Investigating the source of free fetal hemoglobin in preeclampsia. The role of the placenta and the fetus.

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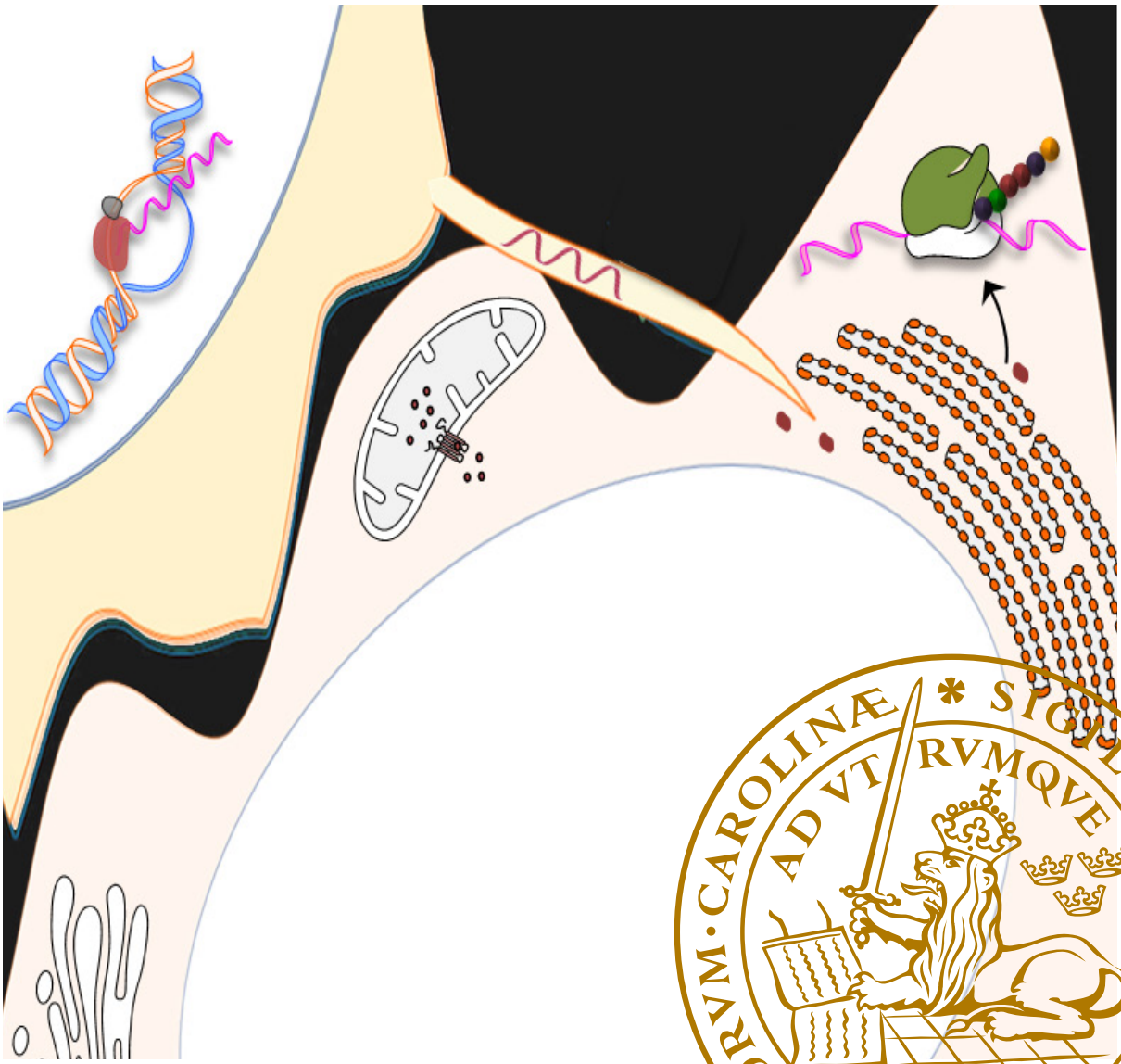

Investigating the source of free fetal hemoglobin in preeclampsia

The role of the placenta and the fetus

Zahra Masoumi

LUND
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Title and subtitle: Investigating the source of free fetal hemoglobin in preeclampsia: the role of the placenta and the fetus

Abstract

Preeclampsia (PE) is an important pregnancy complication that affects 3-8% of women worldwide and is a leading cause of maternal and fetal morbidity and mortality. It is known that PE is associated with placental dysfunction, resulting in hypoxia, oxidative stress, altered metabolism and production of inflammatory cytokines. Hypoxia could enhance erythropoiesis and induce non-erythroid hemoglobin production. Elevated fetal hemoglobin (HbF) levels in term placentas and maternal circulation, as well as higher erythroblast count in the umbilical cord blood (UCB) have been previously described in PE pregnancies. It has been suggested that free HbF damages and leaks through the placental barrier into the maternal circulation and that could trigger inflammation, general endothelial dysfunction and organ damage in the mother. But to date, the source of the increased HbF is unknown.

The aim of this thesis is to elucidate whether altered fetal and placental erythropoiesis and hemolysis could contribute to the excess placental HbF. In addition, the effect of hypoxia-induced oxidative stress on non-erythroid hemoglobin production was analyzed.

Our results indicated an absence of erythropoiesis in term placentas in both PE and normotensive pregnancies. However, hypoxia-triggered oxidative stress induced production of alpha globin protein in non-erythroid cells. Analysis of placental and fetal hematopoietic stem/ progenitor cells (HSPCs) and erythropoiesis (*in vitro* and *in vivo*) did not indicate a significant difference in HSPC migration/ homing capacity or erythroid differentiation. However, proteomics and transcriptome analyses of fetal erythroid precursors indicated an imbalance in mammalian target of rapamycin (mTOR)/ AMP-activated protein kinase (AMPK) pathways. Considering that AMPK pathway activation is required for erythroid maturation, this imbalance is therefore suggested to underlie the higher erythroblast count in the UCB.

In summary, our results suggest that hypoxia-induced oxidative stress may lead to non-erythroid globin production in PE placentas. In addition, we propose that PE could lead to an imbalance in mTOR/AMPK pathways in fetal erythroid cells that could hinder their maturation and lead to higher UCB erythroblast count observed in PE. Further analysis is required to indicate the effect of non-erythroid or hemolysis-induced hemoglobin in PE.

Key words: Preeclampsia, Placenta, Umbilical cord blood, Fetal erythropoiesis, Non-erythroid globin

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To My Family

"Je suis de ceux qui pensent que la science est d'une grande beauté. Un scientifique dans son laboratoire est non seulement un technicien*; il est aussi un enfant placé devant des phénomènes naturels qui l'impressionnent comme des contes de fées."*

-Marie Curie

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List of Abbreviations

AGM	Aorta-gonad-mesonephros
AMPK	AMP-activated protein kinase
ARE	Antioxidant response element
BACH1	BTB domain and CNC homolog 1
BFU-E	Burst forming units-erythroid
BP	Blood pressure
CFU-E	Colony forming units-erythroid
cHbF	Fetal hemoglobin concentration
CTCF	CCCTC-binding factor
ctHb	Total hemoglobin concentration
DE	Differentially expressed
EPO	Erythropoietin
ER	Endoplasmic reticulum
ETC	Electron transport chain
FACS	Fluorescence activated cell sorting
FL	Fetal liver
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA1	GATA binding protein 1
GPA	Glycophorin A
GSEA	Gene set enrichment analysis
H&E	Hematoxylin and Eosin
H3K9me	Histone H3 lysine 9 methylation
Hb	Hemoglobin

HbA	Adult hemoglobin
HBA	Alpha globin
HbE	Embryonic hemoglobin
HbF	Fetal hemoglobin
HBG	Gamma globin
hCG	Human chorionic gonadotropin
HIF	Hypoxia-inducible factor
HO-1	Heme oxygenase 1
HRE	Hypoxia-response element
HS	DNase hypersensitive sites
HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem/progenitor cells
ITFG2	Integrin alpha FG-GAP repeat containing 2
IUGR	Intrauterine growth restriction
KEAP1	Kelch-like ECH associated protein 1
KLF1	Kruppel-like factor 1
KLHL36	Kelch-like family member 36
LCR	Locus control region
MCS	Multispecies conserved sequences
MGG	May-Grünwald-Giemsa
MNC	Mononuclear cell
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NF-E2	Nuclear factor, erythroid 2
NFE2L1 (NRF1)	Nuclear factor, erythroid 2-like 1
NFE2L2 (NRF2)	Nuclear factor, erythroid 2-like 2
PE	Preeclampsia/ Preeclamptic
PGF	Placental growth factor

ROS	Reactive oxygen species
SAM	Surface adhesion molecule
SCF	Stem cell factor
sFLT1	Soluble fms-related tyrosine kinase 1
SWI/SNF	SWItch/Sucrose Non-Fermentable
TF	Transferrin
TNF	Tumor necrosis factor
UBQ	Ubiquitin
UCB	Umbilical cord blood
ULK1	Unc-51-like autophagy activating kinase 1
UPR	Unfolded protein response
VAcHbF	Veno-arterial differences of cHbF
VActHb	Veno-arterial differences of ctHb
VEGF	Vascular endothelial growth factor

Introduction

What is preeclampsia and why does it matter?

Preeclampsia (PE) is an important pregnancy complication affecting 3-8% of women worldwide (1). Despite all the advances in the medical field, PE remains a leading cause of maternal and fetal morbidity and mortality, particularly resulting in extreme fetal prematurity (2). More than a century after eclampsia was distinguished from epilepsy (in 1739) (3), proteinuria (4) and hypertension (5) were determined as premonitory symptoms of eclampsia, giving rise to the concept of "pre-eclampsia" (6). To date, diagnosis of PE is still based on the maternal symptoms; presence of hypertension characterized by two readings of systolic blood pressure (BP) ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg, with a 4- to 6-hour interval, as well as proteinuria recognized by a urine protein level ≥ 300 mg or dipstick of ≥ 1+ per 24 hours mainly after 20 weeks of gestation in a previously normotensive non-proteinuric pregnant woman (7, 8). In more severe cases of PE, sustained systolic BP ≥ 160 mmHg or diastolic BP ≥ 110 mmHg is associated with nephrotic-range proteinuria, pulmonary and cerebral edema as well as other systemic disturbances (8). The classification of the patients as early- or late-onset PE relies on whether the onset of the symptoms is prior or after 34 weeks of gestation, respectively. The number of cases demonstrating objective evidence of placental dysfunction and very severe clinical symptoms is higher in the early-onset category (2, 8).

Diagnosis of PE is followed by a) providing close medical care to the patients in order to decrease the adverse effects on the maternal health, and b) delivery of the fetus and the placenta (8). Whilst remitting the maternal PE symptoms, this does not resolve the systemic influences of PE on the maternal health, including higher lifetime risk of cardiovascular disorders (9, 10), stroke (11, 12) and death (13, 14). Depending on its severity, PE can also have dramatic effects on fetal development and survival. In severe PE, the risk of stillbirth increases up to 21 in 1000 live births (15) while the infant birth weight can decrease up to 12%, a condition known as intrauterine growth restriction (IUGR) (16). But even mild PE leading to late-preterm delivery can increase the risk of neonatal mortality and can expose the infants to various complications during infancy and adulthood, such as higher lifetime risk of endocrine, nutritional, and metabolic disorders (17, 18).

Etiology of the "disease of theories"

Since its first description by Hippocrates in about 400 BC (19), many speculations have been made into the etiology of PE, which earned it the title of "disease of theories" in 1916 (20). To date, PE etiology and pathogenesis remain enigmatic due to the complex multifactorial nature and heterogeneous presentation of this disorder (21). The commonly known risk factors of PE vary from the primigravity, *in vitro* fertilization-induced or multiple pregnancy (22) to maternal obesity, diabetes, kidney disorders, advanced age, familial history of PE, hypertension or African ancestry, and living at high altitude (23, 24). Several epi/genetic (25-27) and paternal (28-30) factors have also been suggested to play a role in triggering PE. This emphasizes the magnitude and variety of the pathways that are activated and eventually translate into the clinical symptoms diagnosed as PE. With increasing knowledge and interest in the field, multiple studies have focused on and reported differences among PE subtypes, particularly between early- and late-onset groups (2, 31, 32). However, PE can occur in the absence of a fetus, as in hydatidiform mole (33-35) and in extra-uterine pregnancies (36, 37). Even retained placenta or incomplete removal of it can result in post-partum PE (36, 38). All of this suggests that a dysfunctional placenta is a mutual central defect in the majority of PE cases.

The first glimpses into defective placentation were provided by Young in 1914. After extensive analysis of placentas from hypertensive pregnancies, he demonstrated the presence of placental infarctions and lesions suggesting "blockage of the decidual veins" as the plausible casual factor (39). The next significant observation was made in 1953 when Browne and Veall reported a prominent reduction in the blood flow in the intravillous space in hypertensive pregnancies (40). Within the next two decades, mounting evidence confirmed pathological changes in the placental bed spiral arteries in hypertensive pregnancies (41-45). Finally, in 1972, Brosens et al. suggested that failure of trophoblast invasion and thereby defective extension of the physiological changes in the placental bed spiral arteries into the myometrium in PE, was an indication of inadequate placentation resulting in reduced blood supply to the intervillous space (46). These significant findings played an essential role in composing the two-stage theory on PE etiology presented in 1991 (47).

The classic two-stage theory on PE suggested that decreased invasion of extravillous cytotrophoblasts into the decidua and defective remodeling of the spiral arteries leads to poor placentation early in pregnancy. This causes a reduction in blood supply to the placental intervillous space and the fetus that can

be further aggravated by acute atherosis (48). Disrupted placental perfusion can result in intermittent placental and fetal hypoxia, particularly when the demand for maternal blood increases during the second trimester of pregnancy. The resulting ischemia-reperfusion injury could affect cellular and metabolic processes and increase placental oxidative stress, senescence and shedding into the maternal circulation (49, 50). The second stage is initiated by the excessive release and leakage of placental factors through the disrupted placental barrier into the maternal circulation, triggering a systemic inflammatory response and general endothelial dysfunction (51), which manifests in the clinical symptoms of PE such as hypertension, proteinuria and edema (49). However, this would mainly explain the PE pregnancies with distinct pathologically defective placentation and incomplete remodeling of the spiral arteries, typically observed in the early onset PE cases (52).

The two-stage theory developed further as the general understanding of placental distress as well as the immunoregulatory and angiogenic disturbances in PE pregnancies advanced (52, 53). The most recent version of the two-stage theory suggests syncytiotrophoblast stress is an important player in PE (53). Syncytiotrophoblast stress can be triggered by various factors such as ischemic reperfusion or inflammation. Placental malperfusion can be caused by deficient spiral artery remodeling (typical for early onset PE) as well as chronic villous congestion in larger than average mature placentas (typical for late onset PE) (Fig 1). Intermittent hypoxia can alter intracellular glucose levels that could affect metabolism as well as protein glycosylation in the endoplasmic reticulum (ER) and Golgi (54). This results in higher ROS and activation of unfolded protein response (UPR) which can lead to cellular stress and apoptosis (54-56). Syncytiotrophoblast stress, determined as presence of apoptotic syncytial knots, oxidative stress and activation of UPR, has been reported in PE placentas (53, 57). This can change the placental production of pro-inflammatory and angiogenic factors (58, 59) which are released into the maternal circulation, generating detrimental effects on both the placenta and the mother.

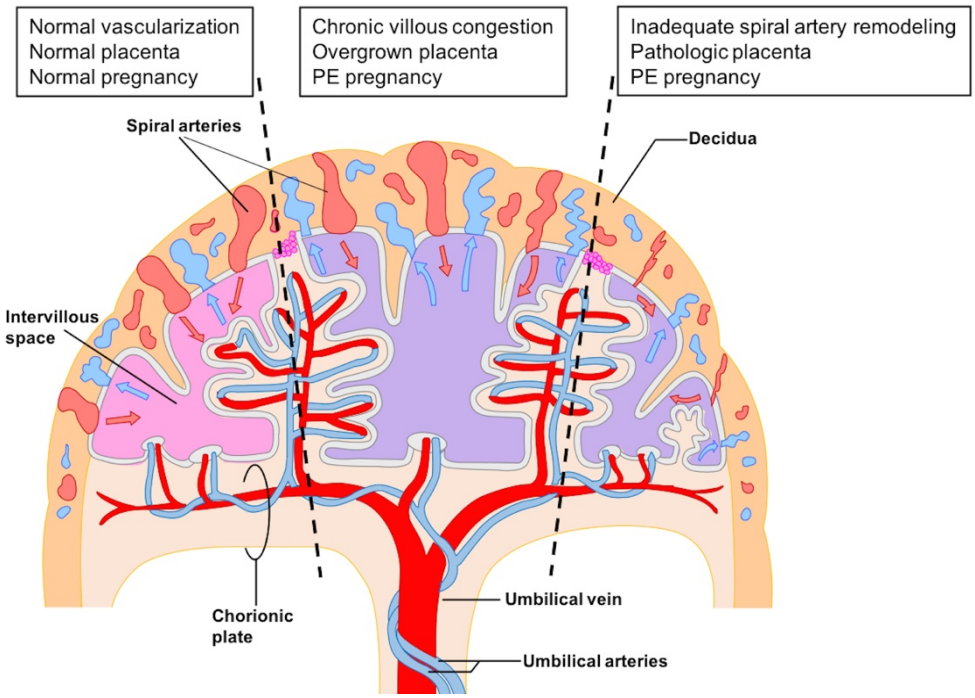

Figure 1. Schematic representation of the placenta in normotensive and PE pregnancies according to the two-stage theory. In a normotensive pregnancy, proper spiral artery remodeling and optimal placenta growth allows appropriate placental perfusion as well as nutrition and gas exchange between the placenta and the fetus. In early- and late-onset PE, primary defective spiral artery remodeling or excessive placenta growth during the pregnancy leads to intermittent hypoxia and ischemic repefusion damaging the placental tissue and preventing sufficient exchange between the placenta and the fetus.

Maternal molecular and cellular changes in PE

In a normal pregnancy, specific maternal anatomical and physiological changes are necessary to accommodate survival and growth of the fetus (60-62). Altered maternal hemodynamics, including increased plasma volume, cardiac output (associated with cardiac remodeling) and glomerular filtration, as well as decreased systemic vascular resistance, allows for proper placental perfusion and waste management. Changes in maternal metabolism, such as decreased insulin sensitivity, act to effectively preserve adequate nutrition to fulfill the needs of the developing fetus. In addition, profound immunological regulations occur as steps in "pregnancy recognition" to adapt the maternal immune system and allow for tolerance towards a semi-allogenic fetus (63-65). These adjustments are in part mediated by various maternal hormones such as human chorionic gonadotropin (hCG), placental lactogen, prolactin and the renin-angiotensin-aldosterone system (61, 64).

In PE, these regulatory mechanisms are adversely affected in the mother (62, 66, 67). Inadequate plasma volume expansion, higher mean arterial pressure along with increased sensitivity of the maternal vascular system to vasopressor factors, such as angiotensin II, are associated with excessive left ventricle hypertrophy that persists postpartum. Despite cardiac remodeling, cardiac output, glomerular filtration rate and renal blood flow decline leading to uteroplacental hypoperfusion. Changes in serum hormones such as lower aldosterone and cortisol and higher brain natriuric peptide could also impact the maternal BP and immune response severely (67). In fact, deficient adaptation of the maternal immune system and the consequent inflammatory response in PE has been investigated and reviewed extensively (68-70). This maladaptation is further accelerated by placental ischemia-reperfusion injury-induced inflammation, affecting the serum levels of various factors such as interferon γ, soluble endoglin, fetal hemoglobin (HbF), soluble fms-related tyrosine kinase 1 (sFLT1) and placental growth factor (PGF) (53, 68).

As the soluble form of the vascular endothelial growth factor (VEGF) and PGF receptor, sFLT1 has been the focus of numerous experiments to elucidate its role in PE. Both VEGF and PGF are pro-angiogenic factors that promote vascular permeability and endothelial cell survival (71, 72). Studies on animal models and trophoblast cell cultures have indicated that FLT1 is a negative regulator of angiogenesis (73) and its expression, particularly in its soluble form, increases in hypoxia (74). Excess sFLT1 has been shown to adversely affect the vascular and glomerular endothelium and cause hypertension and proteinuria in both pregnant

and non-pregnant rats (75). It has also been reported that the expression of PGF decreases in the trophoblasts cultured under hypoxic conditions (76) and in PE placentas (77, 78). In other words, trophoblastic stress in PE may lead to decreased levels of angiogenic factors and excessive release of sFLT1 into the maternal circulation. The resulting imbalance in sFLT-1/(PGF, VEGF) ratio has been suggested to underlie the maternal systemic endothelial dysfunction as well as defective placental angiogenesis as observed in PE. Disproportionate sFLT-1/PGF has been confirmed in pregnant women affected by PE (75, 79) and is considered as a possible clinical predictive biomarker of this disorder (80). Pilot clinical trials on PE pregnancies have also shown that extracorporeal removal of sFLT1 from the maternal circulation stabilizes BP, decreases proteinuria and prolongs gestation (81, 82).

Another factor that has been studied is free hemoglobin (Hb). Hemoglobin is a heterotetramer metalloprotein that consists of four polypeptide subunits. Two of the subunits are from the alpha globin cluster (zeta, mu, alpha1, alpha2 and theta) and the remaining two are from the beta globin cluster (epsilon, G-gamma, A-gamma, beta and delta). Expression of these genes is regulated throughout development but the most common heterotetramers found in erythrocytes include embryonic hemoglobin (HbE: $zeta_2$ $epsilon_2$), fetal hemoglobin (HbF: $alpha_2$ $gamma_2$) and adult hemoglobin ($alpha_2$ $beta_2$). Each globin chain is tightly linked to a prosthetic heme group that consist of an iron bound to four nitrogen atoms in a tetrapyrrole ring. The heme is linked to the protein subunit by coordination of the ferrous iron (Fe^{2+}) to the imidazole ring (of the histidine of the protein), leaving one free coordination site for the ferrous iron to reversibly bind gaseous molecules (83, 84). This structure provides hemeproteins with a "pseudoperoxidase" capacity that could lead to production of unstable ferryl Hb (Fe^{4+}) and protein-based radicals (84-86). Thus, proper compartmentalization of Hb is critical as free Hb can cause oxidative stress, inflammation, vasoconstriction and hypertension, renal failure and also thrombosis, partly by its pseudoperoxidase activity and in part by scavenging nitric oxide (84, 87-89). *Ex vivo* and *in vivo* experiments have demonstrated that free Hb could create PE-like pathologies in the human placenta (90, 91) and in several pregnant animal models (92-94). Clinical studies on plasma samples from pregnant women have reported higher levels of free hemoglobin (both HbF and HbA) and heme scavengers in the maternal circulation in PE (95). An increase in HbF and heme scavengers in pregnant women has been suggested as an early predictive biomarker for PE (96). It has also been suggested that excess heme in the maternal circulation overwhelms the protective scavenger system (97, 98) which may explain the mechanism underlying the beneficial effects of applying heme scavengers in PE animal models (92-94). Despite all this, the source of the excess Hb in the maternal circulation in PE remains unknown.

Hb production: Sources and transcriptional regulation

As the major source of Hb production, erythroid cells have been studied vastly and accurately, providing some insight into the transcriptional regulation of the globin loci. It is known that each locus undergoes epigenetic changes (99-103) and recruits various transcription activators and repressors (104-109) to produce HbE, HbF and HbA during embryonic, fetal and postnatal development, respectively. Transcription of the alpha globin gene cluster on chromosome 16 is controlled via four upstream multispecies conserved sequences (MCS) (107, 110) (Fig 2A). The MCSs contain DNase hypersensitive sites (HS), which are considered to be in open euchromatin conformation due to their sensitivity to cleavage by the DNase I enzyme. The most important regulatory MCS, MCS-R2 or HS40 (111), contains at least six sites for erythroid-specific transcription factors: two recognition sites for GATA binding protein 1 (GATA1) as well as two sites for nuclear factor, erythroid 2 (NF-E2) followed by two additional sites for GATA1 (112). In human, deletion of HS40 results in substantial reduction of alpha globin locus transcription causing α-thalassemia (113-115).

The expression of the beta globin gene cluster on chromosome 11 is regulated through an upstream locus control region (LCR) and a downstream HS1 (116) (Fig 2B). The LCR consists of five HSs (1 to 5) with HS5 being located at the 5' end, furthest from the promoter. The HS5 is recognized by CCCTC-binding factor (CTCF), a transcriptional regulator that mediates chromatin looping and histone modifications (117) to block the beta locus enhancer activity in a developmental stage-dependent manner (118, 119). The other four HSs interact with several proteins and transcription activators such as GATA1 and Kruppel-like factor 1 (KLF1) to regulate gene transcription (106). Interestingly, formation of the HSs 1 to 4, which promotes the switch from initiation to elongation of transcription (120), is considered to be specific to erythroid cells (116). Transcription of the beta globin locus diminishes significantly in the absence of the LCR and may cause $(\varepsilon\gamma\delta\beta)^0$-thalassemia (116, 121, 122).

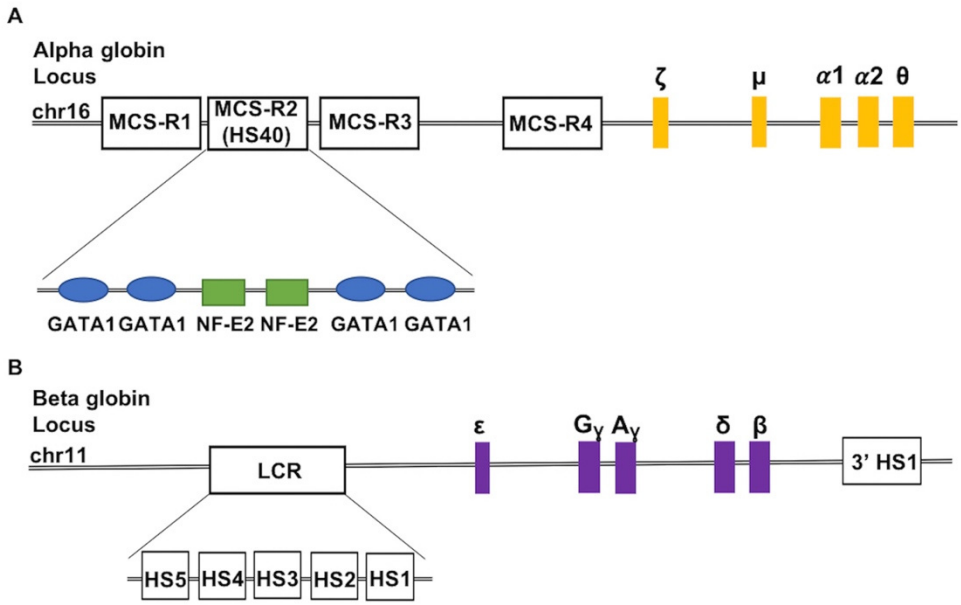

Figure 2. Schematic overview of the general regulatory elements controlling the transcription of alpha and beta globin loci. The regulatory elements up and downstream of the loci are known to inhibit or promote and controle the amount of expression of different globin genes in erythrocytes during development. The details of the role of these elements and their possible interaction with various transcription factors in non-erythroid cells is not well known.

One of the transcription factors considered to enhance the expression of both globin clusters is the nuclear factor erythroid 2-like 2 (NFE2L2/NRF2). But NRF2 pathway is not specific to erythroid cells and is known for its role in promoting cell survival under oxidative stress (123). Under normal conditions, NRF2 bound to kelch-like ECH associated protein 1 (KEAP1) is ubiquitinated and degraded in the cytoplasm. However, cellular and environmental stress leads to release of NRF2 from KEAP1 and its nuclear translocation. In the nucleus, NRF2 induces expression of antioxidants by interacting with antioxidant response elements (AREs), a cis-acting enhancer sequence in the promoter region of several genes. The NRF2-induced expression of globin gene clusters in erythroid cells is regulated by a heme-dependent mechanism (124). In brief, the expression of globin is suppressed due to the interaction of ARE with BTB domain and CNC homolog 1 (BACH1). This protein, like NRF2, is a member of the family of cap'n'colar (CNC) proteins but also contains broad complex, tramtrack, bric-a-brac (BTB) domains. Enhanced production of heme in erythroid progenitors is associated with the translocation of heme from mitochondria to the cytoplasm and nucleus. Heme interacts with and modifies DNA-bound BACH1 so it releases ARE and undergoes proteasomal degradation in the cytoplasm. This is followed by recognition and binding of ARE by NRF2, which promotes globin expression.

Presence of an active ARE upstream of the beta globin locus in primates was first confirmed in the 1990s (125). More recent studies have indicated presence of AREs in the HS2 upstream of the beta globin locus and specifically in gamma globin promoter in human (126). Others have demonstrated that the interplay of NRF2 and BACH1 can not only induce gamma globin expression (127) but also increase alpha globin expression (128).

The concept of erythroid-specific expression of alpha and beta globin clusters has been challenged by an increasing number of studies reporting Hb detection in other cell types such as macrophages (129), cancerous and normal alveolar epithelial cells (130-133), mesangial cells (134), endometrial cells (135), retinal pigment epithelial cells (136), neuronal and glial cells (137-139), hepatocytes (140), vascular endothelial cells (141), and cervical cells (142) from human and animal models. The expression of genes from alpha and beta globin clusters has been determined at mRNA and protein levels in these cells. Induction of hypoxia and treatment of cells with H_2O_2 or inflammatory cytokines have been shown to result in upregulation of globin expression in several of these cell types. The major roles attributed to Hb in non-erythroid cells include antioxidant, nitric oxide scavenger and oxygen homeostasis in hypoxia or hyperoxia. Our understanding of the principal pathway regulating globin expression in non-erythroid cells is very limited, but the transcription factors and mechanisms involved could vary from those in the erythroid cells. For instance, Grek et al. have reported GATA1 to be mandatory for hypoxia-induced Hb upregulation in alveolar epithelial cells (133). On the other hand, Liu et al. have proposed that in the absence of GATA1, Hb expression may be regulated by NRF2 as to protect the cells against oxidative stress triggered by hypoxia, injury or inflammation (140). Moreover, the studies performed in erythroid cells have indicated distinct differences in transcriptional regulation of alpha and beta globin loci. It remains elusive whether, or how, these differences might affect globin expression in non-erythroid cells.

One of the earliest studies that signified the importance of globin expression regulation was performed by Humphries et al. in 1982 (143). In a series of experiments, alpha, beta and delta globin expression was induced in a fibroblast-like cell line from monkey kidney tissue using recombinant plasmid vectors containing a Simian vacuolating virus 40 (SV40) enhancer element. The study demonstrated that transcriptional activity of the alpha globin promoter was independent of the SV40 enhancer while the beta globin promoter activity depended heavily on the presence of the upstream enhancer. Further studies on the alpha globin gene cluster revealed it to be enclosed in a G+C rich area with euchromatin conformation and GpC islands near the promoters (144). In non-erythroid cells, the locus is hypoacetylated and lacks any markers of transcriptional in/activation such as H3K4me3, H3K9me or H3K27me. The significant transcriptional activity of the theta and zeta genes observed in these

cells has been associated with complete coverage of the locus by RNA Polymerase II and BRG1, a member of the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex that facilitates transcription via ATP-dependent chromatin remodeling (145). These studies accentuate the contrast between the alpha globin locus and the A+T rich content of the beta globin gene cluster that lacks GpC islands and was considered to be very tightly packed into inactive heterochromatin in non-erythroid cells (146, 147). These basic differences might underlie the variability in transcription levels of alpha and beta globin genes reported in some non-erythroid cells (140-142).

Placenta: a plausible source of increased Hb in PE

The placenta is crucial during fetal development and as mentioned earlier, it plays a considerable part in triggering PE pathophysiology. In a study by Centlow et al., PE was associated with local overproduction of HbF in the placenta (148). Interestingly, the placenta could have a plausible role in contributing to the excess free Hb in PE by producing it through erythroid and/or non-erythroid pathways.

Placenta: A hematopoietic organ

Studies on placental tissues from human and animal models have indicated that the placenta is an extramedullary hematopoietic organ during fetal development, supporting the possibility of placental Hb production through erythropoiesis. The earliest hematopoietic role of the placenta has been reported in the first trimester placental villi where terminal maturation of the primitive erythroblasts is facilitated through formation of erythroblastic islands (149) consisting of a central macrophage that assists maturation and enucleation of the erythroblasts surrounding it (150). Primitive erythroblasts are nucleated, contain HbE and originate in the yolk sac (151). With further development, the placental hematopoietic role advances beyond providing micro-niches for erythroblast maturation. *De novo* generation of hematopoietic stem/progenitor cells (HSPCs) has been reported in the placenta (152). The HSPCs have also been detected throughout gestation in both placental chorionic plate and vasculature (152-157). Moreover, active placental erythropoiesis has been demonstrated during the first and early second trimester of pregnancy (158). Placental erythropoiesis has been reported based on detection of erythroblastic islands and erythroid precursors expressing glycophorin A (GPA), a protein expressed on the cell surface of erythroid cells from proerythroblasts to mature erythrocytes. However, the residing niche of the HSPCs, including those of the placental origin, changes throughout development (159-162). The developmental journey of the definitive HSCs begins with their migration from the placenta and the aorta-gonad-mesonephros (AGM) region of the embryonic mesoderm to the fetal liver (Fig 3). The liver is the main fetal hematopoietic organ during the second and early third trimester of pregnancy. Eventually, the HSCs migrate from the fetal liver to the bone marrow where they reside throughout neonatal and adult life (Fig 3).

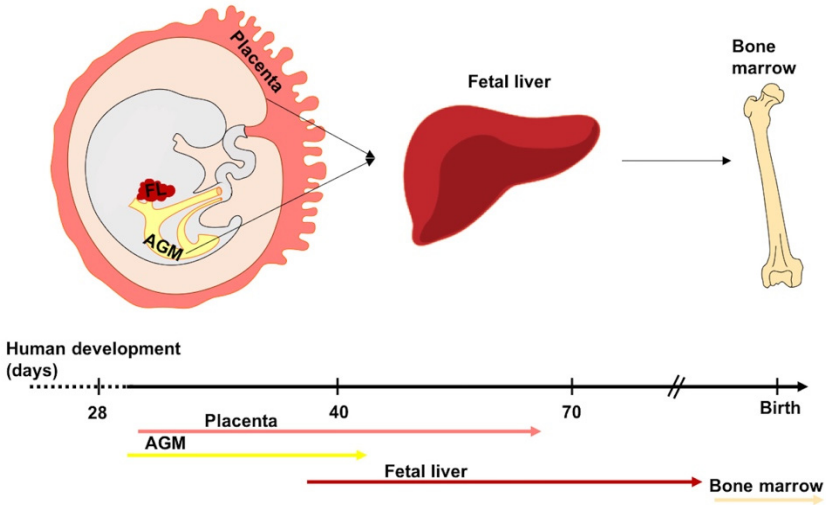

Figure 3. Overview of the developmental journey of the definitive HSCs. An illustration of human embryo and the organs harboring definitive HSCs (Aorta-gonad-mesonephros region: AGM, Fetal liver: FL) and the suggested timeline and process of developmental journey of these cells up to birth (Adapted from Mikkola and Orkin, 2006).

As discussed previously, the placenta changes substantially in PE which might affect placental hematopoiesis during fetal development. For instance, PLGF and VEGF, which are both decreased in PE placentas, have been shown to regulate bone marrow-derived HSC survival and migration (163-165). But the exact effect of these alterations on placental HSPCs is elusive. Ponder et al. have suggested that pregnancy complications, particularly those affected by inflammation, transform the placental niche. In PE, the changes might affect the frequency of HSPCs present in the chorionic plate (166). It remains unclear whether all the differences in the placental niche and localization of HSPCs are associated with altered migration/homing of these cells. Also, it remains to be determined whether the alterations in PE result in enhanced and extended placental erythropoiesis beyond the early second trimester, giving rise to the reported local elevation of HbF production in term placentas (148).

Placenta: Activating the cell survival response

The very primary stages of fetal implantation in a normal pregnancy take place under acute hypoxia which is essential for proper growth and differentiation of trophoblasts (167). In PE, the placental cells are particularly affected by hypoxia and oxidative stress (168, 169). Under stress, adaption of cellular processes such as metabolism is a vital step for

cell survival. This is only accomplished through activation of various gene regulatory pathways. One main family of transcription factors playing an important role in cell adaptation to hypoxia is that of the hypoxia-inducible factors (HIFs), particularly HIF1 (170). The family consists of heterodimer transcription factors including an alpha and a beta subunit. Both subunits are expressed constitutively but while the beta subunits are present in the nucleus, oxygen-dependent proteasomal degradation of the alpha subunits by HIF-specific prolyl hydroxylases in the cytoplasm restricts HIF dimerization in the nucleus in normoxic conditions (171, 172). Cytoplasmic stabilization and nuclear transfer of the alpha subunit in hypoxic conditions allows dimerization of the alpha and beta subunits producing the active form of HIF. HIF-dependent gene transcription includes expression of genes controlled by a hypoxia-response element (HRE), such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transferrin (TF) and nuclear factor, erythroid 2-like 1 (NFE2L1/NRF1) (173). The change in gene expression profile is followed by energy production divergence from mitochondrial electron transport chain (ETC) to glycolysis to limit production of reactive oxygen species (ROS) through defective respiration (174). Moreover, hypoxia has been linked to an increase in cellular heme levels *in vitro* (175) while HIF1 activation facilitates regulation of cellular iron transfer and storage in hypoxia (176). These increased heme levels have been considered to be the outcome of decreased oxygen-dependent enzymatic oxidation and degradation of heme precursors (176). It has also been suggested to have a protective anti-oxidative role by producing biliverdin after being catalyzed by heme oxygenase 1 (HO-1) (176). While the exact mechanism underlying the elevated intracellular heme levels is not clear, the excess heme could still affect globin expression as explained earlier. On the other hand, oxidative stress activates the NRF2 pathway to induce expression of genes, specifically those of antioxidants, to promote cell survival. Despite general agreement on increased NRF2 expression and activation in PE, it is not completely clear whether it has a protective role (177) or it contributes to PE pathogenesis (178, 179). Alteration in NRF2 pathway has also been reported in diabetic pregnancies (180) and twin pregnancies with IUGR (181). The NRF2 pathway is also important due to its effect on enhancing HIF1 signaling in intermittent hypoxia (182). HIF1 is a significant factor in PE (183) and it can change chromatin conformation in stress conditions such as hypoxia and inflammation (184, 185). But it is unclear whether the hypoxia-induced oxidative stress associated with the activity of HIF1 and NRF2 in the placental cells in PE could trigger non-erythroid production of HbF (Fig 4).

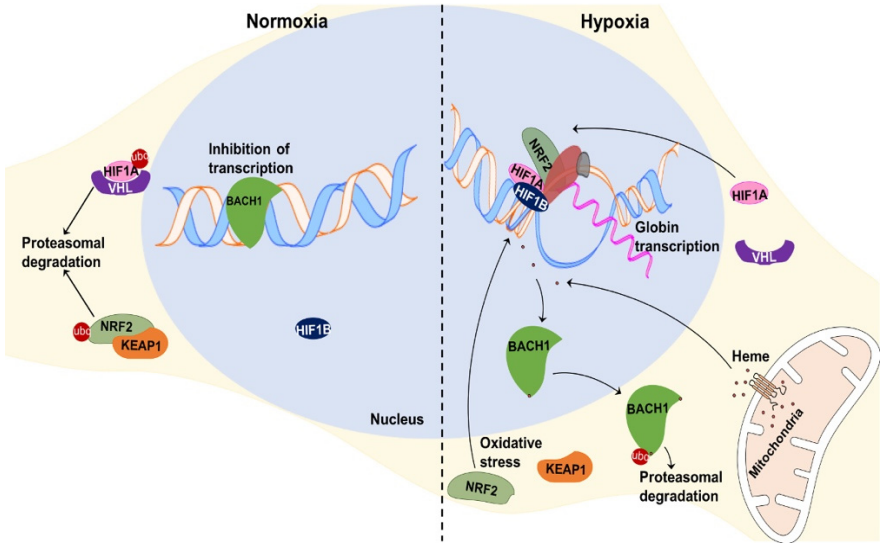

Figure 4. Schematic representation of the pathways suggested to regulate globin transcription in non-erythroid cells. The expression of globin gene in non-erythroid cells has been suggested to require activation of HIFs and NRF2 in reponse to hypoxia-induced oxidative stress. Excess presence of heme, possibly due to lack of oxygen required for its degradation, has been shown in hypoxic conditions. Globin transcription repressor, BACH1, undergoes proteasomal degradation after binding to heme. This process provides access to active transcription factors such as NRF2 to bind regulatory elements such as the ARE in the HS2 of beta globin locus. BACH1: BTB domain and CNC homolog 1, NRF2: nuclear factor, erythroid 2-like 2, KEAP1: kelch-like ECH associated protein 1, HIF1A/B: hypoxia inducible factor1A/B, VHL: von Hippel-Lindau tumor suppressor, ubq: ubiquitin.

The effect of PE on the fetus, particularly fetal hematopoiesis

Adverse *in utero* conditions and fetal growth can impair fetal/ neonatal health and have been associated with the development of different chronic diseases during adulthood (186-188). Studying the changes in umbilical cord blood (UCB) can reflect altered *in utero* conditions affecting fetal growth. The levels of many factors and cytokines are different in the UCB from hypertensive and PE pregnancies (189-191), denoting possible physiological and pathological changes in the fetus. For instance, the elevated proinflammatory cytokines (192-194) and activated natural killer (NK) cells (192, 195, 196) could signify an inflammatory response in the fetuses from PE pregnancies.

One of the most common observations in PE vs normotensive pregnancies is a higher erythroblast (nucleated red blood cell) count in the UCB (197-200). This suggests a major effect of PE on fetal hematopoiesis. Several groups have studied these changes by benefiting from UCB as a source of obtaining fetal hematopoietic cells, particularly HSPCs, and evaluating related regulatory cytokines. Surbek et al. reported that the number of UCB HSPCs and the number of precursor colonies obtained by *in vitro* differentiation of these cells was lower from PE vs normotensive pregnancies (201). However, the expression of surface adhesion molecules (SAMs) related to homing of the UCB HSPCs to the bone marrow was not different between PE and normotensive samples (201). As the decrease in the number of UCB HSPCs and their hematopoietic differentiation capacity could not be explained by altered expression of SAMs and homing, it was suggested that PE led to suppression of hematopoiesis in the fetus (201). While Wahid et al. confirmed a lower UCB HSPC count (202), other reports suggest that PE might not affect (203) or may increase the number of circulating HSPCs (204). On the other hand, a higher fetal erythroblast count has been suggested to result from enhanced erythropoietin (EPO)-induced erythropoiesis that is due to fetal ischemia and hypoxia in PE (205-208). In line with this hypothesis, Santillan et al. associated PE pregnancies with a trend towards higher EPO levels in fetal serum and enhanced *in vitro* erythroid colony formation capacity of the UCB HSPCs (209). However, this hypothesis does not take into account many other changes in the fetus associated with placental dysfunction observed in PE. Altered metabolism (210-212) as well as inflammatory cytokines (213, 214) could promote or hinder erythroblast maturation. All of this highlights the significance of further investigations into the molecular changes of HSPCs and fetal erythropoiesis in PE, to explain the phenotype of higher UCB erythroblast count.

AMPK vs mTOR pathways in regulating HSCs and erythropoiesis

Efficient erythropoiesis requires strict regulation of all the processes from cell cycle, metabolism and DNA replication to cell death and proteolysis at each stage of differentiation (215). Synchronized regulation of iron, amino acids and glucose metabolism (211) as well as various signaling pathways including the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways (216, 217) are essential. The mTOR pathway comprises two complexes including different factors (Fig 5). The role of mTOR complex 2 (mTORC2) is not very clear but it has been suggested to have a role in embryonic emergence of HSCs (218). mTORC1 signaling is important in regulating HSC function as well as differentiation. For instance, mTORC1 elevation is associated with HSC hyperproliferation leading to increased ROS that limits cell survival and eventually results in HSC exhaustion and depletion (219, 220). Enhanced mTORC1 signaling is required for differentiation and proliferation of hematopoietic progenitor cells. In particular, during erythropoiesis, mTORC1 regulates mitochondrial biogenesis and protein expression (221, 222). However, up- and down-regulation of mTORC1 in HSCs *in vivo* leads to macrocytic and microcytic anemia, respectively (223, 224). Furthermore, ROS-induced over-activation of mTOR has been shown to result in erythroblast hyperproliferation and defective maturation (225). This points to the significance and differential regulation of mTORC1 at various stages of erythropoiesis. Functionally opposing mTOR is the AMPK pathway. Interestingly, AMPK regulates the autophagy essential for final stages of erythroid maturation, including degradation of internal organelles and enucleation (217) (Fig 5). Defective autophagy can lead to incomplete enucleation and/or removal of mitochondria from erythroblasts and can result in severe anemia (226). In addition, AMPK activity is important in maintaining erythroid cell integrity (227, 228) and survival (229). The cellular pathways of autophagy or cell growth and division are controlled by mTOR and AMPK through regulating phosphorylation of unc-51-like autophagy activating kinase 1 (ULK1) (230, 231). All these signify the importance of studying the UCB erythroid profile and possible changes of these pathways affecting erythroid maturation in PE fetuses.

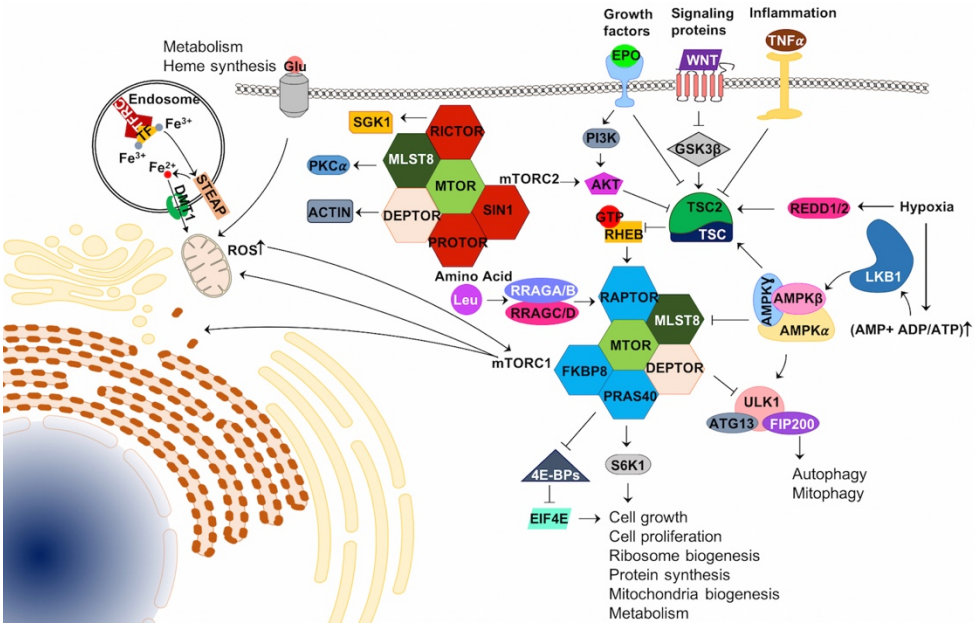

Figure 5. A general overview of the interactions between mTOR and AMPK pathways, with focus on their role in erythropoiesis. The process of erythropoiesis is strictly controlled by various cellular mechanisms, from altering metabolism to regulating different pathways such as mTOR and AMPK signaling. Any deregulation at any of these pathways could adversely affect the erythroid maturation process. MTOR: mammalian target of rapamycin, mTORC1: MTOR complex 1, mOTRC2: MTOR complex 2, AMPK: AMP-activated protein kinase, TNFα: tumor necrosis factor α, WNT: Wnt protein family, GSK3β: glycogen synthase kinase 3 beta, EPO: Erythropoietin, PI3K: Phosphoinositide 3-kinase, AKT: AKT serine/threonine kinase, TSC1/2: Tuberous sclerosis complex subunit 1/2, REDD1/2: DNA damage inducible transcript 4/like, LKB1: serine/threonine kinase 11, ULK1: unc-51 like autophagy activating kinase 1, ATG13: autophagy related 13, RB1CC1 (FIP200): RB1 inducible coiled-coil 1, RHEB: Ras homolog, mTORC1 binding, RAPTOR: regulatory associated protein of MTOR complex 1, MLST8: MTOR associated protein, LST8 homolog, DEPTOR: DEP domain containing MTOR interacting protein, PRAS40: AKT1 substrate 1, FKBP8: FK506 binding protein 8, S6K1: ribosomal protein S6 kinase B1, 4E-BPs: eukaryotic translation initiation factor 4E binding protein family, EIF4E: eukaryotic translation initiation factor 4E, RRAGA/B/C/D: Ras related GTP binding A/B/C/D, RICTOR: RPTOR independent companion of MTOR complex 2, SIN1: mitogen-activated protein kinase associated protein 1, PROTOR: proline rich 5/ like, SGK1: serum/glucocorticoid regulated kinase 1, PKCα: protein kinase C α, ROS: reactive oxygen species, DMT1: solute carrier family 11 member 2, STEAP: six-transmembrane epithelial antigen of the prostate (STEAP) protein family, TF: transferrin, TFRC: transferrin receptor, Glu: glucose, Leu: Leucin.

The Present Investigation

The main purpose of this thesis is to explore the contribution of placental and fetal erythropoiesis and non-erythroid Hb production to the excess HbF previously documented in term placentas and maternal circulation in PE pregnancies. Accordingly, the following general aims were defined:

I To investigate whether the placenta can affect UCB HbF levels in PE

II To explore whether the excess HbF in PE is associated with extreme and extended placental erythropoiesis

III To examine the role of hypoxia and oxidative stress in regulating Hb production in non-erythroid placental cells

IV To study the effect of PE on fetal hematopoiesis, particularly in regulating HSC migration and erythroid differentiation

Material and Methods

In this section, a general description of the materials and methods used in this thesis is provided. The detailed description of each method can be accessed in the respective articles.

Data collection

Arterial and venous UCB samples were collected and analyzed within 15 minutes using Radiometer ABL 735 blood gas analyzers (Radiometer A/S, Copenhagen, Denmark). In order to obtain optical clarity, the blood gas analyzer lyses the blood samples by ultrasonication. The analyzer measures blood gases, the pH as well as various compounds such as the total hemoglobin concentration (*c*tHb) (g/L) and HbF%. As HbF and adult hemoglobin have different molecule structures and light absorbance spectra, they can be measured by spectrophotometry at different wavelengths. All the laboratory and clinical data were paired for the period 2001-2010 after receiving approval from the Regional Ethics Committee in Lund, Sweden (Dnr 2009/222). Eventually, 16,269 samples from normotensive and PE pregnancies were used in the analysis in **Paper I**.

Sample collection

The sample collection in **Papers II, III and IV** was approved by the Ethics Committee Review Board for studies on human subjects at Lund University and Skåne University hospital, Lund, Sweden (Dnr 2014/191). Collection of the placenta as well as arterial and venous UCB from normotensive and PE pregnancies was performed following both Cesarean and vaginal deliveries at Skåne University Hospital. The UCB was collected in flasks containing 10 mL Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and heparin. The UCB and placenta samples, stored at 4°C, were processed within 4 hours after sample collection.

Tissue digestion and mononuclear cell isolation

Placental tissue was cut into fine pieces, rinsed in phosphate buffered saline (without Mg^{2+} and Ca^{2+}) (PBS) and mechanically digested using gentleMACS™ Dissociator. The tissue was further enzymatically digested in a digestion buffer containing Collagenase II, Dispase, DNase, penicillin, streptomycin, Ciprofloxacin and Amphotericin B. After filtration, single cells were resuspended in wash buffer

(5% FBS, 2 mM EDTA in PBS). In **Papers II and III**, the placental cell suspension and the UCB were laid upon Ficoll-Paque PLUS and centrifuged at 400 x g at room temperature (RT) for mononuclear cell (MNC) isolation.

Flow cytometry: surface protein expression analysis and cell sorting

In **paper II**, the placental and UCB MNCs were further enriched using human CD34 MicroBead kit. Fluorescence activated cell sorting (FACS) was used to detect and sort HSPCs, and to evaluate the expression of surface adhesion molecules (SAMs) important to their migration and homing. The UCB HSC profile was also analyzed and $CD34^+$ cells were isolated for colony formation assay analysis.

In **Papers II and III**, MNCs were used to analyze the erythroid profile of arterial and venous UCB from PE and normotensive pregnancies by flow cytometry.

In all analyses, spectral compensation was carried out using VersaComp Antibody Capture Beads kit and the gates were set based on unstained and fluorescent minus one controls. 7AAD was used to confirm viability. Data analysis was performed using FlowJo (V.10.0.8).

In vitro colony formation assay

In **paper II**, $CD34^+$ cells were mixed with Cell Resuspension Solution and Human Methylcellulose Complete Media containing EPO, Granulocyte macrophage colony-stimulating factor (GM-CSF), Interlukin-3 (IL-3) and Stem Cell Factor (SCF). 500 cells/well were plated in triplicate in 6-well plates and placed in a humid chamber at 37°C with 5% CO_2.

Protein extraction and quantitative proteomic analysis

Proteomic analysis was performed at the Proteomics Core Facility at Sahlgrenska Academy, University of Gothenburg. The *in vitro* differentiated BFU-Es were collected, rinsed in PBS and pelleted prior to lysis in SDS Triethylammonium bicarbonate buffer. Sample preparation and nano liquid chromatography-mass spectrometry process were detailed in **Paper II**. Data analysis was performed using Proteome Discoverer version 1.4 against the Human Swissprot Database version March 2017. Mascot 2.5 was used as a search engine with precursor mass tolerance of 5 parts-per-million (ppm) and fragment mass tolerance of 200 millimass units (mmu). The detected peptide threshold in the software was set to false discovery rate (FDR) $\leq$ 1% by searching against a reversed database. Identified proteins were grouped by sharing the same sequences to minimize redundancy. The resulting ratios were normalized in the Proteome Discoverer 1.4 on the median protein value of 1.0 in each sample. Heat maps were generated using XLSTAT software.

RNA extraction

In **Paper II**, total RNA was extracted from sorted UCB HSPCs and *in vitro* differentiated BFU-Es. The cells were lysed in Trizol, and the aqueous phase was isolated after addition of chloroform. The aqueous phase was mixed with 70% ethanol and transferred to RNeasy Mini spin columns for total RNA extraction. Further Poly A^+ RNA purification from the HSPCs was performed using Oligotex Direct mRNA mini kit.

In **Paper III**, sorted erythroid precursors were lysed in RLT Buffer and RNA extraction was performed using AllPrep DNA/RNA/Protein Mini Kit. RNA integrity was analyzed using Agilent RNA 600 Nano Kit on a Bioanalyzer 2100.

Performing cDNA hybridization: library preparation and sequencing

Subtractive hybridization was carried out using the PCR-Select kit. The poly A^+ RNA from PE and normotensive UCB HSPCs were respectively used as tester and driver for cDNA synthesis. Enriched tester-specific amplicons from the second round of subtraction were ligated into pGEM-T Easy Vector System I for insert sequencing (Beckman Coulter Genomics, United Kingdom). All the retrieved sequences were blasted using BLAST analysis on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Performing quantitative gene expression analysis

cDNA library was created from BFU-E RNA using High-Capacity RNA-to-cDNA™ Kit. Quantitative PCR was undertaken using TaqMan® Fast Advanced Master Mix on a QuantStudio 3 Real Time PCR system. The following TaqMan® probes were used for the analysis: Hs04420632-g1 (GAPDH), Hs00218414_m1 (ITFG2), Hs00226863_m1 (KLHL36) and Hs00177504_m1 (ULK1). The threshold cycles (C_T) were obtained by QuantStudio Design & Analysis Software and fold differences were calculated using the $\Delta\Delta C_T$ method with GAPDH as the housekeeping gene.

Performing RNA-sequencing: library preparation and sequencing

The RNA sequencing was performed in collaboration with Center for Human Genetics, KU Leuven. Library preparation and amplification from erythroid precursor RNA samples was performed using the QuantSeq 3' mRNA kit. The quality and sequence length as well as the concentration of the libraries were determined preceding the sequencing in SE50bp mode on an Illumina HiSeq 2500. The reads were adjusted and were mapped to the Human genome (hg38) by STAR 2.5 using Ensembl 87

gene definitions. Reads were only considered in the counting process if the mapping quality was equal to or higher than 10, the strand of the read was the same strand as the gene and the read was not mapped in overlapping gene definitions (the union option). Differential expression analysis was performed using DESeq2 (232) and EdgeR (233, 234). Only genes that were significant with FDR < 0.1 in both tools were considered for further pathway analysis.

Pathway analysis

The significantly differentially expressed (DE) genes were split into up- or down-regulated datasets and each group was compared separately. In **Paper II**, protein-protein interaction prediction in UCB HSPCs and *in vitro* differentiated BFU-Es was carried out by String (V.10.0) (235). In addition, the link between the genes, human phenotype and diseases were evaluated by performing functional gene list enrichment by ToppFun analysis from ToppGene Suite (236). Gene set enrichment analysis (GSEA) in **Paper II and III** was executed using ConsensusPathDB (237).

Placenta explant culture

In **Paper IV**, placenta explants from normotensive pregnancies were cut, rinsed in PBS and cultured in 3 mL of DMEM/Nutrient Mixture F-12 containing FBS, penicillin, streptomycin and Amphotericin-B. The explants were cultured at 37 °C in presence of 21% or 1% oxygen for 24, 48 or 72 hrs.

Placenta tissue fixation and sectioning

The placenta tissue biopsies and explants in **Papers II and IV** were rinsed in PBS and fixed. Fixation was performed on dry ice followed by storage at -80 °C and embedding in OCT or in 4% buffered formaldehyde solution for 24 h before paraffin embedding. The paraffin- and OCT-embedded sections were prepared at 4- and 10-µm thickness, respectively.

Immunohistochemistry and histological staining

Immunohistochemistry analysis of the fixed placental explants and biopsies was performed in **Papers II and IV**. All the procedures were performed at RT unless mentioned otherwise. The OCT-embedded sections were fixed in 4% buffered formaldehyde. The paraffin-embedded sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval for the latter sections was performed in slow boiling 10 mM citrate buffer (pH = 6.0). All the sections were rinsed in PBS, treated with 1% H_2O_2 prior to blocking non-specific protein binding sites. The sections were then incubated with the primary and

secondary antibodies. Signal detection and nuclear counterstaining were performed using DAB and Hematoxylin solutions, respectively.

In addition, **Paper II** contains May-Grünwald-Giemsa (MGG) and Hematoxylin and Eosin (H&E) stainings that were performed on paraffin-embedded sections to detect erythroblastic islands and analyze tissue morphology, respectively.

In situ hybridization analysis

In **Paper IV**, paraffin-embedded sections were used for *in situ* hybridization analysis of HBA1 and HBG1 mRNA by RNAScope® technology. Final signal detection was followed by counterstaining with hematoxylin and a quick rinse in ammonium hydroxide solution.

Statistical analysis

The Mann-Whitney U test and the Wilcoxon signed-rank tests were used to compare various variables among the samples in **Papers I, II and III**. The correlation analysis and comparisons of categorical variables in **Paper I** were performed using Spearman's rank correlation (rho) and the Chi-Square test, respectively. P values < 0.05 were considered statistically significant.

Methodological considerations

Specific considerations should be taken into account prior to studying hematopoiesis and Hb production in a highly vascularized tissue like the placenta. Excessive PBS rinse of the tissue allows removal of most of the blood from the placental vasculature. However, the arduous time-consuming rinsing process may not result in complete and utter removal of all blood cells, particularly in PE placentas where congestion of vasculature by blood cells has been previously documented. Thus, to study placental Hb production in PE and hypoxic conditions, it is necessary to use methods such as immunostaining and *in situ* hybridization. Although these methods did not allow us to quantify the amount of Hb, they facilitated visualization and distinction of Hb mRNA and proteins in non-erythroid placental cells from any Hb contamination originating from erythroid cells trapped within the tissue.

Summary of Results

Paper I

Fetal hemoglobin in umbilical cord blood in preeclamptic and normotensive pregnancies: a cross-sectional comparative study

***Rationale*:**

Preeclampsia has been associated with elevated HbF in term placenta and maternal circulation. Free HbF is cytotoxic, can cause oxidative stress and damage various organs. One of the factors affecting maternal adaptation to pregnancy and neonatal Hb levels is fetal gender. The source of the enhanced free HbF in PE was studied with regards to fetal gender.

***Aims*:**

- To evaluate the placental contribution to the increased synthesis and accumulation of free HbF previously observed in PE
- To explore the possible role of fetal gender on HbF levels in PE

***Results*:**

The 16,269 UCB samples with *c*HbF data included 444 (2.72%) patients diagnosed with PE; 393 (2.41%) with mild-moderate and 51 (0.31%) with severe PE. All the PE patients were merged and compared as one group against the normotensive pregnancies. Gestational age differences were not statistically significant between the two groups (Median: 39; interquartile range: 38 – 40 weeks, in both groups).

The changes in the arterial UCB was considered to have fetal origin while the venous UCB reflected placental alterations. Compared to normotensive pregnancies, the PE UCB samples demonstrated statistically significant higher arterial and venous *c*tHb and venous *c*HbF. The differences in arterial *c*HbF or the veno-arterial differences (VA) of *c*tHb or *c*HbF were not statistically significant between PE and normotensive pregnancies.

The higher arterial and venous UCB *c*tHb and *c*HbF in male versus female neonates were statistically significant ($P < 0.001$) in normotensive pregnancies

and a similar pattern was observed among PE pregnancies. Intra-gender analysis indicated statistically significant ($P < 0.02$) higher arterial and venous *c*tHb and venous *c*HbF in both male and female neonates affected by PE. However, the differences between normotensive vs PE arterial *c*HbF, VA*c*tHb and VA*c*HbF were not statistically significant among male or female newborns.

Paper II

Disrupted fetal erythroid maturation in preeclampsia: beyond fetal hypoxia-a new etiological perspective

***Rationale*:**

Preeclampsia is associated with placental dysfunction, resulting in hypoxia that accelerates erythropoiesis. Elevated HbF levels in term placentas and maternal blood, increased frequency of the HSPCs in the placenta, and a higher erythroblast count in the UCB has been reported in PE.

***Aims*:**

- To investigate the changes in migration/homing of placental and UCB HSPCs in PE
- To explore extended erythropoiesis in term PE placentas as a source of elevated HbF
- To examine the changes in erythroid differentiation capacity of UCB HSPCs as a source of fetal HSPCs
- To investigate the effect of PE on intracellular pathways in fetal HSPCs and during erythroid maturation
- To study the possible changes caused by EPO-induced erythropoiesis in the UCB erythroid precursor profile

***Results*:**

The expression of SAMs was not significantly different between PE and normotensive placental or UCB HSPCs. No erythropoiesis was detected in term placentas. No significant difference was observed in the *in vitro* erythroid colony differentiation capacity of PE or normotensive UCB HSPCs. However, the UCB HSPC gene expression and the proteomics profile of the *in vitro* differentiated erythroid cells differed between PE and normotensive samples. The UCB erythroid precursor profile does not differ between PE and normotensive groups.

Paper III

Preeclampsia causes transcriptional changes in fetal erythroid precursors in a gender-dependent manner

***Rationale*:**

Preeclampsia has been associated with placental dysfunction, fetal hypoxia and increased EPO-induced erythropoiesis leading to higher erythroblast count in the UCB. In addition to hypoxia, placental dysfunction can also cause nutritional and oxidative stress as well as inflammation. However, the effect of PE on fetal erythropoiesis is not well known.

***Aims*:**

- To examine gender-specific changes of arterial and venous UCB erythroid precursor profile in PE and normotensive pregnancies
- To investigate gender-specific alterations in the transcriptome of the UCB fetal erythroid precursor profile in PE vs normotensive pregnancies

***Results*:**

The analysis of the erythroid populations in PE vs normotensive male and female fetuses was not significantly different between the groups. Transcriptome analysis indicated that PE affects erythropoiesis in male fetuses more severely than female fetuses. Pathway analysis suggested a deregulation in mTORC1/AMPK signaling pathways controlling cell cycle, differentiation and protein synthesis that may affect erythroid maturation.

Paper IV

Non-erythroid globin expression due to hypoxia-induced oxidative stress response mimics the pattern observed in preeclamptic placentas

***Rationale*:**

Preeclampsia has been associated with enhanced levels of HbF early in pregnancy in the maternal circulation as well as in term placentas. This increase is not associated with erythropoiesis in term placentas. Activation of transcription factors like HIF1A and NRF2 have been suggested to trigger non-erythroid production of hemoglobin in various cell types.

***Aim*:**

- Investigating the role of hypoxia-induced oxidative stress in altering globin loci transcription in non-erythroid placental cells as a source of elevated HbF in PE

***Results*:**

Our results indicated increased HBA, but not HBG, protein levels and NRF2 nuclear translocation in non-erythroid stromal cells in PE placentas and hypoxia-induced *in vitro* cultured placenta explants from normotensive pregnancies. Detection of nuclear HIF1A was also higher in hypoxia-induced *in vitro* cultured placenta explants from normotensive pregnancies. *In situ* hybridization analysis showed that HBA and HBG mRNA were only present in macrophage-like cells.

General Discussion

Placental contribution to increased HbF in PE (Paper I, II, IV)

The placental contribution to elevated HbF levels, documented in the maternal circulation and in term placentas in PE, was investigated by analyzing the venous and arterial UCB *c*tHb and *c*HbF in PE and normotensive pregnancies, as presented in **Paper I**. The *c*tHb was higher in the fetuses from PE pregnancies but, the *c*HbF was only significantly higher in the venous UCB. This suggested a possible role for the placenta in regulating the HbF levels in PE. Considering that the placental niche is altered in PE (166), these results emphasized the importance of elucidating whether the elevated venous *c*HbF was associated with extension of placental erythropoiesis into the third trimester in PE pregnancies. To achieve this goal, appropriate methods were used to compare HSPC migration/homing and erythropoiesis within term placentas from normotensive and PE pregnancies in **Paper II**. No difference was observed in the migration/homing of the placental HSPCs and no erythropoiesis was detected in term placentas. Consequently, hypoxia-induced oxidative stress modulation of Hb production in non-erythroid placental cells was evaluated in **Paper IV**. Hypoxia-induced metabolic changes can trigger oxidative stress and affect cellular heme levels and iron homeostasis (174, 238). *In vitro* hypoxic conditions have been shown to lead to higher cellular heme deposit (175) that may be due to decreased oxygen-dependent enzymatic oxidation and degradation of heme precursors (176). Despite its cytotoxic and pro-oxidative effects, heme can also have regulatory effects on the expression of ARE-controlled genes such as HO-1 and globin (124, 176). Accordingly, induction of hypoxia (1% oxygen) in normotensive placenta explants led to increased nuclear translocation of HIF1 and NRF2 as demonstrated in **Paper IV**. Higher nuclear translocation of NRF2 was also observed in PE vs normotensive placenta samples. The HBA and HBG mRNAs were mainly expressed in macrophage-like cells with long extensions, but the protein expression was observed in many other non-erythroid stromal cells within the placenta. This implied possible transfer of mRNA from the macrophage-like cells to other cells. Microvesicle-associated transfer of (micro)RNAs is a well-known process that has been studied extensively (239, 240), but a recent study reported that intercellular transfer of full-length

mRNA between mammalian cells requires nanotube-like structures similar to those observed in our analysis (241). The analyses used in our study allowed specific localization of globin mRNAs and proteins but did not provide us with quantitative values. Thus, it remains unclear whether activation of the HIF1-NRF2 axis was associated with increased HBA and HBG mRNA expression. Moreover, HBA protein expression was higher in PE vs normotensive samples and also in placenta explants cultured in hypoxic vs normoxic conditions. However, HBG protein was not detected in either PE vs normotensive placentas or explants cultured in hypoxic vs normoxic conditions. Differential expression and detection of HBA and HBG mRNA and proteins have been previously reported (140-142) and can be due to the distinct transcriptional regulation at alpha and beta globin loci. Considering that it was not possible to confirm presence of full-length functional mRNA in our analysis, it would be interesting to use an *in vitro* translation system to investigate the functionality of the HBA and HBG mRNA transcripts. In addition, the process of translation is regulated at various levels by recognition of the mRNA by ribosome and also other non-coding RNAs. For instance, microRNA-96 has been shown to inhibit HBG mRNA translation, leading to lower HBG protein expression in erythroid cells (242). Further analyses are required to determine the effect of PE and hypoxia-induced oxidative stress on transcription and translation of HBA and HBG within the placental stromal cells. Non-erythroid Hb has been previously suggested to decrease cellular oxidative stress in macrophages (243) and regulate nitric oxide signaling in vascular endothelial cells (141). It would be important to indicate whether globin translation and Hb production in non-erythroid placental cells has a protective or destructive role in PE.

Fetal erythropoiesis in PE and its possible contribution to free HbF (Paper I, II, III)

The higher venous and arterial UCB *c*tHb in the PE vs normotensive pregnancies observed in **Paper I** implied enhanced fetal erythropoiesis. The concept of hypoxia-induced EPO-dependent increase in erythropoiesis in the fetuses from PE pregnancies is widely accepted and is considered to underlie the higher UCB erythroblast count (205-208). However, some studies suggest a different image of fetal hematopoiesis in PE (201, 202) with many aspects of the changes in molecular pathways remaining unknown. Thus, to investigate the changes in fetal erythropoiesis in PE and to understand whether these changes contribute to elevated free HbF, the migration/homing of fetal (UCB) HSPCs, their transcriptome and their erythroid differentiation capacity was analyzed in **Paper**

II. In addition, venous and arterial UCB erythroid precursor profile was compared between PE and normotensive pregnancies, demonstrated in **Papers II and III**. The results indicated no significant difference in the migration/homing or erythroid differentiation capacity of fetal HSPCs in PE. Moreover, the erythroid precursor profile was not significantly different between the venous and arterial UCB or PE and normotensive groups. Absence of significant differences in venous and arterial erythroid precursor profiles confirmed our conclusion in **Paper II**; that the excess venous HbF was not due to placental erythropoiesis. But changes in the transcriptome of the HSPCs signified alterations in cellular and molecular pathways that could affect erythropoiesis. Consequently, in **Papers II and III**, proteomics and transcriptome analyses were performed on *in vitro* differentiated erythroid cells and UCB-isolated erythroid precursors, respectively. These analyses suggested an imbalance in the mTOR pathway, metabolism and cellular response to stress, with greater impact on the male fetuses.

Over-activation of mTOR can lead to ER stress and UPR activation (244, 245) as well as hyperproliferation and disruption of erythroid maturation (219, 220, 225). As mentioned earlier, various factors including nutrients, growth factors, signaling proteins and inflammatory cytokines could regulate mTOR activation in different ways. Higher glucose, glucose/insulin ratio (246, 247), lactic acid (248) and inflammatory cytokines, such as tumor necrosis factor α (TNFα) (194), have been previously reported in fetuses from PE pregnancies. All these factors could promote mTORC1 signaling (249-252) which is significant in regulating the cellular mechanisms controlling erythropoiesis (221, 222, 225). These changes may underlie the mTOR over-activation, affecting erythroid maturation resulting in higher number of UCB erythroblast count observed in the fetuses from PE pregnancies. Although our study is the first to suggest differential mTOR/AMPK activation in erythropoiesis, increased mTOR signaling (253), impaired autophagy (254, 255) and elevated ER stress (54, 55) has been previously reported in other fetoplacental cells in PE pregnancies. Also, the gender specificity of the effect on erythropoiesis is in line with the more severe clinical outcome amongst male neonates born to PE pregnancies (256, 257). Moreover, mRNA expression of CD99 and ERMAP antisense were significantly different in PE vs normotensive groups in a gender-specific manner. Both CD99 and ERMAP are glycoproteins expressed on the surface of erythroid cells that can act as adhesion molecules. Additional analysis is required to indicate whether the changes observed in our analyses contribute to elevated free HbF by promoting congestion of placental vasculature and erythroid cell rupture (hemolysis).

Concluding Remarks

Our results suggest that the effect of hypoxia and oxidative stress in PE pregnancies on the fetoplacental cellular processes may be more profound than previously proposed. We reported a hypoxia-triggered oxidative stress-induced globin expression in non-erythroid placental cells that may contribute to the documented higher placental Hb production in PE. Also, exploring the effect of PE on fetal erythropoiesis implied a deregulation in mTOR/AMPK balance that could explain the higher UCB erythroblast count in these pregnancies. In addition, confirming gender-specific differential expression of CD99 and ERMAP proteins on the surface of UCB erythroid cells is essential and may provide some insight into fetal erythrocyte integrity and viability in PE.

Future Perspectives

Accumulating evidence has demonstrated metabolic and proinflammatory changes in the fetal and placental cells in PE pregnancies. These changes could affect cellular signaling pathways and *in utero* fetal development. To elucidate the full extent of the effect of PE on fetal erythropoiesis and erythroid maturation, further examination of mTOR and AMPK protein phosphorylation is required. Considering that mTOR inhibition can lead to IUGR, it would be important to study how mTOR and AMPK signaling might differ in fetuses born to early- vs late-onset PE.

Furthermore, it is essential to verify the identity of placental macrophage-like cells harboring HBA and HBG mRNAs. Additional *in vitro* analyses could allow verification of full length-mRNA transfer from these to other placental cells. Considering that the HBA and HBG protein expression was only observed in hypoxia-induced and PE placentas, it would be important to study the changes in hypoxia-triggered oxidative stress-induced protein translation.

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"Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great."

-Mark Twain

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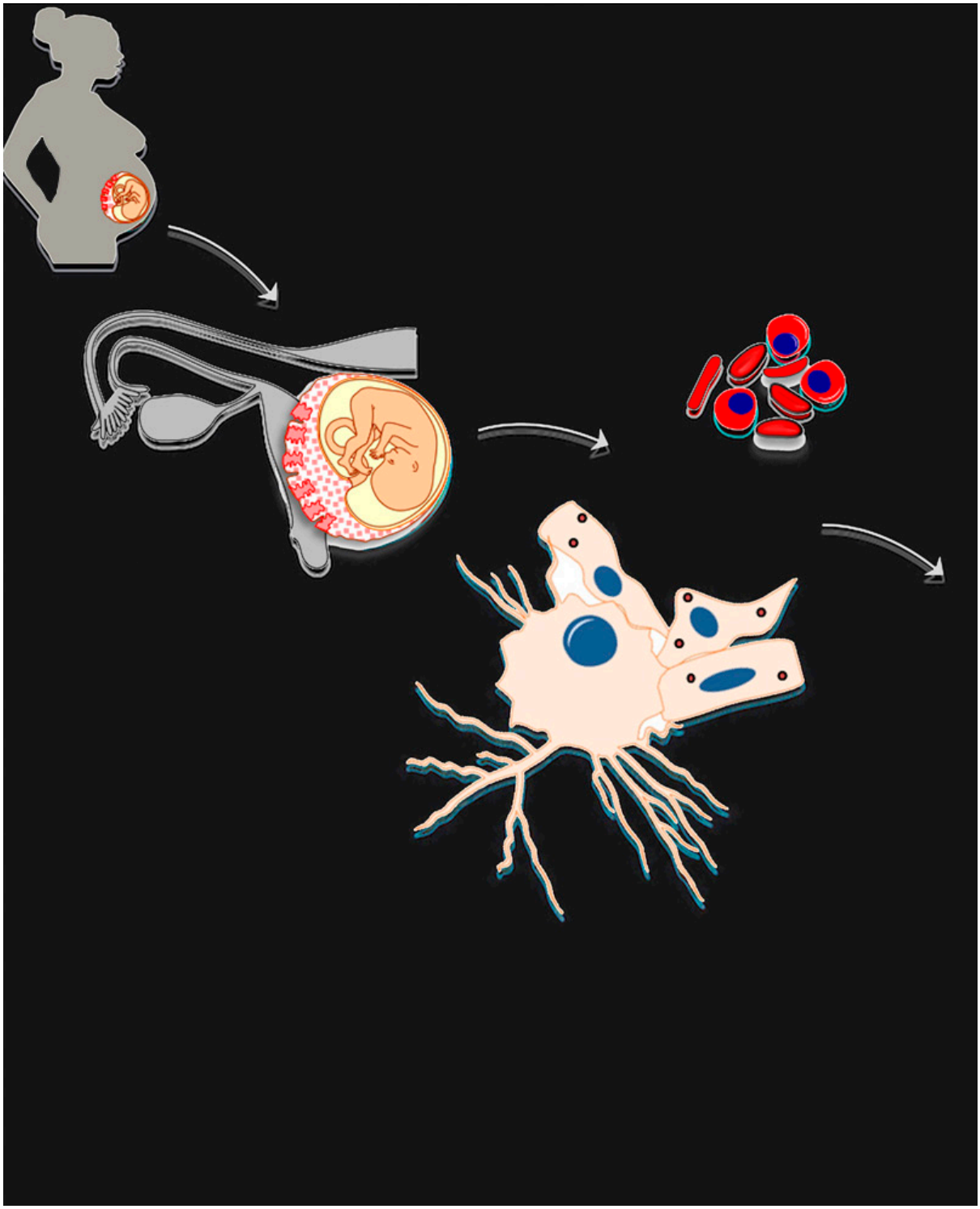

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