Chapter 4: Results

Plasmodium falciparum, the causative agent of cerebral malaria, is responsible for millions of deaths every year. Annotation of *P. falciparum* complete genome sequence revealed a number of genes with unknown functions. There is a need for a rapid and an efficient tool to study the function of these genes. Recently, RNAi has emerged as an efficient tool to study the function of the genes in number of organisms. We thought of developing and applying RNAi in malaria, an intracellular parasite. To carryout RNAi in malaria parasite, we chose to silence the expression of cysteine protease genes, *falcipain 1&2*. These genes were selected as the morphological effects on parasites due to cysteine proteases inhibitors have been well described and moreover, cysteine proteases have been shown to play an important role in the hemoglobin degradation pathway, an essential step in parasite life cycle. We used both dsRNA as well as siRNA to carryout RNAi in *P. falciparum*.

4.1 DsRNA mediated gene silencing in *P. falciparum*

4.1a Synthesis of dsRNA corresponding to *falcipain1&2* and their uptake by *P. falciparum*

To prepare dsRNAs corresponding to *falcipain 1&2* we followed the protocol by Clemens *et al.* (2001). Briefly, the cysteine protease genes, *falcipain1* (1.6 Kb) and *Falcipain 2* (1.4 Kb) were PCR amplified from the genomic DNA of 3D7 and cloned in pGEM-T vector. Using T7 and SP6 primers, cysteine protease genes from the pGEM-T vector were amplified along with the flanking T7 and SP6 promoter sequences (Fig.1). With the help of T7 and SP6 Polymerase, both sense and antisense strands were prepared. dsRNAs were obtained by annealing both the complementary strands. These dsRNA were analyzed on 1% agarose gel to ensure that they were predominantly double-stranded (Fig.2).

The hallmark of RNAi is its specificity, simplicity, and the ease with which it can be applied to an organism. RNAi was first demonstrated in *C. elegans* by soaking the worm in dsRNA solution (Fire *et al.*, 1998). Later, RNAi was applied in various organisms by using dsRNA as well as siRNA to study the function of the genes. In *Drosophila* S2 cell lines; RNAi was used for dissecting the signal transduction pathway by just adding dsRNA to the

culture medium (Clemens *et al.,* 2000). Previous studies have shown that DNA/oligonucleotides molecules can be introduced into parasitized RBC by either electroporation or by their simple addition to the culture medium (Rapaport *et al.*, 1992, Bakers *et al.*, 1996). Since, electroporation procedure has been shown to cause death of large number of the parasite, we decided to use the soaking procedure i.e., by adding dsRNA to the culture medium. To determine the efficiency of this procedure, we first studied the uptake of labeled dsRNA by malaria parasite. In order to carryout this, synchronized *P. falciparum* cultures were incubated with 10 nM ^{32}P labeled dsRNA made from either of the *falcipains*. After 24 h of incubation, parasitized erythrocytes were collected by centrifugation, lysed using saponin and washed three times to obtain a pure parasite pellet. Level of radioactivity was measured in different fractions of parasitized red blood cells. Approximately 0.1-0.15% of the input labeled dsRNAs were found to be associated with purified parasite pellet, suggesting that dsRNA was taken up by the parasites.

4.1b Effect of *falcipain 1&2* dsRNA on the growth of parasites

To determine the effect of *falcipain 1&2* dsRNA on the growth of parasite, synchronized *P. falciparum* parasites were incubated with dsRNA for each of the *falcipains* separately and in combinations at three different concentrations (10 µg/ml, 20 µg/ml, 50 µg/ml). Treatment of parasites with *falcipain 1&2* dsRNA led to the distinct morphological changes, which included swollen food vacuole with the accumulation of malarial pigment (Fig.3). Similar morphological effects were also seen when parasitized erythrocytes were treated with E-64. Rosenthal and co-workers have also reported such kind of food vacuole abnormalities on treatment with E-64 (Rosenthal, 1995). Maximum effects were seen, when the parasites were incubated with *falcipain1&2* together at a concentration of 25 µg/ml and 50 µg/ml than at 10 µg/ml concentration of either of *falcipain1*or *2* separately and in combination. Approximately, 60 % of the parasites showed swollen food vacuole abnormalities on incubation with both the *falcipains* dsRNA at 50 µg/ml concentration where as 30 % of parasitized erythrocytes showed food vacuole abnormalities on treatment of the parasites separately with each of the *falcipains* at both 25 and 50 µg/ml concentrations. Addition of the two

dsRNAs together to the parasite culture also drastically reduced the formation of new rings as well as total parasitemia. E-64 at a concentration of 100 nM was used as a positive control and dsRNA made from *Aminopeptidase-N* (insect origin) was used as a negative control (Table 1). We also studied the effect on the growth of the parasite by using ^{3}[H]-hypoxanthine uptake assay, as the inhibition of growth is proportional to the uptake of hypoxanthine. We followed the protocol described by Rosenthal *et al.* (1995). The parasites at ring stage were treated with dsRNA and incubated for 24 h. Approximately 1μCi of ^{3}[H]-hypoxanthine was added to the cultures and further incubated for 24 h. We observed 50-60 % reduction in the uptake of hypoxanthine in the case of *falcipain 1&2* dsRNA treated parasites at 50 μg/ml concentration, whereas only 30 % reduction was observed when the parasites were incubated separately with *falcipain 1&2* dsRNA (Fig.4). Parasites treated with E-64 gave complete inhibition in the uptake of ^{3}[H]-hypoxanthine and *aminopeptidase* dsRNA treated parasites showed no inhibition in the ^{3}[H]-hypoxanthine uptake, thus exemplifying the specificity of RNAi.

4.1c Inhibition of hemoglobin degradation by *falcipain* dsRNAs in *P. falciparum*

It has been shown that the morphological effects observed in parasites after the treatment with E-64, a broad-spectrum cysteine protease inhibitor are due to the accumulation of toxic hemoglobin (Rosenthal *et al.,* 1995). It has also been shown that recombinant *falcipain 1&2* have the ability to degrade hemoglobin. To investigate whether the observed morphological abnormalities in *falcipain 1&2* dsRNA treated parasites were predominantly due to the accumulation of hemoglobin, parasites were treated with both the *falcipain 1&2* dsRNAs separately and in combination at 50 μg/ml concentration and the total parasite proteins were subsequently analyzed by SDS-PAGE. Incubation of parasites with *falcipains* dsRNAs resulted in the accumulation of hemoglobin (Fig.5, lane 2-4). E-64 treated parasites were used as a positive control (Fig.5, lane 6). Parasites treated with DEPC water showed almost complete degradation of hemoglobin (Fig.5, lane 1). These results suggested that the addition of *falcipain 1&2* dsRNA to the *P. falciparum* culture leads to

the inhibition of hemoglobin degradation and subsequent accumulation of hemoglobin in the food vacuole of the parasite.

4.1.d *Falcipains* dsRNA mediated cleavage of its corresponding mRNA

By definition, RNAi is a process by which dsRNA brings about the silencing of a gene by cleaving its cognate mRNA. To determine whether the effects seen on *P. falciparum* were due to the *falcipain 1&2* mRNA degradation, we assessed the levels of *falcipains* mRNA in *falcipains* dsRNA treated parasites by Northern analysis. Parasites treated with *falcipain 1* dsRNA showed the *falcipain 1* specific mRNA degradation as compared to the untreated, *falcipain 2* dsRNA treated and *aminopeptidase* dsRNA treated parasites (Fig.6, panel A). Similarly, parasites treated with *falcipain 2* dsRNA showed loss of *falcipain 2* mRNA in comparison to control parasites or parasites treated with nonspecific dsRNA (Fig.6, panel B). These results clearly showed that the effects seen on parasites upon treatment with *falcipains* dsRNA treatment were because of degradation of its corresponding mRNA

4.1e RNAi machinery is conserved in *P. falciparum*

Several studies on the mechanism of RNAi have suggested a two-step mechanism for RNAi. The first step involves, degradation of dsRNA by RNAse III like enzyme, referred to as DICER, into small RNA species of lengths 21-25nt (siRNA). The second step involves, binding of siRNA to RNA induced silencing complex (RISC), subsequent activation of RISC, which is mediated by unwinding of siRNA, followed by the degradation of mRNA complementary to one of the strands of siRNA in the RISC (Agrawal *et al.*, 2003). To determine whether *falcipain 1&2* dsRNAs were also processed into small RNA, the cultured parasites were treated with ^{32}P–labeled dsRNA corresponding to either of the two *falcipains* used in this study. After 24 h of incubation, parasite pellets were processed to get total RNAs and, subsequently, small RNA species were isolated from the total RNA. These were then analyzed on 15 % denaturing PAGE. Both *falcipain 1&2* dsRNAs were processed to 25 nt siRNA species (Fig.7, lane1 and 2) whereas treatment of normal RBCs with radiolabeled dsRNA did not generate 25nt siRNA

species (Fig.7, lane 3-4). We also observed an additional RNA species of approx 70nt, which could have been generated as intermediates during the processing of dsRNA.

In *Drosophila*, an *in vitro* system was developed to understand the different steps involved in the mechanism of RNAi (Elbashir *et al.,* 2001; Zamore *at al.,* 2000). In this system, it has been demonstrated that preincubation of the dsRNA with the *Drosophila* lysates results in potential nuclease activity for the target mRNA degradation (Tuschl *et al.,* 1999; Zamore *et al.,* 2000). To find out whether similar nuclease activity can be generated in *P. falciparum*, the *P. falciparum* culture was treated with *falcipain 2* dsRNA. The extracts prepared from treated vs. untreated cultures were analyzed for nuclease activity at different time points. Extracts from *falcipain 2* dsRNA treated parasites efficiently degraded *falcipain 2* mRNA whereas extracts from untreated parasites did not degrade mRNA at all (Fig.8). The degradation process was completed within 30 min of incubation of labeled *Falcipain 2* mRNA, whereas extracts from untreated parasites did not show any degradation of mRNA even after 3 h of incubation.

In summary, all these results showed that RNAi operates in *P. falciparum* and can be used to carryout functional genomic analysis in *P. falciparum.*

4.2 siRNA mediated gene silencing of *P. falciparum*

4.2a Effect of siRNA on the growth and morphology of *P. falciparum*

The hallmark of RNAi and the related phenomenon are generation of small RNA species of approximately 21-25 nt; named small interfering RNAs (siRNA), which in association with protein complex functions as a guide for the degradation of target transcripts. Elbhashir *et al.* (2001) provided the first evidence for the siRNA mediated sequence specific gene silencing and showed that the dicing step can be bypassed by the introduction of siRNA into the cells. Tuschl and coworkers for the first time applied siRNA to silence the genes in mammalian cells (Tuschl, 2002). Since then, siRNA is being used efficiently for wide scale functional genomic studies. Considering the above facts, we initiated studies using siRNA corresponding to *falcipain 1&2* and investigated their effects on the growth as well as morphology of *P.*

falciparum parasites *in vitro*. The sequences for *falcipain 1&2* siRNA were carefully selected by using the software on the Ambion site. Fig.9. shows the target sequence and its corresponding siRNA sequence.

4.2a.1 Growth Inhibition

We have previously shown that silencing of *falcipain1 and 2* genes using corresponding dsRNAs brings about substantial inhibitory effects on the parasite growth. To determine whether similar effects could also be produced by siRNAs corresponding to these genes synchronized *P. falciparum* parasites at the late ring stage were treated with siRNAs corresponding to *falcipain1&2* genes separately, as well as in combination at two different concentrations of 10 µg/ml and 100 µg/ml. Unrelated siRNA corresponding to insect aminopeptidase gene was used as a negative control. Reduction in [^{3}H]-hypoxanthine uptake was observed at both, 10 µg/ml and 100 µg/ml concentrations in comparison to the untreated parasites. However, at 100 µg/ml concentration of individual *falcipain* siRNA, pronounced reduction in the uptake of [^{3}H]-hypoxanthine was observed (50-60%). Maximum inhibition (~85%) in [^{3}H]-hypoxanthine uptake was seen when the parasites were treated with both the *falcipain* siRNAs at 100 µg/ml concentration (Fig. 10). No reduction in [^{3}H]-hypoxanthine uptake was observed when parasites were treated with *aminopeptidase-N* siRNA.

4.2a.2 *Falcipain 1&2* siRNAs treatment affects total parasitemia and the merozoite release

We also investigated the effects of *falcipains* siRNAs (*falcipain 1& 2*) on the parasite development as well as on the total parasitemia after 56 h of treatment. Treatment of parasites with either of the two *falcipains* siRNAs considerably reduced the total parasitemia (~60-65 %) (Fig. 11 A), thereby suggesting that these enzymes play an important role in the parasite survival. Different stages of the parasite i.e. ring as well as schizonts stages were also analyzed separately in the treated cultures. In the control culture, most of the parasites were at the new ring stage. *Falcipain 1* siRNA treated culture showed 60 % reduction in the formation of new rings in comparison to the control

culture (Figs.11 B and C). Few or no schizonts were seen in the *falcipain* 1 siRNA treated culture. However, cultures treated with the *falcipain 2* siRNA showed large number of schizonts along with some new ring stage parasites. These schizonts appeared as spherical clusters of merozoites, enclosed in a delicate membrane covering (Fig. 12). Parasites in the *Falcipain 2* siRNA treated culture seemed to be arrested at the schizont stage. Similar kind of morphological clusters have been reported on treatment of *P. falciparum* culture with different protease inhibitors (Wickham *et al.,* 2003; Salmon *et al.,* 2001; Lyon and Haynes, 1986).

4.2a.3 Silencing of *falcipain 2*

To determine whether the effects seen upon addition of *falcipain 2* siRNA were due to RNAi, we performed Western analysis of the total protein extract from treated and untreated parasites to assess the reduction in Falcipain 2 protein. To assess the level of Falcipain 2, total protein was extracted from the parasite and separated on 12% SDS- PAGE. Western analysis was done by using Falcipain 2 specific antibodies. As shown in the (Fig.13), *falcipain 2* siRNA treated parasites showed 3-4 fold reduction in the amount of Falcipain 2 in comparison to the *aminopeptidase-N* (insect origin) siRNA treated parasites and untreated parasites. Anti HRPII antibody was used to confirm equal loading in different wells.

These results indicated that *Falcipain 2* siRNA treatment specifically reduces the Falcipain 2 production in *P. falciparum, which* in turn inhibits the growth of the parasite and merozoite release from RBCs.

4.2b Determination of origin of membrane surrounding the incompletely ruptured schizonts

Previously it has been shown that the treatment of parasite with different cysteine protease inhibitors results in the accumulation of a large number of incompletely ruptured schizonts, which are either surrounded by PVM or erythrocyte membrane depending on the type of cysteine protease inhibitor used for the study. Since, we observed a large number of incompletely ruptured parasites in *falcipain 2* siRNA treated cultures we decided to determine the origin of the limiting membrane surrounding these

incompletely ruptured schizonts. To do so, we followed the experimental design of Wickham *et al.* (2003). These authors used GFP expressing lines (3D7+His) to ascertain the selective inhibition of PVM or erythrocyte membrane rupture by different protease inhibitors. In these parasite lines when PVM rupture was inhibited and the RBC membrane rupture proceeded, the GFP fluorescence was lost. However, when PVM was cleaved and the RBC membrane remained intact, GFP fluorescence diffused through the whole RBC around the merozoites. Upon treatment with *falcipain 2* siRNA, these transgenic parasites exhibited diffused fluorescence throughout the infected RBC, thereby suggesting that the RBC membrane in these parasites remained intact (Fig.14A). This result showed that RBC membrane rupture is inhibited in parasite lines treated with *falcipain 2* specific siRNA. To further confirm the erythrocytic origin of the membrane surrounding the merozoite clusters in *falcipain 2* siRNA treated parasites, immunofluorescence assays using antibodies to one of the parasite protein (PfEMP-1), and an erythrocyte membrane protein (Band 3), were carried out. PfEMP-1 is a parasite protein that has been shown to be localized on the host erythrocyte membrane during trophozoite and schizont stages. Incompletely ruptured schizonts in *falcipain 2* siRNA treated cultures showed the presence of PfEMP1, as well as Band 3 in the membrane surrounding them (Fig.14, B and C). This indicated that the limiting membrane surrounding the incompletely ruptured merozoite clusters in *falcipain 2* siRNA treated parasite is indeed the erythrocyte plasma membrane. Results of these experiments showed that Falcipain 2 plays an important role in the rupture of the erythrocyte membrane and the release of merozoites.

4.2c Localization of Falcipain 2 protein in different stages of parasite growth by immunofluorescence assay

Earlier studies conducted by Rosenthal's group on the localization of Falcipain 2 have shown that Falcipain 2 protein is predominantly localized in the food vacuole (Shenai *et al.,* 2001). Recently, Dhawan *et al.* (2003) showed the localization of the Falcipain 2 protein in the PVM as well as in the erythrocyte cytosol. In order to gain insight into the functional role of Falcipain 2, we

carried out immunolocalization studies using Falcipain 2 specific antibodies raised in mice. If Falcipain 2 is involved in the erythrocyte plasma membrane rupture, it probably should be transported to the erythrocyte cytoplasm from the parasite. Immuno-localization studies were carried out using confocal microscopy on the untreated parasites using Falcipain 2 specific antibodies. To obtain highly specific antibodies against Falcipain 2 protein, we first treated the Falcipain 2 specific mice antisera with recombinant Falcipain1 protein in order to eliminate the cross-reacting antibodies, common to Falcipain 1&2 proteins. Synchronized parasite cultures at the ring, trophozoite and schizont stages were immuno-stained to localize the Falcipain 2 protein. At the trophozoite stage, Falcipain 2 labeling was confined to the parasite cytosol and it co-localized with the endoplasmic reticulum resident protein (PfERC) (Fig. 15, panel A). At an early segmenter stage (schizont stage), intense Falcipain 2 labeling appeared in the food vacuole as well as around individual merozoites. Remarkably, Falcipain 2 labeling appeared comparable to the merozoite surface protein 1 (MSP-1) labeling in each segment (Fig. 15, panel B and C), although MSP-1 and Falcipain 2 did not merge totally; labeled Falcipain 2 appeared as a red rim circumventing the green MSP1 label at certain places. Absence of Falcipain 2 staining in the free merozoites suggested that it is not associated with the merozoite surface (Fig. 15, panel D). Considerable reduction in Falcipain 2 labeling in the parasites treated with *falcipain 2* siRNAs was observed (Fig. 16).

4.2d Fractionation of infected erythrocyte to localize Falcipain 2 protein

If Falcipain 2 is transported to the erythrocyte, it should either be associated with the membrane network or it should lie free in the cytoplasm. To further confirm the localization of Falcipain 2, infected erythrocytes were treated with Saponin and Streptolysin O, which was followed by western analysis using Falcipain 2 specific antibody. Saponin and Streptolysin O treatment allows the analysis of membrane–associated proteins as well as the exported proteins. Saponin dissolves both the erythrocyte plasma membrane and parasitophorous vacuolar membrane, while Streptolysin O permeabilises only the erythrocyte membrane, leaving the parasitophorous membrane intact. Upon lysis of parasitized erythrocyte with Saponin, Falcipain 2 was found both in the supernatant and in the pellet while in Streptolysin O treated

parasites, Falcipain 2 was present only in the pellet but not in the supernatant (Fig.16. panel A). These results clearly provided evidence for the association of Falcipain 2 protein with the tubolo-vesicular network (TVN) in the erythrocyte cytosol (Fg.16, panel B). These studies revealed that Falcipain 2 protein at schizont stage is exported from the parasite to the RBC cytosol through the vesicular network and remains associated with the TVM in the cytosol of the RBC.

4.2e Effect of Brefeldin A on the trafficking of Falcipain 2

In eukaryotes, the proteins, which are either secreted outside the cell or to the surface of the plasma membrane, are transported by a vesicle-mediated pathway or also known as classical pathway, which involves the Golgi apparatus. Treatment of eukaryotic cells with Brefeldin A leads to the collapse of Golgi network, which in turn blocks the transport of secretory proteins. In *Plasmodium*, it has been previously shown that most of the parasite proteins like KHARP, PfEMP-1 and PfEMP-3 that are exported out from the parasite through the vesicle mediated pathway are blocked by treatment with Brefeldin A. To determine whether the Falcipain 2 protein is transported to the cytosol by the vesicle-mediated pathway i.e., "classical pathway" or some other alternate pathway; we treated infected erythrocyte with Brefeldin A at the early trophozoite stage and studied the localization of Falcipain 2 after 24 h of treatment by using immunofluorescence microscopy. Trafficking of Falcipain 2 in the treated parasite was affected and it was found to be associated to a contracted compartment in the parasite cytosol, which co-localized with PfERC, suggesting that Falcipain 2 was retained in the ER in the treated parasite (Fig.18) From these results we can conclude that Falcipain 2 is transported to erythrocytic cytosol via the classical pathway and its transport is affected by the treatment with Brefeldin A.

4.2f Immuno-electron microscopy confirming the localization of Falcipain 2

To further confirm the sub cellular localization and transport of Falcipain 2, immuno-electron microscopy was performed using anti Falcipain 2 specific antibody. The Falcipain 2 protein showed localization in the food

vacuole of the parasite as well as in the cytosol of the infected erythrocyte (Fig.19). Many of the immunogold particles were associated with the membrane structure in the erythrocyte cytosol and in the Maurer's clefts, which are considered to be the transient depot during the export of the parasite protein (Fig.20, panel A and B; Fig.21). Thus, these studies provide substantial evidence regarding the transport of Falcipain 2 to the erythrocyte membrane through a tubulo-vesicular network and the Maurer's cleft.

Based on the results presented in this study, it can be suggested that in addition to its role in hemoglobin degradation, Falcipain 2 plays an important role in erythrocyte membrane rupture.

4.3. *In vivo* RNAi in mouse malaria model- *P. berghei*

Over the years RNAi has evolved into a powerful tool for manipulating gene expression in number of organisms with a potential utility for investigating gene function, for high–throughput, function based genetic screens and potentially for development as a therapeutic tool. Recently, a number of studies have shown the use of siRNA for genetic based therapies especially in viral infections, cancers and inherited genetic disorders. A strong evidence for the therapeutic potential of RNAi has come from a recent publication describing the prevention of Fas-mediated Hepatitis in mice following the intravenous delivery of naked siRNA by high-pressure injection. However, the effects of siRNAs in adult animal and their potential to treat or prevent disease are yet to be fully investigated. In this present study, we explored the possibility to carry out RNAi on circulating parasite *in vivo.*

4.3a Uptake of siRNA by circulating *P. berghei* parasite in mice

To investigate the role of falcipains' orthologues from rodent malaria parasite *P. berghei* and to study the *in vivo* efficacy of siRNA to trigger RNAi in circulating parasites, we used siRNA of *berghepain1&2* (Selected from Ambion website; Fig.22) to silence the *berghepain* genes in the circulating *P. berghei* parasites. The major obstacle in the use of siRNA in mice model is the route of delivery. The reported routes of delivery tried in different organisms are soaking, feeding, microinjection, viral vector, and intravenous injection.

We followed intravenous route for the delivery of siRNA to assess the effect of *bergheipains'* siRNA on the circulating parasites. Initially, we assessed the uptake of ^{33}P-labeled siRNA by the circulating parasites in the blood of the mice. Eighteen hours after the injection of ^{33}P-labeled siRNA, parasitized RBCs were collected by centrifugation; lysed using saponin and low molecular weight (LMW) RNAs were isolated. These RNAs were blotted to a nylon membrane and the uptake of labeled RNAs was assessed by autoradiography. We could detect a small amount of input labeled siRNA in these parasites (Fig.23). Presence of labeled siRNA in the parasitized erythrocyte indicated their uptake by the parasites but at a very low amount.

4.3b Effect of *berghepain* siRNA on *P.berghei* parasite

In the previous section, we have demonstrated the effect of *falcipains'* siRNA on the morphology and growth of the *P. falciparum* culture. To determine whether *berghepains'* siRNA trigger similar effects *in vivo,* we conducted a series of experiments using two different concentrations of *berghepains'* siRNAs, 5 µg and 25 µg. In all these experiments, siRNA was dissolved in 200 µl 1X PBS and injected intravenously into the *P. berghei* infected mice. SiRNAs were introduced into the mice at the onset of 1% parasitemia or just after 8 h of inoculation of parasite into the mice. Mice were treated with siRNA once a day for three days consecutively. *GFP* siRNA was used as a negative control. Forty-eight hours after the last injection, Geimsa stained smears were made and examined for morphological abnormalities in *P. berghei* parasite. Mice injected with 25 µg of *berghepains'* siRNA produced maximum abnormalities in circulating parasites, which included swollen food vacuole with the accumulation of malaria pigment in *P. berghei* parasites (Fig.24). These effects were similar to those observed in *P. falciparum cultures* after treatment with either E-64, a cysteine protease inhibitor or *falcipain 1&2* dsRNA.

4.3c Accumulation of hemoglobin in *berghepain* siRNA treated parasites

It has been previously reported that the food vacuole abnormalities seen in malaria parasites after treatment with cysteine protease inhibitors are mainly due to blockage of hemoglobin degradation. To assess the effects of

berghepains' siRNA on the accumulation of hemoglobin, *bergheipains'* siRNA treated parasitized erythrocytes were collected from mice blood, lysed using saponin and the parasite proteins were analyzed on 15 % SDS-PAGE. A 2-3 fold increase in the hemoglobin level was observed in *berghepains'* siRNA treated parasites in comparison to the control parasites (Fig.25). These results showed that Berghepains like Falcipains are involved in the degradation of hemoglobin and siRNAs can be effectively used to study the function of genes in *P. berghei*-a mouse malaria parasite.

4.3d. Northern analysis to show the reduction of *berghepain* mRNA level in *berghepains'* siRNA treated parasites

To determine whether the effects observed due to the treatment of parasites with *berghepains'* siRNA were due to the degradation of *berghepain* mRNA, we performed Northern analysis by using *berghepains'* DNA as a probe. For Northern analysis, total RNA was extracted from the treated and untreated parasites fractionated on 1.2 % denaturing agarose gel and transferred onto the nylon membrane. The membrane was hybridized with ^{32}P labeled *berghepain 1&2* DNA. The parasites treated with *berghepains'* siRNAs resulted in the reduction of >40 % of *berghepain 1* mRNA (Fig.26, panel A) and >50 % of *bergheipain 2* mRNA (Fig.26, panel B) as compared to the *GFP* siRNA treated and untreated parasites. Ethidium bromide stained gel showing rRNA was used as a loading control (Fig. 26, panel C).

4.3e Generation of Low molecular weight (LMW) RNA species

As mentioned earlier the hallmark of RNAi reaction is the generation of 25nt RNA species. Earlier, we have reported the generation of small RNA species resulting from the degradation of dsRNA. To investigate whether small RNA species were also formed in *berghepain* siRNA treated parasite, we isolated small RNA species from *berghepains'* siRNA treated parasite and analyzed them on 15 % denaturing PAGE, followed by their detection using labeled *berghepain* RNA probe. We observed generation of 25nt species in *berghepain* siRNA treated parasites, while untreated and *GFP* siRNA treated parasites did not show the generation of any small RNA species (Fig.27).

Along with 25nt species we also observed additional small RNA species >25nts. These longer species might represent the intermediates during mRNA degradation as reported in the previous studies in different organisms. From these results, it is clear that RNAi is functional in *P. berghei* parasite and siRNA can be used to silence genes of *P. berghei* in the mice by intravenous injection of siRNA.

In conclusion the results of the present study have shown that it is possible to carry out RNAi in malarial parasite both *in vitro* as well as *in vivo*. The RNAi can be an important tool for functional genomic studies for malarial parasites.

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