Chapter 7: Summary and Conclusion

Malaria remains a public health problem of enormous magnitude, affecting over 500 millions people every year. In the fight against malaria, there is an urgent need to develop new antimalarials and effective malaria vaccine because of wide spread resistance to common antimalarials. The progress in the development of effective antimalarials/ vaccine has been slow due to the lack of understanding of the functions of different parasite proteins. Reverse genetics is one of the ways to assess the function of parasite genes. Gene function elucidation by antisense approach, ribozyme technology and gene disruption technology has met with limited success. Even though transfection technology to knock out different genes has been developed for different malaria parasites, they are not being applied routinely due to extensive selection procedures. With the availability of malaria genome sequence, there is an urgent need to develop an effective molecular tool to carry out functional genomic studies in malaria.

Recently, RNAi has emerged as a powerful and quick tool to study the gene function in a wide variety of organisms. RNA silencing is a sequence specific RNA degradation process mediated by double stranded RNA. It differs from other methods of reverse genetics in terms of specificity and simplicity. Extensive genetic and biochemical studies have revealed a three-step mechanism for RNAi induced gene silencing. The first step involves degradation of dsRNA in to small interfering RNA (siRNA) of 21-25nt long. An RNAse III like enzyme called DICER has been shown to be involved in this step. In the second step, RISC (**R**NA **i**nduced **s**ilencing **c**omplex) is activated by forming a complex with siRNA. The third step involves recognition of corresponding mRNA by siRNA-RISC complex and degradation of that mRNA by nucleases present in the complex. Currently RNAi is being used extensively in *C.elegans*, *Drosophila*, in cultured insect and mammalian cell lines to elucidate the function of the genes. In *C.elegans* RNAi has been successfully used to carry out large-scale functional analysis of several genes.

Effect of dsRNA on *in vitro Plasmodium falciparum culture*

To carry out RNAi in malaria parasite, initially procedures to make dsRNA corresponding to falcipain genes were developed. Double-stranded RNAs for these genes were synthesized using T7 and SP6 polymerases. Agarose gel electrophoresis was carried out to analyze the synthesized dsRNA. Since malaria parasite is an

intracellular parasite, in the beginning, uptake of labeled dsRNA by the malaria parasite was examined. Careful analysis of different fractions of malaria parasite showed that approximately 0.1-0.15 % of dsRNA was taken up by the parasite. To determine the effects of *falcipains* dsRNA on *P. falciparum cultures*, synchronized parasites at late ring stage were incubated with dsRNA for each of the two *falcipains* separately, as well as in combination, at different concentrations. Inclusion of dsRNAs at conc. 50 μg/ml in the parasite culture showed distinct morphological changes, the most notable of which was the abnormally swollen food vacuole that contained an accumulation of malaria pigment. Addition of each dsRNA as well as in combination also brought reduction in the formation of new rings and as well as total parasitemia. These studies showed that cysteine proteases play an important role in parasite development and growth. The effect of *falcipain* dsRNA on the parasite growth was also confirmed by ^{3}[H] hypoxanthine uptake assay. To investigate whether the observed morphological abnormalities in *falcipains* dsRNA treated parasites were indeed caused by a blockage in hemoglobin degradation, protein extracts were prepared from the treated as well as untreated parasite cultures and subsequently analyzed on SDS-PAGE. Parasites treated with *falcipains* dsRNAs showed significant accumulation of hemoglobin, when compared with the untreated parasites. The fate of *falcipain* mRNAs in dsRNA treated parasites was examined by Northern blot analysis. Parasites treated with *falcipains* dsRNA showed significant degradation of endogenous *falcipain* mRNAs in comparison to untreated parasites. One of the salient features of RNAi machinery is the formation of small RNA species called siRNAs. Analysis of small RNA species in the *P. falciparum* culture, treated with labeled *falcipain* dsRNAs showed the generation of 25 nt labeled RNA species. This shows that the mechanism of RNAi is conserved in *Plasmodium*. Parasite extracts prepared from the *P. falciparum* culture treated with *falcipain* dsRNA showed ability to cleave *falcipain* mRNA, while untreated parasite extract did not show any such nuclease activity. These results demonstrated the conservation of RNAi machinery in the malaria parasite.

SiRNA mediated gene silencing in *P. falciparum*

SiRNA are the true intermediates of an RNAi reaction and have been used as an effective initiator for an RNAi reaction. SiRNA of *falcipain 1&2* were also used for an efficient RNAi in malaria parasite. Parasites treated with *falcipains* siRNAs

showed considerable growth reduction as evident from geimsa staining and ^{3}[H] hypoxanthine assay. After 56hrs of treatment with *facipains'* siRNAs, individually and both together, considerable reduction in the number of new ring formation was observed. *Falcipain*2 siRNA treated parasite culture also showed large number of abnormal parasites arrested at schizont stage. Morphological examination of such parasites under light microscopy showed spherical clusters of merozoites, each enclosed in a delicate membranous covering. The delicate membrane surrounding these spherical clusters of merozoites was found to be of RBC origin based on immunofluorescence studies using PfEMP1 antisera. Previous studies have suggested that Falcipain 2 is only localized in the food vacuole. If Falcipain 2 plays a role in the merozoite release from RBCs, it should also be transported to RBC cytoplasm. Immunofluorescence studies using confocal microscopy and electron microscopy showed that Falcipain 2 is transported to erythrocyte cytoplasm in the parasitized RBC through Maurer's cleft, a pathway shown to be responsible for the export of parasite proteins. These results thus demonstrate the potential use of siRNA to study the function of the genes and also suggest the role of Falcipain 2 in the release of merozoites besides hemoglobin degradation.

RNA interference in *P. berghei*, a mouse malaria model

Recently, siRNA have been shown to hold great potential as gene therapeutic agents. In cultured cell lines, siRNA have been successfully used to inhibit expression of onco-proteins. Moreover, RNAi has also been used to silence the expression of genes in adult mice. Based on these facts, silencing effects of siRNAs corresponding to *berghepain* genes on *Plasmodium berghei* in adult mice were investigated. Three siRNA corresponding to *bergheipain 1&2* and GFP were used in the present study. GFP siRNA was used as a negative control for these studies. Mice were intravenously injected with siRNA once a day for three days and after 48hrs of last injection, Geimsa stained blood smears were examined for the morphological abnormalities. We observed similar kind of abnormalities as in the case of *falcipain* dsRNA treated *in vitro P. falciparum* culture. To assess the effects of *berghepains'* siRNAs on the accumulation of hemoglobin, treated parasitized erythrocytes were collected from mice blood, lysed with saponin and parasite extracts were analyzed on 15% SDS-PAGE. Accumulation of undegraded hemoglobin in comparison to the control parasites was observed in siRNA treated parasites. These siRNA treated parasites also

showed generation of 25mers. These results thus provide evidence that RNAi holds the potential for an attractive therapeutic strategy for the control of parasitic diseases. Based on the above observations, following conclusions can be drawn.

1. RNAi mechanism exists in *Plasmodium.*
2. dsRNA/ siRNA can be used in *P. falciparum* to study the functions of the genes.
3. Cysteine proteases are essential for survival of the parasite.
4. Falcipain 1 and 2 act at the initial stage of the hemoglobin degradation pathway.
5. Falcipain 2 is exported out from the parasite to the erythrocyte cytosol and resides in the maurer's cleft
6. siRNA mediated silencing of Falcipan 2 revealed that Falcipain 2 other than its role in hemoglobin degradation also helps in the release of the merozoites from the RBC by the rupture of erythrocyte membrane.
7. siRNA can be used as a potential therapeutic agent against malaria.
8. Bergheipains like Falcipains are involved in hemoglobin degradation.