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Invited Review

Genomics and epigenetics of sexual commitment in *Plasmodium*

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ABSTRACT

Malaria is the disease caused by the apicomplexan parasites belonging to the genus *Plasmodium*. Expanding our arsenal to include transmission-blocking agents in our fight against malaria is becoming increasingly important. Such an implementation requires detailed understanding of the biology of the *Plasmodium* life cycle stages that are transmissible. *Plasmodium* gametocytes are the only parasite stage that can be transmitted to the mosquito vector and are the product of sexual development in a small percentage of parasites that continually proliferate in host blood. The critical decision made by asexual erythrocytic stages to cease further proliferation and differentiate into gametocytes, as well as the first steps they take into maturity, have long remained unknown. Recent studies have contributed to a breakthrough in our understanding of this branch point in development. In this review, we will discuss the findings that have allowed us to make this major leap forward in our knowledge of sexual commitment in *Plasmodium*. We will further propose a model for the mechanism triggering the switch to sexual development, constructed around the proteins currently known to regulate this process. Further insight into sexual commitment and gametocyte development will help identify targets for the development of transmission-blocking malaria therapies.

1. Introduction

Malaria is a mosquito-borne parasitic disease caused by apicomplexan protozoa of the genus *Plasmodium*. Human malaria is caused by five *Plasmodium* spp. and has a dramatic health and economic impact worldwide. The most lethal of these, *Plasmodium falciparum*, is responsible for approximately 500,000 deaths annually, largely in sub-Saharan Africa, disproportionately affecting the poor, children under 5 years old and pregnant women. Despite the fact that control interventions over the last decades have saved the lives of millions, the disease remains an intractable problem, with approximately 214 million new cases reported in 2015 (World Health Organization, 2015).

The success of malaria parasites could be attributed to their complex life cycle, comprising a range of asexual and sexual developmental stages, as well as their sheer numbers coupled with wide genetic diversity among and within populations (Manske et al., 2012). To date, anti-malarial agents have largely targeted the pathology-causing, asexual, blood stages of the parasites, often ineffectively clearing sexual stages and allowing transmission from asymptomatic hosts (Bousema et al., 2006; Butterworth et al., 2013; Miller et al., 2013; Abdul-Ghani et al., 2015). Furthermore, the obligatory phase of sexual reproduction in the mosquito midgut is not only prerequisite for transmission, but also enables meiotic recombination between genomes, with the consequent production of parasites with re-assorted genotypes. It results in new combinations of polymorphic genes, thus facilitating evolutionary adaptation and the emergence and spread of drug resistance (reviewed in Babiker and Walliker, 1997).

For these reasons, attention of the scientific community is shifting to include development of transmission-blocking agents (reviewed in Alonso et al., 2011). Transmission is initiated by sexual commitment that leads to the production of mature circulating gametocytes and it comprises a series of processes: gametocyte activation, gamete fertilisation, ookinete development and progression through the midgut wall, generation of oocysts (the sites of sporogony) and sporozoite migration to the salivary gland. Infective sporozoites are then delivered into the host during the blood meal taken by the mosquito, eventually colonising the liver and initiating the mammalian phase of the parasite life cycle. Following hepatocyte rupture, parasites enter the circulation, where the potential for replication is vast. Processes relating to transmission represent biological bottlenecks of varying severity, resulting in a substantial reduction in the number of parasites (reviewed in Sinden, 2010; Wu et al., 2015). Thus, targeting these bottleneck

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processes would reduce transmission from symptomatic and asymptomatic hosts, it would prevent sexual recombination within the vector, decreasing the likelihood of drug resistance spreading, and would also provide an efficient strategy in the fight to eliminate the disease as smaller populations can be targeted much more efficiently.

Blocking transmission to the anopheline vector requires disruption of the production or functionality of gametocytes, the gamete precursors and the only sexual stage developing in host blood. Since the initial description of gametocytes in 1880, their morphology and metabolic profiles have been examined and there has been progress in identifying genes involved in their differentiation (reviewed in Alano, 2007; Dixon et al., 2008; Baker, 2010). However, many aspects of their biology remain unknown, including the initiation of their development. Intriguingly, only a small proportion of the parasites per erythrocytic cycle commit to gametocytogenesis (Ponnudurai et al., 1982; Graves et al., 1984). All merozoites from a sexually committed schizont develop into either male or female gametocytes, a developmental decision made at a point prior to the release of the committed merozoites (Silvestrini et al., 2000). Once committed, the *P. falciparum* gametocyte develops over 10–12 days through five distinct morphological stages (I-V), the last of which is taken up by the feeding mosquito (Josling and Llinás, 2015). Gametocytes of other *Plasmodium* spp. develop much more quickly, albeit typically slightly slower than the asexual stage (Galinski et al., 2013).

The mechanism underlying commitment to sexual differentiation has until recently remained elusive, but there is recent evidence that it is an epigenetically regulated process. Epigenetic regulation allows differential gene expression during development, the pattern of which can be inherited via DNA or histone post-translational modifications (PTMs), without alteration of the nucleotide sequence (Jaenisch and Bird, 2003). A *Plasmodium* genome has a typical nucleosomal organization (Fig. 1A) and epigenetic phenomena are indeed mediated by PTMs of histones, replacement of core histones by histone variants (H2A, H2B, H3, H4 by H2A.Z, H2B.Z, H3.3, CenH3, respectively) and chromatin remodelling (Fig. 1B) (Cui and Miao, 2010; Duffy et al., 2012; Voss et al., 2014). While the biological importance of DNA methylation as an epigenetic modification has been widely recognised, only recently has there been evidence provided that it is likely involved in regulating gene expression in *Plasmodium*, through the identification of a functional DNA cytosine methyltransferase and genome-wide mapping of methylated cytosines in *P. falciparum* (Ponts et al., 2013).

Epigenetic regulation is critical in many processes in *Plasmodium* biology, including the demarcation of functional elements in the genome and life cycle progression (reviewed in Merrick and Duraisingh, 2010; Cortés et al., 2012). Control at the epigenetic level also regulates clonally variant gene expression, a process that results in transcriptional heterogeneity among a clonal parasite population. This spontaneous transcriptional switching of certain genes and gene families, also known as bet-hedging, offers a method of adaptation that is alternative to traditional gene regulation. It is key for the success of the parasite as it generates a phenotypically heterogeneous population, thus making the parasite fitter in response to varied environmental challenges, before these even present. Several genes are variantly transcribed and contribute to bet-hedging, including the main high-copy gene families and, most importantly, the *var* gene family (Rovira-Graells et al., 2012). This family encodes for *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), the antigens responsible for sequestration of infected red blood cells (RBCs) in the microvasculature and persistence of infection. An intricate epigenetic strategy, which involves reversible histone modifications, chromatin remodelling and gene repositioning, regulates monoallelic expression of the 60-member *var* gene family and results in antigenic variation of PfEMP1 (Guizetti and Scherf, 2013).

Renewed interest in transmission blocking interventions underlies the importance of deciphering sexual commitment in *Plasmodium*. Recent studies have contributed to a breakthrough in our understanding of this branch point in development; a transcription factor (TF) essential for commitment to gametocytogenesis has been elucidated, which, in turn, provided the basis for unravelling the role epigenetics plays in this process. In this review we will discuss the recent findings that have allowed the development of a model for the mechanism triggering the switch to sexual development. The model is derived from research on *P. falciparum*, the blood stages of which are readily cultured in vitro, and *Plasmodium berghei*, a rodent parasite which provides an in vivo model.

2. AP2-G, the master regulator of gametocytogenesis

The identification of AP2-G as a regulator of sexual differentiation in *Plasmodium* by two independent studies has provided a key piece of the unresolved puzzle of commitment to gametocytogenesis (Kafsack et al., 2014; Sinha et al., 2014). AP2-G is part of the apiAP2 family of DNA binding proteins, which belongs to the larger Apetala2/ethylene response factor protein family found in apicomplexan protists and *Plantae* (Balaji et al., 2005; Campbell et al., 2010). Many of the ApiAP2 members characterised to date regulate major developmental transitions throughout the *Plasmodium* life cycle (Balaji et al., 2005; Iyer et al., 2008; Campbell et al., 2010). *Pfap2-g* stood out as the most variantly transcribed member of the AP2 family in a study analysing transcriptional heterogeneity within several clonal parasite populations. Its detection among differentially transcribed genes linked to early gametocyte development (Rovira-Graells et al., 2012) and its conservation throughout the phylum was consistent with a role in commitment to sexual development.

Forward genetic approaches which identified *ap2-g* as the only mutated gene in a number of gametocyte non-producing (GNP) *P. falciparum* and *P. berghei* lines strengthened this hypothesis (Kafsack et al., 2014; Sinha et al., 2014). Evidently, *ap2-g* inactivation has repeatedly occurred in vitro and after continuous blood passage. However, no loss-of-function mutations in the specific locus have been found in approximately 300 *P. falciparum* field isolates, where generation of gametocytes is required for transmission (Kafsack et al., 2014). The link between AP2-G and the ability to produce gametocytes was corroborated by the generation of deletion mutants (Δpfap2-g and Δpbap2-g), both of which lost their ability to create gametocytes. Genetic complementation of Δpbap2-g and correction of the mutations in the previously identified *P. berghei* GNP lines rescued the phenotype (Sinha et al., 2014). Attempts to generate complementation constructs were unsuccessful for Δpfap2-g, but Kafsack et al. (2014) verified the link between *pfap2-g* deletion and the Δpfap2-g phenotype by creation of a PfAP2-G knockdown line using the destabilisation domain (DD) system (Banaszynski et al., 2006). Accumulation of PfAP2-G, as a result of addition of the shield compound, lead to a 30-fold increase in gametocytogenesis relative to the control compound-treated parasites, restoring the levels of commitment to those observed in the high-gametocyte-producing parental line (Kafsack et al., 2014).

The potential role of AP2-G as a transcriptional regulator of sexual-specific gene expression was substantiated by comparison of the transcriptome of Δpfap2-g and its parent line. Downregulated genes included many expressed in the initial stages of gametocytogenesis and some of the known early markers of sexual commitment (Pfs16, Pfg27/25 and Pfg14.744) (Kafsack et al., 2014). Comparative microarray analysis of *pbap2-g* deficient

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