Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization

Relja Popovic¹, Laurie E. Riesbeck¹, Chinavenmeni S. Velu², Aditya Chaubey², Jiwang Zhang³, Nicholas J. Achille³, Frank E. Erfurth³, Katherine Eaton³, Jun Lu⁴, H. Leighton Grimes², Jianjun Chen⁵, Janet D. Rowley⁵ and Nancy J. Zeleznik-Le¹,³,⁶*

¹Molecular Biology Program, Loyola University Medical Center, Maywood, IL, USA; ²Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; ³Oncology Institute, Loyola University Medical Center, Maywood, IL, USA; ⁴Broad Institute of MIT and Harvard, Cambridge, MA, USA; ⁵Department of Medicine, University of Chicago, Chicago, IL, USA; ⁶Department of Medicine Loyola University Medical Center, Maywood, IL, USA

Running Title: MLL regulates mir-196b expression

*Corresponding author

Nancy J. Zeleznik-Le
2160 South 1st Avenue
Bldg 112, Room 337
Maywood, IL 60153
Tel: (708) 327-3368
Fax: (708) 327-3342
Email: nzelezn@lumc.edu

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Abstract

Chromosomal translocations involving the MLL gene produce chimeric proteins which cause abnormal expression of a subset of HOX genes and leukemia development. Here, we show that MLL normally regulates expression of mir-196b, a hematopoietic microRNA located within the HoxA cluster, in a pattern similar to that of the surrounding 5’ Hox genes, Hoxa9 and Hoxa10, during ES cell differentiation. Within the hematopoietic lineage, mir-196b is most abundant in short-term hematopoietic stem cells and is down-regulated in more differentiated hematopoietic cells. Leukemogenic MLL fusion proteins cause overexpression of mir-196b, while treatment of MLL-AF9 transformed bone marrow cells with mir-196-specific antagonir abrogates their replating potential in methylcellulose. This demonstrates that mir-196b function is necessary for MLL fusion-mediated immortalization. Furthermore, overexpression of mir-196b was found specifically in patients with MLL associated leukemias as determined from analysis of 55 primary leukemia samples. Overexpression of mir-196b in bone marrow progenitor cells leads to increased proliferative capacity and survival, as well as a partial block in differentiation. Our results suggest a mechanism whereby increased expression of mir-196b by MLL fusion proteins significantly contributes to leukemia development.

INTRODUCTION

The Mixed Lineage Leukemia (MLL) gene is commonly involved in chromosome translocations that cause leukemia.1,2 MLL-associated leukemias can be myeloid, lymphoid or bi-phenotypic, depending on the partner gene to which MLL is fused.3 There have been more than 60 different MLL fusion partners isolated to date and in most cases overexpression of a subset of HOX genes is a hallmark of the disease.4 HOX genes are
transcription factors which play an important role during development and hematopoiesis.\textsuperscript{5,6} Humans have 13 paralogous groups of \textit{HOX} genes clustered on four different chromosomes. Expression of \textit{HOX} genes is spatially and temporally regulated with 3’ genes expressed earlier and having a more anterior boundary of expression.\textsuperscript{5} Similarly, expression of \textit{HOX} genes is tightly regulated during hematopoiesis. Genes located at the 3’ end of the cluster are downregulated as CD34+ cells become lineage specific progenitors while 5’ genes, like \textit{HOXA10}, are turned off only after cells progress to the more differentiated CD34- stage.\textsuperscript{7} MLL regulates expression of some of the \textit{HOX} genes at the chromatin level by binding to the promoters and recruiting various transcriptional regulators.\textsuperscript{8-10} However, what happens at the molecular level in the presence of the leukemogenic fusion proteins to cause \textit{HOX} overexpression is still poorly understood.

Among 6800 genes analyzed by expression microarrays, overexpression of \textit{HOXA9} was the most correlative marker of poor prognosis in acute myeloid leukemia patients.\textsuperscript{11} Immortalization of bone marrow progenitors by the MLL fusion protein MLL-ENL is dependent on the presence of \textit{Hoxa9} and \textit{Hoxa7} genes.\textsuperscript{12} Like other \textit{HOX} genes, the \textit{HOXA9} locus gives rise to a number of alternatively spliced transcripts.\textsuperscript{13-15} The precise role of each one of these transcripts is still unclear, but some of them do not code for proteins and are more likely to have a strictly regulatory role.

MicroRNAs (miRNAs) are small RNA transcripts (~22nt) that function in posttranscriptional regulation of gene expression. The mature miRNAs are processed from much longer primary precursor molecules through a multistep process that involves a number of proteins. MiRNA precursors are transcribed by RNA Polymerase II and
initial processing is performed in the nucleus by Drosha. Subsequent transport into the cytoplasm and additional processing by Dicer and helicase give rise to the mature miRNA that is incorporated into the RISC complex, guiding it to the target mRNAs.\textsuperscript{16} It is now clear that miRNAs play an important role during development and disease and many of the genes encoding microRNAs are found at sites frequently deleted or mutated in cancers.\textsuperscript{17} While the role of miRNAs in various systems is starting to be elucidated, the mechanism by which their expression is regulated is still poorly understood.

We report here the regulation of a miRNA, mir-196b, by wild type MLL as well as its dramatic overexpression by leukemic MLL fusion proteins. Mir-196b is located in a highly evolutionarily conserved region between \textit{HOXA9} and \textit{HOXA10} genes, at chromosome band 7p15.2 in human and 6qB3 in mouse. Our studies indicate that MLL regulates mir-196b expression in a pattern similar to that of the surrounding genes. Mir-196b is overexpressed specifically in primary leukemia samples from MLL patients, but not from other types of leukemia. Furthermore, expression of MLL fusion proteins in primary bone marrow cells causes overexpression of mir-196b. Treating these cells with specific antagonir targeting mir-196b significantly compromises the increased replating potential of MLL-AF9-expressing cells. Mir-196b expression increases proliferation and survival, and also partially blocks differentiation of normal bone marrow hematopoietic progenitor cells. Overexpression of mir-196b is an important component in leukemia development caused by MLL fusion proteins.
Materials and methods

Cell Lines

*Mll* wild-type and -/- MEFs and ES cells have been described.\(^{18,19}\) *Menin* wild-type and -/- MEFs were previously described.\(^{20}\) *MLL* and *MLL-AF4* add back clones were previously described.\(^{21}\)

miRNA Detection

RNA was isolated using TRI Reagent (Sigma) according to the manufacturer’s protocol. Reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed in triplicate on an ABI 7300 machine using Taqman analysis according to the manufacturer’s instructions (miR196b probe set 000496, normalized to RNU6B probe set 001093)(Applied Biosystems).

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin Immunoprecipitation Assay was performed using EZ-ChIP Kit (Upstate) according to manufacturer’s protocol. Chromatin was immunoprecipitated using anti-dimethyl H3 K79, anti-dimethyl H3 K4, anti-trimethyl H3 K4 and anti-dimethyl H3 K9 (Upstate) and analyzed by qPCR in triplicate on an ABI 7300 machine using iTaq SYBR Green Supermix with Rox (BioRad). Percentage enrichment was normalized to input DNA and calculated as previously reported.\(^{22}\) Amplification of the AB region was performed with primers previously described.\(^{21}\)

Embryonic Stem Cell Differentiation and isolation of CD41+ cells

Embryonic stem (ES) cells were maintained, differentiated, and day 10 embryoid bodies collected as described previously.\(^{23}\) Isolation of hematopoietic progenitors was
performed using anti-CD41 antibody (BD Pharmingen) Cells not bound by the
nanoparticles were collected as CD41- population.

**Bone Marrow Cell Sorting**

Bone marrow was harvested from wild type C57BL6/J mice using a Loyola
University IACUC-approved protocol. After red blood cell lysis, BM nucleated cells
were dispersed into a single-cell suspension. The Long-term HSCs (Lin–Sca1+C-Kit+Flk2–,
LSKF–), short-term HSCs (Lin–Sca1+C-Kit+Flk2+, LSKF+), and the committed progenitors
(Lin Sca1 C-Kit+, LK) were enriched by lin+ cell depletion (EasySep Mouse Hematopoietic
Progenitor Cell Enrichment Kit, Stem Cell Technologies), and purified by FACS Aria flow
cytometer (BD Biosciences) sorting after FITC-Lin cocktail, PE-Sca1, APC-c-Kit and PE-
Cy5.5-Flk2 staining. Gr1+ myeloid cells and B220+ B cells were sorted from BM cells
after FITC-Gr1 and APC-B220 staining. All fluorescent antibodies used were purchased
from e-Bioscence.

**RT-PCR and qRT-PCR**

RT-PCR reactions were performed in a Hybaid PCR Express machine. Each 50μl
reaction contained 1.5mM MgCl2, 100ng of each primer, 0.125mM dNTP mix, 5μl of
10X PCR Buffer (Fermentas) and 2.5 units of Taq DNA Polymerase (GenScript). PCR
products were analyzed with ethidium bromide by electrophoresis on a 2% agarose gel.

For quantitative RT-PCR, cDNA was analyzed using ABI Prism 7300. For SYBR
Green, each 20 μl reaction contained 2μl of 10X iTaq SYBR Green Supermix with Rox
(BioRad) and 0.25 μM forward and reverse primers. All reactions were performed in
triplicate and gene expression levels were normalized to hprt expression and fold
difference calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences are available upon request.
Cloning mir-196b Expression Vector

A 200bp DNA fragment surrounding mir-196b was amplified using the primers: forward 5’ GAAGATCTTTCCTTGGCGGCGACA 3’ and reverse 5’CCCAAGCTTGA TGGCCCGCCTA 3’. Gel purified PCR product and vector were both digested with HindIII and BglII and ligated into pSuper.retro vector (OligoEngine).

Retrovirus Production and Colony Assay

Retroviruses were produced as described previously. Bone marrow colony assays were performed as we have previously described with minor changes.24 Briefly, bone marrow cells were collected from 4-12 week old C57Bl6 mice and c-kit positive progenitors were selected using an Easy Sep Selection kit (StemCell Technologies). Lineage depleted cells were separated using Negative Selection Kit (StemCell Technologies). Following the second infection, cells were plated in methylcellulose with cytokines IL6, IL3, SCF and (+/-) GM-CSF, in the presence of puromycin (8.75 micrograms/ml).

Antagomir treatment of MLL-AF9 transduced bone marrow cells

Antagomir oligoribonucleotides were synthesized (Dharmacon)(2’-O-Me on each ribonucleotide, phosphorthioate on the first two and last four ribonucleotides, and a 3’ cholesterol modification linked through a hydroxyprolinol linkage) (miR-196, 5’-CCCAACAACAGGAAACUACCUA-3’; control (mutant) miR-196, 5’-CCCAAGAAACAGGUAGACGUACGUAGUA GUACGUAGU-3’). Wild type Lin bone marrow cells were treated with 100 nM antagomir. To confirm efficacy, RNA was extracted after 72 h using TriZol (Sigma), then analyzed for steady-state mature miR-196b expression. The level of mature miR196b was not detectable by TaqMan assay after antagomir treatment. For
colony assay, wild type Lin- bone marrow cells were transduced with an MLL-AF9 expressing retroviral vector and treated with 100nM control antagomiR-196 or antagomiR-196 before 15,000 cells were plated in duplicate in methylcellulose medium containing IL-3, IL-6 and SCF (M3534, Stem Cell Technologies). Colonies were enumerated after 8 days of culture and the average number plotted (+/- SD). For serial replating, colonies were pooled and 15,000 cells were treated with control antagomiR or antagomiR-196b and replated.

**Bead-Based miRNA Expression Profiling Assay**

A large-scale, genome-wide miRNA expression profiling analysis was performed using a bead-based flow cytometric method. A total of 55 AML primary patient samples, including 29 MLL-associated acute leukemias (10 ALLs and 19 AMLs) and 26 AMLs with other abnormalities, along with 3 normal bone marrow controls were included in the expression assay. To control for data quality, only samples with total miRNA signals equal to or greater than 15000 were analyzed further. All patient samples were obtained with informed consent given, in accordance with the Declaration of Helsinki, with approval of the Institutional Review Board at the University of Chicago. After normalization, only probes for human miRNAs with maximum expression in any sample being greater than or equal to 7.25 were retained for further analyses. TIGR Multiple Array Viewer software package (TMeV version 4.0) was used to perform data analysis and to visualize the results. The details of this assay were reported elsewhere.

**Myeloid and B-Cell Differentiation Assay**

Bone marrow cells harvested after one week culture in the methylcellulose colony assay (see above) were used for the differentiation assays. 35,000 mir-196b or vector-
infected cells were plated on 20,000 OP9 cells per well to a 24-well dish in RPMI, 20% FBS, 1% Pen/Strep. Wells were supplemented either with 10ng/ml of GM-CSF (myeloid) or 10ng/ml of IL-7 and 10ng/ml of FLT3L (B cell). After five days cells were stained with APC conjugated CD117 and PE conjugated CD11b or PE conjugated B220 antibodies. Percentage of cells expressing these surface markers was determined using flow cytometry.

Results

MLL-dependent expression of mir-196b

MLL is a known regulator of HOX gene expression. We have recently described expression of an Mll-dependent transcript from a region highly conserved across species located approximately 4.5kb upstream of the canonical Hoxa9 promoter. Mll protects this DNA region from becoming methylated thus allowing expression of the transcript. Mir-196b is located in the center of the conserved region and we hypothesized that the transcript encodes the precursor of mir-196b (Figures 1A and B). Our initial approach used ligation-mediated PCR to isolate the mature form of mir-196b (data not shown). While our method was successful, we were not able to determine the expression levels quantitatively. To overcome this problem, we used a microRNA assay specific for mir-196b which utilizes TaqMan probe chemistry thus allowing for quantitative measurement. Analysis of mouse embryonic fibroblasts (MEFs), wild type and null for Mll, showed a 3.5-fold higher level of expression of mir-196b in the presence of Mll (Figure 1C). As previously reported and confirmed in our lab (data not shown), canonical Hoxa9 transcript expression shows a similar Mll dependency. Dependence of mir-196b
expression on MLL was further confirmed by exogenous expression of wild type MLL in 
\textit{Mll-/-} MEFs. MLL restored expression of mir-196b in these cells (Figure 1D).

The tumor suppressor protein menin was previously biochemically purified as part of an MLL protein complex and subsequently shown to be an essential cofactor in MLL dependent gene expression.\textsuperscript{10,28} Binding of Mll to some of its target genes that have been studied requires menin; absence of menin leads to decreased expression of Mll-target genes, including \textit{Hoxa9}.\textsuperscript{10,29} Thus, we hypothesized that if mir-196b is regulated by Mll, then the absence of menin should also diminish mir-196b levels. To test this hypothesis \textit{Men-/-} MEFs were assessed for mir-196b expression. As predicted, mir-196b expression in \textit{Men-/-} MEFs was 4-fold lower than in the wild type counterparts (Figure 1C).

Several studies have documented that Mll regulates expression of its target genes at least in part by altering chromatin structure.\textsuperscript{8-10,30} Previous studies have shown that the SET domain of MLL possesses histone methyltransferase activity specific for lysine 4 on histone H3.\textsuperscript{8,9} Using chromatin immunoprecipitation (ChIP) we investigated histone modifications in the mir-196b region in wild type and \textit{Mll} null cells. We and others have previously reported binding of endogenous Mll to this region.\textsuperscript{21,31} Surprisingly, we did not observe any differences in H3K4 methylation between the two cell types (Figure 1E). However, we did notice dramatically higher levels of H3K79 dimethylation in the \textit{Mll} wild type cells as compared to \textit{Mll-/-} cells (Figure 1E). H3K79 methylation by the protein Dot1 has been linked to active transcriptional elongation suggesting that this mark may also represent transcriptional elongation in the conserved region only in the wild type but not \textit{Mll-/-} cells.\textsuperscript{32,33}

\textbf{Mll-dependent mir-196b expression during ES cell differentiation}
Mll regulates expression of a subset of genes in the Hox cluster during embryonic development. Mice lacking Mll are embryonic lethal and display gross defects in the early stages of hematopoiesis. Absence of Mll leads to deficiencies in proliferation and survival of hematopoietic progenitors in the yolk sac. To investigate the dependency of mir-196b expression on Mll during development, we used wild type and Mll-/ murine embryonic stem (ES) cells. Upon removal of LIF, ES cells form embryoid bodies (EBs) and the pattern of gene expression in the embryoid bodies generally follows that of early hematopoiesis in a developing embryo. Previous studies have shown that the expression of a number of Hox genes depends on the presence of Mll during ES cell differentiation. We wanted to determine if Mll regulates the timing of mir-196b expression in a manner similar to that of the neighboring Hoxa9 and Hoxa10 genes. As previously reported, and confirmed in our lab, Mll-/ cells form embryoid bodies indistinguishable from those derived from the wild type cells. Levels of Hoxa9 and Hoxa10 genes are similar in both cell types in undifferentiated ES cells and during the first few days of differentiation (Figure 2A and 2B). At the later stages of differentiation, transcription of both of these genes is upregulated in the wild type cells. Cells carrying homozygous Mll mutations show lower levels of Hoxa9 and Hoxa10 expression.

Similarly, levels of mir-196b are decreased up to 14-fold in the absence of Mll. Mir-196b is expressed during ES cell differentiation in a pattern of expression similar to that of Hoxa9 and Hoxa10 (Figure 2C). Sharing of cis-regulatory elements has been previously described for genes in the HoxB and C clusters, however it remains unclear if mir-196b shares regulatory elements with the neighboring Hox genes or if its expression is controlled by Mll independently of Hoxa9 and Hoxa10.
Embryoid bodies contain a highly heterogenous population of cells. To more specifically investigate hematopoietic cells we isolated CD41+ cells from Day 10 embryoid bodies. This marker was shown to be present on definitive hematopoietic progenitor cells giving rise to all blood lineages.\textsuperscript{40} As reported by Ernst et al., wild type and \textit{Mll-/-} ES cells form a comparable number of CD41+ cells however \textit{Mll-/-} CD41+ cells are less efficient at producing hematopoietic colonies.\textsuperscript{37} We found that both the canonical \textit{Hoxa9} as well as \textit{mir-196b} are expressed at a lower level in \textit{Mll-/-} CD41+ cells in comparison to the wild type cells (Figure 3A and 3B). Additionally, expression of \textit{mir-196b} was also \textit{Mll} dependent in the CD41 negative population, suggesting that \textit{Mll} is also required for expression of \textit{mir-196b} in other cell types and tissues (Figure 3B).

**Mir-196b expression in hematopoietic progenitors**

MiRNA expression patterns vary between cell types. We wanted to examine if \textit{mir-196b} is generally expressed in hematopoietic progenitor cells or if it is limited to expression in specific stages of hematopoiisis. Mouse bone marrow cells were separated into more differentiated versus less differentiated populations. Expression of \textit{mir-196b} was on average 2.5-fold higher in lineage negative cells (data not shown). Similarly, the c-Kit+ population of bone marrow cells expressed 6-fold higher levels of \textit{mir-196b} than the c-Kit- cells (data not shown). These results suggest that \textit{mir-196b} is indeed expressed in the hematopoietic progenitor population and that it may be playing a role at early stages of hematopoiisis.

To determine which population(s) of hematopoietic progenitors expresses \textit{mir-196b}, mouse bone marrow cells were sorted into long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells (ST-HSC), multipotent progenitors (MPP) and more...
mature myeloid (GR1+) and lymphoid (B220+) cells. Expression levels of mir-196b are the highest in ST-HSC and decrease as cells become more differentiated (Figure 3D). The tightly regulated pattern of mir-196b expression suggests that misregulation of mir-196b expression may lead to drastic defects in normal hematopoiesis.

**MLL fusion proteins induce expression of mir-196b, which is required for increased proliferative capacity**

Overexpression of certain HOX genes is a hallmark of MLL-associated leukemias, however the exact mechanism by which MLL fusion proteins exert their effect in the leukemic cells is still poorly understood. Because MLL regulates expression of various HOX transcripts we wanted to investigate whether an MLL fusion protein causes a change in mir-196b expression. Transfection of constructs expressing the MLL-AF4 fusion protein into Mll-/- MEFs reactivated expression of mir-196b to levels higher than transfection of constructs expressing wild type MLL (Figure 1D). We also utilized a bone marrow (BM) progenitor colony assay to investigate the effect of MLL-AF9 on mir-196b expression. C-Kit+ bone marrow cells infected with MLL-AF9 retroviruses show on average more than a 100-fold increase in mir-196b expression in comparison to vector infected cells (data not shown). Expression of canonical Hoxa9 is also greatly increased (> 300-fold) (data not shown).

Although MLL fusion proteins caused increased mir-196b expression, we next determined whether mir-196b expression was necessary to manifest the increased in vitro proliferative capacity observed for MLL fusion-transduced bone marrow progenitor cells. For this experiment, MLL-AF9 transduced progenitors were treated with antagonim specific to mir-196b or with control (mutant) antagonim and assessed for replating
activity. We found that mir-196b function is required to maintain the increased proliferative capacity of MLL fusion-expressing bone marrow progenitors in vitro (Figure 4). The role of mir-196b in immortalization and enhanced proliferation of cells transduced by MLL fusion proteins was intriguing and suggested that further investigation of mir-196b in MLL leukemia was warranted.

Mir-196b is overexpressed in the majority of MLL-associated leukemias

To investigate mir-196b expression in primary leukemia patient samples, we utilized bead-based expression profile technology. To this end, 55 different patient samples (10 ALL and 45 AML) and 3 normal bone marrow samples were analyzed for expression of mir-196b. The 29 MLL leukemia samples (10 ALL and 19 AML) expressed consistently higher levels of mir-196b than non-MLL leukemia samples (Figure 5). It is of interest that multiple cell lines derived from MLL leukemia patients also express high levels of mir-196b (data not shown). As a molecule that has the potential of regulating expression levels of many different proteins, high levels of mir-196b in MLL leukemias may play a crucial role in the disease process.

Mir-196b causes increased proliferative capacity and decreased differentiation

To examine possible roles of mir-196b overexpression in hematopoietic progenitor cells we cloned a 200bp region surrounding the mature miRNA into a retroviral expression vector. Increased mir-196b expression was confirmed after transfection in MEFs (data not shown). In vitro serial replating colony assays have been used extensively to determine the leukemogenic potential of MLL fusion proteins. Because we found mir-196b overexpressed in primary MLL leukemia samples, we wished to
determine whether overexpression of mir-196b could contribute to the immortalization
process by providing increased proliferative capacity to progenitor cells. Bone marrow c-
kit+ cells were transduced using retroviruses that express mir-196b precursor or with the
empty vector alone and serially replated in methylcellulose containing either SCF, IL6,
IL3 and GM-CSF (referred to as +GM-CSF) or SCF, IL6 and IL3 (referred to as – GM-
CSF). Transduced cells formed colonies of various sizes after the first week in culture
for both vector and mir-196b under both conditions, as expected (Figure 6B). Mir-196b
was expressed approximately 2-fold higher in the mir-196b-transduced cells as compared
to controls(data not shown).In the presence ofGM-CSF, mir-196b expressing cells
survived into the third week (after the second re-plating) and increased in cell number on
average 5-fold, in contrast to control-infected cells which did not survive following the
first re-plating (Figure 6A). Overexpression of mir-196b was not sufficient to completely
immortalize cells under these conditions. However, using cytokine mix - GM-CSF, a
more dramatic effect of mir196b expression on increased colony forming ability and
proliferative capacity was observed. Under these conditions, mir-196b expressing cells
formed tight colonies even after the fourth plating of cells, in contrast to the vector-
transduced cells which formed primarily loose colonies after the second plating and
almost no colonies after the third plating (Figure 6A,B). Mir-196b-expressing cells after
4 platings in methylcellulose became a relatively homogenous population resembling
blast cells and expressed intermediate levels of CD117 and Sca1, with about 50% also
expressing Gr-1, whereas vector-transduced cells after the second and third plating (very
few cells remaining after the third plating) became CD117 and Sca1 high, but negative
for lineage markers and resembled mast cells (Figure 6B and data not shown). To rule
out the possibility that simply activating the microRNA processing pathway was responsible for the observed increased proliferative capacity, we also transduced cells with retroviruses which express short hairpin RNAs that target two different molecules, CtBP and Bmi-1. Cells transduced with either of these retroviruses formed colonies in the first week, confirming effective infection of the cells; however the colony forming ability of both of these were similar to vector-transduced cells (data not shown). Our data demonstrate that mir-196b expression causes an increased survival and proliferative capacity of bone marrow progenitor cells.

The normal process of terminal differentiation is often abrogated during development of leukemia. We investigated whether mir-196b helps hematopoietic progenitor cells retain a more undifferentiated state. To this end, equal numbers of bone marrow progenitor cells cultured in methylcellulose with puromycin selection for one week after transduction with vector or mir196b-expressing constructs were re-plated on OP9 feeder cells in the presence of GM-CSF or IL7/FLT3L. The two conditions support myeloid and lymphoid differentiation, respectively. After five days the cells were analyzed for the expression of CD117/CD11b or CD117/B220 markers. Under both conditions the mir-196b infected cells retained a significant percentage (approximately 20%) of CD117 positive cell population, whereas vector expressing cells did not (Figure 6C). This suggests that overexpression of mir-196b can partially block differentiation in hematopoietic progenitor cells.

Discussion

MLL, a large multi-domain protein, is a master regulator of gene expression during development and hematopoiesis. MLL positively regulates expression of genes by
Translocations in \textit{MLL} give rise to in-frame fusions with various partner genes and the formation of chimeric proteins leading to misregulation of MLL target genes. While transcriptional activation and histone methyltransferase domains of MLL are both lost in the chimeras, the fusion proteins still cause overexpression of downstream MLL targets, including members of the \textit{HOX} clusters.\cite{41, 42} Dysregulation of certain \textit{HOX} genes plays a central role in MLL-associated leukemogenesis, however the mechanism is still very poorly understood.\cite{12, 28} In our study we demonstrate that MLL and MLL fusion proteins regulate expression of a microRNA located in the \textit{HOX} cluster, mir-196b. Mir-196b is an attractive target of MLL regulation for various reasons: 1) mir-196b is situated between \textit{HOXA9} and \textit{HOXA10}, two MLL targets that are frequently overexpressed in MLL leukemias; 2) some of the mir-196b targets are \textit{HOX} genes themselves and by regulating mir-196b expression, MLL can simultaneously regulate boundaries of \textit{HOX} expression\cite{43}; and 3) as with any other miRNA, strict regulation of mir-196b expression is essential for its proper function and possible misregulation by MLL fusion proteins may lead to misregulated expression of mir-196b target mRNAs, and thus contribute to large-scale changes in gene expression often seen in leukemic cells.

In this study, we provide several lines of evidence to support mir-196b as a target of MLL regulation. Our previous findings demonstrated that the presence of Mll selectively prevents DNA methylation of a region adjacent to mir-196b which allows for expression of transcripts that are mir-196b precursors.\cite{21} Using a method that detects the mature form of miRNA, we demonstrate that \textit{Mll-/-} MEFs express lower levels of mir-196b than wild
type cells. We and others have previously shown that the expression of other Mll targets, such as Hoxa7, a9, a10, are also decreased in these cells. One of the components of an MLL protein complex is menin, a tumor suppressor protein often deleted in multiple endocrine neoplasms. Genome wide mapping of menin and MLL binding to chromatin shows that the two proteins are often bound to the same loci. Our data show that mir-196b expression is also dependent on menin and that in the absence of menin, mir-196b levels fall to similar low levels as measured in Mll-/- cells. Transfection of MLL or MLL-AF4 into the Mll-/- cells boosts expression of mir-196b to and above wild type levels respectively, which is accompanied by the reversal of DNA methylation.

The SET domain of MLL contains histone methyltransferase activity specific for lysine 4 on histone H3 and this function contributes to activation of MLL target genes. Surprisingly, ChIP data does not show any changes in H3K4 methylation between wild type and Mll-/- cells in the region surrounding the microRNA. It has been previously noted that trimethylation of H3K4 is a mark usually found close to the transcription start site and it is possible that the mir-196b precursor transcript starts further upstream. Interestingly, our ChIP data show that the mir-196b region is greatly enriched in H3K79 dimethylation only in the presence of Mll. H3K79 methylation is a mark associated with transcriptional elongation suggesting that Mll is necessary for proper transcriptional elongation in this region. Dot1 is the only H3K79 histone methyltransferase isolated to date. It is still unclear if any of these histone marks are altered in the presence of MLL fusion proteins, however several MLL partners, including AF10, AF9, ENL and AF4 interact with Dot1 in a multiprotein transcription elongation complex with pTEFb.
is possible that this interaction may also play a role in overexpression of MLL target
genes in the MLL-associated leukemias.

Using *in vitro* differentiation of wild type and *Mll*−/− murine embryonic stem cells
we show that Mll regulates expression of mir-196b similarly to other 5′ Hox genes. These
data suggest that Mll regulates mir-196b temporally and studies by Mansfield *et al.*
showed that the paralogous mir-196a is also regulated spatially in a Hox-like pattern
during embryogenesis.47 Considering that a subset of mir-196 targets are also members of
the HOX cluster, it is likely that the pattern of mir-196b expression plays a role in fine-
tuning the levels of HOX gene expression. In a developing organism, this mechanism
would help generate the boundaries of HOX expression without the need for turning off
the genes completely.

Microarray profiles of miRNA expression patterns have shown that mir-196b is
expressed at the highest levels in bone marrow and spleen suggesting it has a role in
hematopoiesis.48 Our analysis of c-Kit+ hematopoietic progenitors demonstrates that mir-
196b is expressed at higher levels in this population than in the more differentiated c-Kit-
population. Additionally, separation of mouse bone marrow into lineage negative and
lineage positive cells confirms that mir-196b is expressed at higher levels in less
differentiated cells. Of the early hematopoietic progenitors, expression of mir-196b is the
highest in ST-HSCs. Isolation of CD41+ definitive hematopoietic progenitors from
differentiated embryoid bodies confirms mir-196b expression in hematopoietic progenitor
cells only in the presence of Mll. Aberrant hematopoietic colony formation of *Mll*−/−
CD41+ cells was rescued by the addition of certain Hox genes or the homeodomain
containing transcription factor Cdx4.37 Cdx4 positively regulates expression of Hox genes
and, similar to MLL, binding of Cdx4 to the Hoxa9 locus is dependent on menin.\textsuperscript{49} In a mouse model, Cdx4 overexpression generates acute myeloid leukemia while dysregulating Hox expression.\textsuperscript{50} It would be of interest to determine if Cdx4 also regulates mir-196b expression and if this regulation plays a role in Cdx4-induced leukemogenesis.

A hallmark of MLL-associated leukemia is overexpression of specific HOX genes.\textsuperscript{41,42} Likewise, our study shows that MLL fusion proteins also drastically increase levels of mir-196b. C-Kit+ bone marrow cells transduced with MLL-AF9 retroviruses express on average more than 100-fold increased levels of mir-196b than cells infected with control retrovirus. The increased proliferative capacity of the MLL fusion-expressing progenitors was dependent on mir-196b expression because antagomir specifically targeting mir-196b abrogated this effect. Importantly, expression analysis of primary patient leukemia samples confirms high levels of mir-196b expression specifically in MLL leukemias. This increase in expression is observed in both acute myeloid as well as acute lymphoid MLL leukemia samples. Significantly, non-MLL leukemias do not express high levels of mir-196b. Abnormal expression of mir-196b influences expression of many downstream targets which likely contributes to the development of leukemia. Targets of mir196b include Hoxb8 and Hoxa7 along with an extensive list of additional putative mir-196b targets. In order to either rule-in or rule-out a direct contribution of mir196b regulation of Hox target expression in the transformation process, we quantitatively assessed levels of multiple Hox genes after expression of mir196b in bone marrow progenitor cells. These include Hoxa5, Hoxa7, Hoxa9, Hoxa10, Hoxb3, Hoxb4, Hoxb6, Hoxb8, Hoxc8 and Meis1. Hoxc8 and Hoxb8 were below the
level of detection in both control- and mir196b-expressing cells, suggesting that they do not play a role in the observed phenotypes. Of the other genes measured, Meis1 and Hoxc6 do show decreased expression when mir196b is overexpressed, however, these are not predicted to be direct targets of mir196b. Further studies are needed to determine which mir196b targets are relevant in MLL leukemogenesis.

Using a serial replating hematopoietic colony assay we show that increased levels of mir-196b stimulate the proliferative capacity of hematopoietic progenitor cells, but are not sufficient to completely immortalize cells. Furthermore, overexpression of mir-196b in hematopoietic cells also results in a partial differentiation block such that a significant percentage of progenitors retain expression of c-Kit under conditions that cause differentiation of cells not expressing this miRNA. Increased proliferation and block in differentiation are essential to leukemia development. Our results suggest that misregulation of mir-196b expression by MLL fusion proteins plays an important role in the disease. This evidence establishes a connection between misregulation of miRNA expression and increased proliferation, survival, and differentiation block in leukemia (Figure 7). In this model, expression of mir-196b is tightly controlled by MLL in hematopoietic precursor cells. The presence of MLL fusion proteins leads to aberrant mir-196b expression causing increased proliferation, abnormal hematopoietic differentiation and contributes to leukemogenesis. MiRNAs may be used as new molecular targets for the development of novel therapeutic strategies.
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Wild type and 

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Authorship

Contribution: R.P. designed and performed research, wrote the manuscript. L.E.R., C.S.V., A.C., J.Z., F.E.E. and J.L. designed and performed research. N.J.A. and K.E. performed research. H.L.G and J.C. designed research, analyzed data, and edited manuscript. J.D.R. analyzed data and edited manuscript. N.J.Z-L. designed research, wrote and edited manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Nancy Zeleznik-Le, PhD. Department of Medicine, Loyola University Medical Center, Maywood, IL, 60153, USA; email:nzelezn@lumc.edu
References


Figure Legends

**Figure 1. Expression of mir-196b is dependent on Mll and Menin.** (A) Schematic illustration of the murine *Hoxa9* locus on chromosomal band 6qB3. Open boxes represent three exons and grey boxes indicate CpG islands. Exon II is the homeodomain (HD) containing exon. The canonical *Hoxa9* is encoded by exons CD and II. Regions of high homology between species are noted as dark boxes and the region amplified by ChIP primers is indicated by arrows. The location of mir-196b is labeled above exon AB with an arrowhead. (B) Sequence alignment of the conserved mir-196b sequence among different species. The mature mir-196b sequence is highlighted in black. (C) Quantitative RT-PCR of mir-196b levels in WT, *Mll-/-* and *Men-/-* MEFs. The experiment was run in triplicate and the results represent average expression ±SD. (D) Quantitative RT-PCR of mir-196b expression in individual clones expressing an empty vector, MLL or MLLAF4 in *Mll-/-* MEF background. Transfection of *Mll-/-* MEFs and clone selection was performed as previously described. Mir-196b expression was determined from total RNA. (E) ChIP assay performed in WT and *Mll-/-* MEFs. Chromatin was precipitated using indicated antibodies and qPCR performed with primers spanning mir-196b region. All samples were run in triplicate and were normalized to the input chromatin.

**Figure 2. Mir-196b is regulated by Mll during ES cell differentiation, similar to *Hoxa9* and *Hoxa10*.** (A and B) Quantitative RT-PCR of *Hoxa9* (A) and *Hoxa10* (B) mRNA using SYBR green. All values are compared to WT day 0 and this value is set to 1. Experiments were performed in triplicate and the results represent average fold change
±SD. (C) Quantitative RT-PCR of mir-196b expression using mir-196b Taqman primers and probe.

**Figure 3. Mll regulates mir-196b expression in hematopoietic progenitors.** (A) RT-PCR for canonical *Hoxa9* in CD41+ ES cells. Day 10 EBs were disrupted and CD41+ cells isolated using a magnetic column. Total RNA was isolated from the CD41+ cells and used for cDNA synthesis. *Hoxa9* primers used span the junction between exon CD and exon II of the gene. *Gapdh* is used as a loading control. Space between panels indicates repositioned gel lanes (B) Quantitative RT-PCR of mir-196b expression in WT and *Mll-*/- CD41+ and CD41- murine embryonic stem cells from day 10 embryoid bodies. WT levels were set to 1 for comparison. Experiment was performed in triplicate and the results represent average expression ±SD. (C) Quantitative RT-PCR of mir-196b expression in sorted mouse bone marrow cells. Mouse bone marrow cells were sorted in various populations based on the expression of cell surface markers. Expression of LT-HSCs is set to 1 for comparison. The results represent average fold change ±SD.

**Figure 4. MLL fusion proteins induce expression of mir-196b in primary bone marrow progenitors, which is required for their MLL fusion-dependent increased proliferative capacity in vitro.** Wild type Lin- bone marrow cells were transduced with MLL-AF9 retroviral vectors and treated with either control antagomiR-196 or antagomiR-196 then plated in duplicate. Colonies were enumerated after 8 days and the average number was plotted. Cells were then replated after additional antagomir
treatment. The cycle was repeated twice. Data shown is representative of two independent experiments with similar results.

**Figure 5. Mir-196b is overexpressed in the majority of MLL-associated leukemias but not in non-MLL leukemias, irrespective of their phenotype.** Heat map of relative mir-196b expression of 55 leukemia samples and 3 normal controls using bead-based technology.

**Figure 6. Mir-196b expression enhances colony formation and partially blocks hematopoietic progenitor cell differentiation.**

(A and B) Serial replating myeloid colony assay using c-Kit+ bone marrow cells. Cells were transduced with retroviruses producing mir-196b construct or with an empty vector and plated in methylcellulose with two different cytokine mixes. The number of colonies (A) and colony and cell morphology (B) at one and four weeks are shown. Only mir-196b expressing cells are able to form colonies at the fourth week. Scale bars on cytospin pictures represent 10µm in top panel and 25µm in lower panel. (C) Representative FACS profiles of *in vitro* differentiated bone marrow cells. After one week of growth and selection in methylcellulose, mir-196b or vector-expressing bone marrow cells were plated on the OP9 cell line in the presence of GM-CSF or IL7/Flt3L. After five days, expression of CD117 and CD11b (top panels-cells on GM-CSF) or B220 (bottom panels-cells on IL7/Flt3L) was determined. Under both conditions, overexpression of mir-196b causes a partial block in differentiation.
Figure 7. Model for the role of mir-196b in hematopoietic cells and MLL fusion-mediated leukemia.

MLL regulates expression of mir-196b and MLL fusion proteins cause a block in differentiation of progenitor cells. This effect may be due partially to high levels of mir-196b expression caused by the MLL fusion protein. High levels of mir-196b may also provide a survival advantage to cells expressing the MLL fusion protein.
Figure 2

(A) **Hoxa9**

(B) **Hoxa10**

(C) **Mir-196b**
Figure 3

A

WT  Mll-/-

gapdh

Canonical Hoxa9

B

Relative Expression

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C

Relative Expression

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Figure 4
Figure 7

MLL fusion protein

mir-196b

Survival
Proliferation
Survival
Proliferation

LT-HSC

ST-HSC

MPP

Differentiated cell

mir-196b levels
Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization