Altering microRNA miR15a/16 Levels as Potential Therapy in CLL: Extrapolating from the *De Novo* NZB Mouse Model

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1. Introduction

1.1 Chronic lymphocytic leukemia (CLL)

CLL is a hematological malignancy characterized by accumulation of B-1 cells in peripheral lymphoid organs, bone marrow and peripheral blood. It is the most common lymphoid malignancy in the Western World, accounting for 30% of all leukemias. Although the median age at diagnosis is 73, our ever increasing lifespan has put the lifetime risk of developing CLL at 1 in 210 people (NCI, 2011). In addition, since its first description more than 150 years ago, the etiology of CLL remains largely unknown. Hence, it is imperative to study this largely geriatric and incurable disease in more detail.

Diagnosis is made based on the presence of B-lymphocytosis (>5000/ul of peripheral blood), and in particular the expansion of $CD5^+CD19^+CD20^{dull}CD23^+IgM^{dull}$ B cells [Reviewed by (Hallek et al., 2008)]. The disease is usually asymptomatic and as a result in most cases it is diagnosed during a routine blood test. Clinically CLL is most commonly classified using the modified Rai Staging System or Binet Classification (Hallek et al., 2008). With recent advances in screening procedures, increasing number of patients are been diagnosed at Rai Stage 0 (Shanafelt, 2009). The current treatment protocol adopts the 'wait and watch' policy until the disease progresses or becomes symptomatic since treatment does not offer any survival advantage (Mhaskar et al., 2010). Based on the rate of disease progression, CLL can be classified as either Aggressive or Indolent, with either type exhibiting a characteristic molecular signature. Grossly, aggressive CLL is characterized by high ZAP70 (a kinase not normally expressed in B cells which is detected by flow cytometric techniques) and unmutated IgH V_H whereas indolent is characterized by low ZAP70 and mutated IgH V_H [Reviewed by (Gribben and O'Brien, 2011)].

The circulating B-CLL cells have an apoptosis defect and are hence long lived. Spleen, bone marrow and lymph nodes are believed to be proliferating centers and replenish the peripheral B-CLL cells [Reviewed by (Damle et al., 2010)]. Although traditionally B-CLL was described as accumulation of quiescent B-1 cells in the periphery, recent in vivo kinetic studies using deuterium (Messmer et al., 2005) or deuterated glucose (van Gent et al., 2008) have shown that 0.08-1.76% of new CLL cells are generated per day.

1.2 NZB as a mouse model of CLL

Mouse models are very crucial for the study of human malignancies since unlike in vitro cell culture systems they allow the study of complex interplay of cells involved in tumor formation and maintenance. Currently there are several transgenic mouse models of CLL available, for example: Tcl1 transgenic, TRAF2DN/Bcl2 transgenic, miR155 transgenic [Reviewed by (Pekarsky et al., 2010). Although transgenic models can be used to ascertain the oncogenic potential of candidate genes, they make poor models for a more holistic study of the tumor development and progression since cancer is a multifactorial disorder. Hence, de novo mouse models that can faithfully mimic human malignancy are a better system for the latter purpose. Our lab has long been interested in the study of CLL biology using the NZB mouse model. The disease penetrance is near 100% in these mice indicating the presence of a strong genetic bias. Similar to CLL patients, NZB mice exhibit age associated spontaneous development of CD5$^+$B220^{dull}IgM$^+$ B-1 cell malignancy [Reviewed by (Scaglione et al., 2007)]. These mice also exhibit an underlying autoimmunity characterized by the presence of anti-RBC and anti-DNA antibodies and hence are used as a model for Sytemic Lupus Erythematosus (SLE) [Reviewed by (Scaglione et al., 2007)]. The underlying autoimmunity makes these mice an even more faithful model of human CLL since 10-25% of patients develop Autoimmune Hemolytic Anemia (AIHA) and 2% of patients develop autoimmune thrombocytopenia (Kipps and Carson, 1993).

Recent evidence suggests that almost all cases of CLL are preceded by an asymptomatic precursor stage of monoclonal or pauci-clonal B cell lymphocytosis (<5x10^9/l) termed MBL (Shim et al., Caporaso et al., 2010, Rawstron et al., 2002). Most subjects possess MBL whose immunophenotype is similar to CLL. Although the incidence of expression of prognostic markers like Zap70 and CD38 was less than that observed in CLL, approximately 70% of MBL cases in families with a history of CLL possess the 13q14 deletion (Lanasa et al., 2011). We have recently shown that NZB mice also exhibit this pre-cursor MBL stage, further validating it as a true model for human CLL (Salerno et al., 2010).

1.3 Genetic abnormalities in CLL

In their seminal review, Hanahan and Weinberg proposed that genetic abnormalities underly the six hallmarks of cancer: Constitutive proliferative signaling, Immunity to tumor suppressors, Apoptosis Evasion, Limitless Replicative Potential, Sustained Angiogenesis and Metastasis (Hanahan and Weinberg, 2000). With the advent of High Throughput DNA Sequencing, this theory has gained further credence and it is now widely accepted that cancer arises due to a series of genetic hits (Reviewed in (Hanahan and Weinberg, 2011)). Some of the frequently observed chromosomal abnormalities in CLL include 11q23 deletions (contains ATM and miR34b/miR34c cluster), trisomy 12 (increase in MDM2), 17p deletions (contains p53) (Dohner et al., 2000). However, the most common chromosomal abnormality observed in CLL patients (50-60%) is 13q14 deletion (contains miR15a/16-1) (Dohner et al., 2000). This region is also deleted in 50% of Mantle Cell Lymphomas and 40% of Multiple Myeloma indicating that it harbors critical tumor suppressor genes (Chang et al., 2004, Chen et al., 2007, Flordal Thelander et al., 2007). Detailed characterization of the 13q region in CLL patients led to the discovery of a 130kb Minimal Deleted Region (MDR) centromeric to the marker D13S272 (Corcoran et al., 1998, Migliazza et al., 2001). Potential CLL-associated tumor suppressor genes in the MDR identified by earlier studies include Exon 1 of Dleu1,

Dleu2, Dleu5, Dleu7 and Kcnrg. However, currently only Dleu2 and Dleu7 have been demonstrated to have tumor suppressive functions in CLL (Klein et al., 2010, Palamarchuk et al., 2010).

1.3.1 Role of 13q14 locus in CLL

In a *Blood* plenary paper, we reported the linkage of three loci - D14Mit160, D18Mit4, and D19Mit6 – to the presence of lymphoproliferative disease (LPD) in NZB mice (Raveche et al., 2007). Due to the homology to human Chr.13, we further analyzed the candidate genes in the D14Mit160 locus and discovered an association between miR15a/16-1 and CLL.

The highly conserved large non-coding RNA, Dleu2, is the host transcript for the biscistronic microRNAs miR15a/16-1 (Calin et al., 2002). It is located within intron 4 of the Dleu2 transcript in mouse (See Fig.1A) and within intron 3 in human (See Fig.1B). Dleu2 has not been shown to encode any protein, yet there is high degree of sequence homology between mouse and human suggesting that it is biologically very important (See Fig.1C). Currently it is unclear whether the full length Dleu2 transcript is functionally important; however critical functions have been assigned to two genes encoded within Dleu2. In addition to miR15a/16-1, Dleu2 transcript also encodes an anti-sense for Dleu5 (also called Rfp2 of Trim13) (Corcoran et al., 2004). Dleu2 is transcribed from the reverse strand while Dleu5 is transcribed from the forward strand in Chr.13 in humans and Chr.14 in mice. In humans, there is partial overlap between Dleu5 and Dleu2 genes leading to the formation of a sense-anti-sense pair. In mice a region of Dleu5 has been duplicated and inserted upstream into Dleu2 giving rise to a sense-anti-sense pair even in the absence of physical overlap. In humans miR15a/16-1 is upstream of Dleu5 antisense whereas in mice it downstream of the Dleu5 antisense. The interaction between Dleu5 and Dleu2 is represented schematically in Fig.1D. Dleu5 protein contains a tripartite Ring finger B-box coiled-coil domain (RBCC) and thus belongs to the RBCC or Trim family of proteins. It is frequently deleted or downregulated in various malignancies. It functions as a novel E3 ubiquitin ligase (Lerner et al., 2007) and can cause proteosomal degradation of MDM2 and ATM thereby enhancing DNA damage induced apoptosis (Joo et al., 2011).

1.4 MicroRNA as oncomiRs or tumor suppressor miRs

microRNA genes are frequently located at cancer associated loci or fragile sites making them vulnerable to genetic lesions (Calin et al., 2004). Similar to other regulatory elements like transcription factors, dysregulation of microRNAs has been implicated in the pathogenesis of different types of cancer. Based on the genes they target, their up or down regulation could have an oncogenic effect. miR17-92 cluster was the first reported oncomiR and its upregulation accelerated lymphoma development in a mouse model of B cell lymphoma [Reviewed by (van Haaften and Agami, 2010)] indicating that it possesses direct oncogenic potential. Since then a number of other microRNAs like miR21, miR155, miR29 etc have been shown to function as oncomiRs in a number of tumor types. On the opposite end of the spectrum are tumor suppressor microRNAs like miR15a/16-1 whose down-regulation is associated with CLL pathogenesis (Calin et al., 2002). The expression of miR15a/16-1 is also frequently reduced in prostrate cancer and exogenously increasing the level of these microRNAs had a therapeutic effect on xenograft models of prostrate cancer (Bonci et al., 2008).
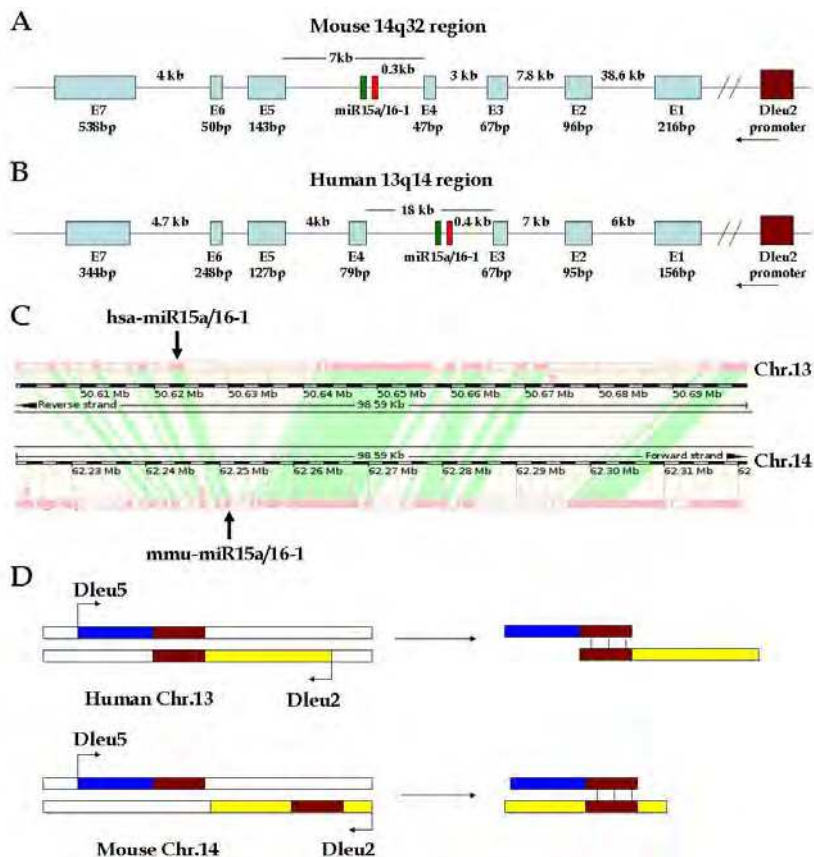
Fig. 1. Genomic Organization of Dleu2 Cluster in Mouse and Humans: Schematic representation of Dleu2 gene in mouse – Transcript ID ENMUST00000152279 (A) and in human – Transcript ID ENST00000416253 (B). In both humans and mice, miR15a/16-1 is found in the large intronic region of this large non-coding RNA (lncRNA), DLEU2. C) Human and mouse Dleu2 genes are aligned against each other. The homologous regions are shown by connecting green bars. D) Schematic representation of sense-anti-sense pairing of Dleu2 and Dleu5 transcripts in human (upper) and mouse (lower).

1.5 Serum microRNAs as biomarkers

MicroRNA levels in serum can serve as noninvasive biomarkers for diagnosis of hepatitis B, cardiovascular diseases, various cancers and a potential host of other diseases. For example, serum levels of miR-141 can be used to differentiate patients with prostate cancer from normal healthy controls with elevated levels of this miRNA in patient's serum (Mitchell et al., 2008). In another study, four miRNAs, miR-21, miR-210, miR-155, and miR-196a, were assayed in plasma and shown to be associated with pancreatic adenocarcinoma thus offering blood-based biomarkers (Wang et al., 2009). In a recent study it was shown that elevated miRNA levels in serum may also offer early CLL

detection and differentiation between Zap70 status (Moussay et al., 2011). Earlier studies were mainly focused on serum microRNA levels in solid tumors; however, this study also showed that hematological malignancies also harbor increased serum microRNA levels. The authors further concluded that increased expression of miR-150, miR-29a, miR-222 and miR-195 can be used as a highly sensitive diagnostic test for CLL. Interestingly the level of miR16-1 was elevated in CLL patients as compared to healthy controls. We have observed a similar increase in the plasma level of miR15a in our NZB mouse model as compared to wild type mice. This finding is intriguing since the cellular level of miR15a/16-1 is reduced by up to 50% in NZB mice and CLL patients. The finding that serum levels of miR15a/16-1 are somewhat increased in NZB mice and CLL patients as compared to control was opposite to anticipated. There seems to be a disconnect between the cellular and serum levels of miR15a/16-1. However, enhanced exosomal secretion could be responsible for this disconnect. Exosomes are cargo containing nano-vesicles (30-100 nm) that are secreted by numerous cell types and proteomic studies have shown that they harbor an abundance of microRNAs, mRNAs and proteins characteristic of their particular cellular origin. Exosomes have been shown to transfer both cellular and viral microRNAs that can give rise to pathological consequences like malignant transformation (Meckes et al., 2010, Valadi et al., 2007). Studies have demonstrated that malignant cells have increased microvesicle formation than do non-malignant cells (Ghosh et al., 2010). We speculate that the elevated serum miR15a/16-1 in NZB mice may be the result of increased exosomal packaging of all the microRNAs in the malignant cells, including the reduced level of miR-15a/16.

2. Results

2.1 Mutated miR15a/16-1 loci in CLL patients and NZB mice

We recently reported the presence of a mutation and a deletion in the 3' flanking region of miR15a/16-1 gene of NZB mouse model of CLL (See Fig.2A) (Raveche et al., 2007). This mutation in NZB is at a nearly identical location as a C to T point mutation found in CLL patients (Calin et al., 2005). However, this mutation is rare in patients (Calin et al., 2005),(Yazici et al., 2009). We have also shown that this mutation and deletion is associated with almost a 50% reduction in the cellular level of mature miR15a/16-1 in the NZB mice (See Fig.2B) and the NZB derived cell line, LNC (See Fig.2C). Although the expression of miR15a/16-1 is reduced in both B-1 and B-2 cells in the NZB (as compared to non-NZB strain), pathologic consequences are observed only in the B-1 cells. A number of targets have been validated for miR15a/16-1; however, targeting of critical anti-apoptotic and cell cycle regulatory proteins like Bcl-2 and Cyclin D1 respectively is of particular importance in CLL pathogenesis (Salerno et al., 2009). Thus, the reduced expression of miR15a/16-1 confers an anti-apoptotic phenotype to the cells. Recently a transgenic mouse having conditional knock-out of the entire MDR region or the miR15a/16-1 region was generated (Klein et al., 2010). 42% of the MDR-/- mice and 26% of the miR15a/16-/- mice developed a lymphoproliferative disorder at 15 to 18 months of age. Moreover, CD19-cre driven knockout gave rise to an apoptosis defect in the B cells. In summary these findings show that the miR15a/16-1 locus plays a critical tumor suppressive role in CLL. Therefore, we hypothesized that increasing the miR15a/16-1 levels can serve as a novel therapeutic strategy for CLL.
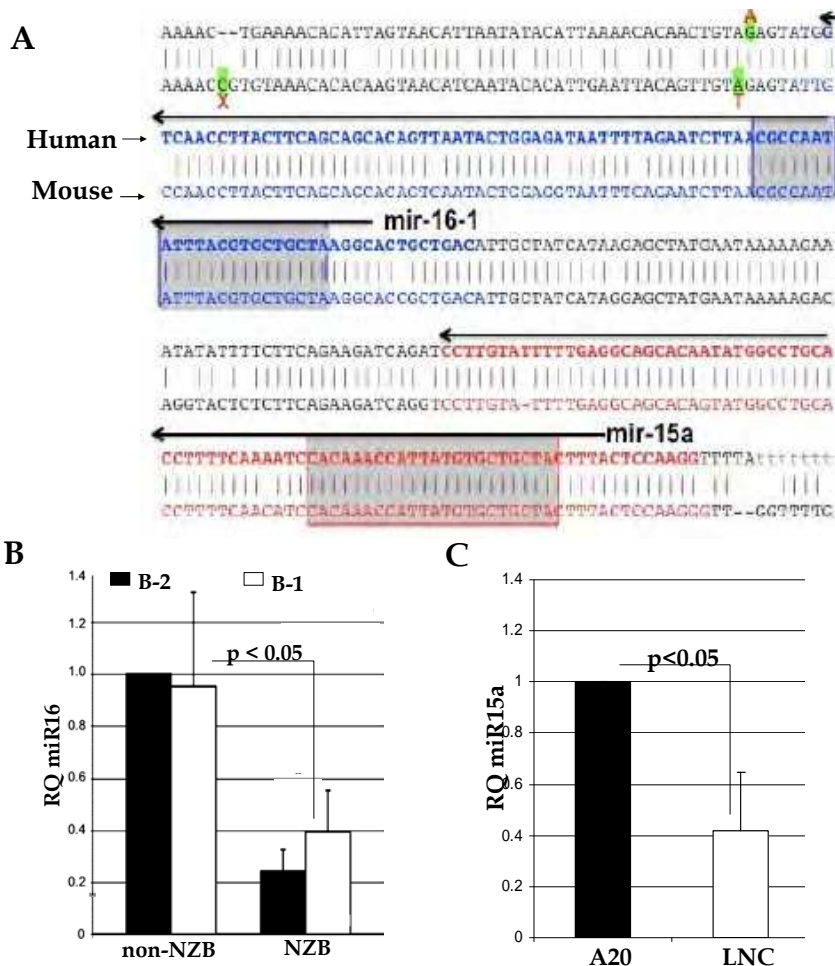
Fig. 2. Identification of Point Mutation and Deletion in miR15a/16-1 Flanking Region: A) Wild type sequence of human (top row) and mouse (bottom row) miR15a/16-1 and its flanking region is shown. The mature miR15a sequence is in red and mature miR16-1 sequence is in blue. Sequencing of this region led to the discovery of an A to T point mutation and deletion of C (40bp upstream of miR16-1) in NZB mice. A homologous A to G point mutation was found in a subset of CLL patients. The substituted nucleotide is written below the corresponding wild type base and the deletion is indicated by an 'X'. B) Spleen cells from NZB and non-NZB mice were sorted into B-2 (IgM+B220+) and B-1 (IgM+B220dull). The expression of mature miR16 in the sorted cells was measured using TaqMan miR-16 Assay according to manufacture's instructions. Data from three independent sorts was analyzed using student's t test (p<0.05). C) The expression of mature miR15a was compared between the NZB derived cell line LNC and a non-NZB derived B cell line A20 using TaqMan miR-15a Assay. Data from three independent experiments was analyzed using student's t test (p<0.05).

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MicroRNAs are usually present in intergenic regions and may possess their own promoter or use the host gene promoter. Whether miR15a/16-1 is transcribed from its own promoter or from Dleu2 promoter or a combination of both, is controversial. However, data from our lab supports that it depends on the Dleu2 promoter since there is a strong positive correlation between the level of Dleu2 and miR15a/16-1 transcripts. RNA pol II transcribes the host gene to form a long primary transcript (pri-miR). In the nucleus the pri-miR transcript is processed to a 60-70nt long stem loop precursor transcript (pre-miR) by the RNase III enzyme Drosha. The pre-miR is then exported to the cytoplasm via Exportin 5 where it is further cleaved by Dicer to give the 22nt long mature microRNA duplex. Preliminary data from our lab suggests that the mutation and deletion leads to defective processing of pri-miR15a/16-1 to mature miR15a/16-1. We speculate that the mutation and deletion may lead to the formation of an unstable stem loop structure or inhibit the binding of Drosha.

2.2 In vitro miR15a/16 upregulation in NZB derived malignant CLL cell line

We hypothesized that the reduced miR15a/16-1 levels observed in CLL lead to an apoptosis defect. Our lab has previously developed the cell line LNC (CLL cell line derived from a NZB mouse lymph node) (Peng et al., 1994). LNC cells make a great in vitro system for studying the effect of mutated miR15a/16-1 loci since they have retained the NZB miR15a/16-1 genotype. Similar to the NZB mice, as compared to a non-NZB B cell line, the level of mature miR15a/16-1 in LNC cells is reduced by as much as 50% (See Fig.2C). In order to test our hypothesis, we employed 1) microRNA mimics or 2) replication incompetent lentiviruses; to artificially increase the level of mature miR15a/16-1.

2.2.1 Effect of miR15a/16 mimics on LNC

microRNA mimics are commercially available double stranded RNA oligonucleotides that resemble endogenous mature microRNA molecules. They are commonly used to transiently increase the expression of microRNA in vitro and more recently in vivo as well (Trang et al., 2011). Transfection of miR15a or miR16 mimics led to a significant increase in the percentage of cells in G1 and a decrease in the S phase as compared to negative control mimics (See Fig.3A). miR15a/16-1 targets cyclin D1 and hence we hypothesized that the observed cell cycle arrest could be in part attributed to cyclin D1 degradation. microRNAs have been shown to reduce target gene expression either by mRNA decay or instabilty or by translational repression. microRNAs can interfere with protein translation by blocking initiation or elongation stage, as well as by promoting pre-mature termination and co-translational protein degradation [Reviewed in (Huntzinger and Izaurralde, 2011)]. Although we did not see a difference in the mRNA level of cyclin D1, we observed a reduction in the protein levels in NZB cell line expressing high level of miR15a/16 (See Fig.3B). Thus, miR15a/16-1 seems to interfere with translation of cyclin D1 mRNA and not cause mRNA degradation. However, since the effect of microRNA mimics is transient, we then derived stable LNC sub-lines having increased miR15a/16 levels using a lentiviral approach.
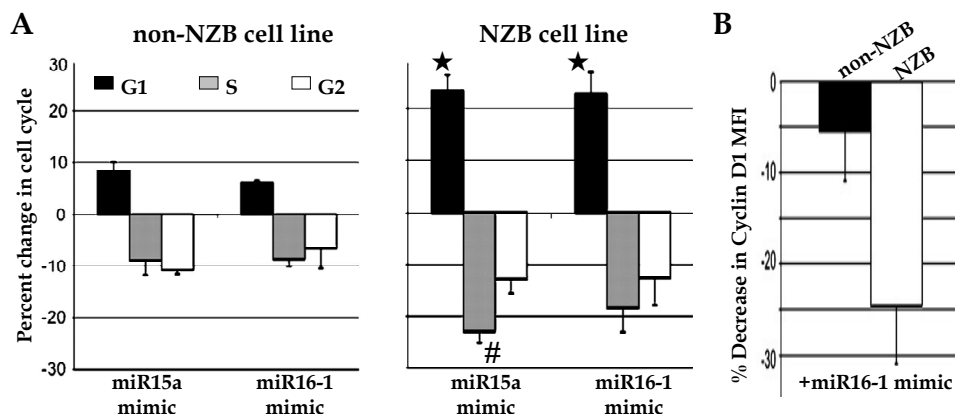
Fig. 3. Growth Inhibitory Effect of miR15a and miR16-1 mimics: NZB cell line and non-NZB cell line were transfected with 3ug of mmu-miR15a or mmu miR16-1 mimic or a non-targeting negative control mimic (Dharmacon) using Amaxa Nucleofection and analyzed 24hrs later. A) The transfected cells were stained with hypotonic prodium iodide and acquired on BD FACS Calibur to analyze the cell cycle distribution. Y-axis is the change in the percentage of cells in different cell cycle phases relative to the negative control mimic. As compared to a non-NZB cell line, a significant increase in cells in G1 (★ $p<0.05$) and a significant decrease in cells in the S phase (# $p<0.05$) was observed. Similar results were obtained with both miR15a and miR16-1 mimic. B) Cyclin D1 protein level was measured using intracellular flow cytometry. The percent decrease in mean fluorescence intensity (MFI) of cyclin D1 in miR16-1 mimic treated cells relative to the negative control mimic is plotted on the Y-aixs.

2.2.2 Effect of stable miR15a/16 increase using lentivirus

HIV-1 derived lentiviruses as a tool for stable delivery of genetic material were first described by Naldini et al (Naldini et al., 1996a, Naldini et al., 1996b). They are a type of retrovirus and can be used to target up to 8kb of genetic material to a broad variety of cell types (proliferating as well as quiescent). Lentiviruses are safer gene delivery vehicles than earlier viral vectors like adenovirus, gamma-retrovirus and adeno-associated viral vectors. Lentiviruses are less immunogenic than adenoviruses [Reviewed by (Nayak and Herzog, 2010)]. Early gene therapy trials for the treatment of X-linked SCID utilized gamma-retroviruses to deliver the therapeutic gene to patient stem cells ex vivo. Two out of the nine children treated successfully developed T cell Acute Lymphoblastic Leukemia (T-ALL) 3 years post treatment due to insertional activation of the LMO2 proto-oncogene (Hacein-Bey-Abina et al., 2003). Gamma retroviruses primarily integrate into 5' region of genes and the strong enhancers present in the viral LTR can hyper-activate the adjoining gene promoters leading to tumorigenesis [Reviewed by (Bushman et al., 2005)]. In contrast, inspite of greater integration load lentiviruses did not enhance tumorogenesis since they target other areas of gene rich regions (Montini et al., 2006). The lentivirus employed in our experiments is Self-Inactivating (SIN) and hence its LTR lacks strong viral promoters, thereby further reducing the risk of insertional gene activation (Zufferey et al., 1998). Owing to their broad tropism they pose a potential

biosafety hazard. However the 3rd generation of lentiviruses has been engineered to be replication incompetent and the amount of HIV genome has been reduced to 20%.

We utilized a custom made lentiviral vector encoding miR15a/16-1 from System Biosciences, to stably increase the expression of these two microRNAs in vitro and later in vivo. The lentiviral vector also has a puromycin resistance and a GFP expression cassette for selection of transduced cells. LNC cells were transduced overnight with miR15a/16-GFP-puro lentivirus (miR lentivirus) or an empty GFP-puro lentivirus (GFP lentivirus) at an MOI of 10 in the presence of 4ug/ml polybrene. The miR lentivirus transduced cells were then sorted on the basis of GFP (See Fig.4A, left) and maintained in media containing puromycin to obtain stable sub-lines (GFP low and GFP hi) (See Fig.4A, right). The cells transduced with miR-lenti exhibited 50% increase in the sub-G1 or apoptotic population as compared to those transduced with GFP-lenti (See Fig.4C). The sub-lines were characterized further for the expression for miR15a and its effects on cell cycle. The expression of miR15a was significantly higher in GFP low and GFP hi as compared to untransduced LNC cells (See Fig.4B). A strong positive correlation exists between intensity of the GFP signal and miR15a/16-1 expression. The sub-lines also exhibit a significant reduction in the percentage of cells in the S phase indicating reduced proliferation in response to increase miR15a/16-1 levels (data not shown).
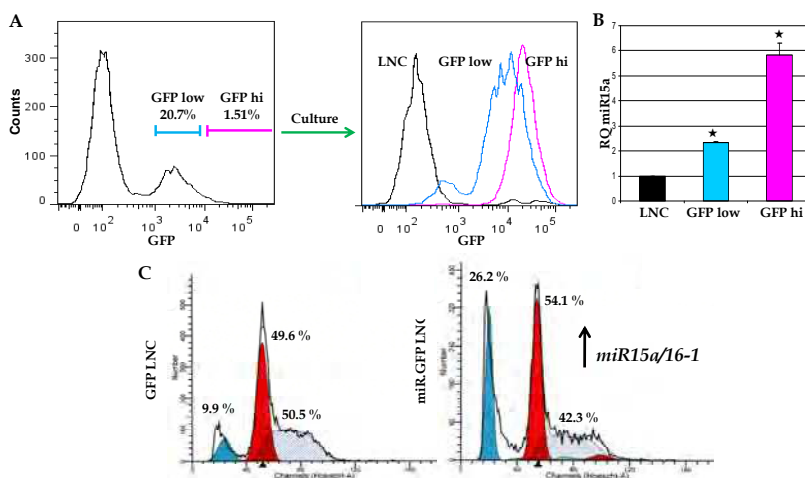

Fig. 4. In vitro Effects of Lentiviral Delivery of miR15a/16 Levels: The NZB derived B cell line LNC was transduced with lentivirus encoding bicistronic miR15a/16-1 (miR lentivirus) under the control of a CMV promoter or with a control empty lentivirus (GFP lentivirus). A) 48hrs post transduction of the miR lentivirus, cells were sorted based on GFP expression into GFP low and GFP hi populations according to the gating strategy indicated (left). The cells were then cultured and the GFP expression was measured using the BD LSR II instrument (right). Thus, two stable LNC sub-lines – GFP low and GFP hi - were established. B) The expression of miR15a was compared between LNC and the two new sub-lines using TaqMan microRNA Assay ($p < 0.05$). C) 48hrs post transduction cells were stained with Hoescht dye and their cell cycle was analyzed. A considerable increase in the sub-G1 peak is observed in the miR lentivirus transduced cells (right) as compared to the GFP lentivirus transduced cells (left).

2.3 Potential Triggers for regulation of miR15a/16 and B-1 clonal expansion

A single microRNA can critically regulate a number of genes in a cell type specific manner. Hence they can serve as very efficient effectors for master regulators like c-Myc. A single genetic hit involving this network could potentially lead to tumorigenesis since it can simultaneously disrupt multiple pathways. A recent report by Chang et al, gives credence to this theory since they showed that c-Myc induced expression of miR17-92 cluster and a more global repression of other microRNAs led to the development of B cell lymphomas (Chang et al., 2008). An increased c-Myc transcript level is associated with disease progression and severity in CLL patients (Halina et al., 2010). Interestingly miR15a/16 expression is negatively regulated by c-Myc via repression of the Dleu2 promoter (Lerner et al., 2009). However little is known about other transcription factors that can regulate microRNA transcription.

2.3.1 miR15a/16-1 Increase as a Consequence of BSAP knockdown

BSAP is a transcription factor considered to be a key regulator of B-lymphocyte development and is encoded by the PAX-5 gene. It plays a critical role in early B-cell lymphopoiesis and for progression beyond the pro-B-cell stage [Reviewed by (Cobaleda et al., 2007) (Nutt et al., 1998)] On the other hand, overproduction of BSAP in a late B-cell line was shown to suppress differentiation into plasma cells (Nera et al., 2006, Morrison et al., 1998). Malignant B-1 cells have been found to have increased BSAP levels (Chong et al., 2001). As normal B cells have been shown to react to IL-2 stimulation by BSAP downregulation continued BSAP expression in CLL could explain their blocked differentiation stage (Wallin et al., 1999).

We have previously shown that BSAP levels are higher in LNC cells as compared to a non-NZB cell line and normal B-1 cell. BSAP knockdown gives rise to a growth inhibitory effect in LNC cells but not in non-NZB cell line (Chong et al., 2001). A recent report showed that BSAP negatively regulates the promoter of Dleu2, the host gene for miR15a/16 in a lymphoma cell line Myc5 (Chung et al., 2008). However, since gene regulation is highly cell type specific, we first wanted to test whether a similar loop exists between BSAP and miR15a/16 in our system, especially due to the presence of the mutated miR15a/16 locus. siRNA mediated knockdown of BSAP (See Fig.5A) led to an increase in the level of mature miR15a/16 in LNC cells (See Fig.5B). Next we wanted to examine whether this increase in miR15a/16-1 expression was sufficient to give rise cell cycle arrest. Indeed, we observe an increase in the percentage of cells in the G2 phase in the siRNA treated cells as compared to the controls (See Fig.5C).

2.4 In vivo augmentation of miR15a/16 via lentivirus in NZB mice with CLL

Having successfully demonstrated the inhibition of malignant cell growth in vitro by increasing miR15a/16 levels by different strategies, we next wanted to test its therapeutic potential in vivo. In the current clinical trials involving lentiviruses, they are only used for adoptive transfer of ex vivo transduced cells or for intra-tumoral delivery. This is a serious drawback in the treatment of systemic diseases like leukemias. To overcome this caveat we attempted to employ lentivirus for systemic delivery of miR15a/16 in our murine model of B-CLL.
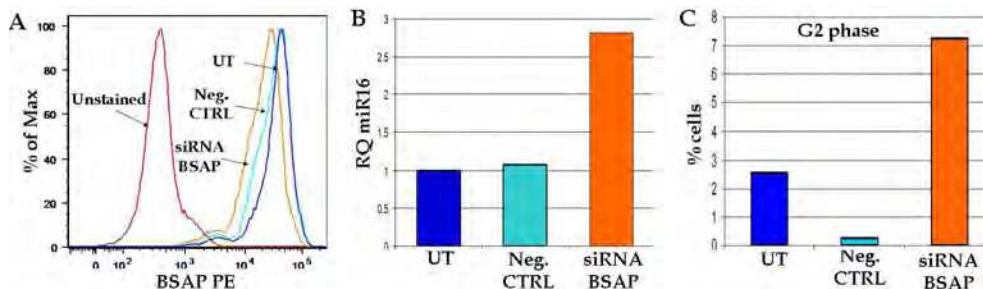
Fig. 5. In vitro effects of BSAP Silencing: NZB derived B cell line, LNC was transfected with 200nM siRNA against BSAP or with 200nM negative control siRNA using HiPerFect (Qiagen) according to manufacturer's instructions or were transfected (UT) and analyzed 24hrs later. A) Cells were stained with BSAP-PE antibody (eBiosciences) and acquired on BD LSR II. Single color PE histograms of different treatment groups have been overlayed. B) The levels of miR16-1 were measured using TaqMan MicroRNA Assay (Applied Biosystems). The reduced expression of BSAP observed by flow cytometry translated into increased miR16-1 expression in the siRNA treated groups. C) Cells were stained with hypotonic PI and the cell cycle distribution was assessed. Shown above is the percentage of cells in G2 phase of the cell cycle in the different treatment groups. Data is from a representative experiment. Similar trends were observed in the replicates.

Aged NZB mice were injected with lentivirus at day 0 and sacrificed on day 8 for the short term group or administered a second dose on day 24 and sacrificed on day 29 for the long term group. We were able to successfully increase the expression of miR15a/16 in NZB mice following intravenous (i.v) and intraperitoneal (i.p) injections of the lentiviral prep. Interestingly, the expression of miR15a/16 is elevated only in the transduced B-1 population. This could be due to cell type specific regulation of microRNA levels. In line with the in vitro data, systemic delivery of miR15a/16 led to a considerable reduction in the percentage of B-1 cells in the spleen as compared to control lentivirus treated mice both in the short term and long term study (See Fig.6A). Similar reduction was observed in the peritoneal cavity of these mice (Data not shown). In order to confirm that the lentiviral delivery led to an increase in miR15a/16-1 expression, we sorted the cells into B-1/GFP+ and B-2/GFP+ and quantified the levels of miR15a/16-1 using 100 cell RT-PCR. The expression of miR15a/16-1 was significantly increased in mice injected with miR-lentivirus as compared to those injected with the control lentivirus (See Fig.6B). Interestingly only B-1/GFP+ cells and not B2/GFP+ cells exhibited an increase in the miR15a/16-1 levels. B-1 cells were preferentially transduced in comparison to other cell types like B-2 and T cells (Data not shown).
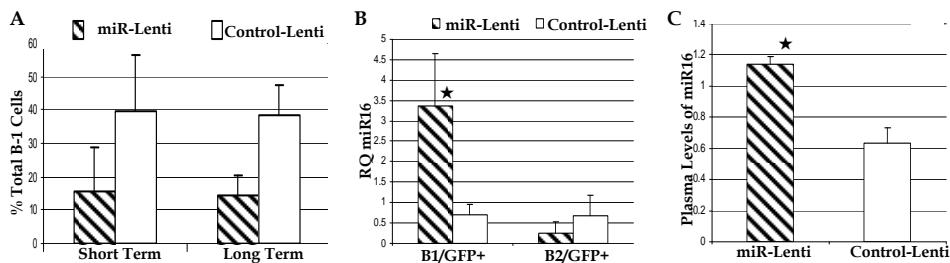
Fig. 6. In vivo effects of miR15a/16 increases on CLL in NZB mice: A) Percentage of total B-1 cells in the short term (n=3 per group) and in the long term (n=4 per group). B-1 cells were gated as CD5^{dull}B220$^{+/dull}$. B) Spleen cells from the short term treatment were sorted into B1 (CD5^{dull}B220$^{+/dull}$) and B-2 (CD5$^-$B220$^+$) and then further sorted based on GFP expression. 100 cell RT-PCR was performed to quantitate the level of miR16 in the sorted cells. n=3 per group, ★ B1/GFP+ from miR-Lenti Vs B1/GFP+ from Control-lenti, p<0.05. C) Mice were bled retro-orbitally into EDTA tubes; RNA was extracted using Trizol-LS reagent and used to perform TaqMan miR16 Expression Assay (Applied Biosystems). n=3 per group. ★ miR16 in miR-Lenti group Vs control-Lenti group, p<0.05.

With the advent of microRNA based therapy, it is critical to devise means to study its pharmacokinetic properties. Just before sacrificing, plasma was collected and miR16 levels were measured in the short term group. Even 8 days post injection, the level of miR16 was significantly elevated in the miR-Lenti mice as compared to the control-Lenti mice (See Fig.6C).

2.5 Alteration of miR15a/16 in human CLL using BSAP knockdown

Next, we wanted to extrapolate the findings from the mouse model presented above to patient cells. CLL patient PBMCs were purified using Ficoll-Hypaque Density Gradient Centrifugation. Malignant B-1 cells isolated from patient blood are quiescent and BSAP was knocked down using siRNA and its effects were studied at 24, 48, 72 and 96hrs. The reduced levels of BSAP protein translated into increased miR15a/16-1 as well as an increase in the percentage of cells undergoing apoptosis (See Fig.7). However, although the BSAP levels were greatly reduced even at 96 hrs post transfection, the miR15a/16 levels returned to baseline after 48hrs (data not shown), indicating the presence of a compensatory mechanism for maintaining lower levels of miR15a/16. Moreover, the microRNA levels peaked at different time points in different patients.
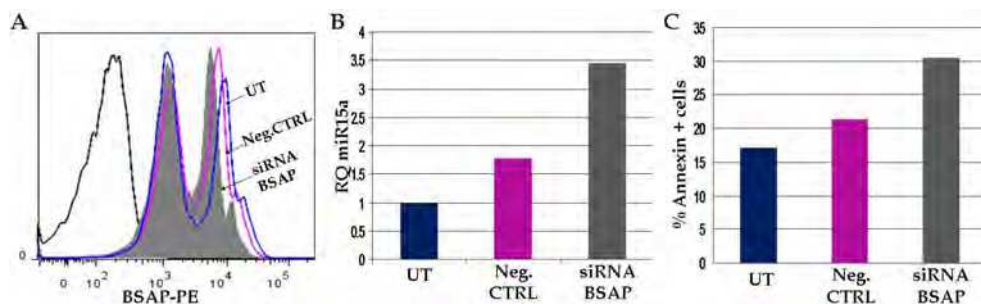
Fig. 7. Effect of Increasing miR15a/16-1 in Ex Vivo CLL Cells: $2x10^6$ patient PBMCs were transfected with BSAP siRNA or negative control siRNA using Human B cell Nucleofection kit (Lonza) or were untransfected (UT) and analyzed at different time-points.A) PBMCs were stained with BSAP-PE antibody and acquired on BD LSR II. B) Total RNA was extracted using Trizol and used to measuring the level of miR15a by TaqMan miR15a Assay (Applied Biosystems). C) Cells were stained with Annexin-V PE (BD Biosciences) and apoptosis was measured as the percentage of Annexin-V+ cells.

3. Conclusion

NZB mice faithfully mimic human CLL in phenotype, disease development and progression. The mutation and deletion in NZB mice leads to a significant reduction in the cellular level of mature miR15a/16-1 as compared to wild type mice. Similar reduction in miR15a/16-1 is observed in 50-60% of CLL patients [Reviewed by (Pekarsky et al., 2010)]. Yet patients harboring 13q14 deletions alone exhibit a more indolent disease as compared to patients having 17p and or 11q deletions in combination with 13q deletions. Moreover, in comparison to patients having a normal FISH profile, patients having 13q14 deletions have a shorter survival period that correlates with the percentage of nuclei with the deletion (Van Dyke et al., 2010, Chena et al., 2008).

We have also explored the therapeutic potential of systemic lentiviral delivery of miR15a/16-1 in the NZB mouse model of CLL. We propose that in addition to the direct cytotoxic effect of lentivirus mediated miR15a/16-1 increase; other indirect mechanisms may be responsible for the reduced percentage of B-1 cells post treatment. We observed a significant increase in the level of plasma miR16 in miR-Lenti mice as compared to control-lenti mice. However, the transduction efficiency was only 5-10%. This is consistent with reports from other labs that B cells and T cells are not amenable to efficient lentiviral transduction [Reviewed by (Frecha et al., 2010)]. We speculate that the few cells that were transduced secreted miR16 into circulation that was taken up by the non-transduced cells leading to their apoptosis. Another possibility is that the lentivirus transduced and killed supporting cells thereby reducing the amount of growth factors.

BSAP negatively regulates the Dleu2 gene promoter (Chung et al., 2008), and hence we hypothesized that its knockdown would lead to enhanced transcription of Dleu2 and in turn of miR15a/16-1. Although the mutation and deletion slows down the processing of pri-miR15a/16-1, its effect can be compensated by correspondingly increasing the transcription of its host gene. This strategy holds true even in CLL patients since although 13q14 region is

frequently deleted, the size of the deleted region varies and may or may not include miR15a/16-1 (Mosca et al., 2010). In addition the deletion is usually heterozygous and does not affect all the malignant cells.

miR15a/16-1 levels seem to be very tightly regulated in CLL cells. BSAP knockdown (removing the repressor of the miR-15a/16 host gene) in ex vivo patient cells gave rise to an initial increase in miR15a/16-1 levels and apoptosis (See Fig.7). However, the miR15a/16-1 levels were decreased shortly thereafter. This initial transient increased miR15a/16-1 levels could lead to the repression of an activator like p53. Fabri et al showed that p53 and miR15a/16-1 form a feedback inhibition loop (Fabbri et al., 2011). p53 acts as a transactivator of miR15a/16-1 and increases its expression. However, p53 is a target of miR15a/16-1 and is degraded in the presence of increased miR15a/16-1. Future studies will involve the transfection with BSAP siRNA followed by p53 knock-in at 48hrs to see whether miR15a/16-1 levels can be elevated for longer time.

In conclusion, we have successfully demonstrated that miR15a/16-1 levels can be modulated by different strategies – mimics, lentiviral delivery of the microRNAs and BSAP knockdown (removal of a repressor) – both in vitro and in vivo/ex vivo (See Section 2.2, 2.3, 2.4, 2.6). We have also presented evidence that increasing the level of miR15a/16-1 in B-CLL cells leads to cell cycle arrest (See Fig.3A, 5C), reduced proliferation (See Fig.4C) and increased apoptosis (See Fig.5C, 8C), which in effect leads to reduced malignancy (See Fig.6A). Future studies will be directed at further exploring the therapeutic potential of exogenous miR15a/16-1 delivery alone or in combination with siRNA BSAP using novel delivery vehicles like lipidoids that have been shown to be very efficient for systemic in vivo delivery (Goldberg et al., 2011). microRNAs have multiple targets and the net outcome of their up-regulation is very difficult to predict and may give rise to serious side-effects. However, we have shown here that exogenous increase of miR15a/16-1 has a net positive effect on disease outcome. These findings validate miR15a/16-1 as a promising therapeutic target for the treatment of CLL.

4. Acknowledgements

5. References

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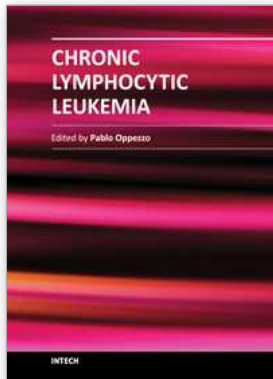
Chronic Lymphocytic Leukemia

Edited by Dr. Pablo Oppezzo

B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancitopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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