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Gametocytogenesis in malaria parasite: commitment, development and regulation

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Summary

Malaria parasites have evolved a complicated life cycle alternating between two hosts. Gametocytes are produced in the vertebrate hosts and are obligatory for natural transmission of the parasites through mosquito vectors. The mechanism of sexual development in *Plasmodium* has been the focus of extensive studies. In the post-genomic era, the advent of genome-wide analytical tools and genetic manipulation technology has enabled rapid advancement of our knowledge in this area. Patterns of gene expression during sexual development, molecular distinction of the two sexes, and mechanisms underlying subsequent formation of gametes and their fertilization have been progressively elucidated. However, the triggers and mechanism of sexual development remain largely unknown. This review aims to provide an update of our understanding of the molecular and cellular events associated with the decision for commitment to sexual development and regulation of gene expression during gametocytogenesis. Insights into the molecular mechanisms of gametocyte development are essential for designing proper control strategies for interruption of malaria transmission and ultimate elimination.

Keywords

malaria; gametocyte; sexual development; commitment; gene expression; transcriptional regulation; translational repression

Malaria remains a major public health issue in much of the tropical and subtropical regions worldwide. According to World Malaria Report 2010 [201], the estimated annual malaria incidence in 2009 was 225 million cases, resulting in ~781,000 deaths, with the majority of deaths occurring in children under five years old in sub-Saharan Africa. Economic loss in African countries to malaria exceeds $12 million annually [202]. The causative agents of malaria are the protozoan parasites of the genus *Plasmodium*. Four species *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* commonly infect humans, and zoonotic infection by the simian parasite *Plasmodium knowlesi*, 'the fifth

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human malaria parasite', has recently emerged as a public health threat in some Southeast Asian countries [1,2]. Whereas *P. falciparum* causes the most serious form of malaria and is responsible for most malaria-related human deaths, the so-called 'benign tertian' malaria caused by *P. vivax* has been increasingly recognized as associated with severe forms of the disease in the tropics [3]. Furthermore, *P. vivax* is the most widely distributed malaria parasite outside Africa, and its ability to produce dormant hypnozoites in the liver that are responsible for relapses of the disease presents additional difficulties to control. With the recent progress in malaria control and amelioration of financial support, many endemic countries are considering malaria elimination as their national goal [4]. However, achieving this goal requires realistic assessments of the technical, operational and financial feasibilities. The recent malERA (Malaria Eradication Research Agenda) initiative has comprehensively evaluated the knowledge base, strategies and tools required for malaria elimination or eradication [5]. Vaccines and drugs that interrupt malaria transmission are among the key areas where critical research is needed.

Plasmodium has a complex life cycle involving a vertebrate host and a mosquito vector [6]. In the human host, following infection by a malarial sporozoite, the parasite undergoes asexual multiplication, first in the liver (pre-erythrocytic schizogony), then in the erythrocytes (schizogony). Sexual development starts with a small proportion of asexual parasites making this irreversible decision and differentiating into female (macrogametocytes) and male gametocytes (microgametocytes), a process termed gametocytogenesis. Gametocytes are obligatory for parasite transmission from an infected human host to a female *Anopheles* mosquito. Shortly after ingestion by the mosquito, gametocytes in the blood meal undergo gametogenesis to produce male and female gametes. For male gametocytes, the genome is replicated three times and eight flagellated microgametes are produced. The breakout of the male gametes, called exflagellation, is such a phenomenal event that has led to the historic discovery of malaria parasite by Alphonse Laveran in 1880. Fusion of the gametes to form a diploid zygote and subsequent sporogonic development on the mosquito midgut epithelium produce thousands of haploid sporozoites. This meiotic recombination process is essential for the exchange of genetic materials among parasite strains and generation of genetic diversity in the parasite population. The biology and molecular biology of gametocytes have been the topic of several recent reviews [7–13]. Here, we attempt to review the biology of sexual development in malaria parasites with a focus on recent understanding of the molecular mechanism of gametocytogenesis and discuss future efforts towards a deeper understanding in this field, which is deemed critical for designing innovative tools for the disruption of parasite transmission.

Gametocyte morphogenesis and metabolism

Whereas this review focuses on the human malaria parasite *P. falciparum*, some contrasting features in gametocyte biology of two model systems (*P. falciparum* and the rodent parasite *P. berghei*) are compared in Table 1. In most *Plasmodium* species, gametocytes are round and mature in a few hours longer than the asexual cycle, whereas gametocytes of *P. falciparum* are crescent shaped and reach full morphological maturity in 8 – 12 days [14–16]. A sexually committed merozoite undergoes a sequence of morphological changes to become a mature gametocyte. Gametocyte maturation in *P. falciparum* has been classified

into five distinctive morphological stages (I–V) [17]. Whereas with Giemsa staining stage I gametocytes resemble young asexual trophozoites, stage II–V is easily distinguished in a blood smear. A major morphological marker of developing gametocytes is the emergence of a pellicular complex consisting of an inner pellicular membrane vacuole and microtubules underneath the gametocyte plasma membrane in late stage I, which creates the crescent shape [18,19]. Sex dimorphism is apparent in stage VI–V gametocytes. Females are characterized by bluish stain with Giemsa, a relatively small nucleus and concentrated pigment, whereas males are stained pink with a larger nucleus, reticular chromatin distribution and more diffuse pigment. Mature female gametocytes contain extensive endoplasmic reticulum and numerous ribosomes, and are prepared for rapid, subsequent protein synthesis [16,18,20]. In males, the presence of kinetochores of the chromosomes and microtubule organizing center is consistent with the requirement for rapid cell division and extensive organelle assembly during gametogenesis. Osmiophilic bodies are found in the cytoplasm of both male and female gametocytes, but the number is much higher in macrogametocytes [18]. Recently, genetic analysis of a resident protein of the osmiophilic bodies suggests that they are important for the emergence of female gametes from red blood cells [21,22]. It is noteworthy that mitochondrion undergoes drastic morphological changes during gametocytogenesis. Both male and female gametocytes contain only one each of mitochondrion and apicoplast [23]. Fluorescence microscopy reveals that mitochondrion becomes a branching structure clustered around the small apicoplast from stage II throughout the gametocyte development. This structure explains why multiple mitochondria were seen in earlier electron micrographs of gametocytes [24], and suggests the existence of metabolic cooperation (e.g., synthesis of heme) between the two organelles [25].

Dramatic morphological changes during gametocytogenesis are accompanied by major physiological and biochemical changes. Like asexual stages, young gametocytes digest hemoglobin to provide amino acids for protein synthesis. In stage III–IV gametocytes, hemoglobin digestion ceases [26]. Earlier studies detected elevated, between haploid and diploid level, DNA contents in *P. falciparum* and the rodent malaria parasite *Plasmodium berghei* gametocytes, suggesting of DNA synthesis during gametocyte formation [18,27]. These earlier observations might be artifacts since gametocytes in *P. berghei* do not take up tritiated hypoxanthine, indicating the absence of DNA synthesis [28]. The triple genome replication in microgametocyte for the production of eight microgametes occurs only after activation in the mosquito midgut and just before exflagellation, implying that mature gametocytes are arrested in the G_0 phase of the cell cycle [29]. Synthesis of RNA can last up to the sixth day of gametocyte development and terminates in the mature gametocytes [9].

Genome-wide gene expression and protein synthesis in gametocytes

Although the sexual forms of *P. falciparum* was first seen by human eyes over a century ago, progress in understanding the biology of gametocytes has been slow. It is the advent of the "-omics" era that has revolutionized research on malaria sexual development [30,31]. Global analytic technologies such as microarray and proteomics and functional genomics tools have enabled a much deeper understanding of the cell biology of malaria parasites including gametocyte stages [32–35]. Following an initial microarray analysis of gene expression in mature *P. falciparum* gametocytes [32], Young et al. conducted a more detailed

transcriptome analysis during the ~ two-week period of gametocytogenesis and detected expression of ~3410 genes, among which a cluster of 246 genes exhibits highly correlated expression patterns specific for gametocyte development [35]. In *P. berghei*, analysis of the transcriptomes of immature (24 h) and mature gametocytes (30 h) identified up-regulation of 977 genes [31], of which 504 have orthologues in *P. falciparum*. Whole cell-based mass spectrometry analyses of *P. falciparum* mature gametocytes detected ~1000 proteins [33,34], and ~1/3 of which are unique for gametocytes. Similarly, a total of 733 proteins are detected in *P. berghei* gametocytes [31]. Proteins that function in ribosomes, translation, cell cycle/DNA processing and energy metabolism are highly represented in the gametocyte proteome. Silvestrini et al. have overcome the difficulty in purifying early *P. falciparum* gametocytes and determined a proteome of 1427 proteins in early stage gametocytes, of which 251 proteins are enriched [36]. Proteins named PfGEXPs (*P. falciparum* gametocyte-exported proteins) that are putatively exported and involved in erythrocyte remodeling are the most overrepresented proteins, suggesting an important requirement for protein export during early gametocytogenesis. Interestingly, nearly 40% of the PfGEXPs belong to PHISTs (*Plasmodium* helical interspersed subtelomeric family) [37], a gene family with 72 paralogues in *P. falciparum.* Sex dimorphism of *Plasmodium* gametocytes is also reflected in sex-specific gene expression. Using transgenic lines expressing green fluorescent protein (GFP), Khan et al. separated male and female *P. berghei* gametocytes by flow cytometry [38]. A total of 650 and 541 proteins were detected in male and female gametocytes, respectively. Of the 305 and 170 proteins specifically produced in gametocytes, only 69 proteins are shared by both sexes, highlighting the independent roles of the two sexes. Female gametocytes contain significant amounts of mitochondrial and ribosomal proteins, which are prepared for active protein synthesis at the onset of gametogenesis [38]. In male gametocytes, proteins associated with axonemes and flagella and those involved in DNA replication are enriched, since they will undergo three rounds of DNA replication following activation, producing eight thin, highly motile, flagellum-like gametes. These transcriptome and proteome studies have provided the essential information for future functional analysis of gametocyte genes.

Gametocytogenesis: sensing the triggers and enhancing gametocytogenesis

While the gametocyte formation trait is genetically determined, it also shows tremendous plasticity and can be influenced by many environmental factors [39]. Although gametocytogenesis can happen early before the appearance of the disease symptoms [40], it is often considered as a stress response of the parasite to deteriorating conditions in the infected blood. In infected hosts, asexual multiplication of the parasite leads to “stress” environments presumably resulted from the released malarial toxin, hemozoin, induced host immunity, and hematological response from erythrocyte destruction. These events often precede peak gametocytemia in patients. Host immunization is shown to have a stimulatory effect on gametocytogenesis in rodent malaria models [41,42]. Similar stimulatory effect is found in *P. falciparum* culture in the presence of lymphocytes and immune serum from malaria patients, and anti-parasite antibodies [43,44]. Likewise, hematological disruption like anemia, lysis of parasitized red blood cells, and increased production of erythropoietin

and reticulocytes are associated with increased gametocyte production [45–49]. Similarly, dilution of *P. falciparum* culture to reduce hematocrit (mimicking anemic conditions) also enhances gametocytogenesis [50]. Beside host factors, parasite-derived (autocrine) cues are found to influence gametocyte conversion. Spent media from *P. falciparum* culture contain soluble factors that appear to stimulate gametocytogenesis [51], which perhaps has motivated the incorporation of conditioned media for synchronous production of *P. falciparum* gametocyte [52]. However, experiments demonstrating the role of conditioned media in enhancing gametocytogenesis are difficult to reproduce and the nature of these factors is unknown. Among antimalarial drugs, chloroquine and antifolates are shown to promote gametocyte production [50,53–56].

How does the parasite sense and respond to the environmental stimuli and commit to sexual development? The malaria parasites possess a large suite of genes encoding components of conserved signal transduction pathways and multiple lines of evidence indicate that these pathways operate during parasite development [57–59]. In earlier studies, pharmacological agents interacting with the signaling pathway such as phorbol diesters, cAMP and cholera toxin affect the sexual conversion rates [60–62], suggesting the involvement of cAMP-dependent and phorbol-ester-inducing pathways in gametocytogenesis. For the cyclic nucleotide signaling pathway, the *Plasmodium* genomes encode two adenylyl cyclases (ACs), two guanylyl cyclases (GCs), four cyclic nucleotide-specific phosphodiesterases (PDEs)(α, β, γ, and δ), and the effector kinases – two cAMP-dependent protein kinases (PKAs) and one cGMP-dependent protein kinase (PKG). Putative G-protein coupled receptors and serpentine receptor-like proteins are found in *Plasmodium* genomes, but their roles in signaling pathways are not determined [63,64]. Cholera toxin treatment, which catalyzes the ADP-ribosylation of G proteins, increases *P. falciparum* gametocyte conversion, implying the presence of trimeric G-proteins in the parasite [65]. However, the *Plasmodium* genomes do not have direct homologues of the mammalian G proteins [66]. The second messengers cAMP and cGMP are synthesized by the ACs and GCs, respectively, and degraded by the four PDEs [67,68]. The involvement of cAMP signaling pathway in the induction of gametocytogenesis has been implied from studies showing that treatments of parasite culture with cAMP, cAMP agonists or PDE inhibitors such as caffeine and 8-Br-cAMP result in increased gametocyte formation [60,61,69]. Further, AC activity and cAMP levels have been correlated with the parasite's ability to produce gametocytes [70], but the significance of ACs for gametocytogenesis remains to be established genetically. In *P. berghei*, ACβ is an essential gene for asexual development, whereas ACα is important for exocytosis in sporozoites and infection of hepatocytes [71]. On the other hand, cGMP does not seem to affect gametocytogenesis, but is essential for subsequent stages. *PfGC*α gene is transcribed in both sexual and asexual blood stage parasites [35] and is essential for asexual blood stages. In contrast, *GCβ* is transcribed at relatively high levels in mature gametocytes, and it can be disrupted in both *P. falciparum* and *P. berghei* without obvious effect on gametocytogenesis and gametogenesis in spite of the resultant reduction of the cGMP level [72,73]. In *P. berghei*, gliding activity of ookinete is sensitive to PKG inhibitor and is impaired by the deletion of *GCβ* [74]. PfPDEα and PfPDEβ are expressed in asexual and sexual stages, whereas PfPDEγ and PfPDEδ are highly expressed in gametocytes. PfPDEα is a cGMP-specific PDE and its genetic ablation does not result in obvious defect in asexual

development [75,76]. Similarly, deletion of *PfPDEγ* or *PfPDEδ* has no discernable effect on parasite development through gametocyte stage [73,74]. Disruption of *PfPDEδ* dramatically reduces ability for gametogenesis [73], whereas disruption of *PbPDEδ* results in the rounding up of the ookinetes, which are defective in motility and unable to cross the midgut epithelium [74]. The major downstream effector kinases for cAMP and cGMP are PKA and PKG, respectively. Whereas PfPKA (with two genes encoding the catalytic and regulatory subunits, respectively) is implicated in multiple cellular functions, it role in gametocytogenesis has not been evaluated [64]. *PfPKG* is expressed in both asexual and sexual blood stages, and is refractory to deletion [77]. PfPKG activity is found essential for blood stage schizogony and gametogenesis [77,78]. In addition, conditional knockout of the *PbPKG* in sporozoites resulted in stalled development of the liver schizonts, indicating essential function of PKG in liver stage [79]. Furthermore, calcium-dependent signaling pathway controls many critical steps in the life cycle of malaria parasite [80], including xanthurenic acid-induced exflagellation [81], ookinete gliding activity [82], and sporozoite motility, cell traversal and transformation [83–85], but its role in gametocytogenesis is not known. Despite the evidence suggesting the involvement of cyclic nucleotide signaling pathways (cGMP or cAMP) in the induction of gametocytogenesis, their significance needs to be further verified. A recent test of a variety of these gametocytogenesis-enhancing conditions did not show similar effects as claimed in earlier studies [86]. Therefore, there is a need to reevaluate some of the earlier conditions for enhancing gametocyte conversion rates, and to determine the signaling pathways by which the parasites sense the triggers for sexual development. A standardized gametocytogenesis procedure such as the one described by Fivelman et al. should be helpful for comparison between experiments [52].

Commitment to gametocytogenesis

Plasmodium gametocytes form mostly during each of the asexual erythrocytic cycles, whereas in some species such as the human parasite *P. vivax* and the rodent parasite *Plasmodium yoelii* gametocytes can also be derived from the merozoites emerging from the pre-erythrocytic schizonts [87,88]. In human volunteers experimentally infected with *P. falciparum* by mosquito bites, *pfs16* mRNA was detected within 48 h after detection of 18S rRNA, suggesting that commitment to gametocytogenesis can also occur early during *P. falciparum* infection [40]. Earlier gametocytogenesis in *P. vivax* before the manifestation of clinical symptoms certainly benefits parasite transmission, but makes malaria control difficult. During its development in an erythrocyte, a parasite must make two decisions: to follow either the asexual or the sexual pathway, and in the latter case to become either a male or female gametocyte. It is clear now that all merozoites from a single schizont are committed to either the asexual or the sexual pathway [50]. Moreover, gametocytes produced from all merozoites of a single committed schizont are either all females or males, suggesting that sex is also determined during maturation of a schizont [89,90]. It is well known that the capability of producing gametocytes is a genetically inherited characteristic of the malaria parasites [20,91], and parasite isolates vary considerably in gametocyte conversion rates [92]. In addition, spontaneous loss of the ability to produce gametocytes during continuous blood passage in experimental animals or blood culture of parasites without selection pressure for mosquito transmission, is often associated with accumulation

of chromosomal aberrations [93–95]. Multiple *P. falciparum* lines that are defective in gametocytogenesis have been used to find the genetic determinants of sexual development.

During in vitro culture adaption, field isolates rapidly undergo deletion in a subtelomeric portion of chromosome 9, which is associated with reduced cytoadherance and gametocyte production [93,96]. This region encompasses 15 annotated genes including *pfgig* [97]. *Pfgig* mRNA is most abundant in schizonts, consistent with possible commitment at this stage. Genetic disruption of *pfgig* led to reduced gametocyte production [97], suggesting that this gene may play a role in regulating the commitment step. Two other gametocyte-nonproducing lines derived from 3D7 have been used to catch the early events of gametocytogenesis through comparison of genome-wide gene expression patterns [98,99]. Similar to the transcriptome analysis on enriched early-stage gametocytes [35], these studies found relatively small numbers of genes as sexual stage specific, which are quite distinctive from gene expression in late stage gametocytes. In one study, 117 genes have been identified to be up-regulated in induced 3D7 as compared with the gametocyteless, isogenic clone [99]. These studies have identified several genes including *Pfpeg3/mdv1*, *Pfpeg4*, *Pfg14.744*, *Pfg14.745*, *Pfg14.748*, *Pfg14.752*, and *Pfg14.763*, among which *Pfpeg3/mdv1* and *Pfpeg4* share similar expression profiles with the early gametocyte markers *pfs16* and *pfg27* [98,99]. It is worth noting that the genes located on the subtelomere of chromosome 14 are members of the *PHIST* gene family, whereas *Pfpeg3/mdv1* and *Pfpeg4* are members of the early-transcribed membrane protein (*etramp*)/*P. falciparum* small exported protein (*pfsep*) family. Follow-up studies showed that Pfg14.748 is trafficked to the periphery of the parasite and parasitophorous vacuole membrane (PVM), while Pfg14.744 and Pfpeg3/mdv1 are exported to the red cell cytosol [98]. Interestingly, the determinant in the male gametocyte defect in Dd2 clone derived from W2 was mapped to an 800 kb region on chromosome 12 [95,100], and *Pfpeg3/mdv1* was eventually incriminated as responsible for this defect through genetic disruption analysis [101]. However, given their possible functions in host cell remodeling, it is unlikely that these early structural proteins are the key regulators of gametocytogenesis.

Although several early gametocyte markers are available and we know that the time of the sexual commitment occurs one cycle before the formation of gametocytes, we still do not know the exact time of sexual commitment and the molecular basis for making this transition. One interesting observation might hold the answers to these questions. In transgenic parasites expressing GFP reporter driven by the 5′ flanking regions of a few early gametocyte genes such as *Pfg14.748*, *pfs16* and *pfg27*, GFP expression is found not only in early gametocytes, but also in a small fraction of schizonts [7,98,102]. Similar findings were observed in a nucleosome assembly protein SET under its gametocyte-specific promoter in both *P. falciparum* and the rodent parasite *P. berghei* [103]. In addition, the protein products of some early gametocyte genes such as pfs16 and pfpeg3/mdv1 have been found in a small fraction of schizonts using immunofluorescence [102,104]. Altogether, these findings imply that these schizonts expressing early gametocyte genes could be those that are committed to gametocyte development. Further investigation into these schizonts may eventually identify the master switch for sexual differentiation.

Sex determination

A comprehensive proteomic study of the *P. berghei* female and male gametocytes has highlighted the dramatic sexual dimorphism [38]. How a single haploid malaria parasite produces both female and male gametocytes is a mystery. The gametocyte sex ratio is typically female-biased in *Plasmodium*. Each female gametocyte only produces a single gamete and each male gametocyte can give rise to up to eight male gametes. Therefore, a female-biased sex ratio would help maximize fertilization in the mosquito midgut and eventually encourage transmission [9]. Yet, gametocyte sex ratio is variable during an infection, between individual patients carrying gametocytes, between clones, and between regions [105–111]. Using the chicken malaria model *Plasmodium gallinaceum*, Paul et al. found that anemia and induction of erythropoesis are associated with increases in the proportion of male gametocytes and the hormone erythropoietin was implicated in the increased allocation of male gametocytes [112]. However, the significance of erythropoietin in sex allocation needs further evaluations in other malaria parasites. Sex allocation theory has been applied to explain the female-biased ratio and adaptive changes observed in malaria parasites [107,113]. This theory predicts that the sex ratio reflects the inbreeding rate. Female-biased sex ratio is expected when inbreeding occurs in a monoclonal population, thus reducing competition for mates between related males and maximizing the number of females available to be fertilized [107]. In contrast, when outbreeding occurs in a population composed of several genotypes, the less female-biased sex ratio will be favored by natural selection. In their elegant experiments with defined strains of the rodent parasite *Plasmodium chabaudi*, Reece et al. have demonstrated that key assumptions of the sex allocation theory apply equally well to this protozoan parasite [114]. Even more surprisingly, the parasites seem to be able to use kin discrimination to evaluate the genetic diversity of their infections and adjust their sex allocation accordingly. Despite these findings from an evolutionary point of view, the molecular mechanism governing sex differentiation remains poorly understood.

How are sexual commitment and sex determination controlled? We now know that both decisions are made before the nuclear division of the schizont [89,90]. Lacking a sex chromosome suggests that the sex determination is regulated by gene expression [91]. There is evidence suggesting that the sexual commitment and sex determination pathways are distinct. For example, certain gametocyte-inducing factors such as chemotherapy affect the gametocyte conversion rates but not sex ratios [9,115]. Whereas the clues for the parasite to choose the male or female pathway remain elusive, Khan et al. have made the first step in deciphering the gender-specific proteomes of *P. berghei* gametocytes [38]. Of the 305 and 170 gametocyte-specific proteins detected in male and female gametocytes, only 69 proteins are shared between the two sexes. To date, only a small selected subset of the sex-specific genes has been validated. In addition to a number of sex-specific structural proteins (e.g., α-tubulin II), the gametocyte proteomes also include many protein kinases and phosphatases, of which a large proportion are gametocyte-specific. Promoter tagging of 10 selected genes in transgenic parasites confirmed the designation of stage and gender specificity of several proteins, including two female-specific LCCL domain containing proteins, one female-specific NIMA-related kinase NEK4, and two male-specific hypothetical proteins. Reporter

gene expression under the sex-specific promoters also confirmed the male specific expression of *α-tubulin II* and *pfsMR5* promoters [102]. To date, several sex-specific proteins have been characterized, α-tubulin II was described as a male specific protein [116], but this protein has promiscuous expression during early gametocytogenesis as detected by antibodies [117]. Our study using GFP-tagged α-tubulin II confirmed this observation (Miao J., unpublished). Pfs47 and Pfg377 are female specific: Pfs47 is dispensable for gametocytogenesis and female fertility [118], whereas the pfg377 is needed for the formation of osmiophilic bodies, which are required for the emergence of female gametes from red blood cells [21]. It is noteworthy that male gametocytes contain fewer osmiophilic bodies, but the pfg377 deletion does not affect exflagellation of male gametocytes. Altogether, there is strong evidence indicating the presence of sex-specific transcription programs in male and female gametocytes, but what the master regulator(s) is and how it controls sex-specific gene expression are not determined. While studying gene regulation during gametocytogenesis, Miao et al. found that the Puf (Pumilio and fem-3 binding factor) family translational regulator PfPuf2 plays an important role in regulating gametocytogenesis [119]. Disruption of *PfPuf2* resulted in increased male/female sex ratio, whereas *PfPuf2* overexpression in both 3D7 wild type parasites and a PfPuf2 knockout clone repressed male gametocyte formation, resulting in even lower sex ratios. Yet, given PfPuf2 is expressed in both sexes, it is unlikely to be the most upstream regulator of the sex determination process.

Gene expression in gametocytogenesis

There are about 200–300 gametocyte-specific transcripts and more than 900 proteins in gametocytes [31–35]. Table 2 lists gametocyte genes for which functional studies have been performed. Gene expression during gametocytogenesis is dynamic [12], but it does not seem to follow the cascade-like pattern of asexual stages [120]. A number of genes expressed at the onset of gametocytogenesis including *pfs16*, *pf14.744*, *pf14.748*, *pfpeg3/mdv1* and *pfpeg4* may control the cellular transformation required for early sexual differentiation [35,98,99]. Among them, Pfs16 is the earliest marker of gametocytogenesis, and its expression is detected at 24 h post-merozoite invasion and lasts throughout gametocyte development [121]. Noticeably, all these five proteins mentioned above are produced at the onset of sexual differentiation and are localized in the PVM [98,99,104]. Disruption of *Pfs16* resulted in reduced gametocyte production and transmissibility to mosquitoes [122]. *Pfmdv1*-disrupted parasite lines show developmental arrest in early gametocytes, resulting in a significant reduction in mature gametocytes, particularly functional male gametocytes [101]. Intriguingly and possibly reflective of evolutionary divergence between *Plasmodium* species, deletion of the *mdv1/peg3* gene in *P. berghei* does not affect the production of gametocytes, but reduces the capacity of female gametocytes to emerge and form gametes and zygotes [123,124]. This result is consistent with the localization of MDV1/Peg3 protein in osmophilic bodies, which are 4- to 5-fold greater in number in female than male gametocytes. *Pfg27* is another early gametocyte specific gene and is transcribed at 30 h post invasion [125]. The product of *Pfg27* is an abundant phosphoprotein in the cytoplasm, which can specifically bind single-stranded RNA and interact with recombinant Src homology-3 domain *in vitro* [125]. Interestingly, *Pfg27* is only found in *P. falciparum* without

orthologues in other malaria parasites except *P. reichenowi,* suggesting that it might be responsible for the extended period of gametocyte development process of *P. falciparum*. The requirement of Pfg27 for gametocytogenesis has not been conclusively determined, since an earlier functional analysis showed that deletion of *pfg27* completely abolishes early gametocytogenesis [126], whereas subsequent work shows that *pfg27* is dispensable for gametocyte formation with only a fraction of *pfg27*-defective gametocytes showing subcellular abnormalities [127]. However, none of these disruptants has been genetically complemented, thus weakening the functional claims from the gene disruption studies. Given that reduced gametocyte formation often occurs during prolonged culture, phenotypic analyses, especially those showing reduced gametocytogenesis, need to be strengthened with complementation [128]. So far, none of these early gametocyte markers appears to be the major regulator of gametocytogenesis.

P. falciparum genome encodes a cysteine motif gene family with 10 members (*pf12*, *pf12p*, *pf36*, *pf36p*, *pf41*, *pf38*, *pfs47, pfs48/45*, *pfMR5*, and *Pfs230*). Interestingly, nine of the genes are arranged on chromosomes in pairs or arrays, probably originated from gene duplication events [129,130]. Pfs230 and Psf48/45 have been studied in greater detail because of their potential as transmission-blocking vaccines (TBVs) [131–134]. Pfs230 has 14 copies of the Cys motif and encodes a protein of ~360 kDa. It is associated with the parasite plasma membrane and forms a stable complex with the GPI-anchored Pfs48/45 [135]. Upon the emergence of gametes, Pfs230 is processed to membrane-bound proteins of ~307 and 300 kDa with the release of ~47 and 35 kDa polypeptides [136]. Disruption of *pfs230* does not affect the surface localization of other known sexual stage-specific antigens including pfs48/45, but the disruptant males are unable to interact with erythrocytes to form exflagellation centers, thus leading to reduced fertilization and oocyst formation [137]. Pfs48/45 contains three Cys motifs and is expressed on the surface of gametocytes starting from stage II and gametes. Disruption of this gene in both *P. falciparum* and *P. berghei* results in reduced formation of zygotes [138]. I addition, disruption of *pfs48/45* prevents *pfs230* from being held on the surface of the gamete. Whereas macrogametes of *pfs48/45* disruptant are normal, the microgametes are defective and unable to penetrate the female gametes. *Pfs47*, a paralogue of *pfs48/45*, is female-specific and located on the surface of female gametes following emergence from red blood cells. However, disruption of this gene does not cause any defect in female fertility [118]. Recently, a comprehensive study of the 6-Cys protein family members in *P. berghei* verified the significance of these three 6-Cys proteins in gamete fertilization [139]. It has been demonstrated that pb230 and pb48/45 are male fertility factors involved in recognition of and attachment to females, whereas pb47 is a female fertility factor involved in recognition and adherence by male gametes. Meanwhile, this study further emphasized potential differences between the human and rodent malaria parasites in gene functions. Although *pb230* is important in male gamete fertility, deletion of this gene did not affect the interaction of male gametes with red blood cells. Further, *pb47* was found essential for female fertility [139].

Members of the *PfCCp* family (or the *PbLAP* family for the *P. berghei* proteins) is characterized with the presence of a *Limulus* coagulation factor C (LCCL)-like adhesive domain [140,141], and this protein family is conserved in Apicomplexa [142]. PfCCp proteins are abundantly expressed in gametocytes starting from stage II, and PfCCp1,

PfCCp2, and PfCCp3 are associated with the plasma membrane of mature gametocytes. These adhesion proteins appear to form multiprotein complexes, and the lack of one PfCCp protein leads to the loss of other family members [143,144]. Genetic disruption of *pfCCp2* and *pfCCp3* results in parasites capable of forming oocyst sporozoites but blocked in the salivary gland transition [140], whereas disruption of *pfCCp4* does not affect parasite development in the mosquitoes [145]. In *P. berghei*, genetic disruptions of *PbLAP1*, *2*, *4*, 5 and *6* also show that they are critical for oocyst maturation and sporozoite formation [146–148].

The other multi-gene families that have received special attention include *var*, *stevor* and *rif,* since their protein products (PfEMP1, STEVOR and RIFIN, respectively) are potential candidates mediating cytoadherence of gametocytes. To avoid immune clearance, *P. falciparum* has evolved the mechanisms to sequester parts of asexual and sexual forms to specific tissues or organs of the host [149]. Therefore, microscopic examination of peripheral blood from typical *P. falciparum* patients only finds ring stage and mature stage V gametocytes. In contrast to the relatively short period of sequestration of asexual stages (trophozoites and schizonts), immature *P. falciparum* gametocytes are sequestered in host tissues for more than seven days. Although this has not been investigated in detail, immature gametocytes were found in bone marrow and spleen [150]. Several host receptors including the glycoprotein CD36 and intercellular adhesion molecule-1 (ICAM-1) are implicated in cytoadhesion of gametocytes [151–154], but parasite ligands mediating these interactions are yet to be determined. PfEMP1 is a family of clonally variant proteins encoded by ~60 *var* genes [155]. PfEMP1 is localized to small protrusions on the surface of infected erythrocytes called “knobs”, and has been found to bind to many host receptors. A number of *var* genes are found expressed in gametocytes. Regardless of the *var* expression patterns in asexual stages, a transcriptional program switch occurs in gametocytes with the preferential expression of a subset of type C *var* genes [156]. Yet, PfEMP1 is only found on the surface of early gametocytes (stage I and IIa) and mediates cytoadherence to C32 melanoma cells via CD36 [151,157,158]. This result is consistent with the observation of knob structure on the surface of early but not late gametocytes. Although *var* expression continues during gametocytes maturation, PfEMP1 is localized inside the parasites and does not seem to play a role in mediating cytoadherence of stage IIb–IV gametocytes. Instead, stage III–IV gametocytes might bind to the host cells through candidate host receptors including ICAM-1, CD49c, CD166, and CD164 with lower avidity adhesion [154]. So far, the gametocyte ligands for these interactions have not been identified. STEVOR (subtelomeric variable open reading frame) proteins are encoded by a multigene family with 30–40 members depending on the parasite strains. In asexual erythrocytic stages, *stevor* transcripts reach peak levels in mid trophozoites ~28 h after invasion, and the proteins are localized in Maurer’s clefts [159]. STEVOR variants are also expressed in early gametocytes but are transported to the infected erythrocyte membrane in stage III gametocytes via a trafficking pathway independent of Maurer’s clefts, implying a possible role in gametocyte cytoadherence [156,160]. However, compared with the *var* expression profiles, the *stevor* transcription pattern does not change between gametocytes and trophozoites [156]. Finally, another protein family that might be involved in cytoadhesion is RIFIN, which are encoded by the largest gene family *rif* (repetitive interspersed) with ~200 genes localized

predominantly at the subtelomeric regions. RIFINs are found to be expressed in both asexual stages and gametocytes. Based on the presence or absence of 25 amino acids in the semi-conserved domain, RIFINs are divided into two types [161]. The A type RIFINs are exported via the Maurer's clefts to the erythrocyte, whereas the B type RIFINs seem to accumulate mainly inside the parasite [162,163]. During gametocytogenesis, *rif* gene expression displays a stage-specific pattern with one B-type *rif* gene, *PF13_0006*, being up-regulated in mature gametocytes [164]. Its high-level expression in mature gametocytes makes it unlikely to be responsible for sequestration of immature gametocytes. Taken together, there is no solid evidence of involvement of members of these three gene families in the sequestration of stage III–IV gametocytes.

From the available evidence, none of these multi-gene family proteins has been proved to be the major adhesive molecules mediating sequestration of gametocytes. As can be seen, elucidation of the mechanisms underlying the adhesive interactions between gametocytes and host tissue receptors encounters some technical difficulties. Early stage gametocytes cannot be easily distinguished or isolated from asexual stage parasites. The development of transgenic parasites expressing markers may overcome this problem. Further, for in vitro adhesion studies, current quantitation methods are not sensitive enough to detect and quantify the low levels of gametocytes that adhere to the endothelial cells. For potential "adhesins" expressed on stage IIb–V gametocyte surface, Sutherland postulated some searching criteria based on gene expression profiles and features of encoded proteins and identified several putative gametocyte surface antigen genes, which remain to be evaluated [165]. Similarly, detailed proteomic studies during gametocytogenesis might provide further clues to this question.

Transcriptional regulation during gametocytogenesis

Gene regulation can be achieved at different levels, from transcription, translation, protein modification, to protein degradation. Genome-wide transcriptional profiling of the intraerythrocytic development cycle (IDC) of two human malaria parasites shows a highly regulated transcription program with genes expressed in a "cascade-like" pattern [120,166]. Thus, transcriptional regulation has been postulated to be a major way of controlling gene expression in the malaria parasites. Compared with free living eukaryotes, *Plasmodium* genomes are relatively deficient in transcription-associated proteins (TAPs). The most recent *in silico* and biological survey has identified 202 TAPs in *P. falciparum* genome, which are divided into general, chromatin-related, and specific transcription factors [167]. At least 52 of these TAPs are expressed in stage V gametocytes and 13 are specific to this stage. In *Plasmodium*, CCCH-type zinc-finger proteins are the most abundant TAPs [168]. Overall, the general transcription factors associated with RNA polymerase II are well conserved [169], and chromatin-related factors involved in epigenetic regulation of gene expression are also highly represented [170]. Whether gametocyte development involves epigenetic mechanisms remains to be investigated. A SET protein involved in chromatin dynamics is expressed in both asexual stages and male gametocytes of *Plasmodium*, and this gene is under the control of stage-specific promoters [103]. The discovery of a large family of transcription factors containing the plant Apetela2/ethylene response factor (AP2/ERF) domain in the apicomplexan parasites is highly significant, since it has filled the deficiency

of specific transcription factors in this group of parasites [171,172]. There are 27 members in the *P. falciparum* AP2 gene family. Recently, Campbell et al. have comprehensively assessed the *in vitro* DNA-binding specificities of the PfAP2 proteins and predicted their potential binding sites in the parasite genome [173]. Yet, the functions of most of these AP2-domain genes in *Plasmodium* biology remain to be determined. The AP2 member, PfSIP2, which binds to the SPE2 motifs upstream of the subtelomeric *var* genes, is involved in heterochromatin formation and genome integrity [174]. The AP2-O gene in *P. berghei*, an orthologue of PF11_0442, is found to activates expression of a number of genes in ookinetes by binding to specific six-base motif TAGCTA at the promoters of these target genes [175]. Similarly, the *P. berghei* AP2-Sp (orthologue of Pf14_0633), expressed in late oocyst to salivary gland sporozoite is a specific activator of sporozoite-specific gene expression [176]. ApiAP2 proteins are found to associate with other TAPs such as chromatin-modification enzymes histone deacetylase [177] and GCN5 histone acetyltransferase [178,179], demonstrating the involvement of AP2-domain proteins in a transcription regulatory network. A few AP2 members including *pff1100c*, *pf11_0091*, *pfd0985w*, *pf11_0442*, and *pff0200c* are expressed during the early stages of gametocytogenesis, suggesting possible involvement in regulating gene expression in gametocytes.

Both computational and experimental approaches have been used to locate the *cis*-regulatory elements involved in the transcription of gametocyte-stage specific genes. Computational analysis identified a number of enriched *cis*-regulatory elements associated with a variety of parasite processes including sexual development [180,181]. However, the functions of most putative *cis*-regulatory motifs are yet to be determined. Motif PfM4.1 (WAGACA) is contained within the sequence CAGACAGC present in the promoter of *pgs28,* which is important for the *pgs28* promoter activity in *Plasmodium gallinaceum* [182]. The core sequence AGACA is also present in the promoters of 14 of the 21 *P. falciparum* male-specific gametocyte genes, but only 2 of the 25 *P. falciparum* female-specific gametocyte genes were identified from *P. berghei* male and female gametocytes [38], suggesting that PfM4.1 may play a role specific to male gametocytes. Using transfection and in vitro DNA-binding assays, the *cis*-regulatory elements for a number of gametocyte-specific genes have been identified. The regions in the *pfs16* and *pfs25* promoters that are essential for high transcriptional activity have been defined and a DNA-binding protein, termed PAF-1, binds to the DNA element AAGGAATA within the *pfs25* promoter region [183]. Similarly, a 140 bp region upstream of the *pfg27* transcription start sites is able to drive stage-specific gene expression [184]. To date, how specific transcription factors interact with *cis*-regulatory elements to dictate gametocyte-specific gene expression is yet to be investigated. With the availability of the genomic information and technological advances in parasite transfection and genome-wide analysis, we envisage that the mechanisms of transcriptional regulation in gametocytogenesis will be elucidated in the near future. Focused studies on certain AP2-domain transcription factors expressed in sexual stages will shed light on the functions of these proteins in gametocytogenesis. In addition, integration of researches in the malaria field with those in model organisms for eukaryotic gene expression is needed to make further advancement in the field of gene regulation in malaria parasites [185].

Post-transcriptional regulation during gametocytogenesis

There is growing evidence demonstrating the essential role of post-transcriptional regulation in malaria parasite gene expression. First, transcripts are degraded at different rates during the IDC with the average mRNA half-life significantly increased during the late schizont stage [186], suggesting differential post-transcriptional regulation of mRNA stability. Further, malaria parasite ribosomal RNAs (rRNAs) are characterized by two structurally distinguishable A and S types and are developmentally regulated, which may affects the translation efficiency [187,188]. The A-type rRNA pools dominant in the asexual stage parasites are replaced by S1-type rRNA in gametocytes and S2-type rRNA in mosquito stages [189]. Comparison of the transcriptome and proteome data during the IDC reveals a positive correlation between mRNA and protein abundance, but the abundance of most proteins peaks significantly later than the corresponding transcripts [190–192], indicating that post-transcriptional regulation is at work. In addition, most proteins in the parasite are subjected to post-translational modifications, the significance of which is yet to be explored [192]. Translational regulation is especially important for the “quiescent” mature gametocytes and sporozoites, which await activation and initiation of subsequent developmental transitions. In these stages, “maternal” transcripts needed for subsequent developmental stages are made and stored in translationally repressed forms. It has long been recognized that the major ookinete surface antigen gene *Pfs25* is transcribed in gametocytes, but proteins are predominantly synthesized in gametes and ookinetes [193]. This phenomenon appears to be quite common; a systematic global analysis in the rodent parasites has detected that a large portion of the gametocyte transcriptome is not translated until gamete formation and fertilization in the mosquito [31].

How these transcripts are kept untranslated in gametocytes? Translational repression is generally achieved through the interactions of RNA-binding proteins with specific sequences in the 5′- and 3′-untranslated regions (UTRs) of the target transcripts, which influences the mRNA localization, stability, accessibility to the translation apparatus and translational capacity. To date, only a few RNA-binding proteins with potential roles in translation regulation in gametocytes have been identified. Pfg27, a highly abundant early gametocyte stage proteins that accounts for almost 5–10% of the total cell content [194], forms a novel protein fold and binds RNA without any apparent specificity [125]. Pfg27 forms dimers and oligomers, and interacts with other RNA-binding proteins such as EF1a and RNA helicase 1 [195]. At present, we do not know what the Pfg27 target transcripts are and whether Pfg27 regulates transcript stability or translation. A female-specific RNA helicase, which shares homology to the DDX6 family of DEAD-box RNA helicases, has been identified in the sex-specific proteome of *P. berghei* gametocytes [38]. In eukaryotic cells, these helicases are components of messenger ribonucleoprotein (mRNP) particles (P granules) and involved in the storage and silencing of mRNAs [196]. The *Plasmodium* gene is named *dozi* (development of zygote inhibited), since *P. berghei* zygotes deleted for *dozi* failed to progress through meiosis to develop into ookinetes [197]. PbDOZI is found to form an mRNP complex with multiple target mRNAs including *Pbs25* and *Pbs28*, and plays a role in the storage and translational repression of these mRNAs. Disruption of *pbdozi* resulted the reduction of 370 transcripts including seven of the nine translationally repressed genes found

from global microarray and proteomic studies [31]. Unexpectedly, 92 transcripts were upregulated in the Δ*pbdozi* mutant, but functional differences seem to exist between these gene pools. The down-regulated subset contains a group of genes that are linked with ookinete motility and invasion. The PbDOZI mRNP has been affinity-purified and found to contain 16 major factors, including homologues of components of metazoan P granules such as the Sm-like factor CITH (CAR-I and fly Trailer Hitch), the mRNA 5′ cap-binding protein eIF4E, homologues of BRUNO and Musashi [198]. In addition, this mRNP contains poly(A)-binding protein and Alba (Acetylation Lowers Binding Affinity) domain proteins; the latter has been found to bind RNA in Archaea. Consistent with Δ*pbdozi,* disruption of *pbcith* also resulted in more than two-fold down regulation of 183 mRNAs, of which 117 mRNAs were common between the *pbdozi* and *pbcith* data sets [198]. Therefore, P granule as an ancient mRNP to keep the integrity of stored translationally repressed mRNA is evolutionally conserved in *Plasmodium*. One interesting observation from this study is that deletion of *pbdozi* and *pbcith* does not result in precocious translation of P28 protein in gametocytes, albeit it led to destabilization of *pbs28* mRNA [198], suggesting that other factors are involved in translation repression. It is noteworthy that certain ApiAP2 transcripts expressed in the gametocyte are associated with the translation repression complex DOZI [198]. This observation suggests that gene expression in gametocytes involves an intricate interplay between transcriptional and translational factors.

In *P. falciparum* gametocytes, two specific translational regulators, members of the Puf family RNA-binding protein family, Puf1 and Puf2, are expressed [199,200]. The Puf RNA-binding motif consists of eight tandem imperfect repeats of ~36 amino acids, which form a curved structure [201]. PfPuf proteins displayed conserved binding to the *Drosophila* NRE (Nanos responsive element), the target sequence of Pumilio. Recently, functional studies have shown that PfPuf1 and PfPuf2 may participate in the two pathways of sexual development and sex determination. Whereas deletion of *PfPuf1* leads to dramatic reduction of gametocyte formation (Cui et al., unpublished), disruption of *PfPuf2* promotes the formation of gametocytes and differentiation of male gametocytes [119]. Although deletion of *Puf2* in *P. berghei* produced similar phenotypes in gametocytogenesis [202], the deletion of *PbPuf1* results in no dramatic phenotypic changes [202,203]. Furthermore, consistent with up-regulated expression of Puf2 in sporozoites, deletion of *PbPuf2* impairs liver infection, since *puf2(−)* sporozoites prematurely undergo exo-erythrocytic form-like transformation in the mosquito salivary glands [202,203]. These studies have demonstrated additional roles of Puf2 protein in regulating expression of proteins that are destined for development in hepatocytes.

Global microarray and proteomic comparisons in *P. berghei* have detected nine genes as translationally repressed in gametocytes, including genes encoding the ookinete antigens P25 and P28 [31]. Deletion of important mRNP components *pbdozi* and *pbcith* detected a large proportion of the gametocyte transcriptome as post-transcriptionally regulated [197,198]. There is also clear evidence showing the association of a number of these translational repressed mRNAs with the DOZI mRNPs. To define the underpinning mechanisms of translation repression, analysis of consensus motifs located in the UTRs of these transcripts has identified that 3′ UTRs of the nine translational repressed mRNAs all harbor a 47-base U-rich motif [197]. Detailed analysis of the 5′- and 3′-UTR of a selected

subset of the translationally repressed genes has demonstrated that the U-rich element plays an essential role in mediating translational repression [204]. Interestingly, this U-rich element is found in the 5′ UTR of *pb25* gene and it mediates silencing of the transcript regardless of its position in the 5′ or 3′ UTR. Although translational repression of *pfs25* and *pfs28* has also been documented in *P. falciparum*, no similar U-rich motif has been found in the *P. falciparum* genome, which further highlights inter-specific difference. Similarly, *in silico* analysis found 95 genes with NRE-like sequences [190], but the target mRNAs for the Puf proteins remain to be experimentally identified. Collectively, translational control is an important mechanism regulating gene expression in malaria parasites. Yet, there is still a disconnection between the identified RNA-binding proteins and characterized sequence motifs present in the translationally regulated mRNAs, precluding a mechanistic understanding of this process.

Conclusion & future perspective

With the availability of the genomic information [30], advances in genome-wide analytic tools [32,33] and reverse genetic approaches [205], research on gametocytes has entered an inspiring stage with a markedly increased discovery pace in recent years. Studies of transcription regulation have led to the identification of gametocyte-specific genes and characterization of promoter elements of some sexual stage-specific genes. The discovery of a large population of “maternal” mRNAs deposited and stored as translationally repressed forms in gametocytes has shed new light on the significance of translational regulation in malaria parasites. Yet, though a number of molecules are shown to enhance gametocytogenesis, only a selected set of these genes have been functionally characterized and we still do not know the master switch(s) that governs the transition from asexual multiplication to sexual differentiation. Equally less understood is the control mechanism underpinning the selection of either the male or female-specific pathway [119]. Genetic screen using forward genetics may lead to eventual breakthrough in the identification of these master switches [206,207].

In the study of sexual development of malaria parasites, the rodent parasite *P. berghei* has played a key role because the entire life cycle can be analyzed *in vivo* under laboratory conditions. In addition, *P. berghei* is more amenable for genetic studies. However, *P. berghei* and *P. falciparum* have drastically different gametocyte biology, therefore data from the rodent parasite may not be fully applicable to *P. falciparum* and verification is needed. Nonetheless, recent functional studies using reverse genetics for large-scale knockout of parasite genes have been performed in both *P. berghei* [148] and *P. falciparum* [208]. Since knockout of sexual stage genes is unlikely to affect the asexual erythrocytic cycle, such high-throughput functional studies should be highly feasible for studying sexual development. In the end, the knowledge gleaned from studying the malaria parasite gametocyte biology will need to be translated into potential gametocytocidal drug targets and TBV candidates, which are desperately needed for malaria elimination/eradication. This is especially true for vivax malaria, since earlier gametocytogenesis before the manifestation of clinical symptoms allows transmission of the parasite before treatment.

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Executive summary

- The biology of gametocytes is still in its infancy, but recent microarray and proteome data provided the blueprint for accelerated functional studies in this area.
- The molecular mechanisms of gametocytogenesis and sex determination remain largely unknown, but functional studies at higher throughputs are expected to provide the needed information.
- The expression of multigene family proteins in gametocytes has been explored, but their possible roles in gametocyte sequestration await further analysis.
- Regulation of gene expression in gametocytes is achieved by multiple mechanisms, at both transcription and translation levels.
- A number of sexual stage-specific promoters have been identified and their regulation by specific transcription factors such as the AP2 domain proteins remains to be established.
- Translational regulation is recognized as an essential way of controlling gene expression in gametocytes. A large portion of the “maternal” mRNAs is stored in gametocytes as translationally repressed forms. An ancient mRNP has been identified as an evolutionarily conserved mechanism of mRNA stability.
- Comprehensive understanding of the gametocyte biology is urgently needed for developing new gametocytocidal drugs and TBVs.

Table 1

General comparison of characteristics of gametocytes between human parasite *P. falciparum* and rodent parasite *P. berghei*.

Feature	*P. falciparum*	*P. berghei*
Genome size	23.3 Mb	18–20 Mb
Commitment to gametocytogenesis	From merozoites	From young trophozoite?
Sequestration of gametocytes	Bone marrow and spleen (?)	No evidence
Development period of gametocytes	8–12 days	26–30 hr
Shape of mature gametocytes	Crescent	Round to oval
Gametocyte mitochondria	Tubular cristae	Acristate
Subpellicular complex	Complete with microtubules	Incomplete or lack of a third membrane under the plasma lemma; absent or few microtubules
Nucleus	With a nucleolus in macrogametocytes; Lack a nucleolus in microgametocytes	Lack a nucleolus

Table 2

Functional studies of genes expressed in gametocytes of *P. falciparum* and *P. berghei*.

Gene	Function	Knockout phenotype	Reference
Signaling pathways			
PbACα	Adenylyl cyclase	Defect in exocytosis of sporozoites and infection of hepatocytes	[71]
PbACβ	Adenylyl cyclase	Essential for asexual growth	[71]
PfGCα	Guanyl cyclase	Essential for asexual growth	[74]
PfGCβ	Guanyl cyclase	Affects gliding of ookinetes	[72–74]
PfPDEγ	Cyclic nucleotide-specific PDE	No discernable effect through gametocytogenesis	[73,74]
PfPDEδ	Cyclic nucleotide-specific PDE	No discernable effect through gametocytogenesis; reduced gametogenesis	[76]
PfPKG	cGMP-dependent protein kinase	Essential; also for gametogenesis and liver stage development	[77–79]
Pfgig	Gametocytogenesis regulator	Reduced gametocytogenesis (Pf)	[97]
Early Proteins			
Pfs16	Gametocyte formation?	Reduced gametocyte production, decreased transmissibility to mosquitoes	[122]
Pfpeg3/mdv1	Defect in male gametocyte	Significantly reduced male gametocyte production (Pf); egress of gametes from erythrocytes inhibited (Pb)	[101,123, 124]
Surface/organelle proteins			
Pfs230	Fertilization	Reduced fertilization and oocyst formation (Pf, Pb); mediates red cell binding of male gametes (Pf only)	[137,139]
Pb230p	Paralogue of p230	No obvious effect on fertilization	[139]
Pfs48/45	Fertilization	Reduced zygote formation	[138]
Pbs47	Female specific	No obvious defect (Pf), female gamete fertility reduced (Pb)	[113,139]
Pfg377	Osimiophilic body protein	Defect in formation of osimiophilic bodies in female and egress of female gametes	[21]
LCCL domain family			
PfCCp1/PbLAP2		Normal gamete and exflagellation appearance (Pf); blocked sporozoite formation or transition from oocyst to salivary glands (Pb)	[143,147]
PfCCp2/PbLAP4		Blocked sporozoite formation or transition from oocyst to salivary glands	[140,147]
PfCCp3/PbLAP1		Blocked sporozoite formation or transition from oocyst to salivary glands	[140,146]
PfCCp4/PbLAP6		No inhibition of parasite development in mosquito (Pf); blocked sporozoite formation or transition from oocyst to salivary glands (Pb)	[145,147]
PfFNPA/PbLAP5		Normal gamete and exflagellation (Pf); reduced sporozoite formation, failure of transmission (Pb)	[143,138]
RNA binding proteins			
Pfg27	Gametocyte formation/integrity	Aborted early gametocytogenesis; subcellular abnormality	[126,127]
PbDOZI	RNA stability	Defect in zygote development	[197]
PfPuf1/PbPuf1	Translational regulator	Dramatically diminished gametocyte production (Pf), no phenotype change (Pb)	[202,203]

Gene	Function	Knockout phenotype	Reference
PfPuf2/PbPuf2	Translational regulator	Increased gametocytogenesis and differentiation of male gametocytes; premature exo-erythrocytic form-like transformation in salivary glands (Pb)	[119,202, 203]