Chapter 5: Discussion

The malaria parasite is an intracellular organism, which has co-evolved in mosquitoes and vertebrates for millions of years. Designing drugs or vaccines that substantially and persistently interrupt the life cycle of this complex parasite will require a comprehensive understanding of its biology. Annotation of *P. falciparum* genome led to the identification of 5,267 genes, out of which 65 % are hypothetical. It is essential to understand the function of these genes in order to design a better drug /vaccine. With the development of resistance against most of the currently used antimalarials, there is an urgent need to identify new drug targets and design new drugs based on these targets. To design better/new drugs, better understanding of parasite biology is required. Hence, there is an urgent need to develop a molecular tool to study the function of the genes in less time and with less labour. The reverse genetic tools represent one of the best tools to decipher the function of the genes. The existing reverse genetic tools like gene knockouts using homologous recombination, ribozyme technology and antisense approaches have worked with some limitations. RNAi has emerged as an efficient tool to silence the genes and study their phenotypes. In the present study, we have successfully demonstrated RNAi in *P. falciparum* by using dsRNA of cysteine proteases, *falcipain1&2* of *P. falciparum*, which are involved in hemoglobin degradation pathway. Even though protease inhibitors have been shown to inhibit parasite growth since 1980, Rosenthal *et al.,* in 1989, for the first time reported a cysteine protease activity in the malaria parasite. Subsequently, cysteine protease gene (*falcipain1*) was cloned, expressed in *E. coli* and baculo vectors and was shown to have hemoglobinase activity (Salas *et al.*, 1995).

Rosenthal and co-workers, based on cysteine protease inhibitor studies, have proposed that Falcipains is involved in the initial step of hemoglobin degradation (Gomboa-de-Domingoez and Rosenthal, 1996). Involvement of Falcipains in the first step of globin digestion was inferred from the observation that the treatment of parasite with E-64 results in the accumulation of undigested globin in the food vacuole of parasites (Rosenthal, 1995). However, for the active cleavage of hemoglobin, cysteine proteases need reducing environment (Salas *et al.*, 1995) which was suggested to be provided by the uptake of glutathione from erythrocyte cytosol during the formation of food vacuole (Rosenthal et al., 1998). Goldberg and co-workers showed that the cysteine proteases isolated from the food vacuole cannot degrade native hemoglobin under non reducing conditions, but under same conditions

can degrade denatured hemoglobin (Francis *et al.*, 1996). These results clearly indicated that the denaturation of hemoglobin is an essential step for the cysteine proteases activity on hemoglobin and the reducing environment is brought about by the glutathione present in the food vacuole. These authors also suggested that the levels of catalase present in the food vacuole may be sufficient to protect hemoglobin from thiol mediated denaturation and the initial degradation was accredited to another class of proteases, Plasmepsin I & II belonging to the family of aspartic proteases (Goldberg *et al.*, 1991). By using aspartic protease inhibitors, it was shown that these proteases are essential for the degradation of hemoglobin (Goldberg *et al.*, 1990). With the completion of genome sequencing and its annotation, large number of new proteases (Cysteine, aspartic and metallo proteases) in the genome of *P. falciparum* has been identified. Ten different aspartic proteases have been described now and of these four have been implicated in hemoglobin degradation. Like wise, four different cysteine protease have been described and all have been implicated in hemoglobin degradation. Two different models have been proposed for the process of hemoglobin degradation. Goldberg and co-workers believe that the hemoglobin degradation is an ordered process in which initial cleavage event is performed by aspartic proteases and subsequent digestion within the food vacuole is the result of the synergistic action of Plasmepsin I, Plasmepsin II and Falcipains. Small peptides generated because of the digestion of cysteine and aspartic proteases are transported to the parasite cytosol, and are further digested to amino acids by the action of cytosolic exopeptidases. On the contrary, Rosenthal's group demonstrated that the hemoglobin can be cleaved by cysteine protease under non reducing conditions but at a slow rate and proposed that both the proteases (aspartic and cysteine proteases) act together on the native hemoglobin in a co-operative manner leading to the initial cleavage of hemoglobin. Due to the lack of gene disruption studies of individual genes, the precise role of these proteins is highly speculative.

In this present study, we made an attempt to pinpoint the exact role of two cysteine protease genes in the hemoglobin degradation by using RNAi. We initially tried different methods for the uptake of dsRNA by the parasite, which include 1) transfection by using different transfection reagents like oligofectamine, lipofectamine and Exygen-500, 2) Electroporation 3) Soaking. Since, transfection by using transfection reagents and electroporation at high voltage led to the excessive death of parasite, we decided to carry out RNAi experiments using soaking procedure

by incubating parasite with dsRNA in absence of serum. Another important aspect is the uptake of dsRNA by the parasite, which was initiated by addition of dsRNA to the parasite culture in serum free medium. We assessed the entry of radiolabeled dsRNA into the parasite which was surrounded by three membranes. Our results clearly showed that dsRNA can be taken up by the parasite at very low levels.

In present investigation, the addition of each of the two *falcipain* dsRNA to *P. falciparum* culture resulted in the inhibition of parasite growth, development and enlargement of food vacuoles. These effects were more pronounced when both the *falcipains* dsRNA were used together. Moreover, each of the *falcipain* dsRNA produced a significant block in the degradation of hemoglobin in the parasite. A non-specific dsRNA from *aminopeptidase-N* gene of insect origin did not produce any of these effects. The additive effect produced by two *falcipains* dsRNA suggested that hemoglobin hydrolysis in the parasites is probably a co-operative process involving a number of different enzymes together. The inhibitory effects produced by dsRNAs treatment of parasites coincided with a marked reduction in the levels of endogenous mRNA homologous to the dsRNA in these parasites, which is one of the hallmarks of RNAi. We also compared the effects produced by *falcipain* dsRNAs on malaria parasite with the effects of E-64, a well known cysteine protease inhibitor. Our study showed that various morphological as well as biochemical effects seen on malaria parasite after treatment with *falcipain1&2* dsRNAs were similar to those shown by E-64 (Rosenthal, 1995). These results indicate that RNAi can be an important tool in investigating metabolic events in the parasite life cycle and adds *P. falciparum* to the list of organisms in which RNAi has been shown to work successfully. The block in hemoglobin degradation observed by silencing the activity of the *falcipain* genes in the present study suggested that both the Falcipain proteins are required for initial cleavage event.

Based on various studies related to the RNAi mechanism in *C. elegans* and *Drosophila*, a two-step mechanism for RNAi has been proposed (Bernstein *et al.*, 2000; Hammond *et al.*, 2001). The first step involves cleavage of dsRNA to siRNA of 21-25 nt length by RNaseIII like enzyme, called as DICER. In a second step, which is also referred as an effector step siRNA produced in the first step serve as a guide for a ribonuclease complex, RISC (RNA induced silencing complex), which cleaves the homologous single stranded mRNA (Agrawal *et al.*, 2003). We wondered whether the mechanism of RNAi is similar in *P. falciparum*. Analysis of small RNA species in *P.*

falciparum culture treated with labeled *falcipain* dsRNAs showed the generation of 25nt labeled RNA species. We also showed that parasite extracts prepared from the *P. falciparum* culture treated with *falcipain2* dsRNA had the ability to cleave *falcipain2* mRNA whereas untreated parasites did not show this nuclease activity. These results supported the two-step mechanism and the components of dsRNA–induced gene silencing in a diverse group of organisms.

As siRNAs are the true intermediates of RNAi reaction, they are now being used successfully to study the function of the genes in large number of organisms including mammals where dsRNA cannot be used. We also assessed the efficiency of siRNA in an RNAi reaction in *P. falciparum*. In the present study, we used siRNA of *falcipains* 1& 2 genes to silence the corresponding *falcipain* genes. Treatment of parasites with *falcipains* siRNA individually and in combination resulted in drastic reduction in the growth of the parasite and the effects seen were similar to that of *falcipain's* dsRNA treated parasites. The effects of these siRNAs were better than those reported earlier with *falcipain* dsRNAs. This might be due to the efficient uptake of siRNA as compared to dsRNA. In addition to the effects seen on the parasite growth after the *falcipains* siRNAs treatment, we also observed accumulation of merozoite clusters in *falcipain2* siRNA treated cultures indicating abrogation of RBC's rupture. These merozoites appeared morphologically normal but were locked within a transparent membrane. These effects were not seen with *falcipain1 siRNA* treated parasites. Similar kind of morphologically abnormal structures have been earlier reported upon treatment of parasites with <10uM of E-64 or upon treatment with Leupeptin or Leupaptin+antipain. Based upon the E-64 inhibitor studies, Salmon *et al.,* (2001) proposed a two step mechanism for the merozoite release that involves erythrocyte membrane rupture followed by PVM rupture. Later, Wickham *et al.*, (2003) proposed a model, which is contradictory to the above proposed model. They showed by using different protease inhibitors that different proteases are involved in selective inhibition of PVM as well as erythrocyte membrane. Based on studies using transgenic parasite lines (-His GFP and +His GFP parasite lines), they proposed a two step egress model for the release of merozoites, which involved an initial rupture of PVM followed by erythrocyte membrane rupture. Since we have observed a block in erythrocyte rupture in *falcipain 2* siRNA treated parasites, we decided to probe it further by studying the origin of membrane surrounding the incompletely ruptured merozoites in *falcipain 2* siRNA treated parasites. To know whether the membrane

surrounding these merozoites is PVM or erythrocyte membrane, we followed the approach of Wickham *et al.,* (2003) using transgenic parasite line, +His GFP. Treatment of parasites with falcipain2 siRNA resulted in the accumulation of incompletely ruptured schizonts showing diffused GFP fluorescence throughout RBC cytoplasm, thereby suggesting that erythrocyte membrane remains intact in these parasites. We confirmed these observations by carrying out immunofluorescence studies using antibodies against PfEMP1 and band 3. PfEMP1 is a parasite protein, which is exported out to the surface of the erythrocyte membrane whereas Band3 is a major erythrocyte membrane protein which is also referred as "Anion channel protein".

Our results using *falcipain 2* siRNA taken together with the studies using cysteine protease inhibitors suggest that falcipain2 is involved in the second step of merozoite release i.e., in the rupture of RBC membrane. In order to rupture RBC membrane, it is essential for Falcipain 2 to be transported to RBC. To confirm this, we carried out immunolocalization studies using Falcipain 2 specific antibodies as well as antibodies raised against some well known parasite proteins (MSP-1_{19}, ERC). Results based on co-localization studies using confocal microscopy showed that Falcipain 2 exists in the food vacuole as well as in the parasite cytosol at the trophozoite stage, whereas in the segmenter stages, Falcipain 2 is localized in the parasitophorous vacuole and also in the erythrocyte cytoplasm. Detergent solubility studies using saponin and streptolysin O showed association of Falcipain 2 with membrane network. These results indicate that Falcipain 2 is transported to erythrocyte cytoplasm and remains associated with the membrane network. In higher eukaryotes, Brefeldin A has been shown to block the export of proteins by disrupting the organization of Golgi complex (Lippincott-schwartz *et al.*, 1998). Even in malarial parasite, it has been shown that Brefeldin A treatment of parasitized erythrocyte results in the collapse of Golgi like vesicular network in the parasite and the Golgi markers were localized within the compartment made up of ER (Elmendorf and Haldar, 1993). The trafficking of Falcipain 2 protein was affected in Brefeldin A treated parasites and it showed the same distribution pattern as seen with other parasite exported proteins, PfEMP-1 and KHARP. These studies thus provide clear evidence regarding the trafficking of Falcipain 2 via membrane network.

Immunolocalization studies using electron microscopy further confirmed trafficking and localization of Falcipain 2 to erythrocyte cytoplasm. Falcipain 2 was

found to be associated with the membrane as well as with the Maurer's clefts. Recent evidences suggests that the Maurer's clefts are parasite-derived membranous system in the cytosol of the RBC and are crucial components for parasite protein sorting as well as trafficking of proteins outside the parasite into the host cell. It has also been proposed that the Maurer's cleft are associated with the host cytoskeleton (Wickham *et al.*, 2003, Przyboski *et al.*, 2003). Based on our results, we propose a model for the new role of Falcipain 2. According to this model, Falcipain 2 is transported to the erythrocyte via PVM through the classical secretory pathway and finally resides in the Maurer's cleft. During the time of RBC lysis, Falcipain 2 in the Maurer's cleft might be re-orienting in such a way that it comes in contact with the cytoskeletal proteins without being released from the Maurer's cleft and then degrades the cytoskeletal proteins leading to the lysis of RBC membrane, an essential step in the merozoite release.

Molecular analysis of erythrocyte membrane has revealed that erythrocyte cytoskeleton forms dense fibrillar shell under plasma membrane. This cytoskeleton network is attached to integral membrane proteins present in the erythrocyte plasma membrane at many points that provide strength and flexibility to the plasma membrane. Recently, Hanspal and co-workers showed that recombinant Falcipain 2 cleave ankyrin and band 4.1 proteins, which are associated with erythrocyte plasma membrane. They also showed that the peptides made from the ankyrin binding site of Falcipain2 can inhibit the cleavage activity. Addition of this peptide to the *P.falciparum* culture at the ring stage blocked the release of merozoites from the RBC (Dhawan *et al.*, 2003). Based on the work of Hanspal's group and the results presented in this study, it can be proposed that Falcipain 2 has dual function i.e., hemoglobin degradation and rupture of RBC membrane. Recently, it was shown that Plasmepsins, which are aspartic proteases were also involved in the cleavage of proteins present in the RBC membrane (Goldberg *et al.*, 2002). Thus it appears that not only cysteine proteases but aspartic proteases are also involved in the lysis of RBC membrane. It will be interesting to see whether these proteases act together or individually on the erythrocyte membrane proteins for the rupture of RBC membrane.

In conclusion, we have shown that RNAi is functional in *Plasmodium falciparum* and can be used as an efficient tool to study the function of genes at a faster rate in comparison with the existing techniques. By using dsRNA/siRNA of *falcipains*, we showed that the Falcipains are involved in hemoglobin degradation and

Falcipain 2 is also involved in the rupture of RBC membrane other than hemoglobin degradation.

Invivo gene silencing of *berghepain* genes in *P. berghei* using *berghepain siRNA*

Earlier, RNAi was shown to have limited applicability. DsRNA were shown to produce non-specific inhibition in mammalian cells. One of the main reasons for the non-specific effects of dsRNA was the induction of γ-interferon response, which activates protein kinase-R (PKR). The activated PKR induces RNAse L, which in turn brings about the degradation of mRNA (Clarke and Mathews, 1995). However, discovery by Tuschl's group that the small interfering RNAs of 21-25 nt length do not produce interference response led to the large scale application of RNAi in mammals (Elbhashir *et al.*, 2002). At present siRNAs are not only being used as a tool to study gene functions, they have also been shown to have therapeutic potential. SiRNAs have been used successfully to block viral replication as well as prevent leukemia in culture cell lines. Recently, RNAi has also been successfully applied in the adult mice to silence Fas receptor genes, which in turn prevented liver cell inflammation and death in mice carrying fulminant hepatitis (McCaffery *et al.*, 2001). Based on these studies, we made an attempt to use siRNAs in adult mice against *P. berghei*-mouse malaria. We intravenously injected siRNAs of *berghepains,* an orthologues of *falcipains* into infected mice to evaluate the *in vivo* efficacy of siRNAs to trigger RNAi in circulating parasites. The effects of these siRNAs were found to be similar to the one seen with *falcipains* dsRNA/siRNA. However, fewer parasites showed morphological abnormalities in comparison to the *in vitro* cultures of *P. falciparum* treated with *falcipain* dsRNA. These results confirmed that Berghepains like Falcipains are also involved in the degradation of hemoglobin. As shown in dsRNA study on *in vitro P. falciparum* cultures, we also showed generation of 25 nt species in *berghepains'* siRNA treated parasites. These results thus demonstrated that RNAi can be applied *in vivo* in *P. berghei*–a mice malaria model.

Thus, through *in vivo* experiments on a mouse model, we have provided evidence that RNAi holds the potential for an attractive therapeutic strategy for the control of parasitic diseases. Nonetheless, many caveats still exist for the therapeutic use of siRNAs in the control parasitic diseases. One of our major concerns is the

efficacy of RNAi under *in vivo* conditions. We could observe only 40-50% reduction in *berghepains'* mRNA levels in the treated parasites. This could be due to the poor delivery of siRNAs to all the circulating parasites or due to the choice of siRNA sequences. It has been shown that all siRNAs along the length of the gene are not equally effective for RNAi (Holen *et al.*, 2002). Even though there was significant reduction in mRNA levels, we did not observe any significant reduction in parasitemia in siRNA treated parasites in comparison to the control. This could be due to the redundancy existing in the hemoglobin degradation pathway. Three different classes of enzymes: aspartic proteases (ten Plasmepsins), cysteine proteases (four Falcipains), and metallo proteases (one Falcilysin) have been shown to be involved in hemoglobin degradation (Padmanaban *et al.*, 2002). *In vivo* studies using *P. vinckei* murine malaria model have shown that inhibitors of any of the three classes of enzymes alone were not effective in curing malaria. However, the combined protease inhibitors cured the majority of the infected animals, suggesting that the combination of these inhibitors act synergistically in the inhibition of *Plasmodium* hemoglobin degradation *in vivo* and in the treatment of murine malaria (Semenov *et al.*, 1998). Based on these facts, it can be argued that mixture of siRNA corresponding to all three classes of enzymes involved in hemoglobin degradation may be required to show efficient antiparasitic effect in this model system.

The use of RNAi in *P. falciparum* to dissect gene function has several advantages over methods requiring the introduction of DNA into cells. Transfection experiments require knowledge of the full sequence of the gene and its flanking regions, whereas for RNAi, dsRNA corresponding to any gene fragments is sufficient to confer the interference effect. Particularly, it is difficult to carry out knockouts in intracellular parasites like *Trypanosomes* and *Plasmodium* (Ullu and Tschudi, 2000). Moreover use of RNAi in *P.falciparum* as well as in other organisms is technically simple and quick, and result of gene silencing can be obtained within 2-3 days. This contrasts sharply with the time necessary to produce selective gene knockouts by transfection technology. With the availability of large amount of sequence information for *P. falciparum,* RNAi-based functional genomics can thus be useful technique for investing the biological functions of novel genes. Finally, given the gene specific feature of RNAi and the ease of inducing RNAi, this methodology may also play an important role in the development of therapeutic applications against malaria.

In conclusion, the present study demonstrates that the RNAi can be a useful tool to study gene functions in *Plasmodium*. In case of pathways, which are essential for the parasite survival, gene targeting by homologous recombination is not possible. RNAi can be an important tool to study the function of genes involved in such pathways. RNAi can also be used in case of redundant pathways where gene-knock out may not permit any useful functional information. In addition to its use in functional studies in *Plasmodium*, our study also demonstrated potential applicability of siRNA as a therapeutic agent. But in this regard, one of the major issues to be taken care of is the delivery of siRNA to the specific tissue, while ensuring appropriate level of efficacy with minimum toxicity.