High efficacy in vitro selection procedure for generating transgenic parasites of Plasmodium berghei using an antibiotic toxic to rodent hosts

Akira Soga1, Hironori Bando2, Mami Ko-ketsu1, Hirono Masuda-Suganuma3, Shin-ichiro Kawazu1 & Shinya Fukumoto1

The malaria parasite Plasmodium berghei is one of the main rodent malaria models. A shortcoming of this model parasite is its low flexibility in genetic manipulation. As this parasite cannot be continuously propagated in cell cultures, in vivo drug selection procedures are necessary to isolate genetic mutants. Drugs harmful to rodents therefore cannot be used for drug selection, which restricts the range of genetic manipulation. In this study, we addressed this problem by establishing a novel in vitro culture drug selection method, which we used in combination with other established methods to successfully isolate genetically manipulated parasites. The target mutants were enriched to the desired level within two weeks. We show that our system can also be used for sequential genetic manipulation of parasites carrying the traditionally used selection markers, demonstrate the procedure’s versatility, and show its use in isolating specific genetically manipulated parasites. This novel in vitro selection method increases the number of available selection markers, allowing more extensive genetic manipulation in malaria parasite research.
results thus be used as a secondary marker in sequential manipulation experiments. However, sequential manipulation is challenging because hdhfr only confers slight resistance to WR992106, and only two reports describe sequential gene disruptions using these markers7,9,10. A marker recycling method using negative selection was developed to address this issue7,11,12, but did not prove to be an effective solution.

In this study, we developed a novel selection method to overcome the genetic manipulation problems in P. berghei. We focused on the puromycin- N-acetyltransferase (pac)-puromycin system, which is commonly used in mammalian cells13,14. pac can be isolated from Streptomyces alboniger and shows resistance to the aminonucleoside antibiotic, puromycin15. A previous study reported that pac might be used as a secondary marker for blood stage long-term in vitro cultures of P. falciparum, the causative agent of tropical malaria in humans15. As puromycin is severely toxic to rodents and thus cannot be used for P. berghei in vivo, we developed a pac-puromycin system based on a novel short-term in vitro culture method.

**Results**

**Expression of pac confers resistance to puromycin.** To determine whether pac can confer puromycin resistance to P. berghei, parasites transfected with pXL/hdhfr-pac-e GFP carrying pac under the control of hsp70 promoters (HSP70-PAC) were obtained using in vitro pyrimethamine selection. IC50 values of wild type and HSP70-PAC were 0.17 ± 0.06 µM and 5.69 ± 0.70 µM (mean ± SD), respectively (Fig. 1). IC50 values of wild type and the mutant parasites were 0.55 ± 0.14 µM and 9.39 ± 1.07 µM (mean ± SD), respectively (Fig. 1).

We identified a suitable puromycin concentration for drug selection by selecting pXL/hdhfr-pac-eGFP-transfected parasites at various puromycin concentrations. The empirical optimal concentration was 1.0 µg/ml (1.8 µM) and the target mutant was not obtained at concentrations above 1.5 µg/ml.

**Generation of transgenic parasites using pac markers through piggyBac transposon.** To determine whether pac can be used as a positive selection marker for genetically manipulated parasites, we used it to select pXL/hdhfr-pac-eGFP-transfected parasites (Fig. 2a). Three selections were performed after transfection: the first after two days, the second after 8–9 days, and the third after 13–14 days. After the second selection, over 95% of parasites were expressing enhanced green fluorescent protein (eGFP) (Fig. 2b,c). The target mutant ratio increased significantly from first to second selections. There was no significant difference between second and third selections (Fig. 2b). A typical transfectant line was also analyzed using flow cytometry. Over 95% of parasites were expressing eGFP after the third selection (Fig. 2f). Three clones were obtained and analyzed using Southern blot analysis (Fig. 2d) to confirm genomic integration. The fragments of integrated pac cassettes (4.8, 5.7, 6.2 kbp) were detected with a pac probe. We did not detect a signal corresponding to plasmid size. Plasmid loci (Table 1). To determine the inhibitory effect of Pac expression on parasite growth, the growth of the three

**Generation of transgenic parasites using pac through double homologous recombination.** We investigated whether pac can be used for gene targeting by assembling constructs that contained pac under the control of an hsp70 promoter and terminator (Fig. 3a). We used a GFP-expressing parasite16 under the control of hsp70 promoter and terminator as a parental strain for transfection. As shown in Fig. 3a, the gfp expression cassette would be replaced by the pac expression cassette after the homologous recombination event, and GFP expression would be lost.

Three selections were performed after transfection: the first after 2 days, the second after 8 days, and the third after 12 days. After the second selection, more than 99% of parasites were GFP-negative (Fig. 3b,d). The mutant ratio increased significantly from first to second selections, but there was no significant difference between second and third selections (Fig. 3b). A typical transfectant line was also analyzed using flow cytometry. More than 99%
Figure 2. Generation of mutants using the *pac* marker and *piggyBac* transposon. (a) Schematic diagram of the *piggyBac* transposon vector containing *hdhfr*, *pac* and *egfp* expression cassettes (pXL/hdhfr-pac-egfp). This plasmid was also used in the experiment of Figs 1 and 6. ITR: inverted terminal repeat. (b) eGFP-positive parasite ratio after each puromycin selection. The ratio was analyzed using fluorescence microscopy. Each bar represents the mean ± SD of three independent experiments. **p < 0.01, n.s.: not significant (paired t-tests). (c) Fluorescence images of parasites after each selection. Parasites were stained with Hoechst 33342. The scale bar represents 10 μm. (d) Southern blot analysis of three mutant clones. Genomic DNA was digested using Eco RV and hybridized with a *pac* probe. The predicted sizes of restriction fragments are shown. WT: wild type. cl.: clone. (e) Growth assay of three clones. WT: wild type. *p < 0.05 (two-tailed unpaired t-tests). (f) Flow cytometry analysis of eGFP positive mutant ratio after each puromycin selection. Numbers above bracketed lines indicate percent parasites with eGFP expression.
of parasites were GFP-negative after the second selection (Fig. 3e). From two of these experiments we obtained two independent clones, which were analyzed using Southern blot analyses (Fig. 3c). The 8.9-kbp fragment containing the gfp cassette was only detected in the parental GFP expressing parasite with the gfp probe. The identical 8.9-kbp signals were detected in both clones, but not in the wild type, with the pac probe and the 5′ hsp70 probe. Based on these results, the in vitro pac-puromycin system is suitable for gene targeting as well as random insertion by transposon.

**Sequential genetic manipulation of pac to pbdhfr-ts parasites.** We demonstrated sequential genetic manipulation using pac and pbdhfr-ts markers for gene targeting. To this end, we assembled a targeting vector composed of a pac cassette flanked by sporozoite microneme proteins essential for the cell traversal 2 (spect2, PBANKA_1006300) gene sequence (Fig. 4a). A GFP-expressing parasite containing pbdhfr-ts marker was used as the parental strain for transfection. The parasite was transfected with the targeting construct, after which puromycin selection was applied. The ratio of the target mutant was determined using genomic Southern blot analysis (Fig. 4b). KO locus signals (3.7 kbp) appeared after the first selection but became dominant after the second selection. We confirmed that GFP was continuously expressed from before transfection to after third selection (Fig. 4c). The KO of spect2 was confirmed by PCR (Fig. 4d). These results show that the in vitro pac-puromycin system can be used in combination with traditional pbdhfr-ts markers to establish double mutant parasites.

**Generation of mutants using the pac::egfp fusion marker.** We investigated whether the pac::egfp fusion gene could be used as a marker by assembling a piggyBac transposon vector that contained the fusion gene under the control of an hsp70 promoter and terminator (Fig. 5a). To confirm that pac::egfp functionally expressed eGFP, in vivo pyrimethamine selection of pXL/hdhfr-pac:egfp transfected parasites was performed prior to in vitro puromycin selection. The expression of eGFP was confirmed by fluorescence microscopy.

After transfection of pXL/hdhfr-pac::egfp, in vitro puromycin selection was conducted three times. Fluorescence analyses showed that more than 60% of parasites expressed egfp after the second selection (Fig. 5b). The mutant ratio increased significantly between first and second selections, but there was no significant difference between second and third selections (Fig. 5b). Fluorescence microscopy confirmed the expression of the eGFP signal (Fig. 5c). Based on these results, pac::egfp is suitable as a dual marker for fluorescence and drug selection.

**Application of the in vitro selection method to the hdhfr-WR99210 system.** To examine the universality of our in vitro selection method, we used the hdhfr-WR99210 system as a model. Preliminary experiments showed that the optimal WR99210 concentration for selection was 6.25 ng/ml (1.6 × 10⁻⁶ M). A wild type parasite was transfected with pXL/hdhfr-pac:egfp, after which WR99210 selection was performed. Proportions of EGFP-positive parasites increased significantly up to the fourth and fifth selections, and the mutant ratio reached more than 90% after the fifth selection (Fig. 6a,b). A typical transfecant line was also analyzed using flow cytometry. Over 90% of parasites were expressing EGFP after the fifth selection (Fig. 6c). These results show that the in vitro selection method may be suitable for application in drug selection systems other than the pac-puromycin system.

**Discussion**

Reverse genetics has been a powerful approach in studying the biology of malaria parasites. However, the limited number of selection markers has hampered the development of the method. To provide further selection markers, we developed a pac marker in P. berghei based on a novel in vitro selection method.

We established this method by combining repeated short-term in vitro cultures with parasite recovery in vivo. The pac marker conferred sufficient resistance to the P. berghei parasite for puromycin selection. Its resistance made it possible to isolate the mutant parasites using piggyBac transposon mutagenesis and gene targeting by homologous recombination. We showed that pac::egfp was also an effective drug selection marker and might constitute a useful tool for imaging experiments. Blood stage parasite growth analyses of Pac-expressing clones indicated that our pac marker cassette did not inhibit parasite growth. While currently available positive selection markers for this parasite only confer resistance to antifolates, pac is independent of antifolate pathways. Thus, the pac-puromycin system can be used in conjunction with the traditional antifolate resistant markers (hdhfr and pbdhfr-ts). The small size of pac (600 bp) is one of its advantages; in Plasmodium, assembling targeting constructs causes a transfection bottleneck because large-sized constructs often result in instability in E. coli caused by the AT-rich Plasmodium genome.

We confirmed the universality of this in vitro selection method by using the traditional hdhfr-WR99210 system as a model. The results indicate that our in vitro method has potential for use in other drug selection systems already established for P. falciparum. In addition, this system could also be used for negative selection in vitro, to

| Clone | Locus | Insertion site |
|-------|-------|---------------|
| 1     | Chromosome 13, 136517–136538 | TTATTCATTTAA-piggyBac- hsp70-TAAATTTAC |
| 2     | Chromosome 13, 2294432–2294433 | AAATATTAA-piggyBac- TAAAAAGAAA |
| 3     | Chromosome 14, 1045361–1045362 | TAATTTGTTAA-piggyBac- TAAATTTATG |

**Table 1.** piggyBac transposon insertion sites.
recycle markers. A further benefit of our method is its relative cheapness, as WR99210 is approximately 100 times more expensive than pyrimethamine (based on Sigma Aldrich prices). The new system significantly reduces the amount of WR99210 required (0.001%) relative to the conventional in vivo method.

Figure 3. Generation of mutants using the pac marker by double-crossover homologous recombination. (a) Schematic diagram showing the targeting of gfp by pac through double crossover homologous recombination. (b) GFP negative parasite ratio after each puromycin selection. The ratio was analyzed using fluorescence microscopy. Each bar represents the mean ± SD of three independent experiments. **p < 0.01, *p < 0.05, n.s.: not significant (paired t-tests). (c) Southern blot analyses of two independent clones. Genomic DNA was digested with Eco RI and hybridized with gfp, pac, and 5’hsp70 probes. The predicted sizes of restriction fragments are shown. WT: wild type, GFP: GFP-expressing parasite (parental line), cl.: clone. Full-length blot images are presented in Supplementary Figure S2. (d) Fluorescence images of parasites after each selection. Parasites were stained with Hoechst 33342. The scale bar represents 10 µm. (e) Flow cytometry analysis of GFP positive mutant ratio after each puromycin selection. Numbers above bracketed lines indicate percent parasites with GFP expression.
Our method can enrich mutant parasites by more than 90% within 2 weeks after transfection, using 2 mice. This level of efficacy has great advantages for cloning mutant parasites, and may reduce the number of mice required to obtain the desired production. Traditional cloning procedures involve using the in vivo limiting dilution method. In our experience, the percentage of target KO parasites obtained when using the conventional method has been lower. Our method, on the other hand, achieves a greatly improved efficacy.

**Figure 4.** Generation of double mutants using pac and pdbhfr-ts markers. (a) Schematic diagram showing the targeting of spect2. pac was inserted into the spect2 locus via double crossover homologous recombination. pdbhfr-ts was used to obtain the parent GFP transgenic parasite by in vivo pyrimethamine selection. (b) Southern blot analysis showing the ratio of spect2 KO mutants after each selection. Genomic DNA was digested using Eco RI and hybridized with a spect2 probe. WT: wild type, KO: spect2 KO. (c) Fluorescence images of parental GFP-expressing parasites and a spect2 KO mutant parasite population after the second selection containing pac and pdbhfr-ts markers. Parasites were stained with Hoechst 33342. The scale bar represents 10 µm. Before: parental GFP-expressing mutant. (d) PCR analysis of genomic DNA isolated from mutant parasites recovered after each selection. Location of PCR primers and the expected products size are shown in (a). Confirmation of the predicted recombination events was achieved with primer combinations specific for 5′ (P3 + P4) or 3′ integration (P5 + P6). An additional primer combination (P1 + P2) was used to assess integration of the pac cassette. The primer sequences can be found as Supplementary Table 1. P: primer.
selection method is around 10–20%, so double limited dilution procedures and at least 20 mice are required to isolate a single cloned parasite. Our in vitro pac-puromycin system, however, allows us to isolate a clone using fewer than five mice. This reduction may save significant expense, time, and labor in rearing mice, and has obvious animal welfare benefits. Furthermore, the method allows the ready generation of several mutants in parallel. In some cases, the drug-selected parasites can be used directly without cloning or flow-sorting steps.

A further advantage of this system is its flexible application for sequential manipulation to mutant parasite generated using traditional antifolate-resistant markers. While various strategies have successfully used negative selection in the P. berghei model, the disadvantage of the traditional marker recycling method is its complexity and the time requirement of several months for the isolation of a double mutant parasite. The "gene out marker out" (GOMO) strategy was developed to address this problem. However, this method is inapplicable.
Figure 6. Application of the *hdhfr*-WR99210 system to *in vitro* selection. (a) eGFP-positive parasite ratio transfected with pXL/*hdhfr*-pac-egfp (Fig. 2a) after each WR992210 selection, analyzed using fluorescence microscopy. Each bar represents the mean ± SD of three independent experiments. *p* < 0.05, ***p* < 0.001, n.s.: not significant (paired t-tests). (b) Fluorescence images of WT and parasites after the fifth selection containing the *hdhfr* marker. Parasites were stained with Hoechst 33342. WT: wild type. The scale bar represents 10 µm. (c) Flow cytometry analysis of eGFP positive mutant ratio after each WR99210 selection. Numbers above bracketed lines indicate percent parasites with eGFP expression.
to parasites already carrying antifolate resistant markers as it requires positive selection using antifolates. Our in vitro pac-puromycin system can readily be used in sequential manipulation experiments (for example when employing gene complementation methods) with the mutant parasites established in past malaria research, using any type of selection method including traditional antifolate markers. In our experiment, the target cloned parasite site was obtained within 3 weeks using less than 10 mice.

In summary, we succeeded in establishing a novel in vitro selection method using the pac marker in *P. berghei*. This in vitro pac-puromycin selection system exhibited high selection efficacy relative to the conventional in vivo method. The method should allow the generation of multiple mutants with greater flexibility and help develop our in-depth understanding of malaria parasite biology.

**Methods**

**Ethics statement.** This study was carried out in strict accordance with the recommendations in the Guide for Laboratory Animals of the Obihiro University of Agriculture and Veterinary Medicine. The protocol was approved by the Committee of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (permit number 28–91).

**Experimental animals and parasites.** ICR and BALB/c (five-week old) mice were obtained from CLEA (Tokyo, Japan). We used the *P. berghei* ANKA strain (obtained from Dr M. Torii, Ehiime University) and a strain constitutively expressing GFP obtained from Dr M. Yuda, Mie University.

**Plasmid construct.** Elements of pXL/hdhfr-pac-egfp and pXL/hdhfr-pac:egfp plasmids were sequentially ligated into a pXL-BacII-DHFR (obtained from the BEI Resources Repository, MRA-916, deposited by John Adams) plasmid backbone20, 21. Firstly, the *hdhfr* expression cassette was excised from pXL-BacII-DHFR, namely pXL-BacII-DHFR (-). Next, *hdhfr*, under the control of elongation factor 1 alpha (ef-1α) promoter and *pbdhfr-ts* terminator, was cloned into pXL-BacII-DHFR (-). Then, for pXL-BacII-DHFR-pac-egfp, egfp was excised from pCX-EGFP Vector22 under the control of an hsp70 promoter and terminator and was then cloned into the plasmid pXL-BacII-DHFR (-) containing the *hdhfr* expression cassette. *pac* was then excised using a PMXS-Puro retroviral vector (Cell Biolabs, San Diego, CA, USA) under the control of an hsp70 promoter and terminator, and cloned in the pXL-BacII-DHFR (-) containing the *hdhfr* and egfp expression cassettes. For pXL-BacII-pac:egfp, *pac* was cloned into a pEFP-N1 vector (Clontech, Palo Alto, CA, USA), then the resulting *pac*:egfp fusion gene was cloned into the plasmid pXL-BacII-DHFR (-) containing the *hdhfr* expression cassette, under the control of another hsp70 promoter and terminator. The promoter and terminators were excised from a Y2 plasmid (obtained from Dr M. Yuda, Mie University).

The transposase expression vector EGF-pgT contained a transposase gene excised from pHTH (obtained from the BEI Resources Repository, MRA-912, deposited by John Adams), under the control of an *ef-1α* promoter and hsp70 terminator, cloned into a pBlueScript (pBS) vector20, 21.

Elements of pBS/spect2 KO were generated into a pBS backbone. The *pac* expression cassette was flanked by 5′ and 3′ sequences of spect2 (pBS/spect2 KO) amplified by PCR (Table S1).

**Parasite transfection and drug selection in vitro.** Parasite transfection experiments were run following standard protocols23. Briefly, about 1 ml of infected blood (1.0–3.0% parasitemia) collected from an ICR mouse by heart puncture was cultured in 50 ml of RPMI 1640 medium (cat no. 23400–021, Thermo Fisher Scientific, MA, USA,) supplemented with NaHCO₃ (0.85 g/l), neomycin sulfate (50 mg/l), and 20% fetal bovine serum for 5 days. The parasites were incubated for 20 h (36.5 °C, 5% CO₂, 5% O₂, 90% N₂). After incubation, the parasites were separated by centrifugation at 500 g for 5 min at room temperature (RT). The supernatant was discarded and the parasites were resuspended in 0.5 ml of culture medium. The suspension (450 μl) was put into a 24-well plate (Thermo Fisher Scientific), and centrifuged at 500 g/ml (1.8 μM) was added. The parasites were incubated for 20 h (36.5°C, 5% CO₂, 5% O₂, 90% N₂). After incubation, the parasites were separated by centrifugation at 500 x g for 5 min at RT, and resuspended in 100 μl of PBS. They were then injected intravenously into a naive ICR mouse. When parasitemia reached 0.5–3.0% (about 5 days post-injection), the same selection procedure was repeated for the 2nd and 3rd selection using about 7 μl of tail blood. Cloning of parasites was done by in vivo limiting dilution method using male BALB/c mice.

In the first selection of double crossover homologous recombination experiments, 200 μl of infected blood was collected in 5 ml of culture medium by heart puncture under anesthesia, using a syringe containing heparin solution. After centrifugation at 500 x g for 8 min at RT, the supernatant was discarded. The blood was resuspended in 10.8 ml of culture medium. The suspension was put into a 25 cm² flask (Thermo Fisher Scientific) with 1.2 ml of...
puromycin (10 μg/ml, diluted by culture medium) solution to make a final concentration of 1.0 μg/ml and a total volume of 12 ml. This suspension was then incubated for 20 h. After drug selection, the sample was centrifuged at 500 × g for 8 min at RT and resuspended in 100 μl of PBS. The suspension was injected intravenously into a naïve ICR mouse. When parasitemia reached 0.5–3.0% (about 5 days post-injection), the selection procedure was repeated. In the 2nd and 3rd selection, about 7 μl of tail blood in 0.5 ml culture medium was used as described above for the piggyBac experiment.

A schematic diagram of pac-puromycin in vitro selection procedures is shown in Supplementary Figure S3.

Mutant ratio calculation by microscopy. About 10 μl of infected blood were collected in 200 μl of PBS, and 2 μl each of heparin solution and Hoechst33342 (1 mM) were added. The sample was then incubated at 37 °C for 5 min. After incubation, the sample was harvested at 500 × g for 5 min, and resuspended in 25–30 μl of RPMI 1640. The suspension was placed on a glass slide, and the number of eGFP-positive parasites stained with Hoechst. At least 1,000 Hoechst-positive parasites were counted in several fields under 400× magnification. The mutant ratio was calculated by dividing the number of eGFP-positive parasites by the number of Hoechst-positive parasites. In experiments where transfection was performed with GFP-positive parasites, the mutant ratio was calculated by dividing the number of GFP-negative parasites by the number of Hoechst-positive parasites.

Flow cytometry analysis. About 20 μl of infected tail blood (5 to 10% parasitemia) was collected in 1 ml of serum-free RPMI 1640 medium containing 1 μM of cell-permeant DNA Dye SYTO59 (Thermo Fisher Scientific) for counter staining, and incubated for 45 min at 20 °C 25. The stained cells were analyzed with an EPICS ALTRA flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with 488 nm argon lasers for GFP or eGFP, and 633 nm HeNe lasers for SYTO59. Parasites were gated using logarithmic forward/side scatter dot plots. The mutant ratio was analyzed by counting the number of GFP or eGFP-positive parasites among gated SYTO59-positive parasites. At least 19,000 SYTO59-positive parasites were analyzed. Data analysis was performed using program Kaluza ver. 1.5 (Beckman Coulter).

Fluorescence analysis. Once parasite nuclei had been stained with Hoechst 33342, fluorescent images were obtained using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and were analyzed using a BZ-II Analyzer (Keyence).

Drug sensitivity test. Wild type P. berghei were transfected with pXL/hdhfr-pac-egfp and EGFP-pgT, selected using pyrimethamine in vivo 23, and named HSP70-PAC. Drug sensitivity tests were performed as previously described 23, 26. Briefly, infected blood (parasitemia 1.0–3.0%) was resuspended in a culture medium to a final cell concentration of 2%. A 500-μl cell suspension was cultured with various concentrations of puromycin (0–15 μM) for 20 h in a 24-well plate. The parasites were harvested by centrifugation and analyzed using Giemsa-stained thin blood smear films. The number of mature schizonts per 300 parasites was counted and normalized to the control. Three experiments were performed in triplicate. IC50 values were calculated using program GraphPad Prism ver.5 (GraphPad, San Diego, CA, USA).

Southern blot analysis. Two micrograms of extracted DNA were digested using Eco RI or Eco RV, separated on agarose gel, transferred onto a Hybond N+ membrane (GE Healthcare, Chalfont St. Giles, UK), and then hybridized with probes labeled using an AlkPhos Direct Kit (GE Healthcare). The signal was detected using a CDP-star detection reagent (GE Healthcare).

Identification of piggyBac insertion site. In summary, genomic DNA of mutant parasites was digested using Nde I and then ligated with T4 DNA ligase. Inverse PCRs were performed using the primer F: ATGTCCACGGAGGAAGAGGC, R: GCCCGCAAAATAAATCTTGC. The sequences of these products were obtained using an ABI3100 Analyzer, and the insertion sites were identified using the PlasmoDB database.

Statistical analyses. Statistical analyses comparing each pac-integrated parasite clone against the wild type parasite were performed using two-tailed unpaired t-tests. The mutant ratio of each selection was compared using paired t-tests. IC50 values were calculated using program GraphPad Prism ver.5 (GraphPad).

References
1. WHO. World Malaria Report 2014, http://apps.who.int/iris/bitstream/10665/144852/2/9789241564830_eng.pdf?ua=1 (Date of access: 09/05/2017) (2014).
2. Mats, J. M. & Kooij, T. W. Towards genome-wide experimental genetics in the in vivo malaria model parasite Plasmodium berghei. Pathog Glob Glob Health 109, 46–60, doi:10.1179/2047773215Y.0000000006 (2015).
3. de Koning-Ward, T. F., Janse, C. J. & Waters, A. P. The development of genetic tools for dissecting the biology of malaria parasites. Annu Rev Microbiol 54, 157–185, doi:10.1146/annurev.micro.54.1.157 (2000).
4. Janse, C. J. et al. A genotype and phenotype database of genetically modified malaria-parasites. Trends Parasitol 27, 31–39, doi:10.1016/j.pt.2010.06.016 (2011).
5. Carlton, J. M. et al. Comparative genomics of the neglected human malaria parasite Plasmodium vivax. Nature 455, 757–763, doi:10.1038/nature07327 (2008).
6. de Koning-Ward, T. F. et al. The selectable marker human dihydrofolate reductase enables sequential genetic manipulation of the Plasmodium berghei genome. Mol Biochem Parasitol 106, 199–212 (2000).
7. Braks, I. A., Franko-Fayard, R., Krooee, H., Janse, C. J. & Waters, A. P. Development and application of a positive-negative selectable marker system for use in reverse genetics in Plasmodium. Nucleic Acids Res 34, e39, doi:10.1093/nar/gnj033 (2006).
8. Goldberg, D. E., Janse, C. J., Cowman, A. F. & Waters, A. P. Has the time come for us to complement our malaria parasites? Trends Parasitol 27, 1–2, doi:10.1016/j.pt.2010.06.017 (2011).
25. Russo, I., Oksman, A., Vaupel, B. & Goldberg, D. E. A calpain unique to alveolates is essential in
22. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. ‘Green mice’ as a source of ubiquitous green cells.
24. Janse, C. J. & Waters, A. P.
23. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the
18. Mamoun, C. B., Gluzman, I. Y., Goyard, S., Beverley, S. M. & Goldberg, D. E. A set of independent selectable markers for transfection
17. Ishino, T., Chinzei, Y. & Yuda, M. A calcium-dependent protein kinase regulates Plasmodium ookinete access to the midgut epithelial cell. Mol Microbiol 59, 1175–1184, doi:10.1111/j.1365-2958.2005.05014.x (2006).
17. Ishino, T., Chinzei, Y. & Yuda, M. A Plasmodium sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. Cell Microbiol 7, 199–208, doi:10.1111/j.1462-5822.2004.00447.x (2005).
16. Ishino, T., Orito, Y., Chinzei, Y. & Yuda, M. A calcium-dependent protein kinase regulates Plasmodium falciparum. Mol Biochem Parasitol 117, 155–160 (2001).
15. de Koning-Ward, T. F., Waters, A. P. & Crabb, B. S. Puromycin-A: an efficient and drug-selectable marker for transfection of rodent malaria parasites. Proc Natl Acad Sci USA 96, 8716–8720 (1999).
14. Lin, J. W. et al. A novel ‘gene insertion/marker out’ (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS One 6, e20928, doi:10.1371/journal.pone.0020928 (2011).
13. Mielke, C., Tummler, M. & Bode, J. A simple assay for puromycin N-acetyltransferase: selectable marker and reporter. Trends Genet 11, 258–259 (1995).
12. Manzoni, G. et al. A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent parasites. Insect Mol Biol 14, 17–30, doi:10.1111/j.1365-2583.2004.00523.x (2005).
11. Li, X. et al. piggyBac internal sequences are necessary for efficient transformation of target genomes. Insect Mol Biol 14, 313–319 (1997).
10. Annoura, T. et al. The development of P. berghei liver stages is dependent on the presence of Class I histocompatibility complex.
9. Jobe, O. et al. Genetically attenuated Plasmodium berghei liver stages induce sterile protracted protection that is mediated by major histocompatibility complex Class I-dependent interferon-g-producing CD8+ T cells. J Infect Dis 196, 599–607, doi:10.1086/519743 (2007).
8. Mamoun, C. B., Gluzman, I. Y., Goyard, S., Beverley, S. M. & Goldberg, D. E. A set of independent selectable markers for transfection
7. Ishino, T., Chinzei, Y. & Yuda, M. A calcium-dependent protein kinase regulates Plasmodium ookinete access to the midgut epithelial cell. Mol Microbiol 59, 1175–1184, doi:10.1111/j.1365-2958.2005.05014.x (2006).
6. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat Protoc 1, 346–356, doi:10.1038/nprot.2006.53 (2006).
5. Manoun, C. B., Gluzman, I. Y., Goyard, S., Beverley, S. M. & Goldberg, D. E. A set of independent selectable markers for transfection of the human malaria parasite Plasmodium falciparum. Proc Natl Acad Sci USA 96, 8716–8720 (1999).
4. Lin, J. W. et al. A novel ‘gene insertion/marker out’ (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS One 6, e20928, doi:10.1371/journal.pone.0020928 (2011).
3. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat Protoc 1, 346–356, doi:10.1038/nprot.2006.53 (2006).
2. Janse, C. J. & Waters, A. P. Plasmodium berghei: the application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitology today 11, 138–143 (1995).
1. Russo, I., Oksman, A., Vaupel, B. & Goldberg, D. E. A calpain unique to alveolates is essential in
Acknowledgements
The authors thank Dr. M. Trii and Dr. M. Yuda for donating the two P. berghei strains used in this study. We also thank Dr. M. Yuda for donating the Y2 plasmid, and the BEI Resources Repository for donating the pXL-BacII-DHFR and pHTH. This study was supported by JSPS KAKENHI (Grant Numbers JP25292168, JP25660223, JP6H05026 to SF).
Author Contributions
A.S. and S.F. designed the experiments. A.S., M.K. and S.F. carried out the experiments and analyzed the data. H.M. and S.K. contributed to the data analysis and discussion. A.S., H.B. and S.F. wrote the manuscript, which was edited by all other co-authors. S.F. supervised the study.
Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-04244-0
Competing Interests: The authors declare that they have no competing interests.
Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.
© The Author(s) 2017