CHAPTER TWO

Materials and Methods
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2.1.1. Cell culture

K562, KCL22 cells were obtained from Dr. Soumen Chakraborty lab (ILS Bhubaneswar). The cells were cultured in 10% RPMI media with foetal calf serum (v/v) and 0.1 mg/ml PenStrep at 37 °C and 5% CO2 conditions. Cells were passaged every 2-3 days and used for study till passage 20. The cells were tested for mycoplasma routinely and found negative consistently. Short term TK inhibitor treatment was performed at inhibitory concentration 50 (IC50) - 0.75 μM of Imatinib Mesylate (purchased from Santa Cruz biotechnology) and incubated for 48 hrs. The dead cells were washed and live cells were scored for Bcr-Abl activity by measuring the expression of p-Crkl, a downstream target molecule of Bcr-Abl tyrosine kinase. TK inhibitor resistant K562 cell line was generated by gradual increase in the dose of TK inhibitor. The first treatment dose was 0.05 μM TK inhibitor, and gradually TK inhibitor dose was increased every week. The treatment was continued for 3 months till achievement of 5 μM and the cells were then characterized as TK inhibitor resistant K562 cells. The TK inhibitor resistant cells were then studied for further analysis.

2.1.2. Isolation of mono-nuclear cells from peripheral blood, and bone marrow

Bone marrow from CML patients was isolated and mononuclear cells were separated from the whole blood by Ficoll Histo-paque density gradient method. RBCs were lysed from the mononuclear cells by treatment with RBC lysis buffer containing ammonium chloride. The cells were washed with PBS twice. The cells were counted and their viability was checked by the trypan blue method with every thawing. 1 million mononuclear cells were cultured overnight in a 6 well culture plate at 37 °C and 5% CO2. This initial 24 hrs culture helped the cells to revive and expand before they were being subjected to any treatment or used in further experiments.
2.1.3. Primary cell culture of peripheral blood cells and bone marrow

Primary cells were cultured in serum free IMDM media supplemented with 1% glutamine (100 mM) and 1% penicillin-streptomycin (100 mM). A five growth factor cocktail comprising 100 ng/ml Flt3-ligand, 100 ng/ml stem cell factor, 20 ng/ml each of interleukin IL-3, IL-6 and granulocyte macrophage colony stimulating factor (GMCSF) was added to the media. Growth factors were purchased from Peprotech.

2.1.4. Small RNA sequencing

K562- the cell line from a late stage CML patient expressed BCR-ABL fusion gene b3-a2. Cells were treated with 0.2 μM concentration of TK inhibitor for 72 hrs. Total RNA was isolated from inhibitor treated and untreated cells, and ligated with 3’, and 5’ adapters for small RNAs. The adapter conjugated RNA was reverse transcribed with 3’ adapter specific primers, and amplified by PCR. The PCR products were run on a 8% polyacrylamide gel, 100 base pair band was cut and eluted for purification. The purified cDNA from drug treated cells was given for sequencing on Illumina hiseq platform. The miRNA sequence data was mapped on to human genome (version hg18) and mature miRNAs were identified using RNA fold and miRDeep to confirm their stability. Sequences were read as frequency i.e. how many times the miRNA had been read and normalized as TPM (transcripts per million). MiRNAs from drug treated K562 cells were compared with miRNA dataset from untreated K562 cells (Prof. Alok Bhattacharya, JNU).

2.1.5. Gene expression analysis

Briefly, total RNA was isolated from CML cells using Trizol method following the manufacturer’s instructions. 20 ng RNA was used to synthesize a specific cDNA of hsa-miRNA182-5p using stem-loop miRNA specific RT primer. qRT-PCR was performed using Sybr green dye (purchased from Kappa) on Applied Biosystems 7500 fast detection system. Expression of miRNA was normalized using the expression of the housekeeping genes 18s r-RNA. Relative quantification of miRNAs was calculated with the delta delta Ct method. Data
is shown as mean of three independent experiments. Error bars represent standard error of three independent experiments with p-values of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001. The Bcr-Abl/Abl ratio was determined for each patient sample (supplementary table 1).

2.1.6. Methylcellulose colony formation assay

K562 cells were counted for cell viability and an equal number of live cells were seeded in 15% FCS with IMDM culture media mixed with 1% methylcellulose. Colonies were determined for their lineage based on morphological characteristics\(^8\)\(^3\)\(^8\)\(^7\). Clonal colonies emerged after 3 weeks were counted and scored for contribution to granulocyte, macrophage, erythrocyte and megakaryocyte lineages. The CFU-Es were progenitors with one or two clusters with 10-100 hemoglobinized erythroblasts in each cluster. The BFU-E colonies were characterized as small (3-8 clusters), intermediate (10-16 clusters) or large (more than 16 clusters) of primitive erythroid progenitors. The CFU-Gs were progenitors of homogeneous population of granulocytes and exhibit the presence of refractive granules in their cytoplasm. The CFU-Ms were clonogenic progenitors of macrophages that display ground glass appearance. The CFU-GEMM displayed heterogeneous population of granulocytes, erythrocyte, macrophage and megakaryocyte. The CFU-GM displayed heterogeneous population of granulocyte and macrophage. Erythroid colonies displayed haemoglobin production by their red brown colour. The granulocytic colonies displayed large refractive granules. Images were taken under fluorescence microscope (Nikon Eclipse TE2000-S; Meville, NY, USA). Colonies of 5-10 different areas were counted per dish. The images were taken at 4X and 10X. The myeloid cells were calculated by adding CFU-G and CFU-M colony numbers. The erythroid cells were calculated by adding CFU-E and BFU-E colonies. The ME% was used to show a shift in lineage distribution. Data is shown as mean of 3 independent experiments. Error bars represent as standard error of three independent experiments with p-values of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001.
% of myeloid-erythroid cells (ME%) = ((CFU-G + CFU-M)/(CFU-E + BFU-E))*100

2.1.7. LNA anti-miRNA

LNA based oligonucleotides are designed to block miRNA182-5p expression. In LNA oligos, the furanose ring of selected ribose sugars is chemically locked into an RNA-mimicking conformation by the introduction of an O2′, C4′-methylene Bridge. The LNA oligos were purchased from GeneX India Biosciences Pvt Ltd.

Scramble Tm-32°C

5′CATCGTCGATCGTAGCGCA3′

Anti-miRNA182-5p Tm-42°C

5′AGTGTGAGTTCTAACCATTGCCAAA3′

Anti-hsa-let7c-5p Tm-33°C

5′AACCATAACAACCTACTACCTCA3′ Bold letters represent LNA modification.

The LNA anti-miRNAs bind to their complementary miRNA and prevent them from binding to their mRNA sequences. Hence, translational block of target mRNA is released in anti-miRNA transfected cells. Transfection of K562 cells with anti-miRNA182-5p was performed using Lipofectamine2000 as per manufacturer’s protocol. Three different concentrations used were unit 1 for 10 nM, 2 for 20 nM and 5 for 50 nM concentration. Scramble oligos were used along with the treatment. After 24 hrs, cells were cultured with TK inhibitor at 0.75 μM for another 48 hrs. Absorbance was measured after addition of WST1 cell proliferation reagent (Roche) and normalised to 100 after 72 hours post transfection of the scramble control. Data is shown as mean of three independent experiments. Error bars represent as standard error of three independent experiments with p-values of the mean. *p < 0.05, **p < 0.01, and ***p < 0.001.
2.1.8. MiRNA mimics

MiRNA mimics were purchased from Sigma. The transfection of K562 cells was performed according to the manufacturer's protocol. Three different concentrations were used 1 for 10 nM, 2 for 20 nM and 5 for 50 nM concentration. Scramble oligos were used along with the treatment89.

2.1.9. Hes1 Knock-down and overexpression

Briefly, K562 cells were nucleofected with Hes1 overexpression and knock-down plasmids on Amaxa 4d nucleofector from Lonza and incubated for 3 days. Hes1 was overexpressed by miGR1-Hes1 plasmid90. Hes1 knock-down was performed by shRNA against Hes1 open reading frame. ShRNA-Hes1 plasmids were a kind gift from Dr. Adolfo Ferrando, Columbia University91. MigR1 control and MigR1-Hes1 plasmids were a kind gift from Avinash Bhandoola lab.

2.1.10. Flow cytometry

Briefly, K562 cells were washed twice in 1X- PBS, fixed in 2% PFA for 10 min at room temperature. Then cells were permeabilized by Saponin followed by blocking with 5% blotto at room temperature for 30 min. Rabbit anti-Hes1 antibody (abcam) was incubated as per manufacturer's protocol. Secondary antibody incubation was done at RT for 30 min and washed with PBS. Cells were assessed for Hes1 expression on FACS Calibur flow cytometer90. The data was analysed using CellQuestPro software and shown as histograms and dotplots.

2.1.11. CRISPR mediated miRNA182 Knockout

Designing of MIR182 constructs- The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated (Cas) system is RNA based genome engineering platform. Recently shown in vitro reconstitution of the Streptococcus pyogenes type II CRISPR system demonstrated that crRNA fused to a normally trans-encoded tracrRNA was sufficient to direct the Cas9
protein to sequence-specifically cleave target DNA sequences matching the crRNA. Here, we engineer a single system of this bacterial type II CRISPR system in human cell lines. For designing the guide RNAs, we identified all the NGG sites (represents Protosporer Adjacent Motif- PAM sequence) in MIR182 cluster region +/- 1000 bp of the target site. To direct Cas9 to cleave sequences of interest, we designed crRNA-tracrRNA fusion oligonucleotides, referred to as guide RNAs (gRNAs) under the human U6 polymerase III promoter. To identify CRISPR target sites around MIR182 cluster that should be cleavable without off-target cuts, we therefore examined all 23bp sequences of the form 5’GBBBBBBBBBBBBBBBBBBBNGG3’, where the B’s represented the bases at the genome location, for which no sequence of the form 5’NNNNNNBBBBBBBBBBBBNGG3’ existed at any other location in the human genome. Identification of CRISPR target sites across MIR182 locus was done using the Zhang lab tool, MIT. A single plasmid system expressing Cas9, eGFP, and GuideRNA was synthesised to target MIR182 loci. The CRISPR plasmids induced double strand breaks in HEK 293T cells assessed by surveyor nuclease assay represented as indel percentage. In HEK293 cells, CRISPR plasmids transfection generated deletion of the MIR182 locus as shown by agarose gel analysis. Each off-target site was assessed for indels. The top five off-targets were chosen based on their quality score and examined for their potential to generate indels. The off-targets of Cas9+0482 and Cas9+9678 did not reveal indels while Cas9+0425-05 off-target present in the non-coding genomic region induced indels. 0.5 Million K562 cells were nucleofected with CRISPR MIR182 plasmids and cultured for 72 hours. Further, expression of plasmids was monitored by eGFP expression. FACS Sorting of 0.5% high eGFP positive cells was performed on FACS ARIA with an anticipation of higher editing efficiency. 30-50 cells were cultured in 48-well plate for 10 days and analysed for deletion clones by PCR method.

2.1.12. **Surveyor mutation detection assay**

Briefly HEK293T cells were transfected with CAS9-GFP-GuideRNAs targeting MIR182 flanking regions. After 48 hrs, genomic DNA was extracted from the pool of cells, and analysed for Indels using Surveyor mutation detection kit
Guide RNAs to target MIR182 locus

129409678: GGAGGGCACGCCGTGCATCA
129410482: CCACTCCCAAGGGAAACCCGA
129410425: GAAGGACCTTGTGCCAGTTG

Top 1
TTTTACTAGTTGTACAAAAACGAGGCTTTAAGGAAACAAATTTCAGTGACTGGATCCGG
Bottom 1
TCATGGGAAATAGGCCCTCTTCCTGCCCAGACCTTTGGTACCAGAGATCCAGTCTGAGATAT
Top 2
GGAAGAGGGCTATTCTCCATGTATTCCCTTCATATTTCATATACGATACAAGGCTGTTAG
Bottom 2
TCTTTGTGTTTACAGTCAAATTCTTATAATTCTCTCTCTCAAAGCCTTTGTATCGTATA
Top 3
TTTTTGGACTGTAACACAAAAAGATATTAGTACAACACAGGGAAATACGTCGAGTAGAAATGATAATTT
Bottom 3
TTTTAAACACATATAATTTAAAACTGCAAACATACCAAGAAATTATAACCTTTCTACGTCACG
Top 4
AGTTTTAAAATATTGTACAAAAATGGAACATATCATATAGCTTACCAGTAACCTTTGAAGATTT
Bottom 4
TCCGTCCTTTCATCAAGTATATAAAGCCCAAGAACATCTTTCTACGTCGAGTAGAAATGATAATTT
Top 5
TTTTTCATTCTGAGAAAGCAGAAACACC(N20)GTGTGTAGCTAGAAATAGCAA
Bottom 5
AGTTGATAACGGACTAGCCTATATTACCTTTGCTATTACACTTTCAACGTCGAGTAGAAATGATAATTT
Top 6
TTTTAAAACAAAATATTTAAAACTGCAAACATACCAAGAAATTATAACCTTTCTACGTCACG
Bottom 6
AAAAGCTAGCTAATGCCAACCTTTGTACAAAGAAAGCTGCTGCTTAGAAAGAAAGCACCAGCT

Primer sequences to amplify MIR182 target sites

9678-FP
TCTCACCCCTCTCCCTGGAAA
9678-RP
CCACTCAGCTGACCCTGTTG
425-FP
AAATAGCAAAAACCCAGCCAC
425-RP
CCAGTGTGAGTTTCTGACACAC
pBSK-FP
TTCCCTTCCAGTCCCGCCT
pBSK-RP
AAGCCGAAGGCCCAGCAGTTCTTC

Primer sequence to amplify the MIR182 locus

182gDNA.FP
GGTTGTTTTAGCTTTGCCACATTCT
182gDNA.RP
TGCAAGACAAACGCTGTTGGGAGGT
(Transgenomic) as per manufacturer's protocol. Briefly, PCR amplicons from mutant (test) and wild-type (reference) DNA were hybridized by heating and cooling the mixture to form hetero- and homoduplexes. The annealed heteroduplex/homoduplex mixture was treated with Surveyor Nuclease. DNA fragments were analysed by agarose gel electrophoresis. The formation of new cleavage products due to the presence of one or more mismatches was indicated by the presence of additional bands. The relative size of these cleavage products indicated the location of the mismatch or mismatches. Intensity of individual DNA bands was measured by ImageJ software. Indel percentage was calculated as per reference protocol\textsuperscript{93}.

**Engineering CRISPR constructs for gene targeting**

- The two halves of the gRNA constructs were synthesized.
- The two halves were merged via PCR.
- gRNA construct were cloned into pCas9-eGFP vector.
- DNA cleavage efficiency of CRISPR constructs were determined by performing Surveyor Nuclease assay.

2.1.13. **The Inoue method for preparation and transformation of competent E.coli. Preparation of Cells**

Inoue transformation buffer (chilled to 0\(^\circ\)C before use) was prepared.

a. 0.5 M PIPES (pH 6.7) (piperazine-1,2-bis[2-ethanesulfonic acid]) was prepared by dissolving 15.1 g of PIPES in 80 ml of pure H2O (Milli-Q, or equivalent). The pH of the solution to 6.7 was adjusted with 5 M KOH, and then pure H2O was added to bring the final volume to 100 ml. The solution was divided into aliquots and stored frozen at -20\(^\circ\)C.

b. Inoue transformation buffer was prepared by dissolving in 800 ml of pure H2O and then 20 ml of 0.5 M PIPES (pH 6.7) was added. The volume of the Inoue transformation buffer was adjusted to 1 litre with pure H2O.

c. Inoue transformation buffer was sterilized by filtration through a prerinsed 0.45-mm Nalgene filter and divided into aliquots and store at -20\(^\circ\)C.
A single bacterial colony (2-3 mm in diameter) was picked from a plate that had been incubated for 16-20 hours at 37°C. The colony was transferred into 25 ml of LB broth or SOB medium in a 250-ml flask. The culture was incubated for 6-8 hours at 37°C with vigorous shaking (250-300 rpm).

This starter culture was used to inoculate three 1-liter flasks, each containing 250 ml of SOB. The first flask received 10 ml of starter culture, the second received 4 ml, and the third received 2 ml. All three flasks were incubated overnight at 18-22°C with moderate shaking. Next day, the OD600 of all three cultures were read and continued to monitor the OD every 45 minutes. When the OD600 of one of the cultures reached 0.55, the culture vessel was transferred to an ice-water bath for 10 minutes. The two other cultures were discarded. The cells were harvested by centrifugation at 2500g (3900 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C. The medium was poured off and the open centrifuge bottle was stored on a stack of paper towels for 2 minutes. The cells were resuspended gently in 80 ml of ice-cold Inoue transformation buffer.

The cells were best suspended by swirling rather than pipetting or vortexing. The cells were harvested by centrifugation at 2500g (3900 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C. The medium was poured off and the open centrifuge bottle was stored on a stack of paper towels for 2 minutes.

### Freezing of Competent Cells

The cells were resuspended gently in 20 ml of ice-cold Inoue transformation buffer. 1.5 ml of DMSO was added. The bacterial suspension was mixed by swirling and then stored in ice for 10 minutes. Working quickly; aliquots of the suspensions were dispensed into chilled, sterile microfuge tubes. Immediately the competent cells were snap-frozen by immersing the tightly closed tubes in a bath of liquid nitrogen. The tubes were stored at -70°C until needed. When needed, a tube of competent cells was removed from the -70°C freezer. The cells were thawed by holding the tube in the palm of the hand. Just as the cells thawed, the tube was transferred to an ice bath. The cells were stored on ice for 10 minutes. A chilled, sterile pipette tip was used to transfer the competent cells to chilled, sterile 17 x 100-mm polypropylene tubes. The cells were stored on ice.
Transformation

All of the appropriate positive (a characterized plasmid) and negative controls (either water control or a plasmid without insert) were included. The transforming DNA (up to 25 ng per 50 ml of competent cells) was added in a volume not exceeding 5% of that of the competent cells. The tubes were swirled gently several times to mix their contents. At least two control tubes were set up for each transformation experiment, including a tube of competent bacteria that received a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that received no plasmid DNA at all. The tubes were stored on ice for 30 minutes. The tubes were transferred to a rack placed in a preheated 42ºC circulating water bath. The tubes were stored in the rack for exactly 90 seconds. The tubes were rapidly transferred to an ice bath. The cells were allowed to cool for 1-2 minutes. 800 ml of SOC medium was added to each tube. The cultures were warmed to 37ºC in a water bath, and then the tubes were transferred to a shaking incubator set at 37ºC. The cultures were incubated for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, the cells were gently agitated (<225 cycles/minute) during the recovery period. The appropriate volume was transferred of transformed competent cells onto agar SOB medium containing the appropriate antibiotic. When selecting for resistance to antibiotic, the entire transformation mixture was spread on a single plate (or plated in top agar). IMPORTANT a bent glass rod was sterilized by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod had cooled to room temperature, the transformed cells were spread gently over the surface of the agar plate. When selecting for resistance to ampicillin, transformed cells were plated at low density (<104 colonies per 90-mm plate), and the plates were not incubated for more than 20 hours at 37ºC. The plates were inverted and incubated at 37ºC. Transformed colonies appeared in 12-16 hours. The plates were stored at room temperature until the liquid had been absorbed.
2.1.14. Plasmid DNA Isolation

A microcentrifuge tube was filled with saturated bacterial culture grown in LB broth + antibiotic. The tube in microcentrifuge was spin for 1 minute. The supernatant was dumped and drained off briefly on paper towel. Step 1 was repeated in the same tube, filling the tube again with more bacterial culture. 0.2 ml ice-cold Solution1 was added to cell pellet and cells were resuspended as much as possible using disposable transfer pipet. Solution1 contained glucose, Tris, and EDTA. 0.4 ml Solution2 was added, tubes were capped and inverted five times gently. Tubes were allowed to sit at room temperature for 5 minutes. Solution2 contained NaOH and SDS (a detergent). 0.3 ml ice-cold Solution3 was added, tubes were capped and inverted five times gently. Tubes were incubated on ice for 10 minutes. Solution3 contained a mixture of acetic acid and potassium acetate. The *E. coli* chromosomal DNA, a partially renatured tangle at this step, was also trapped in the precipitate. The plasmid DNA remained in solution. The tubes were centrifuged for 5 minutes. The supernatant was transferred to fresh microcentrifuge tube using clean disposable transfer pipet. This fractionation step separated the plasmid DNA from the cellular debris and chromosomal DNA in the pellet. The remainder of centrifuge tube was filled with isopropanol. The tube was allowed to sit at room temperature for 2 minutes. The tubes were centrifuged for 5 minutes. A milky pellet should be at the bottom of the tube. The supernatant was poured off without dumping out the pellet. The tube was drained on paper towel. This fractionation step further purified the plasmid DNA from contaminants. 1 ml of ice-cold 70% ethanol was added. The tubes were capped and mixed by inverting several times. The tubes were spin for 1 minute. The supernatant was poured off and tubes were drained on paper towel. The tubes were allowed to dry for ~5 minutes. 50 ul TE/H2O was added to tube. If needed, tubes were centrifuged briefly to pool TE at bottom of tube. Optional: RNAse could be added to either in first step with Solution1 or with TE at final concentration of 20 ug/ml.
2.1.15. Purification of DNA by phenol extraction and ethanol precipitation

An equal volume of phenol was added to the DNA containing reaction mixture and vortexed gently. The aqueous phase was separated which contained the DNA from the organic phase by centrifugation in the microfuge, at 2000 rpm for 5 min or at 8000 rpm for 1 min. The aqueous phase was removed with care into a fresh microfuge tube and an equal amount of 24:1 (v/v) chloroform-isooamyl alcohol was added. In order to precipitate the DNA, a 0.1 volume of 3 M sodium acetate, pH 5.5 was added to the aqueous phase and then 2 volumes of absolute ethanol. The solution was incubated at −20 °C overnight or for shorter periods at −80 °C (e.g. 20–30 min). The precipitated DNA was recovered by centrifugation in the microfuge at 10000 rpm for 5–15 min. The ethanol was removed with care and dried the pellet in a desiccator or 50 °C oven for 5 min. An extra wash with 70% (v/v) ethanol was included to remove excess salt from the pellet. The dried DNA was resuspended in sterile TE, pH 8.0, or water, and stored at 4 °C for further manipulation or at −20 °C for long-term storage.

2.1.16. Primer designing

All the primers were designed in NCBI primer blast. The melting temperature for all the primers was kept 60°C.

2.1.17. Off-target analysis

Off-targets were identified by the Zhang lab, MIT tool. The top five off-targets for each guideRNAs were analysed by the surveyor nuclease mutation detection system\textsuperscript{94}.

2.1.18. Genotype for \textit{MIR182} deletion

\textit{MIR182} genomic site flanking the target sites (1560 bp) was amplified using PCR and was run on a 3% agarose gel containing EtBr. The product length was matched to the expected product and deletion was confirmed by presence of 756, 819 and 1497 bp DNA fragments. Each PCR product was purified using
Qiagen PCR purification kit as per manufacturer’s instructions and given for Sanger sequencing analysis. Each chromatogram was read using FinchTV software.

2.1.19. **Statistical Analysis**

Data points are expressed as mean ± standard error. Student t-test was performed to find the significance of differences. P values of less than .05 were considered to be significant.

2.1.20. **MiRNA Sequencing data analysis**

From the sequencing reads, we trimmed TruSeq small RNA adapters using customized perl script and cutadapt (1) program. We then mapped these reads to rRNA database and unaligned reads were taken for further analysis. We then segregated reads that are 18-24 nucleotides and mapped to hg19 Genome and miRNA databases (2) using bowtie v1.0.0 (3) Customized perl script was used to obtain count data for all the miRNAs. The count data was normalized using DESeq and the normalized data was used for further analysis. MiRNAs which have adjusted p-value < 0.05 were considered for further analysis. We identified 84 miRNAs as significantly expressed. The fold change in the expression of these 84 miRNAs was determined for Imatinib versus control sample. Heatmap was generated in R to show the change in the expression of the identified miRNAs.
Pipeline for miRNA sequencing data analysis