MONASH University

HtrA4-induced endothelial dysfunction in early-onset preeclampsia

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Doctor of Philosophy

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Implantation and Placental Development Laboratory

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&

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Copyright notice

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Summary

Preeclampsia (PE) is a life-threatening complication of human pregnancy that is characterized by wide-spread endothelial dysfunction. Excessive placental factors released into the maternal circulation as a response to placental stress are believed to contribute to endothelial dysfunction. Our laboratory has previously reported that high temperature requirement factor A4 (HtrA4) is a placenta-specific serine protease that is secreted into the maternal circulation and significantly up-regulated in PE, especially in early-onset PE. We have also demonstrated that HtrA4 can disrupt the normal cellular function of human umbilical vein endothelial cells (HUVECs). Therefore, it was hypothesized that excessive circulating HtrA4 that is released by the placenta can adversely affect the maternal endothelium and contribute to the development of early-onset PE.

The first aim of this thesis, Chapter 2, was to investigate the impact of HtrA4 on HUVECs as an endothelial cell model. It was demonstrated that HtrA4 significantly increased inflammatory responses of HUVECs and altered the expression of a range of genes related to endothelial cell biology. In particular, HtrA4 significantly increased the mRNA expression of anticoagulant factor thrombomodulin (THBD), and severely down-regulated the mRNA expression of thrombospondin (THBS)*1*, which is involved in many regulatory processes of endothelial cells. These data provided experimental evidence that HtrA4 could alter endothelial cell biology.

The second aim of this thesis, Chapter 3, was to investigate the impact of HtrA4 on endothelial cell proliferation and repair. It was found that high levels of HtrA4 inhibited HUVEC cell proliferation and significantly down-regulated a number of genes that are critical for cell cycle progression. Furthermore, because circulating endothelial progenitor cells (EPCs) are critical for endothelial repair and regeneration, yet in PE, these cells are reduced in number and functionality, Chapter 3 also investigated the impact of HtrA4 on primary

human EPCs isolated from term umbilical cord blood. HtrA4 significantly inhibited the proliferation of EPCs and impeded their differentiation into mature endothelial cells. These data suggest that circulating HtrA4 may prevent endothelial repair, not only by halting endothelial cell proliferation, but also by inhibiting the proliferation and differentiation of circulating EPCs.

The third aim of this thesis, Chapter 4, investigated the molecular mechanisms by which HtrA4 affects endothelial cells. It was found that HtrA4 could cleave the main receptor for vascular endothelial growth factor A (VEGF-A), the kinase insert domain receptor (KDR), thereby inhibiting VEGF-A action. HtrA4 cleaved the recombinant KDR *in vitro*, significantly reduced the amount of KDR in HUVECs, and inhibited the VEGF-A-induced phosphorylation of Akt kinase, which is essential for downstream signalling. HtrA4 also prevented the VEGF-A-induced tube formation by HUVECs, and dose-dependently inhibited the VEGF-A-induced angiogenesis in explant culture of mouse aortic rings. The data strongly suggests that high levels of HtrA4 in the maternal circulation could impair the VEGF-A action to inhibit angiogenesis in endothelial cells.

In summary, this thesis provided strong compelling *in vitro* evidence that excessive HtrA4 is a possible causal factor of endothelial dysfunction associated with early-onset PE, and that HtrA4 is a potential therapeutic target for treatment.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

Print Name: Yao Wang

Date: 26/02/2019

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals. The core theme of the thesis is the involvement of HtrA4 in endothelial dysfunction associated with early-onset preeclampsia. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Hudson Institute of Medical Research and Department of Molecular and Translational Science, Monash University under the supervision of Professor Guiying Nie and Associate Professor Craig Harrison.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, 3 and 4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N
2	High levels of HtrA4 observed in preeclamptic circulation drastically alter endothelial gene expression and induce inflammation in human umbilical vein endothelial cells	Published ***Placenta*** **(2016)**	80%. Experimental work, contribution to project design, data analysis and preparation of manuscript	1) Guiying Nie, input into project design and manuscript 20%	No

3	HtrA4 may play a major role in inhibiting endothelial repair in pregnancy complication preeclampsia	Published ***Scientific Reports*** **(Feb 2019)**	70%. Experimental work, contribution to project design, data analysis and preparation of manuscript	1) Rebecca Lim, provided cord blood sample 5% 2) Guiying Nie, input into project design and manuscript 25%	No
4	High levels of HtrA4 detected in preeclamptic circulation may disrupt endothelial cell function by cleaving the main VEGFA receptor KDR	Published ***Federation of American Societies for Experimental Biology*** **(Jan 2019)**	70%. Experimental work, contribution to project design, data analysis and preparation of manuscript	1) Mylinh La, 2) Tam Pham and 3) George O Lovrecz, generated HtrA4 5% 4) Guiying Nie, input into project design and manuscript 25%	No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:** 26/02/2019

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:** 26/02/2019

Publications arising from this thesis

1. Yao Wang and Guiying Nie (2016). **High levels of HtrA4 observed in preeclamptic circulation drastically alter endothelial gene expression and induce inflammation in human umbilical vein endothelial cells.** Placenta, 47: 46-55

2. Yao Wang, Rebecca Lim and Guiying Nie (2019). **HtrA4 may play a major role in inhibiting endothelial repair in pregnancy complication preeclampsia.** Scientific Reports, published online on 25th February 2019

3. Yao Wang, Mylinh La, Tam Pham, George O Lovrecz and Guiying Nie (2019). **High levels of HtrA4 detected in preeclamptic circulation may disrupt endothelial cell function by cleaving the main VEGFA receptor KDR.** FASEB J [ePub ahead of print]

Other publications during enrolment

1. Sonia Soo Yee Teoh, Min Zhao, Yao Wang, Qi Chen, Guiying Nie (2015) **Serum HtrA1 is differentially regulated between early-onset and late-onset preeclampsia.** Placenta, 36: 990-995

2. Qi Chen, Yao Wang, Min Zhao, Jonathan Hyett, Fabricio da Silva Costa, Guiying Nie. (2016) **Serum levels of GDF15 are reduced in preeclampsia and the reduction is more profound in late-onset than early-onset cases.** Cytokine, 83: 226-230

3. Yao Wang, Ying Li, Jonathan Hyett, Fabricio da Silva Costa, Guiying Nie. (2016) **HtrA3 Isoform–Specific ELISAs for Early Detection of Preeclampsia.** SLAS Discovery, 1-8

4. Yao Wang, Maree Bilandzic, Guck T. Ooi, Jock K. Findlay, Kaye L. Stenvers (2016) **Endogenous inhibins regulate steroidogenesis in mouse TM3 Leydig cells by altering SMAD2 signalling.** Molecular and Cellular Endocrinology 436: 68-77

5. Qi Chen, Yao Wang, Ying Li, Min Zhao, Guiying Nie (2017) **Serum podocalyxin is significantly increased in early-onset preeclampsia and may represent a novel marker of maternal endothelial cell dysfunction.** Journal of Hypertension, 35(11):2287-2294

6. Yao Wang, Qi Chen, Min Zhao, Kelly Walton, Craig Harrison, Guiying Nie (2017) **Multiple Soluble TGF-β Receptors in Addition to Soluble Endoglin Are Elevated in Preeclamptic Serum and They Synergistically Inhibit TGF-β Signaling.** The Journal of Clinical Endocrinology and Metabolism 102(8):3065-3074

7. Mary Mansilla, Yao Wang, Jonathan Hyett, Fabricio da Silva Costa, Guiying Nie (2018) **Serum podocalyxin for early detection of preeclampsia at 11-13 weeks of gestation.** Placenta, 71:13-15

8. Esther Tseng, Sonia Soo Yee Teoh, Yao Wang, Guiying Nie (2019) **Elevated protease HtrA4 in the maternal circulation of preeclampsia may contribute to endothelial barrier disruption by cleaving key junctional protein VE-cadherin.** Placenta, [Epub ahead of print]

Conference proceedings and presentations

1. Yao Wang, Ying Li, Fabricio Costa, Jon A Hyett and Guiying Nie (2015). **Serum HtrA3 for early detection of preeclampsia and small for gestational age.** International Federation of Placenta Association Meeting, Brisbane, Australia. Poster presentation.

2. Yao Wang, Ying Li, Fabricio Costa, Jon A Hyett and Guiying Nie (2015). **The potential utility of serum HtrA3 for early detection of preeclampsia and small for gestational age (SGA).** Monash Health Research Week, Melbourne, Australia. Poster presentation.

3. Yao Wang and Guiying Nie (2016). **High levels of circulating HtrA4 seen in preeclampsia drastically alter expression of endothelial genes important for vessel biology and significantly induce inflammation.** Society for Reproductive Biology Annual Scientific Meeting, Gold Coast, Australia. Oral presentation.

4. Yao Wang and Guiying Nie (2017). **High levels of HtrA4 observed in preeclamptic serum induce endothelial cell cycle arrest and senescence and inhibit endothelial progenitor cell differentiation for repair.** Society for Reproductive Biology Annual Scientific Meeting, Perth, Australia. Oral presentation.

5. Yao Wang and Guiying Nie (2017). **High levels of HtrA4 observed in preeclamptic serum induce endothelial cell cycle arrest and senescence and inhibit endothelial progenitor cell differentiation for repair.** International Federation of Placenta Association Meeting, Manchester, United Kingdom. Poster presentation.

6. Yao Wang, Mylinh La, Tam Pham and Guiying Nie (2018). **High levels of circulating HtrA4 may contribute to the development of preeclampsia by cleaving the main receptor of VEGFA to inhibit its signalling.** Society for Reproductive Biology Annual Scientific Meeting, Adelaide, Australia. Oral presentation. (**ANZPRA/SRB New Investigator Award)**

Awards and Prizes

2015

YW Loke New Investigator Travel Award (US$500)

2016

Society for Reproductive Biology (SRB) Travel Grant ($200)

2017

Society for Reproductive Biology (SRB) Travel Grant ($250)

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YW Loke New Investigator Travel Award (US$500)

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Abbreviations

AcLDL	acetylated low density lipoprotein
ADMA	asymmetric dimethylarginine
Akt	protein kinase B
ALOX5	arachidonate 5-lipoxygenase
AMD	age-related macular degeneration
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BAD	Bcl-2 associated death promoter
BCL2	B-cell lymphoma 2
BIRC5	baculoviral IAP repeat containing 5
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CCL2	C-C motif chemokine ligand 2
CD45	protein tyrosine phosphatase, receptor type C
CDK1	cyclin dependent kinase 1
CDKN3	cyclin dependent kinase inhibitor 3
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DAPI	4’,6-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco’s Modified Eagle Medium

DNA	deoxyribonucleic acid
EBM-2	endothelial basal medium
ECFCs	endothelial colony-forming cells
ECM	extracellular matrix
EDN1	endothelin 1
EDTA	ethylenediaminetetraacetic acid
EGM-2	endothelial growth medium-2MV BulletKit
ELISA	enzyme-linked immunosorbent assay
EPCs	endothelial progenitor cells
Erk ½	extracellular regulated kinase 1 and 2
EVT	extravillous cytotrophoblast
FBS	fetal bovine serum
FGF2	basic fibroblast growth factor
HCl	hydrochloride
HELLP	hemolysis, elevated liver enzymes, low platelets
HRP	horseradish peroxidase
HtrA	high temperature requirement factor A
HUVECs	human umbilical vein endothelial cells
IAPs	inhibitor of apoptosis proteins
ICAM-1	intercellular adhesion molecule 1
IGF	insulin growth factor
IL1B	interleukin 1β

IL6	interleukin 6
IL11	interleukin 11
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intrauterine growth restriction
KDR	kinase insert domain receptor
LTBP-1	latent TGF-β binding protein 1
MCP1	monocyte chemoattractant protein 1
MEK	MAPK/Erk kinase
MKI67	marker of proliferation Ki-67
MMP1	matrix metalloproteinase 1
NaCl	sodium chloride
NaF	sodium fluoride
NP	neuropilin
OCLN	occludin
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate-buffered saline
PDZ	postsynaptic density protein 95-Discs large-Zona occludens
PE	preeclampsia
PECAM1	platelet and endothelial cell adhesion molecule 1
PI3K	phosphoinositide 3-kinase
PlGF	placental growth factor
PSC	protein stabilise cocktail

PTGIS	prostaglandin I2 synthase
PTGS2	prostaglandin-endoperoxide synthase 2
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERPINE1	serpin family member 1/endothelial plasminogen activator inhibitor
sFlt-1	soluble fms-like tyrosine kinase 1
TBS	tris-buffered saline
TGF-β	transforming growth factor β
THBD	thrombomodulin
THBS1	thrombospondin 1
TNF-α	tumor necrosis factor-α
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule 1
VE-Cadherin	vascular endothelial cadherin
VEGF-A	vascular endothelial growth factor A

Units of measurements

h	hour/s
g	gram
kDa	kilo Dalton
L	litre
µg	microgram
min	minute/s
mm	millimetre
mM	millimolar
ml	millilitre
ng	nanogram

Chapter 1

Introduction and literature review

1.1. Preeclampsia (PE)

1.1.1. Overview of the disease

PE is a serious pregnancy complication that affects 2-8% of all pregnancies, and it is one of the leading causes of maternal fatality worldwide (Duley, 2009, Steegers et al., 2010, Abalos et al., 2013). In recent years, timely diagnosis, proper management and availability of prenatal care in developed countries have seen a dramatic reduction of maternal mortality associated with PE (Ghulmiyyah and Sibai, 2012). However, the incidence of PE is reported to be seven times higher in developing countries where access to health care is limited (Osungbade and Ige, 2011). Potential acute maternal complications include stroke, placental abruption, disseminated intravascular coagulation, HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), pulmonary oedema, respiratory distress syndrome, acute renal failure, and death (Ghulmiyyah and Sibai, 2012). Perinatal complications include stillbirth, intrauterine growth restriction (IUGR), neonatal complications and death (Backes et al., 2011).

The aetiology of PE is not fully understood, and currently the only treatment available is delivery, often prematurely, which is associated with high incidence of neonatal death (Young et al., 2007). Therefore, identifying high-risk women is important for providing better management and possibly prevention of the disease (English et al., 2015). Some common risk factors of PE include primigravid women, low socioeconomic class and family history of the disease (Jido and Yakasai, 2013). PE is less frequent in multiparous women, but the risk of PE increases with changed paternity, suggesting that the maternal immune response to foreign fetal antigen may be an important factor (Trupin et al., 1996). This is further supported by evidence of increased incidence of PE in women who become pregnant with donor eggs,

compared to women pregnant with their own eggs (Salha et al., 1999). In addition, several medical conditions including pre-existing hypertension, renal disease, insulin-dependent diabetes and women with previous early-onset PE, are all considered to be risk factors for PE (English et al., 2015).

1.1.2. Definition of PE

Traditionally, PE is defined as *de novo* hypertension and proteinuria at or after 20 weeks of gestation (Steegers et al., 2010). However, in 2014, the International Society for the Study of Hypertension in Pregnancy (ISSHP) redefined PE as blood pressure of 140/90mm Hg or higher after 20 weeks of gestation with one or more of the following symptoms: proteinuria of >300mg/day, maternal organ dysfunction including renal insufficiency, liver involvement, neurological or haematological complications or uteroplacental dysfunction including fetal growth restriction (Tranquilli et al., 2014). Severity of the disease is associated with increased prevalence of adverse maternal/fetal consequences. In 2003, von Dadelszen *et al* proposed sub-classification of PE by gestational age, as it provides most predictability in maternal and perinatal outcomes (von Dadelszen et al., 2003). At present, PE is classified into the following subtypes: (i) early-onset when the disease presents before 34 weeks of gestation, and (ii) late-onset when PE occurs at or after 34 weeks of gestation (Tranquilli, 2014, Tranquilli et al., 2013). Late-onset PE is further classified into preterm PE when the disease occurs between 34-37 weeks of gestation, and term PE if the disease presents after 37 weeks (Tranquilli et al., 2013).

Early-onset and late-onset PE have different maternal and neonatal outcomes, as well as distinct biochemical markers, heritability and clinical features (Raymond and Peterson, 2011). Early-onset PE represents far more significant maternal risks, with a

significantly higher mortality rate and more severe perinatal outcomes compared to late-onset PE (MacKay et al., 2001, Bellamy et al., 2007, Ghulmiyyah and Sibai, 2012, Madazli et al., 2014). The two PE subtypes have different aetiologies: early-onset PE is generally associated with shallow trophoblast invasion and inadequate remodelling of the uterine spiral arteries during early placentation (Huppertz, 2008, Chambers et al., 2001), whereas late-onset PE is more likely associated with maternal factors or predisposition to cardiovascular or metabolic diseases (Steegers et al., 2010, Valensise et al., 2008).

1.1.3. Early-onset PE

In early-onset PE, the extravillous cytotrophoblast (EVT) invasion is restricted to the peripheral decidual segments of the spiral arteries, which retain their smooth muscle and elastic lamina; these less dilated vessels cause intermittent hypoperfusion of the placenta, leading to oxidative stress and the eventual maternal endothelial dysfunction (Pijnenborg et al., 1991, Burton et al., 2009, Chambers et al., 2001). Egbor *et al* (2006) demonstrated that placentas from early-onset PE had significant reduction of terminal villi volume and surface area compared to their gestational matched control placentas (Egbor et al., 2006).

It is well known that the placenta plays a key role in the development of early-onset PE. Proteinuria disappears and blood pressure returns to normal in the majority of preeclamptic women within one week postpartum, even in severe cases (Makkonen et al., 1996). An intact placenta, not the fetus, is required for PE development. Piering *et al* (1993) reported a rare case of a preeclamptic patient with extra-uterine, intra-abdominal pregnancy, where PE persisted even after the delivery of the fetus, whilst the placenta is retained in the abdomen (Piering et al., 1993). The symptoms of PE

are abolished once the placenta is removed, and the patient's kidney function and blood pressure returned to normal, with no further sign of clinical symptoms (Piering et al., 1993). Another example of placenta as the predominant factor in PE pathogenesis is molar pregnancy where no fetal tissue is present, yet the majority of the reported cases are also accompanied by symptoms of early-onset PE (Kanter et al., 2010).

1.1.4. Late-onset PE

Late-onset PE is less likely linked to abnormal trophoblast invasion, suggesting other factors may be involved in the disease development (Myatt and Roberts, 2015). Vatten & Skjaerven (2004) showed that term PE is associated with both small and large birth weight babies, and often represents mixture of maternal syndromes, ranging from mild PE with moderate involvement of the placenta, to hypertension and proteinuria without any sign of placental dysfunction (Vatten and Skjaerven, 2004). Although in some cases, late-onset PE can still be associated with some abnormal spiral artery remodelling compared to normal pregnancy (Soto et al., 2012), the placenta plays a much lesser role in the development of the disease, suggesting that late-onset PE is more likely to be a maternal disorder where metabolic demands of the growing fetus close to term are not met by maternal supplies (Valensise et al., 2008, Erez et al., 2017). Pathological comparison of the placentas revealed that late-onset PE placentas have less lesions associated with under-perfused vasculature (Ogge et al., 2011), significantly less occurrence of abnormal uterine artery Doppler measurement, and are more likely to resemble placentas of normotensive pregnancies (Crispi et al., 2008, Madazli et al., 2014). Nevertheless, both PE subtypes eventually lead to wide-spread endothelial dysfunction as the end-point of the disease (Poston, 2006).

1.2. Pathogenesis of PE

1.2.1. The placenta

The placenta is a critical organ required during pregnancy. It connects the developing fetus to the mother to allow nutrient and oxygen exchange via maternal blood supply and to remove waste products from the fetus (Enders and Blankenship, 1999). Following implantation, cytotrophoblast cells derived from the trophectoderm of the blastocyst undergo extensive proliferation and differentiation into two distinct pathways (Figure 1) (Gude et al., 2004, Staud and Karahoda, 2018). The non-migratory villous pathway involves cell fusion to form the terminally differentiated syncytiotrophoblast that becomes the outer layer of chorionic villi (Gude et al., 2004). The highly invasive EVT cells differentiate into either endovascular trophoblasts or interstitial trophoblasts (Figure 1). Endovascular trophoblasts invade the decidua and as far as the inner third of the myometrium to extensively remodel the uterine spiral arteries, thereby generating a low resistant and high capacity environment to accommodate the increasing maternal blood flow to the placenta necessary for the developing fetus (Ji et al., 2013, Burton and Jauniaux, 2015). Interstitial trophoblasts invade deep into the decidua and differentiate into multinucleated placental bed giant cells, which are important for communication with maternal uterine cells, fetus attachment and immunological responses (Figure 1) (Ji et al., 2013).

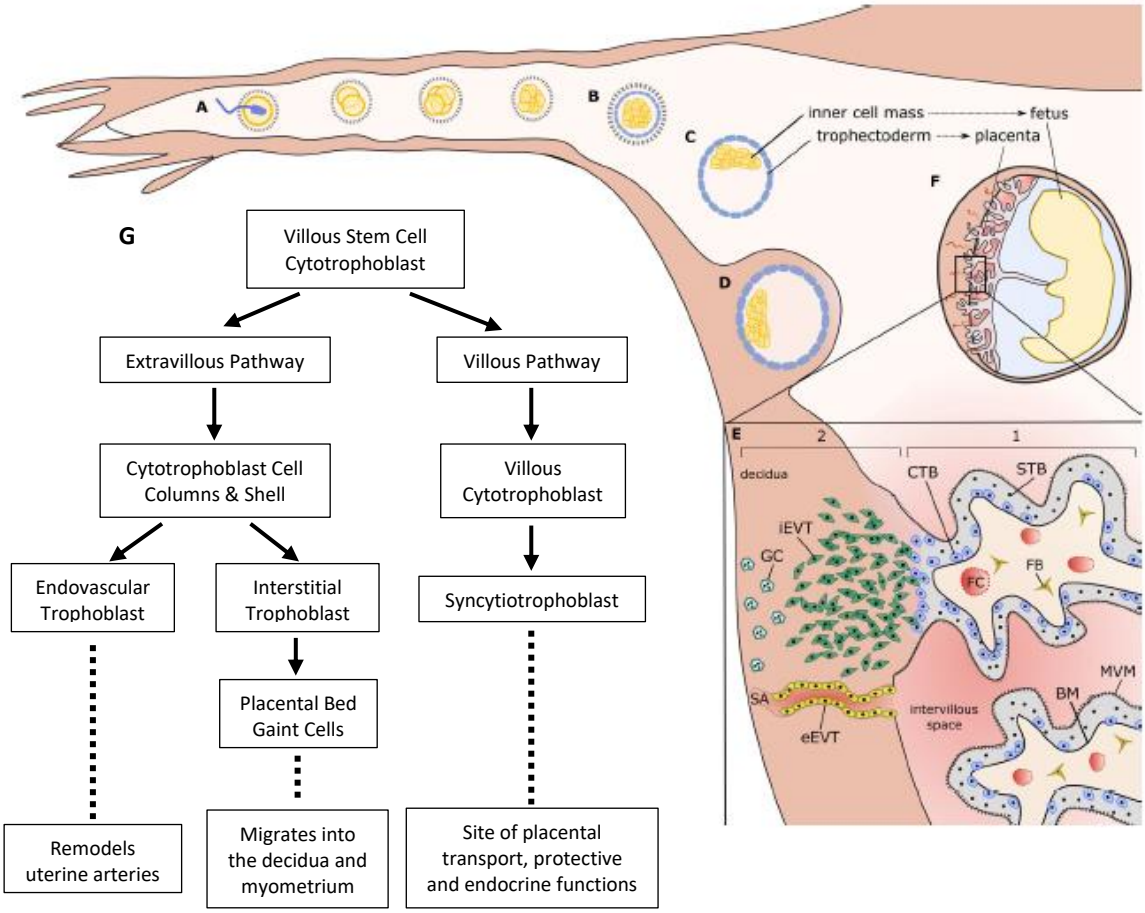

Figure 1. Schematic presentation of human embryogenesis, placenta formation and differentiation pathways of cytotrophoblast cells. A) Following fertilization by a sperm, the egg becomes a zygote and begins dividing. B) The cells differentiate into trophectoderm (blue) and inner cell mass (yellow). C) Formation of a blastocyst, where the inner cell mass clusters at one end, leaving a cavity at the other end. D) The proliferating endometrial lining makes contact with the blastocyst. E1) Proliferation and differentiation of CTB (cytotrophoblast) into multinucleated STB (syncytiotrophoblast) layer, bordered by maternal-facing MVM (microvillous membrane) and fetal-facing BM (basal membrane). E2) Proliferation and migration of CTB into the decidua give rise to two types of extravillous trophoblast cells. The iEVTs (interstitial extravillous trophoblasts) invade the decidua and differentiate into GC (giant cells) as the terminal step of the invasive pathway. The eEVTs (endovascular extravillous trophoblasts) invade the decidua and the myometrium to replace the endothelial cells in the spiral arteries, which gradually become low-resistance and high-capacity arteries to ensure proper oxygen and nutrient delivery to the fetus. F) Following successful placentation, the blastocyst eventually develops into the placenta and fetus. G) The pathways of trophoblast differentiation and function. (Adapted from Staud and Karahoda, 2018 & Gude et al, 2004).

During early pregnancy, cytotrophoblast cells as well as normal embryogenesis require a relative hypoxic condition (Genbacev et al., 1997, Burton et al., 2003), and the placenta is developed in a low oxygen tension environment as the uterine arteries are plugged by EVT cells, restricting the maternal blood flow (Schoots et al., 2018, Chang et al., 2018). The hypoxic environment is essential for the rapid proliferation and differentiation of EVT (Chang et al., 2018). As the pregnancy progresses, the lumens of the spiral arteries gradually lose their endothelium, smooth muscles and elastic lamina to become more dilated vessels, resulting in reduction of the velocity, pressure and pulsatility of the uteroplacental flow (Burton et al., 2009). By the start of second trimester, embryogenesis is completed, and EVT plugs begin to dissolve and the intervillous space gradually changes from a low oxygen tension to a higher oxygen tension environment to support the growing fetus.

In PE, especially early-onset PE, failure of EVT invasion and insufficient remodelling of the maternal spiral artery result in reduction of blood flow to the placenta and increase the formation of reactive oxygen species (ROS), which lead to oxidative stress and release of pro-inflammatory factors from the placenta to induce PE (Ji et al., 2013, Schoots et al., 2018, Redman and Sargent, 2009, Sánchez-Aranguren et al., 2014). However, abnormal placentation and failed vascular remodelling alone are not sufficient to induce the maternal syndrome of PE. This is because reports from other pregnancy complications such as IUGR and preterm birth without any sign of PE have also shown pathological evidence of failed placental vessel remodelling, suggesting that other factors are involved in the manifestation of PE (Roberts and Cooper, 2001).

1.2.2. Maternal predisposing factors

A two-stage model has been proposed to link the dysfunctional placenta to the maternal syndrome of PE (Figure 2) (Roberts and Hubel, 2009). Stage one of PE occurs in the first half of the pregnancy where either poor placentation or maternal predisposing factors including genetic, behavioural and environmental factors may set off a cascade of placental oxidative stress and inflammation that become stage two of PE (Figure 2) (Redman and Sargent, 2009, Steegers et al., 2010). Consequently, an abnormal amount of factors are released from the placenta in response to placental/fetal stress, inducing endothelial dysfunction and manifesting the clinical symptoms of PE (Figure 2) (Roberts and Hubel, 2009, Roberts and Cooper, 2001, Staff et al., 2013).

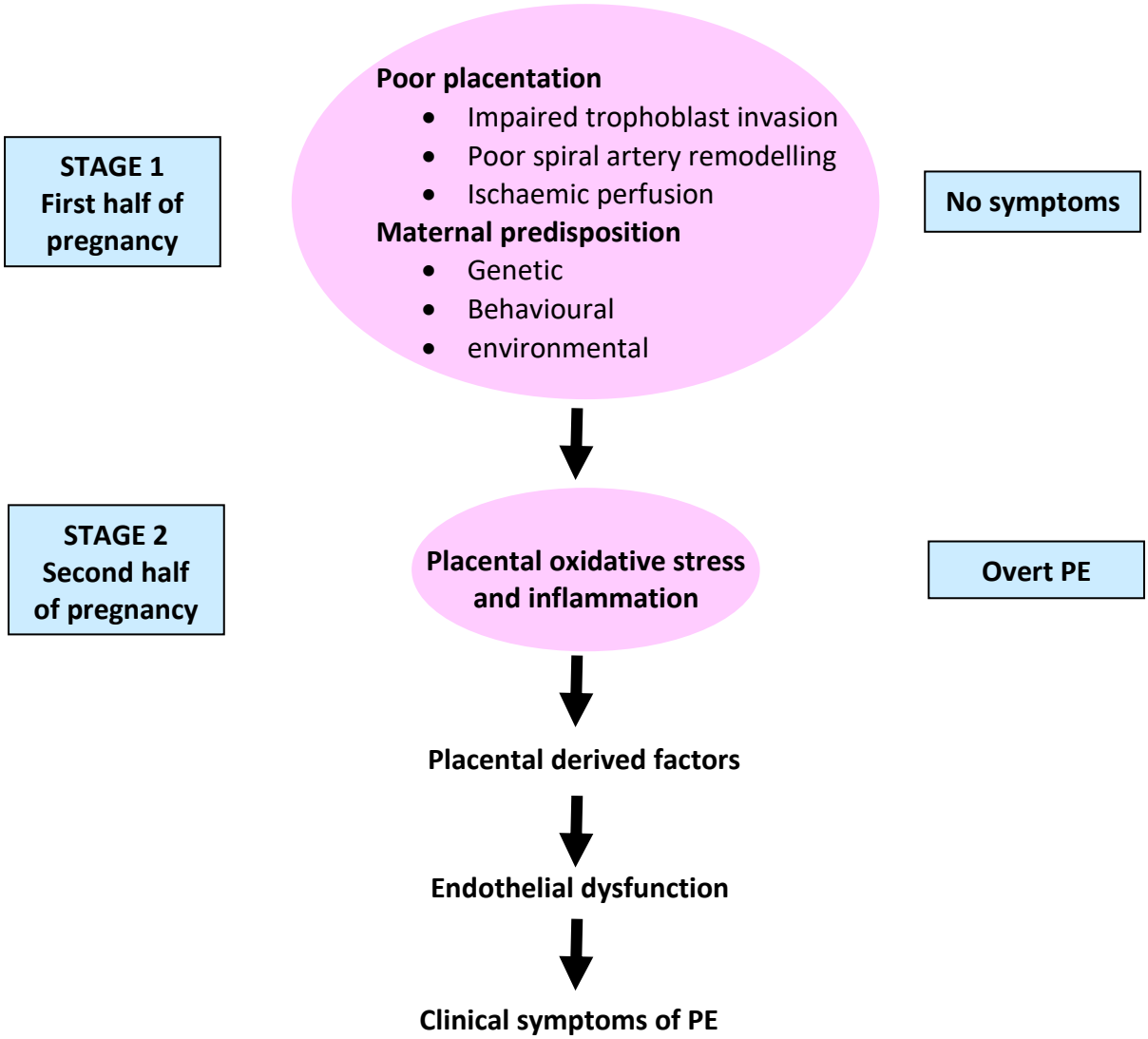

Figure 2. Two-stage model of PE (Adapted from Redman and Sargent, 2009 & Steegers et al, 2010).

Maternal risk factors for PE development include hypertension, diabetes, increased insulin resistance, increased testosterone, metabolic syndrome and vascular diseases (Roberts and Cooper, 2001, Sánchez-Aranguren et al., 2014). Most of these factors are also closely associated with impaired endothelial dysfunction and represent the common predisposing factors to other endothelial diseases (Roberts and Cooper, 2001, Sánchez-Aranguren et al., 2014). One study demonstrated that patients who had PE or eclampsia before 30 weeks of gestation or with recurring PE, have 2.6 times higher incidences of chronic hypertension from a 10-year follow up investigation (Sibai et al., 1986). Other studies have reported that women with PE have almost 4-fold increased risk of developing cardiovascular disease later in life (Cirillo and Cohn, 2015, Ray et al., 2005). The risk of developing ischaemic heart disease, stroke, venous thromboembolism, and overall mortality rate is more than doubled in women who had PE compared to women who had uncomplicated pregnancies (Bellamy et al., 2007, Jonsdottir et al., 1995, Smith et al., 2001, Irgens et al., 2001). Women who had early-onset PE are often accompanied by more severe symptoms and have even higher chances of developing cardiovascular disease and death (Lykke et al., 2010). In addition, children exposed to PE *in utero* also have increased risk of high blood pressure and almost double the risk of stroke later in life (Davis et al., 2012). Evidence suggests that PE is the critical insult during pregnancy that predisposes the fetus to pathological responses later in life, since siblings born to mother with PE in a different pregnancy have normal vascular function (Jayet et al., 2010). However, it is unclear whether PE itself is a predisposing factor or women who develop PE share common underlying risk factors for cardiovascular disease later in life (Poston, 2006).

1.2.3. Placental factors

In normal pregnancy, many factors are released by the placenta and are considered as a heightened maternal response to pregnancy, which itself is a low grade systemic inflammatory response (Redman et al., 1999). Woman who cannot tolerate this modification of placental response or with an excessive influx of placental factors, ends up developing PE (Roberts and Hubel, 2009). Some of the factors found to be significantly elevated in PE include cytokines, antiangiogenic factors, syncytiotrophoblast microparticles and activated leukocytes (Conrad et al., 1998, Maynard et al., 2005, Schipper et al., 2005, Goswami et al., 2006, Mellembakken et al., 2002). Many of these factors are specifically involved in PE as they are not associated with any other pregnancy complications (Shibata et al., 2005, Goswami et al., 2006). To date, there is no definitive answer to whether a single factor or multiple factors are responsible for PE, but at least in early-onset PE, endothelial dysfunction appears to be a common pathophysiological occurrence (Roberts and Hubel, 2009). One study showed that serum from preeclamptic women is cytotoxic to endothelial cells *in vitro*, and the clinical condition improves after 24-48 hours postpartum, when the cytotoxicity is dramatically reduced (Rodgers et al., 1988).

Currently, antiangiogenic factors released by the placenta are thought to be one of the damaging agents that act on endothelial cells (Myatt and Roberts, 2015). Angiogenic factors are not only important for angiogenesis, they are also essential for vasoactivity, endothelial cell proliferation and control of vascular permeability (Myatt and Roberts, 2015). Imbalance of pro- and anti-angiogenic factors secreted by the placenta have been shown to occur well before the onset of PE (Levine et al., 2004). Anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin are

reported to be elevated in the sera of PE women (Maynard et al., 2003, Venkatesha et al., 2006, Shibata et al., 2005), whereas pro-angiogenic factor such as placental growth factor (PlGF) is reported to be lower in PE women with degree of suppression correlates to the severity of PE (Levine et al., 2004, Levine et al., 2005).

Endoglin is a transforming growth factor (TGF)-β co-receptor that is expressed in endothelial cells and placental syncytiotrophoblast, it is involved in TGF-β mediated endothelial cell functions (Lebrin et al., 2004). We have previously demonstrated that the serum levels of soluble endoglin are significantly higher in both early-onset and late-onset PE compared to their gestational age-matched controls (Wang et al., 2017). The excessive amounts of soluble endoglin in the maternal circulation may antagonise TGF-β action and contribute to the pathogenesis of PE (Venkatesha et al., 2006). The involvement of Flt-1 and PlGF in the pathogenesis of PE will be discussed in detail in section 1.4.

1.3. Endothelial dysfunction

1.3.1. Endothelial cells

Endothelial cells, or collectively known as the endothelium, covers the inner cellular lining of the blood vessel and is considered as a major endocrine organ that regulates vascular permeability, inflammation, angiogenesis and many other functions that are listed in Figure 3 (Félétou, 2011). Endothelial cells line the entire vascular system from the heart to the smallest capillary, and control the nutrients and other factors from travelling in and out of the bloodstream (Alberts, 2002). Endothelial cells also play a key role in regulating vessel function and structure (Alberts, 2002). Under stress

conditions, endothelial cells send signals to surrounding connective tissues and smooth muscles to allow the blood vessel to adapt to the changing environment by adjusting blood flow that is required by the body (Alberts, 2002). Furthermore, endothelial cells can also facilitate blood vessel dilation by releasing nitric oxide gas to regulate smooth muscle contraction (Alberts, 2002). One of the most important functions of endothelial cells is angiogenesis, and will be further discussed in detail.

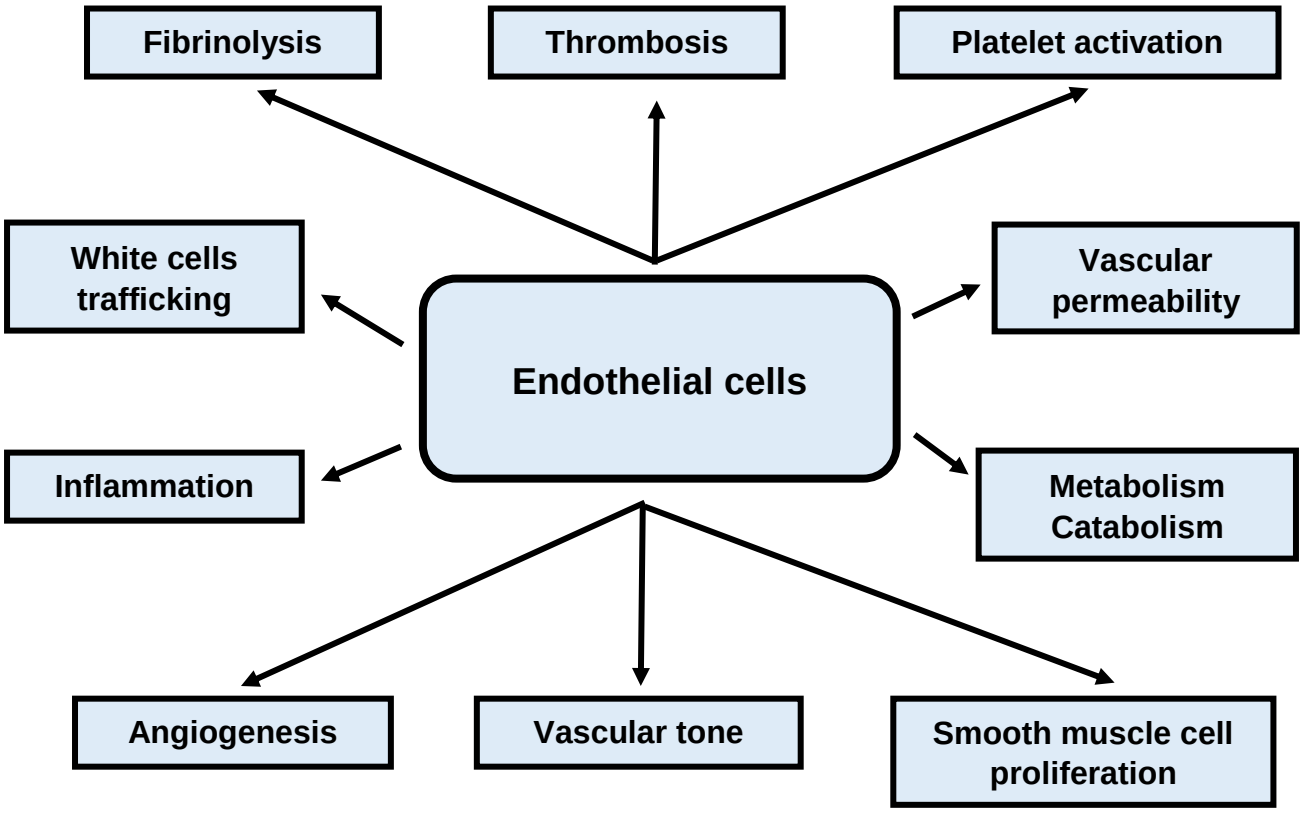

Figure 3. Multiple functions of endothelial cells (adapted from Félétou, 2011).

1.3.2. Angiogenesis

Angiogenesis is the formation of new blood vessels by endothelial cells sprouting from the wall of existing vessels, where the cells continuously hollow out until they encounter another blood vessel, which then forms a connection to allow blood flow (Alberts, 2002). Angiogenesis is a tightly regulated process that is initiated when surrounding tissues demand more oxygen flow and secrete a key protein known as vascular endothelial growth factor A (VEGF-A) that acts on endothelial cells. Following VEGF-A stimulation, endothelial cells undergo a sequential process involving first digestion of the basal lamina of the parental capillary, followed by migration of the cells towards the source of the signal to start proliferation and finally forming a new capillary (Alberts, 2002). Once the new capillary is formed and enough oxygen is delivered to the tissues, the production of VEGF-A is halted and angiogenesis is terminated (Alberts, 2002). Endothelial cells in culture also have the capacity to join up adjacent cells to form a network of capillary-like tubes in the presence of suitable growth factors even in the absence of any other cell types, suggesting that neither blood flow nor pressure are required for the initiation of angiogenesis (Alberts, 2002). The ability of endothelial cells to form tubes *in vitro* provides a great opportunity to investigate the process of angiogenesis (Arnaoutova and Kleinman, 2010), and endothelial cells isolated from various types of arteries and veins have been widely used for the study of endothelial function (Cao et al., 2017, Onat et al., 2011).

1.3.3. Endothelial dysfunction in PE

The endothelium plays an important role in regulating vascular tone, cell growth and crosstalk between leukocytes, thrombocytes and the vessel wall (Konukoglu and Uzun, 2017). The endothelial cells can respond to physical and chemical signals to

produce and release vasoactive molecules that can relax or constrict the vessels to maintain a balance of oxygen supply and metabolic demand (Deanfield et al., 2007). Endothelial dysfunction or a more appropriate term, endothelial activation, occurs when endothelial cells express chemokines, cytokines and adhesion molecules which interact with leukocytes and platelets as a defence response to target inflammation of specific tissues under stress or attack from microorganisms (Deanfield et al., 2007). Endothelial dysfunction is considered as the hallmark of a wide range of cardiovascular diseases that are associated with pathological response of endothelial cells, favouring a vasoconstrictive, prothrombotic and proinflammatory state (Godo and Shimokawa, 2017).

In PE, the evidence of systemic endothelial injury is supported by the appearance of morphological lesions, glomerular endotheliosis, and increased levels of circulating endothelin-1, fibronectin, von Willebrand factor and cytokines, all of which can be secreted by endothelial cells as markers of proinflammatory response to abnormal placental function (Roberts et al., 1989, Roberts and Cooper, 2001). Consequently, reduced production of vaso-relaxing agents and endogenous anticoagulant and increased production of vasoconstrictors and procoagulant by endothelial cells, can lead to further placental hypoperfusion and exacerbate the clinical symptoms of PE (Roberts et al., 1989).

Endothelial dysfunction can precede the clinical presentation of PE, with a rise in markers of endothelial dysfunction in the maternal circulation well before the clinical symptoms appear (Attar et al., 2017). These markers include plasminogen activator inhibitor-1 (PAI-1), asymmetric dimethylarginine (ADMA), which regulate nitric oxide synthesis, and tissue plasminogen activator (tPA) with a correlation to the degree of

proteinuria (Roes et al., 2002, Savvidou et al., 2003, Belo et al., 2002). Furthermore, women who are destined to develop PE are likely to have abnormal uterine artery Doppler waveforms as early as in the first trimester, suggesting that the presence of vascular dysfunction and impaired blood flow in the placenta precede the clinical signs of PE (Giordano et al., 2010, Casmod et al., 2016).

Although the clinical symptoms of PE are generally resolved following delivery, damages to the maternal vasculature can persist in women many years after PE. A study conducted on women who had PE revealed that three years post-pregnancy, exhibited persistent defects in vascular function with more severe impairment in women with recurrent PE (Chambers et al., 2001). Markers of endothelial dysfunction, such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, are also found to be higher in PE women even 15 years after pregnancy (Sattar et al., 2003). Another study also reported the presence of microvascular endothelial dysfunction in women 15-25 years after they had PE (Ramsay et al., 2003). Furthermore, women with a history of PE are more sensitive to angiotensin II and salt (Saxena et al., 2010). All these evidence suggests that endothelial dysfunction as a result of PE may account for the increased risk of cardiovascular diseases in women long after PE, and some other factors independent of the placenta may be involved.

1.3.4. Endothelial progenitor cells (EPCs)

When the endothelium is damaged, a unique population of circulating endothelial cells known as endothelial progenitor cells (EPCs), are recruited to the site of the injury to participate in endothelial cell repair (Steinmetz et al., 2010, Hubel et al., 2011). Human EPCs were first identified and isolated from peripheral blood by Asahara *et al* in 1997,

and these cells were capable of differentiating into endothelial cells *in vitro* (Asahara et al., 1997). More significantly, they were demonstrated to contribute to vascular repair by incorporating into host vessels of ischemic animal models at the site of injury (Asahara et al., 1997). Since then, huge amounts of work have dedicated to study and redefine true EPCs, which are now referred to as a specific subpopulation named endothelial colony-forming cells (ECFCs) (Ingram et al., 2004). These EPCs can be isolated from both adult peripheral circulation and umbilical cord blood. Their identities are confirmed by the expression of endothelial-specific cell surface antigens, but most importantly, they also exhibit self-renewal capacity of high-proliferative potential with over 100 population doubling in culture (Ingram et al., 2004, Patel et al., 2016). Unlike the mature endothelial cells, which are terminally differentiated and have only limited proliferative capacity (Patel et al., 2016), circulating EPCs are highly proliferative and can migrate to the damaged endothelium, where they can differentiate into resident endothelial cells to regenerate the blood vessels and restore endothelial function (Steinmetz et al., 2010, Hubel et al., 2011).

In the normal adult population, reductions in circulating EPCs are associated with increased cardiovascular risks, highlighting the importance of EPCs in the maintenance of endothelial function (Robb et al., 2007). EPC numbers and their migratory activities are reported to be inversely correlated to risk factors of coronary artery disease (Vasa et al., 2001). Notably, EPCs isolated from patients with type II diabetes have impaired proliferation, adhesion and angiogenic activities (Tepper et al., 2002).

In normal human pregnancy, the maternal endothelium undergoes extensive remodelling and repair, where circulating EPCs are suggested to play a major role in

maternal vascular development (Gussin et al., 2002, Sugawara et al., 2005b, Pijnenborg et al., 2006). One study reported that EPC numbers progressively increase in normal pregnancy, and the highest levels are detected in the third trimester (Calcaterra et al., 2014). The same study also demonstrated that circulating EPC numbers in the third trimester are significantly lower in pregnancies complicated by IUGR (Calcaterra et al., 2014). Other studies have reported that maternal as well as fetal/placental EPCs are significantly reduced in PE (Xia et al., 2007, Luppi et al., 2010, Munoz-Hernandez et al., 2014, Monga et al., 2012). EPCs isolated from umbilical cord blood of preeclamptic pregnancies have impaired proliferation, migration and vasculogenesis in culture (Xia et al., 2007). Furthermore, circulating EPCs in early-onset PE are reported to exhibit increased senescence (Sugawara et al., 2005a). These studies suggest that EPCs may play an important role in normal pregnancy, but their numbers and functionality are greatly reduced in PE, which may further disrupt the repair of endothelium to restore normal vascular function and exacerbate the endothelial dysfunction. However, how EPCs are compromised in PE remains unclear.

1.4. VEGF signalling and angiogenesis

1.4.1. Overview of the VEGF family

The main regulators of angiogenesis are ligands that belong to the VEGF family (Otrock et al., 2007). The VEGF family consists of seven secreted glycoproteins known as VEGF-A to VEGF-F and PlGF (Figure 4) (Otrock et al., 2007). Each VEGF protein binds to one of the three tyrosine kinase receptors VEGFR-1/Flt-1, VEGFR-2/kinase insert domain receptor (KDR) or VEGFR-3/Flt-4, with the possible

involvement of co-receptors neuropilins (NP-1 and NP-2), to initiate various intracellular functions (Figure 4) (Otrock et al., 2007). The VEGF-A and PlGF are the two family members that are most relevant to the pathogenesis of PE.

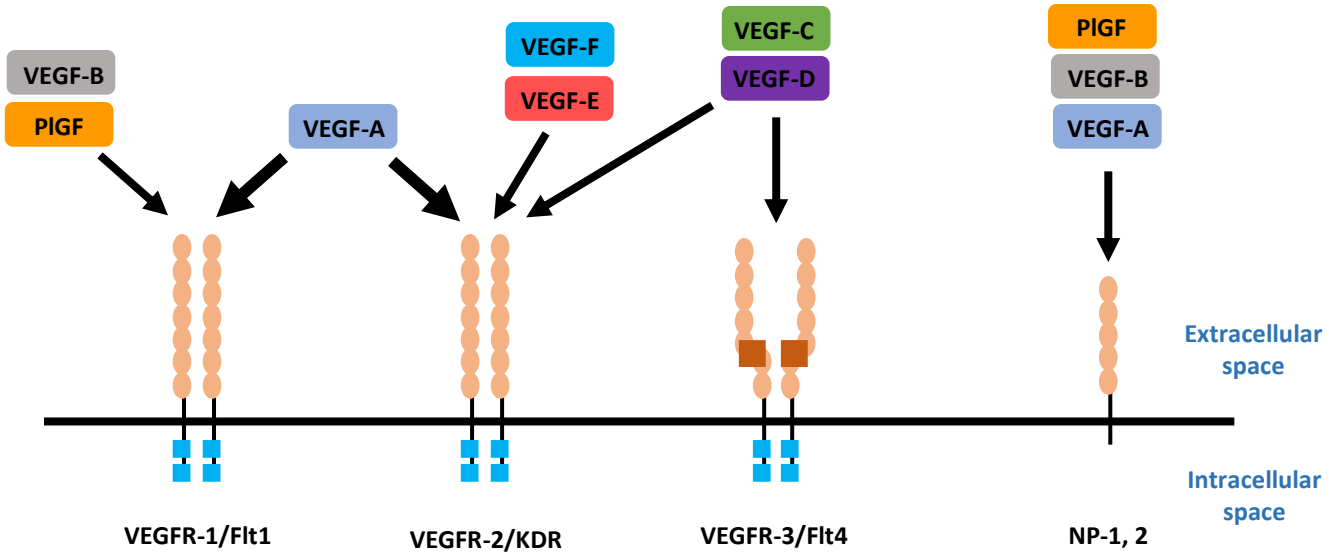

Figure 4. VEGF family members and their receptors. VEGF receptors consist of an extracellular immunoglobulin-like domain (red oval), and an intracellular tyrosine kinase domain (blue square). VEGFR-3 also contains an extracellular signal sequence domain (brown square). (Adapted from Otrock et al, 2007).

1.4.2. VEGF-A

VEGF-A is the most studied and well characterized member of the VEGF family. The human gene encoding VEGF-A is located on chromosome 6p21.3 and consists of eight exons differentially spliced into various mature isoforms (Tischer et al., 1991, Vincenti et al., 1996). To date, six isoforms of VEGF-A have been identified in human: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$, and each number assigned indicates the number of amino acids in the molecules (Robinson and Stringer, 2001, Otrock et al., 2007). All VEGF-A isoforms are secreted as covalently linked homodimers, and in human $VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$ are the predominant isoforms produced by various tissues (Robinson and Stringer, 2001). In comparison, the production of $VEGF_{145}$ and $VEGF_{206}$ isoforms are relatively rare and limited to cells of placental origin (Anthony et al., 1994).

VEGF-A is the most potent pro-angiogenic factor known to date (Otrock et al., 2007). It is also one of the major regulators of endothelial cell proliferation, sprouting and tube formation (Ferrara et al., 2003). VEGF-A is also important for survival of endothelial cells and vascular homeostasis of mature vessels and tissues (Powe et al., 2011). VEGF-A gene deletion in mice is embryonically lethal due to abnormal blood vessel formation (Carmeliet et al., 1996). Inhibition of VEGF-A action in cancer patients often leads to development of hypertension, proteinuria and glomerular endotheliosis, which are similar to the symptoms of PE (Patel et al., 2008). Figure 4 shows that VEGF-A binds to either Flt-1 or KDR, both of which are found on the surface of endothelial cells (Park et al., 1994). However, KDR is the primary mediator for VEGF-A action on endothelial cells (Holmes et al., 2007).

1.4.3. KDR

KDR is a type III transmembrane kinase receptor that has 1356 amino acids, and consists of an extracellular domain (containing a ligand binding site), a short transmembrane domain, and an intracellular part containing two tyrosine kinase domains separate by 70-amino-acid insert (Figure 5) (Holmes et al., 2007). The extracellular domain comprises of seven immunoglobulin-like domains (I-VII) including the VEGF-A binding site at the second and third domain (Figure 5) (Holmes et al., 2007). Downstream intracellular signalling in endothelial cells is activated by binding of VEGF-A to KDR, which then undergoes autophosphorylation at the carboxyl terminal and kinase insert domain (Shibuya, 2006). So far, five tyrosine residues have been identified as the major autophosphorylation sites (Figure 5), which recruit specific intracellular signalling molecules to activate a cascade of downstream cellular functions (Figure 6) (Holmes et al., 2007).

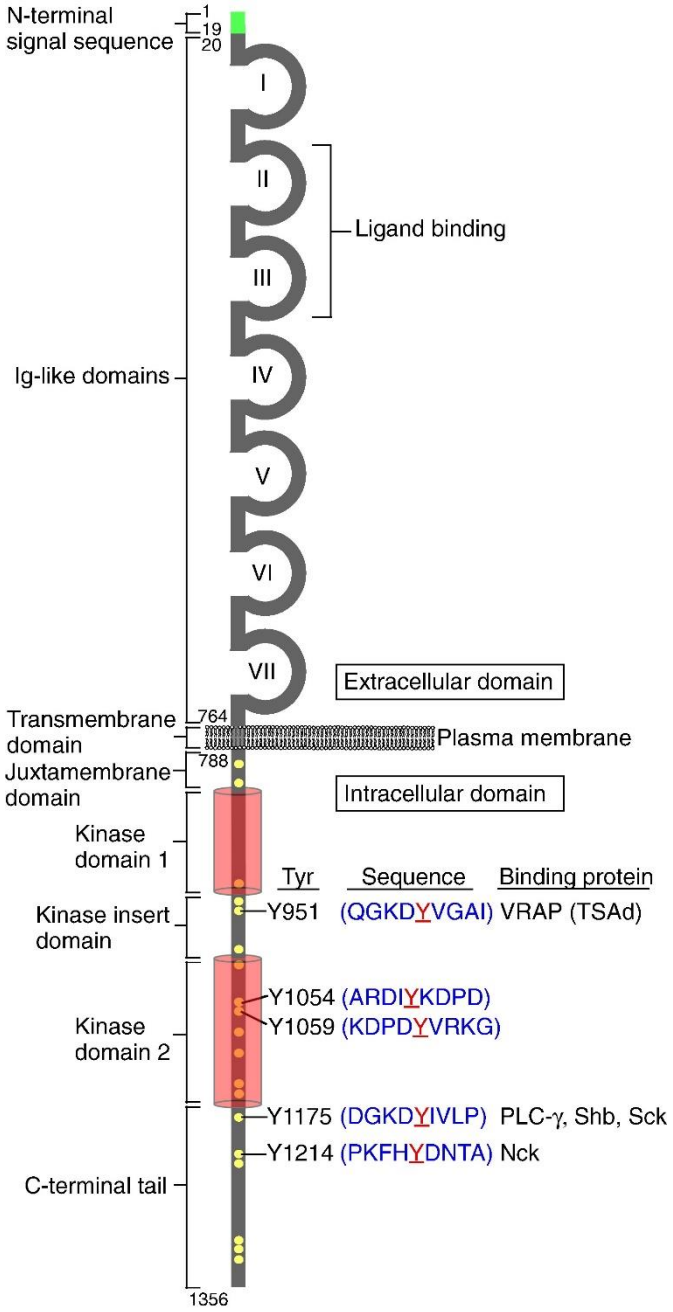

Figure 5. Structure and tyrosine phosphorylation sites of human KDR (Adapted from Holmes et al, 2007).

Activated KDR mediates various physiological and pathological functions in endothelial cells, including proliferation, migration, survival and vascular permeability (Figure 6) (Holmes et al., 2007). Phosphorylation of a specific tyrosine residue generates a consensus sequence that recruits and binds to the Src homology 2 domain of specific signalling molecules, which are then phosphorylated and activated to facilitate specific endothelial cell functions (Figure 6). One of the downstream targets in endothelial cells is the activation of phosphoinositide 3-kinase (PI3K) and subsequent phosphorylation of protein kinase B (PKB/Akt) (Gerber et al., 1998). Akt then directly inhibits the apoptotic activity of Bcl-2 associated death promoter (BAD) and caspase 9 to promote cell survival (Figure 6) (Holmes et al., 2007). Another important role of KDR activation is to induce cell proliferation via the phosphorylation of Y1175 in the C-terminal tail of KDR, which then facilitates a cascade of downstream protein activation that includes extracellular regulated kinase 1 and 2 (Erk 1/2) and MAPK/Erk kinase (MEK) (Figure 6) (Takahashi et al., 2001, Holmes et al., 2007).

KDR plays a critical role in vascular development during embryogenesis and KDR-null mice die *in utero* at embryonic day 8.5-9 due to the absence of structured blood vessels and wide-spread necrosis (Shalaby et al., 1995). In adults, KDR expression is mostly abundant on vascular endothelial cells, although it is also expressed on neuronal cells, megakaryocytes and haematopoietic stem cells (Holmes et al., 2007). Upregulation of KDR has been reported in tumour vasculature and shown to promote VEGF-A action in tumour angiogenesis (Plate et al., 1993). KDR inhibitors developed for cancer therapy have been shown to prevent VEGF-A-induced endothelial proliferation and abolish VEGF-A-induced angiogenesis *in vivo* (Wedge et al., 2005). This evidence suggests that the interaction between VEGF-A and KDR is essential for normal endothelial cell function.

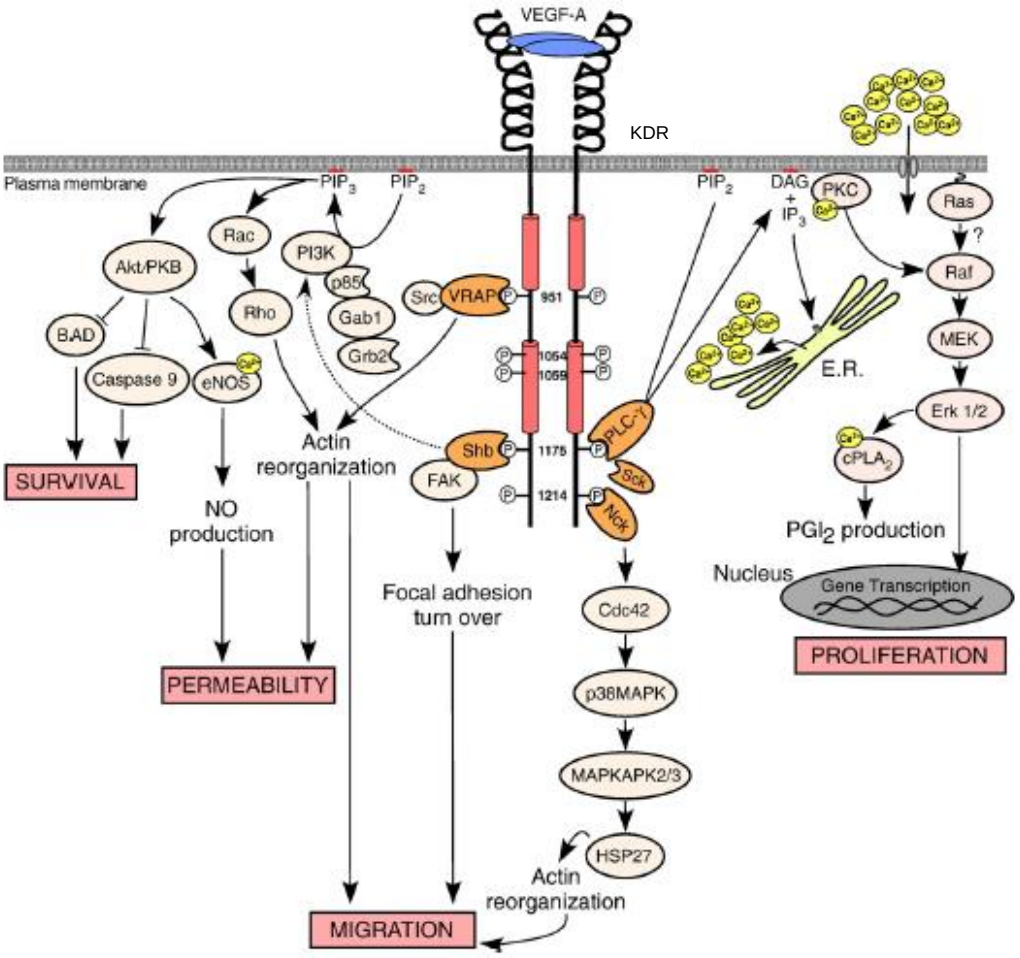

Figure 6. Schematic illustration of KDR signalling. Binding of VEGF-A to KDR induces dimerization and autophosphorylation of specific intracellular tyrosine residues that lead to survival, increased permeability, migration and proliferation of endothelial cells. (Adapted from Holmes et al, 2007).

1.4.4. Flt-1 and its soluble isoform

Although Flt-1 has a very high affinity for VEGF-A, it has a relatively weak tyrosine kinase activity, therefore, the downstream signalling pathway of Flt-1 is unclear (Shibuya, 2006). Flt-1 null mice die during embryonic day 8.5-9 due to overgrowth of endothelial cells and severe blood vessel disarray (Fong et al., 1995). Transgenic mice expressing Flt-1 that lacks the tyrosine kinase domain but with an intact ligand binding domain, appear healthy and have normal blood vessel development, suggesting that the extracellular domain of Flt-1 alone is sufficient to negatively regulate VEGF-A action on endothelial cells, most likely as a decoy receptor by sequestering VEGF-A and thereby preventing activation of KDR (Hiratsuka et al., 1998).

The Flt-1 gene also generates four soluble splice variants that contain only the extracellular domain and in particular sFlt-1 e15a isoform is reported to be highly abundant in the placenta and a major contributor of PE (Palmer et al., 2015, Shibuya et al., 1990). Serum levels of sFlt-1 are abnormally elevated in preeclamptic pregnancies weeks before the first clinical symptom appears (Maynard et al., 2003, Levine et al., 2004). Moreover, intravenous delivery of sFlt-1 in normal pregnant mice can induce hypertension, proteinuria and renal failure similar to PE and directly inhibits VEGF-A action, suggesting that excessive amounts of sFlt-1 may contribute to the abnormal inhibition of VEGF-A action in PE development (Patel et al., 2008, Maynard et al., 2003). Figure 7 shows the schematic presentation of VEGF-A interaction with its two receptors. VEGF-A has a much higher affinity for Flt-1 and sFlt-1 than KDR, which has stronger tyrosine kinase activity (Loges et al., 2009). In PE, excessive amounts of sFlt-1 in the maternal circulation would reduce the availability of free VEGF-A to interact with its main receptor KDR on endothelial cells; sFlt-1 therefore

acts as an efficient antagonist of VEGF-A-induced angiogenesis (Figure 7) (Otrock et al., 2007).

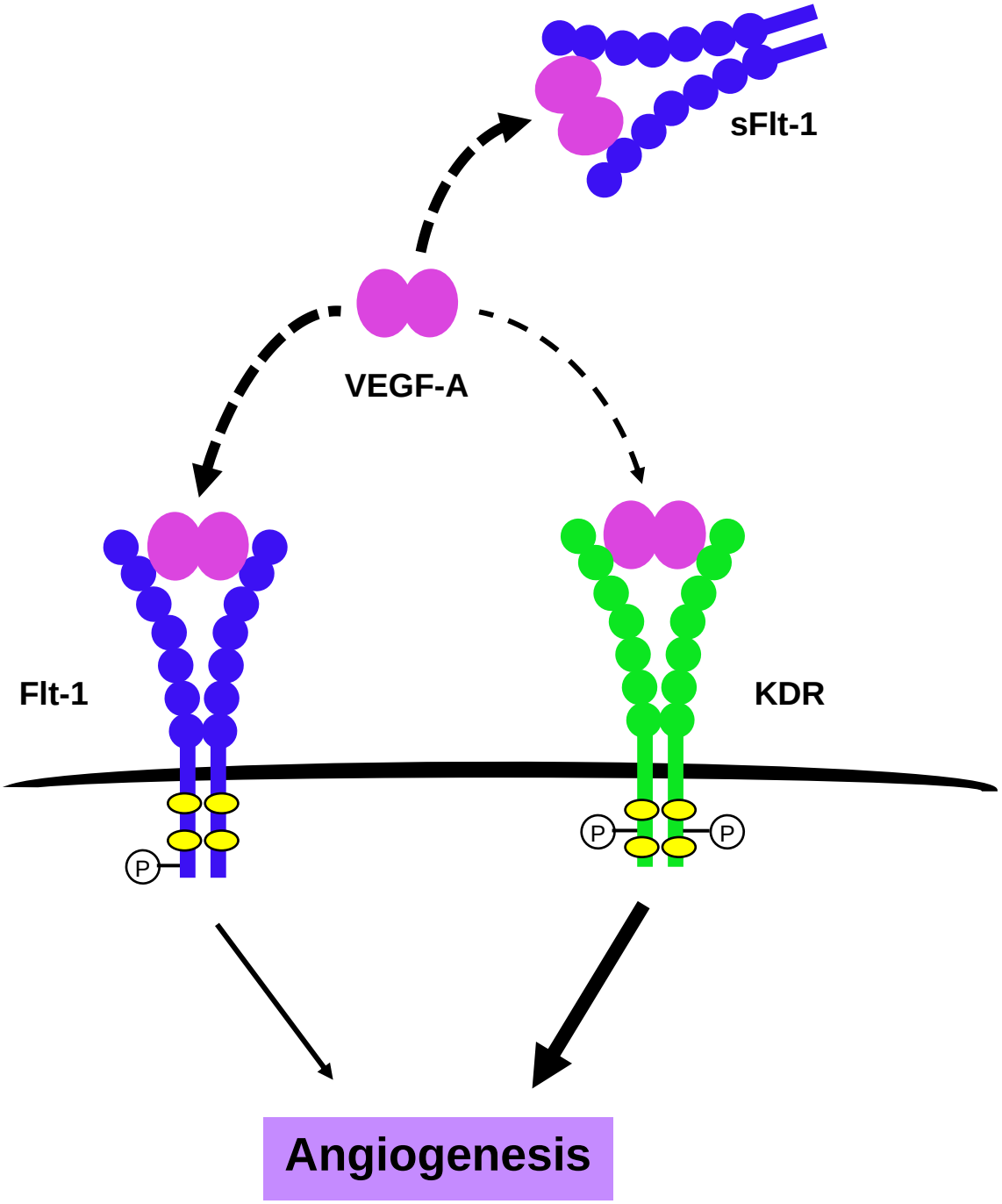

Figure 7. Schematic illustration of VEGF-A interaction with its two receptors. VEGF-A has high affinity for Flt-1 and its soluble isoform. Therefore, excessive amounts of sFlt-1 will reduce the availability of free VEGF-A to bind to KDR, which has stronger tyrosine kinase activity for downstream signalling (Adapted from Loges et al, 2009).

1.4.5. PlGF

PlGF is a VEGF homolog that shares 53% identity and is highly expressed by the human placenta (Maglione et al., 1991). PlGF homodimers bind to the Flt-1 receptor with a high affinity (Powe et al., 2011). PlGF has a very weak mitogenic activity with no effect on endothelial cells *in vitro* alone, but it potentiates the action of VEGF-A in combination; this suggests that PlGF may act by displacing VEGF-A from the Flt-1 receptor, and therefore allowing VEGF-A to bind to the more active KDR receptor (Figure 8A) (Park et al., 1994). PlGF is highly expressed during pregnancy, in particular by trophoblast cells, and is released into the maternal circulation (Khaliq et al., 1996). The levels of circulating PlGF are found to be lowest in women who have early-onset PE, and women who have PE with IUGR have even lower levels of PlGF, possibly as a consequence of abnormal and dysfunctional placenta (Taylor et al., 2003). The current consensus is that circulating sFlt-1 binds to both PlGF and VEGF-A as a decoy receptor, thereby competitively reducing the amount of circulating VEGF-A and PlGF that can bind to their respective cell surface receptors (Cindrova-Davies et al., 2011). In PE where PlGF level is low and sFlt-1 level is high, there is not enough PlGF to compete with VEGF-A for the binding of sFlt-1, therefore, VEGF-A can freely bind to excessively available sFlt-1 in the maternal circulation, thus preventing the activation of KDR (Figure 8B) (Taylor et al., 2003). This imbalance of antiangiogenic and angiogenic factors released from the preeclamptic placenta is a likely contributing factor of endothelial dysfunction in PE.

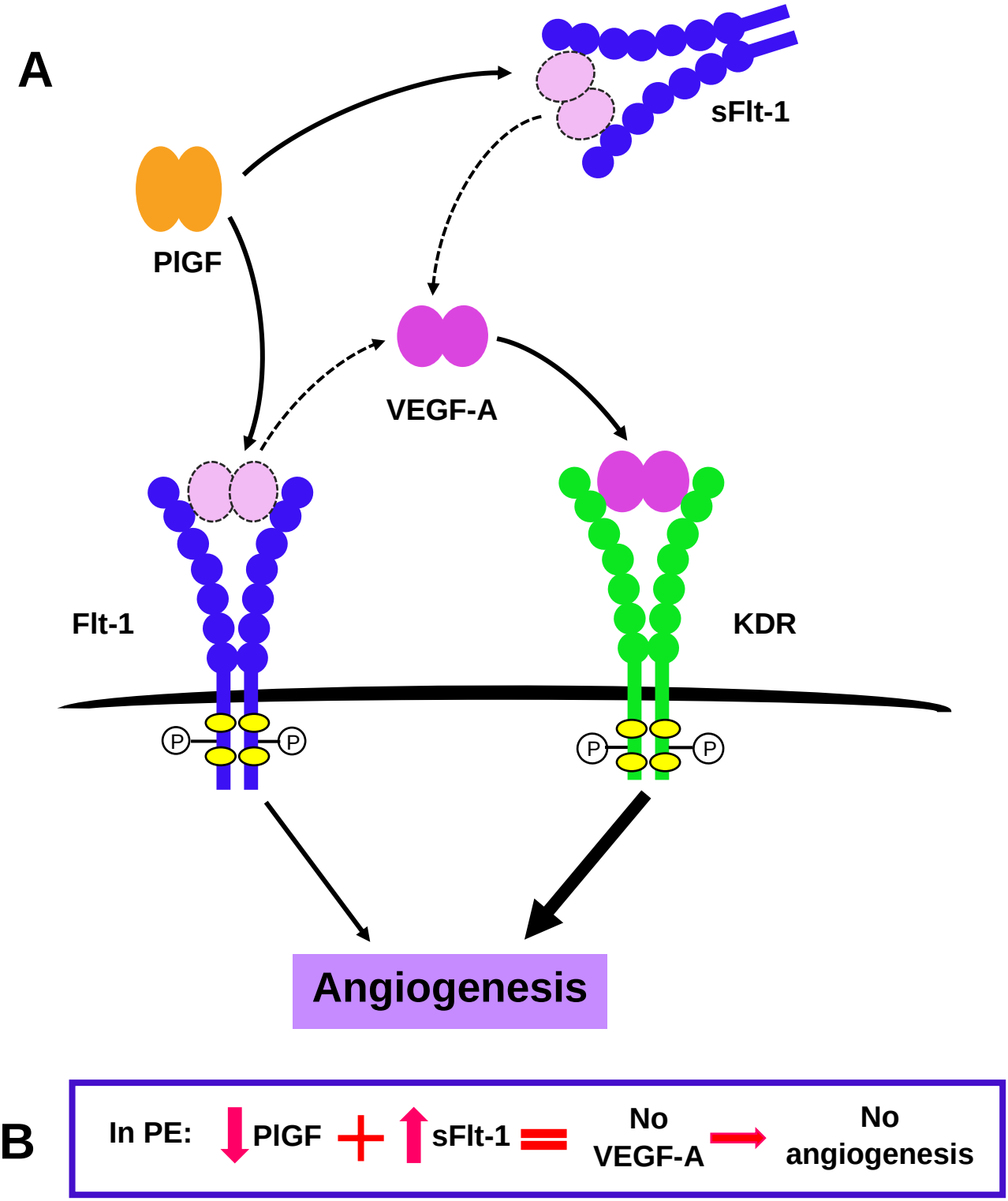

Figure 8. PlGF involvement of proangiogenic signalling and PE. A) Displacement of VEGF-A from Flt-1 and sFlt-1 by PlGF leads to the binding of VEGF-A to KDR to initiate endothelial cell signalling. B) In PE, low levels of PlGF and excessive amounts of sFlt-1 eliminate the bioavailability of VEGF-A to bind to and activate KDR (Adapted from Loges *et al*, 2009).

1.5. High temperature requirement factor A (HtrA) family proteases

1.5.1. Overview of the HtrA family proteases

Proteins are the main machinery required by all living cells whether they are single cell prokaryotes or mammals including human, and they are tightly controlled to ensure normal cell function (Cheregi et al., 2016). Therefore, all cells have the mechanism to produce proteases to remove unfolded, misfolded or damaged proteins as a result of improper folding or environmental stress (Zurawa-Janicka et al., 2017). In mammals, a family of highly conserved serine proteases known as high temperature requirement factor A (HtrA), plays important role in the quality control of cellular proteins (Zurawa-Janicka et al., 2017). The bacterial homolog of HtrA (DegP) was first identified and characterized from *Escherichia coli,* it consists of heat shock-induced protease activity and potential chaperone activity (Chang, 2016). The mammalian HtrA family proteases serve as ATP-independent protein quality control factors that are associated with cell growth, unfolded stress response, programmed cell death and aging (Clausen et al., 2002). So far, four mammalian HtrA proteases (HtrA1-HtrA4) have been identified, and their dysregulation has been implicated in various pathological processes, such as cancer, neurogenerative disorders, arthritic diseases and pregnancy disorders (Zurawa-Janicka et al., 2010, Welsh et al., 2001b, Narkiewicz et al., 2008, Narkiewicz et al., 2009, Lorenzi et al., 2009, Li et al., 2011, Teoh et al., 2015, Singh et al., 2015).

All four HtrAs are structurally similar and contain one or more highly conserved C-terminal postsynaptic density protein 95-Discs large-Zona occludens (PDZ) domain, which binds to target proteins to regulate protein-protein interaction, and a trypsin-like serine protease domain with catalytic activity (Figure 9) (Singh et al., 2014). HtrA1,

HtrA3 and HtrA4 share a similar N-terminal domain structure consisting of a putative signal peptide, an insulin growth factor (IGF) binding domain and a kazal protease inhibitor domain (Figure 9) (Clausen et al., 2002). In contrast, HtrA2 has a completely unique N-terminal consisting of a transient peptide and a transmembrane domain, suggesting that HtrA2 may have different functions compared to the other three HtrAs (Figure 9) (Nie et al., 2003a). Both HtrA1 and HtrA3 have been previously implicated in the development of PE (Ajayi et al., 2008, Teoh et al., 2015, Li et al., 2011).

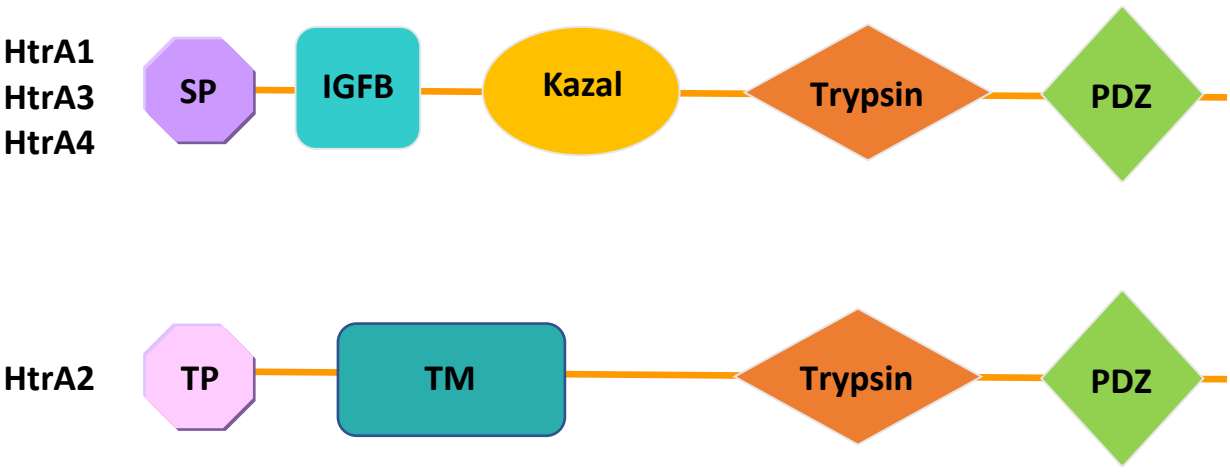

Figure 9. Structure of mammalian HtrA family proteases. SP, signal peptide; IGFB, insulin growth factor binding domain; Kazal, Kazak-type S protease inhibitor domain; Trypsin, trypsin-like serine protease domain; PDZ, postsynaptic density protein 95-Discs larger-Zona occludens domain; TP, transient peptide; TM, transmembrane domain (Adapted from Singh et al, 2015).

1.5.2. HtrA1 and its biological functions

HtrA1 is a secretory protein that is ubiquitously expressed in human tissues with high levels in the placenta (De Luca et al., 2003, Nie et al., 2006a). The human HtrA1 gene was initially identified as a down-regulated gene in SV-40 transformed fibroblasts (Zumbrunn and Trueb, 1996), and the protein has been implicated as a tumour suppressor and promoter of cell death (Zurawa-Janicka et al., 2010). HtrA1 mRNA expression is greatly down-regulated or completely absent in various cancers such as ovarian cancer, metastatic melanomas, breast carcinoma, prostate cancer and many other tumour cell lines (Chien et al., 2004, Baldi et al., 2002, Sotiriou et al., 2006, Welsh et al., 2001a). In addition, overexpression of HtrA1 in metastatic melanoma cell line inhibited cell proliferation *in vitro* and prevented tumour growth *in vivo* (Baldi et al., 2002).

Many growth factors and matrix proteins have been identified as the extracellular substrates of HtrA1 and dysregulation of these factors by aberrant HtrA1 activity has been linked to many other human diseases (Tiaden and Richards, 2013). HtrA1 expression is up-regulated in various musculoskeletal diseases, which coincides with the increased fragmentations of several extracellular matrix (ECM) proteins that are known to be targets of HtrA1, including fibronectin, type II collagen and decorin (Tiaden and Richards, 2013). Increased expression of HtrA1 is also implicated in age-related macular degeneration (AMD), which is also closely associated with increased degradation of various ECM proteins (Lin et al., 2018). On the other hand, decreased expression of HtrA1 is linked to dysregulation of TGF-β signalling in cerebral small vessel disease, which can lead to early-onset stroke and dementia (Beaufort et al., 2014). Latent TGF-β binding protein 1 (LTBP-1), an ECM protein that is processed by

HtrA1 and required by TGF-β signalling, has been shown to attenuate TGF-β signalling in the disease due to lack of HtrA1 activity (Beaufort et al., 2014).

In normal human pregnancy, serum HtrA1 level is increased progressively with increasing gestation (Teoh et al., 2015). In the first trimester placenta, HtrA1 is immuno-localized in both layer of villous trophoblast, the syncytiotrophoblast and cytotrophoblast (Lorenzi et al., 2009). In the third trimester, the HtrA1 staining is more intensified and mainly observed in the syncytiotrophoblast and the maternal decidua cells, suggesting that HtrA1 may be involved in the normal development of the placenta (De Luca et al., 2004). HtrA1 knockout mice are viable and fertile, however, both the placenta and the pups are reduced in size, and the placenta displays impaired artery remodelling (Hasan et al., 2015). The exact role of HtrA1 in placental development is unclear, but it is speculated to be involved in the regulation of trophoblast-decidual interaction and trophoblast invasion, which are crucial for normal placentation (Chen et al., 2014). One study demonstrated that a trophoblast-like cell line, HTR-8/SVneo cells, exhibited reduced migration and invasion in the presence of HtrA1, suggesting that aberrant levels of HtrA1 might directly disrupt placental development by attenuating trophoblast cell migration and invasion (Ajayi et al., 2008). Recently, several studies reported that dysregulation of HtrA1 is associated with PE, in particular early-onset PE. Placental mRNA expression as well as serum levels of HtrA1 have been shown to be significantly elevated in PE cases, making HtrA1 a potential diagnostic marker of PE at the time of disease presentation (Teoh et al., 2015, Ajayi et al., 2008, Inagaki et al., 2012, Zong et al., 2013).

1.5.3. HtrA2 and its biological functions

HtrA2 is the most studied member of this family, and it is structurally different to the other three HtrAs (Wagh and Bose, 2018). While HtrA1, 3 and 4 are all secreted proteins, the precursor of HtrA2 resides in the mitochondrial intermembrane space (Vande Walle et al., 2008). HtrA2 serves as protein quality control factor to maintain mitochondrial homeostasis under normal physiological conditions (Zurawa-Janicka et al., 2010). Loss of HtrA2 in mice leads to accumulation of unfolded proteins in the mitochondria, defective mitochondrial respiration and increased concentrations of ROS which leads to neuronal cell death (Moisoi et al., 2009). Furthermore, mice with a mutation in HtrA2 gene that results in loss of HtrA2 proteolytic activity, exhibit muscle wasting and neurodegeneration very similar to symptoms exhibited in patients suffering from Parkinson's syndrome (Jones et al., 2003). However, in stressful conditions, HtrA2 may switch from a protector to a proapoptotic factor to facilitate cell death (Zurawa-Janicka et al., 2010). Following apoptotic stimulus, HtrA2 is released from the mitochondria, binds and degrades the inhibitor of apoptosis proteins (IAPs), thereby freeing active caspases to induce apoptosis in the damaged or infected cells (Suzuki et al., 2001, Verhagen et al., 2002). Therefore, HtrA2 protein plays a crucial role as a mediator of cell survival as well as cell death, however, unlike the other members of the HtrA family, there is no evidence of HtrA2 involvement in placental development or pregnancy complications.

1.5.4. HtrA3 and its biological functions

HtrA3 is structurally similar to HtrA1, and it was first identified as a pregnancy-related serine protease that is up-regulated in the mouse uterus with a role in the development of the embryo and the placenta (Nie et al., 2003b). In the human, HtrA3 is widely

expressed in the body and the highest levels are detected in the heart, ovary, uterus and placenta (Nie et al., 2003a). So far, two isoforms of HtrA3 produced by alternative splicing have been identified, the long form consists of 453 amino acids, whereas the short form lacks the PDZ domain and consists of 357 amino acids (Nie et al., 2003a). Both HtrA3 isoforms are expressed comparably in human and are both proteolytically active (Singh et al., 2012, Nie et al., 2003a). A recent study demonstrated that both HtrA3 isoforms can interact with cytoskeleton proteins such as actin, β-tubulin, vimentin and TCP1 chaperonin, which are required for actin and tubulin folding (Wenta et al., 2018). Although both HtrA3 isoforms can cleave cytoskeleton proteins while also functioning as chaperone proteins *in vitro*, the short form of HtrA3 has more efficient proteolytic activities, whereas the long form is the most efficient HtrA family protein in facilitating tubulin polymerization (Wenta et al., 2018). Thus, it is likely that the two HtrA3 isoforms may have different roles and both can function either as proteases or chaperone proteins (Wenta et al., 2018). Similar to HtrA1, dysregulation of HtrA3 has been implicated in various cancers and HtrA3 is proposed as a tumour suppressor (Glaza et al., 2015). Down-regulation of HtrA3 has been reported in various cancer cell lines and tumours including ovarian, endometrial and lung cancers (Bowden et al., 2010, Zhao et al., 2014, Bowden et al., 2006, Narkiewicz et al., 2009, Beleford et al., 2010a, Zhao et al., 2016). HtrA3 is further shown to promote lung cancer cell death induced by chemotherapeutic drugs etoposide and cisplatin, and suppression of HtrA3 renders the cancer cells resistant to anti-tumour drugs (Beleford et al., 2010b). Another study demonstrated that HtrA3 can inhibit lung cancer cell invasion and HtrA3 level is negatively correlated to the increased risk of postoperative recurrence of non-small-cell lung cancer (Zhao et al., 2016).

During human pregnancy, HtrA3 is expressed in the placenta in high amounts during the first trimester, especially in the syncytiotrophoblast (Nie et al., 2006b, Li et al., 2011). HtrA3 levels in the maternal circulation reflect placental production, with the highest levels also detected in the first trimester. Placental and serum levels of HtrA3 then drastically reduce from the second trimester onwards, and this is likely to be regulated by changes in oxygen tension from a low to a high oxygen environment (Li et al., 2011). Abundance of placental HtrA3 expression in early pregnancy suggests that HtrA3 may be important for the development of the placenta and the embryo, and *in vitro* studies show that HtrA3 negatively regulates trophoblast invasion during early placental development (Singh et al., 2010, Singh et al., 2011).

HtrA3 knockout mice are phenotypically normal and fertile, however, the placenta displays disorganization of labyrinthine fetal capillaries, which results in fetal growth restriction. The data suggests that maternal HtrA3 is important for vascular organization of the developing placenta and fetal growth (Li et al., 2017). Furthermore, mice born to HtrA3-deficient mothers, irrespective of their own genotype, are significantly heavier with more white fat in their adulthood, indicating that maternal HtrA3 may have a long-term impact on the offspring well beyond *in utero* growth (Li et al., 2017). Dysregulation of HtrA3 in the first trimester may therefore be associated with abnormal placental development. Studies found that pregnant women who were destined to develop PE had significantly higher levels of serum HtrA3 at 13-14 weeks of gestation, which is well before the manifestation of any clinical symptoms (Li et al., 2011, Dynon et al., 2012). Interestingly, serum HtrA3 levels at 11-13 weeks of gestation are significantly lower in women who had IUGR pregnancies (Li et al., 2017), suggesting that optimal concentration of placental HtrA3 is necessary for the optimal

development of a functional placenta. Therefore, this also makes HtrA3 a potential biomarker for early diagnosis of PE and IUGR.

1.6. HtrA4 and its role in early-onset PE development

1.6.1. HtrA4 expression and its biological functions

HtrA4 is the newest and least studied member of the mammalian HtrA family. The HtrA4 gene is located on chromosome 8p11.22 with 8 exons, and it is first identified as a serine protease associated with pregnancy (Clausen et al., 2002). HtrA4 is structurally similar to HtrA1 and HtrA3, however, HtrA4 is highly unique because its expression is almost exclusive to the human placenta (Singh et al., 2015), whereas the other HtrAs are widely expressed in most tissues (Nie et al., 2003a). Analysis of tissue-profiling microarray data showed that HtrA4 mRNA expression is highly placenta specific (Figure 10). One study has immunolocalized HtrA4 to the EVT cells at the maternal-fetal interface (Wang et al., 2012). Little is known about the exact function of HtrA4 in the placenta, but it is believed to be involved in the regulation of trophoblast invasion in placental development (Wang et al., 2012, Chen et al., 2014). A study by Wang *et al* (2014) over-expressed the wild-type and a protease-inactive mutant HtrA4 in trophoblast-like JAR cells and investigated invasion. It was found that JAR cells overexpressing the wild-type HtrA4 had increased invasion, whereas JAR cells overexpressing the mutant HtrA4 had unchanged invasion (Wang et al., 2012). The same study also examined the impact of HtrA4 on another trophoblast-like cell line, the BeWo cells, by knocking down the endogenous HtrA4 expression using shRNA, it was found that the invasion of BeWo cells was suppressed when HtrA4 was

knocked down (Wang et al., 2012). Wang *et al* (2014) then showed that HtrA4 could cleave the extracellular matrix protein fibronectin *in vitro*, suggesting that HtrA4 may facilitate cell invasion by disrupting the interaction between fibronectin and its integrin receptors which would otherwise impede trophoblast invasion (Damsky et al., 1994, Wang et al., 2012). However, whether HtrA4 has a similar regulatory role in trophoblast cells *in vivo* remains to be investigated.

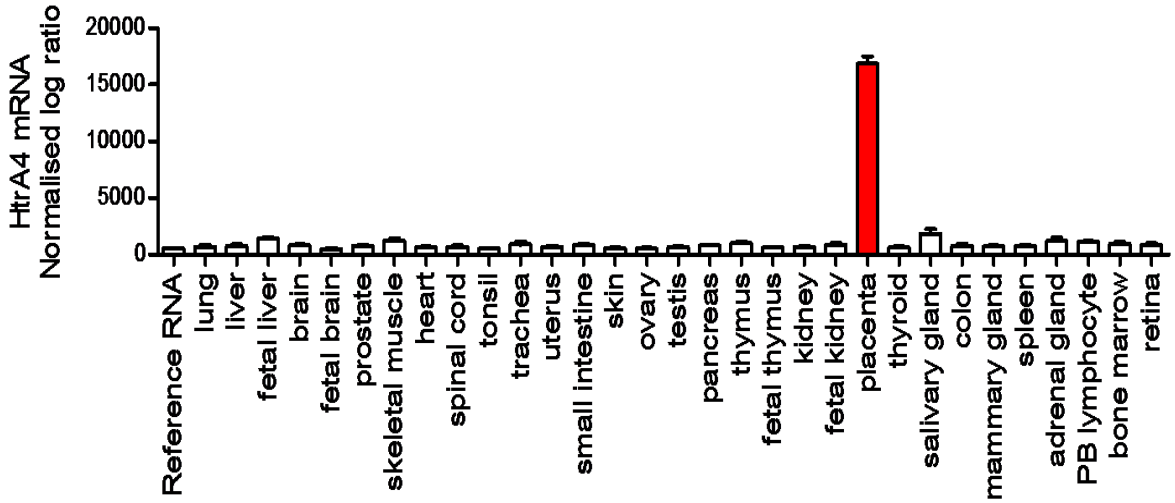

Figure 10. Microarray analysis of HtrA4 mRNA in 31 human tissues. The data set was derived from the National Centre for Biotechnology Information Gene Expression Omnibus database (GDS3113) (Singh et al., 2015).

A recent study by Liu *et al* (2015) reported that HtrA4 null mice have normal embryonic and placental development, with no obvious difference in placental structure or morphology compared to wild-type mice (Liu et al., 2015). In addition, cross breeding of HtrA4 knock-out mice produced similar pup numbers, indicating that fertility is also unaffected (Liu et al., 2015). The authors suggest that the lack of abnormality in HtrA4 null mice is likely because either HtrA4 does not play a significant role in murine placentation or up-regulation of other HtrA family proteins has compensated the lack of HtrA4 expression (Liu et al., 2015). However, their data clearly shows that HtrA4 is not well expressed in mice, including the placenta. This important fact was further confirmed by our laboratory (unpublished). We therefore believe that the lack of a phenotype in HtrA4 null mice is because HtrA4 is not well expressed in mice in the first place.

In contrast, HtrA4 is highly expressed in the human placenta, and it is not well expressed in any other tissues that were examined thus far (Figure 10) (Singh et al., 2015). HtrA4 expression therefore seems to be human placenta-specific, and HtrA4 may have a specific role in human placental development, human pregnancy and human pregnancy complications.

1.6.2. HtrA4 and its potential involvement in PE

Placental HtrA4 is secreted into the maternal circulation. In a normal human pregnancy, the HtrA4 levels in maternal serum increase significantly from 11-13 weeks to 24-25 weeks of gestation, they then remain stable throughout the remainder of the pregnancy (Singh et al., 2015). A number of studies have linked abnormal regulation of HtrA4 particularly to the early-onset PE (Inagaki et al., 2012, Liu et al., 2018, Singh et al., 2015). Gene expression analyses have consistently shown that placental HtrA4

expression is significantly up-regulated in severe PE compared to their gestational-matched controls (Singh et al., 2015, Inagaki et al., 2012, Lapaire et al., 2012, Kaartokallio et al., 2015, Brew et al., 2016, Liu et al., 2018, Nishizawa et al., 2011). Furthermore, HtrA4 levels in the maternal circulation are significantly elevated in the third trimester in early-onset PE at the time of disease presentation compared to their gestational-matched controls (Singh et al., 2015, Inagaki et al., 2012). Moreover, the levels of circulating HtrA4 are shown to positively correlate with PE severity (Inagaki et al., 2012).

Contrary to the norm, study by Wang *et al* in 2012 showed that the intensity of HtrA4 immunostaining was lower in PE placentas (Wang et al., 2012). In this particular study, only two placentas from late-onset PE were examined. These seemingly conflicting reports suggest that HtrA4 expression pattern can be different between early-onset and late-onset PE, and elevation of HtrA4 is very likely a distinct characteristic of early-onset PE. This was supported by a study by Inagaki *et al* (2012) where the expression of all four HtrA family members was compared between placentas from women with severe PE (with an average gestational age of 33.9 weeks) and those from normotensive pregnant women (with an average gestational age of 36.6 weeks), expression of both HtrA1 and HtrA4 was significantly up-regulated in the PE placentas (Inagaki et al., 2012). Placental HtrA4 protein levels were also confirmed to be highly elevated in PE compared to controls (Inagaki et al., 2012). Immunohistochemistry localised HtrA4 protein mainly to the cytoplasm of cytotrophoblasts and syncytiotrophoblasts in placental villi, and more intense signals were observed in PE placentas (Inagaki et al., 2012). The same study also confirmed that HtrA4 level in PE serum was significantly elevated. The study has further demonstrated that the serum HtrA4 level inversely correlate to placental and fetal weight, it was thus suggested that

aberrant levels of HtrA4 may have an adverse impact on fetal and placental development during pregnancy (Inagaki et al., 2012).

Our laboratory has validated the findings of Inagaki *et al* (2012) and demonstrated that HtrA4 mRNA expression is clearly up-regulated in the placenta of early-onset but not late-onset PE (Figure 11A) (Singh et al., 2015). Furthermore, the maternal serum levels of HtrA4 were confirmed to be elevated in early-onset but not late-onset PE (Figure 11B). Taken together, these findings strongly suggest that excessive placental expression of HtrA4 and elevation of serum HtrA4 may be uniquely associated with early-onset PE (Singh et al., 2015).

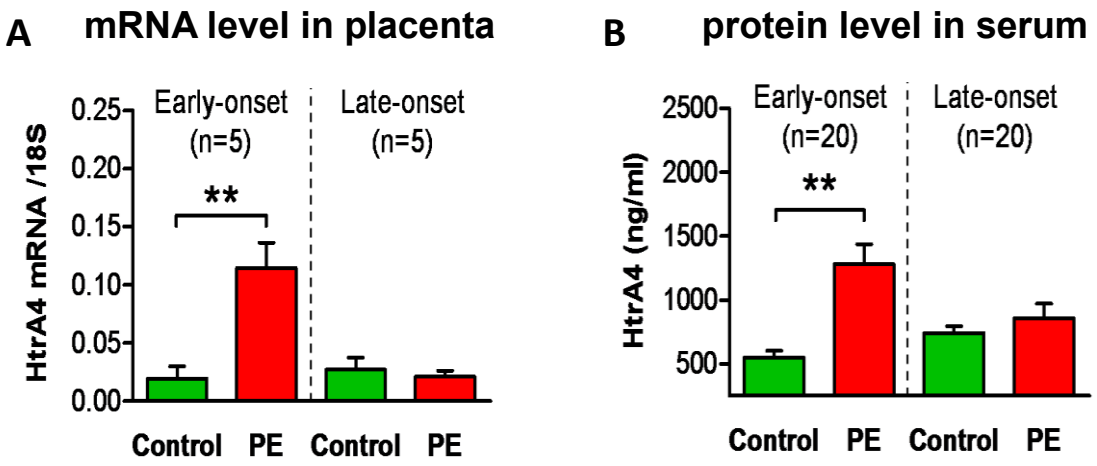

Figure 11. The expression pattern of HtrA4 mRNA in placenta and serum HtrA4 level in maternal circulation. A). HtrA4 mRNA expression is up-regulated in early-onset PE placentas. B). Serum HtrA4 levels are elevated in early-onset PE pregnancies. Mean ± SEM, ** P<0.01. (Data obtained from Singh et al, 2015).

1.6.3. HtrA4 and its impact on endothelial cells

Since HtrA4 is a secreted protease and can be detected in the maternal circulation, HtrA4 may likely act on maternal endothelial cells. Elevated levels of HtrA4 may therefore adversely affect the endothelial cells to contribute to the systemic endothelial dysfunction that is observed in most early-onset PE. A study by our laboratory (Singh *et al* in 2015) demonstrated that HtrA4 could disrupt the normal function of human umbilical vein endothelial cells (HUVECs) as an endothelial cell model. The study has shown that HtrA4 dose-dependently alter endothelial cell integrity and increased cell permeability *in vitro* (Singh et al., 2015). Vascular endothelial (VE)-cadherin, which plays an important role in cell to cell adhesion and the organization of intercellular junction, is greatly reduced in HtrA4-treated HUVECs (Singh et al., 2015). HtrA4 treatment has also altered HUVEC morphology, resulting in a disorderly pattern of F-actin staining and increased number of intercellular gaps (Singh et al., 2015). These findings are consistent with the characteristics of primary HUVECs isolated from PE women, such as disorganisation of endothelial cadherin proteins, reduction in expression of endothelial cell junctional proteins and increases in cell permeability (Wang et al., 2002). HtrA4 also dose-dependently disrupts the formation of tube-like structure by HUVEC *in vitro*, suggesting a potential role of HtrA4 in disrupting the angiogenic activity of these cells (Singh et al., 2015). All the evidence thus presents HtrA4 as a potential contributing factor to the pathogenesis of early-onset PE. However, how extensively the circulating HtrA4 impacts on maternal endothelial cells and the associated molecular mechanisms remain to be investigated.

Research Questions

The specific aims of my PhD study were to investigate:

i. The impact of HtrA4 action on endothelial cells, including the EPCs.
ii. The molecular mechanisms of HtrA4 action on endothelial cells.

Hypotheses:

High levels of circulating HtrA4 that is detected in the early-onset PE circulation, have a detrimental effect on maternal endothelial cells and contribute to the pathogenesis of early-onset PE.

Aims:

(1) To determine the adverse impact of circulating HtrA4 on endothelial gene expression related to vessel biology, using HUVECs as an endothelial cell model.

(2) To determine whether high levels of HtrA4 can affect maternal endothelial repair by

   i. Inhibiting HUVEC proliferation.

   ii. Inhibiting primary EPC proliferation and differentiation into mature endothelial cells.

(3) To determine the mechanism of HtrA4 action on endothelial cells by demonstrating that HtrA4 can

   i. Directly cleave recombinant KDR *in vitro*.

   ii. Reduce total amount of KDR in HUVECs thereby disrupting the VEGF-A action

   iii. Inhibit VEGF-A-induced angiogenesis in HUVECs and explant culture of mouse aortic rings

Chapter 2

High levels of HtrA4 observed in preeclamptic circulation drastically alter endothelial gene expression and induce inflammation in human umbilical vein endothelial cells

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High levels of HtrA4 observed in preeclamptic circulation drastically alter endothelial gene expression and induce inflammation in human umbilical vein endothelial cells

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ABSTRACT

Introduction: Preeclampsia (PE) is a life-threatening pregnancy disorder characterized by wide-spread endothelial dysfunction. Placental factors circulating in the maternal blood are believed to cause endothelial dysfunction. Our previous study identified HtrA4 as a placenta-specific serine protease that is released into the maternal circulation and significantly increased in early-onset PE. In this study, we examined the impact of HtrA4 on expression of endothelial genes related to vessel biology, using human umbilical vein endothelial cells (HUVECs) as a model.
Methods: HUVECs were treated with 0 or 3 μg/ml HtrA4 (highest concentration seen in PE circulation) for 24 h and analysed by an endothelial cell biology PCR array containing 84 genes. HtrA4-induced changes were then validated by real-time RT-PCR and ELISA for time and dose dependency.
Results: High levels of HtrA4 significantly altered the expression of a range of genes related to inflammation, vaso-activity, angiogenesis, cell adhesion, platelet activation and coagulation. In particular, pro-inflammatory genes *IL6*, *PTGS2 (COX2)* and *IL1B* were significantly increased by HtrA4. IL6 protein in HUVEC media was also drastically increased. *THBD*, an anticoagulant factor reported to be increased in PE, was significantly up-regulated by HtrA4. In contrast, *THBS1*, which is involved in many regulatory processes of endothelial cell biology, was severely down-regulated by HtrA4.
Discussion: HtrA4 significantly increased the inflammatory responses of HUVECs, and altered their expression of a number of genes important for vessel biology. These data suggest that placenta-derived HtrA4 that is increased in PE circulation is a potential causal factor of endothelial dysfunction.

1. Introduction

Preeclampsia (PE) is a life-threatening disorder of human pregnancy affecting 2–8% of pregnancies worldwide [1,2]. PE can progress rapidly leading to multi-organ failure and symptoms are closely linked to wide-spread endothelial dysfunction [3]. It is well accepted that in PE, the placenta releases abnormal amounts/types of factors into the maternal circulation and these circulating factors contribute to endothelial dysfunction and the maternal syndrome of PE [4]. Significantly elevated circulating factors in PE include cytokines, antiangiogenic factors, syncytiotrophoblast microparticles and activated leukocytes [5–8]. Changes in many of these factors are believed to reflect an exacerbated maternal response to pregnancy, which itself is considered as a low grade systemic inflammation [9]. One study has shown that serum from preeclamptic women is cytotoxic to endothelial cells *in vitro*, and that clinical condition improves drastically after 24–48 h postpartum when the cytotoxic activity is dramatically reduced [10].

Endothelial injury in PE is evidenced by the appearance of morphological lesions, glomerular endotheliosis, and increased circulating levels of fibronectin, von Willebrand factor and cytokines, all of which can be secreted by endothelial cells as an inflammatory reaction [11,12]. Endothelial cells can also alter their synthesis of vaso-relaxing/vasoconstrictors and pro/anticoagulants

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upon endothelial injury in PE [11], and the damage to the maternal vasculature can persist many years after PE. A study revealed vascular endothelial defects in women even three years after a PE pregnancy, and the data suggested that the impairment was more severe with recurrent PE [13]. PE-induced endothelial injury has long-term and harmful consequences, for instance, women who had PE have a higher risk of developing cardiovascular diseases many years postpartum [14].

PE can be classified into two distinct subtypes, early-onset which occurs before 34 weeks of gestation, and late-onset PE which occurs after 34 weeks [15]. Emerging evidence strongly suggests that the two PE subtypes have vastly different etiologies, and early-onset PE poses a far more significant maternal risk, with a 20-fold higher mortality rate than late-onset PE [16–19]. The risk of cardiovascular disease is also much higher in women who have had early-onset than late-onset PE [20–22], suggesting that endothelial injury is more profound in early-onset than late-onset PE.

We have recently reported that high temperature requirement A4 (HtrA4) is a placenta-specific protease that is significantly increased in the circulation of early-onset PE [23]. HtrA4 belongs to a serine protease family that also includes HtrA1, HtrA2 and HtrA3. These proteins are known to function as ATP-independent protein quality control factors to regulate cellular processes such as proliferation, unfolded stress response, programmed cell death and aging [24]. All HtrAs contain a trypsin-like serine protease domain and are proven to have catalytic activities [25]. HtrA1, HtrA3 and HtrA4 share a similar domain structure and are secreted out of cells, whereas HtrA2 has a transmembrane domain and is localized in mitochondria [26]. HtrA4 is the newest member of the mammalian HtrA family, it is expressed only by the placenta and secreted into the maternal circulation [23]. In a normal pregnancy, HtrA4 serum levels increase progressively to around 24–25 weeks of gestation, then remain stable throughout the remainder of the pregnancy [23].

Our study showed that in early-onset PE, both the placental production and circulating levels of HtrA4 are significantly increased [23]. We further demonstrated that HtrA4 at high concentrations seen in early-onset PE disrupted the tube formation of human umbilical vein endothelial cell (HUVEC), disturbed cellular integrity and increased cellular permeability [23]. These results suggest high levels of circulating HtrA4 of placental origin may contribute to endothelial dysfunction and the development of early-onset PE.

In this study, using HUVEC as a model, we first examined the impact of HtrA4 on expression of endothelial genes involved in vessel cell biology by an array approach, then validated the data by real-time RT-PCR and ELISA.

2. Materials and methods

2.1. Cell culture

HUVECs (ATCC, Maryland, USA) were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, and maintained in DMEM (Thermo Fisher Scientific, VIC, Australia) supplemented with 1% antibiotics (Thermo Fisher Scientific), 2 mM L-glutamine (Sigma-Aldrich, Missouri, USA), 1 mM sodium pyruvate (Thermo Fisher Scientific) and 10% fetal bovine serum (Thermo Fisher Scientific). The starting passage of the HUVECs was 13, and the experiments were completed within eight passages. The cells were cultured in 12-well plates (Thermo Fisher Scientific) at 1×10^5 per well density for 24 h (h), then treated with recombinant HtrA4 (BioTeZ, Berlin, Germany, 1.5 μg/ml or 3.0 μg/ml) or vehicle control for 24 or 48 h. The vehicle control contains 150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 0.05% Brij 35 solution, 50 mM imidazole in ultrapure H_2O. The two doses of HtrA4 were chosen to represent the median and highest levels of HtrA4 found in early-onset PE circulation [23]. After the treatment, media were collected and cells were used for RNA extraction. The experiment was repeated four times.

2.2. RNA extraction

RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and contaminating DNA was removed using RNase-free DNase (Qiagen) according to the manufacturers' protocols. The RNA concentration was determined using Nanodrop ND-1000 (Thermo Fisher Scientific).

2.3. PCR array for endothelial cell biology

RNA samples from vehicle control or 3 μg/ml HtrA4 treatment for 24 h were pooled from three independent experiments, and 500 ng of the pooled RNA presenting control and HtrA4 treatment (3 μg/ml, 24 h) was reverse transcribed into complementary DNA (cDNA) using RT^2 First Strand Kit (Qiagen). A RT^2 Profiler 84 gene PCR array (Qiagen) was screened as per manufacturer's instruction on an ABI 7900 HT Fast real-time machine (Applied Biosystems, VIC, Australia). The results were analysed using Qiagen RT^2 profiler PCR array data analysis software.

2.4. Real-time RT-PCR analysis

Genes showing more than 2-fold differences in expression on the array between the vehicle control and HtrA4 treatment were validated by real-time RT-PCR. RNA (300 ng) from three independent experiments of 24 h and 48 h treatment with 0, 1.5 μg/ml and 3.0 μg/ml HtrA4 was reverse transcribed in 20 μl using SuperScript III First-Strand kit (Invitrogen, VIC, Australia) as per manufacturer's protocol. Real-time RT-PCR was performed with primers specified in Supplementary Table 1 on an ABI 7900 HT Fast real-time machine with the following conditions: 1) 95 °C for 10 min for enzyme activation, 2) 40 cycles of denaturation (15 s at 95 °C), annealing (5 s at 58 °C), extension (10 s at 72 °C), and a single fluorescence measurement at 70–75 °C for quantitation, and 3) dissociation curve assessment between 60 °C and 95 °C with continuous fluorescence measurement. All cDNA samples were diluted 1:80, and PCR reaction was prepared to a final volume of 10 μl with 5 μl SYBR Green PCR Master Mix (Applied Biosystems), 4 μl diluted cDNA sample and 0.5 μM final concentration of forward and reverse primers. All samples were run in triplicates, each gene expression were normalised to 18 S, and fold changes were calculated using ΔΔCt.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The secreted levels of interleukin (IL)6 and monocyte chemoattractant protein (MCP)1 in the above treated cell media were measured by ELISA (Ray Biotech, Georgia, USA) according to manufacturer's instruction.

2.6. Cytokine antibody array

A cytokine antibody array for 36 cytokines (R&D System, Minnesota, USA) was analysed to determine whether HtrA4 alters these cytokines in HUVECs. Conditioned media from cells treated with vehicle control or 3.0 μg/ml HtrA4 for 24 h from four independent experiments were pooled for the array analysis. Dots representing 36 cytokines were analysed by densitometry using ImageJ software (National Institutes of Health, USA).

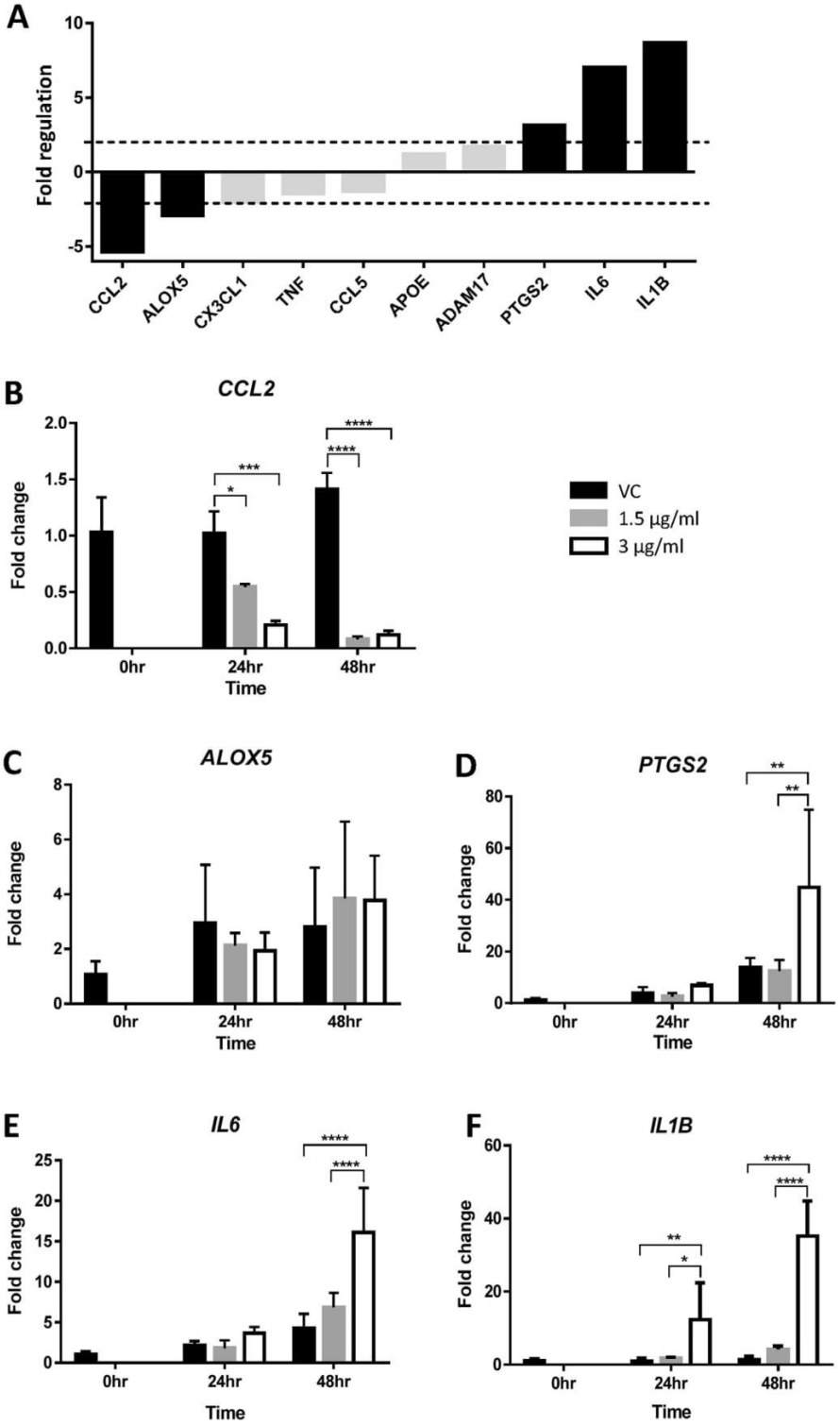

Fig. 1. HtrA4-induced changes in mRNA expression of genes involved in inflammatory responses. A) PCR array data. RNA samples from vehicle control (vc) or 3 μg/ml HtrA4 treatment for 24 h were pooled from 3 experiments for the array. Data is expressed as HtrA4-induced fold changes relative to the control. Genes that showed >2-fold differences (in black bar) were chosen for validation by real-time RT-PCR. (B–F) Real-time RT-PCR analysis of *CCL2* (B), *ALOX5* (C), *PTGS2* (D), *IL6* (E) and *IL1B* (F). Cells were treated with 0, 1.5 μg/ml or 3 μg/ml HtrA4 for 0, 24 or 48 h, n = 3. Data is expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

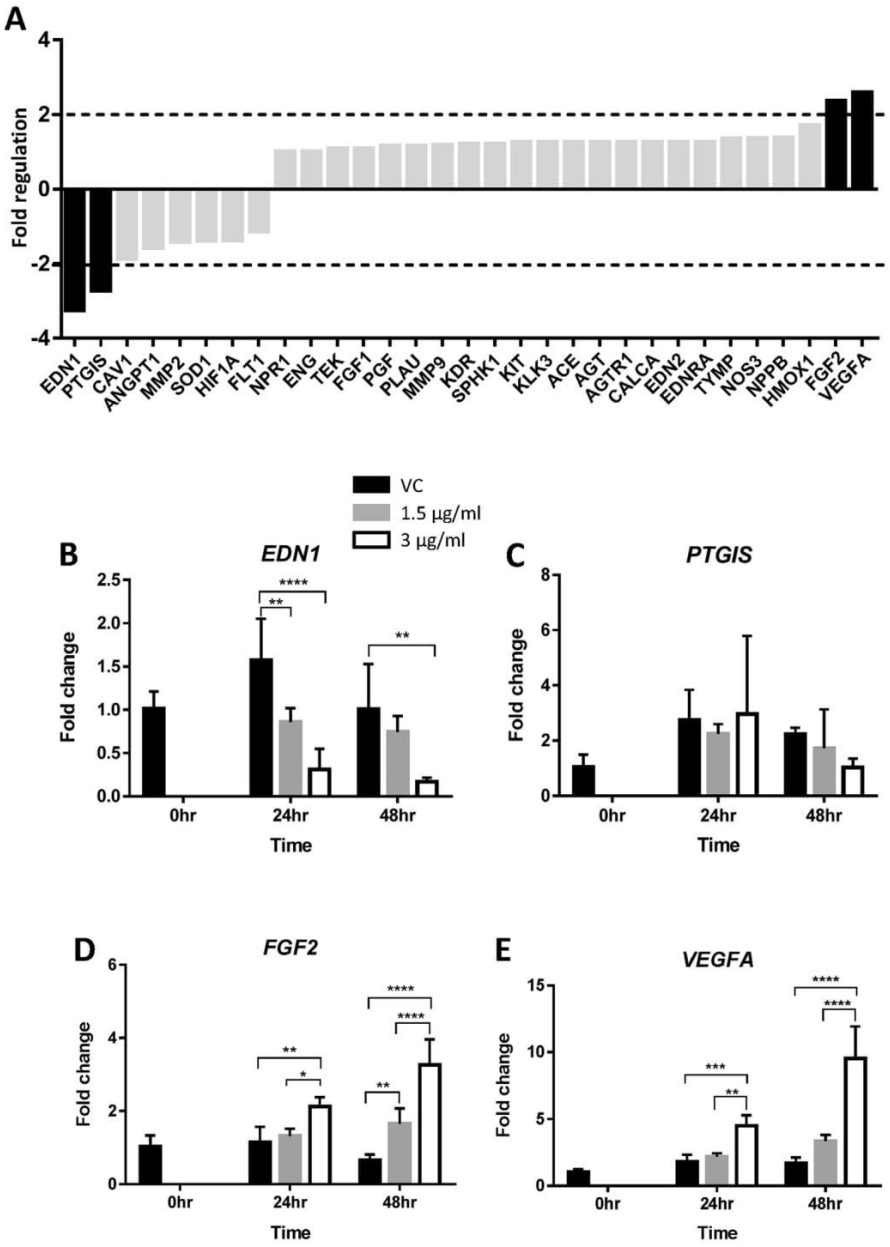

Fig. 2. HtrA4-induced changes in mRNA expression of genes involved in vaso-activities and angiogenesis. A) PCR array data. RNA samples from vehicle control (vc) or 3 μg/ml HtrA4 treatment for 24 h were pooled from 3 experiments for the array. Data is expressed as HtrA4-induced fold changes relative to the control. Genes showed >2-fold differences in mRNA (in black bar) were chosen for validation by real-time RT-PCR. (B–E) Real-time RT-PCR analysis of *EDN1* (B), *PTGIS* (C), *FGF2* (D) and *VEGFA* (E). Cells were treated with 0, 1.5 μg/ml or 3 μg/ml HtrA4 for 0, 24 or 48 h, n = 3. Data is expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

2.7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism (v.6, GraphPad Software Inc., CA). Data are expressed as mean ± SD, comparison was made using two-way ANOVA followed by Tukey's post-hoc tests, and difference was considered significant if $p < 0.05$.

3. Results

We used HUVECs as a model and examined the effect of HtrA4 on the expression of 84 genes that are involved in endothelial cell biology (Supplementary Table 2). These genes were broadly categorised into four groups: inflammation, angiogenesis and vaso-activities, platelet activation and cell adhesion, coagulation and apoptosis. Total RNA from cells treated with vehicle control or 3 μg/ml HtrA4 (highest level detected in early-onset PE serum) for 24 h

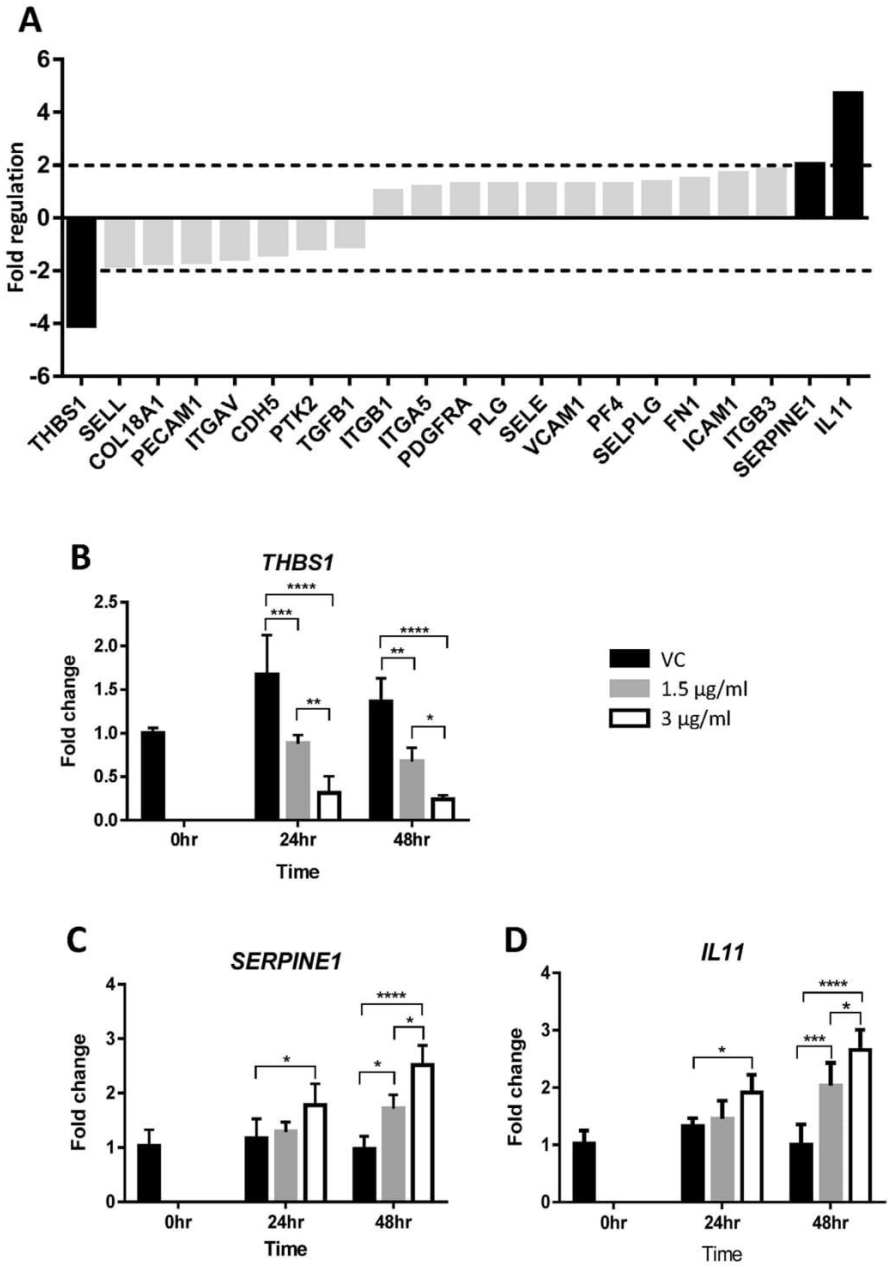

Fig. 3. HtrA4-induced changes in mRNA expression of genes involved in cell adhesion and platelet activation. A) PCR array data. RNA samples from vehicle control (vc) or 3 μg/ml HtrA4 treatment for 24 h were pooled from 3 experiments for the array. Data is expressed as HtrA4-induced fold changes relative to the control. Genes showed >2-fold differences in mRNA (in black bar) were chosen for validation by real-time RT-PCR. (B–D) Real-time RT-PCR analysis of *THBS1* (B), *SERPINE1* (C) and *IL11* (D). Cells were treated with 0, 1.5 μg/ml or 3 μg/ml HtrA4 for 0, 24 or 48 h, n = 3. Data is expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

was analysed on the array. Genes that showed more than 2-fold changes in expression were then validated by real-time RT-PCR for time and dose dependency, using cells treated with vehicle control, 1.5 μg/ml or 3 μg/ml HtrA4 for 0, 24 or 48 h. The array data are presented together with the real-time RT-PCR validation for each of the four gene groups categorised above.

3.1. HtrA4 regulation of genes involved in endothelial inflammation

Among the ten genes related to inflammation, five were altered by HtrA4 by more than 2 folds on the PCR array, two down-regulated and three up-regulated (Fig. 1A). These five genes were further examined by real-time RT-PCR for time and HtrA4 dose dependency. For the two down-regulated genes, *CCL2* (also known as *MCP1*) and *ALOX5*, *CCL2* mRNA was significantly reduced by

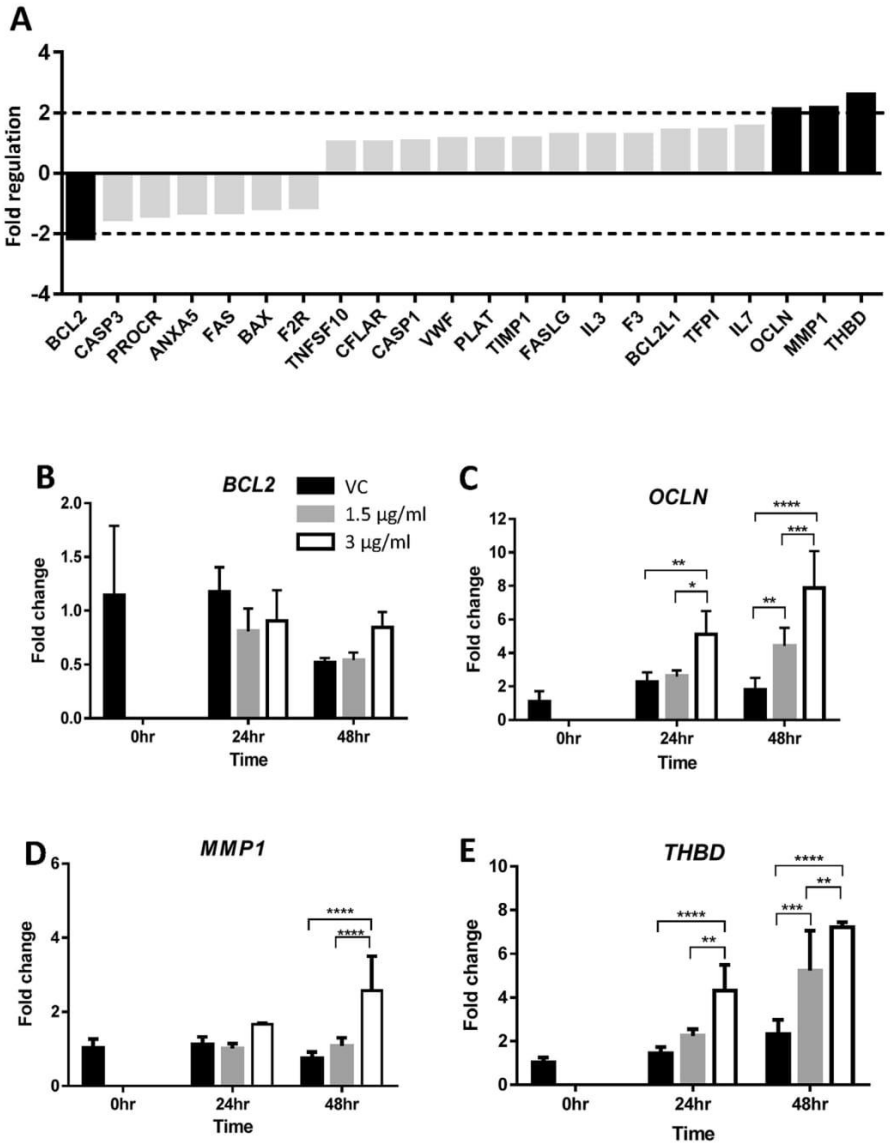

Fig. 4. HtrA4-induced changes in mRNA expression of genes involved in apoptosis and coagulation. A) PCR array data. RNA samples from vehicle control (vc) or 3 μg/ml HtrA4 treatment for 24 h were pooled from 3 experiments for the array. Data is expressed as HtrA4-induced fold changes relative to the control. Genes showed >2-fold differences in mRNA (in black bar) were chose for validation by real-time RT-PCR. (B–E) Real-time RT-PCR analysis of *BCL2* (B), *OCLN* (C), *MMP1* (D) and *THBD* (E). Cells were treated with 0, 1.5 μg/ml or 3 μg/ml HtrA4 for 0, 24 or 48 h, n = 3. Data is expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data shown as mean ± SD.

HtrA4 even at the lower dosage of 1.5 μg/ml; and the reduction was more profound at 48 h than 24 h (Fig. 1B). Although *ALOX5* showed a 2.9-fold reduction on the array, real-time RT-PCR did not find significant changes in *ALOX5* mRNA across all treatment conditions (Fig. 1C). This is likely due to the low expression of *ALOX5* in HUVECs. For the three up-regulated genes (Fig. 1A), *PTGS2* (also called *COX2*), *IL6* and *IL1B* were all validated by real-time RT-PCR. *PTGS2* mRNA was significantly up-regulated by 3 μg/ml HtrA4 at 48 h time point (Fig. 1D). *IL6* mRNA was significantly increased by 3 μg/ml HtrA4 at the longer time point (Fig. 1E). In contrast, *IL1B* expression was increased by HtrA4 dose- and time-dependently (Fig. 1F).

3.2. HtrA4 regulation of genes associated with angiogenesis and vaso-activities

Thirty-one genes on the array were categorised into this group (Fig. 2A). The PCR array analysis identified two down-regulated (*EDN1* and *PTGIS*) and two up-regulated (*FGF2* and *VEGFA*) genes (Fig. 2A). Real-time RT-PCR confirmed that *EDN1* was significantly down-regulated by HtrA4 in a dose- and time-dependent manner (Fig. 2B), but *PTGIS* mRNA was not altered by HtrA4 (Fig. 2C). The two up-regulated genes, *FGF2* and *VEGFA*, were confirmed by real-time PCR to be significantly increased by HtrA4 (Fig. 2D–E). The up-regulation was greatest at 48 h with 3 μg/ml HtrA4 treatment (Fig. 2D–E).

Table 1
List of genes that were validated by real-time RT-PCR to be significantly affected by HtrA4.

Gene category	Gene name	Regulation by HtrA4	Functions in endothelial cells
Inflammatory Response	*CCL2/ MCP1*	↓	Involved in immnoregulatory and inflammatory processes, recruitment of monocytes and macrophages
	PTGS2/ COX2	↑	A key enzyme in prostaglandin biosynthesis, and is involved in inflammation and mitogenesis
	IL6	↑	Major functions in inflammation and the maturation of B cells, highly up-regulated in PE circulation
	IL1B	↑	Mediates inflammatory response and various cellular activities, including proliferation and differentiation
Angiogenesis and Vaso-activities	*EDN1/ ET1*	↓	A secreted peptide that acts as a vasoconstrictor
	FGF2	↑	Involves in ranges of biological processes, including mitogenic and angiogenic activities
	VEGFA	↑	Acts specifically on endothelial cells and has various functions, including angiogenesis and cell growth
Platelet Activation and Cell Adhesion	*THBS1*	↓	Mediates cell-to-cell and cell-to-matrix interactions and plays a role in platelet aggregation and angiogenesis
	SERPINE1	↑	A major inhibitor of tissue plasminogen activator and urokinase, acts on blood vessels to inhibit fibrinolysis
	IL11	↑	Stimulates the proliferation of hematopoietic cells and megakaryocyte to increase platelet production
Coagulation and Apoptosis	*OCLN*	↑	An integral membrane protein that is required for cytokine-induced regulation of the tight junction
	MMP1	↑	Involves in the breakdown of extracellular matrix in many physiological processes
	THBD	↑	An endothelial-specific receptor that binds to thrombin to activate protein C, which degrades clotting factors. It is a marker for endothelial dysfunction

3.3. HtrA4 regulation of cell adhesion and platelet activation genes

Twenty-two genes on the array were in this category (Fig. 3A). The PCR array identified one down-regulated (*THBS1*) and two up-regulated (*SERPINE1* and *IL11*) genes (Fig. 3A). *THBS1* is responsible for cell adhesion, whereas *SERPINE1* and *IL11* are involved in platelet activation. Real-time RT-PCR confirmed that *THBS1* mRNA was significantly down-regulated by HtrA4 in a dose- and time-dependent manner (Fig. 3B). Both *SERPINE1* and *IL11* were also validated by rea-time RT-PCR to be significantly up-regulated by HtrA4 dose- and time-dependently (Fig. 3C–D).

3.4. HtrA4 regulation of genes involved in apoptosis and coagulation

There were twenty-two genes in this group (Fig. 4A). The PCR array identified one down-regulated (BCL2) and three up-regulated (*OCLN*, *MMP1* and *THBD*) genes (Fig. 4A). *BCL2*, which is essential for apoptosis, showed no HtrA4-induced changes in mRNA expression by real-time RT-PCR (Fig. 4B). For the three up-regulated genes, *OCLN*, encoding a cell-junction protein, was confirmed by real-time RT-PCR to be up-regulated by HtrA4, and most prominently at 48 h (Fig. 4C). For the two up-regulated genes that are involved in coagulation, *MMP1* mRNA was significantly increased by 3 μg/ml HtrA4 at 48 h (Fig. 4D). In contrast, *THBD* mRNA was significantly increased by HtrA4 in a dose- and time-dependent manner (Fig. 4E).

In total, thirteen genes were confirmed by real-time RT-PCR to be significantly altered by HtrA4, ten were up-regulated and three down-regulated (Table 1). Functions of these thirteen genes and their potential relevance to endothelial dysfunction and PE are also summarised in Table 1.

3.5. Validation of HtrA4 regulation of pro-inflammatory factors at the protein level

As heightened inflammation is a key feature of endothelial dysfunction and PE, we selected two genes from the inflammation group, one up-regulated (*IL6*, Fig. 1F) and one down-regulated (*CCL2*, Fig. 1B) for validation at the protein level to further confirm the real-time RT-PCR data. The levels of these two cytokines in HUVEC media were measured by ELISA. IL6 protein was significantly increased by 3 μg/ml HtrA4 at 48 h time points, the lower dose of HtrA4 (1.5 μg/ml) and the shorter time point (24 h) had no significant effect on IL6 protein (Fig. 5A). This is highly consistent with the real-time RT-PCR data (Fig. 1E). On the other end of the spectrum, the only down-regulated inflammatory gene, *CCL2*, which encodes MCP1, was significantly reduced at the protein level by HtrA4 in a dose- and time-dependent manner (Fig. 5B), and the pattern is identical to its mRNA changes (Fig. 1B).

As both IL6 and MCP1 are cytokines and only a few cytokines were on the PCR array, we further examined whether HtrA4 affects a broad range of cytokines using an antibody array for 36 cytokines (Fig. 5C). HUVEC media treated with either vehicle control or 3 μg/ml HtrA4 for 24 h were pooled from four independent experiments and applied onto the cytokine array. Of the thirty-six cytokines examined, IL6 showed a 4.6-fold increase and MCP1 displayed a 11-fold reduction by HtrA4 compared to vehicle control (Fig. 5C), the other thirty-four cytokines examined showed no significant difference between vehicle control and HtrA4 treatment (Fig. 5C). These data further confirmed the specific and opposing effect of HtrA4 on IL6 and MCP1.

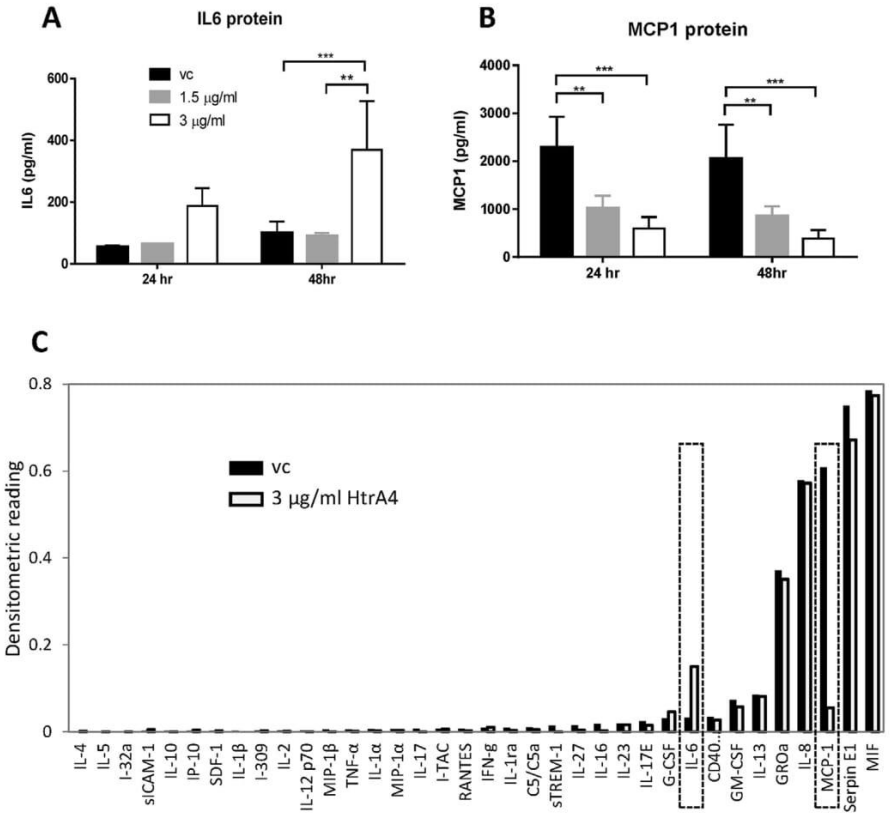

Fig. 5. HtrA4-induced changes in cytokines at the protein level. A-B) ELISA detection of IL6 (A) and MCP1 (B) in media of HUVECs following treatment with 0, 1.5 μg/ml or 3 μg/ml HtrA4 for 24 or 48 h, n = 4. Data is expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. C) Analysis of 36 cytokines by an antibody array. Media from HUVECs treated with vehicle control (vc) or 3 μg/ml HtrA4 for 24 h were pooled from 4 independent experiments and analysed on the array. The graph shows densitometric reading of each cytokine. Significant changes were detected only for IL6 and MCP1, consistent with the ELISA data shown in (A–B).

4. Discussion

This study demonstrated that HtrA4 significantly affected endothelial cell gene expression. At a concentration found in early-onset PE circulation, HtrA4 profoundly altered a range of endothelial genes related to inflammation, vaso-activity, angiogenesis, cell adhesion, platelet activation and coagulation. HtrA4 also significantly increased HUVEC release of pro-inflammatory cytokine IL6.

Several studies reported that maternal serum IL6 is significantly increased in PE patients [27]. One study in particular demonstrated that serum IL6 was much higher in women with early-onset PE compared to late-onset PE or normotensive controls [28]. The same study also showed that placental IL6 expression was much lower in early-onset PE, and suggested that the increase in serum IL6 was more likely resulted from maternal endothelial dysfunction than a direct consequences of defective placenta [28]. Our current study provides strong support for this view. Furthermore, HtrA4 also significantly up-regulated two other major pro-inflammatory genes, *PTGS2* (*COX2*) and *IL1B*, further suggesting that the circulating HtrA4 of placenta-origin can intensify the inflammatory response of maternal endothelial cells, which is frequently observed in early-onset PE [29–31].

Additional inflammatory factors such as TNF-α are reported to be significantly elevated in preeclamptic serum [9,32], it is likely that many of these factors are released directly by the placenta. Endothelial cells are always considered to be the major site of inflammatory response in PE, this study supports this view and suggests that maternal endothelial cells also release multiple inflammatory factors to the circulation as a response to placental factors such as HtrA4.

The only down-regulated gene identified in the inflammation category was *CCL2*, which encodes for MCP-1. The main function of this protein is to recruit monocytes or macrophages to the site of inflammation as a result of tissue injury or infection [33,34]. MCP1 is involved in many human diseases, and is expressed in many cell types including the endothelial cells [33]. MCP1 is reported to be higher in PE women [35], however, the majority of these reports relate to placental production of MCP1, which acts locally to recruit macrophages from the blood stream across the endothelium [36,37]. In particular, MCP1 secretion is reported to be elevated in placental mesenchymal stromal cells isolated from PE patients [38]. However, the regulation of MCP1 expression in endothelial cells and its role in PE has not been investigated. Our data showed a significant reduction of endothelial MCP1 (both mRNA and protein) by HtrA4, this may have important implications in the effectiveness of endothelial cells to recruit macrophages.

Our data also showed that HtrA4 affected several regulatory factors of endothelial cell biology. For Instance, *THBD*, an endothelial cell-specific membrane-bound receptor that functions as an anticoagulant, was highly up-regulated by HtrA4. This is consistent with other studies showing that serum THBD is increased in PE

women compared to normal controls [39,40], and that THBD is a potential marker of endothelial dysfunction to predict PE [39]. Furthermore, increased levels of THBD in the maternal circulation negatively correlate to infant birth weight, and the highest plasma THBD level was detected in PE pregnancy with intrauterine growth restriction, which is often associated with early-onset PE. It was suggested that the damage to endothelium as a result of THBD dysregulation could have a major impact on fetal development [41].

SERPINE1, another gene up-regulated by HtrA4, encodes the inhibitor for fibrinolysis and is also elevated in the circulation of PE women [42]. Dysregulation of SERPINE1 protein is reported to inhibit fibrin degradation, leading to fibrosis and formation of blood clot in the blood vessel [43]. *THBS1*, which is reported to be lower in women with severe PE [44], was significantly down-regulated by HtrA4. THBS1 is involved in many regulatory processes of endothelial cell function such as adhesion, motility and proliferation, and *THBS1* down-regulation can significantly impair the normal cellular function of endothelial cells [44].

Surprisingly, only a small number of genes in the angiogenic and vasoactive group were found to be regulated by HtrA4. This suggest that endothelial genes in this group may not be the major targets of HtrA4 action, and other placental-derived factors may be responsible for their dysregulation and contribute to the endothelial dysfunction.

Overall, our study suggests that high levels of placental-derived HtrA4 that is circulating in early-onset PE women is a potential causal factor of endothelial dysfunction. HtrA4 profoundly altered HUVEC expression of several factors essential for normal endothelial cell function and inflammation responses. As endothelial dysfunction is a major aspect of early-onset PE development, our study provides new insight into the underlying causes of this disease.

One limitation of this study is that HUVEC was used as a model. HUVEC is a well characterized cell line and commonly used for studying endothelial biology, but it may not reflect all the features of human vascular endothelial cells. Future studies will confirm the impact of HtrA4 on endothelial cells using other cell models.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2016.09.003.

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Chapter 3

HtrA4 may play a major role in inhibiting endothelial repair in pregnancy complication preeclampsia

SCIENTIFIC REPORTS

OPEN

HtrA4 may play a major role in inhibiting endothelial repair in pregnancy complication preeclampsia

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Preeclampsia (PE) is a life-threatening complication of human pregnancy with no effective treatment other than premature delivery. It is hallmarked by systemic endothelial injury/dysfunction which is believed to be caused by abnormal levels/types of placenta-derived factors that are circulating in the maternal blood. Emerging evidence suggests that endothelial repair is also dysregulated in PE, as circulating endothelial progenitor cells (EPCs) critical for endothelial regeneration are reduced in number and functionality. However, the underlying mechanisms are poorly understood. HtrA4 is a placenta-specific protease that is secreted into the circulation and significantly elevated in early-onset PE. Here we investigated the impact of HtrA4 on endothelial proliferation and repair. We demonstrated that high levels of HtrA4 halted endothelial cell proliferation and significantly down-regulated a number of genes that are critical for cell cycle progression, including *CDKN3*, *BIRC5*, *CDK1* and *MKI67*. Furthermore, HtrA4 significantly inhibited the proliferation of primary EPCs isolated from term human umbilical cord blood and impeded their differentiation into mature endothelial cells. Our data thus suggests that elevated levels of HtrA4 in the early-onset PE circulation may impair endothelial cell repair, not only by halting endothelial cell proliferation, but also by inhibiting the proliferation and differentiation of circulating EPCs.

Preeclampsia (PE) is a serious disorder of human pregnancy that affects 2–8% of pregnancies worldwide[1,2]. PE is characterized by a *de novo* hypertension accompanied by proteinuria and/or organ dysfunction[3,4]. The condition can progress rapidly leading to multi-organ failure, with symptoms closely linked to wide-spread endothelial dysfunction[5]. Currently, the only effective treatment of PE is the premature delivery of the fetus, along with the problematic organ – the placenta.

PE can be classified into two distinct subtypes: early-onset which occurs before 34 weeks of gestation, and late-onset which occurs after 34 weeks[6]. The two PE subtypes may have different aetiologies. Early-onset PE is associated primarily with inadequate trophoblast invasion during early placentation, which leads to placental ischemia and reduced blood supply to the foetus later in pregnancy[7,8]. Late-onset PE is less likely linked to abnormal trophoblast invasion, suggesting that other factors are involved in the disease development[9].

Early-onset PE poses far more significant maternal risks, with significant higher mortality rate compared to late-onset PE[10,11]. The risk of cardiovascular disease is also much higher in women who have had early-onset than late-onset PE[12–14], suggesting that endothelial dysfunction is more profound in early-onset PE and persists long after the pregnancy[15]. Markers of endothelial dysfunction such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 remain elevated in women even 15 years after their preeclamptic pregnancy[16]. This is consistent with the view that endothelial dysfunction resulting from PE may account for the increased risk of cardiovascular diseases in women with a history of preeclamptic pregnancies[17]. These data

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suggest that early-onset PE has a long-lasting effect on endothelial cells that is not restored after the symptoms of PE have been resolved.

Endothelial progenitor cells (EPCs) are a unique population of cells that circulate in the blood, and are recruited to the endothelium upon endothelial injury, where they then differentiate into resident endothelial cells to regenerate the blood vessels and restore endothelial function[18,19]. In the non-pregnant population, reduction in circulating EPCs is associated with increased cardiovascular risks, highlighting the importance of EPCs in the maintenance of endothelial function[20]. EPC numbers and migratory activities are inversely correlated to risk factors of coronary artery disease[21]. Notably, EPCs isolated from patients with type II diabetes have impaired proliferation, adhesion and angiogenic activity[22]. In normal human pregnancy, the maternal endothelium undergoes extensive remodelling and repair, where circulating EPCs are suggested to play a major role in endothelial repair[23–25]. One study has reported that EPC numbers progressively increase in normal pregnancy and the highest levels are detected in the third trimester[26]. The same study has also demonstrated that circulating EPC numbers in the third trimester are significantly lower in pregnancies that are complicated by intrauterine growth restriction[26]. Other studies have reported that maternal as well as fetal/placental EPCs are significantly reduced in PE[27–29]. EPCs isolated from umbilical cord blood of preeclamptic pregnancies have impaired proliferation, migration and vasculogenesis in culture[27]. Furthermore, circulating EPCs in early-onset PE are reported to exhibit increased senescence[30]. These studies suggest that EPCs may play an important role in normal pregnancy but they are reduced in number and functionality in PE. However, it is unknown how EPCs are compromised in PE.

It is well established that in PE the placenta releases abnormal types/amounts of factors into the maternal circulation, which contribute to endothelial dysfunction and the maternal syndrome of PE[31]. Factors that are significantly elevated in the PE circulation include cytokines, antiangiogenic factors, syncytiotrophoblast microparticles and activated leukocytes[32–35]. Some of these are shown to induce endothelial injury and dysfunction, especially in the case of early-onset PE[31]. However, whether these circulating placental factors compromise EPCs in PE is not well understood.

We have previously reported that high temperature requirement factor A4 (HtrA4) is a placenta-specific serine protease that is released into circulation and significantly increased in early-onset PE[36]. HtrA4 belongs to a serine protease family that serves as ATP-independent protein quality control factors in regulating cell growth, unfolded stress response, and aging[37]. HtrA4 contains a trypsin-like serine protease domain, and a highly conserved C-terminal PDZ domain which regulates protein-protein interaction[38]. In a normal human pregnancy, serum HtrA4 level increases progressively to around 24–25 weeks of gestation, then remains relatively stable throughout the remainder of the pregnancy[36]. However, the exact role of HtrA4 in placental development remains unclear. To date, two studies suggest that HtrA4 may directly regulate trophoblast function, but the results are somewhat conflicting as one shows that HtrA4 promotes trophoblast invasion[39], whereas the other reports that HtrA4 inhibits trophoblast cell migration and growth[40]. In early-onset PE, placental expression as well as circulating levels of HtrA4 are significantly increased at the time of disease presentation[36,40–44]. We have further demonstrated that elevated concentrations of HtrA4 detected in early-onset PE circulation disrupt human umbilical vein endothelial cell (HUVEC) tube formation and induce pro-inflammation[36,45]. These results suggest that high levels of circulating HtrA4 of placental origin may dysregulate maternal endothelial cell function and contribute to the development of early-onset PE.

The aim of this study was to investigate the impact of elevated HtrA4 in the circulation of early-onset PE on maternal endothelial cell proliferation and repair. We first used HUVECs as an endothelial model, then validated the data in primary EPCs. Since EPCs from maternal and fetal origin exhibit similar phenotypes and characteristics once they are in culture[46], we isolated EPCs from umbilical cord blood of term human pregnancy and used as an EPC model for the current study.

Results

HtrA4 inhibits HUVEC proliferation. To assess proliferation, HUVECs were treated with vehicle control or two doses (3.0 ug/ml and 1.5 ug/ml) of HtrA4 for 0, 24 and 48 h, and cell number was counted at each time point (Fig. 1a). The two HtrA4 doses were chosen to represent the median and highest levels of HtrA4 detected in the early-onset PE circulation[36]. Cells treated with vehicle control steadily increased number over the 48 h period as expected (Fig. 1a). While 1.5 μg/ml HtrA4 had no obvious effect on cell growth, 3.0 μg/ml HtrA4 significantly inhibited cell proliferation (Fig. 1a). Cells from all three treatment groups were equally viable (Fig. 1b), suggesting that HtrA4 did not induce cell death while inhibiting proliferation.

We next examined the expression of cell proliferation marker Ki67 by immunocytochemistry (Fig. 1c). The majority of the cells in the control group showed clear positive nuclear staining of Ki67. However, the intensity of Ki67 staining was much lower in HtrA4-treated cells (Fig. 1c). Quantification showed that HtrA4 dose-dependently reduced Ki67 staining, and that 3.0 μg/ml HtrA4 significantly reduced the number of Ki67-positive cells (Fig. 1d).

HtrA4 suppresses cell cycle gene expression in HUVECs. To understand how HtrA4 inhibits HUVEC proliferation, we examined the effect of HtrA4 on cell cycle gene expression using a RT2 Profiler PCR array. This array contained 84 genes which were broadly categorised into four groups (Table 1). Total RNA isolated from cells treated with vehicle control or 3.0 μg/ml HtrA4 for 24 h was analysed on the array, and a total of 35 genes across the four categories showed more than 2-fold down-regulation by HtrA4 (Table 1, Fig. 2a).

These 35 genes were next validated by real-time RT-PCR for time and HtrA4 dose dependency using RNA samples isolated from cells treated with vehicle control, 1.5 μg/ml or 3.0 μg/ml HtrA4 for 0, 24 or 48 h. Validation of the four most highly regulated genes of each category are presented in Fig. 2b–e. *CDKN3*, which is involved in G1 and S phase of the cell cycle, was reduced 4-fold by HtrA4 on the array (Fig. 2a). RT-PCR confirmed that *CDKN3* was significantly down-regulated by HtrA4 dose-dependently at both 24 and 48 h (Fig. 2b). *BIRC5*,

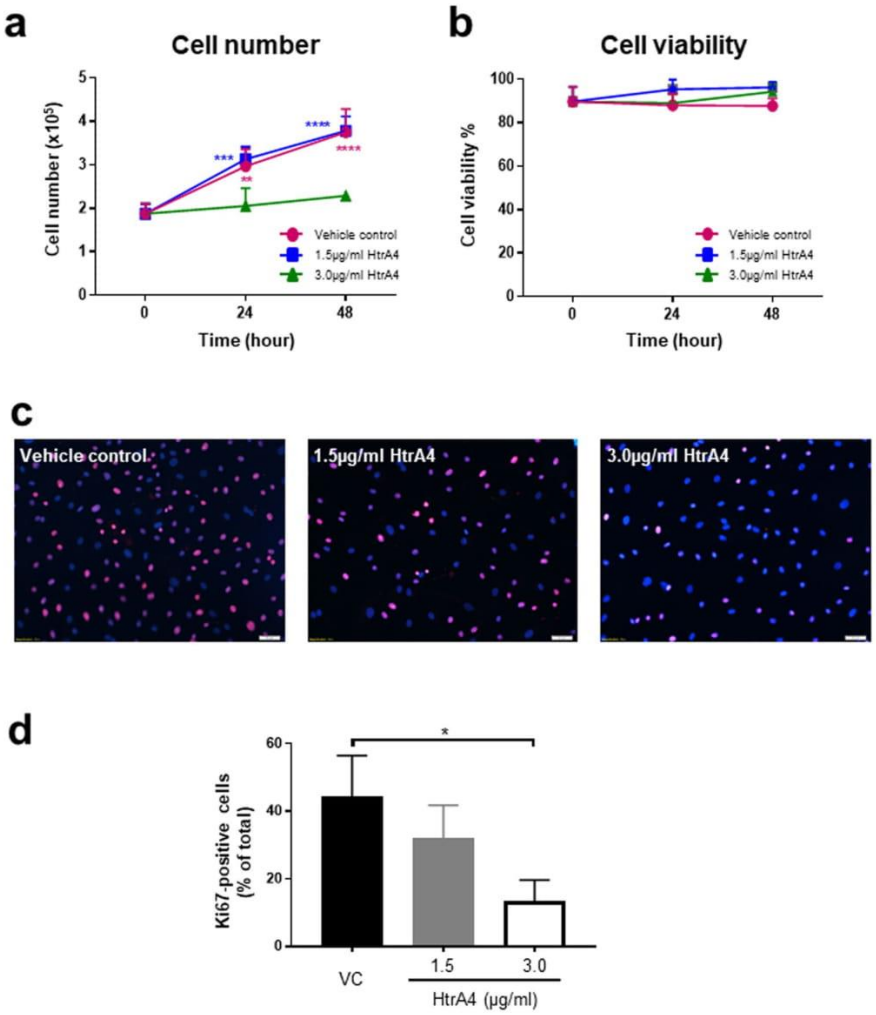

Figure 1. HtrA4 inhibits HUVEC proliferation. HUVECs were treated with vehicle control or two doses of HtrA4 over 48 h. (**a**) Growth curve. (**b**) Percentage of viable cells. (**c**) Immunofluorescence of cell proliferation marker Ki67 at 24 h, representative images at 10x magnification are shown. (**d**) Quantification of Ki67 staining of (**c**). n = 3. Data is expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

which is involved in the G2 and M phase of the cell cycle, showed the biggest reduction by HtrA4 on the array (Fig. 2a). RT-PCR also confirmed that *BIRC5* was significantly down-regulated by HtrA4 both dose-dependently and time-dependently (Fig. 2c). *CDK1*, which is involved in cell cycle checkpoint, was reduced 4-fold by HtrA4 on the array (Fig. 2a). This gene was also validated by RT-PCR, it was significantly down-regulated by HtrA4 in a dose- and time-dependent manner (Fig. 2d). *MKI67*, which encodes Ki67 protein that is essential for cell proliferation and cell cycle regulation, was likewise reduced by HtrA4 on the array (Fig. 2a). *MKI67* expression was again confirmed by RT-PCR to be significantly down-regulated by HtrA4 dose- and time-dependently (Fig. 2e). This data is consistent with the immunofluorescent staining of Ki67 protein presented in Fig. 1c. All these four genes showed most profound reductions in expression when cells were treated with 3.0 μg/ml HtrA4, consistent with cell proliferation data presented in Fig. 1a. All the other remaining 31 genes that were down-regulated by HtrA4 on the array (Fig. 2a), were also validated by real-time RT-PCR and the results are presented in Supplementary Fig. 1A–D.

Isolation and validation of primary EPCs from human cord blood. We next investigated the impact of HtrA4 on primary EPCs. Accordingly, EPC clones were isolated from umbilical blood from four individual pregnancies. Their identity as EPCs was verified through a combination of transcriptional and functional profiles (Fig. 3). First, gene expression of key markers was examined by RT-PCR along with a monocyte sample as a negative control (Fig. 3a). The genes examined included CD144, CD45 and PECAM1. CD144 is an EPC-specific marker, it was exclusively expressed by all four EPC clones but not by monocytes. CD45 is a monocyte-specific

Gene category	Gene name	Full name	Fold change relative to the control
G1 Phase & S Phase	*CDKN3*	Cyclin Dependent Kinase Inhibitor 3	−4.3
	MCM4	Minichromosome Maintenance Complex Component 4	−4.3
	CDC25A	Cell Division Cycle 25A	−3.8
	SKP2	S-Phase Kinase Associated Protein 2	−3.1
	CDC6	Cell Division Cycle 6	−2.6
	MCM3	Minichromosome Maintenance Complex Component 3	−2.6
	MCM2	Minichromosome Maintenance Complex Component 2	−2.4
	MCM5	Minichromosome Maintenance Complex Component 5	−2.4
	CCNE1	Cyclin E1	−2.0
	CDK4	Cyclin Dependent Kinase 4	−1.3
	CCND1	Cyclin D1	−1.2
	CDKN1B	Cyclin Dependent Kinase Inhibitor 1B	−1.1
	CDK6	Cyclin Dependent Kinase6	+1.3
G2 Phase & M Phase	*BIRC5*	Baculoviral IAP Repeat Containing 5	−5.7
	GTSE1	G2 And S-Phase Expressed 1	−4.2
	CCNB1	Cyclin B1	−3.9
	CCNA2	Cyclin A2	−3.6
	STMN1	Stathmin 1	−3.6
	AURKB	Aurora Kinase B	−3.5
	RAD51	RAD51 Recombinase	−3.0
	CKS2	CDC28 Protein Kinase Regulatory Subunit 2	−2.8
	KPNA2	Karyopherin Subunit Alpha 2	−2.8
	MRE11A	MRE11 Homolog, Double Strand Break Repair Nuclease	−2.2
	CCNG1	Cyclin G1	−1.6
	CDK5RAP1	CDK5 Regulatory Subunit Associated Protein 1	−1.5
	ANAPC2	Anaphase Promoting Complex Subunit 2	−1.3
	SERTAD1	SERTA Domain Containing 1	−1.1
	MNAT1	MNAT CDK-Activating Kinase Assembly Factor 1	−1.0
	CCNH	Cyclin H	−1.0
	CDC16	Cell Division Cycle 16	+1.2
	CDK7	Cyclin Dependent Kinase 7	+1.4
Cell Cycle Checkpoint & Cell Cycle Arrest	*CDK1*	Cyclin Dependent Kinase 1	−4.4
	MAD2L1	Mitotic Arrest Deficient 2 Like 1	−3.1
	CDC25C	Cell Division Cycle 25C	−3.0
	BRCA2	BRCA2, DNA Repair Associated	−2.8
	WEE1	WEE1 G2 Checkpoint Kinase	−2.8
	BRCA1	BRCA1, DNA Repair Associated	−2.7
	KNTC1	Kinetochore Associated 1	−2.5
	CHEK2	Checkpoint Kinase 2	−2.0
	CHEK1	Checkpoint Kinase 1	−1.7
	RAD9A	RAD9 Checkpoint Clamp Component A	−1.7
	RAD1	RAD1 Checkpoint DNA Exonuclease	−1.5
	HUS1	HUS1 Checkpoint Clamp Component	−1.5
	MAD2L2	Mitotic Arrest Deficient 2 Like 2	−1.3
	RBBP8	RB Binding Protein 8, Endonuclease	−1.3
	ATR	ATR Serine/Threonine Kinase	−1.3
	CUL1	Cullin 1	−1.2
	CUL2	Cullin 2	−1.2
	CUL3	Cullin 3	−1.1
	CDC34	Cell Division Cycle 34	−1.1
	NBN	Nibrin	−1.1
	CCNG2	Cyclin G2	−1.1
	ATM	ATM Serine/Threonine Kinase	−1.1
	MDM2	MDM2 Proto-Oncogene	−1.1
	RAD17	RAD17 Checkpoint Clamp Loader Component	+1.1
	CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	+1.2
	GADD45A	Frowth Arrest And DNA Damage Inducible Alpha	+1.8

Continued

Gene category	Gene name	Full name	Fold change relative to the control
Regulation of the Cell Cycle	*MKI67*	Marker Of Prolifereation Ki-67	−4.0
	E2F1	E2F Transcription Factor 1	−3.7
	AURKA	Aurora Kinase A	−3.5
	CCNF	Cyclin F	−3.2
	CCNB2	Cyclin B2	−3.0
	CDC20	Cell Division Cycle 20	−2.9
	RBL1	RB Transcriptional Corepressor Like 1	−2.3
	CKS1B	CDC28 Protein Kinase Regulatory Subunit 1B	−2.2
	CDK2	Cyclin Dependent Kinase 2	−2.1
	TFDP1	Transcription Factor Dp-1	−2.1
	CASP3	Caspase 3	−2.0
	CCND3	Cyclin D3	−1.8
	BCCIP	BRCA2 And CDKN1A Interacting Protein	−1.6
	BCL2	BCL2, Apoptosis Regulator	−1.5
	CCNC	Cyclin C	−1.4
	E2F4	E2F Transcription Factor 4	−1.3
	RB1	RB Transcriptional Corepressor 1	−1.1
	RBL2	RB Transcriptional Corepressor Like 2	−1.1
	TFDP2	Transcription Factor Dp-2	−1.1
	CCNT1	Cyclin T1	−1.1
	CDK8	Cyclin Dependent Kinase 8	−1.0
	CDK5R1	Cyclin Dependent Kinase 5 Regulatory Subunit 1	−1.0
	TP53	Tumor Protein P53	+1.0
	ABL1	ABL Proto-Oncogene 1, Non-Receptor Tyrosine Kinase	+1.1
	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	+1.1
	CCND2	Cyclin D2	+1.2
	CDKN2B	Cyclin Dependent Kinase Inhibitor 2B	+1.2

Table 1. Gene list of cell cycle PCR array.

marker, it was negative in all four EPCs but positive in monocytes. PECAM1 is a marker for both EPCs and monocytes, it was detected in all EPC clones and monocytes (Fig. 3a).

We next examined the ability of EPCs to ingest acetylated low density lipoprotein (AcLDL), a well-recognised phenotype of EPC. All four EPC clones ingested AcLDL in culture and showed positive red staining (Fig. 3b). Ishikawa cells, which were derived from human endometrial adenocarcinoma and available in our laboratory, were used as non-EPC control cells[47,48]. These cells did not uptake AcLDL (Fig. 3b). In addition, we confirmed that all four EPC clones formed well-structured tubes (Fig. 3c). These data confirmed that the primary EPC clones isolated here were true EPCs.

HtrA4 inhibits EPC proliferation. EPCs were then treated with vehicle control or two doses of HtrA4 for 0, 24 and 48 h, and cell number was counted at each time point to asses proliferation (Fig. 4a). Cells treated with vehicle control grew steadily over the 48 h period, and cell number roughly doubled at each time point as expected (Fig. 4a). Similar to HUVECs, 1.5 µg/ml HtrA4 had no obvious effect on EPC growth, whereas 3.0 µg/ml HtrA4 significantly inhibited EPC proliferation at both time points (Fig. 4a). HtrA4 again did not affect EPC viability (Fig. 4b).

The expression of proliferation marker, Ki67, was examined by immunocytochemistry (Fig. 4c). The vast majority of EPCs in the vehicle control showed clear positive nuclear staining for Ki67 (Fig. 4c), indicating that cells were in the proliferative state, consistent with our findings in HUVECs. However, the number of Ki67-positive cells was greatly reduced by HtrA4 (Fig. 4c). Quantification from four independent EPC clones confirmed that HtrA4 significantly and dose-dependently reduced Ki67 staining in EPCs (Fig. 4d), and this reduction was apparent even when HtrA4 was at 1.5 µg/ml (Fig. 4d).

HtrA4 down-regulates cell cycle genes in EPCs. We next investigated whether HtrA4 also down-regulates cell cycle genes in EPCs. For this, EPCs were treated with either vehicle control or two doses of HtrA4 for 0, 24 and 48 h, and the four cell cycle genes that were highly regulated by HtrA4 in HUVECs were analysed by real-time RT-PCR (Fig. 5). *CDKN3* expression was significantly suppressed by 3.0 µg/ml HtrA4 at both 24 and 48 h (Fig. 5a). *BIRC5* expression showed a downward trend with 3.0 µg/ml HtrA4 at 24 h time point, but the difference between the treatments was not statistically significant (Fig. 5b). *CDK1* expression was dose-dependently reduced by HtrA4 at 24 h, and the greatest reduction was detected in cells treated with 3.0 µg/ml HtrA4 (Fig. 5c). Cells treated for 48 h had much lower *CDK1* expression in all treatment groups (Fig. 5c). *MKI67* expression was lowest in EPCs treated with 3.0 µg/ml HtrA4 at 24 h (Fig. 5d), and it was greatly reduced across all treatment groups at 48 h (Fig. 5d).

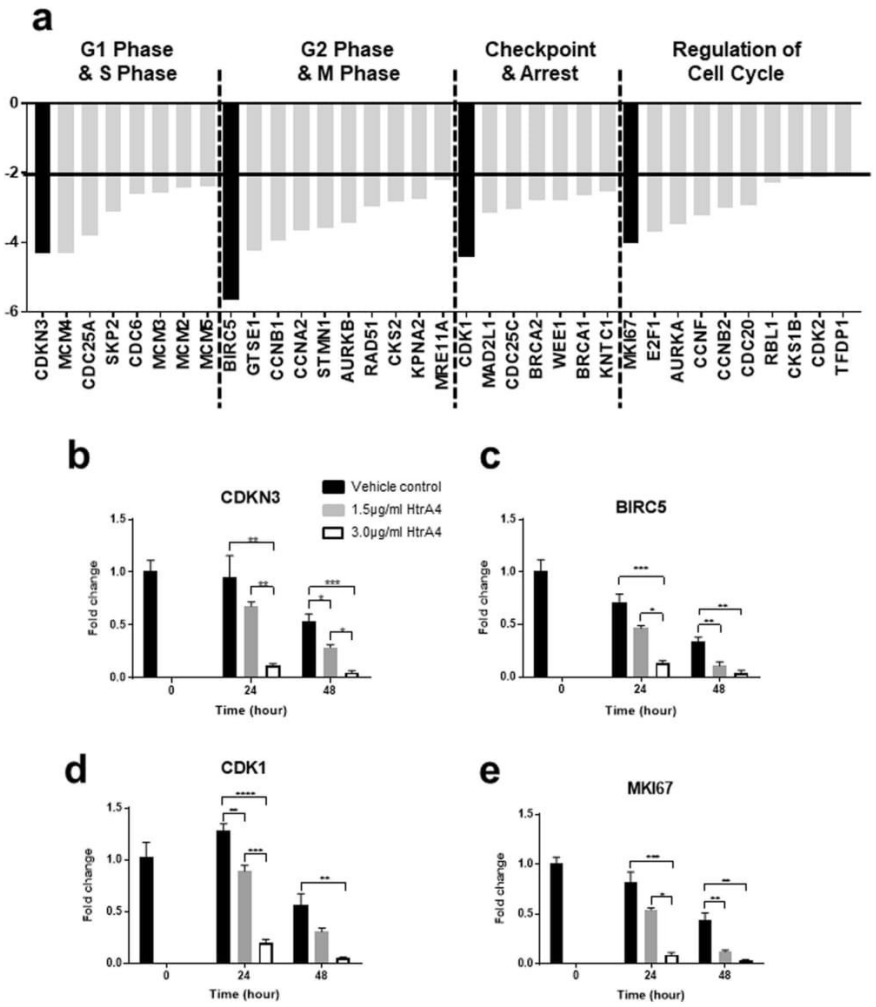

Figure 2. HtrA4 suppresses HUVEC mRNA expression of cell cycle genes. (**a**) Summary of PCR array analysis of 84 cell cycle genes. RNA from cells treated with vehicle control or 3.0 μg/ml HtrA4 for 24 h were pooled from 3 independent experiments and applied to the array. Data is expressed as HtrA4-induced fold changes relative to the vehicle control, and 35 genes that displayed a greater than 2-fold reduction in expression are categorised into four groups. These 35 genes were further validated by real-time RT-PCR, data of those represented in black bars are shown in (**b**-**e**) and the remainder are shown in Supplementary Fig. 1A–D. (**b**–**e**) Real-time RT-PCR validation of the four genes that showed the greatest changes in each category on the array. (**b**) *CDKN3*, (**c**) *BIRC5*, (**d**) *CDK1* and (**e**) *MKI67*. Cells were treated with 1.5 μg/ml or 3.0 μg/ml HtrA4 for 0, 24 or 48 h, n = 3. Data is expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

HtrA4 prevents EPCs from forming endothelial tubes. We further investigated whether HtrA4 would disrupt EPC functionality using the tube formation assay. EPCs treated with vehicle control formed regular tube-like structures (Fig. 6a). EPCs treated with 1.5 μg/ml HtrA4 also formed tubes, but they were thinner and often disjointed compared to the vehicle control (Fig. 6a). In contrast, cells treated with 3.0 μg/ml HtrA4 failed to form any tubes (Fig. 6a). To quantify this result, we measured total tube length (Fig. 6b), branching point (Fig. 6c) and total number of tubes (Fig. 6d) from all four EPCs. All three parameters showed that 3.0 μg/ml HtrA4 significantly suppressed EPCs from forming endothelial tubes.

Discussion

Early-onset PE is associated with wide-spread endothelial injury and dysfunction. Emerging evidence suggests that endothelial repair mechanisms are also compromised in early-onset PE. However, the underlying mechanisms are not well understood. HtrA4 is a serine protease that is specifically expressed by the placenta and significantly up-regulated in early-onset PE[36,40–44]. Data presented in this study suggest that high levels of HtrA4 in the circulation may impede endothelial repair. We demonstrated that high levels of HtrA4 inhibited HUVEC

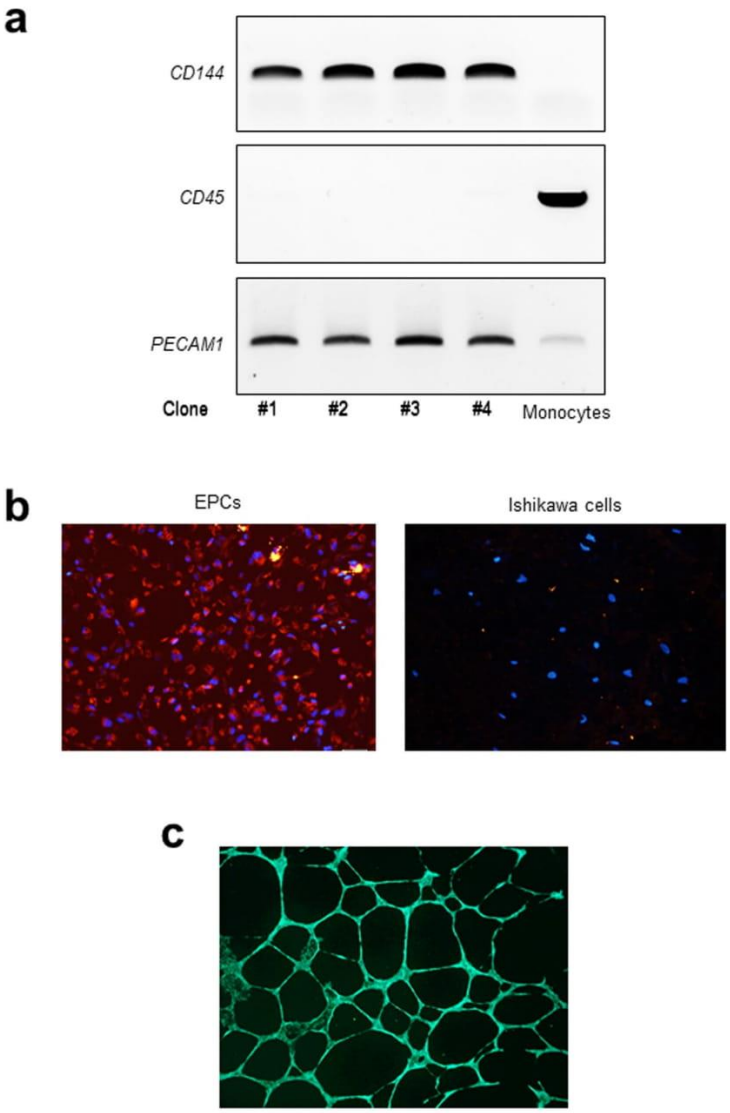

Figure 3. Validation of EPC clones. (**a**) RT-PCR confirmation of gene markers. CD144 and PECAM1 are positive markers of EPC, whereas CD45 is a negative marker of EPCs but a positive marker of monocytes. Full length gels are presented in Supplementary Fig. 2. (**b**) Immunofluorescent staining of Dil-labelled AcLDL (red) in EPCs. Human Ishikawa cells were used as a non-EPC control line, which did not uptake AcLDL. Nuclei were stained with DAPI (blue). Representative images at 10x magnification are shown. (**c**) Confirmation that EPCs form endothelial tubes. All EPCs formed tube-like structures on Matrigel. A representative image at 4x magnification is shown.

proliferation and significantly suppressed a number of genes that are critical for cell cycle. We also showed that HtrA4 inhibited the proliferation and differentiation of freshly isolated primary EPCs.

We first demonstrated that 3.0 μg/ml HtrA4, which represents the highest blood concentration detected in early-onset PE, significantly inhibited HUVEC proliferation. On the other hand, cells treated with either vehicle control or the lower concentration of HtrA4 grew normally. As cell viability was not affected, the high concentration of HtrA4 reduced cell growth not because of cell death but due to inhibition of cell proliferation. This was confirmed by immunostaining for proliferation marker Ki67. Very little Ki67 staining was detected in cells treated with 3.0 μg/ml HtrA4 compared to the vehicle control. Although not statistically significant, cells treated with 1.5 μg/ml HtrA4 also showed less Ki67 staining, suggesting that even lower concentrations of HtrA4 may restrain endothelial cell proliferation.

We further demonstrated that HtrA4 dose-dependently down-regulated a number of cell cycle genes by a PCR array and by real-time RT-PCR. As the original PCR array experiment did not include replicates, statistical

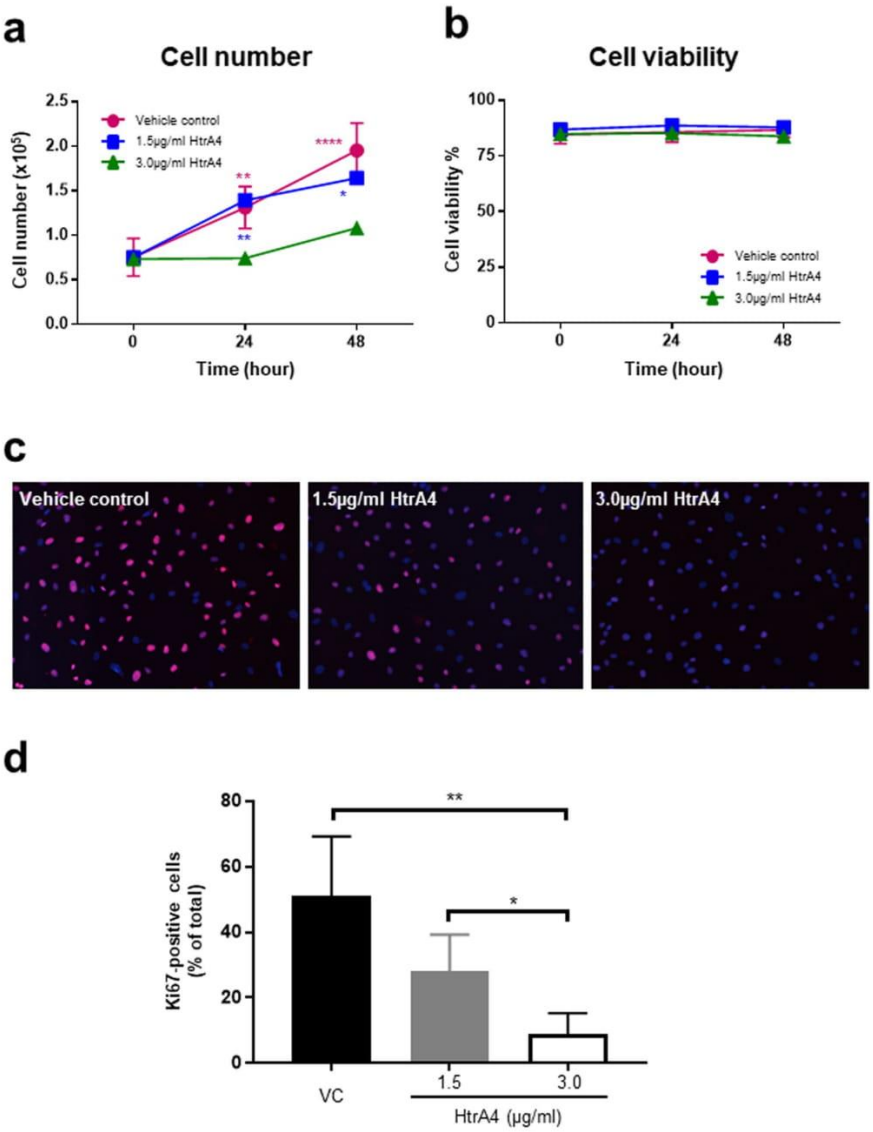

Figure 4. HtrA4 inhibits primary EPC proliferation. EPCs were treated with vehicle control or two doses of HtrA4 over 48 h. (**a**) Growth curve. (**b**) Percentage of viable cells. (**c**) Immunofluorescence of cell proliferation marker Ki67 at 24 h, representative images at 10x magnification are shown. (**d**) Quantification of Ki67 staining of (**c**). n = 4. Data is expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{****}p < 0.0001$.

testing could not be performed. This limitation was, however, mitigated by extensive validation of the findings by real-time RT-PCR on three independent samples per treatment. Among 84 cell cycle genes examined on the array, HtrA4 down-regulated 35 genes by more than 2-fold, these changes were subsequently confirmed by RT-PCR. Overall, HtrA4 dose-dependently down-regulated genes that are critical for cell cycle, and 3.0 μg/ml HtrA4 had the most profound effect on their expression. *MKI67* mRNA expression was significantly reduced by HtrA4 even at the lower dosage at both 24 and 48 h, supporting the immunocytochemistry result. The data presented in this study suggests that at the molecular level, HtrA4 dose-dependently inhibited the expression of endothelial genes that are involved in proliferation. Among the 49 genes that showed less than 2-fold changes on the array and were thus not further examined by RT-PCR, 3 showed an upward (fold change >1.3) whereas 7 showed a downward (fold change >1.5) trend following treatment with HtrA4 (Table 1). These borderline genes may warrant further investigation in the future. Two apoptosis-related genes, *BCL2* and *CASP3*, were not altered by HtrA4 treatment, consistent with the cell viability data.

Our data presented in this study also demonstrated that HtrA4 likewise inhibited EPC proliferation and expression of genes involved in cell cycle. Firstly, we isolated four EPC clones from four individual pregnancies

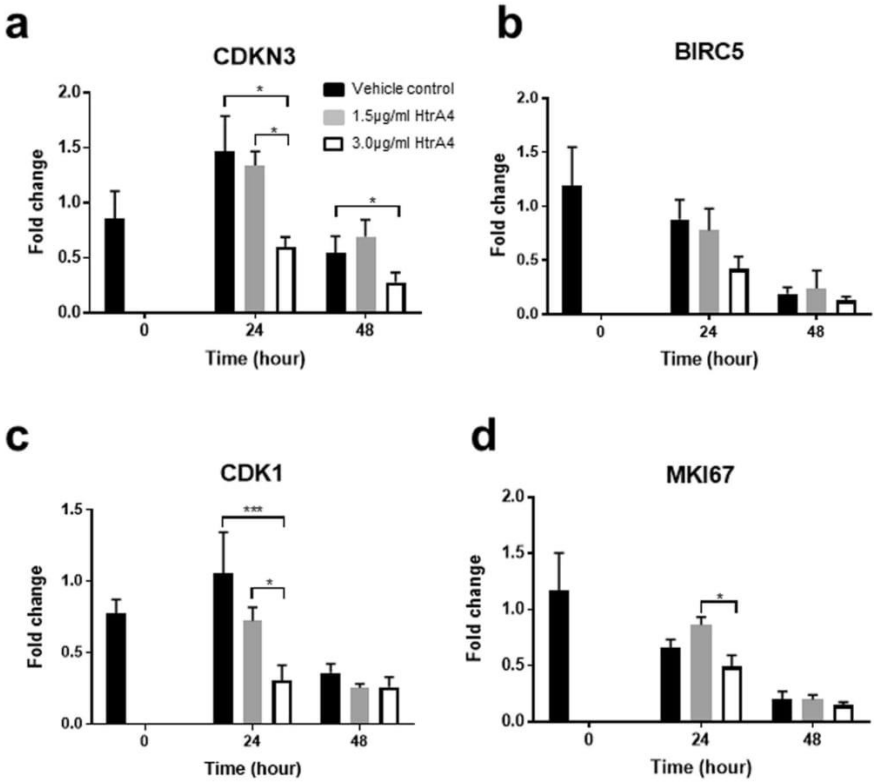

Figure 5. Real-time RT-PCR analysis of cell cycle genes in EPCs. (**a**) *CDKN3*, (**b**) *BIRC5*, (**c**) *CDK1* and (**d**) *MKI67*. Cells were treated with 1.5 μg/ml or 3.0 μg/ml HtrA4 for 0, 24 or 48 h, n = 4. Data is expressed as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

and confirmed their identity. This was achieved by determining the mRNA expression of EPC markers, their ability to uptake AcLDL, and to form endothelial tubes on Matrigel. We then used these primary EPCs and demonstrated that high concentrations of HtrA4 inhibited EPC growth, consistent with the HUVEC result. Similarly, HtrA4 did not affect EPC viability, again indicating that HtrA4 did not induce cell death. Immunostaining for Ki67 showed that HtrA4 reduced the number of Ki67-positive cells in a dose-dependent manner in all four EPCs. Compared to the HUVECs, EPCs appeared to be more sensitive to HtrA4, because a statistically significant reduction in Ki67 staining was observed in EPCs but not in HUVECs when they were treated with 1.5 μg/ml HtrA4. Furthermore, the effect of 3.0 μg/ml HtrA4 on Ki67 staining was more pronounced in EPCs than in HUVECs. This result suggests that HtrA4 even at lower dosages may restrain EPC numbers and function in normal pregnancy; its significance is unclear but it may be a mechanism that permits maternal vascular remodelling/adaptation for pregnancy.

The four cell cycle genes, *CDKN3*, *BIRC5*, *CDK1* and *MKI67* that were highly down-regulated by HtrA4 in HUVECs, were also examined in EPCs. All these genes except *BIRC5* showed a significant reduction in expression when EPCs were treated with 3.0 μg/ml HtrA4 for 24 h. At 48 h, only *CDKN3* expression was significantly down-regulated by 3.0 μg/ml HtrA4. However, at the 48 h time point, expression of all four genes was significantly lower even in the vehicle control. This data suggests that EPCs proliferate more quickly than HUVECs, and they may have stopped proliferating by 48 h. Therefore, the impact of HtrA4 treatment on expression of *BIRC5*, *CDK1* and *MKI67* was less obvious at 48 h. In EPCs, while Ki67 immunostaining was reduced by HtrA4 in a dose-dependent manner, the lower dosage of HtrA4 had no significant effect on *MKI67* gene expression, suggesting that HtrA4 may also regulate Ki67 at the protein level.

One of the main functions of EPCs is to differentiate into resident endothelial cells to repair injured endothelium[49]. An *in vitro* test of this differentiation process is to examine their angiogenic ability to form tube-like structures on Matrigel, which we have successfully confirmed for all four primary EPC clones. However, when these EPCs were treated with 1.5 μg/ml HtrA4, tubes were formed but they were thinner and disjoined in many areas, suggesting that even the lower concentration of HtrA4 affected EPC function. When EPCs were treated with 3.0 μg/ml HtrA4, the tube formation process was completely blocked.

One limitation of our study was the use of HUVECs as a model. As HUVECs may not reflect all features of human vascular endothelial cells, future studies are warranted with endothelial cells of other vascular origin. However, we validated the HUVEC data in primary EPCs in this study. Another limitation was the source of EPCs, which ideally would be from peripheral blood, however, EPCs are rare cell populations in peripheral

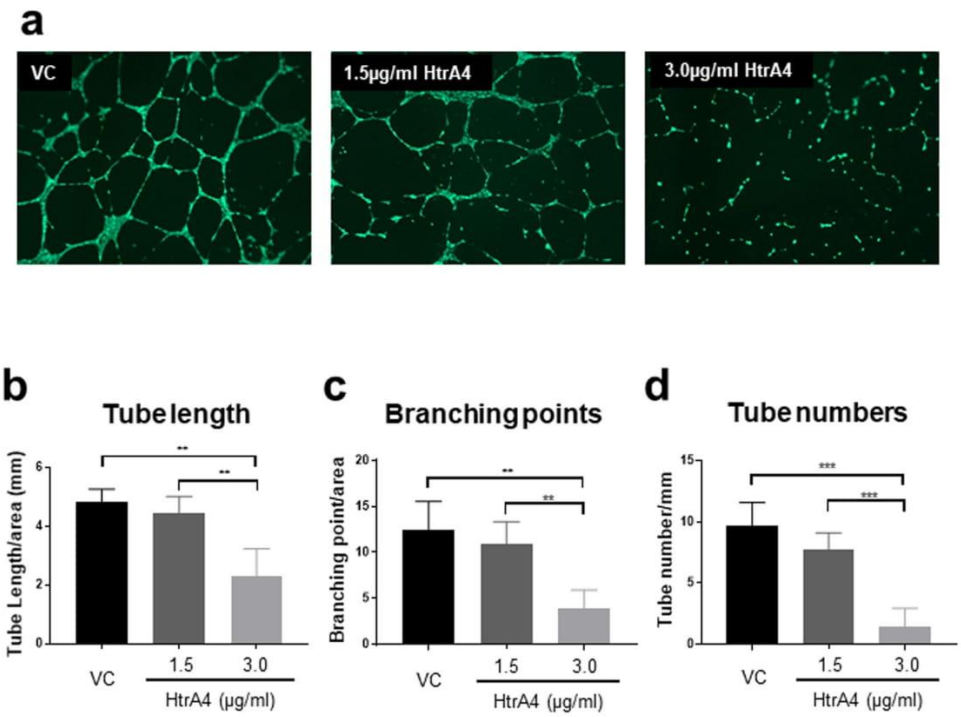

Figure 6. HtrA4 disrupts tube formation in EPCs. Cells were treated with vehicle control or two concentrations of HtrA4, and their abilities to form tubes were assessed. Four independent EPC clones were examined. (**a**) Representative images at 4x magnification. (**b–d**) Quantification of tube formation. (**b**) Total tube length per mm^2 area, (**c**) Total branching point per mm^2 area, and (**d**) Number of tubules per mm^2 area. n = 4 for all. Data are expressed as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

blood and generating EPC clones is a difficult and long process. As EPCs from either maternal and fetal origin exhibit similar phenotypes and characteristics once they are in culture[46], in this study we used umbilical cord blood derived EPCs due to their relative abundance. Furthermore, since HtrA1 is also significantly elevated in early-onset PE circulation[50], whether HtrtA1 has a similar function or work synergistically with HtrA4 should be investigated.

In summary, this is the first study to show that placental-derived HtrA4, which is elevated in the early-onset PE circulation may inhibit endothelial cell proliferation. Furthermore, our study demonstrated that high levels of HtrA4 may also impede endothelial repair by inhibiting the proliferation and differentiation of circulating EPCs. These results suggest that circulating HtrA4 may present a potential therapeutic target for treatment of early-onset PE.

Material and Methods

Cell culture. HUVECs (ATCC, Maryland, USA) were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, and maintained in DMEM (Thermo Fisher Scientific, VIC, Australia) supplemented with 1% antibiotics (Thermo Fisher Scientific), 2 mM L-glutamine (Sigma-Aldrich, Missouri, USA), 1 mM sodium pyruvate (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific). The starting passage of the HUVECs was 12, and the experiments were completed within eight passages. Cells were sub-cultured in 12-well plates (Thermo Fisher Scientific) at a density of 1.0×10^5 per well for 24 hours (h), then treated with two doses of recombinant HtrA4 (1.5 μg/ml and 3.0 μg/ml, BioTeZ, Berlin, Germany) or vehicle control for 24 or 48 h. The vehicle control contains 150 mM NaCl, 5 mM CaCl2, 50 mM Tris-HCl pH 7.5, 0.05% Brij 35 solution, 50 mM imidazole in ultrapure water. The two doses of HtrA4, 1.5 μg/ml and 3.0 μg/ml, were chosen to represent the median and highest levels of HtrA4 detected in the early-onset PE circulation[36].

Cell proliferation assay. HUVECs were plated in 12-well plates and treated with either vehicle control or two doses of HtrA4. Cell numbers and viability in each treatment were determined at 0, 24 and 48 h post-treatment. Cells from each well were trypsinized and re-suspended in 500 μl growth media, 10 μl of the cell suspension was mixed with an equal volume of trypan blue which stains dead cells. The total number of viable (clear cytoplasm) and dead (blue cytoplasm) cells were then counted using an automated cell counter (Countess, Thermo Fisher Scientific). Cell viability was determined by the percentage of viable cells in the total number of cells collected from each treatment. Cells were subsequently collected for RNA isolation and the experiment was repeated independently three times.

RNA extraction. RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and genomic DNA was removed using an RNase-free DNase set (Qiagen) according to the manufacturer's protocols. RNA was isolated from three independent experiments and concentration was determined using Nanodrop ND-1000 (Thermo Fisher Scientific).

Analysis of cell cycle genes using a pathway-specific PCR array. RNA was isolated from HUVECs treated with either vehicle control or 3.0 μg/ml HtrA4 for 24 h. Samples from three independent experiments were pooled, and 500 ng of pooled RNA representing control and HtrA4 treatment was used for the array. RNA was reverse transcribed into complementary DNA (cDNA) using a RT^2 First Strand Kit (Qiagen), and applied onto a RT^2 Profiler 84 gene PCR array (Qiagen) according to manufacturer's instructions on an ABI 7900 HT Fast real-time machine (Applied Biosystems, VIC, Australia). Results were then analysed using Qiagen RT^2 profiler PCR array data analysis software (Qiagen). Genes showing more than 2-fold differences in expression between the vehicle control and 3.0 μg/ml HtrA4 treatment were validated by real-time PCR.

Real-time RT-PCR analysis. For RT-PCR validation, cells were treated with 1.5 μg/ml or 3.0 μg/ml HtrA4 for 0, 24 and 48 h respectively, and RNA (300 ng) from each treatment of three independent experiments was reverse transcribed with a SuperScript III First-Strand kit (Thermo Fisher Scientific) according to manufacturer's instructions. Real-time PCR was performed with primers specified in Supplementary Table 1 on an ABI 7900 HT Fast real-time machine (Applied Biosystems, California, USA) with the following conditions: 1) 95 °C for 10 min for enzyme activation, 2) 40 cycles of denaturation (15 s at 95 °C), annealing (5 s at 58 °C), extension (10 s at 72 °C), and a single fluorescence measurement at 70–75 °C for quantitation, and 3) dissociation curve assessment between 60 °C and 95 °C with continuous fluorescence measurement. All cDNA samples were diluted 1:80 and PCR reaction was prepared to a final volume of 10 μl with 5 μl SYBR Green PCR Master Mix (Thermo Fisher Scientific), 4 μl diluted cDNA sample and 0.5 μM final concentration of forward and reverse primers. All samples were run in triplicates, gene expression levels were normalised to 18S, and fold changes were calculated using ΔΔCt.

Immunocytochemistry. Cells were seeded on 10-mm glass coverslips (Thermo Fisher Scientific) that were placed in 48-well plates, and treated with vehicle control or two doses of HtrA4 for 24 h. Cells were fixed with 4% paraformaldehyde (VWR, Radnor, USA) for 10 min and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min. All procedures from here onwards were carried out at room temperature unless stated otherwise. Cells were blocked with 1% BSA in PBS for 2 h, incubated at 4 °C overnight with an anti-Ki67 rabbit monoclonal antibody diluted 1:250 (cat# ab16667, Abcam, Cambridge, United Kingdom), and then with a donkey anti-rabbit Alexa Fluor 568 antibody diluted 1:200 (cat# A10042, Thermo Fisher Scientific) for 2 h. Nuclei were stained with 5 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) for 10 min, and coverslips were mounted onto glass slides with fluorescent mounting media (Dako, Glostrup, Denmark). Staining was visualised using an Olympus BX60 fluorescent microscope (Olympus, Notting Hill, Australia) and images were taken at 10x magnification using an Olympus DP70 camera and Olympus CellSens software (Olympus). Three to four images per each treatment were taken randomly. Ki67 positive cells were quantified using FIJI software (NIH, Maryland, USA). Experiments were repeated independently three times.

Isolation of primary EPCs from umbilical cord blood of term human pregnancy. Umbilical cord blood from healthy term human pregnancy was collected at Monash Medical Centre (Melbourne, Australia) with approval from the Monash Medical Centre Human Research Ethics Committees (HREC reference number: 13357B) and all experiments were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all study participants. Primary EPCs were isolated from the cord blood as previously described[46]. Briefly, mononuclear cells were separated from blood using Ficoll-Paque PLUS (Amersham Biosciences, Little Chalfont, United Kingdom) and centrifugation. They were then seeded at a $3–5 \times 10^7$ per well density in 6-well plates that were pre-coated with rat tail collagen I (Merck, New Jersey, USA), and maintained in complete EGM-2MV BulletKit media (EGM-2, Lonza, Basel, Switzerland) containing 10% FBS and 1% antibiotics. Media were changed daily till colonies of endothelial cells were observed. Individual colony was then isolated using a cloning cylinder (Sigma-Aldrich) and propagated as monoclonal EPC line in complete EGM-2 media.

Validation of primary EPCs. To confirm the purity and identity of EPC clones, RNA was extracted from each clone and reversed transcribed into cDNA using SuperScript III First-Strand kit. RT-PCR was then performed on a conventional block PCR machine at 58 °C for 30 cycles, together with an EPC-negative cDNA sample of monocytes which were also isolated from the cord blood at the same time. Two EPC markers (CD144 and PECAM1) and one hematopoietic cell/monocyte-specific marker (CD45) were examined using the following primers: CD144, 5′-GCACCAGTTTGGCCAATATA-3′ and 5′-GGGTTTTTGCATAATAAGCAGG-3′; PECAM1, 5′-CACACCAAGAACTCTCCCCA-3′ and 5′-CCCTCACCTGTCCTGCTCAT-3′; CD45, 5′-CTGGGGAGAAGGAAAGCAAA-3′ and 5′-GCAGTGAATGAGTAGAGGTG-3′. Phenotypic characteristics of EPCs were also examined by ingestion of AcLDL. EPCs were seeded in 48-well plates at a density of 0.5×10^5 per well for 24 h, and then incubated with 10 μg/ml Dil-complexed AcLDL (Thermo Fisher Scientific) for 4 h at 37 °C. The cells were counterstained with DAPI and visualised using a fluorescent microscope. Cells that ingested AcLDL fluoresced red. Another major functional characteristic of EPCs is forming tube-like structures on Matrigel, to confirm this, EPCs were cultured for 24 h at a density of 1.0×10^5 per well in 48-well plates that were pre-coated with growth factor reduced Matrigel (BD Bioscience, New Jersey, USA), and stained with 4 μg/ml

calcein AM fluorescent dye (BD Bioscience) for 30 min at 37 °C. Tube formation was visualised using a fluorescent microscope. Four clones were independently verified by all of the above methods.

Treatment of EPCs with HtrA4. For each EPC clone, cells were sub-cultured in 12-well plates at a density of 0.5×10^5 per well for 24 h, then treated with recombinant HtrA4 (1.5 μg/ml or 3.0 μg/ml) or vehicle control for 24 or 48 h. At the end of the treatment, cell number was measured as described per HUVEC proliferation assay protocol, and cell viability was determined by calculating the percentage of viable cells over the total number of cells collected per treatment. RNA was subsequently extracted as described per HUVEC experiment and real-Time RT-PCR was performed using selected primers specified in Supplementary Table 1. EPCs were also seeded at a density of 0.5×10^5 per well on 10-mm glass coverslips that were placed in 48-well plates, treated with vehicle control or two concentrations of HtrA4 for 24 h, and then stained with a Ki67 antibody as per HUVEC experiment. All experiments were repeated with four individual EPC clones.

Assessment of EPC tube formation following HtrA4 treatment. EPCs were seeded at a density of 1.0×10^5 per well in 48-well plates that were pre-coated with growth factor reduced Matrigel, and treated with either vehicle control or two doses of HtrA4 (1.5 μg/ml or 3.0 μg/ml) for 16 h. Cells were washed three times with Hanks' balanced salt solution (Thermo Fisher Scientific) and labelled with 4 μg/ml calcein AM fluorescent dye for 30 min at 37 °C. Tubes were assessed using an Olympus BX60 fluorescent microscope at 4x magnification, and images were taken using an Olympus DP70 camera and Olympus CellSens software. Three images per treatment condition were taken randomly, and the total tube length, branching points and total tube numbers per area were quantified using FIJI software. Experiments were repeated with four individual EPC clones.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism (v.6, GraphPad Software Inc., CA). Data were expressed as mean ± SD, and compared using one-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$ was considered significant.

Data Availability

All data generated or analysed during this study are included in this published article (and its Supplementary information files).

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Author Contributions

Y.W. and G.N. designed the study and wrote the manuscript; R.L. provided umbilical cord blood samples; Y.W. performed experiments and analysed data. All authors reviewed the manuscript.

Additional Information

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Chapter 4

High levels of HtrA4 detected in preeclamptic circulation may disrupt endothelial cell function by cleaving the main VEGFA receptor KDR

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High levels of HtrA4 detected in preeclamptic circulation may disrupt endothelial cell function by cleaving the main VEGFA receptor KDR

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ABSTRACT: Systemic endothelial dysfunction is a key characteristic of preeclampsia (PE), which is a serious disorder of human pregnancy. We have previously reported that high-temperature requirement factor (Htr)A4 is a placenta-specific protease that is secreted into the maternal circulation and significantly up-regulated in PE, especially early-onset PE. We have also demonstrated that high levels of HtrA4 detected in the early onset PE circulation induce endothelial dysfunction in HUVECs. In the current study, we investigated whether HtrA4 could cleave the main receptor of VEGFA, the kinase domain receptor (KDR), thereby inhibiting VEGFA signaling. We first demonstrated that HtrA4 cleaved recombinant KDR *in vitro*. We then confirmed that HtrA4 reduced the level of KDR in HUVECs and inhibited the VEGFA-induced phosphorylation of Akt kinase, which is essential for downstream signaling. Further functional studies demonstrated that HtrA4 prevented the VEGFA-induced tube formation in HUVECs and dose-dependently inhibited the VEGFA-induced angiogenesis in explants of mouse aortic rings. These data strongly suggest that high levels of HtrA4 in the maternal circulation could cleave the main receptor of VEGFA in endothelial cells to induce a wide-spread impairment of angiogenesis. Our studies therefore suggest that HtrA4 is a potential causal factor of early onset PE.—Wang, Y., La, M., Pham, T., Lovrecz, G. O., Nie, G. High levels of HtrA4 detected in preeclamptic circulation may disrupt endothelial cell function by cleaving the main VEGFA receptor KDR. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: early onset preeclampsia · angiogenesis · endothelial dysfunction · HUVEC

Preeclampsia (PE) is a life-threatening disorder of human pregnancy, affecting 2–8% of pregnancies worldwide (1, 2). PE is characterized by *de novo* hypertension after 20 wk of gestation with 1 or more of the following symptoms: proteinuria, maternal organ dysfunction, and uteroplacental dysfunction (3). The condition can be severe, leading to multiorgan failure involving the liver, kidney, and brain and causing swelling, vision disturbances, convulsions, and stroke (4). These symptoms are closely associated with wide-spread endothelial dysfunction (5–8). Currently, the only effective treatment for PE is delivery, which is often performed prematurely.

PE can be divided into 2 distinct subtypes: early onset (before 34 wk of gestation) and late-onset (after 34 wk) (9). The 2 PE subtypes have different etiologies, and early onset PE poses far more significant maternal risks, with a much higher maternal mortality rate compared with late-onset PE (10–12). Early onset PE is associated with inadequate trophoblast invasion into the uterine spiral arteries during early placentation, which increases utero-placental vascular resistance (13, 14), leading to placental ischemia and reduced blood supply to the fetus (13). Furthermore, placental-derived factors which are released into maternal circulation as a response to reduced placental perfusion or as a result of genetic and environmental predisposition have shown to contribute to the maternal syndrome of PE (7). Factors significantly elevated in PE circulation include cytokines, antiangiogenic factors, syncytiotrophoblast microparticles, and activated leukocytes (15–18). To date, it is unknown whether a single factor or multiple factors are responsible for PE; however, damage to the maternal endothelium is a common pathophysiological occurrence at least in early onset PE (7). One study showed that serum from women with preeclampsia is cytotoxic to endothelial cells *in vitro* (19). In the majority of PE cases, the clinical conditions improve after 24–48 h of delivery, although serum levels of IL-6 and TNF-α were reported to remain high even after 12–24 wk postpartum (20).

ABBREVIATIONS: BSA, bovine serum albumin; EBM-2, endothelial basal medium; FBS, fetal bovine serum; FCS, fetal calf serum; Flt-1, FMS-like tyrosine kinase-1; Htr, high-temperature requirement factor; KDR, kinase domain receptor; PE, preeclampsia; TBS, Tris-buffered saline

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We have previously reported that high-temperature requirement factor (Htr)A4 is a placenta-specific serine protease that is secreted into the maternal circulation and significantly increased in early onset PE (21). HtrA4 belongs to a family of serine proteases that include HtrA1–HtrA4. These proteases serve as ATP-independent protein quality control factors that are associated with cell growth, unfolded stress response, programmed cell death, and aging (22). All HtrAs contain a trypsin-like serine protease domain with catalytic activity and 1 or more highly conserved C-terminal PDZ domains, which bind to target proteins to regulate protein–protein interaction (23). Although HtrA1, HtrA3, and HtrA4 share a similar N-terminal domain structure that consists of putative signal peptide, insulin growth factor binding domain, and kazal protease inhibitor domain (22), HtrA2 has a unique N-terminal transmembrane domain. Studies show that HtrA1, HtrA3, and HtrA4 but not HtrA2 are dysregulated in PE (24–26). However, HtrA4 is most unique because its expression is highly specific to the human placenta (21).

Placental HtrA4 expression and its circulating levels are significantly increased in early, but not late onset, PE at the time of disease presentation in the third trimester (21, 26–30). We have also demonstrated that high levels of HtrA4 detected in early onset PE inhibit tube formation and induce proinflammatory factors in endothelial cells (21, 31). These data suggest that HtrA4 may represent a placenta-derived contributor of endothelial dysfunction in early-onset PE development. However, the molecular mechanisms of HtrA4 action in endothelial cells remain unknown.

VEGFA is a key regulator of angiogenesis, and its dysregulation is strongly associated with PE development (32). VEGFA binds and activates 2 tyrosine kinase receptors: FMS-like tyrosine kinase-1 (Flt-1) and kinase domain receptor (KDR). Although Flt-1 has a high affinity to VEGFA, it has low kinase activities for intracellular signaling and thus acts as a decoy receptor (33). In contrast, although KDR has a lower affinity to VEGFA, it has high kinase activities. KDR is therefore the predominant receptor for downstream intracellular signaling of VEGFA in endothelial cells (33, 34). KDR has been shown to mediate various physiologic and pathologic actions of VEGFA in endothelial cells, including proliferation, migration, survival, and permeability (34). KDR is essential for endothelial proliferation and differentiation, and KDR gene knock-out mice die *in utero* due to lack of blood-island formation and vasculogenesis (35). Furthermore, KDR inhibitors developed for cancer therapy inhibit VEGFA-induced endothelial proliferation and abolish VEGFA-induced angiogenesis *in vivo* (36). This evidence suggests that KDR plays an important role in normal endothelial cell functions.

Because Flt-1 can limit the amount of free VEGFA available to bind to the main receptor KDR, Flt-1 acts as a negative regulator of VEGFA signaling. Consequently, up-regulation of a soluble form of Flt-1 has been extensively investigated for PE development (15, 37–39). In contrast, the importance of KDR has been largely overlooked in PE research. In this study, we postulated that high levels of circulating HtrA4 may cleave the main VEGFA receptor KDR to impair VEGFA action and prevent angiogenesis of endothelial cells and thereby contribute to the development of early onset PE.

MATERIALS AND METHODS

Production of recombinant HtrA4

Expi293F cells (Thermo Fisher Scientific, Waltham, MA, USA), derived from the HEK-293 cell line and having a higher transfection capability than the standard HEK-293 cells, were cultured in suspension in Expi293F expression medium (Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. HtrA4 was produced by transient transfection of a full-length human HtrA4 construct (OriGene Technologies, Rockville, MD, USA) into Expi293F cells using linear polyethylenimine (25,000 Mw; BD Biosciences, Franklin Lakes, NJ, USA). After transfection, the culture was fed with 6 g/L glucose (MilliporeSigma, Castle Hill, NSW, Australia), 2 mM Glutamax-I (Thermo Fisher Scientific), 5 g/L LucraTone Lupin (Solabia, Pantin Cedex, France), and 0.2 mM butyric acid (MilliporeSigma). The culture was harvested 3 d after transfection. The conditioned media were centrifuged at 2975 *g* (Beckman Coulter, Brea, CA, USA) at 4°C for 10 min, and the supernatant was clarified using a 0.2-μm filter (Nalgene, Rochester, NY, USA). The supernatant was loaded onto a column containing ANTI-FLAG M2 affinity gel (MilliporeSigma), and protein was eluted with 100 μg/ml FLAG Peptide (MilliporeSigma). The eluted recombinant proteins were buffer exchanged and concentrated using Amicon Ultra-15 (Merck, Branchburg, NJ, USA).

In vitro cleavage assay

Recombinant human KDR (100 ng) was purchased from R&D Systems (Minneapolis, MN, USA) and incubated at 37°C overnight with assay buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 0.25% CHAPS] alone or with 100 or 200 ng recombinant HtrA4 and analyzed by Western blot using a human VEGFR2/KDR goat pAb (AF357; R&D Systems). The experiments were repeated independently 3 times.

Cell culture

We used HUVECs as an endothelial cell model for this study. Because HUVECs have been widely used as a cell model to study endothelial cell functions and angiogenesis (40), they express KDR and respond well to VEGFA, and our previous studies showed that these cells respond to HtrA4 (21, 31). HUVECs (American Type Culture Collection, Rockville, MD, USA) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air in EGM-2MV BulletKit (Lonza, Basel, Switzerland) medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% antibiotics (Thermo Fisher Scientific). Cells were subcultured in 12-well plates (Thermo Fisher Scientific) at a 2.5×10^5/well density or in 48-well plates (Thermo Fisher Scientific) at a 1.0×10^5/well density for 24 h. For experiments that examined VEGFA activities, the cells were changed to endothelial basal medium (EBM-2; Lonza) containing 2% FBS and 1% antibiotics and treated with PBS or 40 ng/ml recombinant VEGFA (R&D Systems) in the absence or presence of recombinant HtrA4 (1.5 or 3.0 μg/ml) or vehicle control for HtrA4 [Tris/glycine buffer containing 3 mM Tris (pH 7.4), 15 μM NaCl, 1 mM glycine, plus 25% protein stabilizing cocktail]. The 2 doses of HtrA4 were chosen to represent the median and highest levels of HtrA4 detected in the early onset PE circulation (21).

Western blot analysis

HUVECs were cultured in 12-well plates overnight and then treated for 24 h with either vehicle control or different doses of

recombinant HtrA4. The cells were then stimulated with VEGFA (40 ng/ml) for 15 min and lysed in lysis buffer [50 mM Tris, 150 mM NaCl, 2 mM EDTA, 25 mM NaF (serine/threonine protein phosphatase inhibitor), 0.2% Triton X-100, 0.3% Nonidet P-40 (MilliporeSigma), and 25 mM glycerolphosphate (a phosphatase inhibitor)] with the addition of Protease Inhibitor Cocktail Set III (Merck). The protein lysates were separated on 10% polyacrylamide SDS-PAGE gels and transferred to PVDF membranes (GE Healthcare, Chicago, IL, USA). The membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA; Bovogen, Keilor East, VIC, Australia) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (5% BSA/TBS-T) (MilliporeSigma) and incubated at 4°C overnight with primary antibodies diluted in 5% BSA/TBS-T. The following primary antibodies were used: human VEGFR2/KDR goat pAb (diluted to 1 μg/ml; R&D Systems), phospho-Akt rabbit mAb (4060, diluted 1:500; Cell Signaling Technology, Danvers, MA, USA), and Akt rabbit antibody (9272, diluted 1:1000; Cell Signaling Technology). The membranes were washed 3 times in TBS containing 0.2% Tween-20 and incubated for 1 h at room temperature with horseradish peroxidase–conjugated appropriate secondary antibodies diluted 1:2000 [a rabbit anti-goat secondary antibody for KDR and a goat anti-rabbit antibody for phospho-Akt and Akt (Dako, Glostrup, Denmark)]. To check loading control, a horseradish peroxidase–conjugated β-actin antibody (diluted 1:2000; Cell Signaling Technology) was incubated at room temperature for 30 min. The membranes were washed 3 times with TBS containing 0.2% Tween-20 and incubated in Lumi-Light Western Blotting Substrate (Roche Molecular, Mannheim, Germany). The bands were visualized using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Densitometry was performed using Fiji software (National Institutes of Health, Bethesda, MD, USA). Western blot analyses were repeated using lysates from 4 independent experiments.

Immunocytochemistry

HUVECs were seeded on 10-mm glass coverslips (Thermo Fisher Scientific) placed in 48-well plates and treated with vehicle control or different concentrations of HtrA4 for 24 h. Cells were then fixed with 4% paraformaldehyde (VWR, Radnor, PA, USA) for 10 min and permeabilized with 0.1% Triton X-100 (MilliporeSigma) in PBS for 5 min. All procedures were carried out at room temperature unless stated otherwise. Cells were blocked with 1% BSA in PBS for 2 h and incubated at 4°C overnight with 5 μg/ml VEGFR2/KDR goat pAb. Cells were then washed 3 times with PBS and incubated with a donkey anti-goat Alexa Fluor 488 antibody (diluted 1:200; Thermo Fisher Scientific) for 2 h; all the following steps were performed in the dark. Nuclei were stained with 5 μg/ml DAPI (diluted 1:2000; MilliporeSigma) for 10 min, and coverslips were mounted with fluorescent mounting media (Dako). Staining was visualized using a fluorescence microscope (BX60; Olympus, Notting Hill, VIC, Australia), and images were taken using an Olympus DP70 camera and Olympus CellSens software. Three to four images per treatment condition were taken randomly, and experiments were repeated independently 3 times.

Tube formation assay

HUVECs were seeded in 12-well plates and treated for 24 h with either vehicle control or VEGFA (40 ng/ml) in the absence or presence of 1.5 or 3.0 μg/ml HtrA4 in EBM-2 media supplemented with 2% FBS and 1% antibiotics. This low serum condition was used because HUVECs would not form tubes unless exogenous VEGFA was added. Cells were then reseeded at a 1×10^5 per well density in 48-well plates that were precoated with growth factor–reduced Matrigel (BD Biosciences) and treated with the same condition as prior to reseeding for another 16 h. Cells were washed 3 times with HBSS (Thermo Fisher Scientific) and labeled with 4 μg/ml calcein AM fluorescent dye (BD Biosciences) for 30 min at 37°C. Tubes were assessed using an Olympus BX60 fluorescent microscope at ×4 magnification, and images were taken using an Olympus DP70 camera and Olympus CellSens software. Each slide per treatment condition was roughly divided into 4 equal subsections, and 1 image was taken from each subsection to avoid overlapping images. The total tube length, branching points, and total tube numbers per area were quantified using Fiji software. Experiments were repeated independently 3 times.

Mouse aortic ring assay

C57BL/6J female mice were housed and handled according to the Monash University animal ethic guideline, and studies were approved by the Animal Ethic Committee (MMCB/2017/14) at Monash Medical Centre, Melbourne, Australia. The aorta was dissected, and fat was cleaned according to a previously published method (41). The aorta was gently flushed with 1 ml Opti-MEM (Thermo Fisher Scientific), cut into 0.5-mm-wide rings, and then serum starved overnight in Opti-MEM at 37°C. Type I rat-tail collagen (Merck) was diluted to 1 mg/ml in DMEM (Thermo Fisher Scientific) and used to coat 96-well plates (50 μl/well). Each aortic ring was then embedded in the center of each collagen-coated well with the lumen of the ring parallel to the bottom of the well and allowed to set at 37°C for 1 h. The rings were treated with 50 μl Opti-MEM supplemented with 1% FBS and 1% antibiotic without or with 40 ng/ml VEGFA in the absence or presence of 2 doses of HtrA4 (1.5 or 3.0 μg/ml). Medium was replaced after 72 h with fresh treatment medium and cultured until 144 h. Aortic rings were then stained with 4 μg/ml calcein AM fluorescent dye for 30 min at 37°C. Images were taken with a confocal microscope (Olympus) at ×10 magnification, and the numbers of microvessels formed and the distance of microvessel outgrowth per ring were determined using Fiji software. Treatments were conducted in duplicate per mouse and repeated in 4 mice.

Statistical analysis

Statistical analyses were conducted using Prism (v.8; GraphPad Software, La Jolla, CA, USA). Means were compared using either unpaired Student's *t* test or 1-way ANOVA, followed by Tukey's and Dunnett's *post hoc* tests, and a value of $P < 0.05$ was considered significant.

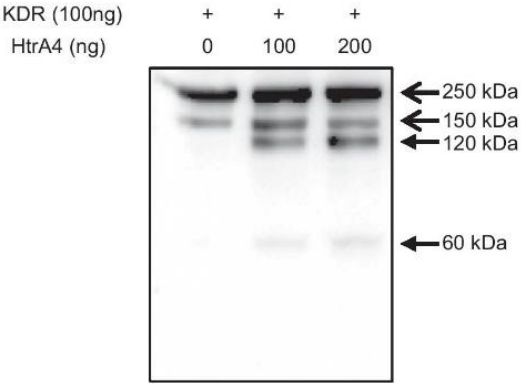

Figure 1. HtrA4 cleaves recombinant KDR *in vitro*. Recombinant KDR was incubated with different amounts of HtrA4 and analyzed by Western blot using an antibody against human KDR. A representative Western blot image is shown.

RESULTS

HtrA4 cleaves recombinant KDR *in vitro*

Recombinant human KDR was incubated overnight without and with recombinant HtrA4, and KDR cleavage was assessed by Western blot analysis using a KDR-specific antibody (**Fig. 1**). KDR alone displayed a main band at 250 kDa and a fainter band at 150 kDa. Incubation with HtrA4 produced additional KDR bands of 120 and 60 kDa (Fig. 1), consistent with KDR being cleaved by recombinant HtrA4.

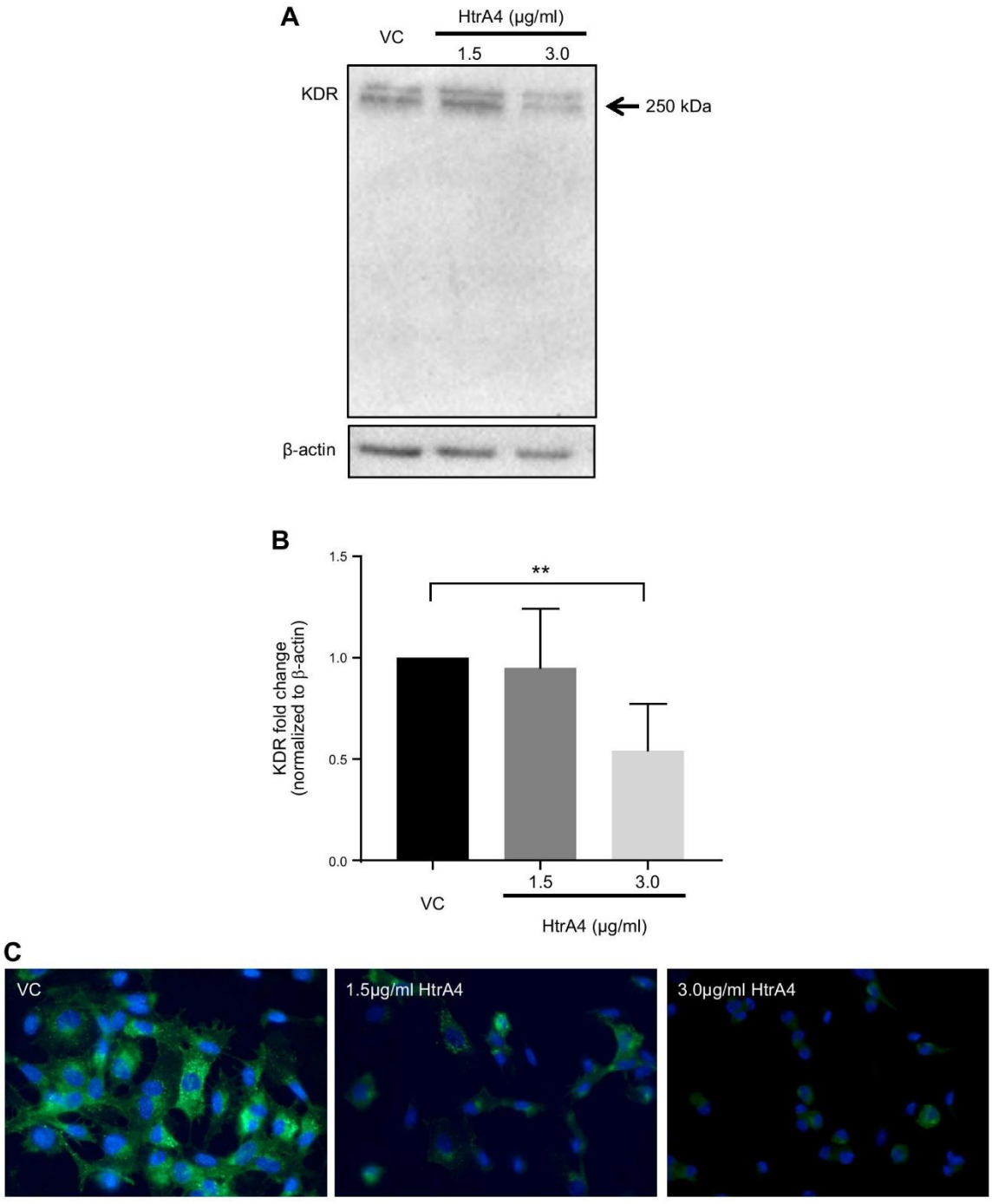

Figure 2. HtrA4 reduces the amount of KDR in HUVECs. *A*) HUVECs were treated with vehicle control (VC) or with 2 concentrations of HtrA4, and the lysates were analyzed by Western blot for KDR. β-Actin was used as a loading control. A representative Western blot image is shown. *B*) Densitometric analysis of Western blots of *A*. The graph represented the total intensity of the 2 KDR bands (normalized to β-actin) relative to vehicle controls ($n = 4$). Data are expressed as means ± SD. $^{**}P < 0.01$. *C*) Immunofluorescence of KDR in HUVECs. Cells were treated as in *A*, and a representative image is shown.

HtrA4 reduces the amount of KDR in HUVECs

To confirm that HtrA4 could also cleave KDR in HUVECs, cells were treated with vehicle control or HtrA4, and lysates were analyzed by Western blot for KDR (**Fig. 2*A***). Vehicle control–treated lysates showed 2 bands of KDR at ~250 kDa. Although no obvious difference was observed with 1.5 μg/ml HtrA4, cells treated with 3.0 μg/ml HtrA4 displayed a substantially lower amount of KDR (Fig. 2*A*). Densitometric analysis of 4 independent experiments showed that treatment with 3.0 μg/ml HtrA4 significantly reduced KDR levels in the HUVECs (Fig. 2*B*). To further confirm these data, HUVECs were similarly treated with the 2 doses of HtrA4, and KDR was examined by immunocytochemistry (Fig. 2*C*). Cells treated with vehicle control showed clear positive staining for KDR, whereas the signal was reduced by HtrA4 treatment, and this reduction was observed at both 1.5 and 3.0 μg/ml HtrA4 (Fig. 2*C*).

HtrA4 inhibits VEGFA-induced phosphorylation of Akt in HUVECs

PKB, also known as Akt, is a downstream signaling molecule that is phosphorylated by VEGFA to regulate various cellular responses (42, 43). To confirm that HtrA4 attenuates VEGFA action, HUVECs were treated without or with HtrA4 and then stimulated with VEGFA, and phospho-Akt and total Akt were analyzed by Western blot (**Fig. 3*A***). Compared with the vehicle control, treatment with 3.0 μg/ml HtrA4 greatly reduced the VEGFA-induced phospho-Akt relative to total AKT (Fig. 3*A*). Densitometric analysis of 4 independent experiments demonstrated that the level of phospho-Akt was significantly reduced in cells treated with 3.0 μg/ml HtrA4 compared with the vehicle control (Fig. 3*B*).

HtrA4 blocks VEGFA-induced tube formation in HUVECs

To further confirm that HtrA4 disrupts VEGFA action in endothelial cells, tube formation assay was performed using HUVECs (**Fig. 4*A***). Previously published work showed that HUVECs form tube-like structures when cultured in Matrigel supplemented with media containing 10% fetal calf serum (FCS) (21). To reduce VEGFA that is present in serum, HUVECs were cultured overnight in EBM-2 containing 2% FCS prior to plating on the Matrigel. HUVECs in this low-serum media did not form tubes, whereas addition of 40 ng/ml VEGFA induced regular tube-like structures (Fig. 4*A*), confirming that VEGFA is essential for HUVECs to form tubes. Cells treated with 1.5 μg/ml HtrA4 also formed tubes upon VEGFA stimulation, but the tubes were thinner compared with the VEGFA controls (Fig. 4*A*). In contrast, cells treated with 3.0 μg/ml HtrA4 failed to respond to VEGFA stimulation and did not form any tubes (Fig. 4*A*). To quantify the above data, total tube length (Fig. 4*B*), branching points (Fig. 4*C*), and total number of tubes (Fig. 4*D*) were measured from 3 independent experiments. All 3 parameters showed that exogenous VEGFA significantly promoted tube formation; however, this VEGF-induced process was completely blocked by 3.0 μg/ml HtrA4.

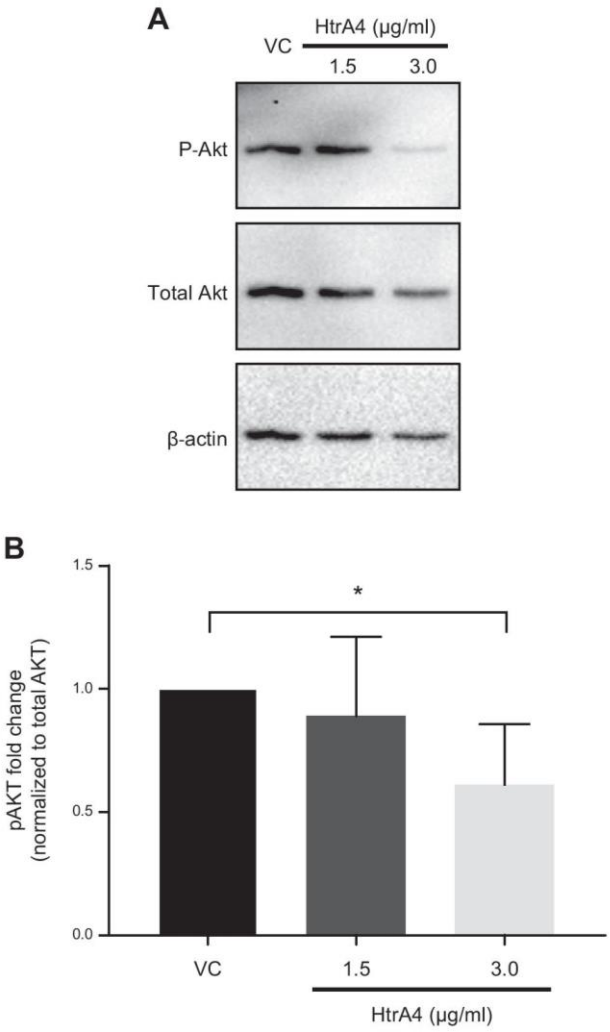

Figure 3. HtrA4 inhibits VEGFA-induced phosphorylation of Akt in HUVECs. *A*) Cells were treated with vehicle control (VC) or different concentrations of HtrA4 and stimulated with 40 ng/ml VEGFA. Lysates were analyzed by Western blot for phospho-Akt (p-Akt), total Akt, and β-actin. A representative image is shown. *B*) Densitometric analysis of Western blots of *A*. The intensity of phospho-Akt was normalized to total AKT ($n = 4$). Data are expressed as means ± SD. $^*P < 0.05$.

HtrA4 inhibits VEGFA-induced angiogenesis in mouse aortic ring explants

To further validate that HtrA4 can disrupt the VEGFA-induced angiogenesis, a mouse aortic ring explant assay was performed. The aortic rings were treated without or with VEGFA in the absence or presence of HtrA4, and the VEGFA-induced angiogenic activity was determined by the number of microvessels sprouted from each ring and the growth rate of the microvessels. Compared with the vehicle control, rings treated with VEGFA showed an increased level of angiogenesis with more microvessels as well as a bigger area of outgrowth (**Fig. 5*A***). This again confirmed the essential role of VEGFA in angiogenesis in this assay. Treatment with HtrA4 dose-dependently inhibited the VEGFA-induced angiogenesis in this assay, and complete inhibition was observed with 3.0 μg/ml HtrA4 (Fig. 5*A*). The aortic

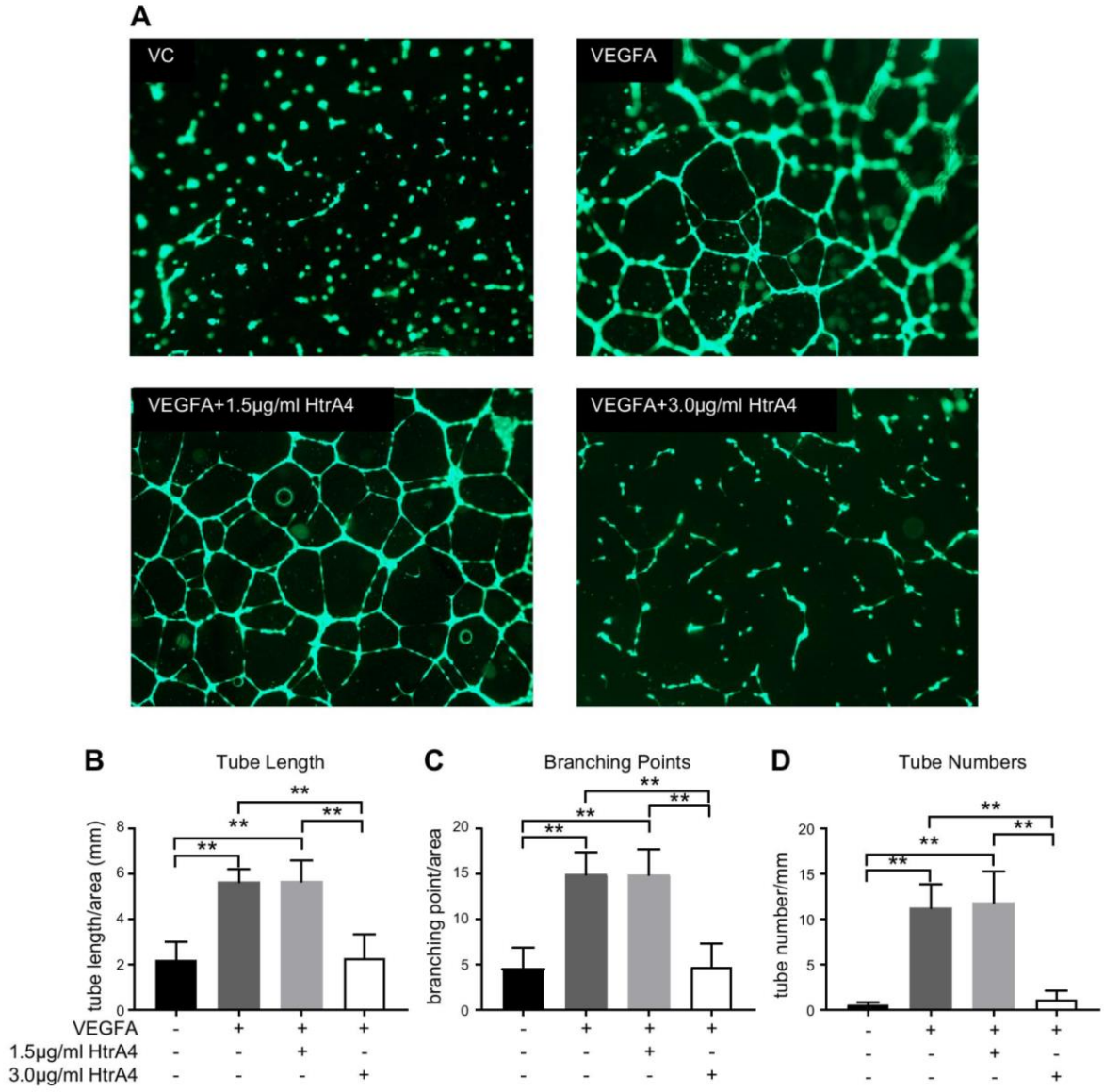

Figure 4. HtrA4 inhibits VEGFA-induced tube formation in HUVECs. *A*) Cells were treated with vehicle control (VC) or 40 ng/ml VEGFA without or with different concentrations of HtrA4. Three fields of view per treatment were randomly photographed, and 3 independent assays were performed. Representative images are shown. *B–D*) Quantification of tube formation. Total tube length per square millimeter area (*B*), total branching point per square millimeter area (*C*), and number of tubules per square millimeter area (*D*) ($n = 3$ for all). Data are expressed as means ± SD. $^{**}P < 0.01$.

rings were quantified from 4 independent experiments by measuring the distance of microvessel growth (Fig. 5*B*) and the number of microvessels protruded from the rings (Fig. 5*C*). Both parameters confirmed that VEGFA promoted angiogenesis, whereas HtrA4 dose-dependently inhibited VEGFA action. A complete inhibition was apparent when the rings were treated with 3.0 μg/ml HtrA4 (Fig. 5*B*, *C*).

DISCUSSION

Systemic endothelial dysfunction is a key feature of early-onset PE. We have previously identified that serine protease HtrA4 is produced specifically by the placenta and significantly up-regulated in early onset PE (21, 26–30). We have further reported that high levels of circulating HtrA4 in PE may be a potential causal factor of endothelial dysfunction (21, 31). Here, we demonstrated that high levels of circulating HtrA4 could cleave the main VEGFA receptor KDR to disrupt endothelial cell function and inhibit the VEGFA-induced angiogenesis.

We first demonstrated that HtrA4 cleaved the recombinant KDR *in vitro*, where extra bands of KDR were observed in the presence of HtrA4. We then demonstrated that the total amount of KDR protein was significantly reduced by HtrA4 in the HUVEC lysate. Unlike the recombinant KDR, HUVEC lysates contained many proteins, and the concentration of KDR was much lower; therefore, the

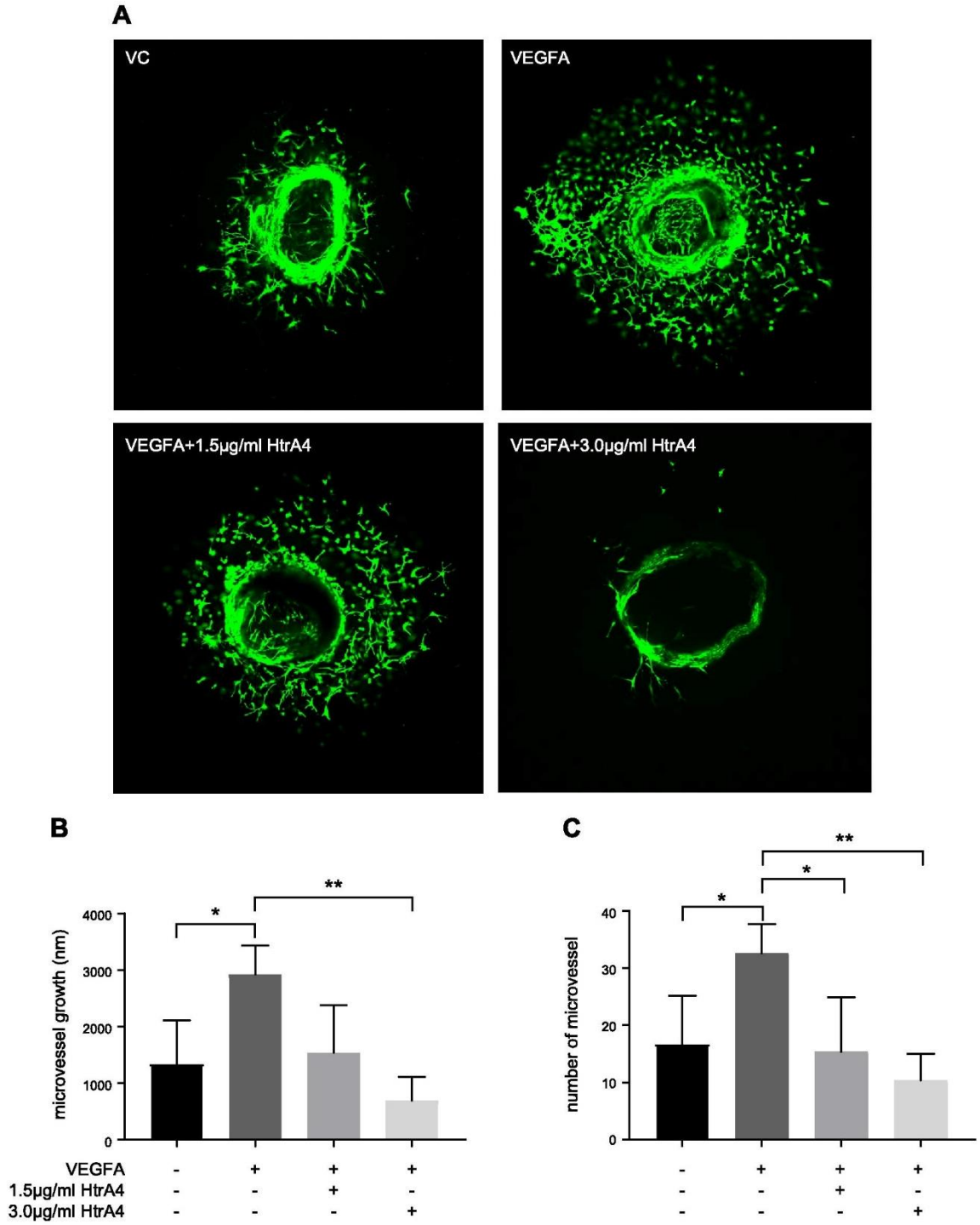

Figure 5. HtrA4 inhibits the VEGFA-induced angiogenesis in mouse aortic rings. *A*) Mouse aortic rings were treated with vehicle control (VC) or 40 ng/ml VEGFA without or with different concentrations of HtrA4. Each treatment was conducted in duplicate, and experiments were repeated in 4 individual mice. Representative images are shown. *B, C*) Quantification of microvessels formed from the aortic rings. Distance of microvessel growth from the aortic ring (*B*) and number of microvessels formed from the aortic rings (*C*) ($n = 4$ for all). Data are expressed as means ± SD. *$P < 0.05$, **$P < 0.01$.

detection of KDR on the Western blot appeared to be much weaker than the purified recombinant KDR. Furthermore, Western blot did not detect smaller cleaved bands in the lysates, likely because of the low amount and/or because the antibody only recognized the full-length KDR remaining in the cell lysates. We further confirmed that HtrA4 reduced KDR by immunocytochemistry, where the intensity of KDR staining was significantly reduced in HUVECs after HtrA4 treatment. This is a significant finding because for the first time the key endothelial VEGFA receptor KDR was identified

as a likely target of placenta-derived HtrA4. This provides a molecular explanation regarding why elevated circulating HtrA4 may adversely affect the systemic endothelium and contribute to the development of early onset PE.

KDR is the predominant receptor for VEGFA, which mediates various downstream functions in endothelial cells (44). Binding of VEGFA to KDR initiates a cascade of downstream intracellular signaling, including phosphorylation of Akt (34). When HUVECs were stimulated with exogenous VEGFA, phospho-Akt was clearly detected by Western blot analysis. However, cells that were treated with 3.0 μg/ml HtrA4 displayed significantly lower levels of phospho-Akt upon VEGFA stimulation, whereas the total Akt or β-actin was not altered by the HtrA4 treatment. These data strongly suggest that VEGFA was less effective in activating downstream Akt signaling when HUVECs were exposed to high levels of HtrA4, consistent with VEGFA action being attenuated by HtrA4 *via* cleaving KDR.

VEGFA is one of the most proangiogenic proteins in its family (45), and mice with deletion of the VEGFA gene are embryonically lethal due to abnormal blood vessel development (46). To further confirm that high levels of HtrA4 could inhibit VEGFA-induced angiogenesis, we examined the angiogenic ability of HUVECs to form tube-like structures. As an endothelial cell model, HUVECs formed regular tube-like structures when plated on Matrigel in normal cultural medium containing 10% FCS (21). However, few tubes formed when the serum levels were reduced to 2%, which minimized the number of growth factors present in the media. When this growth factor–reduced media was supplemented with 40 ng/ml VEGFA, HUVECs formed well-structured tubes, confirming the essential role of VEGFA in promoting tube formation (44, 45). When HUVECs were treated with 1.5 μg/ml HtrA4 and VEGFA together, thinner tubes formed, suggesting that even lower concentrations of HtrA4 impeded VEGFA action. When the HtrA4 concentration was increased to 3.0 μg/ml, the cells failed to respond to VEGFA stimulation for tube formation, suggesting that high levels of HtrA4 could completely block VEGFA action in endothelial cells. One limitation of this study is the use of HUVECs as a model. Although they are commonly used for studying of endothelial cell function (40), HUVECs may not reflect all the features of human vascular endothelial cells. Future studies are warranted with endothelial cells of other vascular origin.

To further confirm the blockade of VEGFA action by HtrA4 in another system, we used a mouse aortic ring explant culture model. Although mouse aorta does not represent microvessels, it is relatively easier to isolate, responds well to VEGFA in culture, and has been used by a number of studies to investigate angiogenesis and PE (39, 41). As reported previously, when mouse aortic rings were stimulated by VEGFA, an increased number of microvessels formed from the outer layer compared with the vehicle control, consistent with VEGFA-promoting angiogenesis (39). However, when the rings were treated with HtrA4, the VEGFA-induced microvessel numbers and the area of outgrowth were dose-dependently reduced by HtrA4, suggesting that HtrA4 inhibited the VEGFA-induced angiogenesis in mouse aortic rings. Furthermore, the rings treated with 3.0 μg/ml HtrA4 showed even less microvessel growth compared with the vehicle control, suggesting that the high dose of HtrA4 inhibited the endogenous VEGFA activities. These data also suggest that explant mouse aortic rings were much more sensitive to HtrA4 than HUVECs.

This is the first study to demonstrate that high levels of HtrA4 observed in the early onset PE circulation could cleave the main VEGFA receptor to cause endothelial dysfunction. The vast majority of studies in PE have focused on sFlt1 as a main antiangiogenic factor (39, 47), and great efforts have been dedicated toward reducing serum sFlt1 levels as a therapeutic treatment (39). Our studies presented here suggest an additional and previously unknown mechanism that may significantly impair angiogenesis in PE. We demonstrated that high levels of HtrA4 reduced endothelial KDR and significantly impeded VEGFA-activated Akt signaling and angiogenesis. Because KDR is the main receptor of VEGFA action in endothelial cells, cleavage of KDR by HtrA4 would impede angiogenesis even when VEGFA is ample. This would suggest that, to fully restore angiogenesis in early-onset PE, increasing circulating VEGFA by reducing sFlt1 may not be sufficient, and inhibition of KDR cleavage may be necessary. Our studies therefore suggest that HtrA4 is a potentially important factor that may be involved in early onset PE development and is a unique target for treatment. Fj

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AUTHOR CONTRIBUTIONS

Y. Wang and G. Nie designed the study and wrote the manuscript; M. La, T. Pham, and G. O. Lovrecz contributed to HtrA4 purification; and Y. Wang performed experiments and analyzed the data.

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Chapter 5

Integrated discussion, future directions and conclusions

5.1. Integrated discussion

PE is a serious complication of human pregnancy and the early-onset subtype poses more serious maternal and neonatal consequences. PE is closely associated with systemic endothelial dysfunction, however, the underlying causes remain unclear and currently the only effective treatment is premature delivery. Previous studies have demonstrated that HtrA4 is a serine protease that is specifically produced by the placenta and significantly up-regulated in early-onset PE (Singh et al., 2015, Inagaki et al., 2012, Lapaire et al., 2012, Kaartokallio et al., 2015, Liu et al., 2018, Brew et al., 2016). We have subsequently demonstrated that high levels of circulating HtrA4 can disrupt the normal endothelial cell function (Singh et al., 2015).

Therefore, it was hypothesized that high levels of HtrA4 observed in early-onset PE circulation could adversely affect the maternal endothelial cells and contribute to the pathogenesis of early-onset PE. The aims of my PhD research were to investigate the impact of HtrA4 on endothelial cell biology and repair, and to explore the mechanisms of HtrA4 action on endothelial cells using HUVECs and primary EPCs as endothelial cell models. The results of my PhD research confirmed that high levels of HtrA4 induced inflammation in HUVECs and altered their expression of genes vital for vessel biology. Furthermore, HtrA4 not only halted endothelial cell proliferation, but also inhibited circulating EPC proliferation and differentiation into mature endothelial cells for repair. Lastly, my research demonstrated that high levels of HtrA4 reduced the main VEGF-A receptor KDR in the HUVECs to inhibit angiogenic-like functions.

Chapter 2 (Wang and Nie, 2016 *Placenta*) examined the impact of HtrA4 on endothelial gene expression using HUVECs as a model. A range of genes that play important roles in inflammation, angiogenesis, vaso-activity, platelet activation, cell

adhesion and coagulation were all significantly altered by HtrA4 (Table 5.1). The study showed that the expression of pro-inflammatory factors interleukin (IL)6, prostaglandin-endoperoxide synthase (PTGS)2 and IL1β were all significantly up-regulated in cells that were treated with 3.0 μg/ml HtrA4, the highest level observed in early-onset PE circulation. The IL6 protein in the media was also significantly elevated, consistent with its mRNA result. Overall, this study found that high levels of HtrA4 detected in the preeclamptic circulation induced inflammation in HUVECs and induced wide-spread changes in gene expression observed in early-onset PE.

Table 5.1 listed genes that were highly regulated by HtrA4 in HUVECs. Thrombomodulin (THBD) mRNA expression was significantly up-regulated by HtrA4, and its protein level is reported to be increased in PE serum, making it a potential marker of endothelial dysfunction (Prochazka et al., 2015, Rousseau et al., 2009). Thrombospondin (THBS)1 protein is closely involved in regulation of endothelial cell functions such as adhesion, motility and proliferation, and its mRNA expression was significantly down-regulated by HtrA4, consistent with reports that THBS1 is lower in women with severe PE (Stenczer et al., 2012). All of this evidence suggests that high levels of circulating HtrA4 detected in early-onset PE could alter endothelial cell biology and induce endothelial dysfunction.

Of all the genes examined by real-time RT-PCR, only three genes (*ALOX5*, *PTGIS* and *BCL2*) showed no significant changes in their mRNA expression between the controls and HtrA4 treatments, inconsistent with the microarray data. Intriguingly, all these three genes were expressed at a low level in HUVECs, and therefore the microarray assessment may not be accurate. Real-time RT-PCR detected the low expression of these genes more accurately, showing large variabilities among

samples with no significant differences between treatments. Due to limitation of time and reagent, only two proteins, IL6 and monocyte chemoattractant protein (MCP)1 were validated by ELISA. A cytokine antibody array was subsequently used to identify more cytokines that might be affected by HtrA4. However, no new candidate was found. Although IL1β, which showed a huge increase in mRNA expression, displayed no difference in protein levels when treated with HtrA4. Explanations for the inconsistency between mRNA and protein may be due to the low levels of IL1β present in the HUVEC media, or a possible unknown mechanism of IL1β regulation at the protein level.

Table 5.1. List of genes that were significantly affected by HtrA4.

Gene category	Gene name	Regulation by HtrA4	Functions in endothelial cells
Inflammatory Response	*CCL2/MCP1*	↓	Involved in immnoregulatory and inflammatory processes, recruitment of monocytes and macrophages
	PTGS2/COX2	↑	A key enzyme in prostaglandin biosynthesis, and is involved in inflammation and mitogenesis
	IL6	↑	Major functions in inflammation and the maturation of B cells, highly up-regulated in PE circulation
	IL1B	↑	Mediates inflammatory response and various cellular activities, including proliferation and differentiation
Angiogenesis and Vaso-activities	*EDN1/ET1*	↓	A secreted peptide that acts as a vasoconstrictor
	FGF2	↑	Involves in ranges of biological processes, including mitogenic and angiogenic activities
	VEGFA	↑	Acts specifically on endothelial cells and has various functions, including angiogenesis and cell growth
Platelet Activation and Cell Adhesion	*THBS1*	↓	Mediates cell-to-cell and cell-to-matrix interactions and plays a role in platelet aggregation and angiogenesis
	SERPINE1	↑	A major inhibitor of tissue plasminogen activator and urokinase, acts on blood vessels to inhibit fibrinolysis
	IL11	↑	Stimulates the proliferation of hematopoietic cells and megakaryocyte to increase platelet production
Coagulation and Apoptosis	*OCLN*	↑	An integral membrane protein that is required for cytokine-induced regulation of the tight junction
	MMP1	↑	Involves in the breakdown of extracellular matrix in many physiological processes
	THBD	↑	An endothelial-specific receptor that binds to thrombin to activate protein C, which degrades clotting factors. It is a marker for endothelial dysfunction

Chapter 3 (Wang et al, 2019 *Scientific Reports*) further examined the impact of HtrA4 on endothelial cells. It was found that high levels of HtrA4 not only inhibited endothelial cell proliferation, but also inhibited circulating EPC proliferation and differentiation for repair. Firstly, it was shown that high levels of HtrA4 completely halted HUVEC proliferation, while cell viability was not affected. At the molecular level, thirty-five genes that are involved in cell cycle regulation were significantly down-regulated by HtrA4, many of which were also affected at a lower dose of HtrA4. This data thus strongly suggests that HtrA4 has a major impact on endothelial cell proliferation. Ki67 is a cell proliferation marker, and HUVECs treated with high levels of HtrA4 showed a significant loss of Ki67 immunofluorescent staining compared with control cells, further confirming that HtrA4 inhibits cell proliferation.

Secondly, Chapter 3 examined the impact of HtrA4 on EPCs, which are circulating endothelial cells that can be recruited to the site of endothelial injury and differentiate into resident endothelial cells to repair the damaged endothelium. HtrA4 likewise inhibited primary EPC proliferation without affecting cell viability. HtrA4-induced reduction in cell proliferation marker ki67 was even more pronounced in EPCs than HUVECs, suggesting that EPCs are more sensitive than HUVECs to HtrA4 treatment. A number of cell cycle genes that were highly down-regulated by HtrA4 in the HUVECs were also examined in primary EPCs, and majority of these likewise showed a significant reduction in mRNA levels following treatment with high doses of HtrA4, confirming that high levels of HtrA4 inhibit EPC proliferation. Interestingly, three out of the four genes examined were affected more significantly by the higher dose of HtrA4 at 24h time point, and at 48h all four genes had lower levels of expression across all treatment groups, including the vehicle control. This data suggests that EPCs may proliferate more quickly than HUVECs in culture and that their proliferation may be

inhibited more severely by 48h, which is consistent with the highly proliferative nature of progenitor cells.

EPCs were further assessed for their ability to form tube-like structures on Matrigel. EPCs treated with lower doses of HtrA4 still formed tubes similar to the control cells, however, the tubes were thinner and disjoined in many areas. In contrast, EPCs treated with high doses of HtrA4 failed to form any tubes, suggesting that high levels of HtrA4 could have a detrimental effect on circulating EPCs, impeding endothelial repair and exacerbating the endothelial dysfunction.

Chapter 4 (Wang et al, 2019 *FASEB*) discovered a novel mechanism of HtrA4 action on endothelial cells: HtrA4 can cleave the main VEGF-A receptor KDR to inhibit VEGF-A-dependent angiogenesis and endothelial cell function. The result showed that high levels of HtrA4 significantly reduced the amount of KDR present in HUVECs, and reduced phosphorylation of downstream signalling molecule Akt, confirming that HtrA4 can inhibit VEGF-A activity.

Chapter 4 further validated that high levels of HtrA4 can significantly inhibit VEGF-A-induced angiogenesis. This was first demonstrated in an HUVEC tube formation assay. HUVECs were cultured in 2%FCS with no growth factor supplement, and no tubes were formed. Addition of VEGF-A promoted the formation of well-structured tubes, confirming the essential role of VEGF-A in angiogenesis. Treatment with lower doses of HtrA4 resulted in thinner tubes, showing that HtrA4 could exert an effect on VEGF-A even at a lower dosage. Treatment with higher doses of HtrA4 completely blocked VEGF-A action as the HUVECs failed to form any tubes.

VEGF-A-induced angiogenesis was then examined in an aortic ring assay using explant culture of mouse aorta. Rings treated with VEGF-A showed an increased

number of micro-vessels forming from the outer layer of the rings, as well as a wider area of outgrowth, compared to the untreated controls. HtrA4 in this assay dose-dependently reduced the VEGF-A-induced micro-vessel numbers as well as outgrowth, further confirming that HtrA4 attenuates VEGF-A action.

Chapter 4 confirmed that HtrA4 cleavage of KDR is detrimental to VEGF-A action in endothelial cells, implicating a previously unknown mechanism that may significantly impair angiogenesis in early-onset PE (Figure 12). The data suggests HtrA4 as a potentially important therapeutic target for treatment of endothelial dysfunction in early-onset PE. Since HtrA4 is not expressed outside the placenta, inhibiting HtrA4 in the maternal circulation may restore VEGF-A function without inflicting major side effects on other tissues.

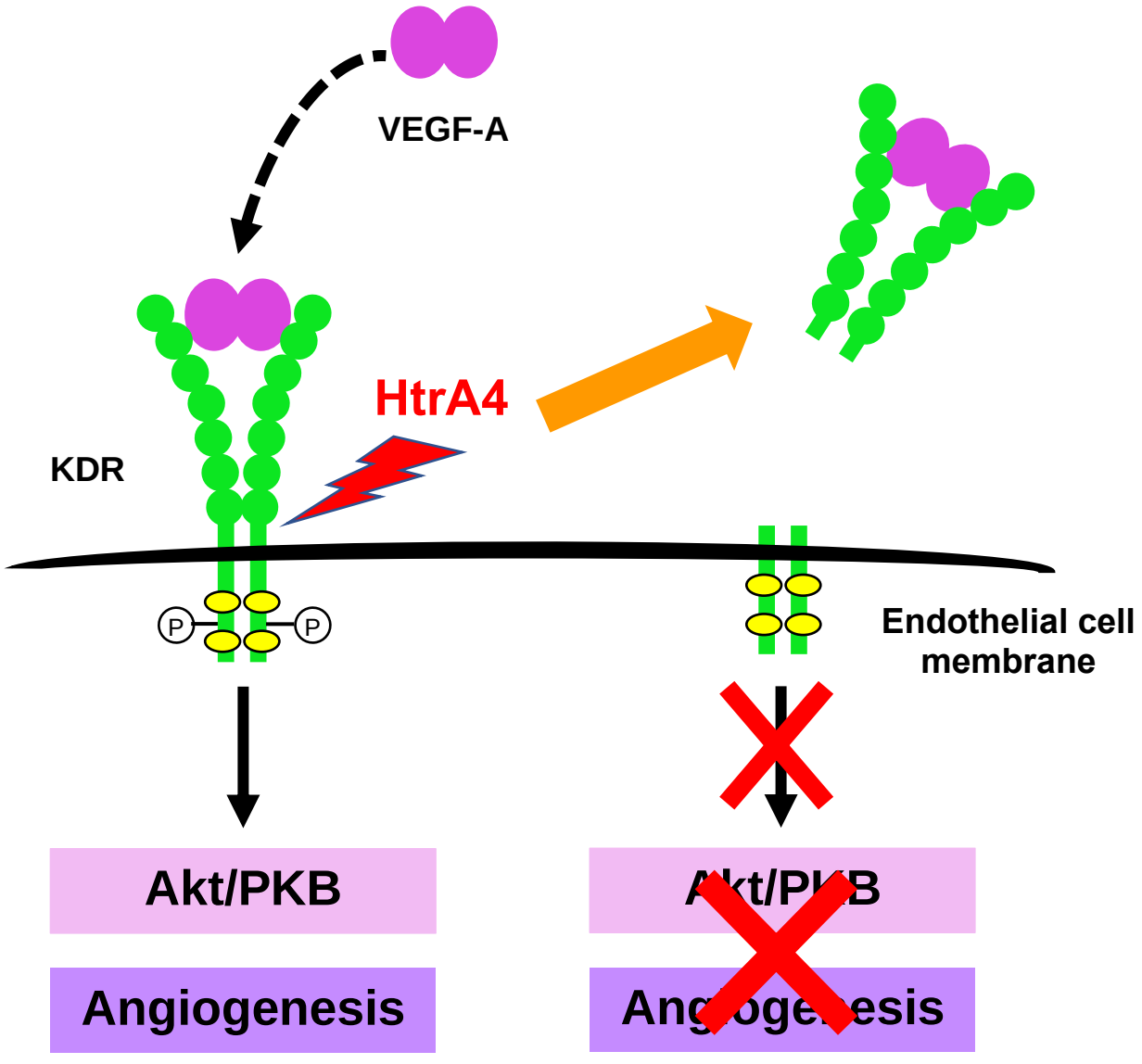

Figure 12. HtrA4 cleavage of VEGF-A receptor KDR has a detrimental effect on downstream endothelial cell signalling and angiogenesis.

In summary, my studies showed that HtrA4 has a major role in the pathogenesis of early-onset PE. My studies suggest that high levels of HtrA4 can adversely impact on endothelial cells, it also can also inhibit circulating EPC proliferation and differentiation to impede their repair function. Reductions in EPC numbers and functionality may explain the persistent endothelial dysfunction that is often observed long after PE, as the endothelial repair mechanism in these women are compromised. Moreover, HtrA4 inhibits angiogenesis in endothelial cells by cleaving the main VEGF-A receptor KDR. Given KDR is a crucial mediator of various endothelial cell functions including proliferation, permeability, migration and survival, HtrA4-induced KDR reduction in endothelial cells will have a detrimental effect on endothelial cell functions.

HtrA4 is the only member of the mammalian HtrA family that is specifically expressed in the placenta, and highly elevated in early-onset PE at the time of disease presentation. Currently, little is known about its exact roles in the normal placenta and its pathophysiological roles in early-onset PE. The other members of the HtrA protease, HtrA1 and HtrA3 are also highly expressed by the placenta, as well as many other tissues, therefore, unlike HtrA4, these two proteases may have significant roles in wide range of tissues and diseases. In contrast, HtrA4 is not widely expressed in other tissues and thus presents as a unique target for treatment of early-onset PE with minimal impact on other organs. My PhD project is the first study to demonstrate that excessive amounts of HtrA4 observed in early-onset PE circulation may have a detrimental effect on endothelial cells, contributing to the pathogenesis of early-onset PE. This is also the first time a novel mechanism of HtrA4 has been identified which can directly impair angiogenesis in endothelial cells.

5.2. Future directions

This PhD study identified HtrA4 as a potential causal factor in endothelial dysfunction associated with early-onset PE. HUVECs were used as the primary model of endothelial cells, but it may not reflect all the features of vascular endothelial cells. Future studies require examination of HtrA4 impact on human vascular endothelial cells, which may be a better representation of the maternal vascular system.

This study identified many cell cycle genes that were significantly down-regulated by HtrA4. When cells stop proliferating, they would undergo senescence and telomere attrition. Future studies thus should investigate whether HtrA4 affects genes related to senescence and telomere function in endothelial cells. One study has already demonstrated that EPCs isolated from early-onset PE women show increased cellular senescence (Sugawara et al., 2005a), it would thus be important to investigate whether HtrA4 facilitates the premature aging of endothelial cells and circulating EPCs in early-onset PE, contributing to the persistent endothelial dysfunction in this disease.

Primary human EPCs used in this project provide a useful model to study the effect of HtrA4 on endothelial cell functions. As this study has demonstrated that HtrA4 can inhibit VEGF-A action by cleaving KDR in the HUVECs, future studies should investigate whether HtrA4 can also cleave KDR from EPCs to block VEGF-A- induced cellular functions. These studies would further confirm the detrimental effect of HtrA4 on endothelial cell repair, and identify the mechanism of HtrA4 action on EPCs.

This PhD study has demonstrated that HtrA4 cleaves recombinant KDR *in vitro* as well as in HUVECs. Future studies should investigate the potential target sequences of

HtrA4 on KDR through N-terminal sequencing to gain a better understanding of the mechanism of HtrA4 cleavage.

Women with early-onset PE often have elevated sFlt-1 and low PIGF level in their blood circulation, and this is thought to be the major contributing factor to the reduced angiogenic activity in their endothelial cells, because excessive sFlt-1 would bind to free VEGF-A to prevent the activation of KDR. Future studies should examine whether high levels of HtrA4 and sFlt-1 would synergistically inhibit VEGF-A action to severely affect endothelial cells.

This PhD study demonstrated the adverse impact of HtrA4 on endothelial cells mainly using purified recombinant HtrA4. Since HtrA4 is released into the maternal circulation, it would be interesting to treat endothelial cells directly with sera collected from preeclamptic women to determine if it can disrupt endothelial cell function. Our laboratory has been working on a neutralising antibody for HtrA4, and if successful, this antibody can be used to block HtrA4 activity, and to examine the specific activity of HtrA4 in preeclamptic serum on endothelial cells.

5.3. Conclusions

This PhD study demonstrated that high levels of HtrA4 observed in the circulation of early-onset PE induced inflammation and profoundly altered the expression of genes important for endothelial cell biology in HUVECs. Furthermore, high levels of HtrA4 inhibited endothelial cell proliferation and hindered EPC proliferation/differentiation, suggesting that HtrA4 may prevent endothelial repair.

Moreover, this PhD study demonstrated that high levels of HtrA4 cleaved the main VEGF-A receptor KDR in endothelial cells, thereby reducing downstream Akt activity, and inhibiting VEGF-A action. Functionally, high levels of HtrA4 inhibited VEGF-A-induced angiogenesis in both HUVECs and explant culture of mouse aorta. This study thus found that high levels of HtrA4 inhibited VEGF-A action, identifying a novel mechanism of HtrA4 action on endothelial cells.

Collectively, this PhD study suggests that high levels of HtrA4 can have a detrimental effect on endothelial cells, and that HtrA4 is a potential causal factor of early-onset PE. These findings provide new insights into the understanding of the pathogenesis of early-onset PE. Since HtrA4 is not widely expressed, inhibiting it in the maternal circulation may offer an attractive therapeutic solution without major side effects for the treatment of early-onset PE.

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Appendix 1

Solutions and buffers

Cell culture media

HUVEC culture media

DMEM

10% (v/v) filtered FBS

2 mM L-glutamine

1 mM sodium pyruvate

1% (v/v) Antibiotic-Antimycotic (containing penicillin, streptomycin & amphotericin B)

Mouse aortic ring culture media

Opti-MEM

1% (v/v) filtered FBS

1% (v/v) Antibiotic-Antimycotic (containing penicillin, streptomycin & amphotericin B)

EPC culture media

Endothelial basal medium per 500ml, supplemented with:

- 75 ml filtered FBS
- 5 ml (v/v) Antibiotic-Antimycotic (containing penicillin, streptomycin & amphotericin B)
- 0.2 ml hydrocortisone
- 2 ml hFGF-B
- 0.5 ml VEGF
- 0.5 ml R3-IGF-1
- 0.5 ml Ascorbic Acid
- 0.5 ml hEGF
- 0.5 ml GA-1000

EPC culture coating solution

0.575 ml glacial acetic acid

Dissolve in 495 ml milliQ H_2O, and filter

Dissolve 25 mg rat tail collagen I to the above solution

Freezing media

95% (v/v) FBS

5% (v/v) DMSO

Immunocytochemistry

4% paraformaldehyde (PFA)

Add 4 g PFA to 100 ml PBS on a heat block until all powders are full dissolved.

Permeabilization buffer (0.1% Triton X-100)

Dissolve 10 µl Triton X-100 in 10 ml PBS

Blocking Buffer (1% BSA)

Dissolve 0.1 g BSA in 10 ml PBS

Cleavage assay

Assay buffer

1.17 g NaCl

0.788 g Tris-HCl

0.25 g CHAPS

Make up to 100 ml milliQ H_2O

Western blot analysis buffers

Universal immunoprecipitation (UIP) buffer (lysis buffer)

0.61 g Trizma base

0.88 g NaCl

0.058 g EDTA

0.076 g EGTA

0.105 g NaF

0.2 ml Triton X-100

0.3 ml Nonidet P-40

0.54 g β-glycerolphosphate

Make up in 100 ml milliQ H_2O

10x Laemmli's buffer

30.3 g trizma base

144.4 g glycine

Make up to 1 L with milliQ H_2O

20% sodium dodecyl sulfate (SDS)

Dissolve 20 g SDS in 100 ml milliQ H_2O

1x running buffer

100 ml 10x Laemmli's buffer

5 ml 20% SDS

895 ml milliQ H_2O

1x transfer buffer

100 ml 10x Laemmli's buffer

200 ml methanol

700 ml milliQ H_2O

10x TBS

60.5 g trizma base

87.6 g NaCl

Make up to 1 L with milliQ H_2O and pH to 7.6

Washing buffer (TBS-T)

2 ml Tween 20

Dissolve in 1 L 1xTBS

Blocking buffer (5%BSA/TBS-T)

0.1 ml Tween 20

5 g BSA

Dissolve in 100 ml 1xTBS

10% ammonium persulfate (APS)

Dissolve 1 g APS in 10 ml milliQ H_2O

Running gel (lower)

4.9 ml milliQ H_2O

2.5 ml 1.5 M Tris

2.5 ml 40% Acrylamide/Bis

50 µl 20% SDS

50 µl 10% APS

5 µl TEMED

Stacking gel (upper)

3.2 ml milliQ H_2O

1.25 ml 0.5 M Tris

0.5 ml 40% Acrylamide/Bis

25 µl 20% SDS

25 µl 10% APS

10 µl TEMED

4x loading buffer

1 ml 1 M Tris pH 6.8

2 ml glycerol

1 ml 20% SDS

Small amount of bromophenol blue

Add 50 µl β-mercaptoethanol to 1 ml of the above solution before use (5%)

Appendix 2

Protocols

Immunocytochemistry

Materials

- 10 mm coverslips
- 48-well plate
- Cell culture PBS
- 4% PFA
- Permeabilization buffer 0.1% Triton X-100/PBS
- 1%BSA/PBS
- Primary antibodies
- Secondary fluorescence Alexa antibodies
- Negative control IgG – same species as primary antibody
- Fluoro-Save mounting media

Procedure

1. Culture cells in 48-well plate containing 10 mm coverslip to approximately 80% confluency.
2. Remove media and rinse the wells 3 times with cold PBS.
3. Fix the cells in 4% PFA for 10 min and rinse again 3 times with cold PBS.
4. Permeabilize the cells with 0.1% Triton X-100/PBS for 5 min and rinse 3 times with PBS.
5. Block with 1% BSA/PBS for 2 hours at room temperature.
6. Dilute primary antibody (eg 1:50, 1:200 and 1:500) in 1% BSA/PBS, add 100 ul to each well and incubate overnight at 4^{o}C.
7. Wash the cells 3 times for 15 min each with PBS.
   Perform all the following steps with the lights off.
8. Dilute secondary fluorescence Alexa detection antibody at 1:200 dilution in 1% BSA/PBS, add 100 ul to each well and incubate at room temperature for 2 hours.
9. Wash the cells 3 times for 15 min each with PBS.
10. Dilute DAPI to 1:2000 with PBS and add to the cells for 10 min at room temperature to stain the nuclei then wash 3 times for 15 min each with PBS.
11. Place flurosave reagent on a slide, carefully remove the coverslip from each well and place with the side with the cells facing down onto the slide.
12. Seal the coverslips with nail polish and leave to air dry.

Western blot analysis

Materials

- Biorad SDS-PAGE and western blot apparatus
- 4x loading buffer
- 1x running buffer
- 1x transfer buffer
- Washing buffer TBS-T
- Blocking buffer 5%BSA/TBS-T
- Roller
- Primary antibodies
- Secondary HRP-conjugated antibodies
- Lumi-light western blotting substrate
- Bio-Rad ChemiDoc MP Imaging System

Procedure

1. Determine the volume of samples needed and add 4x loading buffer to give a final concentration of 1x sample buffer.
2. Incubate the samples on a heat block for 5 min at 95°C, then spin down briefly.
3. Load marker and samples onto SDS-PAGE gel in desired order.
4. Fill the tank with 1x running buffer and run gel at 200 V for approximately 45 min.
5. Pre-soak PVDF membrane (roughly cut to the size of the gel) with 100% methanol, then immerse in ice cold 1x transfer buffer.
6. Following completion of the gel electrophoresis, carefully remove the gel from the enclosed casing and assemble the transfer sandwich according to the following order:
    - Place the white side of the cassette on the bottom.
    - Place one piece of cassette sponge on top, then 2 filtered papers.
    - Place PVDF membrane on top using a forceps.
    - Place gel on top and then two filtered papers, remove any air bubbles with a roller.
    - Place another cassette sponge on top and roll out any remaining air bubbles.
    - Close and clamp the sandwich unit.

7. Place the assembled sandwich unit into the transfer unit, with the black side of the sandwich unit facing the black side of the transfer unit, and fill tank with 1x transfer buffer.
8. Add ice block to the tank and transfer at 100 V for 1h.
9. When transfer is completed, remove the PVDF membrane from the sandwich unit using a forceps.
10. Block the membrane with 5% BSA/TBS-T for 1h at room temperature on a roller.
11. Remove the blocking buffer and add primary antibody (diluted in 5% BSA/TBS-T) and incubate overnight on a roller at 4°C.
12. Discard the antibody and wash membrane 3 times for 10 min each with TBS-T.
13. Incubate membrane with secondary antibody (diluted in TBS-T) for 1h at room temperature on a roller.
14. Discard the antibody and wash membrane 3 times for 10 min each with TBS-T.
15. Prepare the Lumi-light western blotting substrate, and applied directly on the membrane for 1-2 min.
16. Scan the membrane immediately using Bio-Rad ChemiDoc MP Imaging System.

Isolation of EPCs from umbilical cord blood

Materials

- Anticoagulant – Heparin (need 10 U per 1 ml blood)
- PBS
- Ficoll-Paque PLUS
- EBM-2 10:1 (EBM-2 containing 10% FBS & 1% antibiotic-antimycotic)
- Trypan blue solution for cell count
- EGM-2
- 15 ml and 50 ml conical centrifuge tubes, sterile
- Trypsin/EDTA, warm to 37°C
- 20-ml syringe, sterile
- Mixing cannula,
- Transfer pipet, sterile
- 6-well and 24-well plates
- Rat tail collagen I for coating (used at final concentration of 50 µg/ml diluted in 0.02 N sterile glacial acetic acid)
- Cloning cylinders
- Forceps, sterile
- Pasteur pipets, sterile
- 1.5 and 2.0 ml microcentrifuge tubes
- 25 and 75 cm^2 vented tissue culture flasks, coated with rat tail collagen I

Procedure

1. Collect the blood in heparin (use 10 U heparin/ml blood), and aliquot 15 ml of the blood into a 50 ml conical centrifuge tube.
2. Add 20 ml PBS to the blood and pipet several times to mix.
3. Draw up 15 ml Ficoll into a 20 ml syringe and fit the syringe with a mixing cannula.
4. Place the tip of the mixing cannula at the bottom of the tube of diluted blood and carefully underlay the Ficoll. Make sure all air has been removed from the syringe before dispensing the Ficoll.
5. Centrifuge the tubes 30 min at 740 × *g*, room temperature, with no brake.
6. Using a transfer pipet, remove the hazy layer of mononuclear cells (MNCs) that sits at the interface between the clear Ficoll layer below and the yellow serum

layer above. Dispense the MNCs into a 50ml conical tube containing 10 ml EBM-2 10:1.

7. Centrifuge the MNCs 10 min at 515 × *g*, room temperature with a high brake.
8. Carefully aspirate and discard the supernatant. Care should be taken to avoid disturbing the cell pellet.
9. Gently tap the tube to loosen the pelleted cells and resuspend in 10 ml of EBM-2 10:1.
10. Repeat the steps 7 to 9 one time.
11. Remove 10 µl of the cell suspension and mix with 10 µl trypan blue. Count viable cells.
12. Centrifuge the cell suspension 10 min at 515 × *g*, room temperature, with a high brake. Aspirate the supernatant.
13. Tap the tube to loosen the cell pellet and resuspend MNCs in EGM-2 at 1.25 × 10^7 cells/ml.
14. Pipet 4 ml (5 × 10^7 MNCs) into each well of a 6-well tissue culture plate pre-coated with rat tail collagen I and place in a 37°C, 5% CO2 humidified incubator.
15. After 24h (day 1), slowly remove the medium from the well with a pipette. Medium is removed at a rate of 1 ml per 4 to 5 sec. Leave some liquid in the well to prevent drying of the plate surface.
16. Slowly add 2 ml EGM-2 to the well.
17. Slowly remove the 2 ml of medium and add 4 ml cEGM-2 to the well. Return the cells to the incubator.
18. After another 24h (day 2), refresh the medium by slowly removing the medium from the well with a pippet and adding 4 ml cEGM-2 to the well. Repeat medium change daily to day 7 and every other day thereafter.
19. From day 7, medium can be aspirated from the plate, and medium can be added at a moderate rate of 1 ml per 2 to 3 sec.
20. ECFC-derived colonies appear between day 14 and day 28 of culture as well-defined areas of cobblestone-appearing cells.
21. Individual colony is visualized and outlined using an inverted microscope.
22. Prepare the cloning cylinders.
23. Aspirate the medium and wash the culture well two times with 3 ml PBS.
24. After aspirate the final wash of PBS, place the cloning cylinder around each colony and press firmly against the plate using forceps.

25. Using a Pasteur pipet fitted with a bulb, add 1 to 2 drops of warm trypsin/EDTA into each cloning cylinder.
26. Incubate the plate for 1 to 5 min until the cells within the cylinder begin to ball up and detach.
27. Once all the cells within the cylinder have detached, place the tip of a Pasteur pipet containing 200 to 300 µl of cEGM-2 into the centre of the cylinder and pipet up and down vigorously several times. Collect the entire volume into a microcentrifuge tube. Wash the area within the cylinder 1 to 3 more times with 200 to 300 µl cEGM-2 until all cells are collected into the microcentrifuge tube.
28. Seed the cells from each ECFC colony into one well of a 24-well tissue culture plate pre-coated with rat tail collagen I in a total volume of 1.5 ml of EGM-2 and culture in a 37°C, 5% CO2 humidified incubator for expansion.
29. ECFCs can now be propagated as monoclonal cell line.

Mouse aortic ring assay

Materials

- Dissection tools
- Petri dish
- Small ruler
- Opti-MEM
- 27-G needle
- 1 ml syringe
- Scalpel
- 6-well plate
- Rat tail collage I
- Forceps
- DMEM
- 5 N NaOH
- 96-well plate
- Culture medium

Procedure

1. Kill adult mice and open the thoracic cavity from below the diaphragm.
2. Remove the heart and lung to expose the aorta, which is a fat-covered blood vessel sitting along the spine.
3. Gently holding the anterior end of the aorta, and use a small scissors to detach the aorta from the spinal column by running the scissors between the aorta and the spine all the way down toward the posterior end just before the abdomen.
4. Cut the aorta from both end and transfer it to a petri dish containing Opti-MEM.
5. Under the dissection microscope, remove all extraneous fat, tissue and branching vessels with forceps and a scalpel. Be careful not to cut the aorta.
6. When the vessel is visible, hold the aorta at one end with a forceps, and inset a 27-G needle fixed to a 1 ml syringe filled with Opti-MEM into the end of the blood vessel, and slowly passing 1 ml of Opti-MEM through it. (this will also make the aorta more distinguishable from the fat)
7. Further clean up the fat and any existing branching from the aorta to make the aorta as clean as possible.

8. Cut the aorta into rings ~0.5 mm in width with a scalpel and transfer to a 6-well plate containing Opti-MEM.
9. Repeat the proceed 1-8 for each aorta, and maintain in the wells till ready to transport to tissue culture.
10. Transfer the rings to new wells containing fresh, sterile Opti-MEM to serum starve the ring overnight at 37°C before embedding.
11. Next day, on ice, add rat tail collagen I to DMEM to get a final concentration of 1 mg/ml.
12. Adjust the pH with a few drops of 5 N NaOH (~20 µl per 10 ml of mixture) to turn the mixture to pink colour to indicate slightly basic pH.
13. Transfer 50 µl of collage mixture to each well of 96-well plate, only do 4-6 wells at a time, to prevent the matrix from polymerize before adding aortic ring.
14. Transfer one ring at a time to each well carefully with forceps, by placing the ring directly onto the bottom of the wells, completely submerged in the collagen matrix. The lumen of the ring should be parallel to the bottom of the well.
15. Leave the plate undisturbed for 10-15 min at room temperature, and then incubate it at 37°C/5% CO_2 for 1h.
16. Carefully add 150 µl of Opti-MEM media containing 1% (vol/vol) FBS and 1% antibiotic-antimycotic with or without treatment.
17. Change the medium after 72h, and the rings are stained at 144h.

Appendix 3

Supplementary materials

Appendix 3.1 Supplementary tables for Chapter 2

Supplementary table 1. Primer sequences for real-time RT-PCR

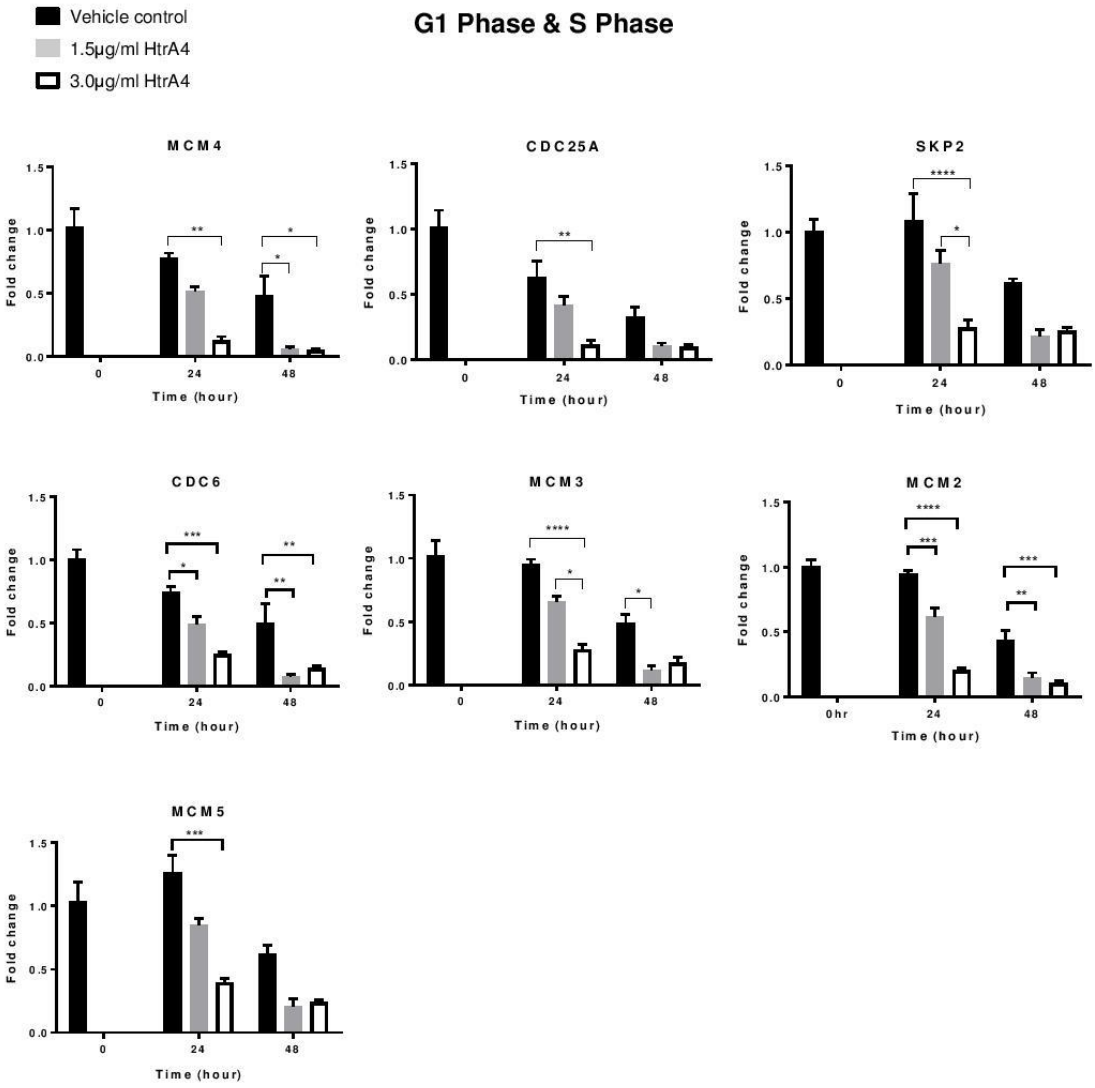
Gene symbol	Accession Number	Primer sequence (5'→3')	Product Size (bp)
ALOX5	NM_000698	Forward CTACATCTACCTCAGCCTCG Reverse CCAGTCGTCATTCAGCCAGT	184
BCL2	NM_000633	Forward ACGCCCCATCCAGCCGCAT Reverse TCACCCCGTCCCTGAAGAGC	262
CCL2	NM_002982	Forward AGTCTCTGCCGCCCTTCTGT Reverse ATCCTGAACCCACTTCTGCT	250
EDN1	NM_001955	Forward CCTTCCTCCATCCCCCATAC Reverse CCAACCTCTTTCATTAGCCG	266
FGF2	NM_002006	Forward CGGATGGGGGTAGTGAGCA Reverse ATCTTGAGGTGGAAGGGTCT	180
IL1B	NM_000576	Forward GGGCTGGCAGAAAGGGAACA Reverse GGGAGCGAATGACAGAGGGT	270
IL6	NM_000600	Forward CCCCTGACCCAACCACAAAT Reverse ACAACAATCTGAGGTGCCCA	165
IL11	NM_000641	Forward CCCAAAGCCACCACCGTCCT Reverse ACCCCAGTCCCCTCCTCCTC	227
MMP1	NM_002421	Forward CAGGTATTGGAGGGGATGCT Reverse ACGCTTTTGGGGTTTGTGGG	267
OCLN	NM_002538	Forward CTCTCTCAGCCAGCCTACTC Reverse GTTCCATAGCCTCTGTCCCA	170
SERPINE1	NM_000602	Forward CCTGCTTCCACCCGTCTCTC Reverse ACATTCACTCTGCCACCTGC	283
THBD	NM_000361	Forward CCTGTGCCTCCTCACCCCCA Reverse CCAATAACGCTCACCCTCCT	266
THBS1	NM_003246	Forward GAACAGGAAGAAGCGTAAAGAC Reverse TGAGCACAAGGGGCAGAGCA	172
PTGIS	NM_000961	Forward ACTCCCCCCTTCCAAATCAG Reverse CTAACCCACTCATCTCTCCC	287
PTGS2	NM_000963	Forward CAGTCTTCTCATCACTTCGT Reverse TTCCAGTCACAAACCCCGTA	316
VEGFA	NM_001171623	Forward GGAGGGGGAGGAGGAAGAAG Reverse GGAGGTAGAGCAGCAAGGCA	317

Supplementary table 2. Genes on the endothelial cell biology PCR array.

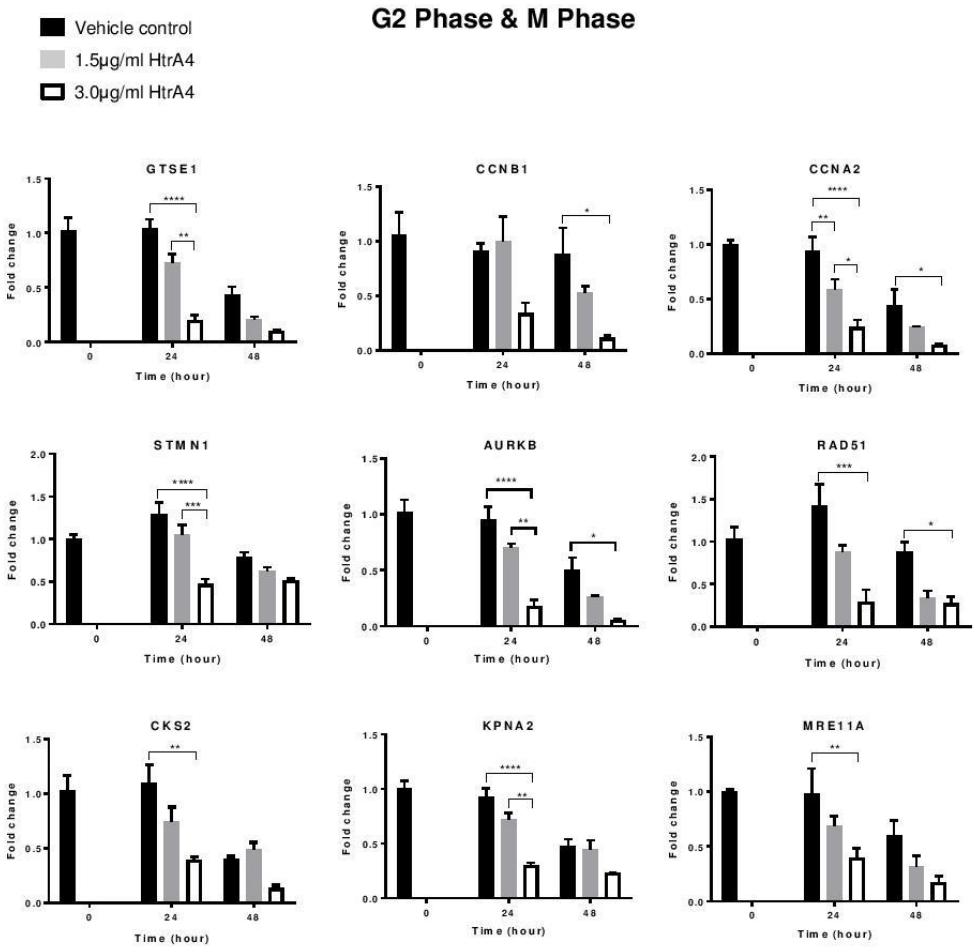
Gene category	Gene name	Full name
Inflammatory Response	*ADAM17*	ADAM Metallopeptidase Domain 17
	ALOX5	Arachidonate 5-Lipoxygenase
	APOE	Apolipoprotein E
	CCL2/MCP1	Chemokine (C-C Motif) Ligand 2/ Monocyte Chemoattractant Protein-1
	CCL5	Chemokine (C-C Motif) Ligand 5
	CX3CL1	Chemokine (C-X3-C Motif) Ligand 1
	IL6	Interleukin 6
	IL1B	Interleukin 1, Beta
	PTGS2/COX2	Prostaglandin-Endoperoxide Synthase 2/ Cyclooxygenase-2
	TNF	Tumor Necrosis Factor
Angiogenesis and Vaso-activities	*ACE*	Angiotensin I Converting Enzyme
	AGT/SERPINA8	Angiotensinogen/Serpin Peptidase Inhibitor, Clade A, Member 8
	AGTR1	Angiotensin II Receptor, Type 1
	ANGPT1	Angiopoietin 1
	CALCA	Calcitonin-Related Polypeptide Alpha
	CAV1	Caveolin 1
	EDN1	Endothelin 1
	EDN2	Endothelin 2
	EDNRA	Endothelin Receptor Type A
	ENG	Endoglin
	FGF1	Fibroblast Growth Factor 1 (Acidic)
	FGF2	Fibroblast Growth Factor 2 (Basic)
	FLT1	Fms-Related Tyrosine Kinase 1
	HIF1A	Hypoxia Inducible Factor 1, Alpha Subunit
	HMOX1	Heme Oxygenase 1
	KDR/ FLK1	Kinase Insert Domain Receptor/Fetal Liver Kinase-1
	KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog
	KLK3	Kallikrein-Related Peptidase 3
	MMP2	Matrix Metallopeptidase 2
	MMP9	Matrix Metallopeptidase 9
	NOS3	Nitric Oxide Synthase 3
	NPPB	Natriuretic Peptide B
	NPR1	Natriuretic Peptide Receptor 1
	PGF	Placental Growth Factor
	PLAU	Plasminogen Activator, Urokinase
	PTGIS	Prostaglandin I2 (Prostacyclin) Synthase
	SOD1	Superoxide Dismutase 1, Soluble
	SPHK1	Sphingosine Kinase 1
	TEK	TEK Tyrosine Kinase, Endothelial
	TYMP	Thymidine Phosphorylase
	VEGFA	Vascular Endothelial Growth Factor A
Platelet Activation and Cell Adhesion	*CDH5*	Cadherin 5, Type 2 (Vascular Endothelium)
	COL18A1	Collagen, Type XVIII, Alpha 1
	FN1	Fibronectin 1
	ICAM1	Intercellular Adhesion Molecule 1
	IL11	Interleukin 11
	ITGA5	Integrin, Alpha 5
	ITGAV	Integrin, Alpha V
	ITGB1	Integrin, Beta 1
	ITGB3	Integrin, Beta 3
	PDGFRA	Platelet-Derived Growth Factor Receptor, Alpha Polypeptide
	PECAM1	Platelet/Endothelial Cell Adhesion Molecule 1
	PF4	Platelet Factor 4
	PLG	Plasminogen
	PTK2	Protein Tyrosine Kinase 2
	SELE	Selectin E
	SELL	Selectin L
	SELPLG	Selectin P Ligand
	SERPINE1	Serpin Peptidase Inhibitor, Clade E, Member 1
	TGFB1	Transforming Growth Factor, Beta 1
	THBS1	Thrombospondin 1
	VCAM1	Vascular Cell Adhesion Molecule 1
	ANXA5	Annexin A5

Coagulation and Apoptosis	*BAX*	BCL2-Associated X Protein
	BCL2	B-Cell CLL/Lymphoma 2
	BCL2L1	BCL2-Like 1
	CASP1	Caspase 1, Apoptosis-Related Cysteine Peptidase
	CASP3	Caspase 3, Apoptosis-Related Cysteine Peptidase
	CFLAR	CASP8 and FADD-Like Apoptosis Regulator
	F2R	Coagulation Factor II (Thrombin) Receptor
	F3	Coagulation Factor III
	FAS	Fas Cell Surface Death Receptor
	FASLG	Fas Ligand (TNF Superfamily, Member 6)
	IL3	Interleukin 3
	IL7	Interleukin 7
	MMP1	Matrix Metallopeptidase 1
	OCLN	Occludin
	PLAT	Plasminogen Activator, Tissue
	PROCR	Protein C Receptor, Endothelial
	TFPI	Tissue Factor Pathway Inhibitor
	THBD	Thrombomodulin
	TIMP1	TIMP Metallopeptidase Inhibitor 1
	TNFSF10	Tumor Necrosis Factor (Ligand) Superfamily, Member 10
	VWF	Von Willebrand Factor

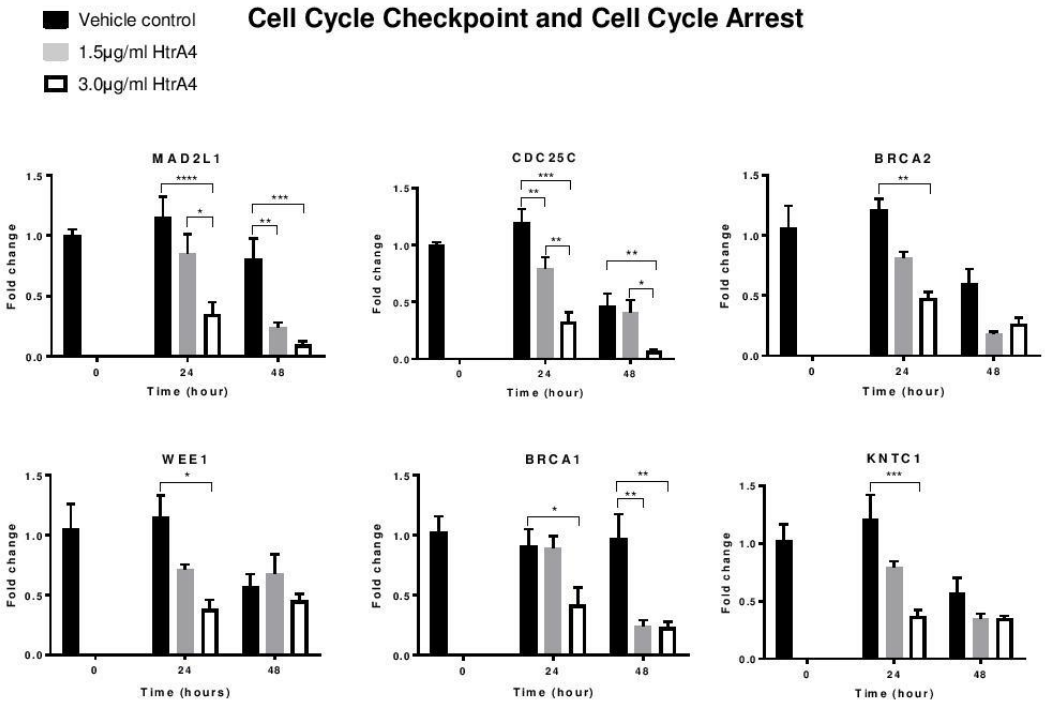
Appendix 3.2 Supplementary material for Chapter 3



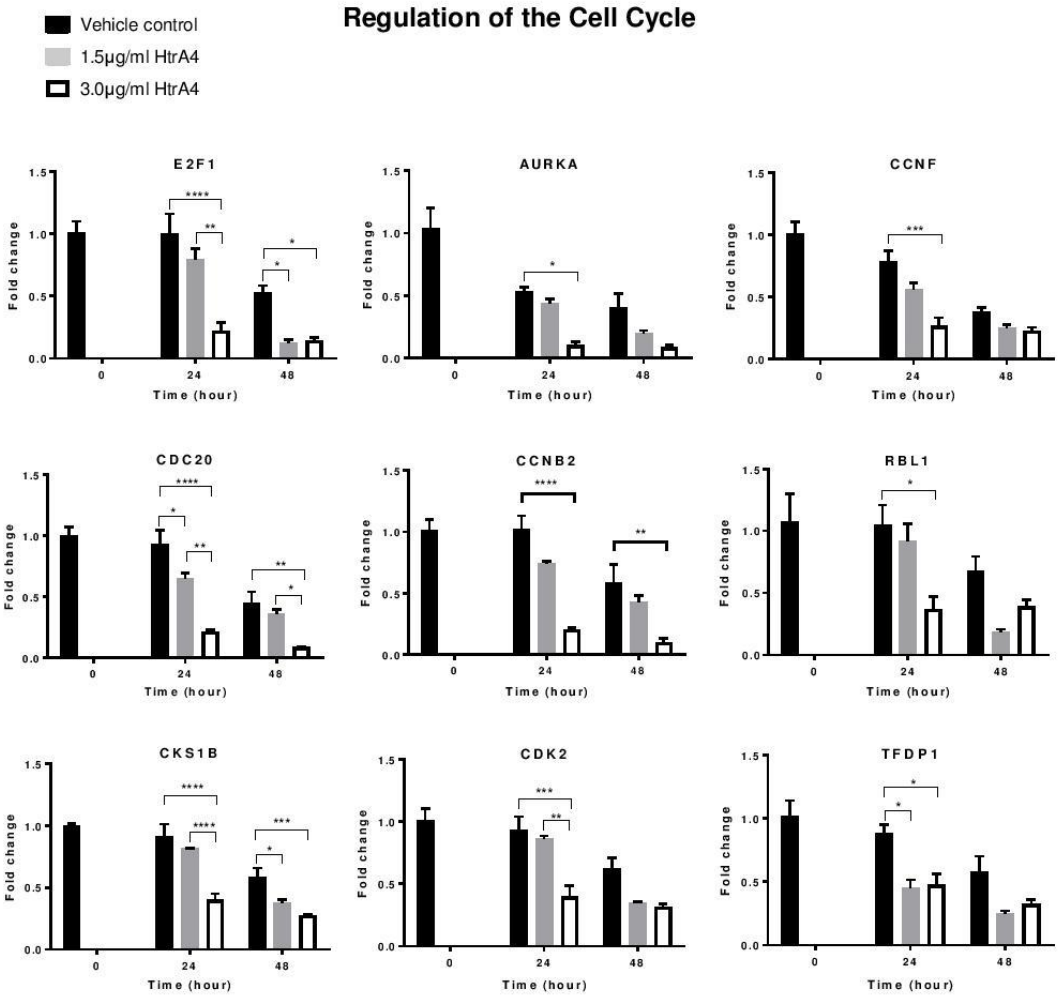
Supplementary Figure 1A



Supplementary Figure 1B

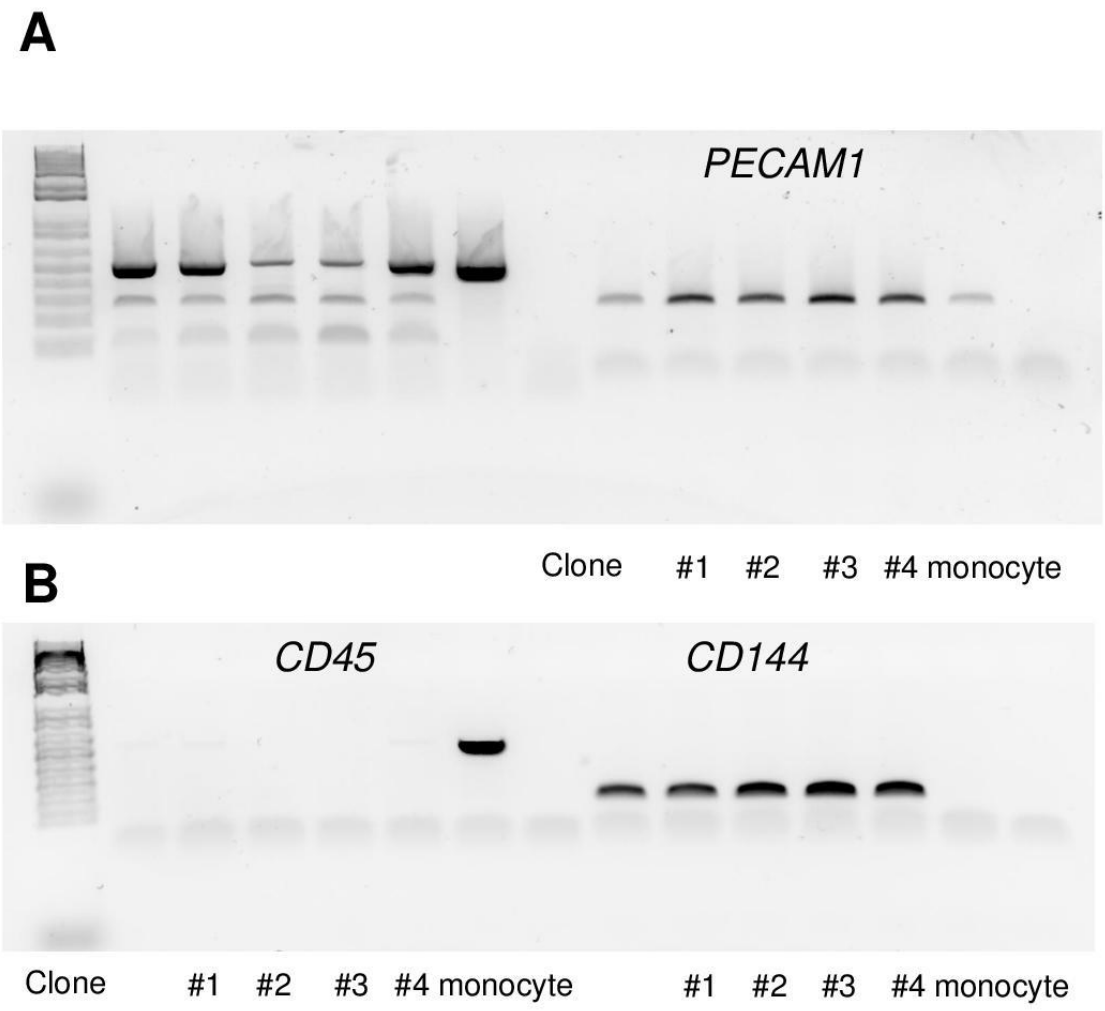


Supplementary Figure 1C



Supplementary Figure 1. Real-time RT-PCR validations of cell cycle genes represented in grey bar in Figure 2A. A) Genes involved in G1 and S phase. (B) Genes involved in G2 and M phase. (C) Genes involved in cell cycle checkpoint and cell cycle arrest. (D) Genes involved in regulation of the cell cycle. Cells were treated with 1.5µg/ml or 3.0µg/ml HtrA4 for 0, 24 or 48h, n=3. Data is expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 1D



Supplementary Figure 2. Full PCR agarose gel electrophoresis images of Figure 3A. (A) Full image of PECAM1 on the right, including negative sample in last lane. (B) Full image of CD45 and CD144. Also included negative sample in last lane for each primer.

Supplementary table 3. Primer sequences for real-time RT-PCR.

Gene symbol	Accession Number	Primer sequence (5'→3')	Product Size (bp)
AURKA	NM_198433.1	Forward TGACCCCGATCAGTTAAGGA Reverse CGGACAGACACACAGCATTC	197
AURKB	NM_004217	Forward TGGACCTAAAGTTCCCCGCT Reverse ACGCACCCGAGTGAATGACA	200
BIRC5	NM_001168.2	Forward CCATCCTTAAAACCAGACCC Reverse TAACCTGCCATTGGAACCTC	218
BRCA1	NM_007294	Forward GCTGTTGCTTTCTTTGAGGT Reverse CAATCAAGTCTTCACTGCCC	196
BRCA2	NM_000059	Forward ATGGAATGAGGTCTCTTAGT Reverse TCAAAAGGAAACACCACTCT	192
CCNA2	NM_001237.3	Forward TGGTGGTCTGTGTTCTGTGA Reverse CCATCTGTTCTGTGATTTTTT	211
CCNB1	NM_031966	Forward TCCTTTTGGTTTACCTGGGG Reverse TAGGGATTCGGTGGTAGACT	216
CCNB2	NM_004701	Forward GCCAAGAATGTGGTGAAAGT Reverse AGAAAAAGGGCACAATGAAG	202
CCNF	NM_001761	Forward CCCCCAAATGCTCTTGAACC Reverse CGACAGGACGGGTCTACAGT	196
CDC6	NM_001254	Forward GAACTCCTGACCCTCAAGTG Reverse CCAGTGTCCTCCAAATCTCC	181
CDC20	NM_001255	Forward GGTGGCTGAACTCAAAGGTC Reverse CTGAGGTGATGGGTTGGTCT	216
CDC25A	NM_001789	Forward CTACTCATCCCTGCCCTCTG Reverse GTCCTCTCCCCCACATTTTT	217
CDC25C	NM_001790	Forward TGGTGGCAGAGTCCTGGAG Reverse AAGAGGGGGAAACAAAGAAG	205
CDK1	NM_001786.4	Forward AAAGCTAACATGAGAGCAT Reverse CGAAAGCCAAGATAAGCAACT	193
CDK2	NM_001798	Forward CTGAGACAGGGATTGTGCTT Reverse CTATGGGGTAGGAGGTGGAC	164
CDKN3	NM_005192.3	Forward ACAGCCTGCGAGACCTAAGA Reverse AACACTGGTGGTTTCATTTC	218
CKS1B	NM_001826	Forward AGTAGAGCCACCACCACCAT Reverse TCCGCAAGTCACCACACATA	220
CKS2	NM_001827	Forward CTGAAGAGGAGTGGAGGAGA Reverse GCACAGGTATGGATGAAAGA	235
E2F1	NM_005225	Forward GCTGTTCTTCTGCCCCATAC Reverse TTCACCACCCCTCCTGCCCA	222
GTSE1	NM_016426	Forward CCTGTTTGGAGCGGGGCATC Reverse TCTGGGGTTGGGTGGGGGAT	268
KNTC1	NM_014708	Forward AGGAGTTTGGGATTTTGGCA Reverse CAGAATTTCACAGGGAGCAG	197
KPNA2	NM_002266	Forward GCCGTGACCAACTATACCAG Reverse TGTCTAAGCCTCCACATTCT	217
MCM2	NM_004526	Forward GCGTATTCAGGCTGCTTTTG Reverse GATAACTGTGGCGAGTGGAT	193
MCM3	NM_002388	Forward TGGGGGCACAACTGTTTTCT Reverse CATCTCGGATATTCATCTGG	218
MCM4	NM_182746	Forward GCACCCAGCCTTTGTTTTAT Reverse GCCAGGTAACCAAGTATTATTCC	196
MCM5	NM_006739	Forward ACTTCACCAAGCAGAAATAC Reverse GCAGAGGTCCCAGCAACAT	205
MAD2L1	NM_002358	Forward CTGTAGATGGAAAAACTTGTGC Reverse AAATGAAGGTCAAAAGGAGC	214
MKI67	NM_002417.4	Forward TTGGTACTGGGGGAGGGAGA Reverse TGGGAGGCGAAAAAGTAAAA	188
MRE11A	NM_005591	Forward GCACTGATGGAATCCCTCTA Reverse AACAGGCTGAACCAAATGAA	312
RAD51	NM_002875	Forward TGTAGCAAAGGGAATGGGTC Reverse GCAGGTAGATGGTGAAGGGC	159
RBL1	NM_183404	Forward GGACCAGTGAAAGAGGAAAG Reverse TGTGCGGGGAAATATAAATG	211
SKP2	NM_005983	Forward ACATTTCAGCCCTTTTCGTG Reverse GAGAATCCAGAACACCCAGA	204
STMN1	NM_203401.1	Forward AAGACGCAAGTCCCATGAAG Reverse CCATTTGTGCCTCTCGGTT	173
TFDP1	NM_007111	Forward CTAAACAGCCCCAAGCAAAC Reverse CTCCATAGCCGCACCAGCAT	220
WEE1	NM_003390	Forward CCCTCCTTTGGAATGCTGTA Reverse TGTGAAAAACTTGGAATGTC	251