Summary of work done

Role of *Drosophila Argonaute-1* in apoptosis

Introduction

Apoptosis is collection of multiple perturbations of the cellular structure and architecture that do not promote only programmed cell death but incubate the unwanted, old or abnormal cells to be removed via phagocytes and reduce innumerous immune responses. It also plays an initial role in tissue homeostasis and proper development of an organism. Organ structure, shape and size determination too depends on programmed cell death. Programmed cell death allows the apoptotic cells to be dismantled in a programmed manner that minimizes damage and disruption of adjacent cells. These events are orchestrated intensely by the activation of series of cysteine proteases family members referred as caspases. A number of diseases including cancer, immune deficiency disorders and neurodegenerative disease develop as a result of deregulated cell death.

Argonaute (AGO) family proteins are highly conserved across both animal and plant kingdom. In mammals, both AGO and PIWI includes four members in their subfamily. Structurally AGO proteins contain four domains- an amino terminal domain which is relatively less conserved in nature and highly conserved mid domain, PAZ domain & PIWI domain. AGO proteins are the direct binding partners of existing small RNA. As member of mammalian and *Drosophila* AGO family, both AGO1 and AGO2 are well studied, because of its role in microRNA (miRNA) biogenesis and as a major representative of miRNA effector complex (RISC complex). miRNAs are generated after processing of long double stranded precursors RNAs and control gene expression by post-transcriptional regulation. By interacting with AGO proteins miRNA guide the RISC (RNA-induced silencing complex) to its mRNA targets to induce translational repression or degradation. As a post-transcriptional regulator for a large number of vital genes, miRNAs play crucial role in controlling a wide range of cellular processes including cell proliferation, migration, cell death and differentiation. Therefore mis expression of miRNAs leads to the development of various diseases including cancer and neurodegenerative disease. miRNAs can act as a tumor inducer or an inhibitor of tumor by altering the expression of tumor suppressor genes or oncogenes. Altered expression of miRNA biogenesis associated proteins such as DROSHA and DICER is frequently observed during tumorigenesis and cancer progression. As AGO is the prime factor for functional regulation of miRNA, it is speculated that its misexpression affect vital cellular processes such as cell growth, cell division, cellular differentiation and programmed cell death.

Statement of problem

When we had planned this study, the role of *Ago-1* on RNAi was well known but no literature was available explaining *Ago-1* as a regulator of apoptosis. This lack of knowledge provokes us to investigate the role of *Ago-1* on the very crucial process of any organism's life, apoptosis. Moreover, at that time no suitable *in vivo* screening platform was there to identify micro RNA inhibitor small molecules, synthesized by our chemists in our institute. That scenario encourages us to develop an efficient *in vivo* screening platform to test micro RNA inhibitor small molecules using *Drosophila* model system which allows us to extend our study into genetic level, instead restricting to conventional screening work.

The first aim of this study was to find, *Ago-1* is related to any kind of apoptosis or not. After getting the answer of this question our next query was how *Ago-1* regulates apoptosis and which pathways are involved there? Then, our next aim was to identify micro RNAs (miRs) which are

involved in this process. After acquiring answers of above mentioned query; our final goal was to develop an efficient *in vivo* screening platform to test micro RNA inhibitor small molecules using *Drosophila* model system and to identify some small molecules to target miRs which has a potential to inhibit *Ago-1* induced apoptosis.

Objectives of the study

1. Identify the role of *Ago-1* as a regulator of apoptosis and dissecting the pathway related to *Ago-1* induced apoptosis.

2. To identify the micro RNAs (miRs) involved in *Ago-1* induced apoptosis and their functional roles.

3. Identifying small molecules that modulate target miRs having potential to inhibit *Ago-1* induced apoptosis.

Methodologies Used and Sample Results

In order to understand the role of *Ago-1* in apoptosis, we developed *Drosophila* lines by using well established and powerful biochemical tool of UAS/GAL4 system to ectopically overexpress *Ago-1* gene in different organs of *Drosophila* using specific GAL4 drivers. GAL4 and the UAS are important for any ectopic expression in *Drosophila* as their ectopic expression or overexpression does not interfere with other processes in the organs. In this method, *Drosophila* modular protein GAL4 is fused under the control of different endogenous driver promoters. GAL4 is therefore only expressed in the cells in which driver promoters are active. GAL4 protein binds to the UAS sequences to activate fused target gene expression. In this study, we used GAL4/ UAS *Ago-1* transgene that is expressed ectopically in a tissue specific manner. *UAS Ago-1* flies were crossed with GMR-GAL4 driver, which expressed GAL4 under the control of GMR promoter, thereby ectopically expressing *Ago-1* in the developing eye. Interestingly, ectopic overexpression of *Ago-1* lead to phenotypic changes in the adult eyes that resembled apoptotic cell death compared to the control flies that contained endogenous copy of *Ago-1*. To understand whether these deformities in the adult eyes were due to any developmental apoptotic cell death we performed Acridine Orange (AO) test that gives a measurement of apoptosis in eye imaginal discs of third instar larvae arising from stocks that contained overexpressed *Ago-1*, mutated *Ago-1* and normal. A large number of positively stained apoptotic cells were observed in the discs arising from larvae in which *Ago-1* is overexpressed (*UAS Ago-1/GMR GAL4*). Suppression of this phenotype by dominant negative mutation of fly JNK, *basket* clearly indicates the involvement of JNK pathway with this process. Followed by real time PCR, Western blotting and immunostaining further confirms the involvement of pro-apoptotic genes, apoptotic inhibitor proteins and caspases. Chromatin immunoprecipitation (ChIP) assay and nuclear extract immunoprecipitation (IP) revealed the interaction between AGO1 and RNA Pol II at the promoter region of *tak-1* (TGF-β activated kinase-1) gene which is an upstream player of JNK signaling.

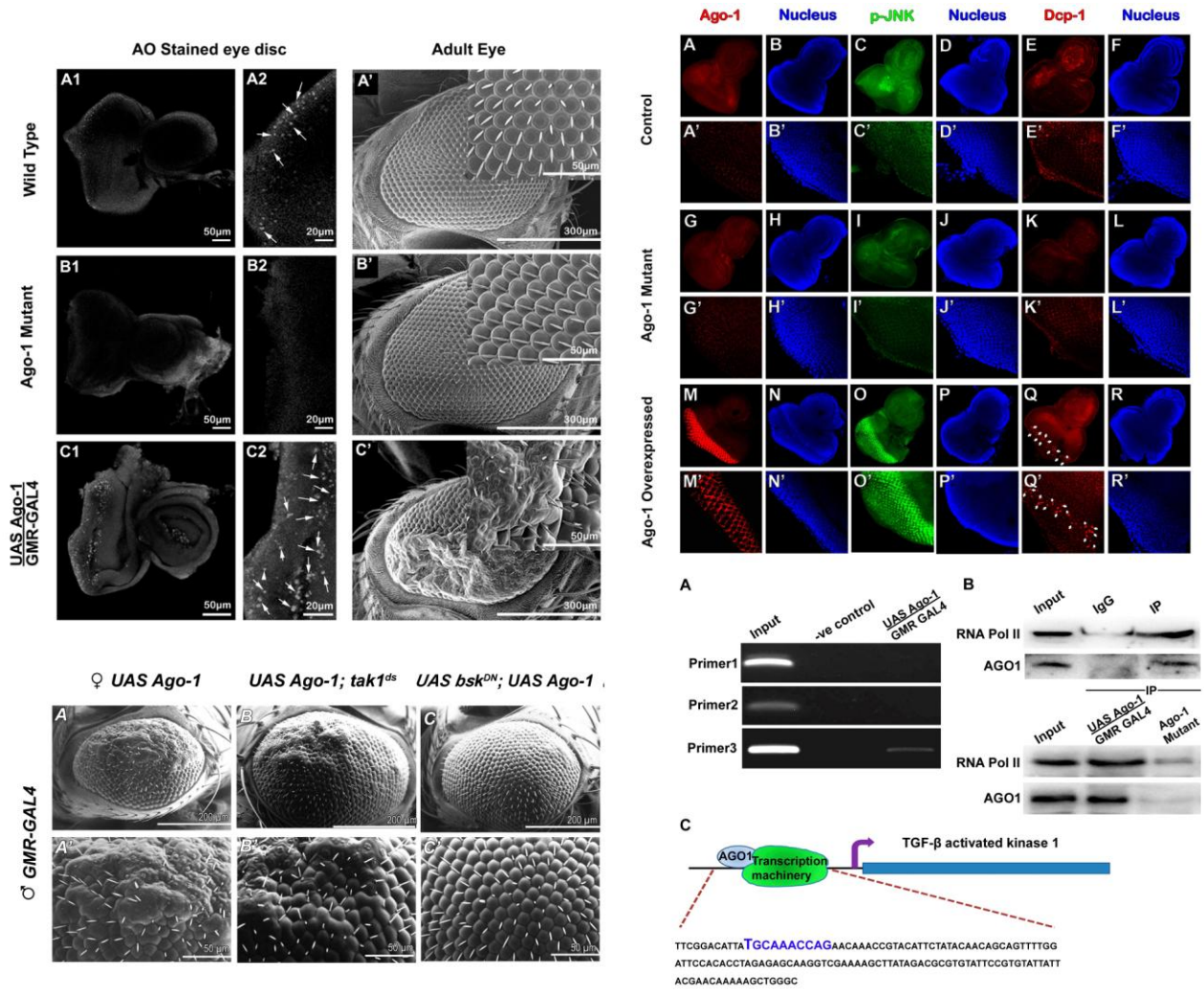

Figure 1: Result figures for objective-1- *Ago-1* induces caspase dependent apoptosis in developing fly organ through phosphorylation of fly JNK, *basket* (*bsk*) by interacting with RNA Pol II at the promoter region of *TGF-β activated kinase 1* (*tak1*).

Micro RNAs (miRs) are endogenously produced tiny (~21nt) non-coding RNAs that are involved in the expression of genes in many developmental processes. A large number of miRNAs have been identified in all organisms including fly and human that control around 30–40% of total genes. Various reports on functional analysis of micro RNA showed their significant role in modulation and fine-tuning of different biological activity including apoptosis. To understand the change of miR expression in response to altered Ago-1 expression micro RNA microarray profiling was carried out. Involvement of dme-miR-14 and bantam was confirmed from array data, realtime PCR and genetic crosses.

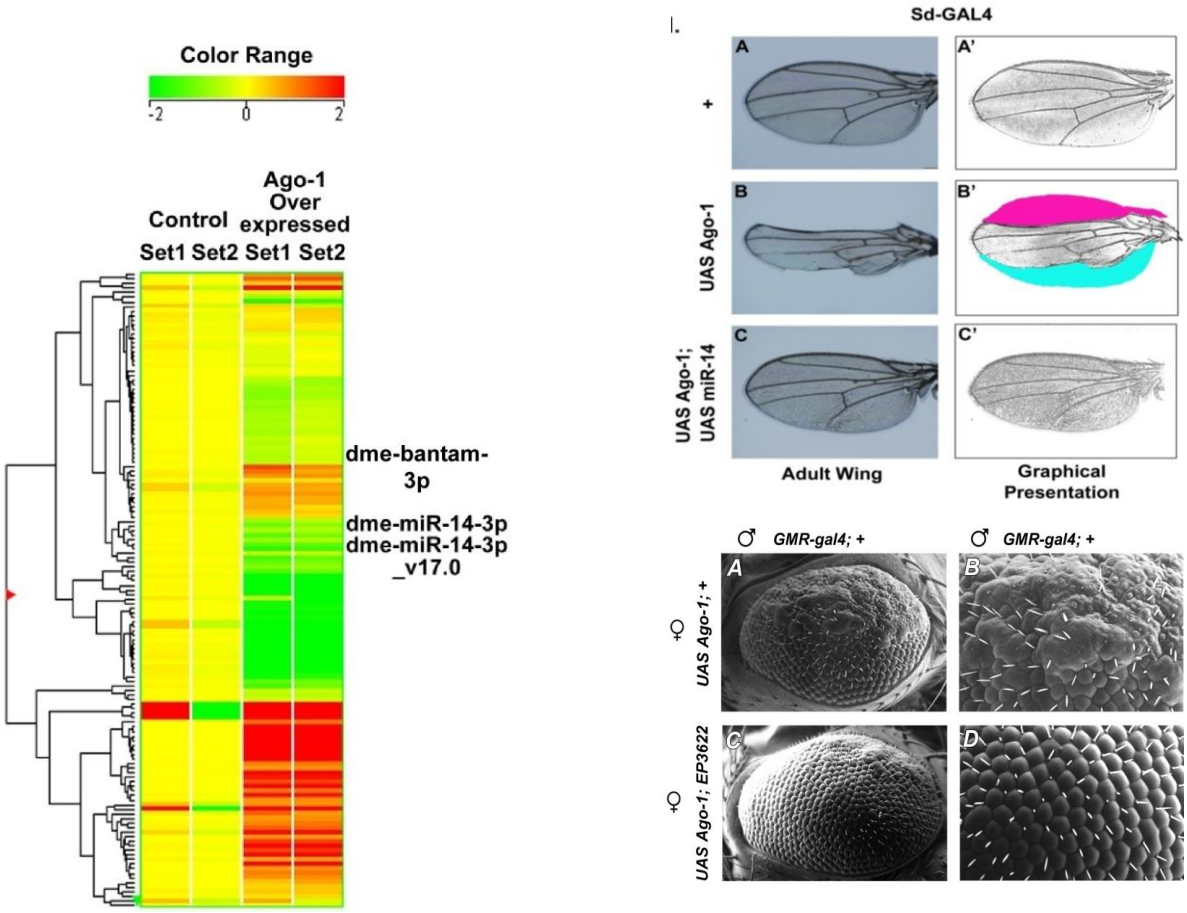

Figure 2: Result figures for objective-2- *Ago-1* down regulates apoptotic inhibitor micro RNA, *miR-14* and *bantam* to induce apoptosis.

For above mentioned reasons, micro RNAs are considered as attractive drug targets against different diseases including cancer. Small molecule mediated targeting of miRNAs, particularly targeted inhibition of oncogenic micro RNAs (oncomiRs) serve a novel approach to discover better therapeutics against cancer.

In our present study, miR microarray analysis was carried out to know the changes of miR level in altered *Ago-1* expression. From that result, *miR-14* and *bantam* has been identified as modulator miR which has a link with *Ago-1* related apoptosis. Next, we have synthesized and characterized a small molecule library to inhibit the function of these oncomiRs for better therapeutics. Triazole derivative small molecule, DCPTN-PT (1-(4-(3, 4-Dichlorophenyl)-1, 2, 3, 4-tetrahydronaphthalen-1-yl)-4-pentyl-1H-1, 2, 3-triazole) and Triazole linked benzoxazole derivatives has been identified as an inhibitor of *bantam* and *miR-14*. These small molecule inhibitors induce apoptosis by targeting microRNAs (*bantam, miR-14, miR-2* and *miR-13*) that suppress apoptotic process in fly. Fly genome sequencing showed that around 77% of human genes that are associated with various diseases are conserved in *Drosophila*. Ectopic expression of human Parkinson's disease (PD) causing mutant alpha-synuclein gene (SNCA) in *Drosophila melanogaster* results in loss of dopaminergic neurons. Fly orthologs of human cancer causing genes can induce cancer-like phenotypes in *Drosophila*, including high rate of cell proliferation, defective apoptosis and deregulation in the process of differentiation. So, it is very obvious that small molecule inhibitors which can inhibit fly oncomiRs will have the potential to inhibit its human counterpart also.

To screen these small molecules as inhibitors of miRs “miR Sensor” approach has developed. Control sensor fly always express GFP under tubulin (tub) control and miR sensor express GFP depending on miR activity, as miR sensor lines carry miR binding site at its 3’ UTR (Un Translated Region). When

one molecule inhibits the activity of any miR; GFP expression of that particular sensor will increase at that time as a result of the release of miR induced repression of GFP trans-gene by that particular miR. In this study, initially we've screened miR inhibitor small molecules using this miR Sensor approach, followed by detailed study has been carried out by over expressing those miRs using UAS/GAL4 system. As miR-14 has a role in the regulation of mitochondrial Reactive Oxygen Species (mtROS) level, so changes in mtROS level was measured using MitoSOX staining. The expression of pro-apoptotic genes and activation of caspases has been determined by semi-quantitative & quantitative PCR and western blot analysis.

In conclusion, an efficient *in vivo* screening method has been developed that not only identifies miR inhibitor small molecules, but also classifies their elaborate biological functions in cellular and developmental pathways. And these newly identified small molecules can function as unique modulator for understanding the role of miRs in apoptosis which probably opens a new way for alternate use of small molecules to regulate oncomiRs and brings a new light of hope in the efficient development of a modern therapeutic strategy to treat cancer.

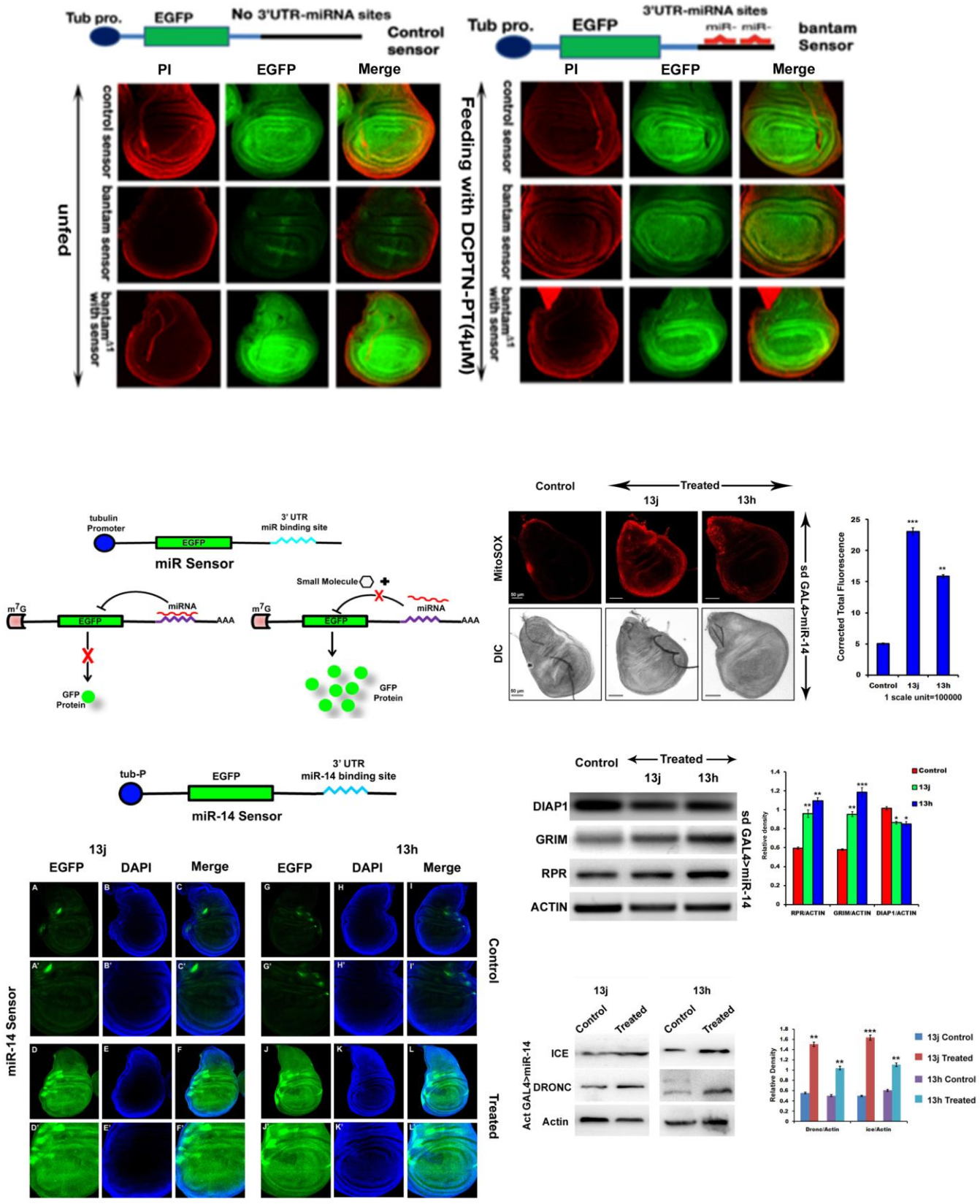

Figure 3: Result figures for objective-3- Small molecule DCPTN-PT, "13j" and "13h" inhibits apoptotic inhibitor miR, *bantam* and *miR-14* to induce apoptosis. "13j" and "13h" also induces *reaper* (*rpr*) induced apoptosis by increasing mitochondrial reactive oxygen species accumulation.