CHAPTER SEVEN

Summary and Discussion
Chapter-07 Summary and Discussion

7.1. MiRNA182 in the development of CML

We investigated chronic myeloid leukemia as a model disease in which hematopoietic stem cells acquire Bcr-Abl fusion gene. The molecular factors responsible for late stage CML progression are poorly understood. The identification of these factors requires integration of high throughput approaches as well as in vitro data. Our initial analysis of RNA sequencing data detected an elevated expression of miRNA182 in K562 cells and primary CML cells in the context of TKI inhibitor. These observations were verified in a panel of CML patients. The elevation of miRNA182 had been of our primary interest as this was the only candidate seen overexpressed out of 83 other deregulated miRNAs. The erythroid commitment of K562 cells after Imatinib treatment further suggested that expression of miRNA182 might be linked with lineage program of K562 cells. Our data showed that miRNA182 transient interference and exogenous overexpression reciprocally shift the ME%. The molecular mechanism underlying erythroid type CML and increased erythroid burden seen in therapeutically resistant cases can be potentially linked to high miRNA182 levels. In order to establish a robust stable system to define this observation and lay the foundation for subsequent analysis, we used CRISPR as a tool to generate a MIR182 deletion. CRISPR has been used to engineer human cells to understand both biological processes as well as their therapeutic implications. The cell line generated shows a clear switch with the emergence of myeloid cells (CFU-G and CFU-M) at the expanse of erythroid cells (BFU-E). Our data not only showed a clear function of miRNA182 but also validates the use of this gene editing technique for MIR deletion. This is consistent with the clinical observation of erythroid cells in the presence of both tyrosine kinase resistance and miRNA182 expression. Collectively, these data suggested that miRNA182-5p upregulation concomitantly contributed to both accumulation of erythroid cells as well as resistance to TK inhibitors. Some of the important questions came from our study are as follows- a) is/are there any intrinsic resistance mechanism/s which is/are universal to all the cancerous/non-cancerous cells, b) how deeply do the miRNAs penetrate in the cellular
Model based on Hypothesis

A. Therapeutic Stress
   - Ectopic Expression of miRNA182
   - Smad3
   - MiRNA182
   - Hes1
   - Erythroid Differentiation
   - TKI Resistant

B. CRISPR/Cas9
   - LNA Anti-miRNA182
   - Cas9
   - MiRNA182
   - Anti-MiRNA 182
   - Myeloid Differentiation
   - TKI Sensitive
decisions and c) could TKI resistance mechanisms be associated with one miRNA or more factors are involved.

7.2. MiRNA182 associates with the TKI resistance mechanisms

Our results reveal a striking switch in the balance of myeloid-erythroid ratio in ΔMIR182-K562 cells, demonstrating a clear role of this microRNA in contributing to lineage decisions and also implicating such choices with tyrosine kinase resistance. Our initial analysis was using established methods that transiently interfere with gene expression and revealed an insight into both the role of miRNA182 in regulating proliferation/viability and the lineage choices. Other studies have also raised the possibility of an important role for miRNA182. For example, miRNA182 has been implicated in chemo resistance in breast cancer cells and in the progression of tumors such as melanomas. Other studies have suggested a role for this miR in the context of development, though the mechanistic roles are currently poorly understood. The molecular pathways that are implicated in mediating the effects of miRNA182 are only partially characterized. Recent reports of miRNA203 (Cancer Cell 2008) regulating ABL1 and BCR-ABL1 oncogene expression and and miRNA328 (Danilo Perrotti et al., Cell 2010) in the pathogenesis, and blastic transformation of CML critically point out their involvement, and impact on cellular networks. There are reports which suggest an oncogenic or tumor suppressor function of miRs. Several miRs have been characterized and one such example is miRNA182. MiRNA182 has been reported in lung, colorectal and liver cancer as an oncomiR by H Xia et al 2012. MiRNA182 has also been shown to affect DNA repair and sensitivity to PARP inhibitors by D. Chowdhury et al 2011. Several miRs such as miRNA223, miR144/451 have been implicated in mediating a role in tyrosine kinase resistance. An unresolved question that remained unanswered in the study is the complementation of the Bcr-Abl with the miRNA182 signalling. Our data predicts that the clear understanding of both the molecular signalling pathways would result in the management of the disease.
7.3. Oncogenic and tumor suppressor miRNAs

As miRNAs have been appreciated as oncogenic miRNAs and laid the groundwork for miRNA based therapies, tumor suppressor miRNAs have been reported in cancers. One of the first tumor suppressor miRNA was miRNA15 and -16. These two miRNAs are transcribed as a cluster (miRNA15-a-miRNA16-1) that resides in the 13q14 chromosomal region. Deletions and point mutations in this region occur at very high frequency in CLL, lymphoma and several solid cancers. These miRNAs of this cluster targets BCL2, CCND1, WNT3A which promote several prostrate tumorigenic features including survival, proliferation and invasion. Interestingly, deletion of miRNA15 and -16 loci in a transgenic mice model generated indolent B-cell autonomous, lymphoproliferative disorders, recapitulating the spectrum of CLL associated phenotypes observed in humans. Another example with tumor suppressor phenotype is let-7 miRNA which has been shown to have inverse relationship with RAS oncogene. Earlier, Ras proteins have been shown to contribute to the pathogenesis of several human tumors\(^{133}\). In addition to above examples, miRNA182 cluster has been found amplified in human cancers. The miRNA182 along with miRNA96 and -183 are frequently found overexpressed in various tumor types\(^{134}\). The miRNA182 LOCUS IS located on human chromosome 7q32.2. The locus is also one of the hotspot for the insertions and deletions. The chromosomal instability that has been mostly attributed to leukemias suggests that miRNA182 locus becomes unstable and amplified during the course of the disease. Surprisingly, the miRNA182 cluster that is conserved for 600 million years and played critical role in ectodermal tissue development becomes predominant candidate in cancer cell signalling. In other report, Let7 has also been shown to downregulate the expression of Myc at mRNA as well as protein level in lymphoma cells. MiRNA34 family of miRNA has three members. While miRNA34-a is transcribed from chromosome 1p36, other two member miRNA34-b and -34-c are generated through the processing of a bicistronic transcript from chromosome 11q23\(^{135}\). The expression of this cluster reflected the p53 status and ectopic expression leads to decrease in phosphorylated retinoblastoma (Rb) protein level. The cluster majorly affects the expression of
CCNE2, MET and CDK4. Among other targets reported later include BCL2 and MYCN in neuroblastoma and NOTCH1, NOTCH2 and CDK6 in glioma cells\textsuperscript{136}. Some other miRNAs which have been associated with p53 dependent tumor suppression include miRNA29 family members, miRNA29-a, -b and –c. The members of this cluster inhibit expression of p85 alpha and CDC42\textsuperscript{137}. Moreover, miRNA122 is seen downregulated in liver cancer cells and it exerts its effect on migration, invasion by suppressing ADAM17, a disintegrin and metalloprotease 17\textsuperscript{138}. Collectively, the data from our study emphasize that genome contribution should be integrated with cellular networks.

7.4. MiRNA182 targets Hes1 and components of the notch signalling in CML cells

To further validate the role of the miRNA182 axis, we followed our initial bioinformatics analysis with experiments to assess the role of Hes1. The results with Hes1 modulation also are consistent with a key role for the miRNA182 axis in modulating myeloid-erythroid ratio. The molecular pathways that are implicated in mediating the effects of miRNA182 are only partially characterized. Hes1 is a key molecular component of the Notch pathway, which has been implicated in both hematopoietic lineage determination and disease. The lineage determinants and their pathways in haematopoiesis are well-defined and serve as a paradigm to understand disease progression. Genes and pathways implicated in this process include Bmi1 and other polycomb genes, the Wnt-beta catenin pathway, the Notch and Sonic Hedgehog signalling pathways as well as the Hox family of transcription factors\textsuperscript{139}. The Notch signalling genes were shown to be expressed in self-renewing tissue and activation of Notch signalling was subsequently shown to potentiate self-renewal of both HSCs and NSCs\textsuperscript{140,141}. The traditional view of the Notch pathway in the context of a disease is based on mutations such examples include leukemias, particularly ALL. Notch1 mutations causing Notch signalling continuously activated have been found in nearly 60% T-ALL patients. The activated Notch signalling results in enhanced self-renewing ability, thus leading to T-ALL\textsuperscript{142}. Additionally, other components of Notch signalling are
involved in the initiation and progression of T-ALL. Such examples include Notch3 and Hes1 which are found highly expressed in T-ALL cells, as well as dramatically reduced or absent in remission\textsuperscript{143,144}. Hes1 being a critical regulator of myeloid differentiation, its increased expression in CML cells is suggestive of promoting both myeloid immaturity and blast crisis transformation through a number of different mechanisms\textsuperscript{145}. However, the classical explanation of their expression fails to reveal their functional details in disease state. The molecular understanding of regulation of the Notch signalling at transcriptional, post-transcriptional and epigenetic level will be useful to find alternate therapeutic options in leukemias. The data generated in our study would suggest a rethinking of the role of this pathway in the context of late stage CML and particularly tyrosine kinase resistance in the absence of mutations in the TK locus.

As a wider implication, we also validate that gene editing of specific locus in established cell lines. Our results reveal a striking switch in the balance of myeloid-erythroid ratio in ΔMIR182 cells demonstrating a clear role of this microRNA in contributing to lineage decisions and also implicating such choices with tyrosine kinase resistance.

7.5. Future Directions

Given the diversity of regulatory signalling pathways upstream and downstream to miRNA182, different stages of CML might require rewiring of the miRNA182-dependent transcriptional programs. The development of modulators of differentiation might synergise with other therapeutic strategies in the management of late-stage CML. CML management could be improved with the addition of miRNA182 and erythroid-myeloid lineage status along with the Bcr-Abl status. The assay performed on K562 cells that displayed limited differentiation potential could be improved by addressing the question in early progenitor cells such as hematopoietic stem cell and using an animal model to track the cells for a longer time period. Given the higher expression seen in the embryonic stem cells, induced pluripotent stem cells and hematopoietic stem cells, miRNA182 function remains unanswered in development. A better and
quicker model system such as drosophila or transgenic mice would endow the functional details. An integrative approach involving the transcriptome/mass spectrometry would help in identifying novel targets of miRNAs. Gene editing on clinically important genomic loci is a useful approach to create reagents for phenotypic analysis in the context of chemoresistance. The use of both the cell line that we have generated and clinical analysis should offer an opportunity to create novel therapies for the relapsing CML cases in the years ahead.

7.6. High throughput approaches and international efforts- An Integrative Approach to Fight Cancers

High throughput approaches have emerged recently as important tools that can be used to find novel therapeutic targets in cancers. Some of the striking example of its clinical use includes identification of rare genetic variants associated with monogenic Mendelian disorders and efficient detection of either inherited or somatic mutations in cancer genes. These deep sequencing based platforms facilitate the study of genome, transcriptome, proteome and epigenome which is used for understanding biological processes deregulated in disease. The major outcome of these studies is accurate measurement of disease specific molecular patterns such as single nucleotide polymorphisms (SNPs), small RNAs such as miRNAs, histone methyl transferases and an integrated map of global gene crosstalk. The high throughput sequencing approach has proven to be a powerful tool to identify recurrent, specific genetic abnormalities in solid cancers and leukemias. The generation of these data and identification of these novel abnormalities are result of international collaborations. The international cancer genome consortium (ICGC) and the cancer genome atlas (TCGA) are some of the initiatives of worldwide collaborative efforts which catalogue the genomic information of thousands of cancer genomes across many diseases.