Plasmodium berghei Brca2 Is Required for Normal Development and Differentiation in Mice and Mosquitoes

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Abstract

Background: Malaria is a major global parasitic infestation caused by *Plasmodium* spp. Its zygotes undergo meiosis and develop into haploid ookinetes which differentiate into oocysts that undergo sporogony. Homologous recombination (HR) occurs during meiosis and introduces genetic variation. However, the mechanisms of HR in *Plasmodium* are unclear. In humans, the recombinases Rad51 and Dmc1 are required for HR and are regulated by BRCA2. Most eukaryotes harbor BRCA2 homologs. Nevertheless, they have not been reported for *Plasmodium*.

Methods: A Brca2 candidate was salvaged from a database to identify Brca2 homologs in *Plasmodium*. To confirm that the candidate protein was Brca2, interaction activity between *Plasmodium berghei* (Pb) Brca2 and Rad51 was tested by a mammalian two-hybrid assay. To elucidate the functions of PbBrca2, it was knocked out and parasite proliferation and differentiation were assessed in mice and mosquitoes. Transmission electron microscopy was used to identify sporogony.

Results: The candidate protein was conserved among *Plasmodium* species and indicated that they harbored critical BRCA2 domains including BRC repeats, tower, and oligonucleotide/oligosaccharide-binding-fold domains. The *P. berghei* BRC repeats interacted with PbRad51. Hence, this candidate was considered a Brca2 homolog. PbBrca2 knockout parasites were associated with reduced parasitemia, gametocytemia, female gametocyte ratio, oocyst number, and ookinete development in both mice and mosquitoes. Nevertheless, gametocyte and ookinete morphology was comparable with that of wild type parasites. Transmission electron microscopy disclosed that sporogony never progressed in *Brca2*-knockout parasites.

Conclusions: Brca2 is implicated in nearly all *Plasmodium* life cycle stages and especially in sporogony. PbBrca2 contributes to homologous recombination during meiosis.

Introduction

Malaria is an infection caused by protozoan parasites of the genus *Plasmodium*. In 2020, there were an estimated 229 million cases and 409,000 deaths worldwide (WHO: <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020>). Malaria is difficult to control; no efficacious vaccine has been developed. Moreover, there is continuous antigenic variation, genetic diversity, and antimalarial drug resistance in *Plasmodium* [1, 2]. This parasite has a unique life cycle [3]. In humans, it is transmitted from host to host via *Anopheles* mosquitoes. When a mosquito takes a blood meal from a host, it injects *Plasmodium* sporozoites into the bloodstream. The sporozoites ultimately migrate to the host liver where they develop into merozoites which are then released into circulation and start an asexual reproduction cycle. Only a small proportion of the parasites exist as gametocytes in the blood. When a female *Anopheles* mosquito takes a blood meal from a host, gametocytes are ingested into the midgut and differentiate into male and female gametes. Fertilization, zygote formation, and meiosis then occur. Next, the zygotes differentiate into motile ookinetes. The latter migrate, traverse the midgut

epithelium, settle under the midgut basal lamina, and differentiate into oocysts at approximately twenty-four hours after blood ingestion. The asexual reproduction cycle then resumes once again. Several thousands of sporozoites form within each oocyst and are released approximately two weeks after blood ingestion. The sporozoites then migrate, invade the mosquito salivary glands, and enable the insect to transmit the pathogen to another host.

Efficacious drugs targeting all *Plasmodium* life cycle stages are required to control malaria. The stages from zygote to ookinete are feasible as drug targets as their numbers are far lower than those of the other stages. Moreover, they are not enclosed in the erythrocyte membrane and are, therefore, relatively more susceptible to direct drug exposure [4]. However, currently available anti-*Plasmodium* drugs only target blood-stage parasites. Sexual reproduction and meiosis occur from the zygote to ookinete stages. In mammals, Rad51 and Dmc1 DNA recombinases regulate homologous recombination (HR) during meiosis [5]. *Plasmodium* Rad51 also contributes to HR [3, 6, 7]. *Dmc1* knockout in *Plasmodium berghei* resulted in reduced numbers and altered oocyst and sporozoite development [3, 6, 7]. Thus, Rad51 and Dmc1 are required for HR during meiosis. The recombinases in mammals interact with BRCA2 and are regulated by it. This mechanism also contributes to HR during meiosis [5, 8]. Mammalian BRCA2 harbors several functional domains. In humans, Rad51 and Dmc1 interact with BRCA2 through BRC repeats. Rad51 and Dmc1 also interact with BRCA2 through the C-terminal Rad51- and DMC1-binding domains, respectively [9–11]. However, Thorslund *et al.* reported that DMC1 interacted with BRCA2 through the DMC1-binding domain but not through BRC repeats [10]. In contrast, Martinez *et al.* demonstrated that BRCA2 does, in fact, interact with BRC repeats [9]. Both recombinases required BRCA2 for effective recruitment to HR sites. BRCA2 also has tower and oligonucleotide/oligosaccharide-binding (OB)-fold domains that bind DNA. They facilitate Rad51 and Dmc1 recruitment to the DNA HR sites. HR also participates in DNA double-strand break (DSB) repair. Most living organisms repair DSBs via HR and/or non-homologous end joining (NHEJ). The HR-related proteins are conserved in *Plasmodium* whereas the NHEJ proteins are not [3]. Thus, most DSBs are repaired by *Plasmodium* HR proteins [12].

BRCA2 orthologs have been detected in eukaryotes such as mammals, *Xenopus*, fish, *Caenorhabditis elegans*, *Ustilago maydis*, *Trypanosoma*, and *Leishmania*. Nevertheless, they are absent in yeast, archaea, and bacteria [13–17]. In yeast, Rad52 is the Rad51-regulating protein. Human BRCA2 cannot interact with yeast Rad51 [18]. Human BRCA2 is a large protein (384 kDa). However, in lower eukaryotes such as *Caenorhabditis elegans*, *Ustilago maydis*, *Trypanosoma*, and *Leishmania*, Brca2 orthologs possess only the vital BRC repeats and the tower and OB-fold domains. In *Plasmodium*, Rad51 and Dmc1 are conserved and their functions resemble those of human Rad51 and Dmc1. However, neither the Rad51 nor the Dmc1 regulatory protein has been identified. Thus, the aims of the present study were to salvage BRC repeats and DNA binding domain-containing proteins from the *Plasmodium* database and clarify the functions of *Plasmodium* Brca2 by generating knockout (KO) *P. berghei*.

Methods

Bioinformatics

The PbBrca2 (PbANKA_1343400) genomic sequences used in the present study were retrieved from PlasmoDB (<http://www.plasmodb.org>). The online hidden Markov model structure prediction tool HHpred (<https://toolkit.tuebingen.mpg.de/tools/hhpred>) was used to predict the features of the Brca2 domain [19, 20]. The online signal peptide prediction tool PSORT II Prediction (<https://psort.hgc.jp/form2.html>) was used to predict the subcellular localization of PbBrca2 [21, 22].

Crystal structure modeling

The crystal structures of human Rad51 and BRC repeat 4 were retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank at <http://www.rcsb.org/> (PDB ID: 1N0W) and analyzed with Chimera software (<http://www.cgl.ucsf.edu/chimera/>) [8]. The OB-fold and tower domains were predicted with AlphaFold2 [23] and their structures were compared via Chimera against those of human BRCA2 helical, OB, and tower domains (PDB ID: 1MIU) or that of the OB domain of human replication protein A1 (RPA1) (PDB ID: 4O0A).

Mice, parasites, and mosquitoes

ICR and BALB/c mice aged 6–8 weeks were obtained from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). The *P. berghei* ANKA strain constitutively expressing GFP was obtained from Dr. M. Yuda of Mie University, Tsu, Mie Prefecture, Japan [24]. *Anopheles stephensi* (STE2 strain) mosquitoes were kept in an insectary at 27°C, 80% relative humidity (RH), and a 14 h/10 h light/dark cycle and fed 10% (w/v) sucrose solution. All experimental procedures were executed following a protocol approved by the Kitasato University Animal Care and Use Committee (Approval Nos. 16-020 and 19-087).

Mammalian two-hybrid assay

The coding regions of the PbBrca2 BRC repeats domain were cloned into pM plasmids (Clontech Laboratories, Mountain View, CA, USA). *Plasmodium berghei* Rad51 and Dmc1, *Saccharomyces cerevisiae* Rad51, and human Rad51 were cloned into pVP16 plasmids (Clontech Laboratories). The methods used for the mammalian two-hybrid assays were previously described [25]. FuGENE HD transfection reagent (Promega, Madison, WI, USA) was used to co-transfect $\sim 2 \times 10^5$ cells 293T cells with pM and pVP16 vectors, the pGluc luciferase reporter, and pRL-tk normalization plasmids (Promega). The luciferase activity of the cell extracts was measured with a Dual-Luciferase Reporter Assay System (Promega).

Generation of PbBrca2-KO parasites

PbBrca2 was knocked out by double-crossover homologous recombination technology as previously described [26]. To produce null-PbBrca2 parasites, a donor plasmid was generated using a pBluescript II-based plasmid containing 1,000-bp homology arms, cloned upstream with the primers 5' PbBrca2 F; 5'-CGGGGTACCTTTTATTGTATCCCTATAA-3' and 5' PbBrca2 R; 5'-GGCGGGCCCCTTTATTTAATTATTAAGATTTTTTTTGTTC-3', and cloned downstream with the primers 3' PbBrca2 F; 5'-CCGTCTAGATATCGTTTAAAGGTTGTTTC-3' and 3' PbBrca2 R; 5'-CCGGCGGCCGCTATGTTGTATTGTTTGTTC-3'. Ten micrograms donor vector was linearized and

electroporated with a Nucleofector 2b™ device (Lonza Group, Basel, Switzerland) into cultured *P. berghei* schizonts. Transfected parasites were selected by puromycin and cloned by limiting dilution as previously described [26].

PbBrca2-KO parasite genotyping and PbBrca2 amplification in cDNA

Blood infected with WT or PbBrca2-KO parasites was collected and its DNA or RNA was extracted with a Gentra Puregene Blood Kit (Qiagen, Düsseldorf, Germany) or a NucleoSpin® RNA Blood Kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan) according to the manufacturers' instructions. To genotype the PbBrca2-KO, the *PbBrca2* locus was amplified by PCR using 5' PbBrca2 F and 3' PbBrca2 R from genomic DNA and the primers 5' PbBrca2 F and 3' PbBrca2 R. The extracted RNA was reverse-transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. *PbBrca2* expression was detected by PCR using PbBrca2 exon 3 F; 5'-GGGAACCACATTTTAAATGA-3' and PbBrca2 exon 4 R; 5'-CCTTTGGGTATGTTCTTAGGG-3'.

Southern blot analysis

Two micrograms extracted DNA was digested with the restriction endonuclease *EcoRV* (Roche Diagnostics, Basel, Switzerland), separated on 0.8% agarose gel, transferred to a Hybond N⁺ membrane (GE Healthcare, Little Chalfont, UK), and hybridized using probes labeled with a PCR DIG Probe Synthesis Kit (Roche Diagnostics). The signal was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics) and CDP-Star Detection Reagent (GE Healthcare).

Phenotypic analysis of PbBrca2-KO parasites

The development of WT and PbBrca2-KO parasites in the BALB/c mice was assessed. Five mice per group were each injected with 1×10^6 WT or PbBrca2-KO parasites. Parasitemia was monitored daily using Giemsa-stained blood smears. When parasitemia reached approximately 10% in five mice per group, the gametocytes per infected red blood cells were enumerated and the gametocyte sex ratios were determined by Giemsa-stained tail blood smears. Male gametocyte exflagellation was quantified as previously described [27]. Briefly, 1 μ L gametocyte-infected blood was drawn from the tail vein and immediately mixed with 1 μ L heparin and 38 μ L complete ookinete culture medium (OKM: RPMI 1640 medium (Gibco, Grand Island, NY, USA) and 20% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO, USA)). The mixture was placed under a coverslip at RT. After 5 min, the exflagellation centers were counted under a phase-contrast microscope (ECLIPSE E200; Nikon, Tokyo, Japan) over the next 10 min. Parasite differentiation into gametocytes and exflagellation were then assessed. *Anopheles stephensi* mosquitoes fed on the infected mice and the oocysts were microscopically examined at days 13–15. At least 30 mosquitoes were dissected. EGFP-expressing oocysts were detected by fluorescent stereoscopic microscopy (Leica M205 FA; Leica Microsystems GmbH, Wetzlar, Germany). The oocysts in each positive midgut were counted to determine infection intensity and the prevalence of infection or the number of infected mosquitoes [28].

Ookinete culture and purification

Blood from mice presenting with 10×10^4 RBCs undergoing exflagellation was obtained by heart puncture. Ten volumes OKM was added to the blood and the suspension was cultured at 19°C for 24 h. Ookinetes were enumerated and their structure was examined using culture suspension smears. They were purified in a MidiMACS Separator System (Miltenyi Biotec, Bergisch-Gladbach, Germany) as previously described [27]. A total of 3 mL culture was passed through the system thrice before removing the column from the magnet. The ookinetes were recovered by passing 5 mL OKM through the column. The purified ookinetes were centrifuged at $1,000 \times g$ and 4°C for 10 min and washed thrice in phosphate-buffered saline (PBS). Ookinete morphology was observed by differential interference contrast microscopy (Olympus IX83; Olympus, Tokyo, Japan).

Transmission electron microscopy (TEM)

Mosquito midguts were collected at 7, 14, and 21 d post-infection to evaluate oocyst differentiation, sporozoite development, and sporozoite maturation, respectively. The midguts were fixed in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde (PFA) in 0.03 M HEPES buffer (pH 7.4) at 4°C for 2 h. The cells were then treated with 1% (w/v) osmium tetrachloride/1.5% (w/v) potassium ferrocyanide at 21–24°C for 2 h to enhance cytoplasmic contrast [29]. The midguts were then dehydrated, embedded in epoxy resin, sectioned to 70 nm thickness with an Ultracut N (ReichertNissei, Wein, Austria), stained with 1% (w/v) uranyl acetate for 18 min and 2% (w/v) lead citrate for 1 min, and examined under a transmission electron microscope (H-7650; Hitachi Ltd., Tokyo, Japan) at 80 kV [28].

Statistical analyses

Student's *t*-test with or without Holm's correction and the *F*-test were used to compare treatments in terms of interaction activity (mammalian two-hybrid assay), parasitemia, gametocytemia, gametocyte and exflagellation ratios, and ookinete numbers. The female and male gametocyte counts were analyzed by a χ^2 test. Oocyst numbers and infection intensity (number of oocysts/midgut) was assessed by a Mann-Whitney *U* test.

Results

Bioinformatic analyses of *Plasmodium* Brca2

Analysis of the *Plasmodium* genome by the online hidden Markov model structure prediction tool (<https://toolkit.tuebingen.mpg.de/tools/hhpred>) suggested that the conserved gene product PBANKA_1343400 is a BRCA2 ortholog in *P. berghei* (Fig. 1A). It was predicted that *P. berghei* has three BRC repeats and OB-fold and tower domains such as *Trypanosoma brucei* and *Leishmania infantum* Brca2 and *Ustilago maydis* Brh2, which was the simplest BRCA2 ortholog harboring essential BRCA2 domains; one BRC repeat, OB and tower domains, and nuclear localization signals (Fig. 1A). The 3D structure of the predicted PbBrca2 OB-fold and tower domains were generated by AlphaFold2

(<https://www.alphafold.ebi.ac.uk>) and compared against human BRCA2 and the typical OB domain of the RPA1 structure (Additional file 1: Fig. S1). AlphaFold2 predicted one tower and two OB domains. We also estimated the subcellular localizing signal using the online subcellular localization prediction tool PSORT II Prediction (<https://psort.hgc.jp/form2.html>). It was predicted that PbBrca2 had three nuclear localization signals and localized to the nucleus with 69.6% probability.

PbBrca2 BRC repeats interacted only with PbRad51

BRC repeats are essential for human BRCA2 to be able to interact with Rad51. Its three predicted BRC repeats aligned with human BRCA2 BRC repeat 4 (Fig. 1B). All three BRC repeats were highly conserved with human BRC repeat 4. The phenylalanine residues F1524 and F1546 were also conserved and they were essential for the Rad51 interaction [30]. We used the mammalian two-hybrid assay to confirm that the predicted BRC repeats interact with Rad51. Human BRCA2 interacts with Rad51 and DMC1 via BRC repeats and the other binding domains. It does not interact with *S. cerevisiae* Rad51 [9–11, 18]. We tested the interaction activity of the PbBrca2 BRC repeats against *P. berghei*, human, and *S. cerevisiae* Rad51 and the meiosis-specific Rad51 paralog *P. berghei* Dmc1 (Figs. 1C and D). Relative luciferase activity increased only in the *P. berghei* BRC repeats and PbRad51 in the transfected samples (Fig. 1C). The other combination had the same luciferase activity as the negative control which was only transfected with Rad51- or Dmc1-expressing plasmids. We also confirmed Rad51 and Dmc1 expression in each species via the mammalian two-hybrid assay (Fig. 1D). Thus, the PbBrca2 BRC repeats interacted only with PbRad51 but not human Rad51. We aligned the amino acid sequences of *P. berghei* and human Rad51 to determine why interaction activity was observed only in PbBrca2 and PbRad51 (Additional file 2: Fig. S2A). The Rad51 homology between *P. berghei* and human Rad51 was > 80%. However, the chemical properties of the amino acid residues M158, Y205, M251, and L255 in human Rad51 differed between human and *P. berghei* Rad51. These residues were associated with the important phenylalanines F1524 and F1546 in the BRC repeats. We predicted the structure using Chimera software, human BRC repeat 4, and the Rad51 3D structure, and estimated the interaction mechanism (PDB ID: 1N0W) (Additional file 2: Fig. S2A). We conducted an in silico analysis to estimate the effects of the *P. berghei* amino acid residues on the interaction between human Rad51 and BRC repeat 4. Substitution of M158L, Y205I, M251G, and L255I reduced the number of contacts between BRC repeat 4 and Rad51 (Additional file 2: Fig. S2B).

PbBrca2-knockout (KO) parasite production

PbBrca2 was conserved in both *Plasmodium* species and PbBrca2 and PbRad51 interacted. Thus, we attempted to elucidate the function of PbBrca2. To this end, we generated a *Brca2* null *P. berghei* (Fig. 2). To generate the *Brca2* KO parasite, we transfected a codon-optimized puromycin resistance gene with up- and downstream *PbBrca2* homology arms into a *P. berghei* ANKA strain expressing green fluorescent protein (GFP) (Fig. 2A). We then subjected the full-length genomic DNA region of the puromycin-selected *PbBrca2* null clones to PCR amplification and Southern blot hybridization (Figs. 2B and C). Two *PbBrca2*-KO parasites were generated. A PCR analysis of the genomic DNA revealed that the amplicons of the KO clones were shorter than that of the WT parasite. Both KO parasites harbored the predicted genomic DNA

fragment size. To confirm the lack of mRNA-level *PbBrca2* expression, we performed RT-PCR using primers amplifying between exons 3 and 4 of *PbBrca2*. As expected, amplification was absent in the *PbBrca2*-KO parasites (Fig. 2D).

PbBrca2 is required for asexual blood stage growth and oocyst formation

A comprehensive gene expression analysis revealed that *PbBrca2* is upregulated in the schizont, female gametocyte, and ookinete. Moreover, its expression gradually increases from the ring to the schizont stage and gradually decreases from the ookinete to the sporozoite stage (<https://www.sanger.ac.uk/science/tools/mca/mca/>) [31]. To elucidate the function of *PbBrca2*, we analyzed asexual blood stage growth (Fig. 3) and found that the *PbBrca2*-KO parasites caused milder parasitemia than the WT (Fig. 3A). The morphology and proportions of the ring to schizont stages in *PbBrca2*-KO parasites were comparable with those in the WT parasites (data not shown).

We then analyzed oocyst formation in mosquitoes because they are susceptible to parasite infestation at that stage. Oocyst formation also decreased in the *PbBrca2*-KO parasites (Fig. 3B). We evaluated the proportions of mosquitoes infected with more than one oocyst to determine whether the *PbBrca2*-KO parasites had low infectivity in the mosquitoes. The WT and *PbBrca2*-KO parasites did not differ in terms of the severity of midgut infestation they caused (Fig. 3C).

Female gametocytes and ookinete formation were inhibited in PbBrca2-KO parasites

As oocyst formation was reduced in the *PbBrca2*-KO parasites, we attempted to identify the disrupted stage. The *PbBrca2*-KO1 and *PbBrca2*-KO2 clones were similar in terms of the parasitemia severity and oocyst number. Hence, we analyzed the *PbBrca2*-KO1 clone in the subsequent experiments. We enumerated gametocytemia and the proportions of female and male gametocytes using blood smears from *PbBrca2*-KO or WT parasite-infected mice. There were no apparent differences between the *PbBrca2*-KO and WT parasites in terms of gametogony morphology (Fig. 4A). However, the *PbBrca2*-KO parasites induced less gametocytemia than the WT parasites (Fig. 4B). The proportions and total numbers of female and male gametocytes differed between the WT and *PbBrca2*-KO parasites in four independent experiments (Fig. 4C and Table 1). The proportions of female and male gametocytes were lower and higher, respectively, in the *PbBrca2*-KO than the WT parasites. The exflagellation ratio indicating mature male gametocytes was similar for both the WT and the *PbBrca2*-KO parasites. Therefore, male gametocyte maturation was normal in the *PbBrca2*-KO parasites (Fig. 4D). Ookinete formation was also reduced in the *PbBrca2*-KO parasites but there were no apparent morphological differences between the *PbBrca2*-KO and WT ookinetes (Figs. 4A and E).

Table 1
Total numbers of female and male gametocytes

Parasite	Female	Male	χ^2 test
Wild type	230	57	$P < 0.01$
PbBrca2-KO1	173	95	

PbBrca2-KO parasite oocysts presented with morphological disorder

Mosquitoes infected with PbBrca2-KO parasites could not transmit them to their secondary hosts. Furthermore, sporozoites were absent from the salivary glands of these mosquitoes (data not shown). Dmc1 KO parasites formed aberrant oocysts and sporozoites. Thus, we examined the morphology of PbBrca2-KO parasite oocysts and sporozoites in the mosquito midguts (Fig. 5). An ultrastructural analysis disclosed that PbBrca2-KO formed oocysts in the midguts on day 7 post-infection. By contrast, PbBrca2-KO revealed no sporozoite formation in the oocysts on day 14 or 21 post-infection. The parasites in PbBrca2-KO remained undeveloped after day 7. On day 14 post-infection, the oocysts regressed and vacuoles appeared in them. Certain mosquitoes shed oocysts from their midgut lumens on day 14 (data not shown). At day 21 post-infection, the PbBrca2-KO parasites in the oocysts were vacuolated and the oocyst capsules shrank. The foregoing results indicate that PbBrca2-KO ookinetes can differentiate into oocysts but not sporozoites in mosquito midguts. Therefore, PbBrca2-KO cannot undergo sporogony and has a defective life cycle.

Discussion

Meiotic HR is a suitable drug target for efficacious *Plasmodium* control. In the present study, we identified the recombinase-regulating protein PbBrca2 and determined the phenotype of PbBrca2-KO parasites. PbBrca2 is well-conserved among various *Plasmodium* species. BRC repeats of human BRCA2 could not interact with *S. cerevisiae* Rad51 [18]. BRC repeats of PbBrca2 interacted with PbRad51 but not with human or *S. cerevisiae* Rad51. Here, we targeted the Rad51 amino acids M158, I205, M251, and L255 because they interact with the phenylalanine residues F1524 and F1546 in human BRC repeat 4. Nevertheless, the foregoing amino acids differed in *P. berghei*. Their substitution reduced the number of contacts between BRC repeat 4 and Rad51 (Fig. S2B). *Plasmodium* Rad51 forms homomultimers and mediates strand exchange [6, 32]. Homology between *P. berghei* and human Rad51 is > 80% but the chemical properties of the four amino acids near the BRC repeat binding site differ between human and *P. berghei* Rad51. Moreover, the amino acid sequences of the BRC repeats also differ between human and *P. berghei* Brca2. For these reasons, the interactions between Rad51 and Brca2 have uniquely evolved in each species and differ between humans and *Plasmodium*. This discrepancy might facilitate the development and application of anti-*Plasmodium* drugs. We also tested the interaction activity between

PbBrca2 BRC repeats and PbDmc1 as it was reported that human BRCA2 interacted with Dmc1 via BRC repeats [9]. However, we detected no such interaction activity in *P. berghei*. Existence of the DMC1-binding domain in human BRCA2 is controversial. Another study reported a Dmc1-specific binding domain and the absence of any interaction with Dmc1 via BRC repeats containing peptides [10]. Thus, interaction analysis using partial PbBrca2 peptides is required to identify the Dmc1-binding domain in *Plasmodium* Brca2.

Here, we endeavored to establish why relatively few PbBrca2-KO oocysts were found in mosquitoes. PbBrca2-KO parasites also presented with reduced numbers of gametocytes. The female gametocyte ratio had decreased whereas the exflagellation ratio (male gametocyte number) did not. *Brca2* was upregulated in female gametocytes but only weakly expressed in male gametocytes [31]. These results suggest that female gametocyte differentiation was altered or destabilized in PbBrca2-KO. Moreover, PbBrca2-KO presented with reduced numbers of gametocytes and even fewer post-meiosis ookinetes. PbBrca2 may contribute to HR during meiosis. Mice or zebrafishes possessing BRCA2 mutations were infertility by inhibiting gamete development and inducing gamete death [33, 34]. Hence, it might induce abnormal meiosis and parasite death. A few PbBrca2-KO parasites were nonetheless able to form oocysts. TEM revealed no sporogony. PbBrca2-KO parasite differentiation was terminated at ~7 d after WT parasite infection. At 14 d post-infection, vacuoles appeared in the oocysts and the latter began to degrade. Thus, PbBrca2 is also required for sporozoite development.

Certain results of the present study aligned with those previously reported for the PbDmc1-KO parasite [7]. *Plasmodium* Dmc1 is a meiosis-specific recombinase [7]. In humans, DMC1 contributes to HR and is regulated by BRCA2 during meiosis [5]. PbDmc1-KO parasites presented with relatively reduced oocyst and sporozoite numbers. However, their phenotype was milder than that of our PbBrca2-KO parasites. Moreover, PbDmc1-KO parasites could nonetheless differentiate into sporozoites. In contrast, PbBrca2-KO parasites displayed reduced numbers of female gametocytes, ookinetes, and oocysts, and presented with no sporozoite development. They also proliferated only very slowly at the blood cell stage. It was expected that PbDmc1 functions only during meiosis and its knockout had limited efficacy. Nevertheless, PbBrca2 might have a broader function than Dmc1. Moreover, PbBrca2 knockout influenced all *Plasmodium* life cycle stages. Further investigations for PbBrca2 function in meiosis are required to determine whether the roles of Brca2 are more important than those of Dmc1 in *Plasmodium*.

In humans, BRCA2 plays pivotal roles in HR repair when DNA is damaged by double-strand breaks and in meiotic HR via RAD51 binding [35]. The present study did not examine the DNA damage response. Hence, the contribution of PbBrca2 to HR after DNA double-strand breaks could be established by evaluating sensitivity to agents inducing DNA damage during asexual reproduction.

Conclusions

Here, we identified *Plasmodium* Brca2 harboring BRC repeats and OB and tower domains, which are the BRCA2 protein fingerprints. Brca2 knockout in *P. berghei* resulted in reduced parasitemia, oocyst

formation, female gametocyte numbers, and ookinete development. Sporogony did not progress in Brca2 knockout. The foregoing results and those of previous studies suggest that Brca2 contributed to HR during meiosis. The absence of Brca2 affected sporogony and nearly every other *Plasmodium* developmental stage. Brca2 might be an anti-*Plasmodium* drug target. To the best of our knowledge, this study is the first step in elucidating the function of Brca2 in *Plasmodium* and helps clarify the mechanism of meiosis in this pathogen.

Abbreviations

BRCA2: Breast cancer susceptibility protein 2; BSA, Bovine serum albumin; DBD: DNA-binding domain; DMC1: DNA meiotic recombinase 1; DSB: Double-strand break; EGFP: Enhanced green fluorescent protein; FBS: Fetal bovine serum; GFP: Green fluorescent protein; HR: Homologous recombination; KO: Knockout; NHEJ: Non-homologous end joining; OB: Oligonucleotide/oligosaccharide-binding; OKM: Ookinete culture medium; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; RAD51: DNA repair protein RAD51 homolog 1; RPA1: Human replication protein A1; TAD: Transactivation domain; TEM: Transmission electron microscopy; WT: Wild type.

Declarations

Acknowledgments

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Availability of Data and Materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Author Contributions

YY, SF, KK, and HI contributed to the study conception and design. SK, AU, HK, KN, JH, KK, AH, TK, and K Orino performed material preparation, data collection, and data analysis. ZZ, YY, and K Ochiai conducted the in silico analysis. AS and SF generated the knockout parasites. MS performed the electron microscopy. YY and MS wrote the first manuscript draft. All authors commented on previous versions of the manuscript and read and approved the final version.

Ethical approval and consent to participate

The protocols of all experiments conducted in this study were approved by the Institutional Laboratory Animal Care and Use of the School of Veterinary Medicine at Kitasato University, Japan (Approval Nos. 16-020 and 19-087) and were performed in accordance with their guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Brca2 was conserved in *Plasmodium berghei*. (A) Schematic representation of PbBrca2 relative to human, *Trypanosoma brucei*, and *Leishmania infantum* BRCA2 and *Ustilago maydis* Brh2. Predicted BRC repeats, tower and oligonucleotide/oligosaccharide binding (OB)-fold domains, and nuclear localization signals are shown. For human BRCA2, PALB2, DMC1, and RAD51-binding domains (BD) are shown. (B) Alignment of human BRCA2 BRC repeat 4 and three PbBrca2 BRC repeats. Magenta boxes indicate that

the phenylalanine residues F1524 and F1546 are required for interaction with Rad51. (C) Interaction activity of PbBrca2 BRC repeats with *P. berghei*, human, and *S. cerevisiae* Rad51 and *P. berghei* Dmc1 was tested by mammalian two-hybrid assay. 293T cells were transfected with DNA-binding domain (DBD) fused with PbBrca2 BRC repeats, transactivation domain (TAD) fused with Rad51 or Dmc1, and luciferase reporter and normalization plasmids. Relative luciferase activity normalized with *Renilla* luciferase activity. ‘*’ indicates $P < 0.05$ vs. paired TAD fused protein-expressing samples. (D) Expression levels of TAD-fused proteins in mammalian two-hybrid assay were confirmed. All TAD-fused proteins were expressed

Figure 2

Generation of *PbBrca2*-knockout (KO) parasites. (A) Schematic representation of targeted *PbBrca2* disruption. Targeting vector (bottom) containing puromycin resistance gene (Puromycin^R) was integrated into *PbBrca2* locus (top) by double crossover. Arrows indicate genomic DNA (B) and RT-PCR (D) primers.

Black bar indicates Southern blot probe. **(B)** *PbBrca2* locus amplified in wild type (WT) and PbBrca2-KO1 (KO1) and PbBrca2-KO2 (KO2) clones. In WT parasite, PCR products were longer than those detected in KO1 and KO2 parasites. M: DNA size marker. **(C)** PbBrca2 knockout was detected by Southern blot. *EcoRV* digested genomic DNA from WT and KO parasites and demonstrated hybridization with upstream *PbBrca2*. KO1 and KO2 clones detected relatively short bands. **(D)** To analyze mRNA-level *PbBrca2* expression, RT-PCR was performed using primers amplifying between PbBrca2 exon 3 and exon 4 region in blood-stage parasites. *PbBrca2* PCR products were detected in WT but not in KO1 or KO2 parasites. Amplification of 18S ribosomal RNA amplification was positive control. M: DNA size marker

Figure 3

PbBrca2 is required for asexual blood stage growth and oocyst formation. (A) Relative parasitemia in mice infected with either wild type (WT) or PbBrca2-KO parasites. Relative parasitemia in mice intravenously infected with WT (blue) or PbBrca2-KO1 (red) and PbBrca2-KO2 (orange) parasites. Each mouse was infected by intraperitoneal injection with 10^6 blood-stage parasites; $n = 5$. (B) Oocysts/midguts of mosquitoes infected with WT or PbBrca2-KO1 or PbBrca2-KO2 parasites. Horizontal

bars are medians. Data for three independent replicates. (C) Proportions of mosquitoes harboring ≥ 1 oocyst. ‘*’, ‘**’, ‘***’, and N.S. indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and non-significance vs. WT parasites, respectively

Figure 4

PbBrca2 is vital for female gametocyte development and ookinete formation. (A) Morphological analyses of wild type (WT) and PbBrca2-KO1 parasites in male and female gametocytes and ookinetes. (B) Gametocytes were enumerated in mice infected with WT or PbBrca2-KO1 parasites at approximately 10% parasitemia in five independent experiments. (C) Proportions of female and male gametocytes in mice infected with either WT or PbBrca2-KO1 parasites. Proportions of female (pink) and male (blue) gametocytes are shown. (D) Exflagellation ratios were measured for in vitro cultures using blood from mice infected with WT or PbBrca2-KO1 parasites and with approximately 10% parasitemia. (E) Ookinete counts for in vitro cultures using blood from mice infected with WT or PbBrca2-KO1 parasites and with approximately 10% parasitemia. Data are plotted in bar graph form. Error bars indicate standard deviation (SD). ‘*’, ‘**’, and N.S. indicate $P < 0.05$, $P < 0.01$, and non-significance vs. WT parasites, respectively

Figure 5

PbBrca2 is essential for sporogony. Ultrastructural analyses of wild type (WT) and PbBrca2-KO1 oocytes at 7, 14, and 21 d post-infection. WT exhibited normal sporogony whereas PbBrca2-KO1 oocysts were small and had indeterminate morphology (day 7). PbBrca2-KO1 parasites did not form sporozoites and some of them regressed to vacuolated oocysts (day 14). PbBrca2-KO1 oocysts showing prominent regression (day 21). Dotted boxes contain enlargements of upper images. Arrowhead indicates abnormal parasite development. Scale bar = 1 μ m

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