GENERATION OF RETICULOCYTES DERIVED FROM HUMAN UMBILICAL CORD BLOOD CD34^{+} HAEMATOPOIETIC STEM CELLS FOR *Plasmodium knowlesi IN VITRO* CULTURE

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GENERATION OF RETICULOCYTES DERIVED FROM HUMAN UMBILICAL CORD BLOOD $CD34^+$ HAEMATOPOIETIC STEM CELLS FOR *Plasmodium knowlesi IN VITRO* CULTURE

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	percentage
°C	degree Celcius
>	more than
<	less than
≥	greater than or equal to
≤	less than or equal to
dL	deciliter
g	gram
g	gravity
kg	kilogram
kDa	kilodalton
mL	mililiter
µL	microliter
µg	microgram
mM	milimolar
M	molar
mg	miligram
ng	nanogram
v	volume
APC	allophycocyanin
BFU-E	burst-froming unit-erythroid
BM	bone marrow
BSA	bovine serum albumin

CCM	complete culture media
CFU-E	colony-forming unit-erythroid
CFU-GEMM	colony-forming unit of granulocyte, erythroid, macrophage and megakaryocyte
CFU-Lymph	colony-forming unit of lymphoid
CO_2	carbon dioxide
DARC	duffy antigen receptor for chemokines
DBP	duffy binding protein
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPO	erythropoietin
ESCs	embryonic stem cells
EtOH	absolute ethanol
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase 3
FSC	forward scatter
HCl	hydrochloric acid
HDS	hydrocortisone
HGM	Hospital Gua Musang
HKK	Hospital Kuala Krai
HPCs	haematopoietic progenitor cells
hrs	hours
HSCs	haematopoietic stem cells

ICCM	incomplete culture media
IL3	interleukin-3
IL6	interleukin-6
IMDM	Iscove’s Modified Dulbecco’s Medium
iPSCs	induced pluripotent stem cells
MACS	magnetic-activated cell sorting
MSCs	mesenchymal stem cells
NaCl	sodium chloride
PB	peripheral blood
PBS	phosphate buffer saline
PE	phycoerythrin
PerCP-Cy5.5	peridinin-chlorophyll protein complex: Cy5.5
RBC	red blood cell
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SCF	stem cell factor
SFEM II	serum-free expansion medium II
SSC	side scatter
SSCs	somatic stem cells
TPO	thrombopoietin
UCB	umbilical cord blood
WHO	World Health Organization

PENGHASILAN RETIKULOSIT DARIPADA SEL STEM HEMATOPOIETIK CD34^{+} YANG BERASAL DARIPADA DARAH TALI PUSAT MANUSIA UNTUK PENGKULTURAN *Plasmodium knowlesi* SECARA *IN VITRO*

ABSTRAK

Kemunculan parasit malaria zoonotik, *P. knowlesi* menjadi parasit paling dominan yang menyebabkan jangkitan malaria pada manusia di Asia Tenggara dan Borneo Malaysia mencatatkan kes malaria tertinggi yang disebabkan oleh *P. knowlesi*. *P. knowlesi* dilaporkan menunjukkan kecenderungan terhadap retikulosit. Walau bagaimanapun, bagi mendapatkan retikulosit dalam jumlah yang besar untuk bekalan berterusan menjadi halangan utama bagi kajian jangka panjang pengkulturan. Ini disebabkan oleh kepekatan retikulosit yang rendah dalam darah periferal, sumsum tulang dan darah tali pusat. Mujurlah, sistem pembangunan dalam kultur secara *in vitro* untuk menghasilkan retikulosit yang berasal daripada sel stem hematopoietik (HSC) dapat menyelesaikan halangan kajian *P. knowlesi*. Dalam kajian ini, darah tali pusat (UCB) digunakan sebagai sumber HSC kerana ia mengandungi kepekatan CD34^{+} tertinggi berbanding darah periferal dan sumsum tulang. Pertama, CD34^{+} HSC yang berasal dari UCB dikembangkan dalam *serum-free expansion medium II* (SFEM) dengan faktor pertumbuhan dan sitokin yang membolehkan sel berkembang biak. Setelah sel membiak selama 5 hari, sel telah dikultur dalam media pembezaan dengan tambahan faktor pertumbuhan dan sitokin yang mencukupi untuk proses pembezaan retikulosit. Pemeriksaan mikroskopik dan analisis FACS telah dijalankan sepanjang tempoh pengkulturan bagi memerhati perubahan morfologi dan memantau ekspresi

antigen permukaan sel. Akhir sekali, *P. knowlesi* telah dikultur dalam retikulosit yang dihasilkan dan kadar jangkitan ditentukan selepas 24 jam pasca-jangkitan. Walaupun jangkitan *P. knowlesi* tidak dapat dijalankan kerana keadaan yang tidak mengizinkan, hasil kajian menunjukkan bahawa retikulosit yang berfungsi dapat dihasilkan dari $CD34^+$ HSC yang berasal dari UCB dan kadar jangkitan *P. knowlesi* dianggarkan sama dengan jangkitan *P. vivax*. Hal ini kerana, *P. knowlesi* mempunyai filogenetik yang hampir sama dengan *P. vivax*. Sebagai kesimpulan, kajian ini menunjukkan kebarangkalian dalam menghasilkan pengeluaran populasi retikulosit homogen secara besar-besaran daripada $CD34^+$ HSC yang berasal dari UCB.

GENERATION OF RETICULOCYTES DERIVED FROM HUMAN UMBILICAL CORD BLOOD CD34^{+} HAEMATOPOIETIC STEM CELLS FOR *Plasmodium knowlesi IN VITRO* CULTURE

ABSTRACT

The emergence of zoonotic malaria parasite, *P. knowlesi* becomes the most dominant parasite causing malaria infection in humans in the Southeast Asia where Malaysian Borneo has recorded to have the highest cases of malaria diseases caused by *P. knowlesi*. It has been reported that *P. knowlesi* shows preference towards reticulocytes. However, obtaining a huge amount of reticulocytes for the continuous supply becomes the major hurdle for a long-term culture study. This is due to the low concentration of reticulocytes circulating in peripheral blood, bone marrow and umbilical cord blood. Fortunately, the development system in *in vitro* culture to generate reticulocytes derived from haematopoietic stem cells (HSCs) could solve the limitation of the *P. knowlesi* study. In this study, umbilical cord blood (UCB) was used as the source of HSCs which contained the highest concentration of CD34^{+} compared to peripheral blood and bone marrow. First, UCB-derived CD34^{+} HSCs were expanded in serum-free expansion medium II (SFEM) supplement with cytokines allowing the cells to proliferate. After 5 days of expansion, the cells were cultured in differentiation media with sufficient growth factors and cytokines for the process of reticulocytes differentiation. Microscopic examination and FACS analysis were performed during the cultivation period to observe the morphological changes and monitor the expression of cell surface antigens. Lastly, *P. knowlesi* was cultured in the generated reticulocytes and the invasion rate was determined at 24-hour post-invasion.

Although the invasion of *P. knowlesi* was not performed due to unforeseen circumstances, the result showed that functional reticulocytes could be generated from UCB-derived $CD34^+$ HSCs and the invasion rate of *P. knowlesi* was expected to be similar with *P. vivax*. This is because *P. knowlesi* has the closest phylogenetically with *P. vivax.* In conclusion, this study shows the possibility to produce massive production and homogenous populations of reticulocytes generated from UCB-derived $CD34^+$ HSCs.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria was discovered over 2000 years ago since Greek and Roman civilisation had fever caused by the malaria parasite from the genus *Plasmodium*. According to the World Malaria Report 2019, the disease occurred mostly in the African region (93% cases) followed by the Southeast Asia region (3.4% cases) and Eastern Mediterranean region (2.1% cases) (World Health Organization (WHO), 2019). The malaria cases were reported to decline to 57 cases from 71 cases per 1000 population from 2010 to 2018 in all of the WHO regions. Despite the declining cases, it was deteriorated in 2014 when it remained at a similar rate until 2018 (WHO, 2019).

The malaria parasite is a vector and host specific. The vectors for the malaria parasites belong to a group of mosquitoes known as *Anopheles leucosphyrus* (Cooper *et al.*, 2020). Humans are the natural hosts for the human malaria parasites such as *P. falciparum, P. vivax, P. malariae* and *P. ovale.* Long-tailed macaque (*Macaca fascularis*) and pig-tailed macaque (*Macaca nemestrina*) are the natural hosts for *P. knowlesi* that has now been recognised as the fifth human malaria parasite (Amir *et al.*, 2018; Singh and Daneshvar, 2013). Common infections by *P. falciparum* and *P. vivax* have been reported yearly (Grüring *et al.*, 2014). But starting in 2004, the highest cases of *P. knowlesi* were reported especially in Kapit Division, Sarawak, Malaysian Borneo (Grüring *et al.*, 2014) and now spread to other parts in Southeast countries (Cooper *et al.*, 2020).

P. knowlesi have the closest phylogenetically connected to *P. vivax* (Moon *et al.*, 2013; Noulin *et al.*, 2014)). They are similar in term of the host cell invasion that depends on the parasite ligand, Duffy binding protein (DBP) (Mohring *et al.*, 2019). *P. knowlesi* has the 24 hours intraerythrocytic life cycle as compared with that of *P. falciparum, P. vivax*, *P. ovale* (48 hours) and of *P. malariae* (72 hours) (Mohring *et al.*, 2019; Moon *et al.*, 2013). The intraerythrocytic cycle begins after sporozoites are transmitted into a human via an infected female *Anopheles* mosquito. The parasites reach the liver and invade hepatocytes before maturing into schizonts. Merozoites released from the schizonts enter the bloodstream and invade erythrocytes. Following erythrocyte invasion, the parasites grow into ring forms and then develop into mature trophozoites and schizonts filled with merozoites (Singh and Daneshvar, 2013).

In vitro continuous culture of *P. falciparum* permits many studies of the biology of this parasite and the testing of compounds for antimalarial activity (Duffy and Avery, 2017). However, no *in vitro* continuous culture of *P. knowlesi* or *P. vivax* has been established thus far (Kumar *et al.*, 2015; Moon *et al.*, 2013). Attempts were made to maintain *P. knowlesi in vivo* in rhesus macaques (*M. mulata*) (Amir *et al.*, 2016). This requirement involved an expensive cost for daily animal care and therefore restricted research to primate laboratory facilities (Grüring *et al.*, 2014; Moon *et al.*, 2013). *P. knowlesi* also has a difficulty in adaptation to an *in vitro* culture by using human erythrocytes as it has a specificity to invade reticulocytes (young erythrocytes) (Grüring *et al.*, 2014; Lim *et al.*, 2013). The low concentration of reticulocytes in peripheral blood has restricted the use of these cells for establishment of *P. knowlesi in vitro* culture (Noulin *et al.*, 2012).

Haematopoietic stem cells (HSCs) have a capacity to expand and differentiate into reticulocytes by using appropriate microenvironments such as erythropoietin (EPO) and stem cell factor (SCF) (Dorn *et al.*, 2008; Trakarnsanga *et al.*, 2017). HSCs have the potential to self-replicate, self-renewal and are able to differentiate into multiple cell lines. High volume of HSCs can be obtained from umbilical cord blood (UCB), a rich source of HSCs to generate enough amount of reticulocytes for *in vitro* culture (Hordyjewska *et al.*, 2015). HSCs can be determined by the expression of a surface marker, CD34 antigen by using flow cytometry (Hordyjewska *et al.*, 2015). Other important markers such as CD235a represented glycophorin A and CD71 is a transferrin receptor are also expressed on reticulocytes (Noulin *et al.*, 2012).

1.2 Rationale of the study

Elimination of malaria is thought to be achieved when malaria cases decreased in number since 2010. It however does not last long when the number of malaria cases stopped to decline in 2014. The emergence of zoonotic malaria has now become a threat when *P. knowlesi* has the capability to cause infection in humans. Research towards this parasite has become more challenging due to its requirement of macaque blood and laboratory facility. Scarce replication rate of *P. knowlesi in vitro* has been shown, which might be due to the parasite's preference towards reticulocytes compared to mature erythrocytes. Therefore, the differentiation of $CD34^+$ HSCs from UCB source was conducted in the present study to generate reticulocyte lines. The CD34 antigen expressed on the surface of UCB-derived HSCs has the closest value with bone marrow than that with peripheral blood (Hordyjewska *et al.*, 2015). Thus,

the higher proportion and ability of UCB-derived HSCs to differentiate into reticulocytes provides an alternative strategy to obtain high amount of reticulocyte for *P. knowlesi* invasion assay and subsequently for *in vitro* culture.

1.3 Objectives of the study

1.3.1 General objective

To determine the invasion of functional reticulocytes derived from human UCB $CD34^+$ HSC line by *P. knowlesi*

1.3.2 Specific objectives

i. To expand and differentiate human UCB $CD34^+$ HSCs into reticulocytes
ii. To characterise functional reticulocytes differentiated from human UCB $CD34^+$ HSCs
iii. To observe the invasion of generated reticulocytes by *P. knowlesi*

1.4 Experimental design

The study was designed to differentiate human UCB $CD34^+$ HSCs into reticulocytes and to determine the function of generated reticulocytes by using *P. knowlesi* invasion assay. The overall study is summarised in Figure 1.1. The HSCs were expanded for five days in serum-free expansion medium II (SFEM) supplemented with SCF, thrombopoietin (TPO), FMS-like tyrosine kinase 3 (FLT3)

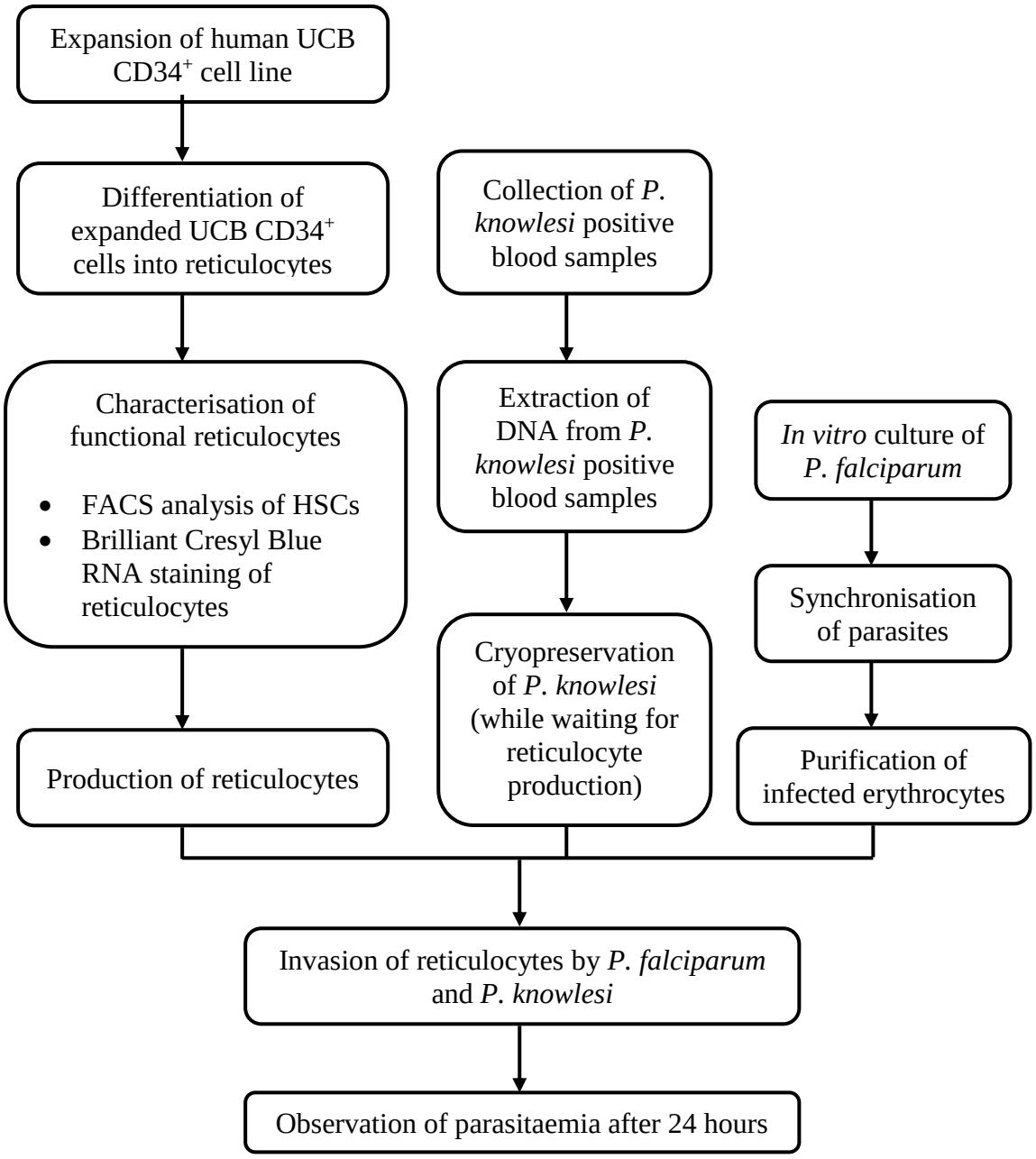

Figure 1.1: Flowchart of the experiment

After 5 days of expansion, the cells were cultured in IMDM allowing cells to differentiate into erythroid line. The functional reticulocytes were characterised by performing FACS analysis and microscopic examination. The collection of *P. knowlesi* samples were performed by qualified nurses at Hospital Gua Musang (HGM) and Hospital Kuala Krai (HKK). The infected RBC with *P. falciparum* was purified using magnetic column separation. The percentage of parasitaemia was observed under 100X light microscope at 24 hours post-invasion.

and interleukin-6 (IL6) at 37ºC in 5% CO_2 incubator. The medium was partially replenished every day and the cells were passaged every three days. On day five of expansion, the medium was changed into Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine, penicillin/streptomycin, inositol, transferrin, monothioglycerol, folic acid, insulin human solution and 10% BSA in IMDM. During the first 8 days of differentiation, the complete culture medium was supplemented with growth factors such as hydrocortisone (HDS), interleukin-3 (IL3), SCF and EPO allowing cells to differentiate into erythroid line. Starting day 8 until 11 of differentiation, the cells were cultured in IMDM supplemented with EPO only and then were maintained in IMDM without any supplements added to the medium until day 14. The medium was partially replenished every two days to supply enough nutrients to the cells and maintained under standard culture conditions in 37ºC of 5% CO_2 incubator.

Fluorescence-activated cell sorting (FACS) was performed on particular days: days 0, 8, 11, 14, 16 and 20 to analyse the expression of CD34, CD45, CD36, CD71 and CD235a surface antigens during differentiation phase. Primary antibodies CD45 PerCP-Cy5.5, CD71 FITC, CD36 APC, and CD235a PE were used. Mouse IgG1 PerCP-Cy5.5, mouse IgG2a FITC, mouse IgM APC, and mouse IgG2b PE isotype controls were used as negative controls. Subsequently, the pellet was incubated in phosphate-buffered saline (PBS) and stained with Brilliant Cresyl Blue solution for 30 minutes before being examined under 100X magnification using light microscope. The morphology of cell derived from human UCB $CD34^+$ HSCs was observed. The differentiation ended on day 14 corresponding to the peak of reticulocyte counts determined by microscopic examination.

P.knowlesi samples were collected from patients at Hospital Gua Musang (HGM) and Hospital Kuala Krai (HKK), while, *P. falciparum* (control) was synchronised at ring stage by using sorbitol treatment to obtain mature stage parasites after 24 hours post-synchronisation. In order to get purified and infected erythrocytes, the mature stage parasites were harvested by using a magnetic column separation. The purified parasites were cultured with reticulocytes derived from human UCB $CD34^{+}$ HSCs. The parasite invasion was determined at 24 hours post-invasion by using cytospin and staining with Giemsa solution.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of malaria

Humans had suffered from malaria disease since years back. The disease is caused by protozoa of the genus *Plasmodium* and occurs mostly in sub-Saharan Africa and throughout the Indian subcontinent, Southeast Asia and South America (Ryan *et al.*, 2015; WHO, 2019). Human malaria parasites undergo sexual development in female *Anopheles* mosquitoes before infecting humans in which the asexual development begins (Roncalés *et al.*, 2015). Various studies such as genetic modification, biology of the malaria parasites and antimalarial drug screening have been carried out to unravel the disease (Moon *et al.*, 2013). The emergence of zoonotic infections caused by *P. knowlesi* is currently a major problem (Ahmed and Cox-Singh, 2015). This malaria parasite has limited laboratory strains that might no longer be representative of current parasite populations (Vythilingam *et al.*, 2018). Therefore, studies on the adaptation of *P. knowlesi* to an *in vitro* culture by using human erythrocytes rather than its natural macaque erythrocytes are urgently needed.

2.2 Epidemiology of malaria

According to the World Health Organization (WHO), 228 million cases of malaria were reported worldwide in 2018 with 85% deaths occurred in 20 countries in the African region and India subcontinent (WHO, 2019). The African region recorded the highest malaria cases (93%) followed by Southeast Asia (3.4%) and Eastern

Mediterranean regions (2.1%) (WHO, 2019). The malaria case incidence rate started to decrease from 71 cases to 57 cases per 1000 population between 2010 until 2018, however, it remained at a similar level since 2014 until 2018 (WHO, 2019). The global malaria case incidence rate for the population at risk is shown in Figure 2.1.

Among all human malaria parasites, *P. falciparum* is the main species reported to be the most life-threatening, causing approximately 99% malaria cases in sub-Saharan Africa (Mohring *et al.*, 2019). Malaria cases outside Africa are predominantly due to *P. vivax* with 200 million global cases reported annually (Dayanand *et al.*, 2018). While, other non-falciparum malaria infections caused by *P. ovale* and *P. malariae* were reported to be malaria-endemic cases in Western Africa, South America, Asia and western Pacific region. These species are less virulent and rarely become the attention because of the scarce cases (Lo *et al.*, 2017; Okafor and Finnigan, 2019). The current emergence of the fifth human malaria parasite, *P. knowlesi* has been widely spread to Southeast Asia, including Indonesia, Cambodia, Philippines, Singapore, Thailand, Vietnam and Myanmar (Barber *et al.*, 2017; Millar and Cox-Singh, 2015). In Malaysia, malaria cases due to this species showed a high number of 100 cases per year in the 2000s, rising to 703 cases in 2011, 1325 cases in 2014 and 2030 in 2017 with six deaths recorded in 2015 until 2017 (Cooper *et al.*, 2020). The high risk populations are children under 5 years old and pregnant women with 24 million cases and 11 million cases, respectively reported in 2018 (Roncalés *et al.*, 2015; WHO, 2019).

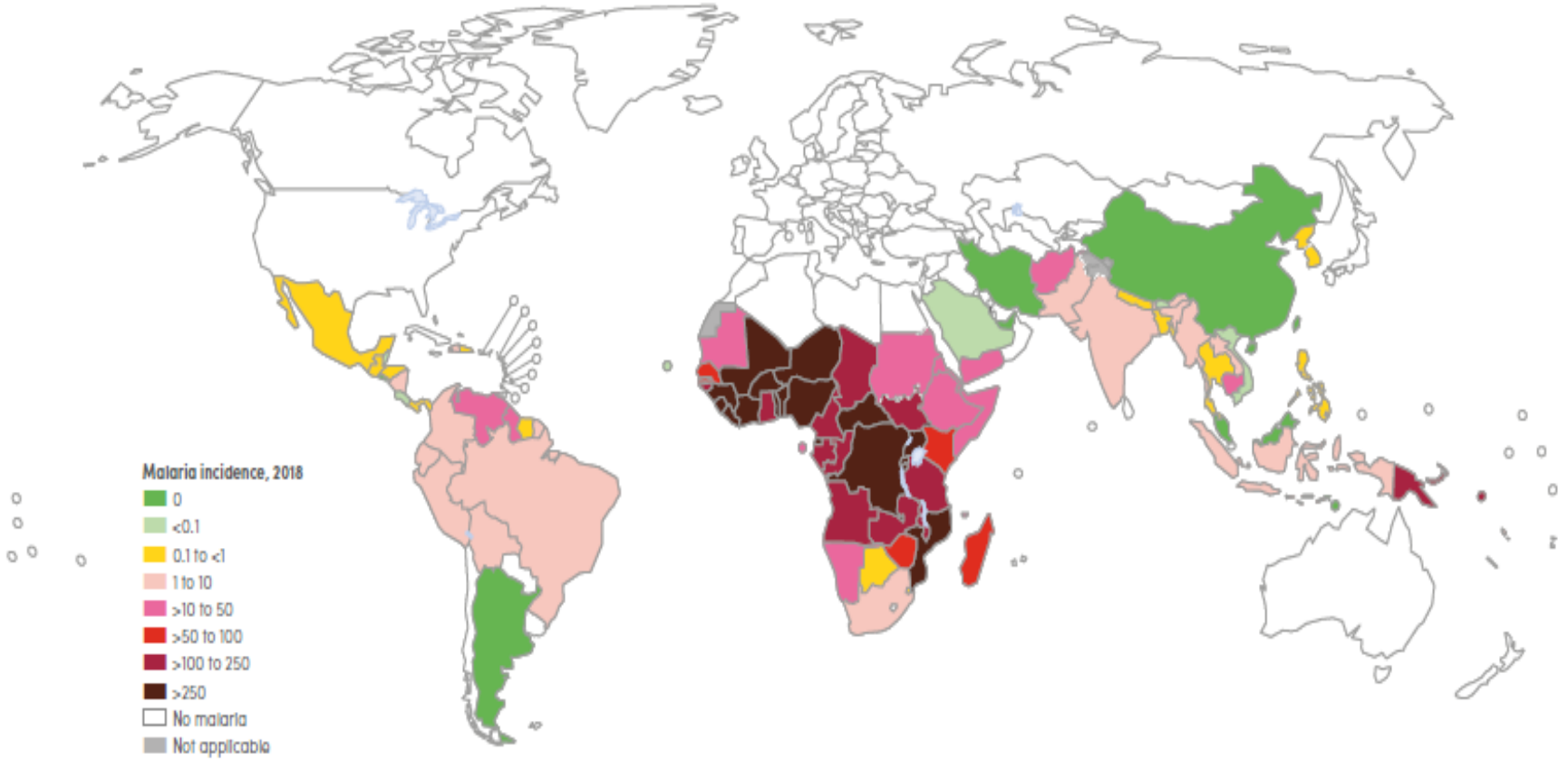

Figure 2.1: The worldwide malaria case incidence rates in 2018

The map shows high malaria cases in the African region. The Eastern Mediterranean region, Western Pacific region, American region and Southeast Asia region showed active transmission of malaria cases in 2018. Adapted from WHO (2019).

2.3 Symptoms of malaria

Patients will show clinical symptoms when the asexual intraerythrocytic cycle of the malaria parasite is initiated (Mace, 2019). The common symptom to all human malaria parasites is a nonspecific mimicking flu-like syndrome and high fever (Cowman *et al.*, 2016; Mace, 2019). The different pattern in fever spike is due to the different period of the intraerythrocytic cycle of the malaria parasites: *P. knowlesi* (24 hours), *P. falciparum, P. vivax* and *P. ovale* (48 hours), and *P. malariae* (72 hours) (Ahmed and Cox-Singh, 2015; Singh and Daneshvar, 2013). Along with fever and chills, patients will experience prodromal symptoms such as malaise, myalgia, anorexia, nausea, occasional vomiting, sweating, headache, fatigue, cough and mild anaemia. The severity of malaria symptoms varies from absent or mild symptoms to severe and deadly. Malaria parasite species, patient's age and immune response to infection are some of the factors contributing to the severity of the disease. Malaria can lead to organ failure and death if it is left untreated (Cowman *et al.*, 2016; Mace, 2019).

2.4 Human malaria parasites

Malaria parasites are transmitted into humans via the bite of female *Anopheles* mosquitoes from the Leucosphyrus group (Roncalés *et al.*, 2015; Singh and Daneshvar, 2013). There are four malaria parasites naturally hosted in humans: *P. falciparum, P. vivax, P. malariae* and *P. ovale*. *P. knowlesi* naturally inhabits in long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) (Amir *et al.*, 2018; Singh and Daneshvar, 2013). *P. knowlesi* has become the fifth

human malaria parasite after the huge cases of zoonotic malaria were reported in the Kapit Division of Sarawak in 2004 (Singh and Daneshvar, 2013). These cases with four deaths were misdiagnosed as *P. malariae*, the malaria parasite that rarely causes fatal (Cox-Singh *et al.*, 2008). Later, it was revealed to be caused by *P. knowlesi* as this malaria parasite is easily misdiagnosed as *P. malariae* due to their morphological similarity when observed by conventional microscopy (Millar and Cox-Singh, 2015). Another case of misdiagnosis of *P. knowlesi* as *P. malariae* was reported in 2007 (Ng *et al.*, 2008).

2.5 Life cycle of the human malaria parasites

The human malaria parasites have a complex life cycle that grow and reproduce through two important phases: sexually and asexually. The malaria parasites undergo the sexual phase within mosquito vectors and the asexual phase within human hosts (Howick *et al.*, 2019; Roncalés *et al.*, 2015).

2.5.1 Sexual cycle of the malaria parasites within mosquitoes

The malaria parasites undergo gametocytogenesis following transmission of gametocytes from humans to mosquitoes (Figure 2.2). After the differentiation of microgametocytes (male) and macrogametocytes (female) into microgametes and macrogametes, respectively, each microgamete exflagellates to produce eight microgametes and fuses with the macrogamete, forming a zygote (Guttery *et al.*, 2015; Siciliano and Alano, 2015). Soon after zygote formation, a motile ookinete is formed and migrates through the mosquito's midgut wall, forming an oocyst. The oocyst

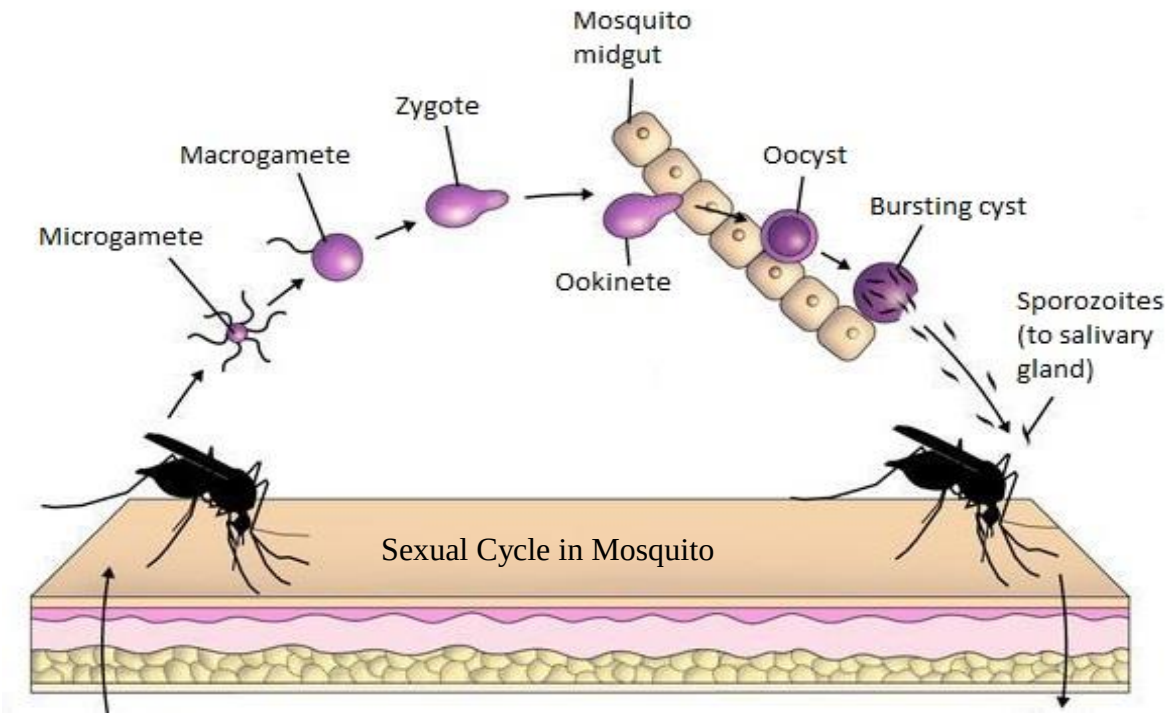

Figure 2.2: The sexual cycle of the malaria parasite within a mosquito

A microgamete (male) fuses with a macrogamete (female) forming a zygote. The elongated zygote transforms into an ookinete. The ookinete migrates through the mosquito's midgut and develops into an oocyst. Upon maturation of the oocyst, the parasite ruptures releasing numerous sporozoites. The sporozoites travel to the mosquito's salivary gland and ready to be injected into a human during a blood meal. Modified from Karanja and Kiboi (2016).

undergoes division and maturation, which gives rise to numerous sporozoites within two weeks of the sporogony. The sexual cycle is completed when thousands of sporozoites invade the mosquito's salivary glands and are injected into humans during blood meal (Kengne-Ouafo *et al.*, 2019; Lampe *et al.*, 2019).

2.5.2 Asexual cycle of the malaria parasites within humans

Sporozoites are injected into venules of a human during a blood meal (Figure 2.3). Within an hour, sporozoites reach the liver and invade the hepatocytes. The parasites undergo the exoerythrocytic schizogony to produce numerous multinucleated schizonts. It takes approximately 15 days for the parasites to mature and develop into schizonts containing merozoites before initiating the intraerythrocytic schizogony. The membrane of the schizonts ruptures, releasing merozoites into the bloodstream to invade erythrocytes (Cowman *et al.*, 2016; White, 2017). Within the erythrocyte, the parasite transforms into ring, trophozoite and schizont stages. The schizont stage-infected erythrocytes rupture, releasing merozoites to reinvade new erythrocytes. The developmental cycle of the malaria parasites is different depending on the species. *P. knowlesi* has the shortest 24-hour life cycle, while *P. malariae* has the longest 72-hour life cycle. Other human malaria parasites, *P. falciparum, P. vivax* and *P. ovale* have the 48-hour life cycle (Ahmed and Cox-Singh, 2015; White, 2017).

2.6 Reported studies on *P. knowlesi*

Almost all countries in Southeast Asia have reported cases of *P. knowlesi* infection (Amir *et al.*, 2018). This malaria parasite has become a highlight when it

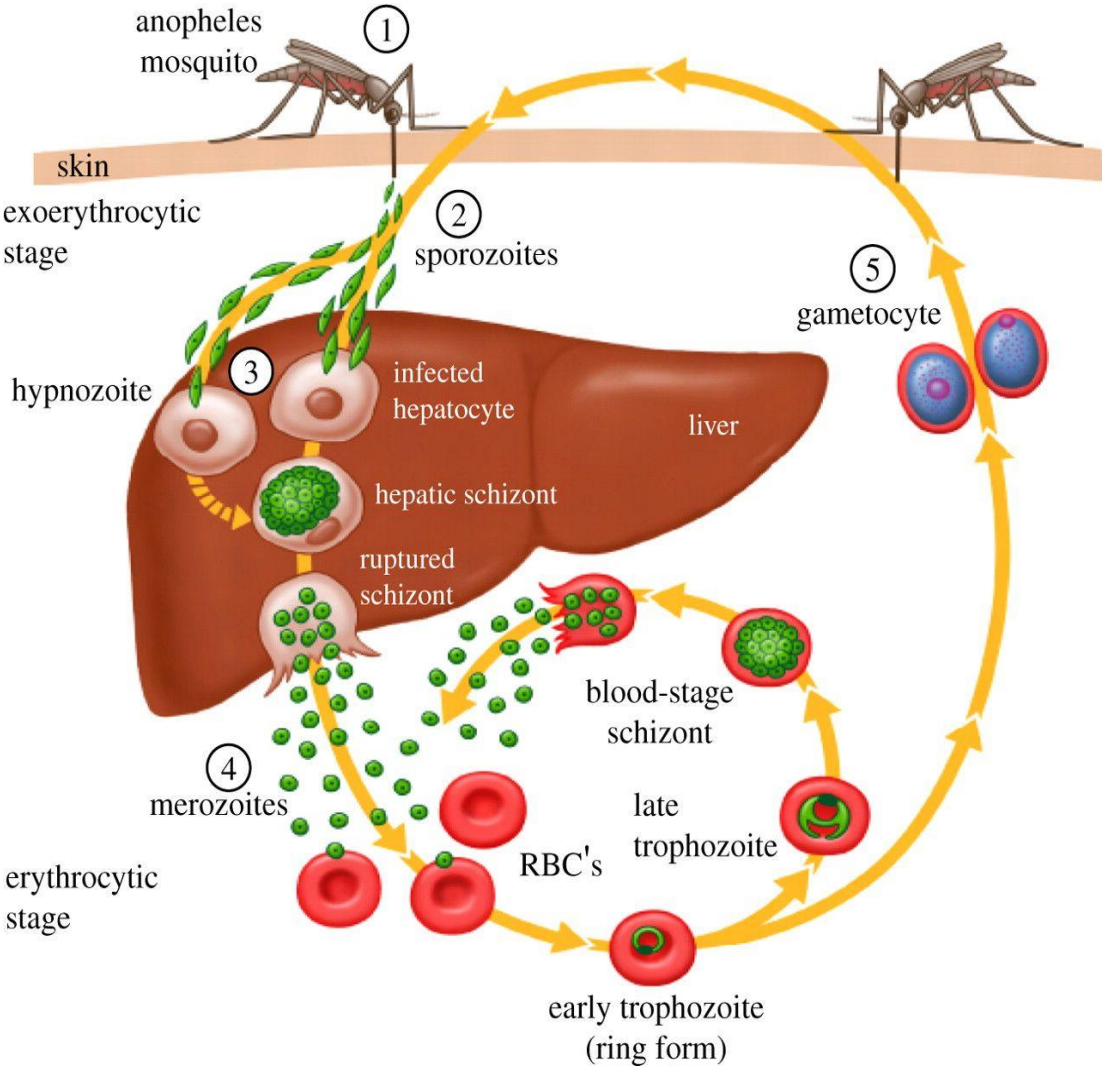

Figure 2.3: The asexual cycle of the malaria parasite within a human

(1) Anopheles mosquito injects sporozoites during a blood meal. (2) Injected sporozoites enter the liver and (3) invade hepatocytes and develop into schizonts. (4) Within 15 days of the incubation, the schizonts mature and rupture releasing numerous merozoites into the bloodstream to invade erythrocytes. The development of the malaria parasite continues inside infected erythrocytes, where merozoites transform into ring, trophozoite and schizont containing merozoites. The infected erythrocytes rupture, releasing merozoites to invade new erythrocytes. (5) Male and female gametocytes differentiated from merozoites were ingested by a mosquito. Adapted from Faust (2016).

shows the ability to infect humans, causing the emergence of zoonotic malaria. This hinders the goal to eradicate malaria and obtain the status of malaria-free countries (Barber *et al.*, 2017). The increasing malaria cases of *P. knowlesi* seem to show that the eradication of malaria is unsuccessful towards zoonotic malaria, thus, different approaches are needed (Cooper *et al.*, 2020).

In vitro culture provides investigation on the development of the malaria parasite as well as other significant research on malaria (Lapp *et al.*, 2015). There are several challenges in culturing *P. knowlesi in vitro*. The use of natural host macaque blood has limited the studies with access to the animal facility (Moon *et al.*, 2013). *P. knowlesi* has also been demonstrated *in vitro* to have a predilection towards reticulocytes (Grüring *et al.*, 2014). The tendency of *P. knowlesi* to invade reticulocytes is higher compared to mature erythrocytes. The Duffy binding protein (DBP) (parasite ligand) on merozoites showed a high interaction to the Duffy antigen receptor for chemokines (DARC) (host receptor) on reticulocytes during invasion (Cowman *et al.*, 2017; Ovchynnikova *et al.*, 2017). Reticulocyte concentration in peripheral blood is, however, very low (0.5-2.5%) with the 24-hour lifespan prior to maturation (Kumar *et al.*, 2015; Noulin *et al.*, 2014), making the establishment of a continuous *in vitro* culture of *P. knowlesi* by using reticulocytes difficult. This becomes a major challenge although there has been a technique known as cryopreservation, which has the ability to store and freeze samples enriched with reticulocytes. Despite that, a high reproducibility of enriched reticulocytes must be obtained to collect sufficient reticulocytes prior to freezing (Kumar *et al.*, 2015). Therefore, alternative methods to produce high volumes of reticulocytes such as

through expansion and differentiation of haematopoietic stem cells (HSCs) are implemented.

2.7 The origin of stem cells

Human body is made up from different types of cells that are originated from stem cells. Stem cells have the ability to regenerate and differentiate into any type of cells for maintaining homeostasis in the body and replacing cells or tissues that are damaged or lost (Zhang *et al.*, 2017). There are three different types of stem cells. Firstly, embryonic stem cells (ESCs) that are the inner cell mass of early blastocyst and pluripotent, which are able to differentiate into all types of tissues. Secondly, somatic or adult stem cells (SSCs), which are multipotent and only able to differentiate into specific cells in the body during cell damage in order to maintain tissue homeostasis. Two types of SSCs are haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Lastly, induced pluripotent stem cells (iPSCs), which are reprogrammed stem cells that are taken from normal mature adult cells to become an ESC-like state (Wanet *et al.*, 2015).

2.8 Haematopoiesis

Haematopoiesis is a process of blood cell formation to replenish the blood system. The first haematopoiesis occurs in the yolk sack, followed by liver and lastly in the bone marrow and thymus (Jagannathan-Bogdan and Zon, 2013). During embryonic development, the blastomeres, known as totipotency, are the first stem cells that are important in developing into any type of cells for human body development

(Figure 2.4). At the gastrulation stage, the totipotency lost the properties of the cell and initiates the process into specialised cells: trophoblast (developed into placenta) and embryonic node (pluripotent cells). Pluripotent cells have the ability to divide continuously and differentiate into any type of cells derived from the ectoderm, mesoderm and endoderm. However, these pluripotent cells are unable to differentiate into germ cells in the placenta once they move to the uterus. These pluripotent cells gradually continue to develop into tissue stem cells (multipotent cells) and subdivided asynchronously into two progenies, which are one HSCs and one haematopoietic progenitor cells (HPCs). HPC is a unipotent cell due to its inability to self-renew and can differentiate into one or more extra lineage only. Myelocytic precursor colony-forming unit of granulocyte, erythroid, macrophage and megakaryocyte (CFU-GEMM) or lymphoid precursor (CFU-Lymph) are the precursors differentiated from HPCs (Hordyjewska *et al.*, 2015).

2.9 Characteristics of Haematopoietic Stem Cells

The unique capability of HSCs is to produce daughter cells continuously while preserving the stem cell characteristics. The HSCs are able to self-renewing, thus do not become specialised or differentiate which resulting in a lifetime source of blood cells (Copelan, 2006). Various sources of stem cells can be used to generate HSCs into different stages of the erythroid lineage. HSCs can be obtained from peripheral blood (PB), bone marrow (BM), umbilical cord blood (UCB) or induced pluripotent stem cells (Giarratana *et al.*, 2011). UCB-derived HSCs have shown a greater capacity to expand as compared to other sources. The high concentration of

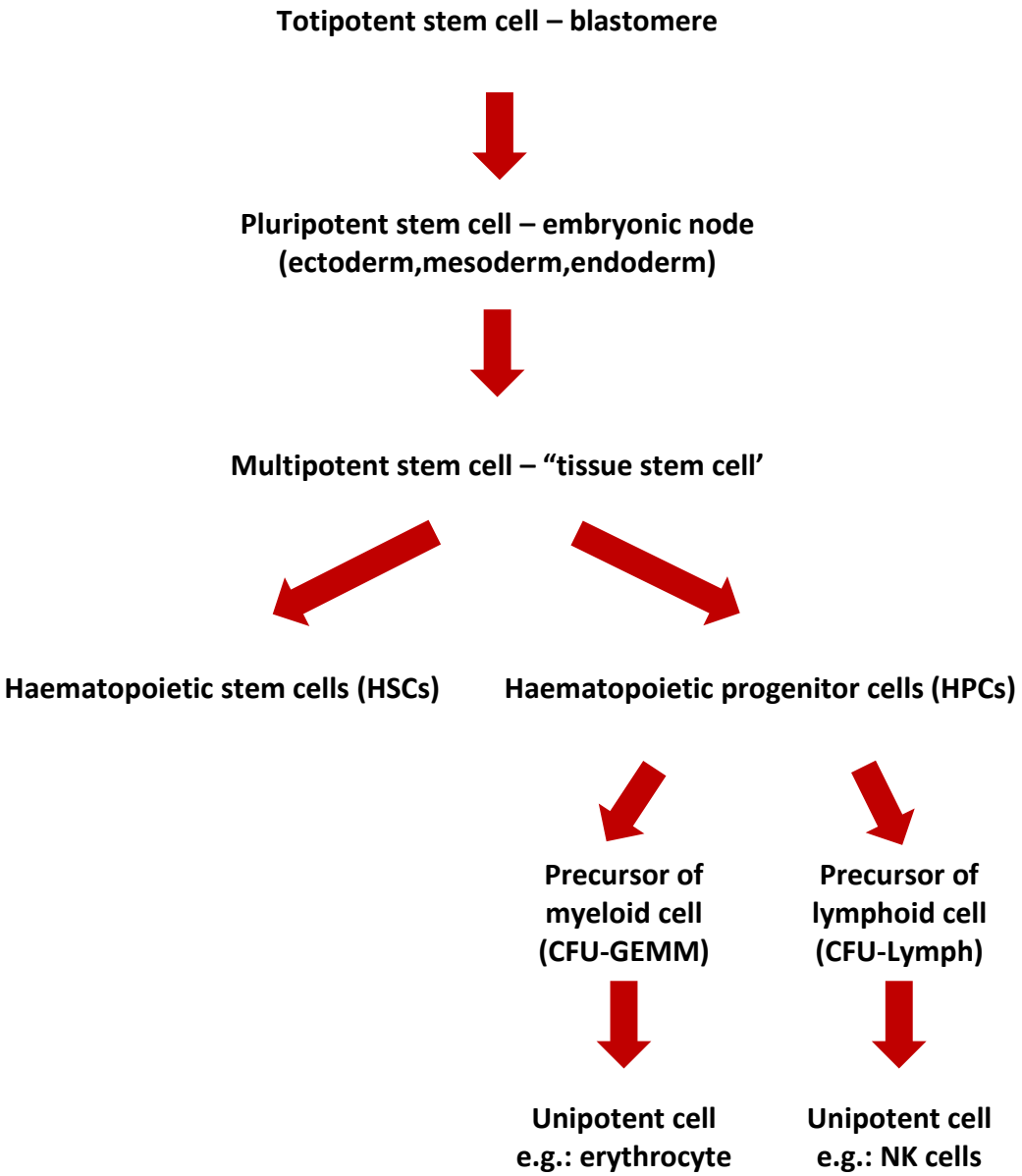

Figure 2.4: The development of haematopoietic stem cells (HSCs)

Blastomeres are the first stem cells known as a totipotent. They are initiated into specialised cells such as embryonic node, which are also called pluripotent stem cells. These pluripotent stem cells start to differentiate into any type of cells derived from the ectoderm, mesoderm and endoderm. After that, the pluripotent stem cells continue to develop into tissue stem cells known as multipotent, which contains two progenies; haematopoietic stem cells (HSCs) and haematopoietic progenitor cells (HPCs). HPCs give rise either to myeloid precursor colony-forming unit of granulocyte, erythroid, macrophage and megakaryocyte (CFU-GEMM) or lymphoid precursor colony-forming unit of lymphocyte (CFU-Lymph). These two precursors arisen from HPCs have the unipotent ability.

HSCs is located at the postpartum placenta and cord of UCB (Trakarnsanga *et al.*, 2017).

HSCs and haematopoietic progenitor cells (HPCs) are known to express specific surface marker, CD34. Specifically, it is a transmembrane phosphoglycoprotein and has approximately 115 kDa of molecular weight. CD34 plays an important role in cytoadhesion and regulation of cell differentiation since most of the ability of an extracellular domain is O-linked glycosylated and certain sites contain N-linked glycosylated (Sidney *et al.*, 2014). Upon the significant function of CD34, recent studies suggested that the appearance of $CD34^+$ is prior to cell division. Therefore, CD34 expression is important as the relatedness to other functions of HSCs such as undergo cell cycle entry, metabolic activation, mobilization and homing (AbuSamra *et al.*, 2017). In addition, *in vivo* cultivation has shown that $CD34^+$ HSCs and HPCs also have the ability to differentiate into other lineages despite haematopoietic lineage and have a great capacity to proliferate (Sidney *et al.*, 2014). Hence, the expression of $CD34^+$ on HSCs is a reliable precursor to generate reticulocytes for parasite culture *in vitro*.

2.10 Erythropoiesis

A healthy human body produces 2 million mature erythrocytes every second through a regulated process called erythropoiesis (Moras *et al.*, 2017). It begins when multipotent HSCs differentiate from myeloid progenitors (megakaryocytic-erythroid progenitor) into burst-forming unit-erythroid (BFU-E) to form mature erythrocytes (Figure 2.5) (Zivot *et al.*, 2018). The erythroid-committed progenitors,

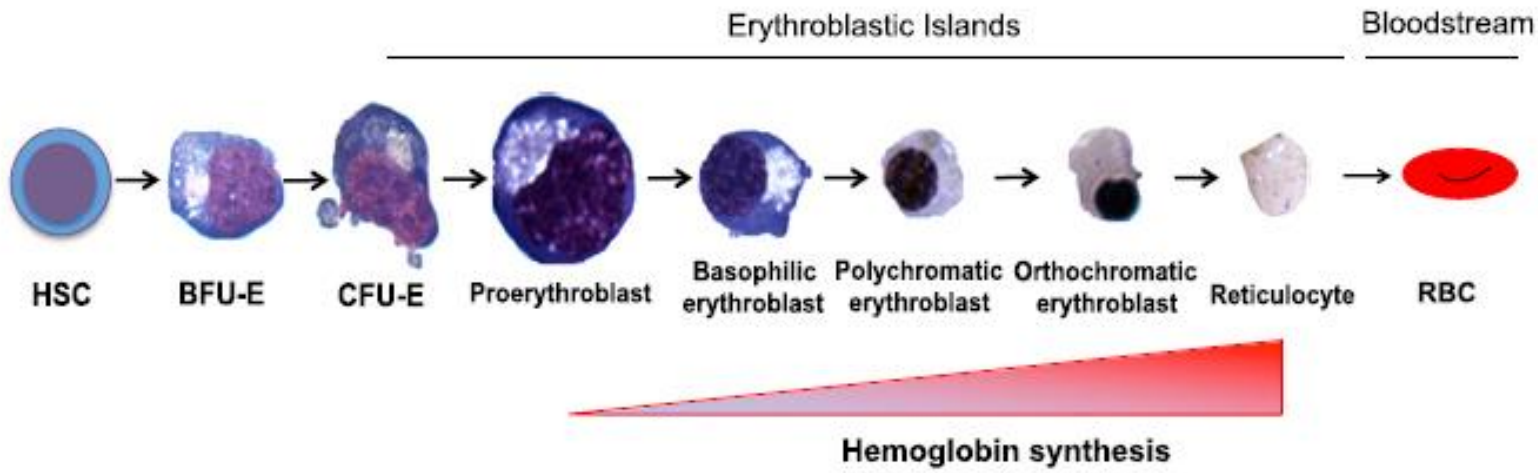

Figure 2.5: The terminal erythroid stage of erythropoiesis

HSCs differentiate into committed erythroid progenitors known as burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E). The nucleated precursors, proerythroblasts differentiates into basophilic erythroblast, polychromatic and orthochromatic erythroblast by undergoing several morphological changes such as accumulation of haemoglobin and cell size reduction. This process occurs in the surrounding of central macrophages in erythroblastic islands before entering the blood stream while continue to achieve maturation and becoming erythrocytes. Adapted from Zivot *et al.* (2018).

BFU-E are the first erythroid lineage that differentiates into colony-forming unit-erythroid (CFU-E) (terminal erythroblast differentiation). Later, the nucleated precursors, proerythroblasts differentiate into basophilic, polychromatophilic and orthrochromatophilic erythroblasts. The differentiation of nucleated precursors undergoes several morphological changes such as haemoglobin synthesis, cell size reduction, chromatin and nuclear condensation resulting in enucleation to form reticulocytes (Rai *et al.*, 2019). At the end of the terminal erythroid stage, erythroblasts lose their nucleus and reticulocytes continue to undergo maturation, forming an erythrocyte-biconcave shape. The erythropoiesis takes place in erythroblastic islands which consists of central macrophages and 30 erythroid cells at a different stage of maturation. The environment in erythroblastic islands provides cellular interactions that are necessary for erythroid differentiation and proliferation (Moras *et al.*, 2017; Zivot *et al.*, 2018).

2.11 Characterisation of reticulocytes derived from HSCs

A successful production of reticulocytes through differentiation of HSCs has been reported previously (Noulin *et al.*, 2012). To produce erythrocytes, HSCs are induced to erythroid differentiation, allowing them to proliferate and differentiate into erythrocyte lines. To produce functional reticulocytes from HSC cultivation, the culture process must mimic the medullar environment. The process of cultivation requires particular erythropoietin cytokines with or without human mesenchymal stem cells as feeder cells to enhance reticulocyte proliferation and differentiation. Reticulocytes derived from HSCs have been shown to be more homogenous. Other

studies had reported an increasing percentage of reticulocyte production from cryopreserved erythroblasts (Furuya *et al.*, 2014; Noulin *et al.*, 2014).

Umbilical cord blood (UCB) is known to be the richest source to obtain HSCs. HSCs appear as small cells with narrow haem cytoplasm and are able to proliferate into different types of lineage. Differentiation of multi-line HSCs can be assessed by the natural characteristic via the expression of specific surface markers throughout the differentiation process (Hordyjewska *et al.*, 2015). Based on the reticulocyte characteristic, there are several cell surface antigens expressed at a different stage prior to maturation of erythrocytes: CD36, CD45, CD71 and CD235a. During preerythroblast until proerythroblast stages, the CD45 is highly expressed but then decreases dramatically, while another surface antigen, CD235a (Glycophorin A) continues to express afterward. The following cell surface antigens, CD71 and CD36 are markers for proerythroblast and mature erythrocytes respectively where they are highly expressed during the young cells stage yet start to decline prior to maturation stages (Noulin *et al.*, 2012). Erythroid cells can also be differentiated by morphology characteristics using microscopic examination.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents and equipment

Chemicals and reagents, antibodies, equipment and software used throughout the study were listed in Table 3.1, Table 3.2, Table 3.3 and Table 3.4, respectively.

3.2 *P. falciparum in vitro* cultivation

3.2.1 Malaria parasite strain

The *P. falciparum* 3D7 strain was used as a control parasite in the study. The parasite was kindly provided by Dr. Khairul Mohd Fadzli Mustaffa from the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM).

3.2.2 Thawing of cryopreserved malaria parasites

Cryopreserved parasites were thawed immediately in a 37°C water bath for 1 minute after removing from the liquid nitrogen tank (Amir *et al*., 2016). The content of thawed parasites was transferred into 15 mL falcon tubes and the volume of content was measured. An equal volume of 3.5% sodium chloride (NaCl) was then added dropwise. The suspensions were centrifuged at 332 × *g* for 5 minutes and