International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

Tools for attenuation of gene expression in malaria parasites

Philip J. Shaw *, Aiyada Aroonsri

National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park, Thanon Phahonyothin, Tambon Khlong Neung, Amphoe Khlong Luang, Pathum Thani 12120, Thailand

ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 15 September 2016
Accepted 28 November 2016
Available online xxxx

Keywords:
Plasmodium
Malaria
Reverse genetics
Transfection

ABSTRACT

An understanding of the biology of *Plasmodium* parasites, which are the causative agents of the disease malaria, requires study of gene function. Various reverse genetic tools have been described for determining gene function. These tools can be broadly grouped as trans- and cis-acting. Trans-acting tools control gene functions through synthetic nucleic acid probe molecules matching the sequence of the gene of interest. Once delivered to the parasite, the probe engages with the mRNA of the target gene and attenuates its function. Cis-acting tools control gene function through elements introduced into the gene of interest by DNA transfection. The expression of the modified gene can be controlled using external agents, typically small molecule ligands. In this review, we discuss the strengths and weaknesses of these tools to guide researchers in selecting the appropriate tool for studies of gene function, and for guiding future refinements of these tools.

1. Introduction

Malaria continues to be a major parasitic disease of humans, although the decline in incidence of the disease in recent years is driving efforts towards elimination in many endemic areas (Tanner et al., 2015; WHO Malaria Policy Advisory Committee and Secretariat, 2015). However elimination could be thwarted by, among other factors, increasing parasite resistance to artemisinin and partner drugs, particularly in southeastern Asia (Woodrow and White, 2016). Successful elimination will thus depend on new parasite control strategies that require better understanding of the parasite's biology so that novel drug and vaccine targets can be identified. This understanding is built incrementally from knowledge of individual gene functions, which can be inferred from the study of mutant phenotypes. In model organisms, gene functions are elucidated using classical forward genetics, which entails mapping genes to phenotypes among the sexual progeny of genetic crosses. *Plasmodium* spp. malaria parasites reproduce asexually in red blood cells, and blood stage parasites are amenable to various experimental approaches including laboratory culture and genetic modification. A subpopulation of blood stage parasites differentiates to the gametocyte form for sexual reproduction. After entry into the mosquito host, sexual reproduction takes place. The terminally differentiated sporozoite progeny complete the life cycle by invading liver cells of vertebrate hosts. The progeny can propagate as blood stage parasites only after liver stage development is complete. The liver developmental stage of *Plasmodium* spp. within narrow host ranges makes classical forward genetics challenging, especially for parasites infecting humans. Forward genetic screening is possible using mutants with random insertion of the *piggyBac* transposon that can be generated during asexual culture (Balu et al., 2009). However, owing to the haploid nature of *Plasmodium* blood stages, parasites with transposon mutations in genes important for growth cannot be propagated for phenotypic assays, and the functions of these genes may not be uncovered by this approach. The alternative is reverse genetics, in which functions are inferred from phenotypes produced using tools designed to perturb gene expression in a controllable fashion.

Here, we review reverse genetic tools described for *Plasmodium* malaria parasites that can be used to control expression of a gene with a hypothesised function, commonly referred to as a gene of interest (GOI). We focus on tools developed in the most widely studied species, the human malaria parasite *Plasmodium falciparum*, and make reference to tools developed in other species (chiefly the rodent malaria parasite *Plasmodium berghei*) where applicable. Some of the material has been covered in previous reviews (Balu and Adams, 2007; Limenitakis and Soldati-Favre, 2011; Pino, 2013; Webster and McFadden, 2014; de Koning-Ward et al., 2015; Matz and Kooij, 2015). More recently, there have been several studies that apply these tools and new technologies have emerged. In addition to the new insights on parasite gene functions, we will focus on the practical challenges and limitations of these tools that have come to light.

* Corresponding author. Fax: +66 2 564 6707.
E-mail address: philip@biotec.or.th (P.J. Shaw).

http://dx.doi.org/10.1016/j.ijpara.2016.11.006
0020-7519/

Before presenting details of the available tools, we should bear in mind the desirable properties. The tool must be specific, such that the expression of only the GOI is directly affected. The tool should be robust such that it works with the same specificity and efficiency for different gene targets. It is also preferable that the control of gene expression by the tool is rapid, reversible and tunable so that phenotypes can be directly correlated with the gene expression level. Finally, the tool should be simple so that it can be genome-scalable.

2. Trans-acting reverse genetic tools

We first review tools for attenuating parasite GOI expression that use trans-acting factors. These tools employ probes designed such that they can modify the expression of the GOI by specifically hybridising with the expressed mRNA. Once engaged with the target mRNA, gene expression is attenuated by parasite post-transcriptional mechanisms. Trans-acting probes are typically synthesised in vitro, delivered to the parasite and gene function inferred from observing the phenotypic effects. Trans-acting reverse genetic tools are attractive due to their simplicity: probes can be designed and synthesised using general methods and they can be delivered into parasites using simple protocols.

In many eukaryotic organisms, the most widely used reverse genetic tool is RNA interference (RNAi; Fire et al., 1998). Target gene expression is attenuated by introduction of a double-stranded (ds)RNA with the same sequence as the target gene. The dsRNA can be introduced into the cell as in vitro transcribed long complementary RNAs, transcribed from a transgene as a single RNA which folds back on itself (short hairpin, shRNA), or as complementary short synthetic RNA oligonucleotides (small interfering, siRNA). dsRNA and shRNA are processed to siRNA duplexes 20–30 bp in length by the RNaseIII enzyme Dicer, and siRNA is loaded into an RNA-induced silencing complex (RISC), a key component of which is the Argonaute (Ago) protein that guides the RISC to the target mRNA. Divergent RNAi machineries with recognisable Dicer and Ago proteins exist in some groups of protozoan parasites, including kinetoplastids and amoebozoans; however, most apicomplexans (including *Plasmodium*) lack RNAi machinery (Kolev et al., 2011). The lack of RNAi in *Plasmodium* was proven by rigorous experiments with siRNA and shRNA, which showed that these RNA probes have no significant effect on target gene expression even at high concentrations (approximately 100 nM for siRNA; Baum et al., 2009).

Although RNAi cannot be applied in wild-type *Plasmodium* spp., it may be possible to introduce foreign RNAi machinery from another species. This approach was taken to make the yeast, *Saccharomyces cerevisiae*, RNAi-competent by introducing genes encoding Dicer and Ago from the related yeast, *Saccharomyces castellii* (Drinnenberg et al., 2009). Alternatively, an RNAi complex can be preassembled in vitro from siRNA and Ago enzyme and then transfected into the parasite. This approach has been demonstrated in the apicomplexan parasite *Cryptosporidium* that also lacks RNAi machinery (Castellanos-Gonzalez et al., 2016).

As an alternative to RNAi, artificial antisense molecules can be used as trans-acting reverse genetic tools. Peptide nucleic acids (PNAs) are oligonucleotides with uncharged backbones that can form stable hybrids with RNA in vivo. PNAs can be delivered into *P. falciparum* and do not appear to trigger target mRNA degradation (Kolevzon et al., 2014). The attenuation of GOI expression using PNA probes in *P. falciparum* thus may occur through a similar mechanism to that described in mammalian cells, in which the antisense pairing of the probe with the target inhibits access of the translation machinery (Doyle et al., 2001). In studies where PNAs have been used in *P. falciparum*, rather high concentrations (0.6–10 μM) were needed to attenuate GOI expression (Dahan-Pasternak et al., 2013; Kolevzon et al., 2014). At these concentrations, attenuation of GOI expression by control PNAs is significant (Kolevzon et al., 2014), suggesting that phenotypic analysis could be confounded by unknown factors including hybridisation of probes to unintended mRNA targets (off-targeting).

Phosphorodiamidate morpholino oligomers (PMOs) are another type of synthetic antisense probe that can be used for attenuating target gene expression. PMOs, similar to PNAs, are more stable than RNA and can be conjugated to chemical moieties such as octa-guanidinium dendrimers (vivo morpholinos, VMO) or peptides (peptide phosphorodiamidate morpholino oligomers, PPMO) to enhance cell penetration. Antisense VMOs can inhibit splicing and translation of target mRNAs in *P. falciparum* (Garg et al., 2015). PPMOs have been applied as tools to mediate trans-cleavage of *P. falciparum* target mRNA through recruitment of the parasite RNaseP (Augagneur et al., 2012). RNaseP riboprotein is a conserved complex essential for tRNA processing, and the *Plasmodium* RNaseP has been described (Piccinelli et al., 2005). By careful design of a PPMO comprising an external guide sequence complementary to the target mRNA, an RNA structure can form that mimics the natural RNaseP substrate. Treatment of parasites with PPMO probes has been shown to reduce target mRNA levels (Augagneur et al., 2012; Garg et al., 2015), consistent with RNaseP-mediated cleavage of the target mRNA. In common with PNA probes, rather high concentrations (1–10 μM) of VMO and PPMO probes are needed to reduce GOI expression. Significant attenuation of GOI expression and parasite growth with control probes is also observed at these concentrations (Garg et al., 2015), pointing to confounding effects such as off-targeting.

All of the current trans-acting tools for *Plasmodium* are not able to attenuate target gene expression efficiently, with no greater than four-fold reduction of target reported. In the reports of trans-targeting leading to a phenotype, e.g. growth inhibition, the marked phenotype is somewhat at odds with the moderate reduction of target expression in these experiments. For instance, a PPMO directed against the 1-deoxy D-xylulose 5-phosphate reductoisomerase (*Pf*DXR) gene leads to complete growth arrest as trophozoites (Garg et al., 2015), yet parasites treated with high doses of fosmidomycin, a specific inhibitor of *Pf*DXR, arrest later as schizonts (Howe et al., 2013). Although the PPMO probe against the *Pf*DXR gene shows some specificity, as demonstrated by rescue with isopentylpyrophosphate, the downstream product (Garg et al., 2015), the growth defect phenotype generated using this probe could be confounded by other factors unrelated to inhibition of the target. Similarly, pronounced growth defects when other gene targets are moderately inhibited by PPMOs point to possible confounding phenotypic effects (Augagneur et al., 2012, 2013; Garg et al., 2015).

The lack of knowledge of how the available trans-acting reverse genetic tools work in *Plasmodium*, in particular confounding phenotypic effects, means that these tools are not yet sufficiently mature, and therefore not appropriate for determining gene functions in a precise manner. A single control probe such as a scrambled random sequence (Kolevzon et al., 2014) may not be adequate. Other controls such as testing multiple non-overlapping probes against the same gene target may be needed to interpret data from phenotypic assays. Although trans-acting reverse genetic tools have the advantage of ease of use, the caveat of off-targeting makes them rather blunt tools. An obvious shortcoming of trans-acting tools would be in determining functions of genes in multigene families, in which it would be extremely difficult to avoid off-targeting. *Plasmodium* spp. contain several multigene families with well-documented roles in immune evasion and pathogenicity (Deitsch and Hviid, 2004). Some members of these

Please cite this article in press as: Shaw, P.J., Aroonsri, A. Tools for attenuation of gene expression in malaria parasites. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2016.11.006