The first step of Plasmodium development in vertebrates is the transformation of the sporozoite, the parasite stage injected by the mosquito in the skin, into merozoites, the stage that invades erythrocytes and initiates the disease. The current view is that, in mammals, this stage conversion occurs only inside hepatocytes. Here, we document the transformation of sporozoites of rodent-infecting Plasmodium into merozoites in the skin of mice. After mosquito bite, ~50% of the parasites remain in the skin, and at 24 h ~10% are developing in the epidermis and the dermis, as well as in the immunoprivileged hair follicles where they can survive for weeks. The parasite developmental pathway in skin cells, although frequently abortive, leads to the generation of merozoites that are infective to erythrocytes and are released via merosomes, as typically observed in the liver. Therefore, during malaria in rodents, the skin is not just the route to the liver but is also the final destination for many inoculated parasites, where they can differentiate into merozoites and possibly persist.

Results and Discussion

P. berghei Develops Inside Skin Cells in the Mouse. We first analyzed the fate of the P. berghei sporozoites that remain in the skin of the host. Anopheles stephensi mosquitoes were allowed to transmit WT green fluorescent sporozoites (14) into the ear of SKH1 hairless mice (15), which display little autofluorescence in the skin, and parasites were imaged daily using spinning-disk confocal microscopy (16). Typically, inside hepatocytes, parasites round up and increase their size to become spherical exoerythrocytic forms (EEF) of ~40-μm average diameter, undergo schizogony, and produce thousands of uninucleate merozoites in ~50–70 h (4). Approximately 11% of the elongated sporozoites detected in the skin soon after the bite were still observed after 24 h (day 1, D1) as brightly fluorescent, round parasites (Fig. 1A and B). The average size and fluorescence intensity of the EEF steadily increased with time (Fig. 1A and C). However, the maximal diameter of skin EEF, typically reached at D3–D4, remained 2–3 times smaller than the maximal diameter of liver EEF, reached at D2 (Fig. 1A and C). When parasite survival was assessed after intradermal injection of ~5,000 sporozoites into the ear skin, only ~1% and 0.2% were found to brightly fluoresce at D1 and D4, respectively (Fig. 1D and C). Despite the ~10-fold decrease in the percentage of parasites...
Fig. 1. *P. berghei* differentiation in the ear skin of a hairless mouse. (A) Parasites (in green) imaged for 4 d ( autofluorescence in red) after sporozoite inoculation by the bite of a single mosquito. Images are maximal Z-projections of 13–21 contiguous pictures separated by 5 μm. Red arrowheads, fluorescent parasites fading over time; white arrowheads, brightly fluorescent parasites until day 4 (D4). The lower-right inset shows a liver stage at the same scale at D1 and D2. (Scale bar, 40 μm.) (B) Cumulative numbers of developing parasites in six different bite sites from two independent experiments. Orange bar (D0), number of sporozoites detected after the bite (n = 258); green bars, number of brightly fluorescent EEF; numbers above the bars, percentages of developing parasites versus sporozoites imaged at D0. (C) Parasite diameter (average ± SD), estimated by the EEF maximum projection area, in the liver (circles) and in the skin (diamond, after bite; square, after injection). (D) Parasites (in green) at D2 after microinjection of 5,200 sporozoites. The image is a maximal Z-projection of 35 pictures covering 70 μm in depth. (Scale bar, 40 μm.) (E) Numbers of developing parasites after intradermal injection. Orange bar (D0), no. of injected sporozoites (5,200); green bars, numbers of brightly fluorescent EEF (average ± SD), in four injection sites; numbers above the bars, percentages of developing parasites vs. sporozoites injected at D0. Similar results were obtained after injection of larger number of sporozoites (75,000–300,000 parasites). (F) Green fluorescent EEF surrounded by a parasitophorous vacuole stained with anti-US4 polyclonal antibody (in red) at D2. (Scale bar, 5 μm.)

Present at D1 after needle injection compared with mosquito delivery, the fate of surviving parasites was similar in the two cases, as judged by the proportion (Fig. 1B and E) and the average size (Fig. 1C) of fluorescent EEF.

We then asked whether parasite maturation in the skin occurred within host cells. In hepatocytes, parasites develop inside a parasitophorous vacuole (PV) formed upon sporozoite entry into the host cell (17). The sporozoite transmembrane protein US4 inserts into the PV membrane and is essential for liver-stage development (18). Staining of skin cryosections with anti-US4 antibodies (Fig. 1F) showed that 53% and 65% of the green fluorescent EEF were delineated by a clear red US4 signal at D1 and D2, respectively.

Fig. 2. Localization of *P. berghei* skin EEF. (A) Schematic view of the epidermis, dermis, and hair follicle of the mammalian skin. Drawn are the keratin5-positive keratinocytes (in red) that rest on the basement membrane separating the dermis from the epidermis and line the invagination of the HF; the Blimp1-positive cells (in green) associated with the superficial layer of the epidermis and the HF; and the vascularization in the dermis (red lines). Ep, epidermis; De, dermis; HF, hair follicle; HS, hair shaft; SG, sebaceous gland; BA, bulge area; HB, hair bulb; DP, dermal papilla. (B) Percentage of dermal (blue), epidermal (red), and hair follicle-associated (green) parasites in the mouse ear estimated by immunofluorescence microscopy at various days after intradermal injection of sporozoites. Number of analyzed EEF for each time point: 33–63. (C) Confocal image showing EEF (in green) in the deep dermis (white arrowhead), the epidermis (yellow arrowhead), and the cartilage (red arrowhead). (D) Confocal images of epidermal EEF (in green) associated with keratin5-positive keratinocytes of the basal layer of the epidermis (Left) or with keratin5-negative keratinocytes of the superficial layers of the epidermis (Right). Abbreviations are as in A. (Scale bar, 10 μm.) (E) Confocal images of hair follicle-associated EEF. EEF (in green) are located in the upper portion of the HF, in keratin5-positive or keratin5-negative cells, often near the sebaceous glands. Abbreviations are as in A. (Scale bar, 10 μm.)
We also analyzed the development in the mouse skin of sporozoites lacking the P30p protein. P30p is important for the formation and/or maintenance of the PV membrane, and a P. berghei P30p knockout clone generates ~5–10% of the EEF produced by WT sporozoites inside hepatocytes (19, 20). We constructed the P. berghei clone P30p-G bearing both the P30p-null mutation and a GFP-expression cassette (Fig. S1), and sporozoites of the clone were injected with red-fluorescent WT sporozoites of the L753 clone (21) into the skin of mice. Approximately 10-fold fewer green P30p-G than red WT EEF were observed at D1 at the injection site (Fig. S1). Together, these data suggested that most WT P. berghei parasites surviving in the mouse skin were developing intracellularly inside a PV.

**P. berghei Develops in the Epidermis and Dermis and in Association with Hair Follicles.** We next localized the EEF in the skin using an immunohistological approach (see Fig. 2A for a schematic representation of the mammalian skin). *P. berghei* sporozoites (20 × 10^4) were injected into the ear skin of hairless mice, and at various days postinoculation ~10-μm cryosections of fixed ear tissues were labeled with DAPI and immunostained using antibody K5, which recognizes keratin5 in keratinocytes of the basal layer of the epidermis (22). EEF were present in multiple sites in the skin. (i) At D1 and D2, ~50% of the EEF were located in the dermis (Fig. 2B and C and Fig. S2A). Dermal EEF behaved similarly to liver EEF, with a sharp decrease between D2 and D3, and only represented ~7% of the skin EEF at D3 and D4 (Fig. 2B). (ii) EEF were also found in the epidermis. At D1 and D2, epidermal EEF were associated mostly with keratin5-positive keratinocytes lying on the basement membrane, whereas at D3 and D4 they were mostly in keratin5-negative cells in the superficial layers of the epidermis (Fig. 2D and Fig. S2B). The number of epidermal EEF did not significantly change up to D3, and only slightly decreased at D4 (Fig. 3A). (iii) More surprisingly, EEF were found in close association with hair follicles, appearing as keratin5-positive keratinocyte-bound tubular invaginations of the epidermis (Fig. 2E and Fig. S2C). In hairless mice, EEF associated with the autofluorescent (and rudimentary) hair follicles were frequently located close to the sebaceous glands (Fig. 2E and Fig. S2C). Their numbers remained stable up to D4 (Fig. 3A), representing 70% of the skin EEF at D4 (Fig. 2B), and parasites were still detected in hair follicles after more than 2 wk postinoculation (Fig. S2B).

Parasite association with hair follicles was also imaged in a Blimp1-GFP mouse (23). In this mouse, a group of cells expressing the transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp1) and residing near the bud site of the sebaceous glands, which act as sebocyte progenitor cells, are green fluorescent (and rudimentary) hair follicles were frequently located close to the sebaceous glands (Fig. 2E and Fig. S2C). As in the hairless mice, hair follicle-associated EEF were observed persisting for several days, in the vicinity of the GFP-fluorescent zone of the sebaceous glands (Fig. 3C, hair follicle). It is still unclear, however, whether these parasites were simply growing slowly in the skin or might include true dormant (growth-arrested) forms.

**P. berghei Developing in the Mouse Skin Generates Merozoites.** We next imaged parasite growth in the skin using bioluminescence. For this, we used sporozoites of the transgenic *P. berghei* clone 676el1 (PhGFP-LUC/C58H) expressing a GFP-luciferase fusion gene via the EEFluc promoter (25), which constitutively produces the fusion protein throughout the parasite life cycle. Sporozoites (2 × 10^4) were inoculated in the ear skin of mice and luciferin injected intraperitoneally at D0 (Fig. S3). The main signal was detected in the liver, peaking at D2, and the signal in the ear skin peaked at D3.

Quantification of the intensity of bioluminescence signals using the Living Image software showed that the signal in the ear skin increased ~threefold between D1 and D2, and ~2.5-fold between D2 and D3 (Fig. 4A). This suggested that, like liver parasites, skin parasites could actively grow.

To investigate schizogony in skin parasites, we examined individual skin EEF at D3 and D4, when most had reached their maximal size, by confocal microscopy. In hairless mice, fluorescent parasites were seen undergoing nuclear divisions (Fig. S4) and generating individual merozoite-like progeny (Fig. 4B). Further, infected cells in the skin were frequently observed giving rise to cell extensions reminiscent of the merosomes that extrude from infected hepatocytes (Fig. S5A). Hepatocyte-derived merosomes contain tens to thousands of merozoites wrapped in the host cell membrane, bud off, and detach from the infected cell to reach the blood in the liver sinusoids (27). Brightly fluorescent merozoite-like extensions were also observed detaching from infected cells (Fig. 4C) and moving in the skin (Fig. 4D and Fig. S5B).

To test whether the fluorescent progeny seen inside skin cells were indeed merozoites, we tested their capacity to invade and multiply in mouse erythrocytes. One day after injection of green fluorescent sporozoites into the skin of mice (1.0–2.5 × 10^4 sporozoites per ear in 2–4 animals), i.e., before the first merozoites are formed in the liver, the skin tissue at the injection site was dissected and treated with collagenase and trypsin to obtain a single cell suspension. Infected fluorescent cells were sorted by FACS (Fig. S4) and incubated for several days at 37 °C in vitro in DMEM 10% FCS. Examination of the sorted cells confirmed the diversity of skin cell types that were infected in situ (Fig. 5B), and after 4 d (1 d in the skin and 3 d in vitro), merozoites were detected inside sorted cells (Fig. 5C). At D4, cells were scratched and the host cell/
parasite mixture injected i.v. into mice. In three independent experiments (Fig. 5D), mice became infected and parasitemia increased at a normal rate (∼10-fold increase/24 h). This demonstrates that P. berghei development in skin cells can generate infective merozoites, and thus that the parasite developmental pathway in the skin indeed reproduced that in the liver.

**In Situ Infectivity of P. berghei Merozoites Generated in Skin Cells.** To test whether merozoites generated in skin cells (skin-derived merozoites) were capable of invading erythrocytes in situ and sufficient for generating a blood stage infection, transplantation experiments of infected skins onto naive mice were complicated by the different timings of maximal release of skin-derived merozoites (D2–D3 post-sporozoite inoculation) and of efficient vascularization of the skin graft. Strikingly, we observed that primaquine, a drug known to inhibit liver EEF development, had a much weaker effect on skin EEF development (Fig. S6A).

After injection of luciferase-producing sporozoites in the ear of mice, treatment with 25 mg/kg primaquine at D0 and D1 abolished the bioluminescence signal in the liver without affecting that in the ear. However, this differential primaquine effect could not be exploited to show the in vivo infectivity of skin-derived merozoites. Indeed, the 25-mg/kg primaquine treatment did not completely prevent liver EEF maturation in all animals (5 of 52 primaquine-treated mice and surgically deprived of the infected ear at D1 became patent; see Fig. S7) and impaired skin EEF development between D2 and D3 (Fig. S6A), whereas 30 mg/kg primaquine significantly affected skin EEF development at earlier time points (Fig. S6A).

In any case, the skin-derived merozoites are clearly outnumbered by their liver-derived counterparts, and their contribution to the onset of blood stage infection would be minimal at best. After injection of luciferase-producing sporozoites in the skin of mice, the maximal intensity signal at the injection site, reached at D3, was found to be ∼2% of the maximal intensity signal in the liver, reached at D2 (Fig. S6B). Not surprisingly, when sporozoites were injected in the ear of mice and the infected or contralateral ear was surgically removed at D1, no statistical difference was detected in the parasitemia of animals lacking or having skin EEF (Fig. S6C).

**P. yoelii Generates Merozoites in the Mouse Skin.** Last, we studied the development of sporozoites of *Plasmodium yoelii*, another species that infects rodents, in the mouse skin. Like sporozoites of *P. falciparum*, the species most lethal to humans, *P. yoelii* sporozoites are known to invade only certain hepatocytic lines in vitro, in a CD81-dependent manner (28). Additionally, quantitative PCR analysis of mouse ear inoculated with *P. yoelii* sporozoites showed that the parasite DNA was still detected 42 h postinjection in the mouse skin (13). We analyzed the fate of *P. yoelii* sporozoites expressing GFP (29) or RedStar (Fig. S8) after intradermal injection in mice. At D1, ∼0.2% and ∼0.15% of the inoculated sporozoites transformed in the ear skin of hairless and Swiss mice, respectively (Fig. S9A). Despite the ∼fivefold smaller
number of *P. yoelii* parasites observed in the skin compared with *P. berghei* at D1. *P. yoelii* EEF, like *P. berghei* EEF, grew both in the epidermis and the dermis of mice (Fig. S9F). *P. yoelii* merozoites were detected from D2 onward (Fig. S9C) and were seen moving across the skin (Fig. S9D), indicating that *P. yoelii* EEF completed their development in the skin of mice.

**Conclusions**

This study shows that the *P. berghei* and *P. yoelii* rodent-infecting species can undergo complete exoerythrocytic schizogony not just inside hepatocytes but also inside skin cells. However, although the *P. berghei* infective merozoites released and moving in the skin in merozoite-like extensions might occasionally invade erythrocytes in the mouse, and thus constitute a potential reservoir of infective merozoites, skin-derived merozoites do not significantly contribute to erythrocyte infection in normal conditions. Whether other mammal-infecting *Plasmodium* species, particularly the human-infecting species, can also develop in the skin of the host remains to be addressed.

The data also suggest that parasites might persist in association with hair follicles, which constitute an immunoprivileged site of the mammalian body characterized by the virtual absence of major histocompatibility complex (MHC) class I expression and a strongly immunosuppressive environment (30). This raises the hypothesis that parasites might become quiescent when associated with hair follicles and act as a source of infection relapses, as proposed for the hypnozoites of *P. vivax* in the liver (31).

Perhaps the most important implication of this work is immunological. Whereas previous data (11, 12) showed that in the mosquito bite site to the blood.

**Materials and Methods**

**Parasites, Mice, and Mosquitoes.** We used the *P. berghei* ANKA clone expressing GFP under the control of the hsp70 promoter (14). The *P. yoelii* expressing parasite clone was obtained from the MRA repository (ATCC no. MRA-817; 17XNL PYGFP). The red fluorescent *P. berghei* line (line 733) contains the RedStar-expressing cassette integrated at the 230 genomic locus. The red fluorescent *P. yoelii* clone contains the RedStar cassette integrated at the *dsu-rrna* locus. We used the *P. berghei* clone 676cl1 (PbGFP-LUCscr) expressing a GFP-luciferase fusion gene via the *EEF1a* promoter (25). C57BL/6J, Swiss and hairless SKH1 mice were purchased from Charles River Laboratories. All experiments were approved by the committee of Institut Pasteur and were performed in accordance with the applicable guidelines and regulations. *A. stephensi* (Sdasseu strain) mosquitoes were reared using standard procedures (16). For intradermal injection of sporozoites into rodents, salivary gland sporozoites were dissected out and a small volume (0.2–10 μL) containing 5 × 10⁶ to 3 × 10⁷ sporozoites was deposited in the dermis of the ear by using a 35- to 36-gauge needle with a NanoFil syringe (World Precision Instruments).

**Intravital Imaging and Immunolabelings.** Intravital imaging was performed as described (16). For immunolabelings, ears were excised, fixed with 4% paraformaldehyde/PBS for 2 h, and dehydrated in 10% and 30% sucrose/PBS before embedding in OCT. Ten-micrometer sections were cut on a CM3050 cryotome (Leica) and adhered to Superfrost Plus Slides (VWR). Sections were air-permeabilized and blocked in PBS containing 0.1% Triton X-100 (Sigma) and 5% FCS, followed by staining with anti-keratin5 polyclonal primary antibody (Covance) or anti-UIS4 polyclonal primary antibody, AlexaFluor 546 conjugate (Molecular Probes), and DAPI (Molecular Probes). Stained slides were mounted with Prolong Gold (Invitrogen), and 3D image stacks were acquired on a SP5 confocal microscope (Leica). Images are displayed as 2D maximum-intensity projections.

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Fig. S1. Generation of P36p-disruptant *P. berghei* clone expressing constitutively the GFP and its development in the skin. (A) P36p gene disrupted *P. berghei* parasites were generated by double-crossing-over homologous recombination using GFP gene as a selectable marker. After transfection of linearized plasmid into schizonts infected erythrocytes, transgenic blood-stage parasites were selected by cell sorting using GFP signal. (B) Numbers of developing parasites 24 h after coinjection of 10,000 L733-RFP (red) and 20,000 ΔP36p-GFP (green) sporozoites in the ear of hairless SKH1 mice. Numbers above the bars indicate the percentages of developing parasites vs. sporozoites injected at D0. This experiment is representative of three experiments performed independently.
Fig. S2. EEF localization in the dermis, epidermis, and hair follicles. (A) EEF localization in the dermis. Confocal images showing EEF in the dermis (arrows). Ep, epidermis; De, dermis; C, cartilage. (Scale bar, 10 μm.) (B) EEF localization in the epidermis. Confocal images showing EEF associated with keratin5-negative cells of the superficial layers of the epidermis. Ep, epidermis; De, dermis. (Scale bar, 10 μm.) (C) EEF localization in the hair follicle. Confocal images showing developing parasites associated with keratin5-negative (D1 and D3) or -positive (D2) keratinocytes lining the hair follicle. Ep, epidermis; De, dermis. (Scale bar, 10 μm.)
Fig. S3. Parasite development visualized by bioluminescence. Developing parasites in the ear (yellow arrowhead) and in the liver (red arrowhead) of C57BL6 mice at D1 and D2 after microinjection of $2 \times 10^4$ GFP::LUC sporozoites.

Fig. S4. Parasite schizogony in the skin. Ten-micrometer cryostat sections of an infected ear imaged by confocal microscopy 3 and 4 d after sporozoite injection. The parasite is in red and the nuclear division is visualized using DAPI staining (white).

Fig. S5. Merosomes in the skin. (A) Images of EEF projecting extensions and merosome-like structures after mosquito bite (Upper) and after intradermal injection (Lower). (Scale bars, 10 μm.) (B) Time lapse of a skin EEF projecting extensions and merosome-like structures after mosquito bite. Each panel is a merge of three contiguous maximal Z-projections pseudocolored in red, green and blue. Each maximal Z-projection is composed of three pictures covering 4 μm. Time after inoculation is indicated at the top of each panel. (Scale bars, 10 μm.)
Fig. S6. Sensitivity to primaquine, development and infectivity of skin vs. liver *P. berghei* parasites. (A) Four- to 6-wk-old C57BL/6 mice were inoculated with \( \sim 10^4 \) GFP::LUC sporozoites in the ear by i.d. microinjection at day 0 (D0), and animals were either left untreated (0) or treated with 25 mg/kg (25) or 30 mg/kg (30) primaquine. Shown are the bioluminescence signals of skin and liver parasites at D2 in untreated and treated animals, and the average radiance of the bioluminescence signals of skin parasites in untreated and treated animals from D0 to D3. (B) Bioluminescence signals of an infected liver at D2 and of an infected ear at D3 (circled) after injection of \( \sim 10^4 \) GFP::LUC sporozoites in the ear and average ratio using six animals. (C) Approximately \( 7 \times 10^3 \) sporozoites were injected into the right ear of mice, and at D1 the infected or noninfected ear was surgically removed and the parasitemia subsequently followed. No statistical difference was observed between the parasitemia in animals having kept or not the infected ear.

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Fig. S7. Testing the infectivity of *P. berghei* skin parasites in situ. (A) Four- to 6-wk-old C57BL/6 mice were inoculated with \( 2-4 \times 10^4 \) sporozoites in the ear by i.d. microinjection at day 0 (D0). The mice were divided in three groups. Group I (positive controls) did not receive primaquine treatment and after anesthesia had the noninoculated ear excised at day 1 (D1). Group II (skin test animals) received 25 mg/kg of primaquine by i.p. injection at D0 and D1. Following anesthesia, the noninoculated ear was excised at D1. Group III (negative controls) received 25 mg/kg of primaquine by i.p. injection at D0 and D1. Following anesthesia, the inoculated ear was excised at D1. The parasitemia of the three groups was followed by FACS analysis until D20. (B) The table shows the number of infected mice out of the number of tested animals per each group in three independent experiments.

Fig. S8. Generation of *P. yoelii* parasites expressing the RedStar fluorescent protein. The RedStar-expressing cassette is integrated at the *d-ssu-rrna* locus. Shown from top to bottom are the targeting plasmid pL1102 (MRA-853, MR4; ATCC), the *d-ssu-rrna* target locus, and the recombinant locus after single crossing-over integration of the plasmid. Plasmid pL1102 contains (i) the RedStar cassette, in which the gene is under the control the *P. berghei* eef1a promoter and followed by 3′ expression sequences from the *P. berghei* dhfr-ts gene, (ii) a pyrimethamine-resistance cassette derived from the *Toxoplasma gondii* dhfr-ts, and (iii) a *d-ssu-rrna* fragment from *P. yoelii* 17XL. Plasmid pPL1121 was linearized at the Apal restriction site (A), and electroporated into *P. yoelii* blood stages.
**Fig. S9.** *P. yoelii* differentiation in the mouse skin. (A) Percentages of *P. yoelii* parasites (red bar) developing at D1 in hairless and in Swiss mice after intradermal injection of ∼5,000 to ∼8,000 sporozoites in 4–6 distinct injection sites for each mouse strain (average ± SD). For comparison, the values of *P. berghei* (green bar) are redrawn from Fig. 1E. (B) Lateral view of the skin showing an autofluorescent hair follicle (in red) and *P. yoelii* EEF (green) in the skin of a Swiss mouse at D3. The skin surface is indicated by the dashed line. (Scale bar, 10 μm.) (Inset) Lateral view of the skin showing a *P. yoelii* EEF with a flat bottom (white arrowhead) located ∼30 μm beneath the skin surface. (C) *P. yoelii* merozoites in the ear skin. (Left) Red fluorescent *P. yoelii* merozoites inside a superficial keratinocyte at D2 after microinjection of sporozoites in the ear skin of a hairless mouse. (Right) Green fluorescent *P. yoelii* merozoites at D3 after microinjection of sporozoites in the ear skin of a Swiss mouse. (Scale bars, 10 μm.) (D) *P. yoelii* merozoite movement in the skin of a BALB/c mouse at D2. The picture is the merge of three different images. The first, recorded at t = 0 s, is pseudocolored in blue; the second, recorded at t = 78 s, is pseudocolored in green; and the last, recorded at t = 108 s, is pseudocolored in red. White arrowhead, trajectory of individual merozoites; asterisks, parasites immotile during the time lapse. (Scale bar, 10 μm.)