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Ensuring transmission through dynamic host environments: host-pathogen interactions in *Plasmodium* sexual development

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Abstract

A renewed global commitment to malaria elimination lends urgency to understanding the biology of *Plasmodium* transmission stages. Recent progress towards uncovering the mechanisms underlying *P. falciparum* sexual differentiation and maturation reveals potential targets for transmission-blocking drugs and vaccines. The identification of parasite factors that alter sexual differentiation, including extracellular vesicles and a master transcriptional regulator, suggest that parasites make epigenetically controlled developmental decisions based on environmental cues. New insights into sexual development, especially host cell remodeling and sequestration in the bone marrow, highlight open questions regarding parasite homing to the tissue, transmigration across the vascular endothelium, and maturation in the parenchyma. Novel molecular and translational tools will provide further opportunities to define host-parasite interactions and design effective transmission-blocking therapeutics.

I. Introduction

The parasite *Plasmodium falciparum* causes the most severe form of malaria with around 600,000 deaths annually, mostly young children and pregnant women in sub-Saharan Africa [1]. Resistance to current drug therapies, the absence of a licensed vaccine, and a large asymptomatic reservoir [2] make the development of effective transmission-blocking therapeutics particularly important to any malaria elimination or eradication program. Given the paucity of known transmission stage-specific biomarkers or drug and vaccine targets, a deepened understanding of the biology of transmissible parasite stages, including their interaction with the host, is essential [3].

P. falciparum has a complex life cycle, in which asexual replication and sexual development take place in red blood cells (RBCs) of the human host and sexual reproduction in the mosquito vector. Though the asexual stages are responsible for all morbidity and mortality, successful transmission is dependent on generation of the sexual stages, termed gametocytes. Gametocytes sequester in deep tissues during their development and once

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mature, are released back into circulation where they can be taken up by the mosquito vector. Once in the mosquito midgut, gametocytes emerge from host RBCs, develop into male and female gametes, and undergo fertilization and further development. Recent discoveries of factors in the human host microenvironment contributing to sexual differentiation and development raise exciting new questions about the biological mechanisms of these processes. In this review, we will examine recent advances in host-gametocyte interactions and discuss open questions and new tools to block malaria transmission.

II. Parasite and host factors drive commitment to gametocytogenesis

Blood stage parasites replicate asexually, with a small fraction diverting away from asexual multiplication and towards sexual development in each replication cycle. Although this process may have stochastic elements, it has long been thought that host environmental or secreted parasite factors may push cell fate decision from asexual to sexual differentiation. MicroRNAs from sickle cell erythrocytes have been associated with increased gametocyte numbers [4] while on the parasite side, genes such as *P. falciparum* gametocyte development gene 1 (Pfgdv1) have been implicated in control of sexual differentiation [5]. In addition, conditioned media (i.e. the supernatant of *P. falciparum* cultures) can stimulate sexual conversion *in vitro* [6,7], implying that the process is induced either by presence of parasite-secreted factors and/or by parasite depletion of nutrients present in the culture media. Several recent studies have identified parasite factors that contribute to the sexual conversion switch.

First, two groups showed that extracellular vesicles (EVs) secreted from malaria-infected red blood cells (iRBCs) can increase gametocytogenesis *in vitro* and hypothesized that EVs can transfer parasite and/or host factors that lead to sexual conversion (Figure 1b) [8,9]. Mantel et al observed that EVs purified from asexual parasite-conditioned media can be transferred between iRBCs and stimulate sexual conversion in a dose-dependent manner. Regev-Rudzki and colleagues demonstrated that drug pressure increased EV release and that EVs could transfer DNA between parasite lines, conferring drug resistance while also increasing sexual conversion in recipient cells. Both studies point towards EVs triggering downstream signaling that modulates the rate of commitment to the sexual pathway; however, given that EV characteristics, including size and timing of release, differed between these studies, further work is needed to validate this central finding. Additional next steps include identifying the EV component responsible for inducing sexual conversion and exploring how this signaling feeds into epigenetic control mechanisms that underlie sexual differentiation (discussed below).

Four recent studies have uncovered a molecular framework by which parasites can integrate signals such as those provided by EVs to commit to sexual development (Figure 1a–b). A transcription factor, AP2-G, has been identified as a master transcriptional regulator for gametocytogenesis, as its deletion or disruption abolished sexual conversion in both *P. falciparum* and *Plasmodium berghei*, a murine malaria parasite. AP2-G expression during schizont stages was linked to upregulation of hundreds of genes, many of which have been implicated in gametocyte development. Positive feedback regulation may also play a role in

commitment, as recombinant AP2-G *in vitro* binds two short recognition sequences frequently found upstream of gametocyte-specific genes including *ap2-g* itself [10,11]. Furthermore, two epigenetic regulators, histone deacetylase 2 (PfHda2) and heterochromatin protein 1 (PfHP1), have been shown to repress sexual development, with disruption of these proteins leading to increased gametocytogenesis and decreased asexual replication [12,13]. Conditional depletion of PfHP1 or PfHda2 in asexual parasites led to de-repression of the *ap2-g* locus and upregulation of gametocyte-specific genes [12,13], implying that epigenetic control of AP2-G regulates sexual commitment. Taken together, these findings support the hypothesis that epigenetic regulation allows *P. falciparum* to adjust developmental decisions promoting survival and transmission based on EVs, nutrients, drugs, and other host or environmental factors (illustrated in Figure 1a–b).

In the murine model, Sinha and colleagues identified an additional transcription factor from the AP2 family, AP2-G2, whose disruption completely blocks development of male gametocytes and reduces numbers of mature female gametocytes [10]. Analogous to regulation of AP2-G expression, it is hypothesized that there are environmental factors that can affect sex ratio by altering AP2-G2 expression. Indeed, mathematical and evolutionary models theorizing that plasticity in gametocyte investment enables parasites to maintain fitness in a changing host environment [14,15] highlight questions of how host factors and drugs interplay to alter male and female sexual commitment. Additional clinical and molecular studies are needed to probe mechanisms of male vs. female gametocyte formation and clearance.

III. Parasites exploit host microenvironments: sequestration in bone marrow

Mature asexual stage parasites are known to avoid splenic clearance by cytoadhering to the endothelial lining of capillaries in many tissues. The extensively characterized remodeling mechanisms that mediate asexual sequestration involve specific ligand-receptor interactions, primarily mediated by binding of the parasite antigen PfEMP1 to host endothelial receptors such as ICAM-1 and CD36 [16]. In contrast to asexual stages, only limited binding of early gametocytes to human endothelial cell lines or to CD36 and ICAM-1 has been demonstrated [17,18]. Further evidence for a gametocyte-specific sequestration mechanism includes minimal levels of PfEMP1 on the surface of early gametocyte-iRBCs and downregulation of *var* genes (responsible for PfEMP1 expression) [18]. Both forward and reverse genetic studies support a role for gametocyte-specific proteins, including the PfGEXPs, which are expressed during early sexual differentiation [19], in gametocyte host cell remodeling [20,21]. Previous qualitative analyses established the presence of immature *P. falciparum* gametocytes in the bone marrow and spleen of infected individuals [22,23], but quantitative information about gametocyte sequestration and remodeling has only been obtained recently.

Gametocytes undergo a marked change in morphology during maturation. Beginning as a round form indistinguishable from asexual stages (termed Stage I), they then develop through several transition stages (Stages II/III) to an elongated spindle form (Stage IV) and finally, the curved sausage-like mature form seen in circulation (Stage V) [24,25]. Three

groups have recently characterized the mechanical properties of these distinct morphological stages, using filtration through a bead matrix, micropipette aspiration and ektacytometry to show decreased gametocyte-iRBC deformability during Stage I–IV and restored deformability during or prior to Stage V [26–28]. Interestingly, the dissociation of polymorphic STEVOR proteins from the iRBC membrane correlates with the rigidity switch from Stage IV to V [28], suggesting a possible role for these proteins in gametocyte deformability. Fluorescence microscopy experiments probing the mechanism for gametocyte morphological and mechanical changes revealed that microtubules elongate from Stage I to IV and collapse from Stage IV to Stage V [29]. Further, an actin cytoskeleton present primarily at the gametocyte poles dissociates during the transition to Stage V [30]. Computational modeling based on iRBC deformability predicts that immature gametocytes cannot pass through sinusoidal slits during splenic filtration [26], agreeing with observations of circulating mature gametocytes vs. sequestering immature gametocytes.

Three recent studies, including a case study of a patient with subacute malaria [31], an autopsy study looking at different sequestration sites in children who died from cerebral malaria [32], and a study of bone marrow aspirates of children with nonfatal malarial anemia [33], together demonstrate by histology and transcript abundance that gametocytes are enriched in the bone marrow parenchyma. In the cerebral malaria study, the majority of bone marrow gametocytes in most patients localized at erythroblastic islands, specialized sites of erythropoiesis, and a minority of gametocytes appeared to be developing inside erythroid precursor cells [32]. These data suggest that gametocytes can develop in the bone marrow parenchyma before returning to circulation as deformable Stage V gametocytes, but they leave open which parasites (asexually or sexually committed) migrate to the bone marrow (illustrated in Figure 1c–e). Transcriptional profiling from malaria-infected patient blood demonstrates quantitative presence of a young gametocyte population in circulation, intimating that at least a subset of these stages are homing to the bone marrow [34]. However, presence of asexual stage parasites in the bone marrow parenchyma and formation of gametocytes in erythroid precursor cells *in vitro* [32,35] suggests that the bone marrow may also represent a reservoir for asexual replication and gametocyte formation. Figure 1c–e illustrates a hypothesized flow of events for parasite sequestration in human bone marrow.

Further research in this exciting new area of gametocyte biology should confirm gametocyte enrichment in the bone marrow parenchyma in other patient cohorts, develop phenotypic assays to characterize the binding and transmigration properties of different gametocyte stages, and replicate the bone marrow microenvironment under *in vitro* or *ex vivo* conditions. Severe anemia, dyserythropoiesis and the presence of the parasite byproduct hemozoin have independently been associated with a higher prevalence of mature gametocytes in the bone marrow [33,36], providing a compelling foundation for future studies on the impact of host pathology on gametocyte sequestration. In addition, advances in *in vivo* live imaging of *Plasmodium* infections in rodent and non-human primate models will enable the study of gametocyte sequestration in the context of the host organism (reviewed in [37,38]).

IV. Discussion

Recent advances in our understanding of *P. falciparum* gametocyte biology and development of molecular, imaging, and drug screening tools provide exciting opportunities to better define the parasite's interaction with its host and design transmission-blocking therapeutics (summarized in Figure 2). Evidence for EV-mediated cellular communication and epigenetic/transcriptional machinery controlling commitment provides a rationale for the systematic dissection of the triggers and downstream targets involved in *P. falciparum* sexual differentiation. Similarly, research building on parasite sequestration in the bone marrow should define mechanisms of homing, transmigration across the vascular endothelium, and development in the parenchyma. Furthermore, it is still unknown whether other tissues of the reticulo-endothelial system, such as the spleen and liver, can also support extravascular parasite development. The application of molecular manipulation tools (most recently CRISPR-Cas-mediated genetic disruption [39,40]) to *P. falciparum* will enable targeted investigation of the developmental pathways involved in sexual commitment and sequestration.

Though recent findings open up numerous possible avenues for drug development, a subset of gametocyte proteins, particularly those involved in epigenetic regulation, signal transduction, metabolism, and cytoskeletal remodeling, likely represent the most realistic points of intervention (Figure 2). Identified in a transposon mutagenesis screen and transcriptional analysis during gametocyte formation and development, putative genes involved in these processes may yield novel drug or vaccine targets [5,21,34]. Several possible drug targets have also emerged from metabolomics approaches indicating increased gametocyte sensitivity to TCA-cycle inhibitors [41] and work implicating the perforin-like protein PPLP2 and sex-specific organelles in membrane permeabilization during parasite egress [42–44]. Further upstream, recent work suggest that lipid metabolism differs between asexual stages and gametocytes [45] and that a female-specific ATP-binding cassette transporter is linked to the accumulation of lipids needed for membrane biogenesis [46]. Despite these advances, there remain many questions about parasite uptake of host nutrients and application of possible gametocyte vulnerabilities to transmission-blocking therapeutics.

Several new platforms for high throughput screening of gametocytocidal drugs could be used to test drugs intervening in the processes mentioned above. Some of these assays rely on fluorescent or luminescent reporters, enabling monitoring of gametocyte-specific drug activity [47,48], while others use DNA dyes, viability dyes, or enzymatic assays to allow screening of all parasite lines including field isolates [49–52]. In addition, new readouts for transmission-blocking activity [53,54] will increase throughput of drug and vaccine testing while the sex-specific proteome of mature gametocytes [55] may help identify stage-specific biomarkers.

Finally, there is still much uncertainty about the nature and extent of transmission-blocking immunity. Numerous epidemiological studies have suggested that transmission-blocking antibodies are short-lived [56]; however, these studies have so far been in limited populations while transmission-blocking immunity likely varies by region. Furthermore, though model simulations suggest that antibodies attacking immature gametocytes would

significantly lower the density of transmissible mature gametocytes [57], it is still unknown what role antibodies play compared to other immune components and if antibodies can target developing gametocytes in addition to mature gametocytes. Whatever gametocyte stage(s) is (are) ultimately targeted by a transmission-blocking drug or vaccine, promising results from a recent vaccine candidate combining the established gamete antigen Pfs48/45 with the asexual antigen GLURP [58] reinforce the value of targeting transmission stages together with asexual stages.

In conclusion, the malaria elimination agenda has driven recent discoveries with applications for novel biomarkers, drugs and vaccines. In particular, advances in illuminating the mechanisms of gametocyte commitment and sequestration uncover new parasite and host targets for transmission-blocking interventions. Further investigation of the knowledge gaps in these areas will both deepen our understanding of host-gametocyte biology and generate new tools to block malaria transmission.

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Highlights

- *P. falciparum* sexual conversion is regulated by epigenetic control of AP2-G.
- Extracellular vesicles and other environmental factors may alter sexual conversion.
- Gametocytes are enriched in the bone marrow parenchyma.
- Insights into sexual differentiation and development reveal potential drug targets.
- New tools will enable better understanding of parasite-host interactions.

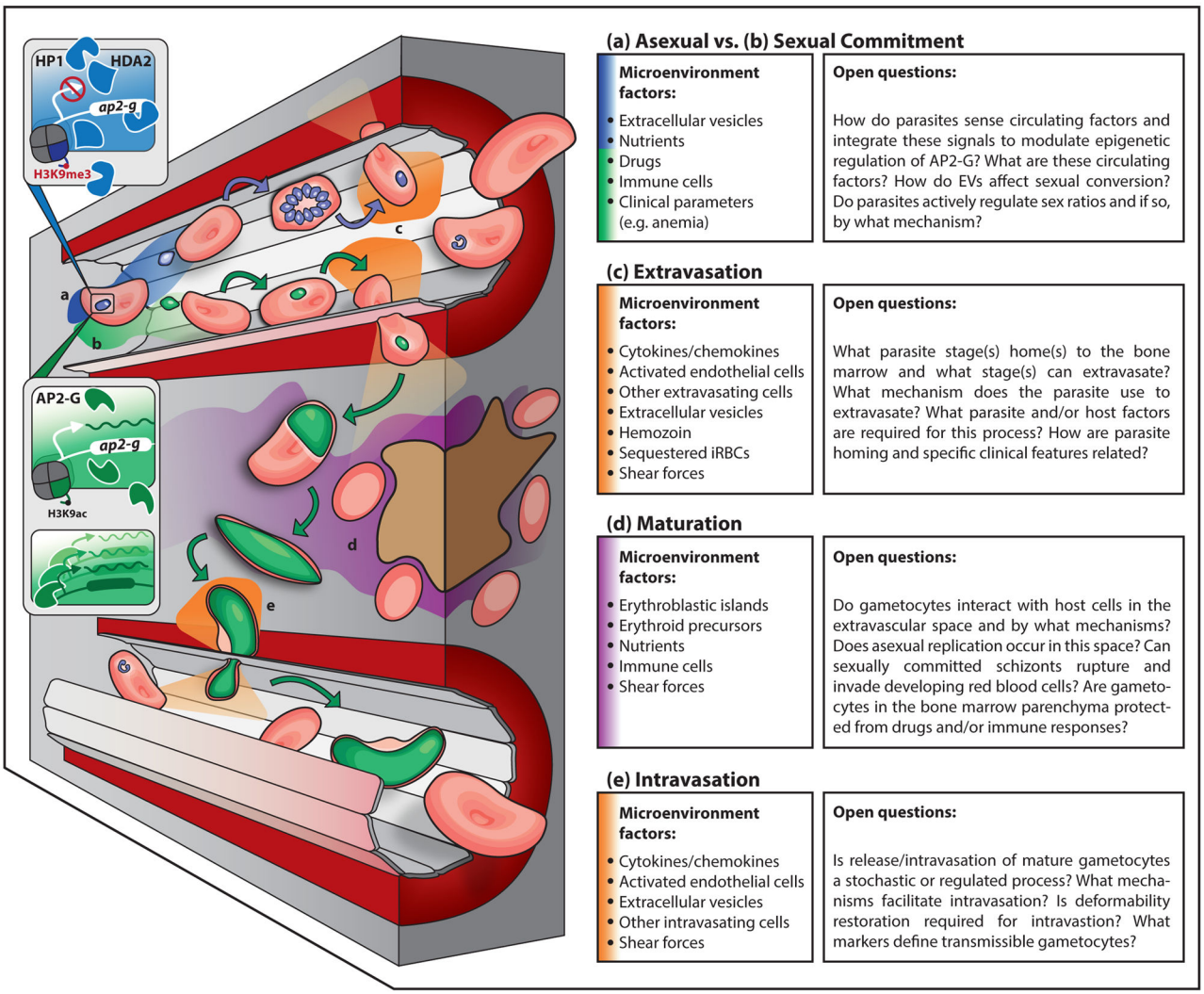

Figure 1. Model for gametocyte commitment and sequestration, including key host-parasite interactions and microenvironment characteristics for each step

In the asexual development pathway (a) (blue), HP1 and HDA2 (and potentially other proteins) inhibit *ap2-g*, and therefore gametocyte gene transcription. Asexual parasites develop in RBCs and may extravasate into the bone marrow parenchyma (c) (orange). In a subset of asexual parasites, *ap2-g* is transcribed, leading to the expression of genes essential for gametocyte development (b) (green). There are multiple parasite stages that may be involved (asexual parasite, merozoite, early gametocyte) in homing to the bone marrow and extravasation through the endothelial lining into the bone marrow parenchyma (c) (orange). Various possible parasite and host factors likely determine homing and extravasation, which may occur in a trans- or para-cellular process, and may be guided by an active endothelial cell process. Local inflammation and endothelial activation stimulated by sequestered parasites, parasite EVs, or hemozoin may contribute to extravasation (c). Upon extravasation, parasite development or ability to remain in this microenvironment may depend on local interactions with host cells, including nurse macrophages or erythroid precursors, or soluble host factors, such as nutrients, that are also present (d) (purple).

Finally, gametocytes must intravasate to return to circulation, with endothelial cells again likely mediating this process (e) (orange).

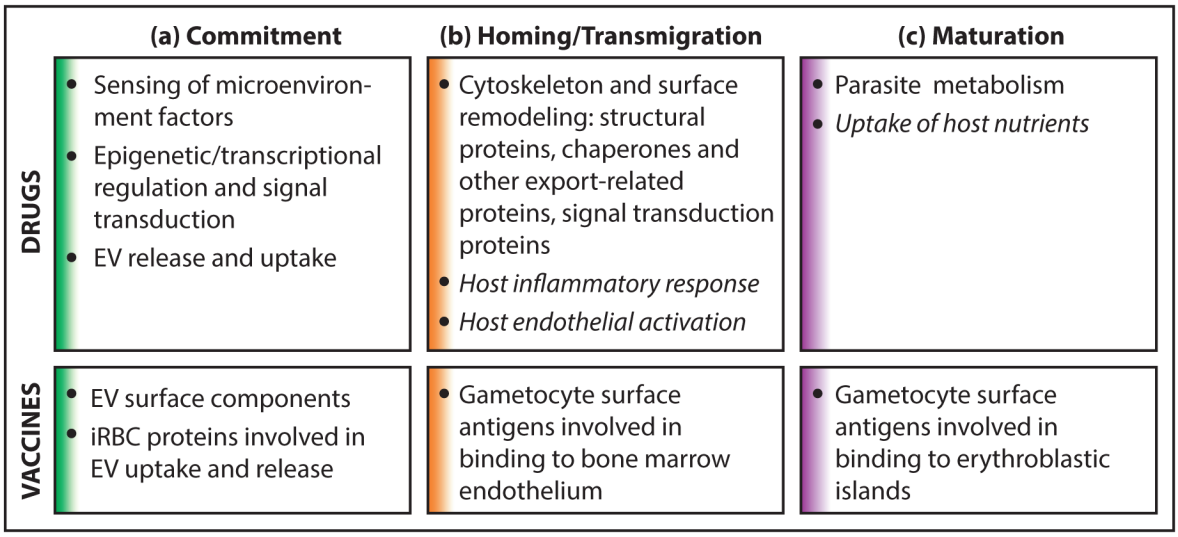

Figure 2. Points of transmission-blocking intervention

Several recently elucidated aspects of gametocyte biology outlined in this review provide potential points of intervention for new clinical tools. Drugs or vaccines could block transmission by targeting (a) commitment to sexual development, (b) homing and transmigration (including extravasation and intravasation), and (c) gametocyte maturation. (a) During commitment, epigenetic regulation and signal transduction involved in sexual conversion and sex ratio determination could be targeted by transmission blocking drugs. Additionally, drugs could target machinery involved in the release or uptake of EVs from iRBCs. Vaccines could similarly target EV surface components or iRBC proteins involved in EV uptake and release. (b) During homing and transmigration, drugs could target parasite-encoded proteins that mediate cytoskeletal or surface remodeling involved in homing or transmigration in the bone marrow. Alternatively, host-targeted drugs could be used to modulate host inflammatory responses and endothelial activation that may drive sequestration. Vaccines could target gametocyte surface proteins required for bone marrow endothelium binding or transmigration. (c) During gametocyte maturation, drugs could target gametocyte metabolic enzymes or proteins involved in host nutrient uptake. Vaccines could target gametocyte surface proteins involved in binding to erythroblastic islands. Host-targeted therapies are indicated by italics. Section colors in this figure correspond to microenvironment colors presented in Figure 1.