the determination of their functional relevance in the systems biology of a specific cancer type. Acute myeloid leukemia (AML) with translocation t(8;21)(q22;22) is characterized by the fusion protein AML1-ETO which is produced by translocation of the AML1 (RUNX1/PEBPα/CBFA2) gene on chromosome 21 to the ETO (MTG8) gene on chromosome 8. This fusion protein consists of the N-terminus of AML1 (1-177 amino

acids) fused to the full length of ETO. Expression of AML1-ETO is detected in 12% of all AML cases and 40% of the FAB subtype M2-AML patients[12].

AML1-ETO blocks myeloid transcription factor C/EBPalpha by downregulating its mRNA, protein and DNA binding activity in t(8;21)-myeloid leukemia and inactivates the myeloid master regulator PU.1 by direct protein-protein interaction in myeloid differentiation[6].Because AML1-ETO disrupts and stops the normal function of myeloid transcription factors and requires other cooperating factors to induce leukemia, we hypothesized that the systematic identification of AML1-ETO target proteins on a global proteome-wide level might lead to novel insights into the pathogenesis and systems biology of AML1-ETO-induced leukemia on a post-genomic functional level[7].

ROLE OF TRANSCRIPTION FACTORS IN NORMAL LEUKEMIC DEVELOPMENT AND THEIR DISREGULATIONS CAUSING VARIOUS ABNORMAL EFFECTS:

Leukemias are spread by leukemic stem cells. The genomic events and pathways involved in the transformation of hematopoietic precursors into leukemic stem cells are increasingly understood. This concept is based on genomic mutations or functional dysregulation of transcription factors in malignant cells of patients with acute myeloid leukemia (AML). Loss of the CCAAT/enhancer binding protein-α (CEBPA) function in myeloid cells in vitro and in vivo leads to a differentiation block, similar to that observed in blasts from AML patients[2]. CEBPA alterations in specific subgroups of AML comprise genomic mutations progress to dominant-negative mutant proteins, transcriptional suppression by leukemic fusion proteins, translational inhibition by activated RNA-binding proteins, and functional inhibition by phosphorylation or increased proteasomal-dependent degradation. The PU.1 gene can be mutated or its expression or function can be blocked by leukemogenic fusion proteins in AML. Point mutations in the RUNX1/AML1 gene are also observed in specific subtypes of AML, in addition to RUNX1 being the most frequent target for chromosomal translocation in AML[1]. These data are persuasive evidence that impaired function of particular transcription factors contributes directly to the development of human AML, and restoring their function represents a promising target for novel therapeutic strategies in AML.

CEBP alpha:

CCAAT/enhancer-binding protein alpha is a protein that in humans is encoded by the CEBPA gene [3].The protein encoded by this intronless gene is a bZIP transcription factor which can bind as a homodimer to certain promoters and enhancers. It can also form heterodimers with the related proteins CEBP-beta and CEBP-gamma[15]. The encoded protein has been shown to bind to the promoter and modulate the expression of the gene encoding leptin, a protein that plays an important role in body weight homeostasis. Also, the encoded protein can interact with CDK2 and CDK4, thereby inhibiting these kinases and causing growth arrest in cultured cells.

Transcription factors play a crucial role in myeloid differentiation and lineage determination. Tumor suppressor protein C/EBP alpha is a key regulator of granulocytic differentiation whose functional inactivation has become a pathophysiological signature of myeloid leukemia [1]. Mechanisms such as antagonistic protein-protein interaction, mutation and post-translational modifications of C/EBP alpha which leads to its transcriptional inhibition and render C/EBP alpha inactive in its functions. The CCAAT enhancer binding protein alpha (C/EBP alpha) is a key transcription factor involved in granulocytic differentiation and myelopoiesis in general. C/EBP alpha belongs to the C/EBP family of proteins first identified from rat liver nuclear proteins. CCAAT enhancer binding proteins encompass a family of transcription factors with structural as well as functional homologies[3]. C/EBP proteins consist of an activation domain at the N terminal, a DNA binding basic region (BR) and a leucine-rich dimerization domain aptly termed the “leucine zipper” (LZ) at the carboxyl terminus. Study of lineage specific transcription factors such as C/EBPs has greatly increased our knowledge of mechanisms underlying the regulation of myelopoiesis and haematopoiesis in general. Although precise etiology of AML is not clearly known, various somatic and genetic alterations in hematopoietic stem or progenitor cells are known to cause malignant transformation which may lead to leukemic phenotype. Recent studies have emphasized that function of lineage specific transcription factors is blocked in AML either due to mutation or their functional inactivation by other inhibitory proteins.

C/EBP alpha and PU.1 are two major regulators of hematopoietic stem cell development. Unlike PU.1, which governs transcription of a wide spectrum of myeloid-specific genes, C/EBP alpha has a more specific function in granulopoietic stem cell development. In many studies, various mechanisms have been suggested through which C/EBP alpha is negatively regulated in certain AML subtypes. Impairments in C/EBP alpha signaling such as reduced mRNA or protein expression, aberrant post translational modifications (phosphorylation) and the presence of (dominant-negative) mutations are often observed in human myeloid leukemias. Since C/EBP alpha is involved with all three hematopoietic checkpoints: cell proliferation, differentiation and apoptosis; it is likely that impairment of either of this may induce leukemia. Therefore, inhibition of C/EBP alpha seems to be epicenter in the pathophysiology of some myeloid leukemias.

NM23 GENE: (Non metastatic gene 23)

This gene encodes a nucleoside diphosphate kinase. It is hexamer of two different subunits(A,B 152 amino acid each ,displaying 88% homology with each other,termed NDP Kinase A(NME1)and NDP Kinase B(NME2). NM23 is called also Metastasis inhibition factor NDP kinase catalyses the phosphorylation of nucleoside diphosphates into triphosphates required for the biosynthesis of nucleic acids. NDP kinase can also phosphorylate GDP in GTP-binding proteins and therefore acts as an activator for such proteins. N-terminal sequence determination of a nucleoside diphosphate kinase isolated from dark-grown oat (Avena) tissue and composed of six 18 kDa subunits shows that 87 % of the 23 amino acids sequenced are identical with human nm23 protein. NM23 shows 78 % homology with a Drosophila

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melanogaster gene product called awd (abnormal wing disk) the disruption of which arrests cells in the metaphase Cell surface expression of NM23 protein is only observed on tumor cell lines, but not on normal cells. Examination can be done which tumor cell line express the cell surface NM23 protein by measuring the cell surface NM23-H1 and NM23-H2 proteins of leukemia line cells on various cellular lineage and differentiation stages. The NM23-H1 was expressed on myeloid leukemia lines but not lymphoid lines, while NM23-H2 was only expressed on erythroleukemia lines.

PU 1:

Transcription factor PU.1,31 kDa-transforming protein.

Function:

Binds to the PU-box, a purine-rich DNA sequence (5'-GAGGAA-3') that can act as a lymphoid-specific enhancer. Also binds RNA and may modulate pre-mRNA splicing. AML1/ETO also directly interferes with recruitment of essential cofactors by a number of crucial hematopoietic transcription factors such as C/EBP alpha and PU.1, thus potentially blocking their differentiation-promoting functions. The transcription factor PU.1 has an essential role in hematopoiesis. New evidence shows that reducing PU.1 expression to 20% of wild-type levels results in an aggressive form of acute myeloid leukemia.PU.1, encoded by the gene Sfpi1, belongs to the Ets family of transcription factors[8]. Its expression is tightly controlled in hematopoietic cells, such that the relative expression level of PU.1 determines whether cells differentiate into lymphocytes, macrophages or granulocytes. Mutations in FLT3 and RUNX1-CBFA2T1translocations, which are generally found in acute myeloid leukemia (AML), were recently found to downregulate expression of PU.1, causing a block in differentiation and implicating PU.1 in leukemogenesis[4][5]. The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. PU.1$^{-/-}$ mice exhibit a complete block in myeloid differentiation. Heterozygous PU.1 mutations were reported in some patients with acute myeloid leukemia (AML), but not in AML with translocation t(8;21), which gives rise to the fusion gene AML1-ETO. A negative functional impact of AML1-ETO on the transcriptional activity of PU.1. AML1-ETO binds to the $\beta_3\beta_4$ region in the DNA-binding domain of PU.1 and displaces the coactivator c-Jun from PU.1, thus down-regulating the transcriptional activity of PU.1. This physical interaction of AML1-ETO and PU.1 did not abolish the DNA-binding capacity of PU.1. AML1-ETO down-regulates the transactivation capacity of PU.1 in myeloid U937 cells, and the expression levels of PU.1 target genes in AML French-American-British (FAB) subtypeM2 patients with t(8;21) were lower than in patients without t(8;21). Conditional expression of AML1-ETO causes proliferation in mouse bone marrow cells and inhibits antiproliferative function of PU.1. Thus, the function of PU.1 is down-regulated by AML1-ETO in t(8;21) myeloid leukemia, whereas overexpression of PU.1 restores normal differentiation.

Both PU.1 (also called SFPI1), an Ets-family transcription factor, and AML1 (also called RUNX1), a DNA-binding subunit of the CBF transcription factor family, are crucial for the

generation of all hematopoietic lineages, and both act as tumor suppressors in leukemia. An upstream regulatory element (URE) of PU.1 has both enhancer and repressor activity and tightly regulates PU.1 expression. AML1 binds to functionally important sites within the PU.1 upstream regulatory element and regulates PU.1 expression at both embryonic and adult stages of development. Dysregulation of PU.1 expression contributed to each of the phenotypes observed in these mice, and restoration of proper PU.1 expression rescued or partially rescued each phenotype. These data demonstrate that PU.1 is a major downstream target gene of AML1.(Data was obtained from papers of Dr sheo mohan singh)

FUTURE RESEARCH:

Intense research to develop a better treatment option is being carried out by various Research groups.

- To research the disease, short segments of DNA, called oligomers, which block the BCR-ABL gene, were developed that suppressed the formation of leukemia cells without affecting the development of the normal bone marrow cells. These, along with other experimental techniques may lead to future treatments for CML.
- Researchers from The University of Texas M. D. Anderson Cancer Center are reporting the pioneer study. According to this study, a vaccine using PR1 peptide can produce a complete molecular remission in some patients. This peptide is found to be abnormally expressed on myeloid leukemia cells. Active clinical trial is being carried out for two other drugs, ceflatonin and nilotinib.These drugs are designed to help CML patients who are resistant to imatinib. Different combinations of treatment modalities are being investigated.

Experiments performed/materials and methods

Experiment performed:

1. COMPETENT CELL PREARATION
2. TRANSFORMATION
3. PLASMID ISOLATION(MINI PREP)
4. TRANSFECTION IN CELL LINES(U937)
5. WESTERN BLOT

EXPERIMENT 1:

AIM:

Preparation of comptent cells of E.COLI DH5alpha.

REAGENTS:

A. LB MEDIA:

Tryptophan -3g+Yeast extract-1.5+Nacl-3g
Mixed in 300ml distilled water ,adjust pH to 7.5 with NaoH,and then autoclaved.

B. 1M Cacl2 PREPARATION:

Cacl2 powder-55.5g+ Distilled water-500ml
Autoclave both of these before use.

C. 0.1M Cacl2:

100ml 1M Cacl2+900 distilled water (autoclaved)
We have use 10ml,so make 1MCacl2+90mld/w.

D. 50% Glycerol:

50ml glycerol+50ml d/w(autoclaved)
E. 0.1M Cacl2+15% glycerol:
1M Cacl2-10ml+50% glycerol-30ml+distilled water-60ml

PROTOCOL:

1. One colony From plate put in 10ml LB
2. Colony taken From Dh5alpha frozen culture and inoculate on LB plates
3. Next day-Colony taken and put in 10ml LB and solution put on shaker at 200 rpm overnight(37 degree C)
4. Next day-2ml LB –for blank(taken)
5. 1ml overnight DH5alpha culture into 100ml of LB media put into shaker at 250 rpm for 2 hours till OD reaches 0.4-0.5(checked at OD600)
6. Stop growth by cooling culture on ice with shaking ,

OD600	TIME
0.229	2 hours
0.291	3 hours
0.411	3.30 hours

7. Spin at 3000 rpm for 10 minutes at 2-4degree C
8. Add 20ml cold 0.1M Cacl2(5ml) to pellet and incubate on ice for 30 minutes

9. Spin at 3000 rpm for 10 minutes at 2-4 degree C(resuspended)
10. 5ml of 0.1M Cacl2+15% glycerol+pellet
11. Store in ependorff stand for atleast 2 hours on ice
12. Use directly in 200ul aliquots for transformation or add 400ul of cold sterile glycerol and mix aliquots in 200ul each
13. Freeze in -70 to -80 degreeC

CHECK COMPETENT CELL:

Spread competent cells on LB plates containing Ampicillin Competent cells show no growth(competent cell are perfect) 60ml LB+6ul Ampicillin-spread in plate –show no growth

EXPERIMENT 2:

Aim:

To perform transformation using AE(aml1-eto plasmid) & competent cells.

Reagents:

LB agar - 3g in 100 ml-.(autoclave)(15psi,15 min)
LB medium- 1.25g in 50 ml-(autoclave)(15psi,15 min)
Competent cells(DH5 alpha)- 2 vials (prepared earlier in Experiment 1.)
Plasmid dna AE(aml1-eto plasmid)- 5 ul.

Protocol:

1. Take 100 ul competent cells(DH5 alpha)(thaw) + 5 ul AE plasmid
2. Keep the mixture on ice for 30 min.
3. Give heat shock treatment at 42 degree C for 40 sec in water bath.
4. Add mixture in LB medium for growth in 50 ml tube.
5. Keep mixture in shaker and maintain 37 degree C ,225 rpm for 1 hour.
6. Spread 50 ul mixture on LB plates containing ampicillin.Incubate overnight at 37 degree C.

EXPERIMENT 3:

Aim:

To purify & isolate plasmid dna by miniprep.

Reagents:

Solution 1:

Prepared in 200 ml distilled water
50 Mm glucose- 1.98 g (Purify by milipore filter)
25 mM tris HCL (pH 8) – 0.6 g.....................(autoclave)(15psi,15 min)
10 Mm EDTA (pH 8) – 0.7 g.....................(autoclave)(15psi,15 min)

Solution 2:

STE (TEN) buffer for 1 litre
0.1 M Nacl – 5.84 g
10 Mm tris HCL (pH 8) -1.21 g
1 Mm (pH 8) – 0.37 g

Solution 3:

5 M Potassium acetate – 9.8 g in 200 ml (neutralizing solution)

Note: Solution 2 is not used in this experiment for better results(as desired by Dr Sheo)

Protocol:

1. Take colony from transformed plate and grow on 2.5 ml LB overnight on shaker.
2. Take 1.5 ml from the above culture and spin at room temperature for 10 min.
3. Add 8 ul lisozyme + 2 ml Solution 1.
4. Resuspend by vortexing at room temperature for 10 min.
5. Leave on ice for 5 min.
6. Add 150 ul of cold ammonium (7.5 M ammonium acetate).
7. Spin at 14000 rpm in centrifuge at 4 degree.
8. Collect 300 ul supernatant from top.
9. Spin again & collect 250 ul supernatant.
10. Add 175 ul isopropanol and leave at room temperature for 5 min.
11. Spin at 14000 rpm in centrifuge at 4 degree.
12. Add absolute 70 % ethanol.
13. Spin and remove supernatant and air dry pellet.
14. Add 30 ul distilled water and resuspend at -20 degree C.
15. And this purifies pasmid can be used for transfection (U937)

Identification of purified plasmid by agarose gel electrophoresis.

1. 18 ul plasmid + 2 ul dye (bromophenol blue)
2. Prepare gel (1% agarose gel) (3 g agarose in 300 ml distilled water)-(autoclave)(15psi,15 min)
3. Use TBE buffer as tank buffer.
4. Mix 5 ul etBr in autoclaved agarose solution before solidification.

5. Pour gel in electrophoretic unit and put comb.
6. Leave the gel for solidification undisturbed and remove the comb..
7. Load the sample (purified plasmid dna) into wells and switch on the the apparatus at 60 V.
8. See the bands under UV trans illuminater .Thus purified plasmid in checked.

Experiment 4:

Aim:

Calcium phosphate transfection of u937 Cells.

Reagents:

DNA Preparation:

Use between 10-20 µg of DNA to transfect $3x10^6$ cells. Q.S the desired amount of DNA in 220 µl of 0.1x TE. Add 250 µl 2x HEPES-buffered saline and vortex briefly to mix. Add 31 µl 2 M $CaCl_2$ slowly over a 30 second period to the DNA solution. Incubate at room temperature for 30 minutes to form a precipitate.

Cell Preparation:

Wash $3x10^6$ cells once in PBS. Resuspend the cells in 0.5 ml PBS.

0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)

For 100 ml:

Transfer 0.1 ml of 1 M Tris-HCl, pH 8.0 to 9.9 ml of sterile water to make a 10 mM solution.

Transfer 0.1 ml of 0.5M EDTA to 9.9 ml of sterile water to make a 5 mM EDTA solution.

Transfer 2 ml of this 5 mM EDTA solution to the 10 mM Tris-HCl solution.

Add 88 ml sterile water to bring the solution to 100 ml.

Filter sterilize and autoclave.

2X HEPES Buffered Saline: (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES, pH 7.5)

2 M Calcium Chloride: Dissolve 14.7 g $CaCl_2$ in 100 ml H2O. Filter sterilize and autoclave.

Transfection protocol:

Pipette the DNA solution once to reduce the size of any large clumps before transfering the DNA to the cells. Incubate the cells and DNA for 20 minutes at room temperature. Add 5.5 ml of complete medium, bringing the cells to a 6 ml volume. Dispense 2 ml of cells per well ($1x10^6$ ce lls/well; $0.5x10^6$ cells/ml) in a Costar 24 well plate. Incubate at 37°C in a 5% CO_2 incubator overnight. The next day, collect the cells and wash once in PBS. Resuspend the cells in 6 ml medium and return to 3 wells of a new 24 well plate. There will still be a moderate amount of the precipitate on the surface of the cells. Selective reagents (G418, etc) may be added at this time. Return cells to the incubator. Feed and split the cells as needed.

Lipofection:

SECOND METHOD: Plasmid DNA Transfection using lipofection:

Transfect DNA into mammalian cells in a 24-well format. All amounts and volumes are given on a per well basis.

Prepare complexes using a DNA (µg) to Lipofectamine™ 2000 (µl)(arranged by Dr Sheo) ratio of 1:2 to 1:3 for most cell lines. Transfect cells at high cell density for high efficiency, high expression levels, and to minimize cytotoxicity.

1. Adherent cells: One day before transfection, plate 0.5-2 x 105 cells in 500 µl of growth medium without antibiotics so that cells will be 90-95% confluent at the time of transfection.

Suspension cells: Just prior to preparing complexes, plate 4-8 x 105 cells in 500 µl of growth medium without antibiotics.

2. For each transfection sample, prepare complexes as follows:

a. Dilute DNA in 50 µl of Opti-MEM® I Reduced Serum Medium without serum (or other medium without serum). Mix gently.
b. Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in 50 µl of Opti-MEM® I Medium. Incubate for 5 minutes at room temperature. Note: Proceed to Step c within 25 minutes.
c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine™ 2000 (total volume = 100 µl). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy). Note:Complexes are stable for 6 hours at room temperature.
3. Add the 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4. Incubate cells at 37°C in a CO2 incubator for 18-48 hours prior to testing for transgene expression. Medium may be changed after 4-6 hours.
5. For stable cell lines: Passage cells at a 1:10 (or higher dilution) into fresh growth medium 24 hours after transfection. Add selective medium (if desired) the following day.

Optimizing Plasmid DNA Transfection

To obtain the highest transfection efficiency and low cytotoxicity, optimize transfection conditions by varying cell density as well as DNA and Lipofectamine™ 2000 concentrations. Make sure that cells are greater than 90% confluent and vary DNA (µg): Lipofectamine™ 2000 (µl) ratios from 1:0.5 to 1:5.

Scaling Up or Down Transfections

To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine™ 2000, nucleic acid, cells, and medium used in proportion to the relative surface area, as shown in the table. With automated, high-throughput systems, a complexing volume of 50 µl is recommended for transfections in 96- well plates. Note: You may perform rapid 96-well plate transfections by plating cells directly into the transfection mix. Prepare complexes in the plate and directly add cells at twice the cell density as in the basic protocol in a 100 µl
volume. Cells will adhere as usual in the presence of complexes.
Culture vessel- 24 well
Surf.area per well-2 cm^2
Vol. Of plating medium-500 ul
Vol. Of dilution medium-2x50 ul
DNA-0.8 ug
Lipofectamine™2000-2 ul

EXPERIMENT 5:

Western blot analysis:

U937 cell clones were harvested and lysed after 8, 12, 24, 36, and 48 hours of Zn^+ induction. Western blot analysis was performed using standard procedures. 24 h after the start of transfection, cells were lysed with radioimmunoprecipitation assay buffer. Equal amounts of totalprotein were separated on 10% SDS-polyacrylamide gels and transferred to nitocellulose membrane. Membranes were incubated with anti-C/EBP alpha antibody or beta-tubulin antibody as an internal control for 60 min and then with Protein A-horseradish peroxidise conjugat for 45 min. Signals were detected with ECL Western blotting detection reagents (Amersham). C/EBP alpha antibody (14AA, SC61) was used for Western Blot and immunoprecipitation .

REAGENTS

ECL Western blotting kit (Amersham Life Science; cat# RPN2108): contains second antibodies for both mouse and rabbit, substrate and milk blocker (the milk blocker is not normally used when using ruminant samples).

Hybond ECL nitrocellulose membrane (Amersham Life Science; cat # RPN2020D)

Kodak X-OMAT (XAR-5, 18X24 cm; cat # 8532665) (Arranged by Dr.sheo).

10X TBS

12.11 g Tris-base (100 mM)
87.66 g NaCl (1500 mM)
1 liter dd H_2O
Adjust pH= 7.6
Washing buffer (TBS-T): 100 ml 10X TBS + 900 ml dd H_2O + 1 ml Tween-20.
Blocking buffer: TBS-T + 1.5% gelatin
Incubation buffer I (for first antibody): TBS + 1.5% gelatin
Incubation buffer II (for second antibody) = blocking buffer (i.e., TBS-T + 1.5% gelatin)

PROCEDURES

1. Immediately after removal from the blotting apparatus, place membranes into blocking buffer for 2 hours. A small plastic gel box is a suitable container. This and all other incubation steps are performed at room temperature and in the rocker platform.

2. Wash membrane in washing buffer: rinse 2 times very briefly, incubate for 15 minutes, then repeat 2 x at 5 minutes each. Use a lot of buffer.

3. Transfer the membrane to a lid of 96-well microtiter plate or similar low volume container. Incubate with first antibody using recommenced dilution in TBS + 1.5% gelatin during 2 hours. Approximately 10 ml of diluted antibody showed to be sufficient.

4. Transfer the membrane into gel box and repeat step # 2.

5. Transfer the membrane back to the small container and incubate with anti-mouse or anti-rabbit IgG horseadish peroxidase diluted 1:8000 in TBS-T + 1.5% gelatin for 1 hour.

6. Repeat step # 4 (wash)

7. Prepare ECL solution for detection: mix equal volume of ECL reagent 1 and 2, (1:1) with final volume regarding of 0.125 ml/cm^2 (for a mini gel- 4 ml of each reagent).

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8. Remove excess buffer from the membrane by draining the membrane over a piece of folded kimwipe paper and briefly touching the edge of the membrane to the paper.

9. Add ECL solution and incubate for 1 minute.

10. Repeat step # 8 (drain excess of substrate)

11. Place membrane down on seran wrap, remove bubbles and wrap membrane completely.

Avoid excess amounts of seran wrap.

1. Tape membrane to the inside film cassette.

2. In the dark, add 1 sheet of x-ray film to the cassette. Expose membrane to film. It will probably be necessary to do several different exposures to find out the best exposure.

3. Develop the film.

MATERIALS AND METHODS

2.1 CELL CULTURE:

Human myeloid cell line U937 stably transfected with AML1-ETO cDNA under the control of human metallothionein promoter in the expression vector pPC18 (U937Z/A-E) was kindly provided by Dr. Sheo Mohan Singh .Parental cell line U937 was purchased from NCCS Pune and used as a control throughout this study. A tet-off inducible U937 cells stably transfected with AML1-ETO under the control of tet-responsive transcriptional repressor tTA (U937T/A-E) and U937 cells containing the empty vector tTA (U937T) was kindly provided by Dr. Sheo Mohan Singh.These cell lines were cultured in 5% co2 at 37 degrees C in CO2 incubator.

2.2 WESTERN-BLOT ANALYSIS:

U937Z/A-E cells were induced with 100 μM $ZnSO_4$ for 6 hrs, lysed in RIPA buffer and analyzed for AML1-ETO expression by Western-blot . Anti-ETO (cat. sc9737), anti-NM23-H2 goat polyclonal antibody (sc14790) and anti-β-tubulin rabbit polyclonal (cat. sc9104) antibodies and anti-Prohibitin antibody (cat. MS-261-P) were provided by Dr sheo.

2.3 LUCIFERASE PROMOTER ASSAY:

Myeloid U937 cell lines were transfected using the effectene kit (Qiagen) . The plasmid constructs used in the transfection assays were; NM23h1-luc promoter (Provided by Dr Sheo.), human C/EBPalpha, C/EBPbeta C/EBPdelta and AML1-ETO plasmids. As an internal control plasmid for cotransfection assays, the pRL0 construct driving the Renilla luciferase gene

(Promega). Firefly luciferase and Renilla luciferase activities were measured in Turner Designs Luminometer using Dual Luciferase Reporter Assay System (Promega). Results are given as means + s.e.m. of at least three independent experiments.[18]

RESULTS

For the purpose of identifying AML1-ETO-regulated proteins, a myeloid cell line system was used where AML1-ETO is stably transfected in U937 cells under Zn-inducible metallothionein promoter (U937Z/A-E) or under the tet-off-inducible promoter (U937T/A-E). AML1-ETO protein expression is induced when Zn is added in the medium (fig.3a lane 5) or when tetracycline is removed from the medium (fig3 b lane 4). In vitro translated AML1-ETO was used as a positive control (fig.3 a+b lane 1). For identificaton of AML1-ETO target proteins, AML1-ETO expression was induced in U937Z/A-E cells with Zn for 6 or 12 hours, cells were lysed in urea lysis buffer. The gels were stained with colloidal coomassie blue stain later.

(a) (b)

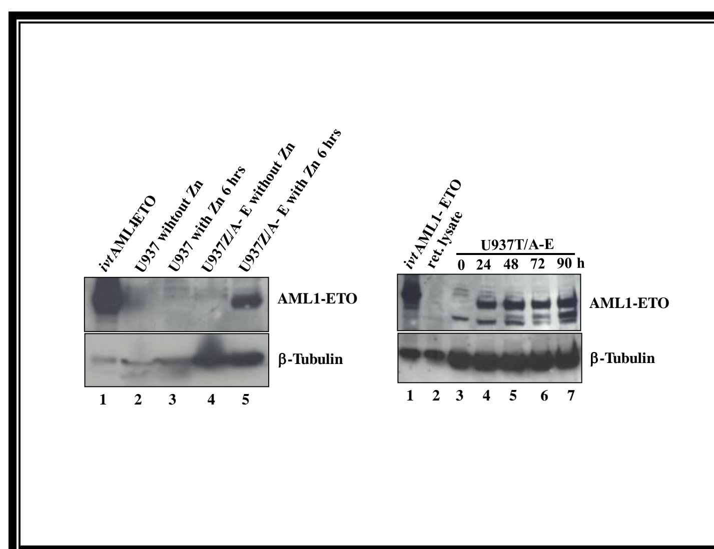

Fig 3: Detection of AML1-ETO in Zn- and Tet-off inducible cell lines

Fig. 3: Western-blot analysis for inducible AML1-ETO expression. (a) U937Z/A-E cells were induced with 100 µM $ZnSO_4$ for 6 hrs, lysed in RIPA buffer and analyzed for AML1-ETO expression by Western-blot. U937 cells were also induced and used as control. In vitro translated AML1-ETO (lane 1) was used as a positive control. As seen in lane 5, AML1-ETO expression is increased after Zn induction. (b) U937T/A-E cells were induced by removing tetracycline from the medium for 24, 48, 72, and 94 hours. Cells were lysed in RIPA buffer and blotted for AML1-ETO. U937T cells containing the empty vector were used as a control. AML1-ETO expression level was increased after 24 hours of tet-off induction (lane 4) and was continuously detectable even after 90 hours of induction (lanes 5-7).

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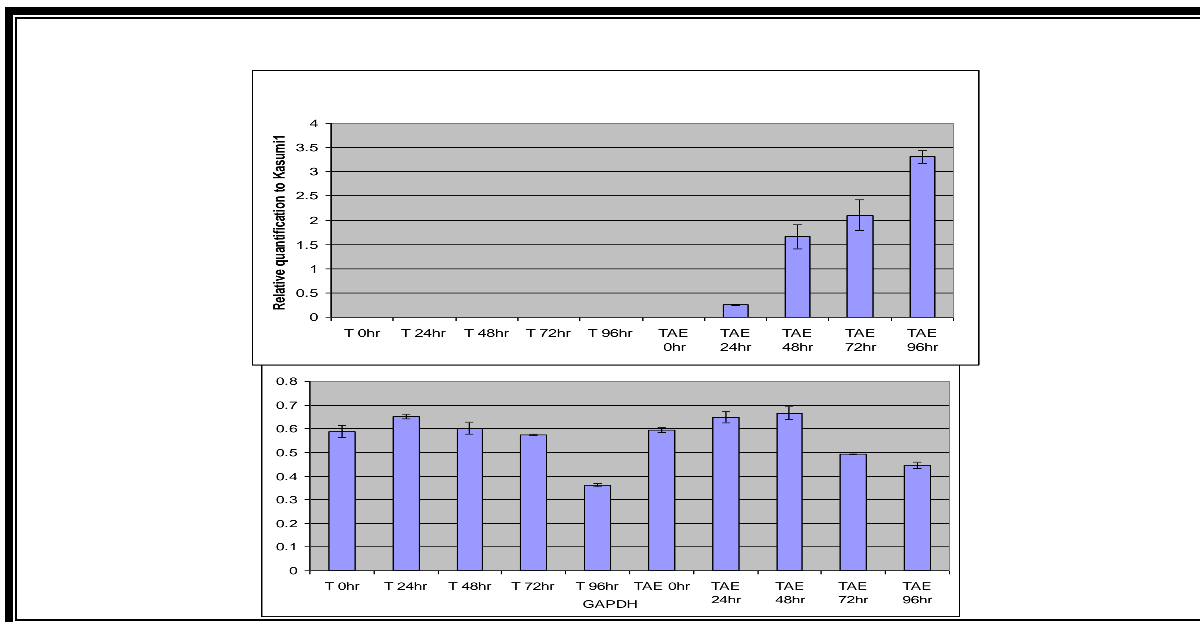

Fig 4 AML1-ETO mRNA expression is increased after tet-off induction in U937T-A/E CELL LINES

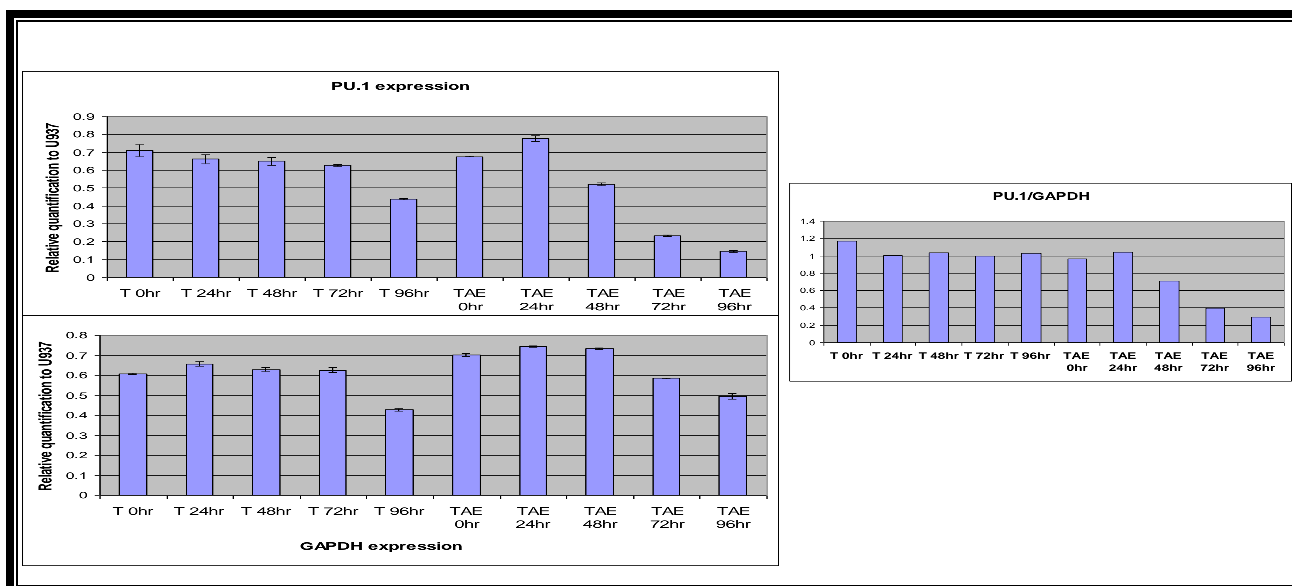

Fig 5 AML1-ETO downregulates PU.1 expression in U937T-AE cells

The protein level of NM23 is increased after AML1-ETO induction and NM23 mRNA expression is elevated in t(8;21)-AML patient samples: To further confirm the proteomics data, AML1-ETO expression was induced in U937T/A-E cells and the expression of NM23 protein was analyzed by Western-blotting. AML1-ETO increases the NM23 protein level (fig6 a lanes 3-5) in comparison with empty vector U937T cells (fig6 a lane 1) and U937T/A-E cells at 0 hour (fig.6 a lane 2). Since the NM23 expression is decreased during induced differentiation of leukemic cell lines MEG-01 and HL60, these data suggest that one possible mechanism of AML1-ETO-induced leukemia could be via the increase of NM23 protein level.

To rule out false positives, it was important to confirm more than one target by an independent method. Therefore, we analyzed the Prohibitin expression by Western-blotting to further validate the proteomics data. Induction of AML1-ETO expression increased the Prohibitin protein level (fig 6 b lane 4).My data suggests that NM23 ia a target of AML-ETO. It is interesting to note that so far the molecular mechanism of how NM23 expression is regulated in AML or how NM23 blocks granulocytic differentiation is not known. Most of the studies have focused on analyzing mRNA or protein levels of NM23 in AML patient samples and correlating it to prognosis. Therefore, further we sought to investigate the mechanism how AML1-ETO might regulate NM23.

(a) (b)

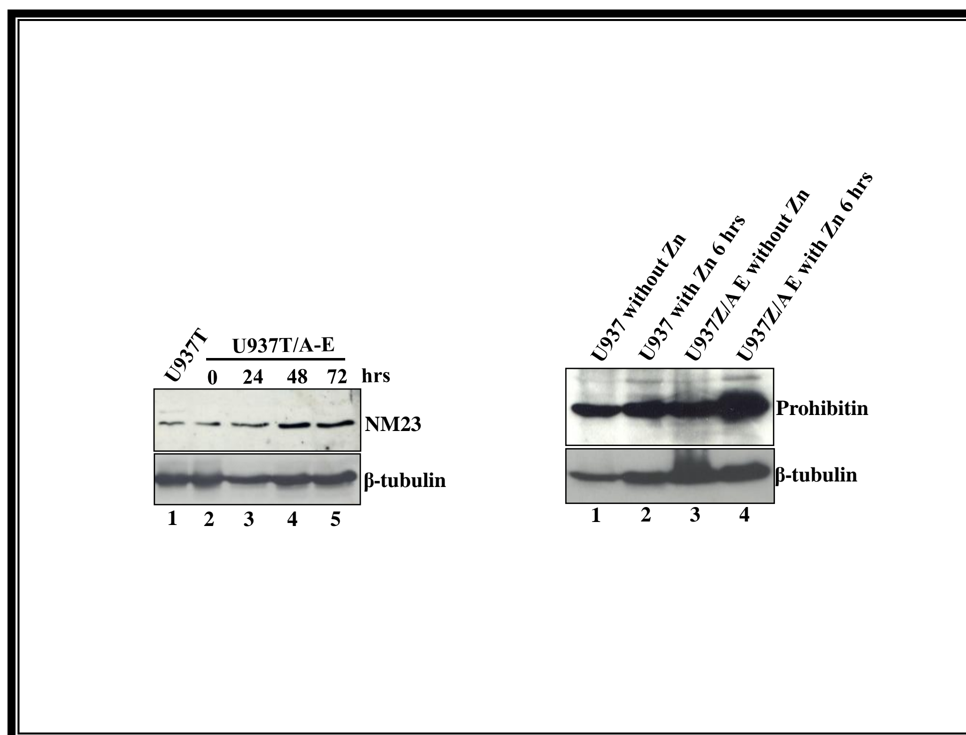

Fig. 6: Confirmation of AML1-ETO-induced pathways (western blot)

Figure 6: AML1-ETO up regulates the protein expression of NM23. (a) U937T/A-E cells were induced by removing tetracycline from the medium for 24, 48 and 72 hours. Cells were lysed in RIPA lysis buffer, 80 µg protein was separated on 12% SDS-PAGE and blotted for NM23 with anti-NM23-H2 goat polyclonal antibody . NM23 expression was upregulated in AML1-ETO induced cells (lanes 3-5) in comparison to empty vector U937T cells (lane 1) and U937T/A-E cells at 0 hour (lane 2) of upper panel. The same blot was stripped and probed for β-tubulin for loading control (lower panel). (b) Western-blot analysis for Prohibitin from U937Z/ A-E cells using anti-Prohibitin antibody shows Prohibitin is upregulated in with AML1-ETO expression after 6 (upper panle). The same blot was stripped and probed for β-tubulin for loading control (lower panel)

AML1-ETO upregulates NM23 by blocking the ability of CCAAT enhancer binding proteins (C/EBP) to downregulate the NM23 promoter: NM23 and AML1-ETO are involved in a block of granulocytic differentiation and C/EBPalpha induces granulocytic differentiation.I hypothesized that C/EBPs may downregulate NM23 expression and AML1-ETO might inhibit this function in order to increase the protein level of NM23 as seen in (fig6 a).I, therefore, transiently transfected myeloid U937 with NM23h1-luc promoter and C/EBPalpha (fig7 a), C/EBPbeta (fig7 b) or C/EBPdelta (fig.7 c). 24h post transfection the promoter-luciferase activity was measured which shows that C/EBPalpha downregulated NM23 promoter 2 fold (fig.7 a), C/EBPbeta 4 fold (fig.3.5b) and C/EBPdelta 5 fold (fig.7 c). When AML1-ETO was cotransfected with C/EBPalpha, C/EBPbeta or C/EBPdelta, the downregulation of NM23 promoter by C/EBPs was blocked.[19]

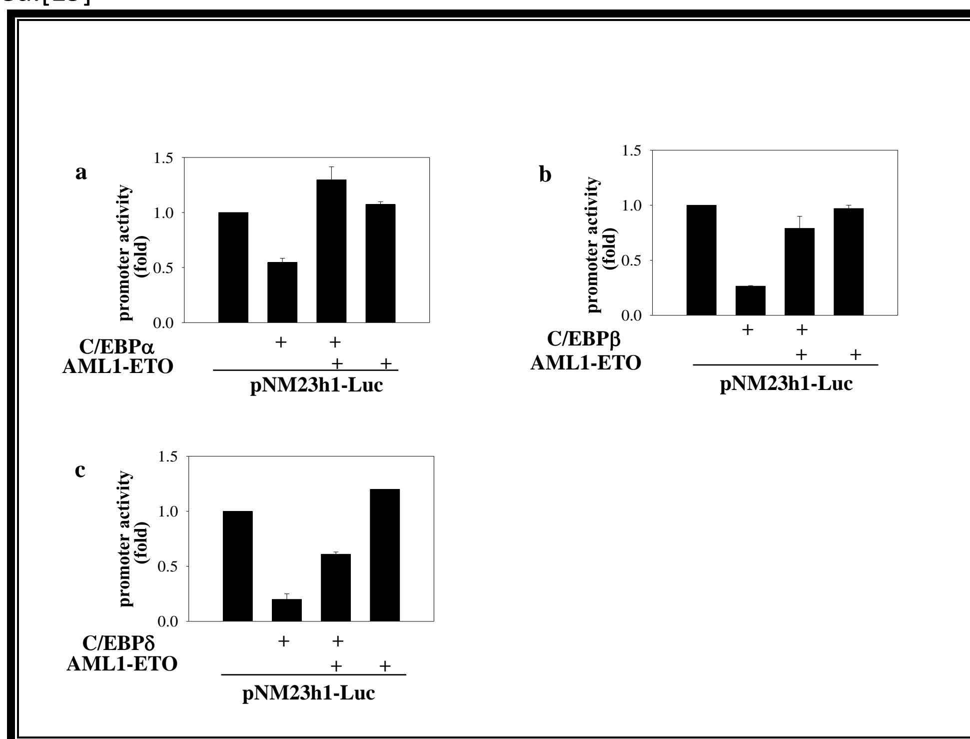

Fig. 7 The AML1-ETO-C/EBP-NM23 pathway

Fig. 7: AML1-ETO blocks the C/EBP proteins mediated NM23 inhibition: U937 cells were transfected with 0.2 µg NM23h1-luc promoter (provided by Dr.Sheo mohan singh), 0.1 µg C/EBPalpha (a), or 0.1 µg C/EBPbeta (b) or 0.1 µg C/EBPdelta (c) 0.2 µg AML1-ETO.

DISCUSSION

The proteomic approach is being routinely applied to the molecular analysis of various human diseases and in particular cancers such as breast, bladder, colorectal, stomach cancers, and so forth. However, few have been reported on the systematic identification of protein profile changes under a given condition of leukemia.NM23 is target of AML/ETO in western

blotting, and further validated the upregulation of Prohibitin ruling out the possibility of identified protein being false positives. Although others have also shown that NM23 is target of AML/ETO, no one has thus far shown the biological relevance of NM23 being a target of AML1/ETO. Apart from NM23, HSP27 another target from identified list was upregulated at mRNA level in various AML samples. Although assessing expression of some of the identified proteins as downregulated both at protein and mRNA level would had been ideal, however, none of them were interesting and hence not considered. Overexpression of NM23 in myeloid precursor 32Dcl3 cells blocks GCSF-induced granulocytic differentiation and its expression increases in proliferating hematopoietic cells, whereas overexpression of C/EBPalpha leads to granulocytic differentiation and downregulation of cell proliferation. Furthermore, since AML1/ETO disrupts myeloid differentiation by inhibiting C/EBP alpha I hypothesized that C/EBPs may inhibit NM23 expression. Using luciferase promoter assay I confirmed that C/EBPs do inhibit NM23 expression and this inhibition was drastically overcome by cotransfectoion of AMl/ETO with C/EBPs proteins. C/EBP beta and delta had more inhibitory effect on NM23 promoter which could be due to the presence of variable protein complexes at the target NM23 promoter. Luciferase assay using NM23 promoter shows that C/EBP alpha indeed binds to NM23 promoter and could possibly mediate its inhibitory upon NM23. Based on my data I propose a hypothetical model (fig.8) suggesting how AML1-ETO might block myeloid differentiation by blocking the ability of C/EBP proteins to downregulate the NM23 promoter[18]. My data suggest that C/EBP proteins downregulate NM23 which might be a prerequisite for normal cell growth and differentiation. AML1-ETO blocks this downregulation, thereby increasing the protein level of NM23 which might lead to a block in differentiation and increase in cell proliferation. Thus, proteomic pathway discovery can identify novel functional pathways in AML, such as the AML1-ETO-C/EBP-NM23 pathway, as key step towards a systems biology and therapy of AML. (Note: Formulation of this model and results were done under guidance of Dr sheo mohan singh and Dr jayeeta Banerjee)

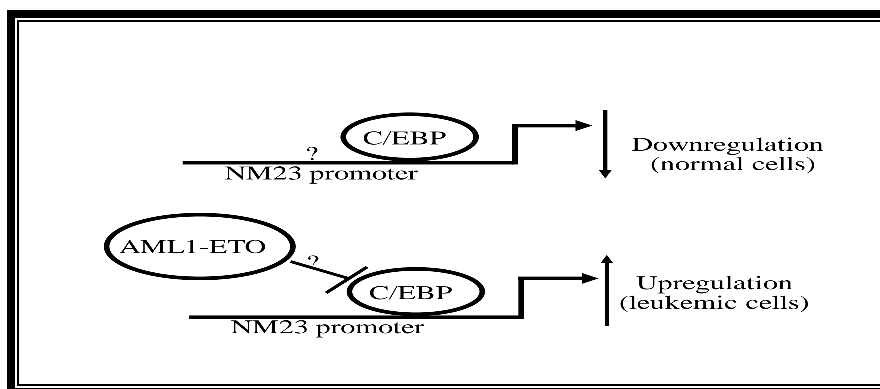

FIG. 8: PROPOSED MODEL: AML1-ETO UPREGULATES NM23 BY BLOCKING C/EBP BINDING TO NM23 PROMOTER

Fig. 8: Model: AML1-ETO might block myeloid differentiation by blocking the ability of C/EBP proteins to downregulate the NM23 promoter. My data suggest that C/EBP proteins downregulate NM23 which might be a prerequisite for normal cell growth and differentiation.

AML1-ETO blocks this downregulation thereby increasing the protein level of NM23 which might lead to a block in differentiation and increase in cell proliferation as seen in AML.

ACKNOWLEDGEMENT:

I thank Dr.Sheo mohan singh for his constant support and guidance.

References: Referneces should be referred to a number [1] in the text and be listed according to this numbering at the end of the paper. The references shoulld comprise the following information and in the given order and with given punctuation as given in the example below: Author name (s) Initials (s), Publication Title Year of Publication; Issue: Page number.

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