

Identification of miRNAs Involved in the Regulation of B Cell Development

Traditionally, gene regulatory networks have been studied at the transcription and translational levels in an attempt to understand the pattern of expression of the encoded proteins. It's only been recently that this dogma has changed by the discovery of microRNAs (miRNAs). miRNAs, short non-coding RNAs, can regulate mRNAs involved in any number of biological processes such as in development, malignant transformation, and neurobiology. Our laboratory is interested in the regulation of lymphocyte development, particularly in pro and pre B cell stages. The goal of this proposal is to identify miRNAs involved in gene regulatory networks influencing B cell development and leukemic transformation.

B cells develop from hematopoietic stem cells into mature B cells with input from various signals and transcription factors. The discovery that specific miRNAs regulate developmental processes such as muscle development and neuronal development led us to hypothesize that miRNAs may also be involved in regulating B cell development and/or oncogenic transformation of B cells.

miRNA processing has been studied in detail and we hope to exploit this knowledge for our purposes. miRNAs originate from 70 nucleotide long pri-miRNA sequences. Pri-miRNA forms a stem loop structure processed within the nucleus by a ribonuclease-III (RNase-III) enzyme, Drosha, and is then exported to the cytoplasm. We propose to utilize this pri-miRNAs processing pathway to modulate the expression of specific mature miRNAs in B cells.

Once in the cytoplasm, the pri-miRNA is processed into a miRNA:miRNA* duplex by another RNase-III, Dicer. The RNA Induced Silencing Complex (RISC) then processes the miRNA:miRNA* duplex producing the mature miRNA¹. The mature miRNA can then regulate its target mRNA by either directed cleavage or translational repression. Many miRNAs have already been identified to have importance in developmental processes. For instance, miR150 expression is critical in mature lymphocytes, but blocks development when expressed prematurely². We will perform a microarray screen for miRNAs that are regulated during B cell development. With the goal of further clarifying the gene regulatory network in B cell development, I hope to examine regulated miRNA "hits" involved in this process and examine their downstream, repressed targets.

Aim 1: To identify miRNAs that may play a regulatory role in B cell development

It is likely that miRNAs are involved in regulating the progression of B cells from one stage to the next. To identify miRNAs involved at the pro-to-pre-B cell transition, I have taken advantage of AMuLV leukemic pro-B cell lines. These cell lines have been transformed by the Abelson Murine Leukemia Virus, blocking development at a pro/pre B-cell like state. To eliminate the block in B-cell differentiation we treated the cells with a drug called Gleevec, a specific inhibitor of the oncogenic kinase v-Abl. Treated cells overcome the block, exit the cell cycle and seemingly continue the progression to the pre-B cell stage³. To ensure that "hits" were not cell specific we used various cell lines. Cells were cultured in the presence or absence of Gleevec. RNA was then purified and used to synthesize labeled probes, then analyzed by hybridization to a miRNA microarray to determine if any miRNAs are up or downregulated as the differentiation block is removed. Preliminary results highlighted an upregulation pattern in a subset of miRNAs once v-Abl was inactivated and B cell progression allowed.

We identified 24 miRNAs that may be involved in regulating B cell development or leukemic transformation at the pro-to pre-B transition. I am currently confirming the expression of a few of these miRNAs by RT-PCR utilizing a published technique that reverse transcribes the miRNAs by using a stem-loop primer and then amplifies the cDNA for Taqman qPCR⁴.

Aim 2: To test the significance of regulated miRNA expression and identify their downstream targets in immortalized B cell lines

I performed a database search for possible downstream targets of my miRNAs of interest utilizing miRBase, TargetScan and the UCSC genome database. To test whether selected miRNAs of interest are affecting the predicted downstream targets, I will overexpress these candidate miRNAs in various cell lines and assay target gene expression.

We are using the miR30 vector as a system for overexpressing my candidate miRNAs in cell lines⁵. Stem-loop structures of my miRNAs were designed to resemble the pri-miRNA prior to processing; the mature miRNA's stem-loop sequence was inserted in place of the known miR30 stem-loop. This chimeric miRNA will then be retrovirally transduced into pro-B cell lines to induce overexpression of the miRNA of interest. The retrovirus is marked with a human CD2 cDNA allowing us to identify transduced cells by flow cytometry. By overexpressing miRNAs of interest using this miR30-based system, we expect the mature miRNAs to diminish the expression of their downstream targets. We can then assay targets predicted by TargetScan to be downregulated by these miRNAs. In order to verify that the predicted targets are being downregulated I would need to examine cells that express both an empty vector control and the miRNA construct. I would expect the target's mRNA level and protein levels to be diminished as analyzed by real-time PCR and western blot, respectively.

Aim 3: To confirm overexpression results in primary B cells and to further examine B cell development in a mouse knock-down model.

We first need to confirm the pattern of expression of our identified miRNAs in sorted B cells from different stages of B cell development. If a different pattern was observed than in the AMuLV cell lines, then we can predict that our target miRNAs are involved in leukemia; a converse observation would indicate that we are looking at development. We can further explore the regulation of the miRNAs and their downstream targets using both overexpression and knock-down strategies. I will infect primary pro-B cell cultures from wild-type mice with various miRNA mir30-based overexpression constructs (see Aim 2) and compare these primary cultures to those infected with an empty vector control. We will examine whether specific marker events of B cell development are altered by miRNA overexpression. If we see an effect of overexpression in primary pro-B cells we can proceed to knockdown the expression of miRNAs of interest in a mouse model to probe their functions.

We will generate a mouse model in which we delete our miRNA sequence by gene targeting. In this way we can examine the B cell development process in mice that inherently lack the miRNAs of interest. We would predict that knocking down our miRNA would give us a phenotype that is the opposite of the phenotype seen when the downstream target is inhibited by the miRNA. In order to examine the B cell developmental process we would need to examine these cells for the hallmarks of B cell development by flow cytometry. The possibility of non-coding short RNAs regulating the B cell development makes the clarification of this gene network an essential process to study for its praxis in development, immunology, and cancer.

Keywords: miRNAs, pro-B cells, gene network

¹ L. He *et al.*, *Nat. Rev Genet.* **5**, 522 (2004).

² B. Zhou *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7080 (2007).

³ S. Muljo *et al.*, *Nat. Immunol.* **4**, 31 (2003).

⁴ C. Chen *et al.*, *Nucleic Acids Res.* **33**, 179 (2005).

⁵ D. Boden *et al.*, *Nucleic Acids Res.* **32**, 1154 (2004).