CRISPR/CAS9 genetic modification of plasmodium falciparum and transgenic parasites in malaria vaccine research

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CHAPTER 7

Conclusions and Discussion

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In this thesis we describe a set of studies that employed novel CRISPR/Cas9 methodologies to improve *Plasmodium falciparum* genetic modification. This was done in order to create novel transgenic parasites, which can be used to better interrogate host-pathogen interactions or used in anti-malarial drug and vaccine research. We first focused on improving *P. falciparum* CRISPR/Cas9 gene editing technology and on identifying a suitable 'neutral' locus to introduce transgenes into the parasite genome. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that express either fluorescent-luminescent reporter proteins or express a major vaccine candidate from the other major human malaria parasite, *P. vivax*.

Improved CRISPR/Cas9 genetic modification of *P. falciparum* (Chapter 3)

The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has transformed genome editing in a wide variety of organisms [1]. This powerful genome editing technique has also been applied to *P. falciparum* and provides an efficient method for manipulating the parasite genome, including site-directed mutagenesis, gene disruption and the introduction of transgenes [2, 3].

In **Chapter 3** we describe further improvements of the CRISPR/Cas9 transfection constructs and selection protocol in order to more rapidly modify the *P. falciparum* genome and to introduce transgenes into the parasite genome without the inclusion of drug-selectable marker genes. This method was used to stably integrate three different GFP-expression cassettes into the *P. falciparum* genome, where GFP is under the control of promoters of three different *Plasmodium* genes. The selection procedure to obtain genetically modified *P. falciparum* parasites before the use of CRISPR/Cas9 methods required 1-3 months of continuous culture to select for parasites where episomal plasmids became integrated into the parasite's genome, either by single or by double cross-over recombination [4]. Moreover, the process of removing introduced drug-selectable markers from genetically modified parasites, which involved multiple steps of cloning, can typically take 5 months or more to complete [5]. With the methods described in **Chapter 3** we describe a protocol where cloned, drug-selectable marker-free parasite lines that stably express reporter proteins can be obtained within a period of 10-12 weeks. The CRISPR/Cas9 *Plasmodium* transfection method requires the introduction of two plasmids into the parasite by electroporation. In one of the plasmids, the sgRNA/donor plasmid, we have placed the selectable-marker cassette outside the donor-DNA cassette, which results in the introduction of a transgene into the parasite genome without the introduction of a drug-selectable marker. Hence the locus can be modified without the inclusion of a drug-selectable marker cassette. These constructs can easily be modified to also perform other genetic manipulations, notably gene-disruptions and gene-mutations, which can

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be used to interrogate gene function and importance. A consequence of the absence of a drug-selectable marker in the genome is that these 'marker-free' parasites can be further modified more rapidly and easily to create parasites that have multiple genetic modifications, for example, 'double' modified parasites that contain both a reporter gene as well as a disrupted or mutated gene. Indeed, such rodent malaria mutants have been used extensively to analyse the phenotypic consequences of a gene disruption/mutation, *in vitro* and *in vivo*, using a variety of imaging technologies.

The GFP-expression cassettes were introduced into the *p230p* gene locus of the *P. falciparum* genome. The *p230p* locus was selected as we predicted it to be a 'neutral' locus based on rodent *Plasmodium* studies, i.e. the loss of this *P. falciparum* gene would not affect parasite development. However, disruption of the *p230p* gene unexpectedly resulted in *P. falciparum* parasites that could not infect and develop in mosquitoes. We describe in **Chapter 4** the phenotype of the '*p230p* gene-deletion' mutants in mosquitoes and the potential role of the P230p protein in mosquito development. In *P. falciparum* the *p47* gene locus has been most frequently used to introduce transgenes into the *P. falciparum* genome [6, 7]. We initially had selected the *p230p* gene locus to introduce transgenes as an alternative to the *p47* gene, since the P47 protein has been shown to be important for limiting defence responses against the parasite in mosquitoes [8, 9]. Consequently, *P. falciparum* parasites lacking P47 expression have a reduced capacity to transmit through some species of *Anopheles* mosquitoes, as they are less capable of escaping the mosquito immune response. In two rodent *Plasmodium* species the male-specific P230p protein appears to be dispensable throughout the parasite's complete life cycle [10-12]. *P. berghei* and *P. yoelii* mutants lacking expression of P230p can develop in the vertebrate host and in the mosquito vector without a discernible phenotype and *p230p* knock-out parasites manifest a wild type parasite phenotype. As rodent *Plasmodium* P230p is not essential, the *p230p* gene is a frequently used locus to introduce additional transgenes into rodent malaria parasite genomes [11]. In contrast, we found that *P. falciparum p230p* cannot be used as a 'neutral locus', in particular for the generation of genetically modified parasites that need to be examined in mosquitoes or in liver stages (see also **Chapter 4**).

Future studies

We generated a set of CRISPR/Cas9 constructs that can be modified and used in future studies to remove or introduce transgenes into the *P. falciparum* genome. For example, these can be used to i) generate reporter lines that express additional (fluorescent/bioluminescent) reporter proteins; ii) to analyse the genetic regulatory elements that control *Plasmodium* gene expression; iii) change the homology regions of the sgRNA/donor DNA construct to permit introduction of transgenes into different *P. falciparum* genetic loci; or iv) to introduce other transgene (e.g. reporter) expression cassettes under the control of different regulatory promoter and transcription terminator (5'- and 3'-UTR) regions.

In this study we generated three different reporter parasite lines, principally to identify strong constitutive promoters that can be used to drive reporter gene expression. We found that the *cam* promoter resulted in relatively weak GFP-expression compared to *gapdh* and *hsp70* in blood stage parasites, which is in agreement with *Plasmodium* RNAseq gene-expression data. The *Plasmodium hsp70* promoter has been used frequently to drive transgene expression in rodent reporter parasites. The *gapdh* promoter has not been extensively examined in either rodent or human parasites. GAPDH is an enzyme involved in glycolysis, the main pathway for ATP production in *Plasmodium* [13], and therefore this protein is likely to be expressed throughout the complete life cycle. Indeed, proteome analyses of oocysts and sporozoites provide evidence for high abundance of GAPDH in these stages (PlasmoDB; www.plasmodb.org). Therefore, we believe that both *hsp70* and *gapdh* promoters may be used to drive transgene expression throughout the complete parasite life cycle.

Further improvements could be made to the CRISPR/Cas9 methodology. In particular the replacement of the 'two plasmid' based transfection system, which results in reduced transfection efficacy and requires double drug selection, with a more efficient and rapid 'single plasmid' transfection method [14].

This study revealed that *230p* gene locus, unexpectedly, was not suitable for creation of transgenic parasite lines that could transmit through mosquitoes. Therefore, identification of additional 'neutral' genetic loci in *P. falciparum* is required to expand the possibilities for creating transgenic reporter lines that can be used at multiple points of the parasite life-cycle. This is even more pressing, since the most frequently used locus for introduction of transgenes is the *p47* gene, whose removal has been shown to compromise *P. falciparum* development in different strains of *Anopheles* mosquitoes.

The phenotype of *P. falciparum* mutants lacking P230p expression (Chapter 4)

The GFP-expression cassettes in the reporter *P. falciparum* lines described in **Chapter 3** were introduced into the genome in the *p230p* gene locus. As we mentioned above, disruption of the *p230p* gene resulted in parasites that could not infect and develop in mosquitoes. In **Chapter 4** we describe studies to analyse the phenotype of the *p230p* 'gene-deletion' mutants in mosquito stages and to identify its potential role in parasite fertilization.

The P230p protein belongs to the s48/45 domain 6-cysteine (6-cys) family of *Plasmodium* proteins, a small family with 14 members that show stage-specific expression throughout the parasite life cycle, with most members localizing to the surface of parasites [15]. Most members have critical roles in parasite development, either in the vertebrate host or in the mosquito vector, and several members are leading targets for malaria vaccines. These include vaccine antigens that target parasites in the mosquito, the so-called transmission blocking vaccine antigens, i.e. P48/45 and P230 [16-18]. In *P. falciparum* these proteins

are expressed in both male and female gametocytes/gametes [19-21]. In two rodent *Plasmodium* species the paralog of P230, the male-specific P230p protein, appears to be dispensable throughout the parasite's complete life cycle [10-12].

We found that *P. falciparum* mutants lacking P230p expression (*Pf*Δ*p230p*) produced wild type numbers of male and female gametocytes that retained expression of P48/45 and P230. Upon activation, male *Pf*Δ*p230p* gametocytes undergo exflagellation and form male gametes. However, these male gametes are unable to attach to red blood cells, resulting in the absence of characteristic exflagellation centres *in vitro*. In the absence of P230p, zygote formation as well as oocyst and sporozoite development were strongly reduced in mosquitoes. These observations demonstrate that P230p, like P230 and P48/45, has a vital role in *P. falciparum* male fertility and zygote formation and identifies P230p as one of a limited number of gamete-specific proteins critical for *P. falciparum* transmission.

The function of P230p in male gamete fertility is in agreement with male-specific expression of *Pf*P230p and concomitant absence in female gametocytes/gametes [19-24]. The important role that *P. falciparum* P230p plays in mosquito transmission does not match the redundant function of P230p in the rodent parasites *P. berghei* and *P. yoelii*. Rodent parasites also express P230p specifically in male gametocytes [25], but mutants lacking P230p have no discernible defect and exhibit normal gametocyte/gamete formation. Activated male gametocytes are fully able to form exflagellation centres and mosquito transmission is similar to WT parasites [10, 11].

In **Chapter 4** we also provide evidence that *P. berghei* P230 is not compensating for the loss of its paralogue P230p, since activated male gametocytes of *P. berghei* mutants lacking expression of both P230 and P230p can still bind to RBCs and form exflagellation centres. These observations demonstrate a critical difference in the function P230p performs in rodent and human malaria parasites. For a few other 6-Cys proteins evidence has been found for functional differences between the orthologs of rodent and human parasites. For example, the female-specific P47 protein is vital for the fertility of *P. berghei* female gametes, while this protein does not appear to be crucial for *P. falciparum* female gamete fertility [10, 26]. Analysis of *P. berghei* mutants lacking P45/48 and P230 demonstrates that these proteins are male-specific fertility factors [10, 27]. In contrast, *P. falciparum* P48/45 and P230 are expressed in both males and female gametes [19-21], which may suggest a role for these proteins in both male and female gamete fertility.

The lack of RBC binding of *Pf*Δ*p230p* male gametes is similar to the phenotype of *P. falciparum* mutants lacking expression of its paralog P230 [28]. These observations suggest that both proteins have a similar, but not interchangeable, function in RBC binding. We provide evidence that *Pf*Δ*p230p* male and female gametes retain expression of both P230 and P48/45, indicating that the lack of RBC binding is not due to the absence of expression of P230 and/or P48/45. These observations would suggest that RBC binding of male gametes is not due to direct interactions of P230 to RBC receptors as was also demonstrated in studies using male gametes lacking expression of P230 [28].

Future studies

P230 and P48 form complexes with several other proteins at the surface of female gametes and zygotes [29-31]. Given that P48/45, P230 and P230p are expressed in male gametes, it is conceivable that comparable complexes, including additional proteins, are also formed at the surface of male gametes. The absence of either P230 or P230p may affect the correct formation of such protein complexes at the gamete surface, which may in turn lead to the same loss of the RBC-binding phenotype observed in mutants that lack either paralog. Future studies are needed to unravel in more detail the molecular interactions between male gametes and RBC and the *Plasmodium* ligand(s) that bind to the putative protein receptors, sialic acid and/or glycophorin on the RBC surface [32].

Since both P230 and Pf48/45 are present on the surface of *P. falciparum* gametes and have a critical role in fertilization they are actively being pursued as malaria vaccine candidates. Immunization with these antigens provoke antibody-based responses in the host that could limit parasite transmission in mosquitoes, so called transmission blocking vaccination [33]. Therefore, our observations of the vital role of P230p in *P. falciparum* fertilization and zygote formation, like P230 and P48/45, would indicate that studies should be performed to investigate its potential as a transmission blocking vaccine candidate antigen; for example, to examine whether immunization with P230p could also generate antibody responses that could limit parasite development in mosquitoes.

A *P. falciparum* NF54 reporter line expressing mCherry-luciferase in gametocytes, sporozoites and liver stages (Chapter 5)

In **Chapter 3** we described studies to generate and analyse different *P. falciparum* transgenic lines expressing GFP from different promoters. This was performed in order to create highly fluorescent reporter parasites that express GFP throughout the complete life cycle. However, given that the insertion of a transgene into the *P. falciparum p230p* locus resulted in parasites that could not infect mosquitoes (**Chapter 3 and 4**), we reverted to using the standard 'neutral' *p47* gene locus for introduction of a novel reporter expression cassette. In **Chapter 5** we describe the creation and evaluation of a reporter line that expresses a fusion of mCherry and luciferase, driven by the *etramp10.3* gene promoter. We selected the *etramp10.3* promoter as this gene has structural similarity to the rodent *Plasmodium uis4* gene and *etramp10.3* and *uis4* have the same syntenic genomic location. In multiple rodent malaria transgenic lines the *uis4* promoter has been used to drive expression of different transgenes, specifically in sporozoites and liver stages, such as genes encoding mCherry, ovalbumin or human malaria proteins [34-40]. We chose to generate an mCherry-expressing *P. falciparum* line in order to visualise interactions of *Plasmodium* sporozoites with host-cells (e.g. immune cells or hepatocytes) that are often labelled with green fluorescent proteins. Moreover, we fused the mCherry gene to the gene encoding firefly luciferase as luciferase expression can be used to quantify parasite numbers (e.g.

sporozoites and liver-stages) using simple and sensitive luminescence assays [41-43]. Using the CRISPR/Cas9 methodology, described in **Chapter 3**, we inserted the *mCherry-luc@etramp10.3* expression cassette into the *p47* gene locus. We demonstrated that this transgenic parasite line (*mCherry-luc@etramp10.3*) expresses mCherry in gametocytes, sporozoites and liver-stage parasites. While we did not detect mCherry above background levels in asexual blood-stage parasites, we were able to measure luciferase expression in asexual blood-stages as well as in gametocytes, sporozoites and liver-stages. The highest levels of reporter expression were detected in stage III-V gametocytes and in sporozoites.

The high activity of the *etramp10.3* promoter in gametocytes is in agreement with the high levels of *etramp10.3* transcripts and ETRAMP10.3 protein levels previously reported in gametocytes by genome-wide analyses of gene expression. The peak *etramp10.3* transcript abundance was observed in stage III gametocytes [44], and ETRAMP10.3 is detected in proteomic analyses of (male and female) gametocytes [20, 21] and is more abundant in gametocytes compared to asexual blood-stages. The expression of ETRAMP10.3 in asexual blood-stages has been reported in proteomic analyses [45, 46] and has been confirmed by immunofluorescence analysis using anti-ETRAMP10.3 antibodies [47]. Moreover, unsuccessful attempts to delete the gene *etramp10.3* indicates that it is essential during asexual blood-stage development [47]. A vital role for ETRAMP10.3 during blood-stage development is in contrast to the rodent *Plasmodium* UIS4 protein, which is dispensable for blood-stage development [48, 49]. In previous studies using rodent parasites, where the *usi4* gene has been replaced with the *etramp10.3* gene, ETRAMP10.3 is unable to complement the essential function of UIS4 in *P. yoelii* liver-stages [47]. These observations indicate that UIS4 and ETRAMP10.4 may have different or only partially overlapping roles in rodent and human malaria parasites. The expression of mCherry and luciferase in gametocytes and sporozoites makes this transgenic parasite line suitable to use in *in vitro* assays to examine the effect of drugs/inhibitors and vaccine-induced immune responses on gametocyte and sporozoite development, as well as to analyse sporozoite biology in mosquitoes, skin and hepatocytes.

Future studies

The development and expansion of reporter lines in *P. falciparum* will increase the range of analyses that could be performed, as has been the case with reporter lines of the rodent parasites *P. berghei* and *P. yoelii*. Studies with *P. falciparum* transgenic reporter parasites, possibly using protocols established with rodent malaria reporter lines, may help to better interrogate *P. falciparum* gene function at different points of development, and will enhance screening of compounds or immune sera in miniaturized, high throughput and rapid assays that inhibit the parasite at different points of development. For example, mCherry-luciferase expressing parasites, such as described in this study, could be used to better understand the interactions of sporozoites and host cells (i.e. in the skin) as well as to examine the action of drugs or vaccines that target gametocyte or liver stage parasites.

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Additional reporter lines are required for *P. falciparum*, in particular those that show high reporter expression throughout liver stage development (i.e. stronger than either *GFP-luc@ef1α* or *mCherry-luc@etramp10.3*), both for sensitive drug tests as well as for the possibility of flow sorting of infected hepatocytes. We are currently creating and testing next-generation *P. falciparum* reporter lines, using data from multi-stage RNAseq transcription analyses to identify suitable strong and constitutive promoters.

Chimeric *Plasmodium falciparum* parasites expressing *Plasmodium vivax* circumsporozoite protein fail to produce salivary gland sporozoites (Chapter 6)

Rodent malaria parasites where the gene encoding circumsporozoite protein (CSP) has been replaced with *csp* genes from the human malaria parasites, *P. falciparum* (*Pf*) or *P. vivax* (*Pv*), are being used as pre-clinical tools to evaluate vaccines targeting CSP *in vivo*. These chimeric rodent parasites produce sporozoites in *A. stephensi* mosquitoes that are capable of infecting rodent and human hepatocytes. The availability of chimeric *P. falciparum* parasites where the *Pfcsp* gene has been replaced by *pvcsp* would open up possibilities for testing *P. vivax* CSP vaccines in small scale clinical trials using controlled human malaria infection (CHMI) studies. Testing the next generation of *P. falciparum* vaccines and vaccine formulations is greatly aided by the ability to vaccinate individuals and then examine vaccine efficacy by infecting immunized individuals with WT malaria parasites in controlled human malaria infections (CHMI)[50-53] Although recently CHMI has also been developed for *P. vivax* [54] and has been applied to assess pre-erythrocytic vaccine candidates [22, 23], *P. vivax* CHMI to screen different *P. vivax* vaccines is extremely limited, due in large part to the lack of methods to continuously propagate *P. vivax* blood stages in culture and to produce gametocytes *in vitro* that can be used to infect mosquitoes and produce sporozoites for challenge infections [54]. *P. vivax* CHMI is largely dependent on sporozoites which have been obtained from mosquitoes fed on infected patients [54]. We reasoned that the availability of chimeric *P. falciparum* parasites that express *P. vivax* antigens would open up possibilities to analyse protective immune responses induced by *P. vivax* antigen-based vaccines followed by CHMI with chimeric *Pf-Pv* parasites, bypassing the need for *P. vivax* sporozoite production and, equally importantly, eliminating the measures required to ensure that *P. vivax* dormant liver stages, hypnozoites, have been removed. In **Chapter 6**, we describe proof-of-concept studies where we generated two chimeric *P. falciparum* parasites using CRISPR/Cas9 gene editing methodologies, in which the *pfcsp* gene was replaced by one of the two major *pvcsp* alleles, either VK210 or VK247 [55].

We found that the two chimeric Pf-PvCSP lines exhibit normal asexual and sexual blood stage development *in vitro* and produce sporozoite-containing oocysts in *A. stephensi* mosquitoes. Expression of the corresponding *Pv*CSP was confirmed in oocyst-derived Pf-PvCSP sporozoites. However, most oocysts degenerate before sporozoite formation and

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sporozoites were not found in either the mosquito haemocoel or in salivary glands. Unlike the chimeric Pf-PvCSP parasites, oocysts of *P. falciparum* parasites lacking CSP-expression do not produce sporozoites. These observations indicate that the *Pv*CSP proteins can be used to initiate sporozoite formation in *P. falciparum* oocysts but are unable to fully complement the function of *Pf*CSP in oocyst maturation and sporozoite development. It seems unlikely that the failure of *Pv*CSP to functionally complement *Pf*CSP is due to incorrect expression of the *Pv*CSP proteins in the Pf-PvCSP lines. We used the same *Pvcsp* genes, as used for successful complementation of CSP in *P. berghei* (*Pb*) [56], to replace *P. falciparum csp*. These *Pvcsp* genes were amplified from the same plasmids that were used for generation of the *Pb-Pvcsp* lines. In addition, the *Pvcsp* gene coding sequence in the genome of the Pf-PvCSP lines was placed under control of the endogenous *Pfcsp* promoter and transcriptional terminator sequences to ensure correct timing and level of CSP expression. It has been shown that the 3' untranslated region (3'-UTR) of *P. berghei csp* plays an important role in accurate CSP expression, and truncation of *Pbcsp* 3'-UTR results in reduced CSP expression, reduced oocyst sporozoite formation and degeneration of oocysts [57]. The inability of *Pv*CSP to replace the *Pf*CSP function in the chimeric Pf-PvCSP lines is therefore most likely due to sequence differences between these *csp* genes resulting in structural differences of the CSP proteins, which may affect interactions with other parasite proteins that are essential for proper sporozoite formation. These can be protein-interactions that influence correct transport of CSP from within the oocyst-cytoplasm to the membrane of developing sporozoites or protein-interactions that affect CSP localization or maintenance on sporozoites [58-61]. As mentioned above, it is possible to create infectious chimeric *P. berghei* parasites expressing *Pv*CSP. It therefore appears that both *Pv*CSP and *Pf*CSP are able to interact with these CSP interacting proteins in *P. berghei*.

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Future studies

Currently, the inability of the two Pf-PvCSP lines to produce salivary gland sporozoites means that these parasites cannot be used for CHMI studies. Further studies are required to investigate if it is possible to create *P. falciparum* parasites that express *P. vivax* CSP molecules; this may consist of creating *P. falciparum* parasites expressing hybrid CSP proteins that contain different domains of *Pf*CSP and *Pv*CSP. Studies using parasites expressing hybrid CSP molecules may also reveal which CSP domains are essential for sporozoite maturation and release and may provide insight into the failure of *Pf*CSP complementation by *Pv*CSP.

While it was not possible to directly create *P. falciparum* parasites where CSP had been replaced with *Pv*CSP, it may be possible to replace in *P. falciparum* other vaccine candidate antigens with the *P. vivax* equivalent, and as such make these parasites suitable for CHMI studies. Finally, it may be possible to create transgenic sporozoites that express the target antigen of both *P. falciparum* and *P. vivax* in the same parasite, and such parasites could be used as an enhanced irradiated or genetically attenuated sporozoite vaccine that could induce protective immune responses against infection with both human malaria parasites.

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