Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes

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Edited by Thomas E. Wellems, National Institutes of Health, Bethesda, MD, and approved November 28, 2012 (received for review September 21, 2012)

Research into the aetiological agent of the most widespread form of severe malaria, *Plasmodium falciparum*, has benefitted enormously from the ability to culture and genetically manipulate blood-stage forms of the parasite in vitro. However, most malaria outside Africa is caused by a distinct *Plasmodium* species, *Plasmodium vivax*, and it has become increasingly apparent that zoonotic infection by the closely related simian parasite *Plasmodium knowlesi* is a frequent cause of life-threatening malaria in regions of southeast Asia. Neither of these important malarial species can be cultured in human cells in vitro, requiring access to primates with the associated ethical and practical constraints. We report the successful adaptation of *P. knowlesi* to continuous culture in human erythrocytes. Human-adapted *P. knowlesi* clones maintain their capacity to replicate in monkey erythrocytes and can be genetically modified with unprecedented efficiency, providing an important and unique model for studying conserved aspects of malarial biology as well as species-specific features of an emerging pathogen.

invasion | transfection

The development of a continuous culture system for asexual blood stages of the most deadly human malaria parasite, *Plasmodium falciparum* (1, 2), proved a milestone in malaria research, enabling genetic modification of the parasite (3), high-throughput drug screening (4), and other fundamental advances in parasite biology. Adaptation of other human malaria parasite species to in vitro culture has proved more challenging, and none of the additional four parasite species that cause human malaria can be continuously maintained in human RBC. This difficulty is a significant obstacle to studying these pathogens, which differ from *P. falciparum* in important aspects of biology and the pathology they cause. Furthermore, although considerable progress has been made in the development of transgenic technologies for *Plasmodium*, *P. falciparum* remains poorly amenable to genetic manipulation, with a typical transfection efficiency of only $\sim 10^{-6}$ (5). Additional in vitro human malaria parasite models that are genetically tractable and that complement the *P. falciparum* system have tremendous potential.

Much of the early work on the mechanics of RBC invasion by the malaria parasite used the simian parasite *Plasmodium knowlesi*. This species has a 24-h erythrocytic life cycle and large, long-lived invasive merozoites, facilitating the use of electron and video microscopy to dissect the dynamics of erythrocyte invasion (6–8). *P. knowlesi* can be cultured in vitro in rhesus monkey (*Macaca mulata*) RBC with rhesus or human serum (9, 10). Importantly, *P. knowlesi* is amenable to genetic manipulation, with reported transfection efficiencies similar to those achieved with the rodent malaria model *Plasmodium berghei* and far surpassing those attained in *P. falciparum* (10, 11). *P. knowlesi* is phylogenetically closely related to *Plasmodium vivax*, the most important cause of malaria outside of Africa (12), so its study can provide insights into unique aspects of the biology of *P. vivax*. *P. knowlesi* has recently been identified as a significant cause of often severe human malaria in southeast Asia (13, 14), where it is likely transmitted as a zoonosis from its natural host the kra monkey or cynomolgus macaque (*Macaca fascicularis*). Collectively, because of the advantages of using *P. knowlesi* to study the cell biology of the parasite, the recognition of *P. knowlesi* as an emerging threat, and the recent publication of the *P. knowlesi* genome (12), *P. knowlesi* could provide an ideal in vitro malaria parasite model. However, previous attempts to adapt *P. knowlesi* to culture in human RBC have failed (15), and the requirement for a supply of macaque RBC and serum has restricted work on this parasite to the very few laboratories worldwide with access to primate facilities.

Here we describe the adaptation of a *P. knowlesi* line to continuous culture in human RBC without requirement for macaque cells or serum. Importantly, the line retains its capacity to infect macaque cells. Clones derived from the human-adapted *P. knowlesi* line were used in a scalable 96-well format FACS-based assay to investigate the importance of major human RBC surface polymorphisms for efficient parasite invasion and growth. Using specifically designed *P. knowlesi* reporter constructs we demonstrate that the human-adapted *P. knowlesi* clone is highly amenable to genetic manipulation, with a 100,000-fold increased transfection efficiency compared with that achieved for *P. falciparum*, and exceeding that achieved with *P. berghei*. This provides an opportunity unique in any malaria parasite species to interrogate the phenotypic consequences of genetic modifications within the first generation of transgenic asexual blood-stage parasites.

Results

Adaptation of *P. knowlesi* to Long-Term Continuous Culture in Human RBC. We initiated in vitro cultures using frozen stocks from 1976 of the *P. knowlesi* A1 strain (derived from the *P. knowlesi* H strain) (16), which previously had been maintained exclusively in vivo in rhesus macaques. The parasites were added to freshly drawn *M. fascicularis* RBC at 2% hematocrit in a modified RPMI medium 1640 containing 0.5% (wt/vol) Albumax II and 10% (vol/vol) human serum in static cultures at 37 °C. The medium was

Author contributions: R.W.M., A.P., A.A.H., and M.J.B. designed research; R.W.M., F.R., Y.S.H., and A.P. performed research; J.H., N.A., and G.H.M. contributed new reagents/analytic tools; R.W.M., F.R., Y.S.H., A.P., A.A.H., and M.J.B. analyzed data; and R.W.M., A.A.H., and M.J.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HF564624, HF564625, and HF564626).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1216457110/-/DCSupplemental.

changed daily for the first 10 d and every 2–3 d thereafter. In line with the ~24-h life cycle of *P. knowlesi* (16), the parasitaemia initially increased on average twofold daily, but after ~8 wk in culture this rate had risen steadily to four- to sevenfold per day, with an erythrocytic cycle length of ~27 h. The resulting culture-adapted line was named A1-O (Fig. 1*A*). Attempts to culture the original A1 stock directly in human RBC, or to passage the A1-O parasites into 100% human RBC failed, with very low growth rates and complete loss of parasites within a week. Indirect immunofluorescence analysis (IFA) of the parasites showed that they maintained a strong preference for cynomolgus cells but were capable of invading and developing to schizont stage within human RBC (Fig. 1*B*). Accordingly, the A1-O line was divided into two separate cultures, one of which we continued to maintain solely in 100% cynomolgus RBC (called A1-C) (Fig. 1*A*); the second, called A1-H(E), was maintained in a 4:1 mixture of human and cynomolgus cells. We reasoned that this ratio of human to cynomolgus cells would be sufficient to sustain parasite growth in the cynomolgus cells yet confer a selective advantage on those parasites able to invade and replicate within human RBC. After a further 8-mo continuous culture of the A1-H(E) line it was possible to remove cynomolgus cells entirely, with the growth rates in 100% human RBC initially averaging twofold per day, before eventually increasing to three- to fivefold per day. The resulting A1-H line was then cloned by limiting dilution, in parallel with the A1-C line (maintained continuously in cynomolgus cells), producing parasite clones A1-H.1 to A1-H.5, and A1-C.1 to A1-C.2.

Clones A1-H.1 (Fig. S1) and A1-C.1 were selected for detailed characterization. To compare their relative invasion and intracellular growth rates in human and cynomolgus RBC, purified schizonts of each clone were added to fresh macaque or human cells to a parasitaemia of 1%, and then the parasitaemia determined at intervals over the course of the ensuing cycle using a FACS-based protocol. Time points (4 h and 24 h from initiation of the assay) were chosen first to obtain estimates of invasion rate by determining the efficiency of new ring-stage parasite formation following schizont rupture, and second to quantify intracellular growth by determining the parasitaemia as the intracellular parasites reached maturity. The results (Fig. 1*C*) demonstrated that for the A1-C.1 clone the main barrier to growth in human RBC was low invasion efficiency, although a slight decrease in parasitaemia following invasion may reflect a small defect in intracellular development. In contrast, the A1-H.1 clone could invade and grow in cynomolgus RBC at rates similar to those in human RBC, a remarkable observation given the fact that the A1-H line from which it was derived had been cultured exclusively in human RBC for nearly a year before these assays.

Human-Adapted *P. knowlesi* Requires the Duffy Receptor for Invasion but Demonstrates No Preference for Different Duffy Haplotypes or Other Major Blood-Group Antigens. It has long been recognized that RBC invasion by both *P. knowlesi* and *P. vivax* requires the presence of the Duffy antigen (Fy), a RBC surface chemokine receptor (17, 18). Three major Duffy alleles exist: Fy^a and Fy^b, which differ by a Gly42Asp substitution, and Fy^-, in which a point mutation in the promoter region abolishes Duffy expression in RBC (19). A recent analysis of *P. vivax* malaria patients has suggested that, in addition to the expected refractoriness of Duffy-negative [$Fy^{(a-b-)}$] individuals to infection, $Fy^{(a+b-)}$ individuals are less susceptible to clinical episodes of *P. vivax* malaria than $Fy^{(a-b+)}$ individuals (20), and that this correlates with relatively poor binding of the major *P. vivax* Duffy-binding protein to the Fy^a Duffy isotype. Although we routinely screened the human blood used during culture-adaptation of the *P. knowlesi* lines for Duffy positivity, we noticed that occasional samples of Duffy-positive blood did not support strong growth of the parasite, raising the possibility that the Duffy phenotype might influence growth rates. The A1-H.1 parasite clone could be readily maintained in 96-well microtiter plates, so we exploited this to examine the Duffy blood-type preference of the clone, as well its dependence on other major blood-type antigens. Purified synchronous A1-H.1 schizonts were added to freshly drawn and washed RBC from 33 volunteers of diverse ethnicity in triplicate (starting parasitaemia ~1%), and the parasitaemia monitored by FACS over the ensuing two intraerythrocytic

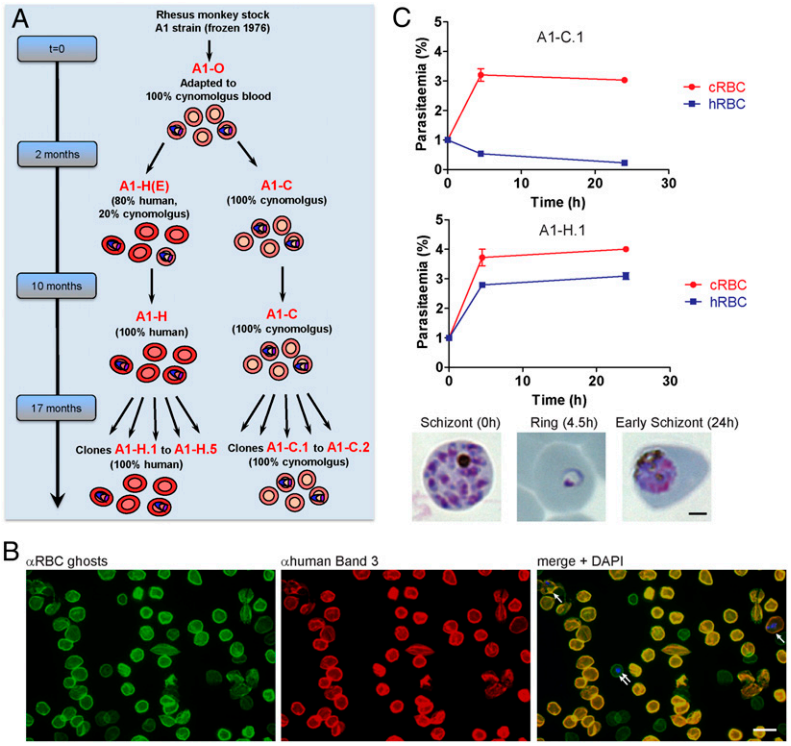

Fig. 1. Adaptation of the *P. knowlesi* A1 strain to continuous culture in human erythrocytes. (*A*) Strategy used to adapt the parasites to growth exclusively in human RBC. (*B*) Analysis of the A1-H(E) line to detect the first signs of stable growth in human RBC. Thin films were probed with an anti-RBC ghost antibody (green), which labels both human cells and cynomolgus cells, plus a monoclonal anti-human Band 3 antibody (red), which labels only human cells. Parasitized human and cynomolgus RBC, identified by staining parasite nuclei with DAPI, are indicated with a single and double white arrow, respectively. (Scale bar, 10 μm.) (*C*) Adaptation primarily involves improvements in invasion of human RBC. Purified schizonts of the nonadapted A1-C.1 clone (*Top*) or adapted A1-H.1 clone (*Middle*) were added to cynomolgus or human RBC to obtain an initial parasitaemia of 1%. Parasitaemia values were determined immediately after schizont rupture was complete and new ring stages had formed (4.5 h), and again at 24 h when the new generation of parasites had reached schizont stage. Note that no residual schizonts were present in any of the cultures by 10 h after initiation of the experiments, demonstrating efficient egress in all cases. Error bars, ± 1 SEM. Giemsa stained images (*Bottom*) show representative parasite stages from 0-, 4.5-, and 24-h time points. (Scale bar, 2 μm.)

cycles (~48 h). Blood-group phenotyping was performed using an agglutination assay to determine the Duffy, ABO, Kell, and Rhesus D, C, c, E, and e antigens of each blood sample. As shown in Fig. 2*A*, no significant association between parasite growth rate and the four major blood types was evident. Similar analyses revealed no dependence on Rhesus antigen polymorphisms or the Kell blood type (Fig. S2). In contrast, there was a clear dependence on the presence of the Duffy antigen, with no parasite replication in Duffy-negative blood, confirming that a Duffy-dependent invasion pathway is essential for the A1-H.1 clone (Fig. 2*B*). In contrast with the indications from the *P. vivax* study referred to above, no significant differences were observed between growth rates in RBC of the three Duffy-positive phenotypes [Fy^a, Fy^b, and $Fy^{(a+b+)}$]. Two Duffy-positive blood samples, one Fy^{a+} and one Fy^{b+}, sustained markedly reduced growth rates (less than sixfold over two cycles). Both of these blood samples produced lower-than-average scores in the agglutination phenotyping assays, suggesting that they were likely heterozygous with a Fy^- allele, resulting in reduced Duffy receptor density on the RBC surface (21), and explaining the reduced invasion rate. Ethical restrictions on this study did not permit donor genotyping to confirm this. The *P. knowlesi* Duffy binding protein-α (DBP-α) plays a key role in invasion of Duffy-positive human RBC (22). Sequencing of the *DBP-α* gene from the A1-O, A1-H.1, and A1-C.1 parasites and comparison with that of the *P. knowlesi* H strain (12) identified just a single nonsynonymous polymorphism unique to the A1-H.1 clone (Fig. S3). Further work will be required to establish whether this plays any role in adaptation to growth in human RBC.

High-Efficiency Transfection with Episomal Constructs Allows Phenotypic Analysis of First-Generation Transgenic Parasites. Previous work has demonstrated that *P. knowlesi* cultured in macaque RBC is amenable to genetic manipulation (10). To explore whether our human-adapted parasites shared similar characteristics, we investigated the efficiency with which the A1-H.1 clone could be transfected with episomal vectors. We initially used vectors based on pHH1 (23), which is in common use for *P. falciparum* transgenesis and uses predominantly *P. falciparum* regulatory sequences to drive transgene and selectable marker expression. Although transfection with these constructs eventually produced drug-resistant parasites, lower than expected transgene expression levels were observed. To improve expression, we replaced the *P. falciparum* promoters with *P. knowlesi* sequences, using the *Pkef1α* 5′ UTR (PlasmoDB ID PKH_111400) to drive the *hdhfr* selectable marker and the *Pkhsp70* 5′ UTR (PlasmoDB ID PKH_093190) to drive GFP expression, resulting in plasmid $PkconGFP_{ep}$ (Fig. S4). Successful transfection in *P. berghei* relies on the electroporation of purified mature schizonts, so to mimic similar conditions, highly synchronous *P. knowlesi* A1-H.1 schizonts were produced by centrifugation on Nycodenz cushions. The Amaxa electroporation system has provided significant improvements in transfection efficiency in *P. berghei* (24), so $PkconGFP_{ep}$ was introduced into the *P. knowlesi* schizonts by electroporation with the Amaxa 4D electroporator. Drug selection (2.5 nM WR99210) was applied ~18 h post-electroporation. To determine transfection efficiencies, parasites transfected in triplicate with either $PkconGFP_{ep}$ or no DNA (mock transfection control) were monitored over the course of 4 d for parasitaemia and proportion of GFP^+ parasites. As shown in Fig. 3, ~30% of the $PkconGFP_{ep}$-transfected parasites were GFP^+ on day 1 before the application of drug selection. By day 3 post-transfection, the drug selection had killed almost all of the control parasites, such that by day 4 nearly 100% of the $PkconGFP_{ep}$-transfected parasites were GFP^+ and at a sufficiently high parasitaemia (~7%) to enable cryopreservation of the parasites and preparation of material for biochemical analysis. In our hands, attaining these numbers of transgenic *P. falciparum* parasites with current transfection methodology (using a similar Amaxa electroporation protocol) takes at least 14–21 d, corresponding to an estimated transfection efficiency at least 1,000-fold lower than that reported here with the *P. knowlesi* A1-H.1 clone.

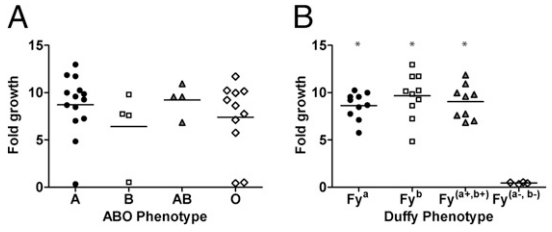

Fig. 2. In vitro growth of human-adapted *P. knowlesi* is Duffy-dependent. Purified *P. knowlesi* A1-H.1 clone schizonts were added in triplicate to washed RBC from 33 volunteer blood donors to attain a parasitaemia of ~1%. Parasitaemia values at were then monitored by FACS over the ensuing two intraerythrocytic growth cycles (48 h), and mean average of fold growth rates plotted against (*A*) ABO blood group, or (*B*) Duffy phenotype [Fy^{a+}, Fy^{b+}, $Fy^{(a+b+)}$, $Fy^{(a-b-)}$]. Black bars indicate mean growth rate in each blood type, and an asterisk denotes Duffy-positive blood groups supporting growth rates significantly different from those in Duffy-negative blood groups (two-tailed *t* test, *P* value ≤ 0.001). Growth was highly dependent on Duffy positivity, but showed no dependence on ABO blood type. Note that the four blood samples in *A* that show low growth rates are the Duffy-negative [$Fy^{(a-b-)}$] samples.

Rapid Genomic Integration by Homologous Recombination of Linear DNA Constructs in Human-Adapted *P. knowlesi*. Disruption, mutagenesis, or tagging of endogenous malarial genes requires integration of DNA constructs into the parasite genome. This integration can currently be achieved with *P. falciparum* but requires transfection with circular plasmid, which is maintained episomally and can only be removed by either repeated cycles of drug selection or the use of negative selection markers (25). To determine whether the observed high-transfection efficiency facilitated genomic modification of our human-adapted *P. knowlesi* parasites, we modified $PkconGFP_{ep}$ to incorporate an ~1.2-kb region of the *Pkp230p* gene (PlasmoDB ID PKH_041110), which is dispensable in *P. berghei* and was therefore considered a suitable targeting sequence for homologous integration (24). The resulting construct, called $PkconGFP_{p230p}$, was linearized using a KpnI site situated within the targeting region to prevent its maintenance as a stable episome and to promote integration through single crossover homologous recombination into the *P. knowlesi p230p* locus (Fig. 4*A*). Parasite cultures transfected with $PkconGFP_{p230p}$ contained ~30% GFP^+ parasites on day 1, similar to those transfected with $PkconGFP_{ep}$ (Fig. 4*B*). Following application of drug selection, however, the proportion of GFP^+ parasites increased more slowly than observed with the $PkconGFP_{ep}$ construct, but the parasitaemia remained approximately the same for the first 10 d of culture, indicating that the majority of the initial GFP^+ parasite population contained only the linear, nonintegrated $PkconGFP_{p230p}$ plasmid. Integration into the *p230p* locus was detectable by PCR as early as day 8 posttransfection, and genotyping of parasites on day 16 demonstrated integration had occurred in a significant proportion of the parasites (Fig. 4*C*). Following limiting dilution cloning of the transgenic parasites in the absence of drug, 40% of the resulting clones displayed the expected integration genotype. These clones displayed strong GFP fluorescence throughout the entire erythrocytic lifecycle (Fig. 4*D*). Our results demonstrate that genome-modified transgenic *P. knowlesi* clones can be readily generated without any requirement for drug cycling or negative selection.

Discussion

We have produced a *P. knowlesi* line and derived clones adapted to robust, continuous growth in human RBC. This process now enables any laboratory with suitable containment facilities to

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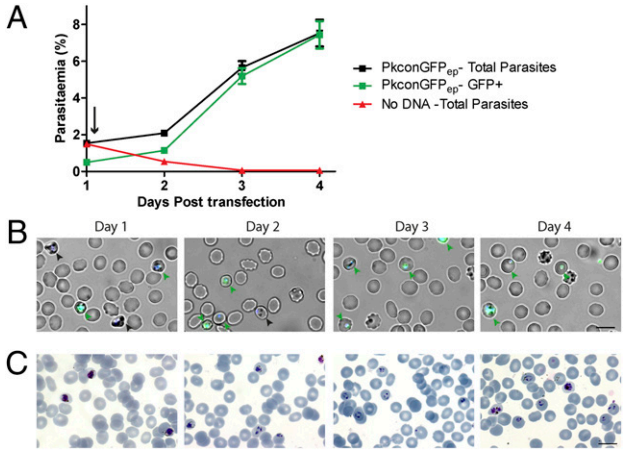

Fig. 3. High efficiency transfection of human-adapted *P. knowlesi*. Purified mature *P. knowlesi* clone A1-H.1 schizonts (~1 × 10^8 per cuvette) were electroporated in triplicate with either PkconGFP$_{ep}$ or no DNA (mock transfection). The transfected parasites were supplemented with 150 μL fresh human RBC, returned to culture, and monitored daily. (*A*) Time-dependent change in total parasitaemia and total proportion of GPF$^+$ parasites determined by fluorescence microscopy. Arrow, point of addition of the selection drug WR99210 (2.5 nM). Over 50% of the PkconGFP$_{ep}$-transfected parasites were visibly GFP$^+$ by day 2, increasing to ~100% by day 4. Error bars denote ± 1 SEM. (*B*) Light microscopic images of live PkconGFP$_{ep}$-transfected parasites taken on the indicated days post transfection, stained with Hoechst 33342 (merge of GFP, Hoechst, and brightfield views). GFP$^+$ and GFP$^-$ parasites are indicated (with green and black arrowheads, respectively). (*C*) Giemsa-stained thin films of the same cultures taken on the same days posttransfection as in *B*. (Scale bar, 10 μm.)

work on *P. knowlesi*, with no requirement for macaque blood or serum. Our achievement provides malarial researchers with access to some of the uniquely tractable features of *P. knowlesi* asexual blood-stage cell biology suitable for studying RBC invasion and the receptor–ligand interactions involved. Analysis of the adaptation process itself should enable us to learn more about determinants of host specificity in malaria. To this end, detailed genome-scale comparisons of the A1-H.1 and A1-C.1 clones are already underway (at both the genomic and RNAseq level) and we anticipate that these will allow identification of the genetic or epigenetic determinants required for growth in human RBC, potentially representing virulence factors important for human *P. knowlesi* malaria infections. Extended maintenance of *Plasmodium* in asexual blood-stage form is well documented to result in loss of capacity to form the sexual gametocyte forms essential for mosquito transmission (26), and indeed none of our culture-adapted lines appears able to produce gametocytes. The genome sequence information may aid in identifying the genetic changes responsible for this, potentially facilitating culture of gametocyte-producing *P. knowlesi* lines in the future.

Using a 96-well microplate-based assay suitable for a medium to high-throughput screen (including drug screens), we were able to demonstrate the requirement for the Duffy antigen in the A1-H.1 clone. Previous single-cycle invasion assays in human RBC using *P. knowlesi* parasites from infected macaques failed to identify enhanced invasion rates in Fy^{b+} cells, despite the demonstration that the parasite RBC receptor, DBP, binds more strongly to Fy^{b+} than Fy^{a+} RBC (22). Our experiments, carried out with a fully culture-adapted clone, allowed for improved sensitivity because of higher invasion rates and multiple invasion cycles, but similarly failed to demonstrate any Fy-dependent difference in growth rate. With the caveat that our data arise from the use of a single human-adapted *P. knowlesi* clone, we suggest that there are fundamental differences between *P. vivax* and *P. knowlesi*, or that the recently observed effect of Duffy polymorphisms on the incidence of clinical *P. vivax* malaria (20) may be because of an increased sensitivity of Fy^{a+} RBC to invasion-blocking antibodies and in some cases allele dosage, rather than a direct effect on invasion efficiency.

Although the A1-H.1 clone displayed greatly improved invasion and growth rates in human RBC compared with the A1 stock from which it was derived, it maintained the capacity to grow well in macaque RBC, often exhibiting slightly higher replication rates than in human RBC. The fact that this characteristic was retained even after many months of culture exclusively in human RBCs presents the exciting possibility of designing experiments that involve shuttling between both macaque and human host cells in vitro. Previous work has demonstrated that it is possible to establish in vivo infections in rhesus macaques from parasites cultured long-term in rhesus RBCs (10), and we aim to explore whether the A1-H clones (and transgenic mutants thereof) may be suitable for similar experiments. The capacity to combine in vitro experiments with in vivo studies in primate models will be invaluable for investigations of parasite pathogenesis and for

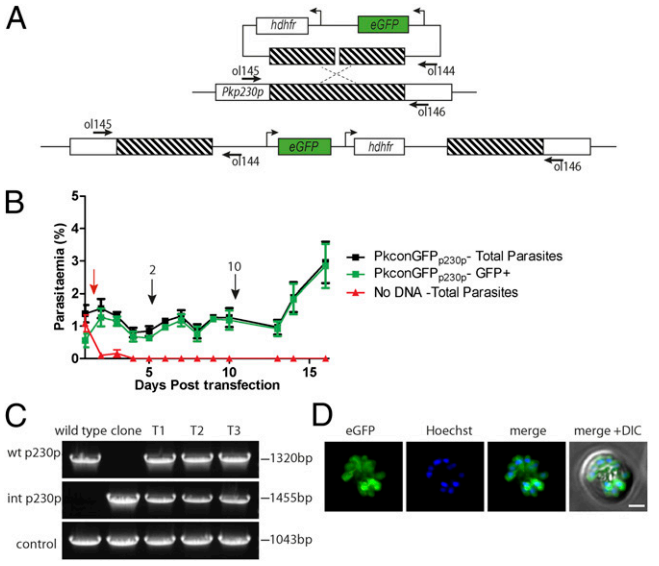

Fig. 4. Rapid genomic integration of linear DNA constructs in human-adapted *P. knowlesi*. *P. knowlesi* clone A1-H.1 schizonts were transfected in triplicate with either linearized PkconGFP$_{p230p}$ or no DNA (mock transfection) and monitored daily. (*A*) Schematic of introduction of linearized PkconGFP$_{p230p}$ into the *P. knowlesi* genome. The construct, containing both *eGFP* and *hdhfr* expression cassettes, is predicted to integrate into the *Pkp230p* locus via single cross-over homologous recombination with the targeting sequence (hatched). Locations of primers used for PCR analysis are marked with thick black arrows. (*B*) Time-dependent change in total parasitaemia and total proportion of GFP$^+$ parasites following transfection. The red arrow indicates the point of addition of the selection drug WR99210, and the black arrows and numbers above indicate points at which cultures were diluted, and the fold-dilution. Over 50% of the PkconGFP$_{p230p}$-transfected parasites were GFP$^+$ by day 2. Parasite replication rate was low for the first 10 d of culture (presumably because of selection of integrants) before increasing to normal rates. Error bars denote ± 1 SEM. (*C*) Diagnostic PCR analysis of three independent PkconGFP$_{p230p}$ transfected lines (T1–T3) on day 16 after transfection, as well a parasite clone derived by limiting dilution. (*Top*) Amplification of a band specific for the wild-type *p230p* locus (primers ol145 and ol146). (*Middle*) Amplification of a band expected only following correct integration of PkconGFP$_{p230p}$ into the *Pkp230p* locus (primers ol145 and ol144). (*Bottom*) Control reaction (with primers ol75 and ol76, specific for an irrelevant gene) expected to produce a product in all parasites. (*D*) Light microscopic image of the *PkconGFP$_{p230p}$* clone showing GFP expression in a segmented schizont, stained with Hoechst 33342. The fluorescent images were produced from a deconvoluted *z*-stack and displayed as extended focus images. (Scale bar, 2 μm.)

vaccine development, particularly vaccines for *P. vivax*, which shares invasion pathways with *P. knowlesi* but lacks a long-term in vitro culture system. Extensive in vitro characterization of parasites used for in vivo macaque experiments will improve the value of the in vivo work, an important goal consistent with the replacement, reduction, and refinement principles of ethical animal experimentation.

Using mature schizonts and an Amaxa electroporation system, we have achieved the highest reported transfection efficiency for any malaria parasite. In both *P. berghei* and *P. falciparum*, the only parasite species previously used extensively for genetic manipulation, relatively low transfection efficiencies [10^{-2} and 10^{-6}, respectively (5, 24)] require parasites to undergo several cycles of replication (taking around 1 wk in *P. berghei* and 14–21 d at best in *P. falciparum*) before they are at levels sufficient for analysis. One consequence of this delay is that modifications that block parasite invasion or intracellular growth are lethal and the transgenic parasites are never recovered. With our human-adapted *P. knowlesi* clone we routinely achieve a transfection efficiency of 30–40%, which not only enables more rapid production of transgenic parasites, but also is unique in allowing analysis of transgenic parasites within the first generation following transfection. Thus, parasites expressing episomal transgenes with dominant negative effects could be examined in the first cycle following transfection, using GFP expression to identify the transgenic parasites. Genomic integration of DNA through single or double homologous recombination has been crucial in the delineation of gene function in malaria parasites. We have shown that this result can readily be achieved with our human-adapted clone, using the nonessential *p230p* gene to target integration into the genome. Recombination efficiencies are highly dependent on the identity and length of the targeting regions used, and increased targeting sequence length has been demonstrated to improve recombination efficiency by up to 10-fold in *P. berghei* (27). We therefore anticipate that further increases in integration efficiency are likely with improvements in transfection construct design, such as use of the recently described pJAZZ vector-based recombineering approach to produce linear malarial transfection constructs with long homology arms (27).

The *P. berghei* malaria model (24) is the current model of choice for reverse genetic studies. However, in vivo infections with this species are asynchronous, the parasite cannot be cultured in vitro long term, and the strict requirement for mouse work (e.g., for producing parasite clones) are major bottlenecks in moves toward high-throughput production of transgenic parasites. The transfection experiments described here with the *P. knowlesi* A1-H.1 clone have the potential to be scaled up considerably, because the Amaxa electroporation device settings used are fully compatible with 96-well plates, and we have already demonstrated the parasites can be maintained in this format. This development therefore provides a tantalizing possibility of a genome-wide gene knock-out project in a human malaria pathogen.

Methods

Human and Macaque Blood. Cynomolgus blood was collected by venous puncture into K_2EDTA vacutainers. Human blood for routine culturing was obtained from the United Kingdom National Blood Transfusion service. Blood for analysis of effect of human blood groups on in vitro growth of *P. knowlesi* clone A1-H.1 was obtained with full consent from volunteers. Venous blood (~10 mL) collected into $CPDA_1$ S-Monovette vials (Sarstedt) was anonymized, and then RBCs washed with RPMI 1640 and stored at 4 °C. Blood typing was carried out using DG Gel cards (Grifols) according to the manufacturer's instructions. For ABO, Rhesus, and Kell antigens the DG Gel ABO/CDE and DG Gel Rh + Kell cards were used. For Duffy antigen typing, the DG Gel Coombs card was used along with anti-Fy^a and anti-Fy^b sera (Lorne Laboratories).

Parasite Adaptation to Growth in Human RBCs. All *P. knowlesi* parasite cultures were initiated from a 1-mL *P. knowlesi* A1 stabilate originally frozen in 1976. The sample was thawed and supplemented with 1 mL 0.6 M NaCl added drop-wise before pelleting the cells by centrifugation at 270 × *g* for 3 min. The supernatant was discarded, a further 1 mL 0.6 M NaCl added drop-wise, and the cells recovered. This process was repeated once more, then the pelleted cells were finally resuspended in complete medium, comprising RPMI 1640 (Invitrogen) with the following additions: 2.3 g/L sodium bicarbonate, 4 g/L dextrose, 5.957 g/L Hepes, 0.05 g/L hypoxanthine, 5 g /L Albumax II, 0.025 g/L gentamycin sulfate, 0.292 g/L L-glutamine, and 10% (vol/vol) human AB^+ serum. Fresh macaque RBCs were added to a 2% hematocrit and the parasites cultured at 37 °C in flasks gassed with a mixture of 90% N_2, 5% O_2, and 5% CO_2. Cultures were monitored by microscopy using Giemsa-stained thin films, and parasitaemia maintained at between 0.5% and 10%. The medium was changed daily for the first 10 d of culture, and every 2–3 d thereafter. For adaptation, A1-C parasites were maintained under the same conditions but in 100% cynomolgus RBC, A1-H(E) parasites were maintained in a mixture of 80% human RBC and 20% macaque RBC, and fully human-adapted parasites (A1-H and derived clones) were maintained in human RBC alone.

Parasite Synchronization. Mature schizonts of culture-adapted *P. knowlesi* were enriched by centrifugation at 900 × *g* for 12 min on a cushion of 55% Nycodenz (Axis-Shield) stock solution [27.6% (wt/vol) Nycodenz powder in 10 mM Hepes, pH 7.0], diluted into RPMI medium 1640. The interface containing schizonts was returned to culture with fresh RBCs in complete medium for at least 1 h at 37 °C to allow schizont rupture and formation of new ring-stage parasites. The culture was then reapplied to a Nycodenz cushion and centrifuged again, this time discarding the residual schizonts. The pellet, comprising newly invaded ring-stage parasites and uninfected RBCs, was returned to culture. Synchronization was repeated as necessary and always performed in the cycle preceding transfection.

Limiting Dilution Cloning and Cryopreservation of *P. knowlesi*. Parasite cultures were diluted in complete medium containing RBC (2% hematocrit) to obtain a suspension containing three parasitized cells per milliliter, then 100 μL of this added to each well of a 96-well microtiter plate. Plates were cultured in a gassed chamber, feeding at 3- to 4-d intervals with medium containing RBCs (1% hematocrit) once a week. After ~2 wk, wells containing growing parasite clones were identified microscopically by Giemsa-stained thin films, then expanded for genotypic and phenotypic analysis. For cryopreservation, 700 μL of freezing solution [111 mM NaCl, 166 mM D-sorbitol, 28% (wt/vol) glycerol] was added drop-wise to 300 μL of pelleted ring-stage parasite-infected blood before freezing in liquid nitrogen.

Invasion and Growth Assays. A flow cytometry (FACS)-based assay was used to determine parasitaemia in growth and invasion assays. To compare invasion and intraerythrocytic growth rates of the A1-H.1 and A1-C.1 clones, schizonts purified from each line were added in triplicate to either human or macaque blood in 24-well plates (500 μL medium per well 2% hematocrit, and starting schizont parasitaemia ~1%). Samples of each culture (50 μL) were taken at once and following incubation at 37 °C in a gassed chamber for at 5 h or 24 h, for FACS analysis. To measure growth in different human blood samples, a similar assay was used except that triplicate 200-μL cultures were set up for each blood sample, in a 96-well plate; starting parasitaemia was 0.8% and samples (50 μL) were taken at time point 0 and after 48 h for FACS analysis.

For FACS analysis, 50 μL of each culture was transferred to wells of a fresh 96-well microtiter plate containing 10 μL hydroethidine solution (50 μg/mL). The plate was incubated at 37 °C for 20 min, then samples fixed by addition of 10 μL of 0.04% (wt/vol) glutaraldehyde with incubation for 1 h at 4 °C. Samples were analyzed within 24 h of fixation on a FACScalibur (BD). For this process, the sample from each well was diluted into 500 μL PBS, transferred to a FACS tube, and analyzed using CellQuest software (BD) for acquisition and analysis, as described previously (28).

DNA Constructs and PCR. DNA constructs for use in *P. knowlesi* transfection experiments were adapted from pHH4, a derivative of plasmid pHH1 (23). To produce vector $PkconGFP_{ep}$, the *PfCAM* promoter was removed by digestion with BglII and BamHI and replaced with ~600 bp of sequence from directly upstream of the *Pkef1α* gene (PlasmoDB ID PKH_111400), amplified by PCR using oligonucleotide primers ol097 and ol098 (Table S1). To drive a GFP reporter gene, an ~1,300-bp fragment upstream of the *Pkhsp70* gene (PlasmoDB ID PKH_093190) was amplified by PCR with ol103 and ol104 and cloned into the construct using the NotI and XmaI sites. Finally the *eGFP* ORF was amplified with primers ol111 and ol112 and cloned between the *Pkhsp70* promoter region and the *PbDT* 3′ UTR already present in the construct using the XmaI and SacII restriction sites. To create plasmid $PkconGFP_{p230p}$, 1,281 bp of the *p230p* locus was amplified using primers ol107 and ol108. The

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fragment was cloned into PkconGFP$_{ep}$ linearized with EcoRI using an In-Fusion HD cloning kit (Clontech). PkconGFP$_{p230p}$ was linearized for transfection using the KpnI site situated within the *p230p* fragment.

Transfection of *P. knowlesi*. Tightly synchronized mature schizonts were purified by centrifugation over a Nycodenz cushion. Transfections were carried out using the Amaxa 4D electroporator (Lonza) and the P3 Primary cell 4D Nucleofector X Kit L (Lonza). For each transfection, DNA (20 μg) was dissolved in 10 μL TE (10 mM Tris•HCl, 1 mM EDTA, pH 8.0), then 100 μL of supplemented P3 primary cell solution added. Approximately 5–10 μL of schizonts (~5×10^7–10^8) were resuspended in the DNA plus P3 primary cell solution and immediately electroporated in a 4D Nucleofector X Kit L cuvette (Lonza) using program FP158. Electroporated parasites were transferred to a 1.4-mL Eppendorf tube containing 500 μL prewarmed complete medium plus 150 μL fresh RBC, and incubated at 37 °C on a thermomixer, shaking at 650 rpm while further transfections were carried out. After 30–40 min, transfected parasites were transferred to wells of a six-well plate, each containing 4.5 mL warm complete medium. Plates were cultured at 37 °C in a gassed chamber. After 24 h, and subsequently at daily intervals, the medium was replaced with fresh medium containing 2.5 nM WR99210.

Transgenic *P. knowlesi* Genotyping and Phenotype Analysis. Parasites transfected with plasmid PkconGFP$_{p230p}$ were genotyped by diagnostic PCR using parasite DNA purified by DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. For diagnostic PCR, three different primer sets were used. The first primer pair (ol145and ol146) was designed to amplify a 1,320-bp fragment only from an intact wild-type *p230p* locus. The second primer pair (ol145 and ol144) was designed to only amplify a 1,455-bp fragment after correct integration of PKconGFP$_{p230p}$ into the *p230p* locus. The third primer pair (ol75and ol76) is a positive control designed to amplify a 1,043-bp region of the *Pkmtip* gene in all parasites. PCR reactions (3 min at 96 °C, then 35 cycles of 25 s at 96 °C, 25 s at 52 °C, and 2 min at 64 °C, and a final extension of 5 min at 64 °C) were carried out using GoTaq Mastermix (Promega) with 1-μL DNA template in a 25-μL reaction with 300 nM each primer.

Microscopy. Macaque and human RBC were distinguished by IFA as described previously (29) using a rabbit antiserum raised by immunization with human RBC ghost preparations (diluted 1:5,000), which detects both macaque and human cells, and mouse monoclonal anti-Band 3 antibody clone BIII-136 (Sigma; diluted 1:5,000), which detects only human cells. Antibody binding was detected with anti-mouse IgG Alexafluor-594 (Invitrogen) diluted 1:5,000, and an anti-rabbit IgG Alexafluor-488 (Invitrogen) diluted 1:5,000. Slides were stained with DAPI (0.5 μg/mL) and mounted in Vectashield (Vector Laboratories).

To determine proportions of GFP$^+$ cells, parasite cultures were labeled with Hoechst 33342 (Invitrogen) (1 μg/mL). Parasitized cells were identified using brightfield and nuclear staining, then scored visually for GFP expression. For live imaging of the *PkconGFP$_{p230p}$* integrant *P. knowlesi* clone, Hoechst 33342-stained schizonts in complete medium were mixed with one volume of Matrigel (BD) on ice. The mixture was placed on a microscope slide, overlaid with a Vaseline-rimmed coverslip and allowed to set at room temperature. Samples were imaged on an Axioimager M1 microscope (Zeiss) with an Axiocam MRm camera and using Axiovision (Zeiss) acquisition software. *Z*-stack images were taken with a 250-nm step size and deconvoluted using Volocity 5.5.1 (Perkin-Elmer) image restoration software.

Ethical Statement. Animal work was approved by the United Kingdom Home Office as governed by United Kingdom law under the Animals (Scientific Procedures) Act 1986. Animals were handled in strict accordance with the "Code of Practice Part 1 for the housing and care of animals (21/03/05)" available at www.homeoffice.gov.uk/science-research/animal-research. The protocol for the collection and use of anonymized blood samples from volunteer donors was approved by the National Institute of Medical Research Ethical Review Panel.

ACKNOWLEDGMENTS. This work was supported by the United Kingdom Medical Research Council (U117532063 and U117532067), the European Community's FP7 Programme under Grant Agreement 242095 (EviMalar), and a Medical Research Council Career Development fellowship (to R.W.M.). A.P. was funded by his faculty baseline support and Office for Competitive Research Funds from the King Abdullah University of Science and Technology.

1. Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. *Science* 193(4254):673–675.
2. Haynes JD, Diggs CL, Hines FA, Desjardins RE (1976) Culture of human malaria parasites *Plasmodium falciparum*. *Nature* 263(5580):767–769.
3. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE (1995) Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* 92(4):973–977.
4. Gamo F-J, et al. (2010) Thousands of chemical starting points for antimalarial lead identification. *Nature* 465(7296):305–310.
5. O'Donnell RA, et al. (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. *EMBO J* 21(5):1231–1239.
6. Dvorak JA, Miller LH, Whitehouse WC, Shiroishi T (1975) Invasion of erythrocytes by malaria merozoites. *Science* 187(4178):748–750.
7. Bannister LH, Butcher GA, Dennis ED, Mitchell GH (1975) Structure and invasive behaviour of *Plasmodium knowlesi* merozoites in vitro. *Parasitology* 71(3):483–491.
8. Dennis ED, Mitchell GH, Butcher GA, Cohen S (1975) In vitro isolation of *Plasmodium knowlesi* merozoites using polycarbonate sieves. *Parasitology* 71(3):475–481.
9. Butcher GA (1979) Factors affecting the in vitro culture of *Plasmodium falciparum* and *Plasmodium knowlesi*. *Bull World Health Organ* 57(Suppl 1):17–26.
10. Kocken CHM, et al. (2002) *Plasmodium knowlesi* provides a rapid in vitro and in vivo transfection system that enables double-crossover gene knockout studies. *Infect Immun* 70(2):655–660.
11. van der Wel AM, et al. (1997) Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. *J Exp Med* 185(8):1499–1503.
12. Pain A, et al. (2008) The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature* 455(7214):799–803.
13. Singh B, et al. (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 363(9414):1017–1024.
14. Cox-Singh J, et al. (2008) *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 46(2):165–171.
15. Kocken CHM, Zeeman A-M, Voorberg-van der Wel A, Thomas AW (2009) Transgenic *Plasmodium knowlesi*: Relieving a bottleneck in malaria research? *Trends Parasitol* 25(8):370–374.
16. Chin W, Contacos PG, Coatney GR, Kimball HR (1965) A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science* 149(3686):865.
17. Miller LH, Mason SJ, Clyde DF, McGinniss MH (1976) The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* 295(6):302–304.
18. Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK (1975) Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science* 189(4202):561–563.
19. Tournamille C, Colin Y, Cartron JP, Le Van Kim C (1995) Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet* 10(2):224–228.
20. King CL, et al. (2011) Fy(a)/Fy(b) antigen polymorphism in human erythrocyte Duffy antigen affects susceptibility to *Plasmodium vivax* malaria. *Proc Natl Acad Sci USA* 108(50):20113–20118.
21. Caren LD, Bellavance R, Grumet FC (1982) Demonstration of gene dosage effects on antigens in the Duffy, Ss, and Rh systems using an enzyme-linked immunosorbent assay. *Transfusion* 22(6):475–478.
22. Haynes JD, et al. (1988) Receptor-like specificity of a *Plasmodium knowlesi* malarial protein that binds to Duffy antigen ligands on erythrocytes. *J Exp Med* 167(6):1873–1881.
23. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* 403(6772):906–909.
24. Janse CJ, et al. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Mol Biochem Parasitol* 145(1):60–70.
25. Duraisingh MT, Triglia T, Cowman AF (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *Int J Parasitol* 32(1):81–89.
26. Ponnudurai T, Meuwissen JHET, Leeuwenberg ADEM, Verhave JP, Lensen AHW (1982) The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitoes. *Trans R Soc Trop Med Hyg* 76(2):242–250.
27. Pfander C, et al. (2011) A scalable pipeline for highly effective genetic modification of a malaria parasite. *Nat Methods* 8(12):1078–1082.
28. van der Heyde HC, Elloso MM, vande Waa J, Schell K, Weidanz WP (1995) Use of hydroethidine and flow cytometry to assess the effects of leukocytes on the malarial parasite *Plasmodium falciparum*. *Clin Diagn Lab Immunol* 2(4):417–425.
29. Olivieri A, et al. (2011) Juxtamembrane shedding of *Plasmodium falciparum* AMA1 is sequence independent and essential, and helps evade invasion-inhibitory antibodies. *PLoS Pathog* 7(12):e1002448.