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Hypoxia Promotes Liver Stage Malaria Infection in Primary Human Hepatocytes In Vitro

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Keywords: Hypoxia, primary hepatocytes, liver stage malaria
Abstract:

Homeostasis of mammalian cell function strictly depends on balancing oxygen exposure to maintain energy metabolism without producing excessive reactive oxygen species. In vivo, cells in different tissues are exposed to a wide range of oxygen concentrations, and yet in vitro models almost exclusively expose cultured cells to higher, atmospheric oxygen levels. Existing models of liver stage malaria that utilize primary human hepatocytes typically exhibit low in vitro infection efficiencies, possibly due to missing microenvironmental support signals. One cue that may influence the infection capacity of cultured human hepatocytes is the dissolved oxygen concentration. We developed a microscale human liver platform comprised of precisely patterned primary human hepatocytes and nonparenchymal cells (MPCC) to model liver stage malaria, but the oxygen concentrations are typically higher in the in vitro liver platform than anywhere along the hepatic sinusoid. Indeed, we observed that liver stage *Plasmodium* parasite development in vivo correlates with hepatic sinusoidal oxygen gradients. Therefore, we hypothesized that in vitro liver stage malaria infection efficiencies may improve under hypoxia. Using the infection of MPCCs with *P. berghei* or *P. yoelii* as a model, we observed that ambient hypoxia resulted in increased survival of exo-erythrocytic forms (EEFs) in hepatocytes, and improved parasite development in a subset of surviving EEFs, based on EEF size. Further, the effective cell surface oxygen tensions (pO$_2$) experienced by the hepatocytes, as predicted by a mathematical model, were systematically perturbed by varying culture parameters like hepatocyte density and media height, uncovering an optimal cell surface pO$_2$ to maximize the number of mature EEFs. Initial mechanistic experiments reveal that treatment of primary human hepatocytes with the hypoxia mimetic, cobalt (II) chloride, as well as a HIF-1α activator, dimethyloxalylglycine, also enhance *P. berghei* infection, suggesting that the effect of hypoxia on infection is mediated in part by host-dependent HIF-1α mechanisms.
Introduction:

Malaria affects 250 million people and causes approximately a million deaths each year (Organization, 2011). The liver stage is an attractive target for the development of antimalarial drugs and vaccines (Prudencio et al., 2006; Mazier et al., 2009), especially with the goal of malaria eradication, but is relatively poorly understood. In vitro models that recapitulate the liver stages of human malaria are needed to identify compounds that have potential antimalarial activity, but most of these models are dependent on cell lines (Gego et al., 2006; Meister et al., 2011) due to limitations in in vitro culture of primary adult hepatocytes. There is evidence that mimicking the in vivo hepatic microenvironment, such as by adding cell-cell interactions, cell-matrix interactions and controlling tissue microarchitecture can improve in vitro models of the liver (Dunn et al., 1989; Sivaraman et al., 2005; Khetani and Bhatia, 2008; Kidambi et al., 2009). For example, micropatterned cocultures (MPCCs) of primary human hepatocytes and supporting stromal fibroblasts result in stable hepatocyte function, including albumin secretion, urea production and cytochrome P450 levels, for several weeks compared to hepatocytes alone (Khetani and Bhatia, 2008). Another feature of the in vivo hepatic microenvironment is the presence of a range of oxygen tensions (Wolfe et al., 1983), which is thought to be a factor that contributes to hepatic zonation, a compartmentalization of functions along the axis of perfusion (Jungermann and Kietzmann, 1996; Jungermann and Kietzmann, 2000). Previous studies have shown that exposing mixed populations of primary rat hepatocytes to physiological gradients of oxygen tension can induce compartmentalization in vitro, render the cells selectively susceptible to zonal hepatotoxins (Allen and Bhatia, 2003; Allen et al., 2005), and recapitulate the zonated patterns of carbohydrate-metabolizing enzyme gene expression in vitro (Wolfe et al., 1983; Jungermann and Kietzmann, 1996; Kietzmann and Jungermann, 1997). Thus, in vitro liver-stage malaria culture platforms might be improved by altering microenvironmental oxygen concentrations.

Ambient oxygen concentrations have a broad spectrum of biological impact, influencing diverse pathways from homeostasis to development (Semenza, 2011). The role of oxygen has been explored in a range of infectious diseases. For instance, hyperoxia reduces certain bacterial and Apicomplexan infections in vitro or in vivo (Park et al., 1992; Tsuneyoshi et al., 2001; Arrais-Silva et al., 2006), whereas hypoxia promotes hepatitis C virus infection in vitro (Vassiliki et al., 2013) and Trypanosoma lewisi infections in vivo (Hughes and Tatum, 1956b). In the malaria field, previous studies have probed the effect of atmospheric oxygen on parasitemia in rodent and avian disease models. In particular, P. berghei-infected rats or P. cathemerium-infected canaries subjected to hypoxia exhibited increased levels of parasitemia (Hughes and Tatum, 1955; Hughes and Tatum, 1956a), whereas hyperoxia decreased P. berghei parasitemia (Rencricca et al., 1981; Blanco et al., 2008) and prevented early death caused by experimental cerebral malaria in the P. berghei-ANKA mouse model (Blanco et al., 2008). Furthermore, continuous in vitro culture of the blood stages of P. falciparum was first achieved by reducing atmospheric oxygen levels (Trager and Jensen, 1976), and subsequent studies have characterized this microaerophilic nature of blood stage P. falciparum (Torrentino-Madamet et al., 2011).
In this study, we explored the influence of cell surface oxygen on liver stage malaria infection of primary human hepatocytes. We used an in vitro model of hepatocyte culture that is phenotypically stable, responsive to ambient oxygen and supports the liver stage of malaria (March, 2013). Using this model system and a mathematical framework to estimate the cell surface oxygen partial pressures (pO2) under a variety of experimental manipulations, we show that oxygen has a profound impact on the Plasmodium liver stage. In particular, both infection efficiency and development of EEFs can be perturbed by altering cell surface oxygen concentrations. We identified an optimal cell surface oxygen level for maximizing infection and demonstrate that host HIF-1α is at least partially responsible for this response.

Results

In vivo EEF development correlates with hepatic oxygen gradients

Oxygen tensions in the hepatic sinusoids vary from 30 – 75 mmHg between the perivenous and periportal regions respectively (Wolfle et al., 1983). To investigate whether this variation in oxygen concentration exerts an influence on liver stage Plasmodium infection in vivo, C57BL/6 mice were infected with GFP-expressing P. yoelii sporozoites, a host-parasite combination that supports robust liver stage infection (Douradinha et al., 2007), and their livers were collected 46h post-infection. Two populations of P. yoelii exoerythrocytic forms (EEFs) were defined to test the hypothesis that the hepatic sinusoidal variation of oxygen concentration correlates with EEF growth. EEFs were defined as periportal EEFs if they were found within 8 cell-lengths of the hepatic portal triad, and perivenous EEFs if they were found within 8 cell-lengths of the hepatic central vein (Figure 1A). This definition minimizes the likelihood of an EEF being simultaneously defined as periportal and perivenous, taking into consideration that the number of hepatocytes between the portal triad and the central vein of a mouse liver is approximately 20. Immunohistochemical analysis of infected liver sections (Figure 1B) revealed that the maximal size of perivenous P. yoelii EEFs were significantly larger than periportal P. yoelii EEFs (Figure 1C), suggesting that oxygen concentrations could be a parameter that influences liver stage Plasmodium infection of primary hepatocytes in vitro.

Ambient hypoxia increases survival and growth of liver-stage malaria parasites in primary human hepatocyte micropatterned cocultures

To investigate whether hypoxia influences P. berghei infection of human liver cells in vitro, micropatterned cocultures (MPCCs) of primary human hepatocytes and supporting stromal fibroblasts were maintained at 4% O2 for 24 hours before infection. A 3 hour exposure to P. berghei sporozoites was followed by an additional 48 hours of hypoxic culture, at which point infection efficiency was determined based on HSP70 immunofluorescence. The number of P. berghei EEFs per hepatocyte island was elevated in response to hypoxic incubation of primary human hepatocytes before, during and after infection (Figure 2A). A significant upward shift in the size distribution of P. berghei EEFs in hypoxic cultures compared to normoxic cultures was also observed (Figure 2C, E). This pattern of improved infectivity was observed in more than one lot of cryopreserved primary
human hepatocytes (Figure 2A, S1A) and also in HepG2 cells (Figure S1C). Hypoxia-treated hepatocytes exhibited a similar increase in susceptibility to P. yoelii infection (Figure 2B, D, F, S1B), suggesting that the observed effect of hypoxia is not restricted to a particular Plasmodium spp.

Since P. berghei liver stage infections mature at 55-65 hours post-infection in vitro (Graewe et al, 2011), P. berghei EEF sizes were quantified at 56 hours and 65 hours post-infection to address the possibility that hypoxia could be speeding up parasite development instead of increasing the potential for parasite growth. P. berghei EEFs were larger in hypoxic cultures at 48, 56 and 65 hours post-infection (Figure S1F). Furthermore, the number of P. berghei EEFs per hepatocyte island was consistently higher in hypoxic cultures at 48, 56 and 65 hours post-infection (Figure S1E). Given that both EEF numbers and sizes are larger in hypoxic cultures throughout the late liver stages of P. berghei, this suggests that the total number of potential merozoites is larger under hypoxia compared to normoxia. Consistent with this prediction, the number of nuclei in P. berghei EEFs at 65 hours post-infection was significantly higher in hypoxic cultures compared to the normoxic control (Figure S1H). P. berghei EEFs were also able to develop normally under hypoxia, as shown by the expression of the mid-liver-stage marker, PbMSP-1, at 65 hours post-infection and the appearance of various EEF morphologies characteristic of late liver-stage EEFs (Figure S2). Moreover, the percentage of MSP1-positive P. berghei EEFs is significantly higher in hypoxic cultures at 56 and 65 hours post-infection (Figure S1G), suggesting that the EEFs progress into the later phases of the liver stage more successfully under hypoxia.

Importantly, the effect of hypoxia on EEF size translated to the human Plasmodium species, P. falciparum, as ambient hypoxia increased the size of P. falciparum EEFs in hepatocytes at both 4 and 6 days post-infection (Figure 2G, H). However, the number of P. falciparum EEFs did not increase in hypoxic cultures maintained at 4% O2 (Figure S1D).

Optimization of cell surface oxygen tension for in vitro liver-stage malaria infection

Given the observed impact of prolonged exposure to a reduced oxygen concentration, we sought an optimal set of conditions that might maximize the elevated infection of primary human hepatocytes (PHHs). By applying a mathematical model of diffusion and reaction solved at steady-state conditions (Yarmush et al., 1992) to PHH MPCCs (Figure 3A, S3), it was estimated that the typical cell surface pO2 when cultures are incubated at normoxia ranges from 110 – 130 mmHg (Table 1). In contrast, in vivo blood pO2 (not at the cell surface) ranges from 30 – 75 mmHg in the hepatic sinusoid (Wolfle et al., 1983). Therefore, culture at ambient hypoxia may improve liver stage malaria infection by reducing cell surface pO2 to a more physiologically relevant level. To test this hypothesis, a Hypoxyprobe™ assay that incorporates a hypoxic marker, pimonidazole hydrochloride (Varghese et al., 1976), was conducted to compare the cell surface pO2 in PHHs incubated at either normoxia or ambient hypoxia. Consistent with our hypothesis, incubation of PHHs at ambient hypoxia results in an increase in Hypoxyprobe™ staining relative to normoxia-cultured MPCCs (Figure 3B), confirming that ambient hypoxia indeed results in a decrease in cell surface pO2 experienced by the hepatocytes.
Cell surface pO$_2$ of MPCCs can also be altered by modifying parameters such as media height and hepatocyte density (Figure 3A). The model predicts that cell surface pO$_2$ decreases as media height increases (Figure S3B). Indeed, elevating the media height in wells of normoxic cultures resulted in an increase in Hypoxyprobe$^\text{TM}$ staining at the cell surface (Figure S4A, B). The greater media height also led to increased numbers of $P$. berghei EEFs at 48 hours post-infection (Figure S4C), collectively supporting the hypothesis that the effects of ambient hypoxia on in vitro liver stage malaria infection efficiencies are mediated by a decrease in the effective cell surface pO$_2$ experienced by the hepatocytes.

Modeling also predicts that cell surface pO$_2$ will decrease as cell density increases (Figure 3C, S2A). However, modifications to hepatocyte density in a conventional monolayer culture may also influence infection efficiency due to the resulting changes in hepatocyte survival, polarization and morphology, rather than in response to changes in cell surface pO$_2$. To vary hepatocyte density while preserving the homotypic interactions necessary for hepatocyte survival and functional maintenance, the density of the hepatocyte island patterning was varied in MPCCs. These modifications led to perturbations of the cell surface pO$_2$ as predicted by the model, based on Hypoxyprobe$^\text{TM}$ staining results (Figure 3C). The simultaneous variation of both hepatocyte island density and atmospheric oxygen level permits fine-tuning of cell surface oxygen levels that span 4 orders of magnitude. Infections with $P$. yoelii across this range of conditions yield a monotonic increase in total EEFs as cell surface pO$_2$ decreases (Figure 3E). However, a threshold cell surface pO$_2$ is observed at 5 – 10 mmHg, below which the number of mature EEFs (> 10 μm) decreases as cell surface pO$_2$ declines (Figure 3D). This biphasic relationship between the number of mature EEFs and cell surface pO$_2$ suggests that there is an optimal cell surface pO$_2$ for maximizing the number of mature EEFs in infected MPCCs. The combination of the optimal hepatocyte island density under ambient hypoxia (4% O$_2$) which gives rise to the optimal cell surface pO$_2$ of 5 – 10 mmHg was hence used for subsequent experiments.

**Kinetics of hypoxic treatment alters liver-stage malaria infection in vitro**

The hypoxia experiments performed thus far have exposed the PHH MPCCs to hypoxia throughout the 24h before infection, during infection (0 – 3h) and after infection (3 – 48h), termed the priming, invasion, and development phases, respectively. To assay whether improved infectivity requires each of these three phases of hypoxic treatment, MPCCs were incubated at ambient hypoxia over varying portions of the assay (Figure 4A). Increased numbers of EEFs at 48 hours post-infection were only observed when the infected MPCCs were cultured under hypoxia during the invasion and development phases (Figure 4B, conditions A, B, E). In contrast, MPCCs pre-treated with hypoxia before infection and subsequently returned to normoxia (Figure 4B, conditions C, D) did not exhibit an increase in EEF number. These findings suggest that hypoxia treatment improves late-stage infection rates by reducing the attrition rate of EEFs rather than promoting the initial susceptibility of
the host hepatocytes to sporozoite invasion. However, hypoxia over varying portions of the assay did not change the proportion of large EEFs 48h post-infection (Figure 4C).

**Hypoxia does not increase sporozoite-dependent or host-dependent invasion**

To examine whether the hypoxia-mediated change in hepatocyte infectivity stems from an impact on sporozoite function, sporozoite gliding motility and sporozoite entry were assayed. Ambient hypoxia did not result in a significant difference in the gliding motility of *P. berghei* sporozoites (Figure 4D), and hypoxic treatment of hepatocytes did not change the number of the sporozoites that successfully entered hepatocytes (Figure 4E), suggesting that hypoxia does not improve late-stage infection efficiencies via sporozoite or host-mediated increases in the initial invasion rate, but rather by affecting the ability of the host cell to support EEF survival and growth.

**Host HIF-1α induction promotes EEF survival in infected hepatocytes**

The hypoxic responses of mammalian cells is largely mediated by the hypoxia-inducible factor-1 (HIF-1) pathway (Semenza, 2012). Consistent with the reported literature, gene set enrichment analysis (GSEA) of PHH MPCCs incubated at ambient hypoxia revealed a marked enrichment for the expression of genes that are transcriptionally regulated by HIF-1α relative to normoxic MPCCs (Figure S6A). Cobalt (II) chloride is a hypoxia mimetic which has been reported to induce the intracellular stabilization of HIF-1α and lead to the transcriptional activation of downstream hypoxia-responsive genes (Jaakkola et al., 2001). To determine whether ambient hypoxia promotes liver-stage malaria infection in PHH MPCCs via host HIF-1α induction, pharmacologic activation of HIF-1α in PHH MPCCs by cobalt (II) chloride was performed at normoxia in three different combinations of the priming, invasion and development phases (Figure 5A). Cobalt (II) treatment of PHH MPCCs at normoxia in any of the three combinations tested resulted in an increased number of *P. berghei* EEFs 48 hours post-infection, with the greatest effect observed if cobalt (II) was present throughout all three phases of priming, invasion and development (Figure 5B). Of note, while ambient hypoxia (4% O₂) consistently led to the emergence of a subset of larger EEFs relative to normoxic controls, cobalt (II) treatment did not fully replicate this outcome (Figure 5C, Figure S5A).

Under normoxia, HIF-1α is constitutively marked for proteasomal degradation by prolyl hydroxylase (PHD). Inhibition of PHD by a small molecule, dimethyloxalylglycine (DMOG), results in HIF-1α stabilization and the associated downstream host hypoxic responses (Jaakkola et al., 2001). GSEA of hypoxic MPCCs also shows a marked enrichment for the expression of a set of genes that are upregulated under DMOG treatment (Figure S6B) (Elvidge et al., 2006). Consistent with the effect of cobalt (II) treatment on *P. berghei* infection at normoxia, PHH MPCCs that were treated with DMOG at normoxia demonstrate increased numbers of *P. berghei* and *P. yoelii* EEFs at 48h post-infection (Figure 5D, E), with the number of *P. berghei* EEFs increasing in a dose-dependent fashion with DMOG concentration (Figure S5B). However, DMOG treatment did not lead to the emergence of a subset of larger EEFs compared to the untreated control, in contrast to ambient hypoxia (Figure
Further increases in DMOG concentration inhibited EEF development (Figure S5C), which is reminiscent of the effect of extremely low levels of pO$_2$ on the number of well-developed EEFs (Figure 3D). Together, these data suggest that intermediate levels of HIF-1α activation in the host hepatocyte support EEF survival but not EEF growth, but higher levels of HIF-1α may inhibit EEF growth and mediate the biphasic effect of pO$_2$ on EEF size observed in earlier experiments.

**Discussion:**

Using an in vitro model of primary hepatocyte culture that stabilizes PHH function, is oxygen-responsive, and infectible with liver stage malaria, we applied a mathematical framework to estimate cell surface oxygen tensions under a variety of experimental manipulations. We show that the cell surface oxygen concentration experienced by primary adult human hepatocytes in vitro influences their ability to support a productive liver-stage malaria infection by *P. berghei*, *P. yoelii* and *P. falciparum*. Moreover, we identified an optimal cell surface oxygen level (predicted cell surface pO$_2$ 5-10 mmHg) for maximizing infection. More extreme levels of hypoxia (predicted cell surface pO$_2$ < 5 mmHg) resulted in increased late-stage parasite survival but arrested parasite development. The effects of hypoxia on late-stage EEF survival, but not EEF development, appear to be regulated in part by host-dependent HIF-1α mechanisms.

Establishing an in vitro model of liver stage malaria has been an ongoing challenge for the field, due in part to the relatively poor maintenance of hepatic functions by existing culture platforms. With the development of the PHH MPCC system, it is now possible to achieve robust liver-stage malaria infection in vitro (March, 2013), but further optimization of infection efficiency remains advantageous. Our mathematical model predicts that conventional MPCCs are hyperoxic under conventional culture conditions, with estimated cell surface pO$_2$ ranging from 110 – 130 mmHg (Table 1), whereas in vivo oxygen tensions in the liver range from 30 – 75 mmHg (Wolfe et al., 1983; Kietzmann and Jungermann, 1997). We have previously shown that achieving more physiological replication of the in vivo environment can improve hepatocyte function and disease modeling capacity in vitro (Allen et al., 2005). Thus, we hypothesized that liver-stage malaria infection might be more robust in vitro in the presence of atmospheric hypoxia. Indeed, the current observations that the sizes of *P. berghei*, *P. yoelii* and *P. falciparum* EEFs increase in PHHs under hypoxia in vitro is consistent with previous observations that primary hepatocytes respond to physiologically relevant oxygen gradients imposed upon them in vitro to recapitulate in vivo zonation phenotypes that are otherwise not observed in vitro (Allen et al., 2005). The observation that *P. berghei* and *P. yoelii* demonstrate increased numbers of EEFs under hypoxia, but not *P. falciparum*, suggests that the kinetics and extent of exposure to hypoxia for increased survival of the human malaria parasite differs from the rodent malaria species. The finding that there is an optimum cell surface pO$_2$ (5 – 10 mmHg) for liver-stage malaria infection in vitro is consistent with the histopathology findings from *P. yoelii* – infected mouse liver sections, which show that EEFs in the perivenous region, which has the lowest sinusoidal oxygen tension of 30mmHg, are larger than those in the periportal region (Figure 1). Intriguingly, this optimum range of cell surface pO$_2$ for primary
human hepatocyte infection in vitro is lower than the 30 – 75 mmHg (Wolfle et al., 1983) reported in hepatic sinusoids in vivo. One possible reason for this discrepancy is due to a lower hepatocyte surface pO2 in vivo than what had been previously measured in the hepatic sinusoid. This could be due either to the unsteady perfusion of the liver which arises from the pulsatile flow that has been observed in vivo (McCuskey et al., 1983), or the significant oxygen consumption by the endothelium in vivo (Santilli et al., 2000). This hypothesis is supported by the observations that liver sections obtained from mice perfused with Hypoxyprobe™ show significant Hypoxyprobe™ adduct accumulation in the pericentral regions (Arteel et al., 1995) and that Hypoxyprobe forms such adducts only at pO2 < 10 mmHg (Varghese et al., 1976). A second possible reason is that the optimal in vitro pO2 for malaria infection could simply be different from in vivo hepatic pO2. This could be because our in vitro model is missing key in vivo microenvironmental cues (growth factor gradients and cycling insulin/glucagon metabolism) that may result in the necessity for more extreme pO2 perturbations to optimize malaria infection in vitro. This disparity is consistent with the fact that in vitro infections, although improved under hypoxia, still require much higher multiplicities of infection than in vivo infections. It is also possible that the in vivo pO2 are not necessarily optimal anyway, since blood stage malaria parasitemia in rodents can be further increased under atmospheric hypoxia that simulates high-altitude atmospheres (Hughes and Tatum, 1956a). A third reason lies in the possibility that our mathematical model underestimates cell surface pO2 in vitro due to the assumption that only diffusion transports oxygen to the cell surface. Furthermore, our mathematical model assumes that hepatocytes exhibit a constant oxygen consumption rate (OCR) (Rotem et al., 1992; Yarmush et al., 1992), which may vary with species, donor, time in culture (Rotem et al., 1994; Bhatia SN, 1996), and culture parameters like density and coculture cell type. The finding that liver-stage malaria infection in vitro has an optimal oxygen tension is also consistent with the microaerophilic nature of the blood stages of *P. falciparum*, which exhibit a propensity for better growth in vitro under ambient hypoxia (Trager and Jensen, 1976; Briolant et al., 2007), and in fact demonstrate optimum growth at an in vitro pO2 (2% – 3%, 15 – 25mmHg) (Scheibel et al., 1979) that is lower than in vivo pO2 levels in the blood (4% – 13%, 30 – 100mmHg) (Tsai et al., 2003). To extrapolate our findings to other in vitro liver-stage models, the appropriate atmospheric pO2 should be determined within a similar mathematical framework as described for MPCCs, and take into account culture parameters such as effective hepatocyte density and oxygen diffusion distance (medium height).

The beneficial effect of hypoxia on in vitro liver-stage malaria infection could be due to changes in the host cell that increase host cell susceptibility to initial parasite invasion or that favor parasite survival or development, or changes in the parasite itself that promotes its own ability to survive and thrive in the host cell. Sporozoite entry assays (Figure 4E) and infection of hepatocytes exposed to hypoxia only prior to invasion but not after infection (Figure 4C) suggest that hypoxia does not increase hepatocyte susceptibility to sporozoite infection. Nonetheless, gene set enrichment analysis of PHH MPCCs incubated at ambient hypoxia versus normoxia showed a marked enrichment for the expression of HIF-1α related genes in hypoxic MPCCs (Figure S6A). HIF-1α plays a major role in the induction of cellular responses that mediate the adaptation of the host cell to hypoxic conditions.
This response includes an increased expression of glucose transporters and multiple enzymes responsible for a metabolic shift towards anaerobic glycolysis (Warburg effect), as well as the downregulation of mitochondrial respiration. The latter in turn reduces mitochondrial oxygen consumption and the resultant generation of reactive oxygen species that occurs due to inefficient electron transport under hypoxic conditions (Weidemann and Johnson, 2008; Semenza, 2012). Among other Apicomplexan infections, host HIF-1α has been shown to be essential for Toxoplasma gondii survival and growth in host cells cultured at physiological oxygen levels (3% O2) (Spear et al., 2006), and is also necessary for the maintenance of Leishmania amazonensis parasitemia in human macrophages in vitro (Degrossoli et al., 2007). In fact, Toxoplasma and Leishmania infection increases HIF-1α protein levels as well as HIF-1α-regulated expression of glycolytic enzymes and glucose transporters (Spear et al., 2006; Degrossoli et al., 2007), suggesting that these Apicomplexan parasites actively activate host HIF-1α presumably to favor their survival or growth. Pharmacological activation of host HIF-1α in infected MPCCs by CoCl2 and DMOG increased EEF survival (Figure 5B, 5D), but did not increase the EEF size distributions (Figure 5C, 5E), suggesting that the effects of ambient hypoxia on liver-stage malaria EEF numbers and EEF sizes may be driven by distinct mechanisms, with host HIF-1α playing a role in maintaining the survival of EEFs but not necessarily driving EEF growth. This hypothesis is supported by the observations that the total number of EEFs increased monotonically with decreasing cell surface pO2 (Figure 3E) but the number of well-developed EEFs exhibited a biphasic relationship with decreasing cell surface pO2 (Figure 3D). However, in the absence of genetic perturbation of host HIF-1α, the possibility that hypoxia, CoCl2 or DMOG are impacting alternate pathways in the parasite that mediate the observed infection phenotype cannot be excluded. One possible mechanism that could explain the effect of hypoxia on EEF size is the activation of the AMPK pathway in the host cell. AMPK activation is known to induce autophagy in mammalian cells (Liang et al., 2007; Kim et al., 2011), whereas autophagy of Plasmodium EEFs in human hepatoma cells is known to occur and may be necessary for the growth of Plasmodium EEFs (Eickel et al., 2013). AMPK activation also mediates mitophagy and mitochondrial biogenesis (Mihaylova and Shaw, 2011), which results in increased mitochondrial renewal, and may promote Plasmodium EEF development. In support of this hypothesis, Toxoplasma gondii, another Apicomplexan parasite, is known to tether host mitochondria to its parasitophorous vacuole membrane (Sinai and Joiner, 2001), suggesting that host mitochondria is necessary for Toxoplasma growth in the host cell. In addition to host-mediated mechanisms, the malaria parasite may contain either oxygen sensors that directly respond to hypoxia or indirect mechanisms that limit their ability to respond to oxidative stress. It is difficult to distinguish the parasite-specific and the host-specific responses to hypoxia. For example, intraerythrocytic P. falciparum is heavily dependent on antioxidant systems despite its almost totally fermentative lifestyle, yet it lacks significant antioxidant enzymes like catalase and glutathione peroxidase which play major protective roles in mammalian cells (Muller, 2004; Vonlaufen et al., 2008). This suggests that the Plasmodium liver stage may also be predisposed to being overwhelmed by environmental oxidants, and that hypoxia may reduce the energy expenditure for the maintenance of redox balance in the EEF.
A caveat of our findings is that changes in atmospheric oxygen may result in modulations beyond simply adjusting cell surface oxygen levels. The modulation of hepatocyte metabolism under hypoxia may result in different rates of nutrient consumption and waste generation, which may lead to secondary effects like changes in pH. This study also does not specifically identify the role of the coculture nonparenchymal cell type in the infection phenotype, and does not use a liver-derived nonparenchymal cell type like sinusoidal endothelial cells or Kupffer cells. The in vivo histopathology findings are correlative and not causal, as the presence of an oxygen gradient along the sinusoid is only one of many other gradients that simultaneously exist in the liver. Thus, it is challenging to decisively untangle the various contributions of oxygen gradients in our observations, but oxygen may be more likely to be the driver of these other sinusoidal gradients than vice versa. More work is required to characterize the role of HIF-1α on Plasmodium infection of PHHs, including performing siRNA-mediated knockdown and overexpression of HIF-1α in primary hepatocytes in vitro, or using a HIF-1α knock-out mouse. Further, the downstream mechanisms of HIF-1α that are ultimately responsible for the effect of hypoxia on Plasmodium infection of PHHs remain to be uncovered. These mechanisms could include increases in glycolysis or iron uptake by hepatocytes, which could lead to an elevation in intracellular glucose or iron levels that are accessible to the Plasmodium EEF. Other mechanisms that may contribute to the effect of hypoxia on infection could include AMPK activation in host cells leading to a starvation response that decreases intracellular ROS levels and frees up resources for the malaria EEF.

In an era of a renewed effort towards global malaria eradication, the finding that oxygen levels influence in vitro Plasmodium liver stage infection of PHHs, in combination with existing literature on the impact of oxygen on the maintenance of in vivo-like hepatocyte functions in vitro, highlights the importance of optimizing oxygen levels experienced by PHHs in vitro so as to develop improved in vitro models of liver-stage malaria for antimalarial drug development.

**Materials and Methods:**

**Reagents and cell culture.** Dimethyloxalylglycine (DMOG) was obtained from Cayman Chemicals (Ann Arbor, Michigan, USA), and cobalt (II) chloride was obtained from Sigma (St. Louis, Missouri, USA). Cryopreserved primary human hepatocytes were purchased from vendors permitted to sell products derived from human organs procured in the United States by federally designated Organ Procurement Organizations. CellzDirect (Invitrogen, Grand Island, New York, USA) was the vendor used in this study. Human hepatocyte culture medium was high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) ITS™ (BD Biosciences), 7 ng/ml glucagon, 40 ng/ml dexamethasone, 15 mM HEPES, and 1% (v/v) penicillin-streptomycin. J2-3T3 murine embryonic fibroblasts (gift of Howard Green, Harvard Medical School) were cultured at < 15 passages in fibroblast medium comprising of DMEM with high glucose, 10% (v/v) bovine serum, and 1% (v/v) penicillin-streptomycin.

**Micropatterned cocultures (MPCCs) of primary human hepatocytes and supportive stromal cells.** 12mm coverslips that were placed into tissue culture polystyrene 24-well plates or glass-
bottomed 96-well plates were coated homogenously with rat tail type I collagen (50 μg/ml) and subjected to soft-lithographic techniques (Khetani and Bhatia, 2008) to pattern the collagen into micro-islands (of diameter 500 μm) that mediate selective hepatocyte adhesion. To create MPCCs, cryopreserved primary human hepatocytes were thawed and pelleted by centrifugation at 100x g for 6 min, assessed for viability using Trypan blue exclusion (typically 70–90%), and then seeded on collagen-micropatterned plates in DMEM. The cells were washed with DMEM 2–4h later and replaced with human hepatocyte culture medium. 3T3-J2 murine embryonic fibroblasts were seeded (40,000 cells in each well of 24-well plate and 7,000 cells in each well of 96-well plate) in human hepatocyte medium 3h after Plasmodium sporozoite infection and medium was replaced every 24h.

**Sporozoites.** *P. berghei ANKA* and *P. yoelii* sporozoites were obtained by dissection of the salivary glands of infected *Anopheles stephensi* mosquitoes obtained from the insectaries at New York University (New York, New York, USA) or Harvard School of Public Health (Boston, Massachusetts, USA). *P. falciparum* sporozoites were obtained by dissection of the salivary glands of infected *Anopheles gambiae* mosquitoes obtained from the insectary at Johns Hopkins School of Public Health (Baltimore, Maryland, USA).

**Infection of micropatterned cocultures.** *P. berghei*, *P. yoelii* or *P. falciparum* sporozoites from dissected mosquito glands were centrifuged at 3000rpm for 5 min on to micropatterned primary hepatocytes cultured without fibroblasts for 2 or 3 days before infection at a multiplicity of infection of 1 to 3. After incubation at 37°C and 5% CO₂ for 3h, the wells were washed twice, and J2-3T3 fibroblasts were added to establish the micropatterned cocultures. Media was replaced daily. Samples were fixed 48h, 56h or 65h post-infection with *P. berghei* and *P. yoelii*, and 4 days or 6 days post-infection with *P. falciparum*.

**Immunofluorescence assay.** Infected MPCCs were fixed with -20°C methanol for 10 min at 4°C, washed thrice with PBS, blocked with 2% BSA in PBS for 30 min and then incubated for 1h at room temperature with a primary antibody mouse anti-PbHSP70 (clone 2E6; 1:200 for *P. berghei* and *P. yoelii*), rabbit anti-PbMSP1 (1:500 for *P. berghei*), or mouse anti-PfHSP70 (clone 4C9, Sanaria; 1:200 for *P. falciparum*). Samples were washed thrice with PBS before incubation for 1h at room temperature with secondary antibody: goat anti-mouse Alexa Fluor 594 or Alexa Fluor 488 or donkey anti-rabbit-Alexa Fluor 488 (Invitrogen; 1:400). Samples were washed thrice with PBS, with nuclei counterstained with Hoechst 33258 (Invitrogen; 1:1000), and then mounted on glass slides with Fluoromount G (Southern Biotech, Birmingham, Alabama, USA). For samples in 96-well plates, 50 μL of Aquamount (Thermo-Scientific, West Palm Beach, Florida, USA) was added per well after counter-staining with Hoechst. Images were captured on a Nikon Eclipse Ti fluorescence microscope.

**Sporozoite gliding assay.** Motility of cryopreserved sporozoites was determined in each batch to define the number of infective sporozoites. Sporozoite gliding was evaluated with 30,000 sporozoites for 40 minutes in complete DMEM, at 37°C on glass cover slips pre-coated for 1h at 37°C with an antibody against *P. berghei* circumsporozoite protein (PbCSP) (clone 3D11, 10μg/mL). Sporozoites
were subsequently fixed in 4% paraformaldehyde (PFA) for 10 minutes and stained with anti-PbCSP antibody. The percentage of sporozoites associated with CSP trails was visualized by fluorescence microscopy. Quantification was performed by counting the average percentage of sporozoites that perform at least one circle.

**Double-staining assay for sporozoite entry.** At 3h post-infection, MPCCs were fixed and stained using a double-staining protocol as previously described (Renia et al., 1988). Briefly, to label extracellular sporozoites, the samples were first fixed with 4% paraformaldehyde for 10min at room temperature, blocked with 2% BSA in PBS, incubated with a primary mouse anti-PbCSP (clone 3D11, 10 μg/mL), washed thrice in PBS and incubated with a secondary goat anti-mouse Alexa Fluor 488 conjugate. This was followed by a permeabilization with -20°C methanol for 10min at 4°C, incubation with the same primary mouse anti-PbCSP, washing thrice with PBS, and incubation with a secondary goat anti-mouse Alexa Fluor 594 conjugate. This second step labels both intracellular and extracellular sporozoites. The samples were counterstained with Hoechst and mounted on glass slides as described above. The number of invaded sporozoites (stained green only) in primary human hepatocytes was quantified.

**Gene expression microarray analysis.** MPCCs established from two different donor lots of primary human hepatocytes were incubated under ambient hypoxia overnight (18-24 h), and total RNA was extracted using TRIZOL and a Qiagen RNA clean-up kit. The RNA was analyzed by a Bioanalyzer, before being labelled with Cy 3 and Cy 5 for the normoxic versus hypoxic samples respectively. The labeled RNA from biological triplicates was loaded onto an Agilent (Santa Clara, California, USA) SurePrint G3 Human Gene Expression Microarray. The microarray data was analyzed by performing a Gene Set Enrichment Analysis (GSEA), which determines whether a pre-defined set of genes shows statistically significant differences between two biological conditions (Subramanian et al., 2005), applying a false discovery rate of 25%.

**Mathematical model.** To estimate the cell surface oxygen tensions in micropatterned cocultures, the transport and consumption of oxygen was modeled as a one-dimensional reaction-diffusion system, which was described previously (Yarmush et al., 1992). The average number of hepatocytes per hepatocyte island in the MPCCs was determined by manual counts with light microscopy. The following assumptions were made in applying this model. Firstly, the oxygen consumption rate of primary rat hepatocytes was used, due to absence of the oxygen consumption rates of primary human hepatocytes. Secondly, as the oxygen consumption rate of fibroblasts is only one-tenth that of primary rat hepatocytes, and the oxygen consumption rate of random cocultures of hepatocytes and fibroblasts was similar to that of hepatocytes alone (Allen et al., 2005), the oxygen consumption of MPCCs was assumed to be that of hepatocytes alone. Thirdly, the oxygen consumption rates were assumed to be independent of culture format and constant throughout the infection experiments.

**Hypoxyprobe assay.** Hypoxyprobe™ (pimonidazole hydrochloride, Burlington, Massachusetts, USA) forms covalent adducts in hypoxic cells at cell surface pO₂ < 10 mmHg (Varghese et al., 1976),
and was used as a hypoxia marker in primary human hepatocytes. Hypoxia was first induced in primary hepatocytes by atmospheric hypoxia, variation of medium heights or variation in hepatocyte island densities. Pimonidazole hydrochloride was then added from a 200mM stock solution (constituted in PBS) into the culture medium (without changing medium to avoid disturbing the steady state oxygen gradient) at a 1:1000 dilution to achieve a final working concentration of 200μM. Cells were incubated at 37°C for 2h, washed twice with PBS, and fixed with chilled methanol for 10min at 4°C. Adduct formation was detected by direct immunofluorescence using the HP-Red549 antibody (Hypoxyprobe™) at a 1:100 dilution.

**Histological analysis.** 50-μm liver slices were obtained from C57BL/6 mice (Charles River, Wilmington, Massachusetts, USA), 46h post-infection with GFP-expressing *P. yoelii* sporozoites. Maximal EEF size of EEFs in the periportal area (up to 8 hepatocytes wide, from portal vein) and in the centrilobular area (up to 8 hepatocytes wide, from central vein) were measured using z stacks of these EEFs acquired via confocal imaging (Olympus, Center Valley, Pennsylvania, USA).

**Statistics.** Experiments were repeated three or more times with triplicate samples for each condition. Data from representative experiments are presented, and similar trends were seen in multiple experiments. Two-tailed t tests were performed for all comparisons between two conditions (e.g. 21% versus 4% O₂) at a single time point. One way ANOVAs were performed for comparisons involving three or more conditions (e.g. 21% versus different periods of 4% O₂) at a single time point with Tukey’s post-hoc test for multiple comparisons. Two way ANOVAs were performed for comparisons involving both simultaneous variation in time points post-infection and oxygen level (e.g. 21% versus 4% O₂ at 48h, 56h and 65h post-infection for *P. berghei*) with Bonferroni’s post-hoc test for multiple comparisons. All error bars represent s.e.m.

**Acknowledgements:**

We thank Robert Schwartz (MIT) for technical help in confocal microscopy, Ana Rodriguez (NYU) and Sandra Gonzalez (NYU) for providing mosquitoes infected with *P. yoelii* and *P. berghei*, Dyann Wirth (HSPH) and Emily Lund (HSPH) for providing mosquitoes infected with *P. berghei*, Photini Sinnis (JHSPH) and Abhai Tripathi (JHSPH) for insightful conversation and providing mosquitoes infected with *P. falciparum*, Charlie Whitaker (Koch Institute, MIT) for help with microarray data analysis and Heather Fleming for critical reading and help with manuscript preparation.

**Competing interests statement:** No competing interests declared.

**Author contributions:**

S.N., S.M., M.M.M., S.N.B. designed research. S.N., S.M., A.G., K.H. performed research. S.N., S.M., K.H. and S.N.B. analyzed data. S.N. and S.N.B. wrote the manuscript.

**Funding:**

This work was supported by the Bill & Melinda Gates Foundation (Award # 51066). SN is supported
by an A*STAR (Agency for Science, Technology and Research, Singapore) National Science Scholarship. SNB is a Howard Hughes Medical Institute Investigator.

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Figure Legends:

**Figure 1:** *Plasmodium* EEF development correlates with hepatic oxygen gradients in vivo.

(A) Schematic of liver sinusoid denoting the definition of periportal (PP) EEFs and perivenous (PV) EEFs used for EEF size quantification. (B) 50μm liver slices were stained with DAPI, and confocal z stacks of GFP-expressing *P. yoelii* EEFs within 8 hepatocyte lengths of either the portal triad (periportal) or the central vein (perivenous) for which the maximal XY area could be determined within the slice were made. (C) Maximal XY areas of *P. yoelii* perivenous or periportal EEFs (as defined in A, above), 46 h post-infection in murine liver. **p < 0.01, two-tailed t-test. Scale bar: 50 μm.

**Figure 2:** Ambient hypoxia increases liver stage malaria infection in vitro.
(A, B) Ambient hypoxia (4% O₂) increases the number of *P. berghei* and *P. yoelii* EEFs in primary human hepatocyte micropatterned cocultures at 48h post-infection. * p < 0.05, ** p < 0.01, *** p < 0.001, two-tailed t-test. (C, D, G) Ambient hypoxia (closed symbols or bars, 4% O₂) increases the EEF size distribution of *P. berghei* and *P. yoelii* at 48h post-infection and *P. falciparum* at 4 and 6 days post-infection in primary human hepatocyte micropatterned cocultures compared to normoxia (open symbols or bars, 21% O₂). ** p < 0.01, **** p < 0.0001, two-tailed t-test. (E, F, H) Representative immunofluorescence images of *P. berghei*, *P. yoelii* EEFs at 48h post-infection, and *P. falciparum* EEFs at 6 days post-infection at either ambient 21% or 4% O₂. EEFs were stained for *Plasmodium* HSP70 (clone 2E6 for *P. berghei* and *P. yoelii*, clone 4C9 for *P. falciparum*). Scale bars: 5 μm.

**Figure 3: Optimal pO2 exists for development of mature Plasmodium EEFs.**
(A) Schematic of steady-state diffusion-reaction model with three parameters that determine cell surface oxygen concentration: atmospheric pO₂, medium height and cell density. (B) Validation of effect of atmospheric pO₂ on cell surface pO₂ by Hypoxyprobe staining. Hypoxyprobe forms covalent adducts with thiol groups at pO₂ < 10 mmHg. (C) Modulation of cell surface pO₂ by varying effective cell density as predicted by the model (red), and on Hypoxyprobe fluorescence intensity (blue). Modulation of cell surface pO₂ by simultaneously varying both atmospheric pO₂ and effective cell density results in (D) a biphasic relationship between the number of well-developed *P. yoelii* EEFs versus predicted cell surface pO₂ in PHH MPCCs but (E) a monotonic relationship between the total number of *P. yoelii* EEFs versus predicted cell surface pO₂ in PHH MPCCs at 48h post-infection. Scale bars: 100 μm.

**Figure 4: Kinetics of hypoxic treatment alters liver-stage malaria infection in vitro.**
(A) Schematic of differential hypoxia treatment regimes. (B) Effect of differential hypoxia kinetic regimes on the number of *P. berghei* EEFs at 48h post-infection. (C) Effect of differential hypoxia kinetic regimes on *P. berghei* EEF sizes at 48h post-infection. (D) Effect of ambient hypoxia on *P. berghei* sporozoite gliding. (E) Effect of ambient hypoxia on *P. berghei* sporozoite entry into hepatocytes at 3h post-infection. ** p <0.01, *** p < 0.001, one way ANOVA with Tukey’s Multiple Comparison Test.

**Figure 5: Host HIF-1α induction increases EEF numbers in infected hepatocytes.**
(A) Schematic of cobalt (II) chloride treatment of primary human hepatocyte micropatterned cocultures (PHH MPCCs) during infection with *P. berghei*. (B) Effect of cobalt (II) treatment of PHH MPCCs at 21% O₂ on the number of *P. berghei* EEFs at 48h post-infection, and (C) on the percentage of *P. berghei* EEFs > 10μm at 48h post-infection. ** p <0.01, *** p < 0.001, one way ANOVA with Tukey’s Multiple Comparison Test. (D) Effect of DMOG treatment of PHH MPCCs at 21% O₂ on the numbers of *P. berghei* EEFs and (E) the number of *P. yoelii* EEFs at 48h post-infection. * p < 0.05, two-tailed t-test.

**Figure S1: Effect of ambient hypoxia on liver stage malaria infection in primary human hepatocytes.**
Ambient hypoxia (4% O₂) increases the (A) *P. berghei* and (B) *P. yoelii* EEF size distributions at 48h post-infection in primary human hepatocyte micropatterned cocultures in a second hepatocyte donor. p < 0.0001 and p = 0.0025 respectively, two-tailed t-test. (C) Ambient hypoxia (4% O₂) increases *P. berghei* infection in human hepatoma HepG2 cells at 48h post-infection. (D) Ambient hypoxia (4%
O2) does not increase P. falciparum infection in in primary human hepatocyte micropatterned cocultures at day 4 and day 6 post-infection. Culture of primary human hepatocyte micropatterned cocultures at ambient hypoxia (4% O2) during infection (E) increases the number of P. berghei EEFs at 48h, 56h and 65h post-infection compared to normoxia (21% O2), * p < 0.05, ** p < 0.01, *** p < 0.001, two way ANOVA with Bonferroni Multiple Comparison Test, F = 32.1, DoF = 1, p = 0.0001 for the effect of O2. (F) increases the P. berghei EEF size distribution at 48h, 56h and 65h post-infection compared to normoxia (21% O2), **** p < 0.0001, two-tailed t-test, (G) increases the number of MSP1+ P. berghei EEF at 56h and 65h post-infection. ** p < 0.01, **** p < 0.0001, two way ANOVA with Bonferroni Multiple Comparison Test, F = 63.5, DoF = 1, p < 0.0001 for the effect of O2 and (H) increases the number of P. berghei nuclei per EEF at 65h post-infection. ** p < 0.01, two-tailed t-test. Scale bars: 10μm.

**Figure S2: Late liver-stage development under ambient hypoxia.** (A) P. berghei EEFs at 65 hpi express PbHSP70 (red) and PbMSP-1 (green) at normoxia (21% O2) or hypoxia (4% O2). (B) Various stages of normal late liver-stage EEF development is observed at hypoxia. MSP-1 is initially expressed on the parasite membrane around all the parasite nuclei, then forms invaginations around groups of nuclei, and eventually surrounds individual merozoites. MSP-1-positive merosome-like structures are also observed breaking off from infected hepatocytes. Scale bars: 10μm.

**Figure S3: Diffusion – reaction model of cell surface pO2.** Mathematical model employed in the study. Table of parameter values used in this study. Graphs showing the modulation of cell surface pO2 by (A) effective cell density and (B) media height as predicted by the mathematical model.

**Figure S4: Modulation of cell surface pO2 by variation in media height.** (A) Increasing medium height at normoxia increases cell surface pO2, as determined by Hypoxyprobe staining (red). (B) Quantification of Hypoxyprobe fluorescence intensity. (C) Decreasing cell surface pO2 by increasing medium height increases P. berghei infection efficiencies in PHH MPCCs, but has no effect on EEF size distribution (D). *** p <0.001, **** p < 0.0001, one way ANOVA with Tukey’s Multiple Comparison Test. Scale bars: 100 μm.

**Figure S5: Effect of hypoxia mimetics on P. berghei infection in PHH MPCCs.** (A) Size distributions of P. berghei EEFs infected at normoxia, ambient hypoxia or with cobalt (II) treatment. (B) Effect of 0.02mM or 0.1mM DMOG treatment on P. berghei infection in MPCCs. (C) Size distributions of P. berghei EEFs infected at normoxia, ambient hypoxia or 0.02mM or 0.1mM DMOG. * p< 0.05, ** p < 0.01, *** p < 0.001, one way ANOVA with Tukey’s Multiple Comparison Test.

**Figure S6: Gene set enrichment analysis comparing PHH MPCCs incubated at normoxia and ambient hypoxia.** In these plots, a positive enrichment score indicates correlation of the queried gene set with the normoxic (21% O2) condition, whereas a negative enrichment score indicates correlation of the queried gene set with the hypoxic (4% O2) condition. The bottom portion of the plot shows the value of the ranked list metric as you move down the list of ranked genes. The ranked list metric measures a gene’s correlation with a phenotype, and its value goes from positive to negative as you move down the ranked list. A positive value indicates correlation with the normoxic condition (21% O2) and a negative value indicates correlation with the hypoxic condition (4% O2). (A) A GSEA
enrichment plot shows enrichment in hypoxia (4% O₂) treated PHH MPCCs for an a priori defined query set of genes that is transcriptionally regulated by HIF-1α, with a negative enrichment score of -0.64, and a normalized enrichment score of -2.01. (B) A GSEA enrichment plot shows enrichment in hypoxia (4% O₂) treated PHH MPCCs for an a priori defined query set of genes that is upregulated by hypoxia mimetic DMOG, with a negative enrichment score of -0.63, and a normalized enrichment score of -2.49.

**Tables:**

**Table 1:** Compilation of predicted cell surface pO₂ in different culture formats in primary hepatocyte in vitro culture models

| Hepatocyte format                          | Predicted cell surface pO₂ (mmHg) |
|--------------------------------------------|-----------------------------------|
| MPCC at normoxia (P_air = 21%)             | 110 – 130                         |
| MPCC at ambient hypoxia (P_air = 4%)       | 5 – 10                            |
| Hepatic sinusoid in vivo                   | 30 – 75 (periportal – perivenous)  |
| Monolayer at normoxia (P_air = 21%)        | 0.1 – 10                          |

1. Wolfle D et al (1983). Eur J Biochem 135(3): 405-412. 2. Siau, A. et al. (2008). PLoS Pathog 4(8): e1000121. 3. Yalaoui, S. et al. (2008). Cell Host Microbe 4(3): 283-292. 4. Mazier et al. (1985) Science. 227(4685): p. 440-2. 5. Bhatia SN et al (1996) Journal of Cellular Engineering, 1:125-135.

**Translational Impact:**

Malaria is a parasitic disease that impacts millions world-wide. The symptoms of malaria are caused by blood cell infection, but before reaching this stage, parasites develop and grow in the patient’s liver. The liver stage, therefore, is an attractive target for drug treatment. To develop drugs that effect malaria parasites in the liver, model systems that mimic normal human liver responses are needed, but existing models are generally hard to infect. The low levels of infection may be partly because cells that are grown in tissue culture are usually exposed to higher levels of oxygen than they would experience inside the body. In this study, malaria parasite growth in the livers of infected mice was observed to correlate with the natural variation of oxygen levels within the liver. Subsequently, several methods were used to expose cultured human liver cells to different levels of oxygen, which led to profound changes in malaria infection efficiency and parasite development. The effect of oxygen was found to be partly due to the activation of a particular intracellular oxygen sensing signaling pathway. This study highlights the importance of optimizing oxygen levels experienced by human liver cells in order to maximize malaria infection rates so as to develop improved models of liver-stage malaria for antimalarial drug development, and suggests that there may be other oxygen-dependent host or parasite mechanisms that influence liver stage malaria parasite development.
Figure 3

A. At $x = 0 \rightarrow P = P_{air}$

$D \frac{\partial P}{\partial x} = \text{constant}$

$d = \text{media height}$

$\rho = \text{effective cell density}$

B. 21%

C. Predicted Cell Surface $pO_2$ (mmHg) vs. Fluorescence Intensity

Hepatocyte density (cells/cm²)

D. # PyEEFs $> 10$ μm/island vs. Predicted cell surface $pO_2$ (mmHg)

E. # PyEEFs/island vs. Predicted cell surface $pO_2$ (mmHg)
**Figure 4**

A. Diagram illustrating the timeline of priming, invasion, and development with h.p.i. (hours post-infection) marked.

B. Bar graph showing the number of HSP70+ EEFs/island with statistical significance indicated by ***.

C. Bar graph displaying the percentage of EEFs > 10 μm with statistical significance indicated by ** and ***.

D. Bar graph depicting the percentage of P. berghei sporozoites gliding, with 'n.s.' indicating no significant difference.

E. Bar graph showing the number of P. berghei sporozoites per island with 'n.s.' indicating no significant difference.
Figure 5

A. Timeline of priming, invasion, and development with hpi (hours post infection).

B. Graph showing the number of HSP70+ EEFs/island with different conditions.

C. Bar chart displaying the percentage of EEFs > 10μm under various conditions.

D. Histogram comparing the number of Pb EEFs/island in control and DMOG treated samples.

E. Graph showing the number of Py EEFs/island in control and DMOG treated samples.