MIR-150, A NOVEL AND POTENT REGULATOR FOR MLL-AF9 LEUKEMIC STEM CELLS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

MiR-150, a Novel and Potent Regulator for MLL-AF9 Leukemic Stem Cells. (May 2013)

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MicroRNAs (miRNAs) are a class of short, non-coding RNAs that post-transcriptionally regulate gene expression, particularly in cancers. MicroRNA-150 has been proven as a critical regulatory for normal hematopoiesis and a potent inhibitor of MLL-AF9 leukemias. To understand the potential mechanism of how miR-150 suppresses MLL-AF9 leukemia through the regulation of leukemic stem cells (LSCs), the gain- and loss-of miR-150 strategies were adopted on an established MLL-AF9 expressing cell line. Gain-of-function will be carried out with miR-150 overexpressing construct and loss-of-function of miR-150 will be performed by utilizing a newly established sponge system to achieve chromosomal recombination. The effect of miR-150 on MLL-AF9 LSCs will evaluated using multiple methodologies.

CHAPTER I

INTRODUCTION

The mixed lineage leukemia (MLL) gene codes for a histone methyltransferase that is crucial for early hematopoiesis. Chromosomal translocation can disrupt these genes to form fusion genes, which can lead to acute myeloid and lymphoid leukemias (AML and ALL). More than 70% of infantile and 10% of adult or treatment related leukemias are related to MLL mutations. One fusion oncogene, MLL-AF9, accounts for 80% of MLL-related infantile leukemias that exhibit mainly AML phenotypes[1]. Given that these leukemias are very aggressive and often have a high mortality rate, it is of great importance to understand their formation, regulatory mechanisms and other properties in order to develop effective treatments.

One major breakthrough in this field of study is the identification of a leukemic-initiated cell population, termed leukemic stem cells (LSC). These cells can be derived from either hematopoietic stem cells or progenitor cells (HSC/PCs) with the recruitment of MLL-fusion genes and wild type MLL proteins[2-4]. Genome wide expression profiles comparing normal HSC/PCs and LSCs revealed that signaling pathways that are important for HSC/PCs survival are also shared by LSCs[5-10]. Characterization and mechanistic studies of LSCs is critical as these cells are responsible for the initiation and potential relapse of leukemias, however the overall framework, including epigenetic regulation, is not yet clear.

MicroRNAs (miRNA) are a class of abundant, short (20-24 nucleotides), non-coding RNAs that have a regulatory purpose. They function by base pairing to sites within their target mRNAs and

prompt mRNA degradation or translational repression. Each miRNA targets multiple mRNAs and almost half of the mRNAs in mammalian cells are targeted by one or more miRNAs[11-21]. Many miRNAs regulate hematopoiesis[22-32] and when abnormally expressed, some function as oncogenes[24,29,33-36] and others as tumor suppressors[34,36-38]. However, the mechanisms and functions of miRNAs in hematopoiesis remain largely unknown.

One miRNA in particular, microRNA-150 (miR-150), has been shown to have an influential regulatory role in hematopoiesis, specifically HSC function and progeny formation[31,39]. When prematurely expressed in HSC/PCs, miR-150 causes specific and severe defects in B cell production from the pre-B cell stage and beyond[31]. The tightly regulated miR-150 expression is crucial for normal hematopoietic differentiation, specifically for HSC/PCs and lineage differentiation. MiR-150 has also been shown to suppress MLL-AF9 leukemia. MLL fusion gene formation is associated with loss of MLL gene function and a global down regulation of miRNAs, one of the most down-regulated being miR-150, compared to other non-MLL leukemia cancers or other hematopoietic progenitors. When miR-150 expression was restored to a physiologically relevant level in MLL-AF9 expression cells, the cells' survival, blast forming ability and LSC colony formation was significantly inhibited.

The impact of a loss of miR-150, however, has not yet been studied. Use of sponges has been proven as an effective method of inhibiting miRNAs. These function by base-pairing with the miRNA so that the miRNA is no longer free to bind its target mRNA, and thereby produce the loss-of-function phenotype[40]. A miR-150 sponge was constructed and tested in comparison with

an overexpression vector to determine the inhibitory effects of miR-150 on LSCs induced by the MLL-AF9 fusion protein.

CHAPTER II

METHODS

Cell culture conditions

All cells were cultured in an incubator to maintain 37ºC with 5% CO_2. 293T cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS); THP-1 cells (human acute monocytic cell line containing the MLL-AF9 oncogene) were obtained from ATCC and maintained in RPMI-1640 with 10% FBS.

Table 1. DNA Oligonucleotide Sequences

Oligos Synthesized by Integrated DNA Technologies	**Sequence**
miR-150 sponge forward	5'TCGAGTATTACACTGGTACAAGGGTTGGGAGAATTAC ACTGGTACAAGGGTTGGGAGAATTACACTGGTACAAGG GTTGGGAGAATTACACTGGTACAAGGGTTGGGAGAATC GCGGGCC 3'
miR-150 sponge reverse	5'CGCGATTCTCCCAACCCTTGTACCAGTGTAATTCTCCC AACCCTTGTACCAGTGTAATTCTCCCAACCCTTGTACCA GTGTAATTCTCCCAACCCTTGTACCAGTGTAATAC 3'
BamHI forward	5'CTCGGATCCACCGGTCGC3'
SalI reverse	5'ATGTCGACCAGCTGGTCTTTCCGCCT3'
HoxA7 forward	5'CGGCCGAAGCCAGTTTC3'
HoxA7 reverse	5'GCGCCGCGTCAGGTAG3'
HoxA9 forward	5'GAATGAGAGCGGCGGAGAC3'
HoxA9 reverse	5'GAGCGAGCATGTAGCCAGTTG3'
Meis1 forward	5'GCAGTTGGCACAAGATACAGGAC3'
Meis1 reverse	5'ACTGCTCGGTTGGACTGGTCTA3'

Plasmids and vectors

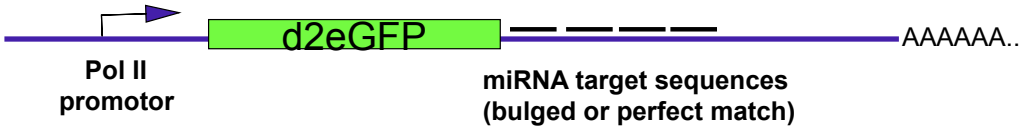
The pcDNA5-CMV-d2eGFP-CXCR4 plasmid contains enhanced green florescence protein (eGFP) coding region and ampicillin resistance gene. The pBabe-puro plasmid contains retroviral backbone and puromycin selection marker. GFP gene with 3' untranslated fragments containing microRNA binding sites was inserted at the cloning sites after digestion with BamHI and SalI at 37ºC overnight.

Construction of the miR-150 sponge retroviral construct

The miR-150 sponge fragment consists of four repeated binding elements complimentary to miR-150 along with one XhoI and one ApaI overhang nucleotide sequences. These oligos (Table 1) were annealed by incubating at 100ºC for five minutes, and then cooled to room temperature for one hour. The pcDNA5-CMV-d2eGFP-CXCR4[40] plasmid was digested with XhoI and ApaI at 25ºC for 2 hours then 37ºC overnight and the large fragment (pCDNA5-CVM-d2eGFP) was gel purified. The annealed sponge fragment was then ligated into the digested vector at 14ºC overnight. The ligation mixture was transformed into DH5α competent cells (Z-Competent™ *E. coli* from Zymo Research) and plated on Luria-Bertani (LB) agar plates containing 50 ug/mL ampicillin. A colony was selected and cultured overnight in 5ml LB broth with 100ug/mL ampicillin. The plasmid was extracted using the Qiaprep Spin Miniprep kit according to the manufacturer's protocol. Enzymatic digestion with XhoI and ApaI confirmed the insertion of the miR-150 sponge fragment. The miR-150sp-eGFP sequence (Figure 1) was then amplified using polymerase chain reaction (PCR) with the BamHI forward and SalI reverse primers, gel purified, then digested with BamHI and SalI and ligated into the digested pBabe-puro[40] plasmid. The product was prepared using the procedure previously described. The

presence of the miR-150sp-eGFP sequence was confirmed using restriction digest analysis and sequencing. The function of the miR-150 sponge was determined by luciferase reporter analysis.

FIGURE 1. MicroRNA sponge gene
The microRNA sponge gene containing the miR-150 binding sites was cloned into the 3'UTR of 2-h destabilized enhanced GFP reporter gene.



Retroviral infection

Mir-150 overexpression was generated using a pLB1-miR150 lentiviral vector. This plasmid was transfected into 293T cells and miR-150 overexpression of the gene was determined by GFP expression and quantitative RT-PCR (qRT-PCR). To generate lentiviral supernatant, the 293T cells were pre-plated at 30% confluency one day before transfection of pLB1-miR150, a VSV-G expression vector (pHCVM-G) and a gag/pol expression vector (pCVMΔR8.9) with Lipofectamine 2000. The supernatant was collected after 48 and 72 hours and used for infection. The THP-1 cells were suspended in the viral supernatant and centrifuged at 720 x *g* for 90 minutes before culturing. The pBabe-miR150sp vector was transfected into the THP-1 cells using a similar procedure.

RT-qPCR

Total RNA was extracted at specific time points from treated THP-1 cells using the Trizol extraction protocol according to the manufacturer's instructions. Gene expression analysis was performed by quantitative RT-PCR with iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) using the primer pairs specified in Table 1. The data presented correspond to the mean of

2-ΔΔCt from at least three independent experimental repeats and normalized to β-Actin or GAPDH reference genes.

Flow cytometry

The effects of miR-150 on THP-1 cells were evaluated by analyzing cell growth, apoptosis, cell cycle, and stem cell populations. The leukemic stem cell population is defined as Lin-(depleted with antibodies against CD4 and CD8 T cells, B220 for B cells, CD11b and CD11c for Myeloid cells) c-Kit+, Sca-1- cells. Fluorescence conjugated antibodies are obtained from eBioscience and flow cytometry analysis was performed using BD Accuri 6. Data were analyzed using FlowJo 7.6.

CHAPTER III

RESULTS

Designing miR-150 sponge primer set

To ensure the binding of miR-150 to the target sponge construct, DNA primers of 120 nucleotide (nt) and 112nt in length, forward and reverse primers respectively, were designed which harbor four miR-150 perfectly complementary binding motifs, each of them 22nt in length connected by a 4nt spacer (Figure 2).

Preparation of the DNA fragment containing the miR-150 sponge sequence

The forward and reverse miR-150 sponge primers were annealed in order to obtain a double-stranded DNA fragment of the sponge with overhanging ends to match Xho1 and ApaI digestion. Two micrograms of both the forward and reverse primers were added to make a 50 uL reaction mixture in annealing buffer (100mM potassium acetate, 300mM HEPES-KOH, pH 7.4, 2mM magnesium acetate). The primers were heated to 100ºC for 5 minutes, and then allowed to gradually cool at room temperature for 1 hour. The annealing product will be used for ligation.

Construction of pcDNA5-CMV-d2eGFP-miR150sp

To prepare the vector construct, the CXCR4 shRNA fragment was first released from pcDNA5-CMV-d2eGFP-CXCR4 (Figure 3). The digested vector was gel purified using the QIAquick Gel Extraction Kit (Qiagen). After purification, the concentration was measured using the NanoDrop-1000® spectrophotometer (Thermo Scientific) and adjusted to 80 ng/uL.

FIGURE 2. miR-150 sponge DNA primer set

2A. Forward and reverse sequences of the miR-150 sponge. Xho1 and Apa1 overhang regions are indicated in green, spacer nucleotides in yellow, and miR-150 binding sites in blue.

Forward:
5’TCGAGTATTACACTGGTACAAGGGTTGGGAGAATTACACTGGTACAAGGGTTGGG
AGAATTACACTGGTACAAGGGTTGGGAGAATTACACTGGTACAAGGGTTGGGAGAA
TCGCGGGCC 3’

Reverse:
5’CGCGATTCTCCCAACCCTTGTACCAGTGTAATTCTCCCAACCCTTGTACCAGTGTA
ATTCTCCCAACCCTTGTACCAGTGTAATTCTCCCAACCCTTGTACCAGTGtTAATAC 3’

2B. Diagram of pcDNA5-CMV-d2eGFP-miR150sp

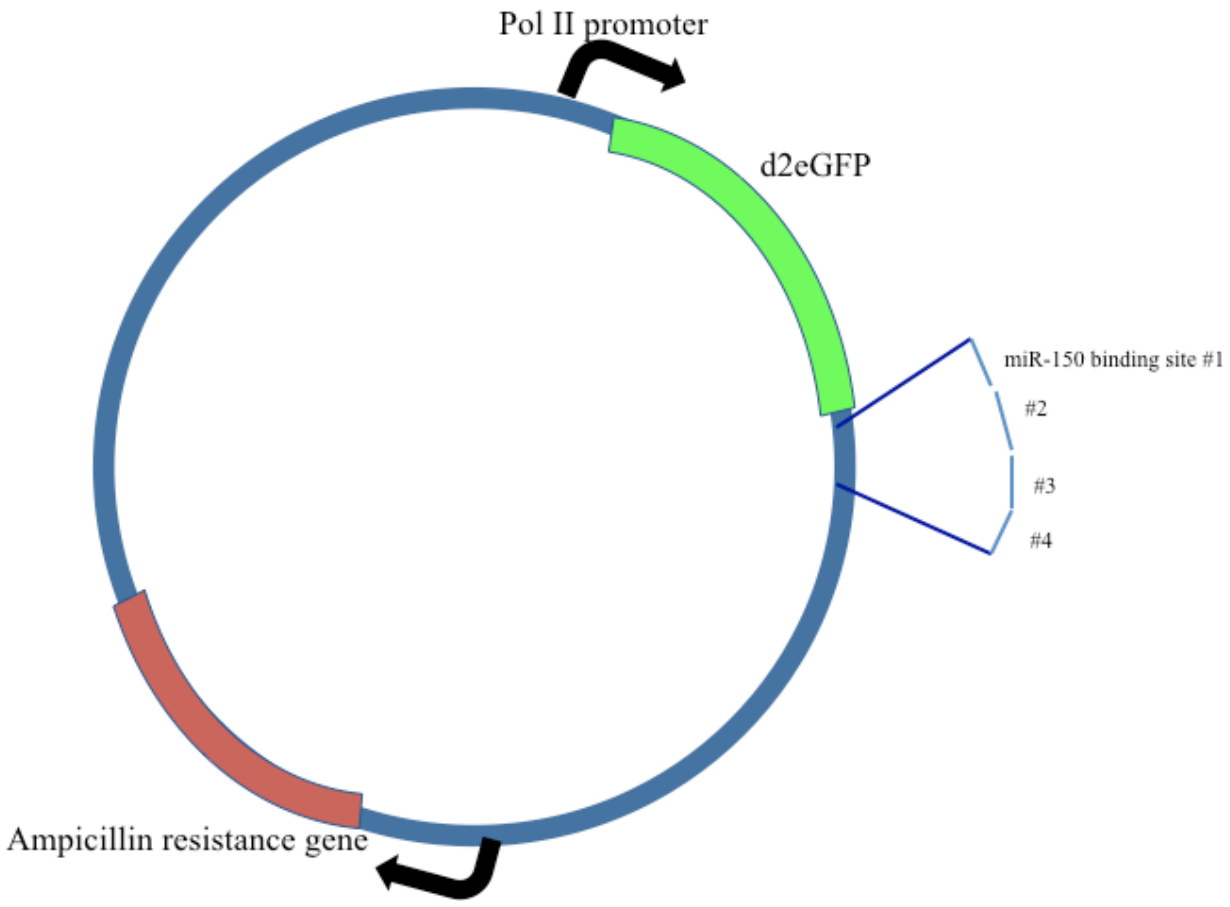
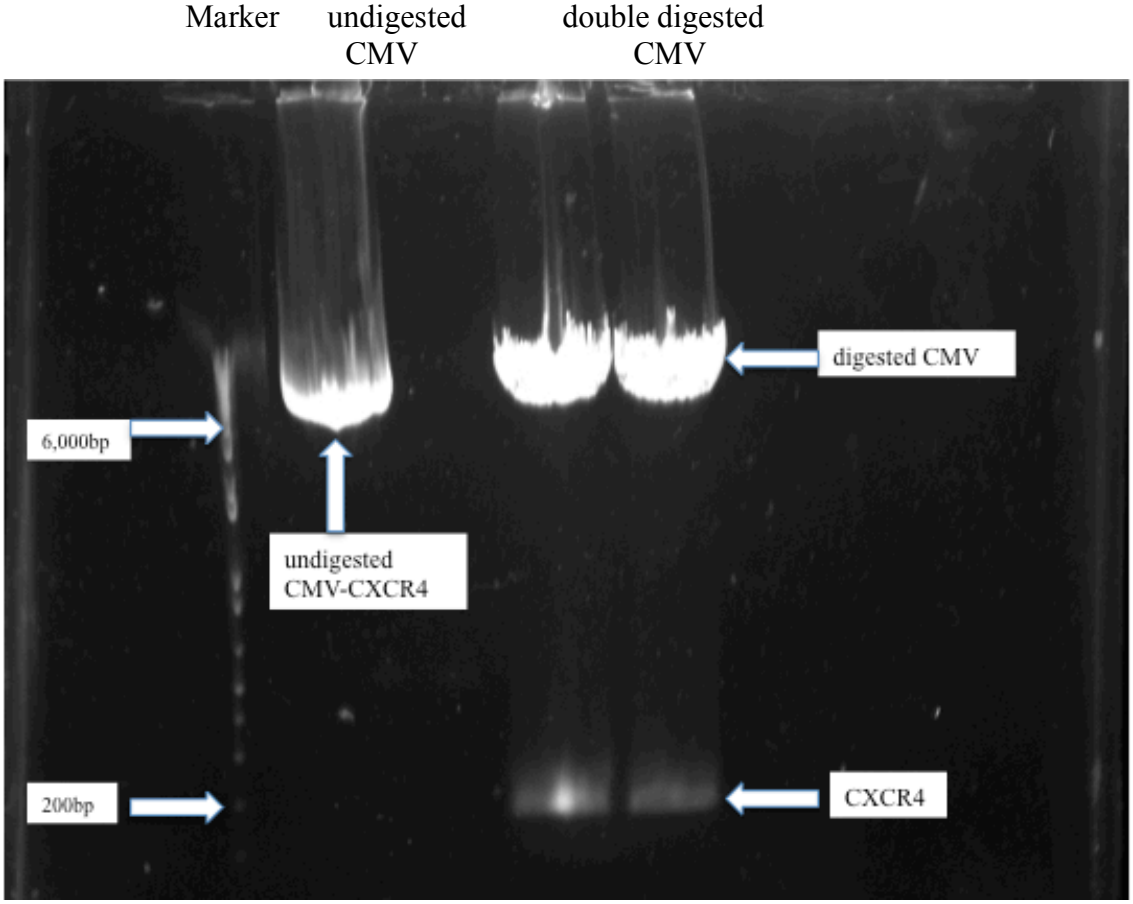


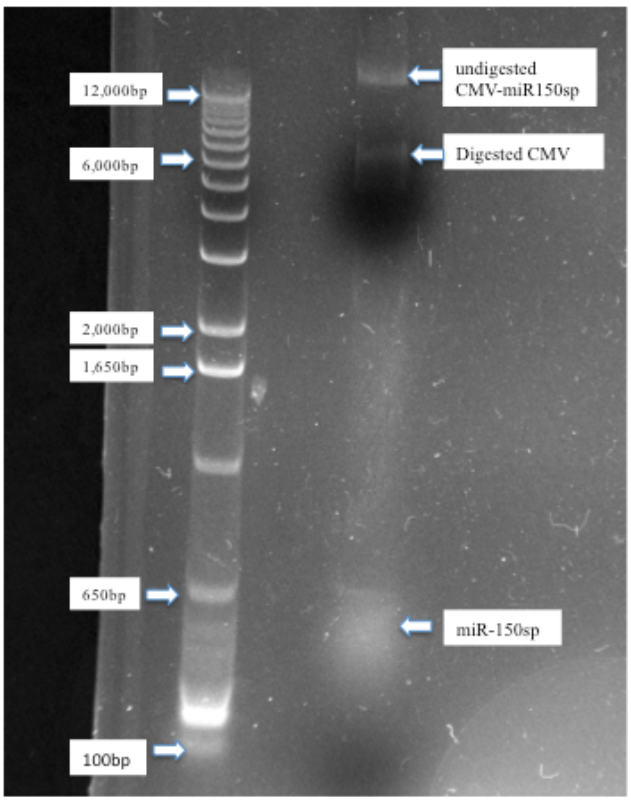
FIGURE 3. pcDNA5-CMV-d2eGFP-CXCR4 after digestion with XhoI and ApaI
After enzymatic digestion, the empty pcDNA5-CMV-d2eGFP (CMV) and CXCR4 fragments were visualized on a 1.2% agarose gel with a 1kb DNA ladder.



The annealed product from the previous step was ligated into the purified vector using T4 DNA ligase (Invitrogen) following the manufacturer's protocol. The ligation product was then transformed into *E. coli* competent cells using a version of the manufacturer's protocol: the ligation product was incubated on ice with 45 uL of cells for 15 minutes, 200 uL SOC was added, and the cells were plated on LB agar containing ampicillin. After 10-16 hours of culture on agar plates containing ampicillin, bacteria containing the successful insertion of the fragment were observed by colony formation. Single colonies were selected and amplified in LB broth

containing ampicillin for 10-16 hours. The plasmid was extracted and the insertion was confirmed by enzymatic digestion with XhoI and ApaI (Figure 4).

FIGURE 4. Confirmation of miR-150 sponge insertion
After enzymatic digestion with XhoI and ApaI, the miR-150 sponge fragment (miR-150sp) and the digested empty pcDNA5-CMV-d2eGFP (CMV) were visualized on a 1.2% agarose gel with a 1kb DNA ladder.



Construction of pBabe-d2eGFP-miR150sp

To transfer the d2eGFP gene coding region with the miR-150sp fragment into a retroviral construct, two primers (Table 1: BamHI forward and SalI reverse) flanking the desired region were applied to amplify the target sequence. The PCR product at approximately 1000nt (Figure 5) was gel purified, concentrated to 5 uL and digested with BamHI and SalI, followed by a

second gel purification. The pBabe-puro vector was digested with BamHI and SalI (Figure 6) followed by gel purification.

FIGURE 5. Purification of PCR product
After PCR amplification, d2eGFP-miR150sp (PCR product) was visualized and purified from a 1.5% agarose gel with a 1kb DNA ladder.

Marker PCR product

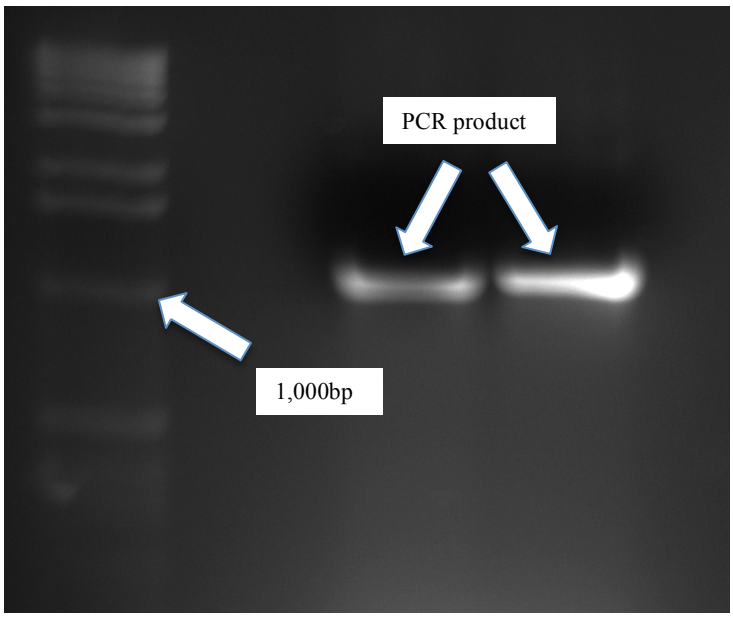
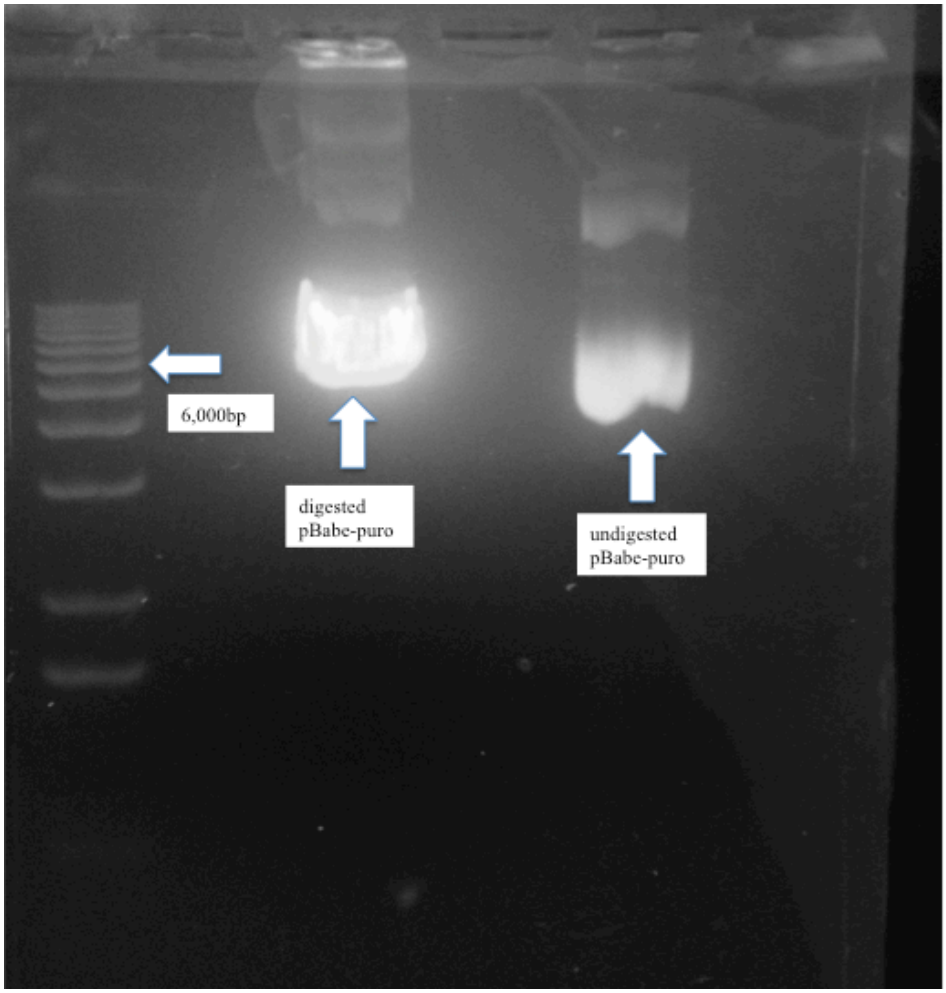


FIGURE 6. pBabe-puro after digestion with BamHI and SalI
After enzymatic digestion the digested empty pBabe-puro vector was visualized and purified from a 1.2% agarose gel with a 1kb DNA ladder.

Marker | undigested pBabe | digested pBabe



Future steps

Complete the construction of the miR-150 sponge system

The PCR product will be inserted into the digested pBabe-puro (Figure 6) by ligation. After transformation and plasmid extraction, the construct will be sequenced to confirm the presence of the d2eGFP-miR-150sp gene.

Evaluation of miR-150 sponge using reporter assays

The knockdown efficiency will be evaluated using luciferase assay to evaluate the efficiency of miR-150 inhibition on a reporter construct containing the luciferase gene-coding region followed by an untranslated region containing a miR-150 binding site. Compared to samples without miR-150sp, we expect to see a recovery of luciferase activity as a result of the miR-150sp neutralization effect.

Investigation miR-150 regulation on LSCs using constructed overexpression and miR-150 sponge systems

The retroviral supernatant will be generated using 293T cells and used to infect MLL-AF9 cells to evaluate the loss of miR-150 on LSC characteristics and survival. Accordingly, gain of miR-150 function on LSCs will be evaluated using similar procedures after transduction of miR-150 overexpression construct, pLB1-miR150. The frequency of LSCs will be determined by flow cytometry analysis using antibodies to identify Lin-, c-Kit+, Sca-1- cells. Lower proliferation rates of LSCs are expected with ectopic miR-150 overexpression and higher rates are expected with the presence of the miR150 sponge.

CHAPTER IV

DISCUSSION

It has been shown that miR-150 expression is down regulated in several cancer types, including MLL-AF9 leukemia. After overexpression, both in vivo and in vitro, miR-150 reduces survival and proliferation of leukemic cells. Whole gene transcriptome analysis revealed that restoration of miR-150 suppressed more than 30% of genes specifically expressed in stem cells (unpublished results). In order to gain a better understanding of the mechanism through which this occurs, the loss-of-function phenotype should be studied, as well. However, it is difficult to knock down microRNA levels effectively in vivo, we adopted a microRNA sponge design that allows investigation of miR-150 using a loss of function strategy.

The rational of a microRNA sponge system utilizes complementary binding sites for a microRNA of interest, specifically miR-150 in this study. However, the efficiency of microRNA knockdown can be improved with several modification options. One option is to introduce a bulge in the binding sequence resulted from mismatch to the mature miR-150 sequence which has been shown to increase the stability of the microRNA sponge[40]. If inefficient transfection or infection occurs, a lentiviral vector might be used instead of the retroviral pBabe plasmid.

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