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Human red blood cell-adapted *Plasmodium knowlesi* parasites: a new model system for malaria research

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Summary

Plasmodium knowlesi is a simian malaria parasite primarily infecting macaque species in Southeast Asia. Although its capacity to infect humans has been recognized since the early part of the last century, it has recently become evident that human infections are widespread and potentially life threatening. Historically, *P. knowlesi* has proven to be a powerful tool in early studies of malaria parasites, providing key breakthroughs in understanding many aspects of *Plasmodium* biology. However, the necessity to grow the parasite either in macaques or *in vitro* using macaque blood restricted research to laboratories with access to these resources. The recent adaptation of *P. knowlesi* to grow and proliferate *in vitro* in human red blood cells (RBCs) is therefore a substantial step towards revitalizing and expanding research on *P. knowlesi*. Furthermore, the development of a highly efficient transfection system to genetically modify the parasite makes *P. knowlesi* an ideal model to study parasite biology. In this review we elaborate on the importance of *P. knowlesi* in earlier phases of malaria research and highlight the future potential of the newly available human adapted *P. knowlesi* parasite lines.

Introduction

Malaria remains one of the most important infectious diseases of humans, causing almost 1 million deaths per year and extensive morbidity (Murray *et al*., 2012). Caused by six species of single cell protozoan pathogens from the genus *Plasmodium*, severe disease and death in Africa is associated with *Plasmodium falciparum* infections, whereas *Plasmodium vivax* also causes a large number of cases and significant morbidity worldwide. *Plasmodium knowlesi* is a simian malaria parasite phylogenetically closely related to *P. vivax* (Pain *et al*., 2008) and has long provided many advantages as an experimental model in the laboratory. It was first identified by Knowles and Das Gupta in 1932, in a cynomolgus macaque (*Macaca fascicularis*). In this first description, they demonstrated that the parasite could also infect humans by blood passage, but the first natural *P. knowlesi* infection was not identified until

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1965 (Chin *et al*., 1965). Today, it is recognized as a major cause of clinical malaria in South East Asia (Singh *et al*., 2013).

Plasmodium parasites have a complicated lifecycle involving both mosquito and specific vertebrate hosts. For most of its development in the vertebrate it is an obligate intracellular parasite, first invading and undergoing asexual multiplication in hepatocytes and then multiple cycles of replication in red blood cells (RBCs). During growth within an infected RBC the parasite replicates to form a schizont, which undergoes segmentation producing invasive forms known as merozoites. These are highly polarized cells with a complex set of organelles and machinery for invasion of RBCs, but are also amongst the smallest of all eukaryotic organisms. Unlike *P. falciparum*, which produces 20 to 30 merozoites each about one micron in diameter, *P. knowlesi* produces fewer (average 10, but up to 16; Coatney *et al*., 1971) but larger merozoites (Fig. 1A).

The laboratory study of malaria has been aided by many critical developments, including the establishment of *in vivo* animal models, laboratory colonisation of mosquito vectors and the *in vitro* culture adaptation of erythrocytic stages. Here, we review the advances in the development of *P. knowlesi* as a model for understanding these various aspects of *Plasmodium* biology. We will highlight the recent technical developments of the *P. knowlesi in vitro* system and provide an outlook for the exploitation of the unique and exciting possibilities this model system offers for future research.

P. knowlesi is a zoonotic infection of humans

After the initial description of a *P. knowlesi* human infection (Chin *et al*., 1965), a subsequent survey suggested that the parasite was a rare zoonosis of humans (Warren *et al*., 1970). However, in 2004, Singh and colleagues were studying a large focus of apparent *P. malariae* clinical cases in Kapit Division, Malaysian Borneo. Genetic analysis revealed that these parasites had been misdiagnosed due to similar blood smear morphology, and that 120 out of 208 cases were *P. knowlesi* (Singh *et al*., 2004). Screening of archival blood smears then showed that *P. knowlesi* had been present in the region since at least 1996 (Lee *et al*., 2009). Most clinical *P. knowlesi* cases are uncomplicated with nonspecific febrile illness and thrombocytopenia. However, the rates of severe malaria are similar to that of *P. falciparum*, reaching 6.5% in a district hospital in Sarawak, Malaysia (Daneshvar *et al*., 2009). The most common complications are respiratory distress and renal failure, and these are often associated with hyperparasitaemia (Barber *et al*., 2013).

P. knowlesi is now known to be the most common cause of malaria in many areas of Malaysian Borneo, and a significant contributor in Peninsular Malaysia. Human cases of *P. knowlesi* have since been discovered throughout South East Asia, including the Philippines, Singapore, Indonesia, Vietnam, Cambodia, Thailand and Myanmar (Singh *et al*., 2013). While malaria cases caused by other *Plasmodium* species are on the decline in Sabah, Malaysian Borneo, *P. knowlesi* cases increased over 10-fold between 2004 and 2011, indicating that this parasite is an emerging threat in the region (William *et al*., 2013). However, there is as of yet no direct evidence for human-to-human transmission by mosquitoes in the field.

P. knowlesi: pioneering malaria research

P. knowlesi offers many important advantages as an experimental model for malaria and this was first demonstrated in several animal studies. While the parasite produces a mild chronic infection in its natural macaque host, *Macaca fascicularis*, it was commonly maintained experimentally in the rhesus macaque, *Macaca mulatta*, in which it produces fulminating infections (Coatney *et al*., 1971). Before *in vitro* culture of *P. falciparum* was achieved and widely adopted, *P. knowlesi* rhesus infections provided a source of highly synchronous parasites for studies in the laboratory.

The *P. knowlesi* animal models provided groundbreaking knowledge on several aspects of *Plasmodium* infection, including the basis of immunity. A landmark finding was the protection provided by passive immunization of newly infected monkeys with serum from chronically infected ones (Coggeshall *et al*., 1937). These transfer experiments and the discovery of antibodies targeting the parasite, demonstrated the importance of the immune response in controlling disease progression (Coggeshall, 1940). Shortly after, the first immunization studies were conducted using killed blood stage *P. knowlesi* or avian *P. lophurae* parasites (Freund *et al*., 1945a, Freund *et al*., 1945b). Both studies showed promising although not entirely protective effects, providing early evidence that a whole parasite vaccine may provide a valuable tool to prevent malaria parasite infection.

Antigenic variation mediated by the successive expression of variant proteins is an important immune evasion mechanism in *Plasmodium* parasites and was first described in *P. knowlesi* (Brown *et al*., 1965). This study paved the way to identify and characterize the corresponding gene family called *SICAvar* in *P. knowlesi* (Howard *et al*., 1983) and later the *var* genes in *P. falciparum*.

The invasion of a RBC by a merozoite is a crucial stage in the course of infection. This highly complex process, involving various receptor-ligand interactions, is thus of great research interest. The first of these receptor-ligand pairs was discovered in *P. knowlesi*, and comprises the Duffy binding protein-α (PkDBP-α) and the Duffy antigen receptor for chemokines (DARC) on human RBCs (Adams *et al*., 1990; Miller *et al*., 1975), and is also shared with *P. vivax* (Horuk *et al*., 1993). In total, the *P. knowlesi* genome encodes three DBPs (Pain *et al*., 2008). While being dispensable for invading rhesus macaque RBCs, PkDBP-α is pivotal for the invasion of human RBCs (Singh *et al*., 2005). The functions of the other two DBPs (DBP-β and -γ) are unknown but since they bind to rhesus but not human RBCs, it is likely that they contribute to rhesus RBC invasion (Chitnis *et al*., 1994). These studies highlight some of the seminal contributions made using *P. knowlesi* in the early days of malaria research.

From *in vivo* to *in vitro* studies

Although research on *P. knowlesi* in non-human primates was of great importance for the understanding of parasite biology, the lack of a robust continuous *in vitro* cultivation system restricted research to laboratories with access to suitable animal facilities. The first successful *in vitro* culture was achieved in 1945 when Ball and colleagues were able to maintain *P. knowlesi* in culture in rhesus RBCs for periods of up to 6 erythrocytic cycles

(Ball *et al*., 1945). Follow-up studies provided insights into the importance of different components of the culture medium (Anfinsen *et al*., 1946; Geiman *et al*., 1966; Trigg, 1968); these detailed efforts were essential for optimizing experimental procedures such as invasion assays.

Although no long-term culture was possible, there were other advantages to studying this parasite. Groundbreaking studies in the 1970s included the first detailed live-microscopy investigation of *P. knowlesi* invasion (Dvorak *et al*., 1975). Dennis and colleagues developed a procedure to isolate live merozoites from infected rhesus RBCs using a polycarbonate sieve (Dennis *et al*., 1975), facilitating detailed electron microscopic studies of the invasion process and the identification of the moving junction between parasite and host cell (Aikawa *et al*., 1978; Bannister *et al*., 1975). While recent advances with *P. falciparum* have now allowed both the real-time imaging of invasion (Gilson *et al*., 2009) and purification of viable merozoites (Boyle *et al*., 2010), *P. knowlesi* still has the significant advantage of size and better viability. The application of new technologies such as super-resolution microscopy (Riglar *et al*., 2011) or long-term live microscopy (Grüring *et al*., 2011) to the *P. knowlesi* system will be particularly fruitful.

Although the achievement of Trager and Jensen to establish culture conditions for *P. falciparum* (Trager *et al*., 1976) diminished the use of *P. knowlesi*, the knowledge gained could be extrapolated for *P. knowlesi* culture. Further developments allowed the extended culture of *P. knowlesi* for several months (Butcher, 1979; Wickham *et al*., 1980), but required frequent media changes or intricate systems and were not widely used. A breakthrough was achieved in 2002 when Kocken and colleagues adapted the *P. knowlesi* H strain to continuous culture, developing an *in vitro* system that produced enough parasites to allow for transfection experiments (Kocken *et al*., 2002). Importantly, the *in vitro* adapted line was readily transferred back into rhesus monkeys, allowing genetic manipulation of parasites *in vitro* and the study of their phenotype *in vivo*. However, the requirement for rhesus macaque RBCs still restricted research to a few laboratories with access to monkey blood; the need for *P. knowlesi* parasite lines able to grow in human blood *in vitr*o was clear (Kocken *et al*., 2009).

Adaptation of *P. knowlesi* to human red blood cells

Although human infections, either artificially induced or naturally acquired, have been known for decades, attempts to adapt *P. knowlesi* to stably proliferate in human RBCs failed, probably due to low replication rates (Kocken *et al*., 2009). This hurdle has now been overcome as our laboratories have reported the successful adaptation of *P. knowlesi* deriving from the H strain to grow in human RBCs (Lim *et al*., 2013; Moon *et al*., 2013). In both studies, parasites initially rapidly died out when grown exclusively in human RBCs but could be slowly adapted by initially growing them in a mixture of macaque (either *M. mulatta* or *M. fascicularis*) and human RBCs (Fig. 1B). Interestingly, after initial adaptation in human RBCs and proliferation rates of approximately two fold per day, the parasites eventually adapted further with rates increasing to up to five fold per day (Moon *et al*., 2013). This suggests two important adaptation steps, first at the level of invasion and second during the intra-erythrocytic growth phase. Whether the two lines underwent the same

genetic or epigenetic changes remains an open question and is currently under investigation. A major step towards adaptation in one of the lines was a change in host RBC preference for invasion. While not restricted to cells of a certain age in macaques, *P. knowlesi* was found to invade mainly young human RBCs. In contrast, the human-adapted line had an increased invasion efficiency into older RBCs, providing access to a greater repertoire of suitable host cells (Fig. 1C). Mathematical modeling suggests that this expansion likely drives the high parasitaemias observed in some clinical infections (Lim *et al*., 2013). However, invasion of both adapted *P. knowlesi* lines was still DARC dependent since no invasion was observed in Duffy-negative human RBCs (Moon *et al*., 2013) or in cells treated with chymotrypsin depleting DARC from the RBC surface (Lim *et al*., unpublished). It will be of great interest to dissect the exact receptor requirements for the human lines as the DARC level on the surface of mature RBCs is diminished compared to reticulocytes (Liu *et al*., 2009). Furthermore, the human RBC-adapted *P. knowlesi* parasites retain their competence to efficiently invade macaque RBCs, showing that the invasion pathways used between the original and human RBC-adapted parasites are not mutually exclusive (Lim *et al*., 2013; Moon *et al*., 2013). The analysis of the adaptation mechanism in these lines could thus be leveraged to understand the basis of the successful zoonosis causing human *P. knowlesi* infections.

These human RBC-adapted lines are now available to the research community and their widespread use will greatly boost research on *P. knowlesi*, which is urgently needed in view of its potential as an emergent human infection.

Transfection of *P. knowlesi*

Genetic modification of the malaria parasite is a cornerstone of modern malaria research and has revealed countless details of parasite biology. However, *P. falciparum* is poorly amenable to genetic manipulation. Until very recently, transfection efficiencies in *P. falciparum* were only around 10^{-6} (O'Donnell *et al*., 2002), rendering the generation of transgenic parasites laborious and inefficient. Certain rodent malaria models, notably *P. berghei*, have higher transfection efficiencies of between 10^{-2} and 10^{-3} (Janse *et al*., 2006), but these parasites cannot be cultured *in vitro*, creating a substantial bottleneck for the high-throughput generation and analysis of transgenic parasites.

The first transfections in *P. knowlesi* were carried out in 1997 using episomal constructs, with *P. berghei* or *P. falciparum* regulatory regions driving the *T. gondii* dihydrofolate reductase-thymidylate synthase selectable marker (van der Wel *et al*., 1997). Schizonts obtained from infected macaques were electroporated and reinoculated into recipient macaques, enabling isolation of transgenic parasites in just over a week of drug selection. Similar methods were used later to integrate constructs by double-crossover homologous recombination to create a circumsporozoite protein (CSP) gene knockout line. Critically, for the first time these authors were able to demonstrate that the whole process could be carried out entirely *in vitro* using a line adapted to grow in culture with macaque RBCs (Kocken *et al*., 2002). Additional selectable markers previously used in other *Plasmodium* species were also demonstrated to work in *P. knowlesi* providing positive selection resistance to

neomycin, Blasticidin and WR99210 as well as negative selection by susceptibility to Ganciclovir (van der Wel *et al*., 2004).

More recently, transfection has been achieved using the human-adapted line. Synchronous late stage schizonts from the A1H.1 line were transfected using the Amaxa electroporation system (Moon *et al*., 2013), which had in the past significantly improved transfection efficiency in *P. berghei* (Janse *et al*., 2006). Expression of transgenes was also improved by the use of *P. knowlesi* specific promoter regions. Transfection with an episomal construct containing a GFP expression cassette resulted in ~30% GFP positive parasites the day following transfection (Moon *et al*., 2013). This indicates the highest recorded transfection efficiency for any human malaria parasite and for the first time, parasites could be analyzed in the first growth cycle after transfection. Using linearized DNA constructs targeted to a genomic locus via single crossover recombination, integrated parasites lines could be obtained in just over a week. Integration efficiency was sufficiently high to allow immediate dilution cloning, meaning that extensive drug cycling is not required. Importantly, the whole procedure could be carried out solely using human RBCs and with 10-fold fewer schizonts per transfection (10^8) than used in previous methods. Subsequent work has demonstrated that this efficiency also holds true for constructs targeting other loci (Moon *et al*., unpublished). The transfection efficiency in the other human adapted line has yet to be tested.

Towards establishing transmission of *P. knowlesi* in the lab

The sexual stages of malaria parasites known as gametocytes form when a subset of replicating asexual blood stages switch to the sexual development pathway. Once induced, *P. falciparum* gametocytes take around 10 days to fully mature, while *P. knowlesi* gametocyte formation requires about 48 hours, which may prove a significant experimental advantage (Coatney *et al*., 1971). Early work demonstrated that *P. knowlesi* could be readily transmitted via mosquitoes in the laboratory from macaque to macaque and even human to human (Chin *et al*., 1968). Unfortunately, none of the *P. knowlesi* lines adapted to *in vitro* growth produce gametocytes (Moon *et al*., 2013; Zeeman *et al*., 2013, Lim *et al*., unpublished) and attempts to induce gametocytogenesis have been so far unsuccessful. It is well established that parasite lines maintained by extended blood passage or in culture may lose their ability to form gametocytes (Janse *et al*., 1992). Therefore, it might be necessary to culture-adapt lines that have recently been transmitted by mosquitoes and retain the ability to produce gametocytes.

There is also a need to identify suitable vectors for use in the laboratory. The most commonly used experimental malaria parasite vector, *Anopheles stephensi*, cannot effectively transmit *P. knowlesi*. While the parasite is able to form midgut oocysts in *An. stephensi*, the sporozoites released from these oocysts are unable to invade the mosquito salivary glands (Coatney *et al*., 1971). Early fieldwork identified *Anopheles hackeri* as a vector for *P. knowlesi* in Malaysia (Wharton *et al*., 1961), but more recent work has implicated other vectors, including *Anopheles cracens* in peninsular Malaysia (Vythilingam *et al*., 2008) and *Anopheles latens* in Malaysian Borneo (Vythilingam *et al*., 2006). Colonies of *An. cracens* have been established in the laboratory and although their maintenance

remains challenging, this species may represent the vector of choice for *P. knowlesi* transmission in the laboratory (Amir *et al*., 2013). While significant challenges remain, *P. knowlesi* may provide a useful alternative to study sexual stages.

The use of *P. knowlesi* as a model for *P. vivax*

Alongside *P. falciparum*, *P. vivax* causes a very high burden of malaria globally, but currently lacks an *in vitro* model. Historically, common aspects of *P. knowlesi* and *P. vivax* invasion, especially their use of DARC as a receptor to invade human RBCs, enabled the understanding *P. vivax* invasion using *P. knowlesi* as a surrogate (Horuk *et al*., 1993; Singh *et al*., 2002). Beside the potential to scrutinize the use of DBP as a vaccine candidate, the *P. knowlesi* system provides the unique opportunity to investigate the role of the reticulocyte binding-like proteins (RBLs), another major ligand family that mediates invasion. The binding properties of the two members present in the *P. knowlesi* genome (PkNBPXa and PkNBPXb) have recently been investigated (Meyer *et al*., 2009; Semenya *et al*., 2012). While both members bind to rhesus macaque RBCs, PkNBPXa also binds human RBCs (Semenya *et al*., 2012). An ortholog of NBPXb has recently been identified in a *de novo* assembly of the *P. vivax* genome (Hester *et al*., 2013). Studies on these invasion molecules could shed light on the adaptation to human RBCs. The sequencing of the *P. vivax* and *P. knowlesi* genomes revealed a near perfect synteny interspersed with expansions of species-specific genes (Carlton *et al*., 2008; Pain *et al*., 2008). The two genomes also share more than 90 genes with no orthologs in *P. falciparum* (Frech *et al*., 2011), indicating that the accessibility of the *P. knowlesi* model may prove beneficial for the study of other aspects of *P. vivax* biology.

However, notable differences between the two parasites limit the use of *P. knowlesi* as a *bona fide P. vivax* model. Unlike *P. knowlesi*, *P. vivax* forms hypnozoites, dormant liver stage parasites that can lead to disease relapses years after the initial infection. Developments in a more closely related simian malaria model, *Plasmodium cynomolgi*, have provided an alternative means to study this phenomenon (Voorberg-van der Weel *et al*., 2013). Nevertheless, as long as *P. cynomolgi* and *P. vivax* lack a robust *in vitro* system, *P. knowlesi* remains a highly accessible and valuable tool to further explore aspects of *P. vivax* biology.

Future studies

The high transfection efficiency obtained in *P. knowlesi*, combined with the ability to culture the parasites in human RBCs means that the system is ideal for the development of both novel reverse genetics and high-throughput genetic techniques (Fig. 2). Conditional knockout systems have proved crucial in determining the functions of essential blood stage proteins. Several conditional knockout/knockdown systems have been developed for use in *P. falciparum*, including the use of ligand-regulatable FKBP protein destabilization domains (ddFKBP) (Armstrong *et al*., 2007), as well as the DiCre conditional recombinase system (Collins *et al*., 2013). These systems could be quickly adapted for use in *P. knowlesi*. Similarly, genome-editing tools such as the zinc-finger nucleases have revolutionized many fields, and recently have been demonstrated to target genomic integration with

unprecedented efficiency in *P. falciparum* (Straimer *et al*., 2012). This approach, and analogous systems such the TALEN (transcription activator-like effector nucleases) (Christian *et al*., 2010) and the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas system (Ran *et al*., 2013), could be readily adapted for use in *P. knowlesi* to facilitate genomic integration of DNA constructs in a matter of days.

Human RBC-adapted *P. knowlesi* also provides exciting opportunities for high-throughput genome-scale transgenesis. The ability to culture *P. knowlesi* in a microplate format, combined with the high transfection efficiency, allows for the generation of pools of recombinant parasites. The development of recombineering techniques for high-throughput generation of DNA constructs for use in *P. berghei* has greatly benefited the field (Pfander *et al*., 2011). Application of such techniques to *P. knowlesi*, which can be cloned and grown in 96-well plates, will provide an important resource for the malaria research community. Taken together, human RBCs-adapted *P. knowlesi* could emerge as an ideal system for forward genetic studies in malaria.

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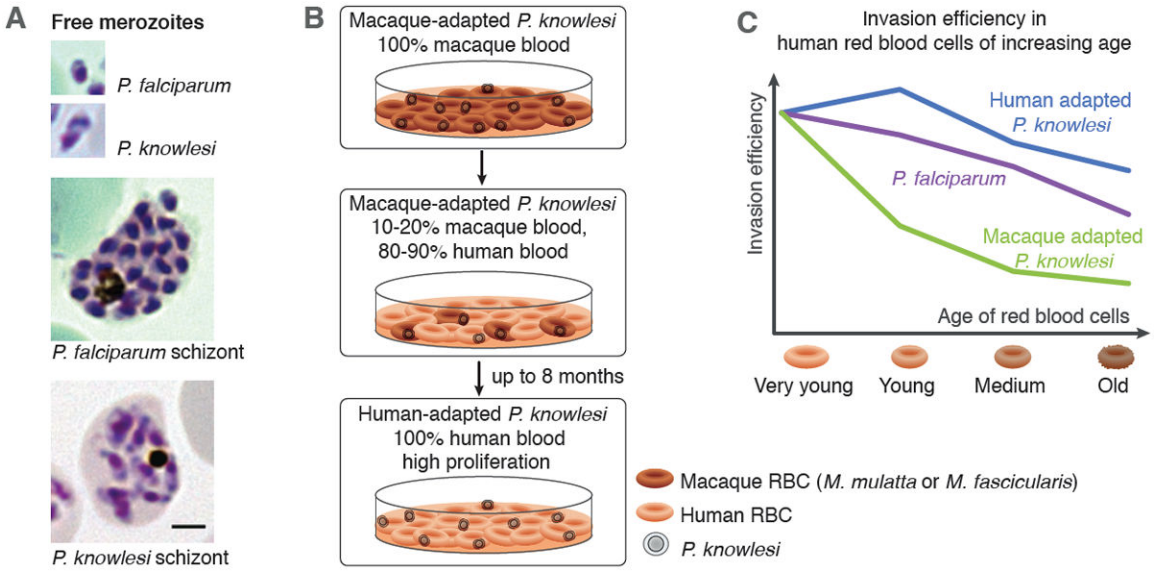

Figure 1. Adaptation procedure and switch in host cell preference of *P. knowlesi*

A. Comparison of *P. falciparum* and *P. knowlesi* schizonts and merozoites. *P. knowlesi* has fewer but larger merozoites in a mature schizont than *P. falciparum*.

B. Schematic representation of the process used to adapt macaque restricted *P. knowlesi* parasites to *in vitro* growth in human RBCs. Macaque RBCs are depicted in dark-red and human RBCs in red. Scale bar represents 2μm.

C. Switch in host cell preference to adapt to growth in RBCs. Graph shows the restriction of *P. knowlesi* grown in macaque blood to very young human RBCs and the subsequent expansion during adaptation to human blood towards a wider range of host cells. The subtle preference of *P. falciparum* for younger cells is also shown as reference.

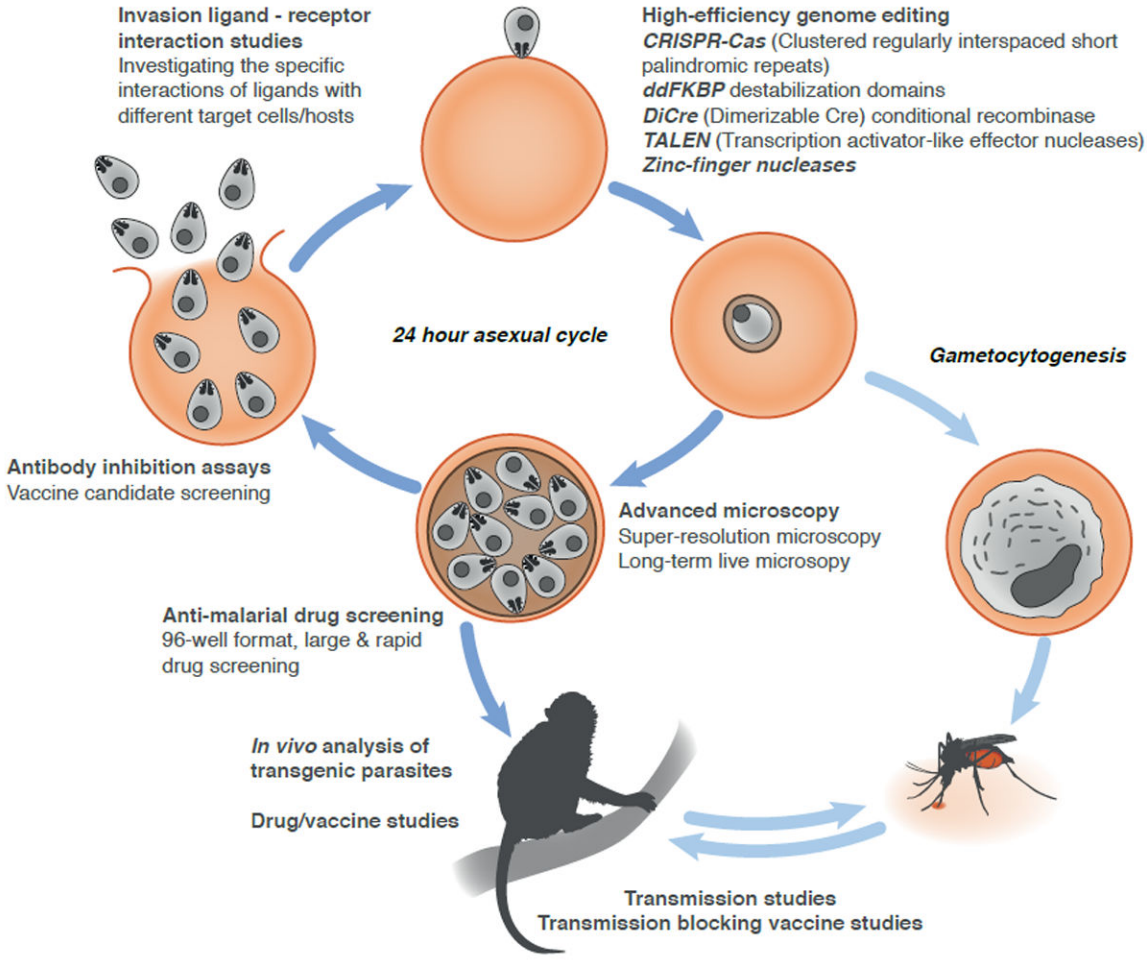

Figure 2. Outline of new tools to advance malaria research using human red blood cell adapted *P. knowlesi*

The 24h *P. knowlesi* life cycle is depicted, starting with the invasion of a merozoite and subsequent development from ring to schizont stage. A selection of tools that can be applied to the human adapted *P. knowlesi* line is shown.

The generation of a gametocyte-producing-line and transmission competent mosquito colonies would allow the research to expand to other life stages.