

Malaria parasite transmission stages: an update

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The Molecular Approaches to Malaria 2004 meeting provided an opportunity to see the impressive progress in all research fields and in the four years since the previous Molecular Approaches to Malaria meeting, when much of the *Plasmodium falciparum* genome sequence was already available. Study of the part of the *Plasmodium* life cycle associated with transmission through the vector, which begins with the commitment of blood-stage forms to sexual development, has been especially fruitful. This success is a result of several reasons including: (i) the availability of the genome sequence; (ii) the availability of good animal models that allow parasite culture and facile *in vivo* studies of many of the life cycle stages involved in transmission; (iii) the availability of genetic manipulation technologies for the animal models of malaria, as well as *P. falciparum*; and (iv) the ability to study lethal gene knockouts at this stage of the life cycle.

The aim of this review is to serve as an update on the recent progress made on the *Plasmodium* transmission stages subsequent to the watershed genome publications [1], and on the work presented at the Molecular Approaches to Malaria 2004 (MAM 2004) meeting held 1–5 February 2004 in Lorne, Australia. This area of work represents several distinct life cycle stages and various research fields, which are too broad to cover in detail here. The reader is referred to the following comprehensive and informative reviews: (i) sexual development and fertilization of the parasite [2]; (ii) growth within the mosquito [3]; (iii) innate immune response of the mosquito to parasite infection [4]; and (iv) successful transmission of infectious sporozoites to the liver [5].

Despite the genomic and phenotypic diversity between different *Plasmodium* spp., they share very similar life cycle strategies. To identify transmission-blocking vaccine candidates, special attention is being given to features of parasite uptake, fertilization, sexual development and egress from the mosquito host. During the asexual cycle in the blood, a small percentage of parasites choose not to follow asexual multiplication and instead develop into sexually committed cells, the female and male gametocytes, which are the precursor cells of the gametes. These cells are arrested in the G₀ phase of the cell cycle and are only activated to produce the gametes in the midgut when ingested as part of the bloodmeal of the female anopheline

mosquito. Gamete formation is followed by fertilization, resulting in the diploid zygote. After one round of meiotic division, the zygote develops into a motile ookinete that penetrates the cells of the midgut and traverses the midgut wall to form an oocyst on the basolateral lamina. These processes are obligatory for successful transmission of the parasite and are amenable to inhibition through vaccination, thus generating antibodies that either block fertilization, zygote development or ookinete penetration of the midgut. After growth and multiplication, > 10 000 sporozoites develop in a single oocyst. The motile sporozoites are released ~17 days after the initial bloodmeal and migrate via the haemolymph to invade the salivary glands, from which they are ready to infect a new victim and continue the cycle.

The slender, haploid sporozoites enter the bloodstream with the saliva of a feeding *Anopheles* mosquito. After invasion of a liver cell, which occurs within minutes of biting, the parasite undergoes a period of growth (G₁ phase of the cell cycle) followed by multiple genome replications and mitotic divisions (S/M phase). This results in the production of 20 000–40 000 daughter parasites, merozoites, that invade red blood cells (RBC) after release from the hepatocyte in the circulation.

Transmission of *Plasmodium*

Malaria parasites of rodents have been particularly useful for the study of the sexual stages and transmission of *Plasmodium*. The experimental advantages have been complemented by the availability of significant amounts of genome data ranging from 5.6x in the case of *Plasmodium yoelii* [6] to 3.5x of both *Plasmodium chabaudi* and *Plasmodium berghei* (N. Hall, Sanger Institute, Cambridge, UK, and The Institute for Genomic Research, MD, USA). The value of the models was emphasized by the fact that the assembled contigs of *P. yoelii* could be aligned with the scaffold of the core, non-subtelomeric regions of the completed 14 chromosomes of *Plasmodium falciparum*. This result had confirmed, in detail, the earlier low-resolution studies [7–9] that had indicated a high level of synteny (a combination of gene orthology and organizational conservation) between the malaria parasites of rodents and *P. falciparum*. Based on this study, a comprehensive list of orthologues could be assembled which includes virtually every gene encoding transmission stage-specific proteins that have been described and/or studied in *P. falciparum* (Pfg25 on chromosome 13

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is the notable exception, but even this gene might ultimately be found when an intact *P. falciparum* locus is sequenced [10]. Indeed, M. Berriman (Sanger Institute) demonstrated that *P. yoelii*, *P. chabaudi* and *P. berghei* could be aligned against the *P. falciparum* scaffold, as well as against themselves. This gives a much more profound representation of a composite genome from the rodent malaria parasite, and of the central chromosomal regions that are orthologous between *P. falciparum* and rodent malaria parasite genomes. Against this background, the use of *P. berghei* and *P. yoelii* as genetically tractable models, and their development as models for the analysis of almost every aspect of transmission, make them the systems of choice for the study of this part of the life cycle. The genome data available are being complemented by increasing amounts of post-genome survey information generated by RNA studies [including DNA microarrays, individual complementary DNA (cDNA) clone sequencing from subtractive hybridization libraries], in addition to proteome studies. The beauty of the genetic orthology in the genes expressed in the transmission stages of *Plasmodium* is that genes identified in one species as being significantly expressed in a particular mosquito stage might be readily investigated in the most amenable study model (Figure 1).

Transcriptomes

The cataloguing of gene expression during transmission has progressed significantly in the past two years. A

recent global DNA microarray study revealed extensive stage-specific gene expression in *P. falciparum* gametocytes and sporozoites [11], confirming earlier studies indicating that as much as 25% of the genome was upregulated in gametocytes [12]. However, when combined with the data from synchronized asexual blood stage forms [11,13], it became apparent that certain classes of transcript, other than those associated with basal house-keeping (related to metabolic and catabolic processes), were shared between stages: 204 genes were shown to be specifically upregulated in gametocytes and 41 in sporozoites [11]. Many of these were in both forms and some were also expressed in merozoites. Several of the parasite forms in this study are polar cells, and share either motility or invasive properties. Their transcriptomes reflect these shared features. Unfortunately, we still lack a global transcriptome based on an array that contains the entire genome which has been used against all possible stages of the life cycle, thus yielding a truly dynamic picture of gene expression throughout the life cycle. This goal might never be realized. However, the available data have already provided rich pickings for the direct experimental investigation of transmission. By using long oligo-based microarray technology, Silvestrini *et al.* [14] examined transcription in gametocytes between two lines of *P. falciparum*, the reference clone 3D7 and a mutant F12 that is unable to undergo sexual differentiation. The authors reported both the transcription of novel gametocyte-specific genes and the upregulation of

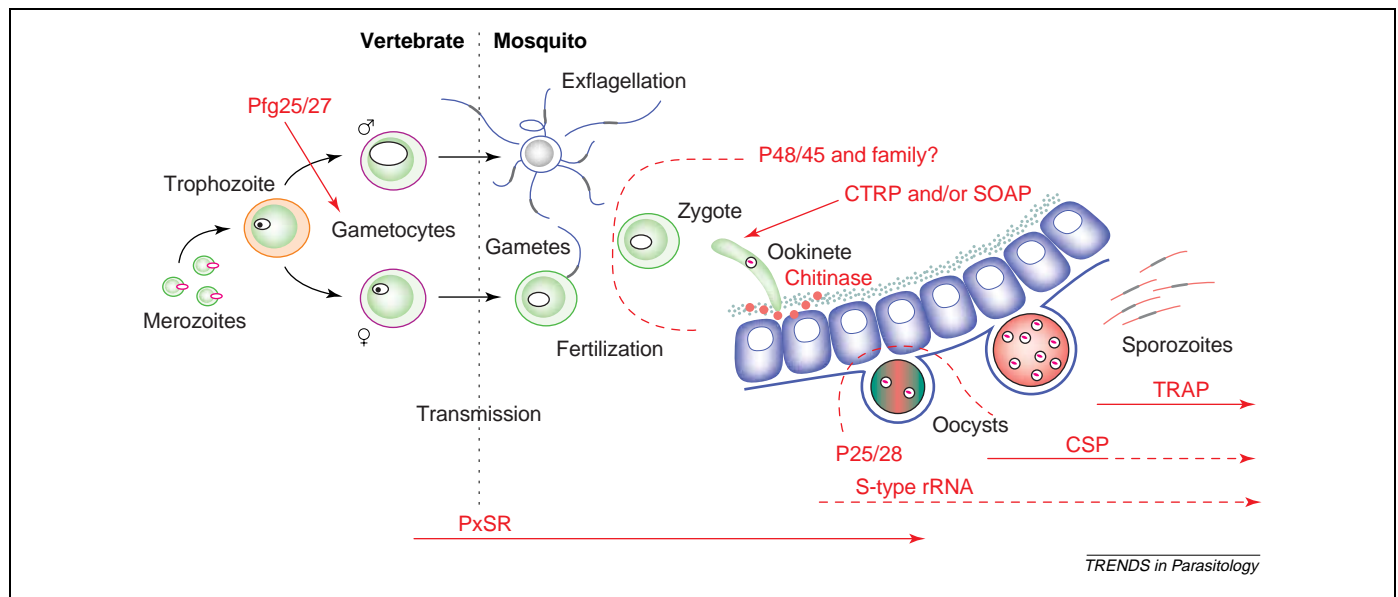


Figure 1. Role of stage-specific genes in transmission of malaria parasites as revealed by genetic manipulation. The expression of several proteins is described; solid arrows indicate the point at which the phenotype is manifest in the disrupted parasite and broken arrows show potential additional phenotypes downstream of lethality. These proteins have been analyzed using transfection technology and their respective roles have been shown to be crucial for development; these are briefly described below. Most of the proteins mentioned in this figure are not described within the body of this review. For more information, please refer to the given references. Mutant *Plasmodium falciparum* parasites lacking the genes for Pfg25/27 [37] are unable to develop into gametocytes. In addition, P48/45 and possibly other members of its gene family affect fertilization of male and female gametocytes [38]. Disruption of the gene encoding CTRP affects the motility and viability of ookinetes [39]. Knockout clones of P25 and P28 also show reduction in oocyst number when compared with those of wild-type parasites, and more so when a double knockout is performed [40]. The *P. falciparum* PxSR knockout is also associated with a dramatic reduction in oocyst number and the oocysts are highly vacuolated [41–43]. The same phenotype is also observed with knockout clones against CSP [44]. Clearly, transfection experiments will only show visible parasites at the life cycle stage before the phase where the gene is essential. Consequently, the exact role and function of the gene cannot always be directly inferred by the phenotype observed, but it will indicate at which stage the protein is essential. However, TRAP mutants will form sporozoites, but with a loss of motility and, consequently, are unable to invade, indicating the role of TRAP in gliding motility [45]. Targeted disruption of the SOAP gene gives rise to ookinetes that are markedly impaired in their ability to invade the mosquito midgut and form oocysts [46]. Similarly, if *Plasmodium* chitinase is disrupted, there is a reduction in infectivity to mosquitoes [47,48]. Disruption of the *Plasmodium* S-type rRNA genes produced parasites that were still viable, but their life span within the mosquito was extended by 10% [49]. Abbreviations: CSP, circumsporozoite protein; CTRP, circumsporozoite and TRAP-related protein; PxSR, *Plasmodium* scavenger receptor protein; SOAP, secreted ookinete adhesive protein; TRAP, thrombospondin-related adhesive protein. This figure is adapted from Ref. [51].

other genes principally expressed in gametocytes on a background of transcription shared with asexual blood stage parasites. It will be of considerable interest to compare the details of these findings to similar conclusions of work carried out on the rodent parasite *P. berghei* (M. Karras, pers. commun.) which has shown good concordance with the published work of Le Roch *et al.* [11].

Proteomes

Published high-throughput proteome analyses of gametocytes and sporozoites have been carried out for *P. falciparum* [15,16], and new studies of midgut and salivary gland sporozoites will shortly become available (E. Lasonder and H. Stunnenberg, Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands; M. Mann, University of Southern Denmark, Odense, Denmark, pers. commun.). These data will soon be complemented by an extensive *P. berghei* proteome survey of the same stages, which will also include an ookinete analysis (D. Raine and R. Sinden, Imperial College, London, UK; J. Yates III, Scripps Institute, San Diego, CA, USA, pers. commun.). These studies identified >2500 parasite proteins from each species, many hundreds of which were stage specific. The stage-specific proteomes of rodent and human *Plasmodium* spp. were in good agreement with each other and reflected specific activities of particular life cycle forms, for example, motility as well as recognition and invasion of various host cell types. Furthermore, all of the proteomes contained common proteins associated with the shared parasite features (e.g. apicoplast, mitochondria and metabolism). In addition, pure populations of *P. berghei* male and female gametocytes have been isolated and subjected to proteome analysis (S.M. Khan, unpublished). Separation was achieved by fluorescence-based sorting using transgenic parasites that express green fluorescent protein (GFP) under the control of different gender-specific promoters. Subsequent high-throughput mass spectrometric analysis generated information on sex-specific events in gametocytes and provided an insight into the readiness of the parasites to produce gametes.

Life cycle switches

It is possible that elements of sexual (male or female) development are controlled by the same signalling pathways, but differ as a result of the temporal and/or environmental context. For example, male gametogenesis is one of the more spectacular events in the parasite life cycle when viewed down the microscope. Upon transfer to the mosquito midgut, gametocytes are activated to leave cell cycle arrest (G0) and emerge from the erythrocyte. The male rapidly undertakes three rounds of nuclear replication, generating eight nuclei that are each packaged into a flagella-like gamete, which then emerges from the gametocyte in a thrashing process termed exflagellation. What are the signals that initiate this process? Previous work by O. Billker *et al.* had established that, in addition to the well-known stimuli of pH rise and (essential) temperature drop, a small molecule [xanthurenic acid (XA)] serves as an essential stimulus of gamete formation superseding a need for a change in pH [17]. XA has been

shown to stimulate Mg^{2+} and/or Mn^{2+} -dependent guanylyl cyclase activity associated with mature *P. falciparum* gametocyte membrane preparations, implicating cGMP as a signalling intermediary in some or all of these developmental processes [18]. Recently, Billker has reported on some downstream aspects of possible signalling pathways. Earlier work had implicated calcium ions as playing an integral role in gamete development [19]. Thanks to both the genome and proteome work, it was clear that there was a class of calcium-dependent protein kinases (CDPKs), two of which were upregulated in gametocytes. Billker first demonstrated that stored calcium was mobilized within *P. berghei* gametocytes upon activation or by XA administration, and could stimulate GFP fluorescence through activation of aequorin, a light-emitting calcium sensor, fused to the GFP transgene in transgenic parasites [20]. Second, an investigation of *P. berghei* clones disrupted in one of the two CDPK genes demonstrated that the CDPK family member designated CDPK4 was responsible for both male gametogenesis and further development of the zygote into a viable ookinete. This defines a pathway that is ripe for drug development and confirms the pivotal role that calcium ions play as secondary messengers at this stage in *Plasmodium* development (Figure 3). This study was notable for being the first occasion where the report of the phenotype of a gene disruption in *Plasmodium* was also accompanied by the demonstration of phenotype recovery associated with restoration of the disrupted gene [20].

Epigenetics

Currently, the control of gene expression throughout the life cycle is an experimental 'black box'. Gametocytogenesis offers the advantage of being a distinct developmental pathway that occurs in blood stages and is relatively tractable, and the gametocytes are obtainable in sufficient numbers to perform biochemical assays. At MAM 2004, Stunnenberg demonstrated that it would be possible to apply cutting edge technologies to these issues. Together with his collaborators, Stunnenberg initiated chromatin immunoprecipitation assays (ChIP) using antibodies directed against the acetylation modification motifs of core histones that commonly indicate an open-chromatin structure associated with regions of the genome that are actively expressed. However, using staged parasites, it proved impossible to discriminate between active and inactive chromatin, despite our knowledge of stage-specific transcription. The working hypothesis is that there are other modifications that have yet to be defined which result in an active chromatin conformation. High-resolution mass spectrometry on purified histones might be the pathway towards their identification.

Insect immune responses

The infection of the mosquito vector by a malaria parasite does not go unnoticed and the mosquito mounts a vigorous, but non-adaptive, immune response initiated at least in part by classic pattern-recognition proteins [21]. Indeed, the parasite undergoes dramatic reduction in numbers at each stage of fertilization, ookinete formation and, in particular, during penetration of the midgut, thus

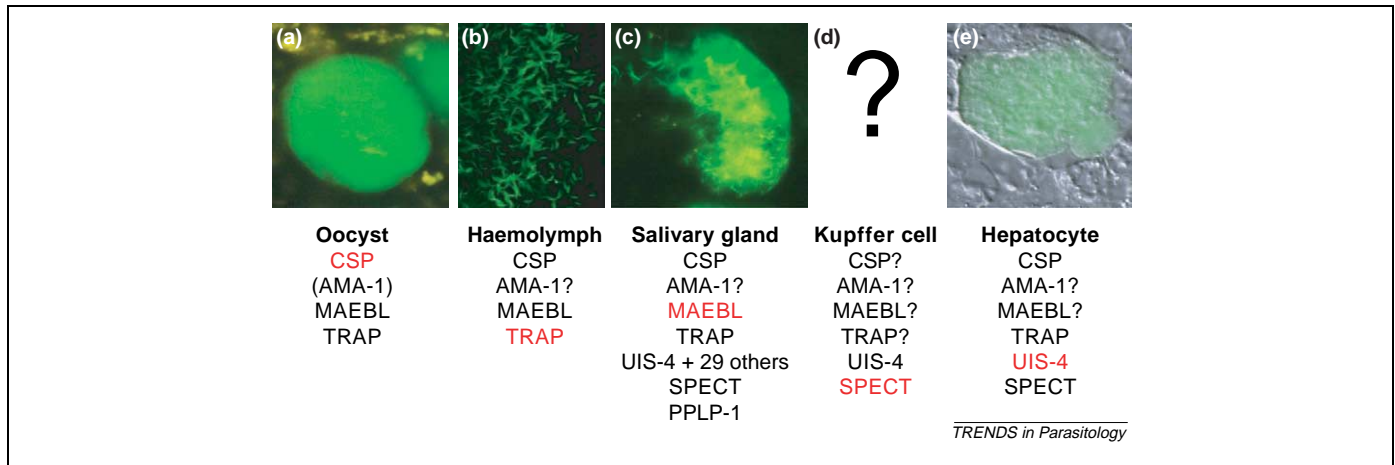


Figure 2. Progress of the developing sporozoite. The several proteins described here have been phenotypically analyzed through methods that involve gene disruption and/or modification. *Plasmodium berghei* parasites that constitutively express GFP are visible in the mosquito [(a) oocyst; (b) haemolymph; (c) salivary gland] and within the mouse liver [(e) hepatocyte] [50]. No images are available of fluorescent parasite either passing through or between Kupffer cells, hence the large question mark in (d). The presence of protein in any particular developmental stage of the sporozoite is indicated by the gene abbreviation. Proteins shown in red indicate at which stage the parasites become arrested when genetic manipulations are performed to knockout this gene. AMA-1 is in brackets to indicate the presence of transcript, but not the protein in the sporulated oocyst. Question marks indicate that the role of the protein at the indicated stage remains unclear. Abbreviation: AMA, apical membrane antigen; CSP, circumsporozoite protein; GFP, green fluorescent protein; MAEBL, apical membrane antigen–erythrocyte-binding-like protein; PPLP, *Plasmodium* perforin-like protein; SPECT, sporozoite microneme protein essential for cell traversal; TRAP, thrombospondin-related adhesive protein; UIS, up in infected sporozoite.

reaching a low point with the number of oocysts generated [22,23]. Vegetative growth and replication within the oocyst amplifies the parasite numbers once more because each oocyst is capable of liberating 13 000 sporozoites [22,23]. Part of the immune response of the mosquito is responsible for the severe reduction in numbers suffered by the invading ookinete as it traverses the midgut wall and emerges at the basolaminal axis [21]. The completion of the mosquito genome sequence [24] and advent of powerful double-stranded RNA (dsRNA)-based methodologies for the analysis of gene function through specific knockdown of any given transcript [25] were employed to examine these processes in the mosquito. Comparative genomics have established that homologues of a family of complement-like thioester-containing proteins (TEPs) known to be involved in the *Drosophila* immune response were also present in the *Anopheles gambiae* genome. Elena Levashina (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) reported her work in collaboration with Fotis Kafatos (The European Molecular Biology Laboratory, Heidelberg, Germany) that demonstrated a role for *An. gambiae* TEP1 associated with the ability of a mosquito to mount a successful melanotic response [26] – one manifestation of insect immunity and pathogen clearance. TEP1 is secreted into the haemolymph by haemocytes possibly in response to injury (i.e. ookinete penetration of the midgut wall) and is able to enter midgut cells. The C-terminal region of TEP1 is capable of binding to the surface of target pathogens including *P. berghei*. There are two lines of *An. gambiae* available that are either susceptible or resistant, and contain distinct alleles of TEP1: TEP1^S and TEP1^R. TEP1^R exhibits faster binding kinetics to the invading ookinete surface, accompanied by complete melanization or parasite lysis. Susceptibility is a relative term in that only 20% of the ookinetes survived the mosquito immune response compared with 0% in the TEP1^R line, such is the fine margin between parasite

success and failure. Knockdown of TEP1^R by dsRNA increased susceptibility of the susceptible line, converted the resistant line to susceptible, and eliminated melanization as a resistance mechanism. Recently, this work has been extended by Kafatos *et al.* [27] and three further genes have been reported (from a pool of ~100 under active investigation, thanks to bioinformatics and a complete *Anopheles* genome sequence). These genes comprise two C-type lectins (CTL4 and CTLM4) and a leucine-rich immune response (LRIM1) gene: all three might act as recognition surveillance molecules with TEP as the likely effector, demonstrating the complexity of the immune response in the mosquito. The details of the pathway between detector and effector molecules will fall into place over the coming years and, although they can be expected to bear a strong resemblance to the increasingly well characterized immune response pathways of *Drosophila melanogaster* [28], no doubt the full *Anopheles* genome will provide a new surprises.

Sporozoite biology

In malaria research, suppression subtractive hybridization (SSH) was originally exploited to generate ookinete-specific libraries [29] and, more recently, libraries from *P. yoelii* sporozoites that are enriched in sequences expressed either in the salivary gland sporozoite [30] or in the pre-erythrocytic stages [31]. Stefan Kappe (Seattle Biomedical Research Institute, WA, USA) and Kai Matuschewski (University of Heidelberg, Germany) reported on a conserved, novel pre-erythrocytic *Plasmodium* protein named UIS-4 (up in infected sporozoite-4), which is expressed in secretory organelles of sporozoites and also in liver stages where it localizes to the parasitophorous vacuole membrane. Targeted gene disruption in *P. berghei* shows that UIS-4 is not involved in hepatocyte invasion, but is crucial for liver stage development. SSH implicates relatively small numbers of genes and, therefore, Kappe and Bill Bergman (Drexel

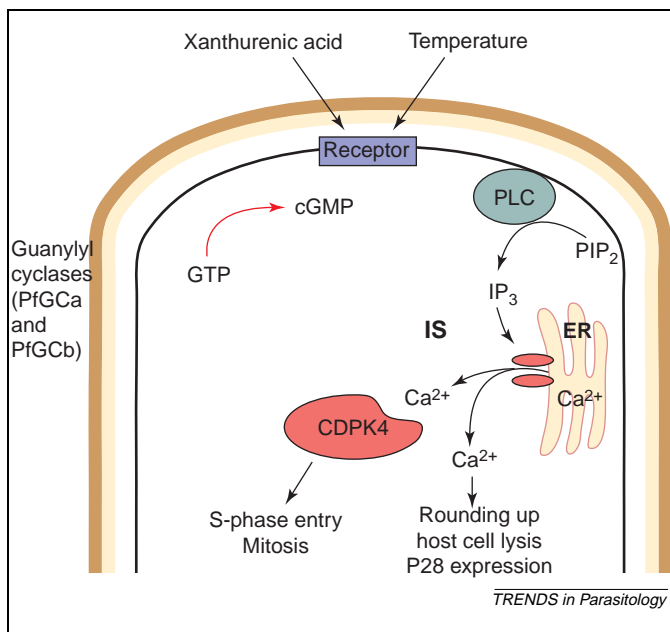


Figure 3. Possible signalling events during gametogenesis. XA stimulates initial Ca^{2+} release, which in turn activates different Ca^{2+} -dependent signalling pathways that control different constituent events of gametogenesis [20]. Ca^{2+} release from the intracellular stores is anticipated to be dependent on the PLC-IP₃ activation pathway. A strictly CDPK4-dependent pathway regulates male-specific events in the cell cycle. Possible substrates for CDPK4 might be found among the cyclins and cyclin-dependent protein kinases, or among components of the replication machinery. In addition, the known stimulation of guanylyl cyclase activity [18] by XA is illustrated, but its precise position and role in the different possible pathways remains obscure, as does the identity of the receptor for XA. This figure is a modified version of an illustration prepared by Oliver Billker who is gratefully acknowledged for this contribution. Abbreviations: CDPK, calcium-dependent protein kinase; ER, endoplasmic reticulum; IS, intracellular stores; IP₃, inositol triphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; XA, xanthurenic acid.

University, PA, USA) described the development and use of long-oligonucleotide microarrays of *P. yoelii* to analyze *in vivo* gene expression in liver stages (Figure 2). The dataset revealed for the first time a global picture of gene expression in liver stages, finding many liver stage-expressed genes that are not expressed in sporozoites or blood stages, and will prove a rich resource for future functional investigations. Subtraction methodologies were also recently reported for the developing oocyst identifying >1100 transcripts that are differentially regulated at different stages of oocyst development from Day 2 to Day 22. The work identified novel genes and confirmed the upregulation of genes such as apical membrane antigen-1 (AMA-1) and apical membrane antigen-erythrocyte-binding-like protein (MAEBL) during sporozoite formation, which were previously thought to be exclusively expressed in merozoites [32].

At MAM 2004, two research groups described different molecular screening methods to identify *P. berghei* proteins that might be of importance in sporozoite infectivity. By using a clone from a subtractive cDNA experiment, Bhanot *et al.* identified a phospholipase (PbPL) as being upregulated in salivary gland sporozoites and present on the sporozoite surface [33]. Knockout experiments on this gene showed a considerable reduction in infectivity compared with that in the wild type when the parasites were inoculated by mosquito bite, but the infectivity was much less affected when the sporozoites

were inoculated by needle. Ishino *et al.* [34] used a clone from the targeted disruption of genes specifically expressed in infective liver sporozoites and described a *P. berghei* sporozoite micronemal protein that they believe is essential for liver sinusoidal cell traversal (SPECT). Targeted disruption of the *spect* gene greatly reduced sporozoite infectivity to the liver *in vivo*. *In vitro* cell invasion assays revealed that these disrupted parasites could infect hepatocytes normally. Clearly, the mutant parasites were affected in their ability to access hepatocytes within the liver, but their apparent liver infectivity was, however, restored by chemically mediated depletion of phagocytic Kupffer cells (which line hepatic capillaries interspersed with endothelial cells). These results show that *Plasmodium* sporozoites chiefly access hepatocytes through the liver sinusoidal cell layer by cell traversal motility mediated by SPECT and strongly suggest that Kupffer cells are main routes for this passage. The salivary gland sporozoite has recently been shown to express one of five homologues of pore-forming proteins of the single membrane attack complex-perforin (MACPF) found in the genome [35]. These secreted proteins could well be involved in host or vector tissue colonization.

Hez *et al.* [36] have recently described a novel method in which they were able to transplant human hepatocytes into a SCID mouse (deficient in both B and T cells) and then establish an infection with *P. falciparum*. The human hepatocytes were transplanted successfully into the mouse spleen when both natural killer cells and monocytes were eliminated. The resulting mouse was receptive to infection by *P. falciparum* sporozoites, as assayed by the production of liver-specific antigens (both human and parasite). This work follows on from previous studies describing the establishment and development of erythrocytic *P. falciparum* stages in immunocompromised mice with constant transfusion of human RBC, and might prove to be a useful, ethically acceptable system for *in vivo* analyses of *P. falciparum*.

Looking to the future

The original MAM 2000 was a landmark meeting, being the first to acknowledge that the field of molecular malaria research was big enough to merit an international meeting. MAM 2004 took this a significant stage further, showing the continuing maturation of the field in the wake of genome and post-genome technologies. It will be fascinating to see at MAM 2008 how much more progress has been made and whether we are able to describe realistic hopes for the new drugs and vaccines, which was the obvious imperative for the investment in the *P. falciparum* genome project in the first place. If we can, then transmission blockade will almost certainly offer very promising candidates. This potential will stem from the use of complete array of technologies of molecular malaria research namely, genome, transcriptome, proteome, genetic engineering and parasite models.

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