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

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## English summary

For rodent malaria parasites the availability of a variety transgenic parasite lines expressing different reporter proteins under the control of stage-specific or constitutive promoters have been of great benefit to studies to reveal parasite gene function and to studies focussed on the evaluation of novel drugs and vaccines. The availability of similar transgenic *P. falciparum* reporter lines would also help to advance gene-function studies and studies aiming at development of new therapies for *P. falciparum* parasites. We review the use of transgenic rodent and human parasites for malaria vaccine research in **Chapter 2**.

In this thesis, we describe a set of studies performed in *P. falciparum* to develop novel CRISPR/Cas9 methodologies to improve *P. falciparum* transgenesis and to create novel transgenic reporter parasites that can be used to analyse host-pathogen interactions and for anti-malarial drug and vaccine research. We first focused on improving CRISPR/Cas9 gene editing technologies and the introduction of transgenes into the *P. falciparum* genome using a new potential 'neutral' locus. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that either express fluorescent-luminescent reporters or express a major vaccine candidate from the other major human malaria parasite, *Plasmodium vivax*.

In **Chapter 3** we describe studies aiming at improving CRISPR/Cas9 genetic modification for introduction of transgenes into the genome of *P. falciparum*. We report on further improvements to both the CRISPR/Cas9 transfection constructs and selection protocol to more rapidly modify the *P. falciparum* genome in order to introduce transgenes into the parasite genome without the inclusion of drug-selectable marker genes. This method was used to stably integrate the gene encoding GFP into the *P. falciparum* genome under the control of promoters of three different *Plasmodium* genes (*calmodulin*, *gapdh* and *hsp70*) to select for constitutive and strong promoters that can be used to drive reporter gene expression. These genes were selected as they are highly transcribed in blood stages. We show that the three reporter parasite lines generated in this study (GFP@cam, GFP@gapdh and GFP@hsp70) have *in vitro* blood stage growth kinetics and drug-sensitivity profiles comparable to the parental *P. falciparum* (NF54) wild-type line. Both asexual and sexual blood stages of the three reporter lines expressed GFP with GFP@hsp70 having the highest fluorescent intensity in schizont stages as shown by flow cytometry analysis of GFP-fluorescence. The improved CRISPR/Cas9 constructs and protocol will aid in the rapid generation of transgenic and modified *P. falciparum* parasites, including those expressing different reporter proteins under different (stage specific) promoters. In addition, this method will make it easier to perform successive gene-deletions and gene-mutations, which will be of value to interrogate parasite gene function and for the development of multiple-attenuated malaria parasites suitable for vaccination. In these studies *gfp*-expression cassettes were introduced into the genome in the *p230p* gene locus. This gene locus was predicted to be a 'neutral' locus (a locus that can be modified without altering the phenotype of the different life cycle stages), since in rodent malaria parasites

the gene encoding P230p is dispensable throughout the complete life-cycle. Unexpectedly, disruption of the *P230p* locus in *P. falciparum* created parasites that could not infect and develop in mosquitoes.

In **Chapter 4** we describe the phenotype in mosquitoes of the *p230p* gene-deletion mutants (*PfΔp230p*) and the potential role of the P230p protein in mosquito development. P230p belongs to the small family of so called s48/45 domain 6-cysteine (6-cys) proteins. Two other members of this family, P48/45 and P230, are important for gamete fertility in rodent and human malaria parasites and are leading transmission blocking vaccine antigens. While P48/45 and P230 are expressed in male and female parasites, P230p is expressed only in male gametocytes and gametes in both rodent and human malaria parasites. The *PfΔp230p* mutants produced normal numbers of male and female gametocytes, which retained expression of both P48/45 and P230. Upon activation male *PfΔp230p* gametocytes undergo exflagellation and form male gametes. However, male gametes were 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 (>98%) in mosquitoes. In contrast, male gametes of rodent malaria parasites lacking P230p show normal attachment to red blood cells and have fertilisation rates and oocyst production that is comparable to wild type parasites. These observations demonstrate that *P. falciparum* P230p, like P230 and P48/45, has a vital role in *P. falciparum* male fertility and zygote formation and warrants further investigation as a potential transmission blocking vaccine candidate.

In **Chapter 5**, we describe the creation and evaluation of a *P. falciparum* reporter line that expresses a fusion of mCherry and luciferase driven by the promoter of the *etramp10.3* gene and we examine these transgenic parasites in blood- and liver-stage cultures as well in mosquitoes. The *P. falciparum* ETRAMP10.3 protein is related to the rodent *Plasmodium* UIS4 protein, which is also a member of the ETRAMP protein family. The promoter of the *uis4* gene has been used to drive high transgene expression in liver-stages of rodent malaria parasites. The CRISPR/Cas9 methodology described in **Chapter 3** was used to insert the *mCherry-luc@etramp10.3* expression cassette into the previously characterised 'neutral' *P. falciparum* *p47* gene locus. This reporter line demonstrates mCherry expression in gametocytes, sporozoites and liver-stages, whereas asexual blood stages and developing oocyst did not show mCherry signals different from background levels. Luciferase expression was demonstrated in asexual blood-stages, gametocytes, sporozoites and liver-stages, with high level reporter expression in stage III-V gametocytes and in sporozoites. The expression of mCherry and luciferase in gametocytes and sporozoites makes this transgenic parasite line a suitable tool to analyse the effect of inhibitors on gametocyte development and to analyse sporozoite and liver-stage biology.

In **Chapter 6** we describe the creation of two chimeric *P. falciparum* parasites (*Pf-PvCSP*), using CRISPR/Cas9 gene editing methodologies, where the gene encoding circumsporozoite protein (CSP), was replaced by two *csp* gene variants (VK210 and VK247) of the human parasite *P. vivax*. The major sporozoite surface protein CSP plays a critical



role both in sporozoite formation and in sporozoite invasion of mosquito salivary glands and liver cells of the host. CSP is the target antigen of the most advanced *P. falciparum* malaria vaccine (RTS,S) and is also an important vaccine target for *P. vivax*. Rodent malaria parasites where the gene encoding circumsporozoite protein (CSP) has been replaced with *csp* genes from the human malaria parasites, *P. falciparum* or *P. vivax*, are used as pre-clinical tools to evaluate CSP vaccines *in vivo*. These chimeric rodent parasites produce sporozoites in *Anopheles stephensi* mosquitoes that are capable of infecting rodent and human hepatocytes. The availability of chimeric *P. falciparum* parasites where the *P. falciparum* *csp* gene has been replaced by the *P. vivax* *csp* would open up possibilities to test *P. vivax* CSP vaccines in small scale clinical trials using controlled human malaria infection (CHMI) studies. The two chimeric *Pf-PvCSP* lines exhibited normal asexual and sexual blood stage development *in vitro* and produced sporozoite-containing oocysts in *A. stephensi* mosquitoes. We confirmed that the oocyst-derived *Pf-PvCSP* sporozoites express the corresponding *PvCSP*. However, most oocysts degenerate before sporozoite formation and sporozoites were not found in either the mosquito hemocoel or salivary glands. Unlike the chimeric *Pf-PvCSP* parasites, oocysts of *Pf* parasites lacking CSP expression do not produce sporozoites. Combined our observations show that while *PvCSP* can partially complement the function of *PfCSP*, species-specific features of CSP govern full sporozoite maturation and development in the two human malaria parasites.