THE ROLE OF KISSPEPTIN AND ITS COGNATE RECEPTOR GPR-54 IN NORMAL AND ABNORMAL PLACENTATION

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TABLE OF CONTENTS

Declaration ........................................................................................................ Page VIII
Acknowledgements ....................................................................................... Page IX-X
Publications and Conference Proceedings ........................................ Page XI-XII
List of Abbreviations .............................................................................. Page XIII-XV
List of Figures .......................................................................................... Page XVI-XVIII
List of Tables .............................................................................................. Page XIX
Abstract ........................................................................................................ Page XX-XXII

CHAPTER ONE – GENERAL INTRODUCTION

1.1 Introduction .......................................................................................... Page 2-4
1.2 The Placenta ...................................................................................... Page 4-7
1.3 Maternal Decidua .............................................................................. Page 7-8
  1.3.1 The Placental Bed ................................................................. Page 9-10
  1.3.2 Decidua Parietalis ................................................................. Page 10
1.4 Trophoblast invasion and trophoblast populations ................. Page 10-12
  1.4.1 Factors affecting trophoblast invasion .................................... Page 12
    1.4.1.1 Oxygen tension .......................................................... Page 12-13
    1.4.1.2 Protease expression .................................................... Page 13-15
    1.4.1.3 Placental Hormones .................................................. Page 15
    1.4.1.4 Angiogenic factors ...................................................... Page 15-17
    1.4.1.5 Kiss1 gene, kisspeptins and GPR-54 ......................... Page 17-19
    1.4.1.6 Kiss1 gene, kisspeptins and GPR-54 in pregnancy .......... Page 19-21
1.5 Transformation of the Spiral Arteries ........................................ Page 21-23
1.6 Failure of Transformation of the Spiral Arteries: 
  Clinical Sequelae ............................................................................... Page 23
1.6.1 Preeclampsia .................................................................Page 23
1.6.2 Intrauterine Growth Restriction .................................Page 23
1.6.3 Aims of Study .................................................................Page 24

CHAPTER TWO – GENERAL MATERIALS AND METHODS

2.1 Tissue Sampling and Collection .....................................Page 26
2.2 Gene Expression Studies ................................................Page 26
   2.2.1 RNA Extraction .........................................................Page 26-27
   2.2.2 Complimentary DNA Synthesis .................................Page 27
   2.2.3 Real-Time Reverse Transcriptase Polymerase Chain
       Reaction ........................................................................Page 27-28
2.3 Protein Expression Studies ..............................................Page 30
   2.3.1 Protein Extraction .....................................................Page 30
   2.3.2 Protein Quantification ..............................................Page 30
   2.3.3 SDS-PAGE and Western Blotting ...............................Page 31
   2.3.4 Immunohistochemistry .............................................Page 32
      2.3.4.1 Kisspeptin Immunostaining ...............................Page 32-33
      2.3.4.2 Kisspeptin Receptor (GPR-54) Immunostaining ....Page 33
      2.3.4.3 MMP-9 Immunostaining .....................................Page 33
      2.3.4.4 VEGF-A Immunostaining .................................Page 34
      2.3.4.5 VEGF-A and Pancytokeratin
          Co-immunostaining .................................................Page 34
      2.3.4.6 VEGF Receptor-1 (VEGF-R1) and VEGF
          Receptor-2 (VEGF-R2) Immunostaining .................Page 34-35
      2.3.4.7 EG-VEGF (Prokineticin-1) Immunostaining .........Page 35
2.4 Enzyme-linked Immunosorbent Assays (ELISAs) ..............Page 35
   2.4.1 Maternal and Cord Serum Kisspeptin-10
       Quantification ..............................................................Page 35-36
2.4.2 Maternal and Cord Serum VEGF-A Quantification........Page 36-37
2.4.3 Maternal and Cord Serum EG-VEGF Quantification......Page 37-38
2.5 Statistical Analysis .............................................................Page 38

CHAPTER THREE – THE EXPRESSION OF KISS-1, MMP-9 AND ANGIOGENIC REGULATORS ACROSS THE MATERNAL–FETAL INTERFACE OF HEALTHY PREGNANCIES

3.1 Abstract.................................................................Page 41
3.2 Introduction.............................................................Page 42-45
3.3 Materials and Methods ................................................Page 45
  3.3.1 Ethics Statement ....................................................Page 45
  3.3.2 Study Participants ..................................................Page 45-46
  3.3.3 Tissue Sampling and Specimen Collection...............Page 46
  3.3.4 RNA Extraction ......................................................Page 46-47
  3.3.5 cDNA Synthesis .....................................................Page 47
  3.3.6 Immunohistochemistry ..........................................Page 47
    3.3.6.1 Kisspeptin Immunostaining .......................Page 48
    3.3.6.2 GPR-54 Immunostaining ........................Page 48
    3.3.6.3 MMP-9 Immunostaining ............................Page 49
    3.3.6.4 PROK-1 Immunostaining ..........................Page 49
    3.3.6.5 VEGF-A immunostaining ..........................Page 49
    3.3.6.6 VEGF-A and Pancytokeratin Co-immunostaining .Page 49
    3.3.6.7 VEGFR-1 and VEGFR-2 Co-immunostaining......Page 50
  3.3.7 Protein Extraction and Quantification .......................Page 50
  3.3.8 Western Blot Analysis .............................................Page 50-51
  3.3.9 Statistical Analysis ................................................Page 51
3.4 Results..............................................................................Page 51
  3.4.1 Clinical Data..........................................................Page 51
3.4.2 Kiss-1 and GPR-54 expression are highest in the placenta .................................................................Page 52-54

3.4.3 MMP-9 expression is highest in the placenta........Page 55

3.4.4 VEGF-A gene and protein expression are highest in the placental bed .................................................................Page 55-58

3.4.5 VEGFR-1 and VEGFR-2 transcripts and protein are differentially expressed across the maternal-fetal interface.....Page 58

3.4.6 PROK-1 and PROK-1R transcript and protein expression are highest in the placenta..............................................Page 59-61

3.5 Discussion........................................................................................................................................Page 62-69

CHAPTER FOUR –THE EXPRESSION OF KISS-1 AND ANGIOGENIC FACTORS ACROSS THE MATERNAL-FETAL INTERFACE OF PREGNANCIES COMPLICATED BY PREECLAMPSIA

4.1 Abstract.................................................................................................................................Page 71-72

4.2 Introduction.............................................................................................................................Page 72-74

4.3 Methods .........................................................................................................................................Page 74

4.3.1 Study Participants ....................................................................................................................Page 74-75

4.3.2 Tissue Sampling...................................................................................................................Page 75-76

4.3.3 RNA Extraction, cDNA Synthesis and RT-PCR ...............................................................Page 76

4.3.4 Protein Expression Studies..................................................................................................Page 77

4.3.4.1 Semi-quantitative analysis of kisspeptin immunostaining ................................................Page 77-78

4.3.5 Statistical Analysis ..............................................................................................................Page 78

4.4 Results........................................................................................................................................Page 78

4.4.1 Clinical Parameters ..............................................................................................................Page 78

4.4.2 KISS1 gene and protein expression across the maternal-fetal interface of healthy and preeclamptic
4.4.3 GPR-54 mRNA transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies.................................Page 80-86
4.4.4 VEGF-A gene and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies........................................Page 87-91
4.4.5 EG-VEGF (PROK1) transcript and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies .......................Page 91-94
4.5 Discussion........................................................................................................Page 94-103

CHAPTER FIVE – THE EFFECT OF KISSPEPTIN STIMULATION ON THE TRANSCRIPT EXPRESSION OF VEGF-A AND ITS RECEPTORS (VEGFR-1 AND VEGFR-2) IN HEALTHY AND PREECLAMPTIC PLACENTAE.

5.1 Abstract........................................................................................................Page 105
5.2 Introduction.....................................................................................................Page 106
5.3 Material and Methods ....................................................................................Page 107
  5.3.1 Study Participants ......................................................................................Page 107-108
  5.3.2 Tissue Sampling and Specimen Collection..............................................Page 108
  5.3.3 Tissue Explant (Kisspeptin Stimulation) Experiments .......................Page 108
  5.3.4 RNA Extraction .......................................................................................Page 108-109
  5.3.5 cDNA Synthesis ......................................................................................Page 109
  5.3.6 Real-Time PCR .......................................................................................Page 109
5.4 Statistical Analysis ........................................................................................Page 109
5.5 Results............................................................................................................Page 110
  5.5.1 The effect of kisspeptin-10 stimulation on placental VEGF-A transcript expression .................................................................Page 110
5.5.2 The effect of kisspeptin-10 stimulation on placental
VEGFR-1 transcript expression.............................................Page 111
5.5.3 The effect of kisspeptin-10 stimulation on placental
VEGFR-2 transcript expression.............................................Page 112
5.6 Discussion........................................................................Page 112-115

CHAPTER SIX – GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion .............................................................Page 117
  6.1.1 The role of kisspeptin in pregnancy health...................Page 117-118
  6.1.2 The role of kisspeptin in preeclampsia..........................Page 118-121
6.2 Conclusions .................................................................Page 121-122
6.3 Future Studies...............................................................Page 122-123

APPENDIX ........................................................................Page 124-125
REFERENCES .....................................................................Page 126-139
DECLARATION

I hereby declare that the work presented in this dissertation is my original work unless otherwise acknowledged in the text. This work has never previously been submitted in any form in application for another degree.

Mushi Matjila

October 2014
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PUBLICATIONS


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AKT</td>
<td>serine/threonine-specific protein kinase</td>
</tr>
<tr>
<td>AMPS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BB</td>
<td>brush border</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchorinic acid</td>
</tr>
<tr>
<td>BCP</td>
<td>1-bromo-3-chloropropane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>c-DNA</td>
<td>complementary DNA</td>
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<tr>
<td>°C</td>
<td>degrees celcius</td>
</tr>
<tr>
<td>CC</td>
<td>cytotrophoblast columns</td>
</tr>
<tr>
<td>cTB</td>
<td>cytotrophoblast</td>
</tr>
<tr>
<td>DAM</td>
<td>donkey anti-mouse</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
<tr>
<td>DAR</td>
<td>donkey anti-rabbit</td>
</tr>
<tr>
<td>Db</td>
<td>decidua basalis</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>dNK</td>
<td>decidual natural killer cell</td>
</tr>
<tr>
<td>DAR</td>
<td>donkey anti-rabbit</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DOHaD</td>
<td>developmental origins of health and disease</td>
</tr>
<tr>
<td>Dp</td>
<td>decidua parietalis</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EG-VEGF</td>
<td>endocrine gland specific VEGF</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular regulated kinase 1/2</td>
</tr>
<tr>
<td>EVT</td>
<td>extravillous trophoblast</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FLT-1</td>
<td>fms-like kinase receptor</td>
</tr>
<tr>
<td>GARP</td>
<td>goat anti-rabbit peroxidase</td>
</tr>
<tr>
<td>GAM</td>
<td>goat anti-mouse</td>
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<tr>
<td>GAMP</td>
<td>goat anti-mouse peroxidase</td>
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<tr>
<td>GnIH</td>
<td>gonadotrophin-inhibitory hormone</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GPR-54</td>
<td>G protein-coupled receptor 54</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HELLP</td>
<td>haemolysis elevated liver enzymes and low platelets</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia Inducible Factor 1-alpha</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IGF-II</td>
<td>insulin-like growth factor II</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>ISSHP</td>
<td>international society for the study of hypertension in pregnancy</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphates</td>
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TGF  transforming growth factor
TMB  3,3’,5,5’- tetramethylbenzidine
TNF-α  tumour necrosis factor alpha
Tris  tris (hydroxymethyl) amino methane
uNK  uterine natural killer cell
uPA  urokinase plasminogen activator
V  volt
vCT  villous cytotrophoblast
VEGF  vascular endothelial growth factor
VEGFR-1  vascular endothelial growth factor receptor -1
VEGFR-2  vascular endothelial growth factor receptor -2
vCv  villous capillary vessels
vM  villous mesenchyme
vST  villous syncytiotrophoblast
vsm  vascular smooth muscle
vT  villous trophoblast
LIST OF FIGURES

Figure 1.1: Coronal section of a placental villus .............................................. Page 5

Figure 1.2: Sagittal section of a placental villus............................................. Page 6

Figure 1.3: Coronal section of the uterus with the placenta in situ .............. Page 9

Figure 1.4: The structure of the human kisspeptin pre-pro-protein (Kp-145) and its posttranslational products ................................................ Page 18

Figure 3.1: Kiss1 and GPR54 (Kiss1 R) expression in the Placenta, Placental bed and Decidua parietalis ................................................................. Page 53

Figure 3.2: GPR54 (GPR-54 ) immunostaining in the Placenta ................. Page 54

Figure 3.3: MMP9 expression in the Placenta, Placental Bed and Decidua Parietalis.......................................................... Page 56

Figure 3.4: The expression of VEGF-A in the Placenta, Placental Bed and Decidua Parietalis.......................................................... Page 57

Figure 3.5: The Expression of VEGF Receptors in the three maternal-fetal compartments .......................................................... Page 60

Figure 3.6: PROK1 and PROK1R expression in the Placenta, Placental Bed and Decidua Parietalis.......................................................... Page 61

Figure 3.7A The anatomy of the maternal-fetal interface ......................... Page 68
Figure 3.7B  The schematic of putative interaction at the maternal-fetal interface. ................................................................. Page 69

Figure 4.1:  Relative Kiss1 transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies. .......................... Page 81

Figure 4.A:  A. The immunohistochemical expression of kisspeptin protein in healthy placentae. .......................................................... Page 82

Figure 4.B:  A. B. The immunohistochemical expression of kisspeptin protein in preeclamptic placentae. ................................................ Page 83

Figure 4.2:  The immunohistochemical expression of kisspeptin protein in the placentae of both Healthy and Preeclamptic Pregnancies .......... Page 84

Figure 4.3: Circulatory kisspeptin-10 levels in the Maternal and Cord Sera of Healthy and Preeclamptic Pregnancies ................................. Page 85

Figure 4.4:  Relative GPR-54 (GPR54) transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies......... Page 86

Figure 4.5:  The Expression of VEGF-A transcript and protein in the Placenta, Placental bed and Decidua parietalis in Healthy and Preeclamptic Pregnancies................................................................. Page 88

Figure 4.6:  Human VEGF-A ELISA Standard Curve .............................. Page 89

Figure 4.7:  Maternal and Cord VEGF-A concentrations in Healthy and Preeclamptic Pregnancies ......................................................... Page 90
**Figure 4.8**: Cord serum VEGF-A concentrations in healthy and preeclamptic pregnancies .......................................................... Page 93

**Figure 5.1**: The effect of kisspeptin-10 stimulation on placental VEGF-A transcript expression in healthy and preeclamptic pregnancies .................. Page 110

**Figure 5.2**: The effect of kisspeptin-10 stimulation on placental VEGFR-1 transcript expression in healthy and preeclamptic pregnancies ............... Page 111

**Figure 5.3**: The effect of kisspeptin-10 stimulation on placental VEGFR-2 transcript expression in healthy and preeclamptic pregnancies .......... Page 112

**Figure 6.1**: The schematic of putative interaction at the maternal-fetal interface of healthy and preeclamptic pregnancies .......................................................... Page 120
LIST OF TABLES

Table 2.1 : Primers and Probe Sequences used for Real-Time (RT) PCR Reactions I ........................................................................................................................................................................... Page 29

Table 3.1 : Clinical Data of Healthy Patients ................................................................................................. Page 52

Table 4.1 : Primers and Probe Sequences used for Real-Time (RT) PCR Reactions II ........................................................................................................................................................................ Page 77

Table 4.2 : Clinical Data of Healthy and Preeclamptic Patients ............................................. Page 79

Table 4.3 : Studies Investigating Maternal and Fetal Tissue VEGF expression in Preeclamptic Pregnancies............................................................................................. Page 102
ABSTRACT

Poor invasion of trophoblast cells in early pregnancy has been associated with preeclampsia and intrauterine growth restriction as well as other adverse pregnancy outcomes such as miscarriage, preterm birth and intrauterine death. Hypertensive disorders of pregnancy, including pre-eclampsia are one of the leading causes of maternal mortality in South Africa (Third report on Confidential Enquiries into Maternal Deaths in South Africa (2002-2004)) and the rest of the world.

The currently accepted mechanism underlying the development of preeclampsia implicates poor trophoblast invasion and inadequate transformation of the maternal spiral arteries. Despite extensive research in this area, the control of trophoblast invasion and early placental development remains poorly understood. A whole host of factors such as oxygen tension, activation of matrix metalloproteinases (MMPs), angiogenic factors (VEGF-A) and immunological factors such as TNF alpha, interleukins and TGFβ have been shown to be involved in the control of trophoblast invasion. Our knowledge of the molecular details of pregnancy is unfortunately limited to in-vitro experiments and animal studies. Recently kisspeptins and their cognate receptor GPR-54 originally involved in tumour metastasis suppression and regulation of puberty, have been implicated in the inhibition of trophoblast invasion. Expression levels of kisspeptin and its receptor in trophoblast cells are highest in the first trimester, when control of trophoblast invasion is critical, and lower towards term.

The placental trophoblast invades maternal tissues so as to attach the conceptus and access the maternal vasculature for nutrient transport and gaseous exchange. In this process fetal trophoblast encounters various maternal cells (including stromal and immune cell components) and hence considerable cross-talk and immune tolerance has to be exercised at the interface between invading fetal trophoblast and native maternal cells of the placental bed. Understanding the expression of factors involved in invasion such as proteases and angiogenic factors across this maternal-fetal
interface is thus crucial in comprehending pregnancy-related disorders.

The hypothesis raised in this study was that kisspeptin gene and protein expression, along with the expression of metalloproteases and angiogenic factors, will be different across the maternal-fetal interface of normal and abnormal placentation (preeclampsia in particular). The expression levels of kisspeptin, its receptor GPR54, metalloproteinases and angiogenic factors were studied across the maternal-fetal interface of healthy and preeclamptic patients. Real-time reverse transcriptase PCR (RT-PCR), immunohistochemistry and western blot analysis were utilized to study gene and protein expression of factors involved in cellular invasion (*Kiss-1*, *GPR-54*, *MMP-9*) and angiogenesis (*VEGF-A*, *VEGFR-1*, *VEGFR-2*, *PROK-1* and *PROK-1R*) in the placenta, placental bed and decidua parietalis of healthy pregnancies and pregnancies complicated by preeclampsia. In addition enzyme-linked immunosorbent assays were employed to investigate circulatory concentrations of kisspeptin, VEGF-A and PROK-1 in maternal and fetal sera.

In the maternal-fetal tissues of healthy pregnancies the expression of invasive genes *Kiss1*, *GPR-54* and *MMP9* were higher in the placenta compared to the placental bed and decidua parietalis. The expression of angiogenic ligand *VEGF-A* was highest in the placental bed and there was reciprocal expression of its receptors with *VEGFR1* most expressed in the placenta and *VEGFR2* in the placental bed. Conversely the expression of the angiogenic ligand *PROK-1* and its receptor *PROK1R* were highest in the placenta.

Pregnancies complicated by preeclampsia demonstrated high placental kisspeptin protein expression in comparison to healthy pregnancies but there was no difference in *GPR-54* expression across the maternal-fetal tissues of preeclamptic and healthy pregnancies. There was reciprocal suppression of mRNA expression of angiogenic ligands across maternal-fetal tissues of preeclamptic pregnancies with *PROK-
suppressed in the placenta and VEGF-A in the placental bed. In addition, mean cord serum VEGF-A concentrations were significantly lower in preeclampsia when compared to healthy pregnancies. There was however no significant difference in mean maternal or cord serum PROK-1 concentrations between preeclamptic and healthy pregnancies. Furthermore placental stimulation with kisspeptin resulted in no difference in the mRNA expression of VEGF-A, VEGFR1 and VEGFR2 in both healthy and preeclamptic pregnancies.

To summarise, in healthy pregnancies, genes associated with trophoblast invasion were highly expressed in the placenta which could suggest fetal control of trophoblast invasive capacity. On the other hand, findings on angiogenic gene expression profiles suggest that angiogenesis may be regulated by two distinct pathways with the PROK1-PROK1R ligand-receptor pair predominantly mediating fetal angiogenesis and VEGFA-VEGFR2 ligand-receptor pair facilitating maternal angiogenesis. In preeclamptic pregnancies increased kisspeptin expression in the fetal (placenta) and suppressed pro-angiogenic gene, (VEGF-A and Prokineticin-1) expression in maternal (placental bed) components of the interface respectively, is consistent with reduced trophoblast invasiveness and compromised angiogenesis and may represent a molecular mechanism that explains the development of preeclampsia.
CHAPTER ONE – GENERAL INTRODUCTION

1.1 Introduction........................................................................................................Page 2-4

1.2 The Placenta ......................................................................................................Page 4-7

1.3 Maternal Decidua ..............................................................................................Page 7-8
  1.3.1 The Placental Bed.......................................................................................Page 9-10
  1.3.2 Decidua Parietalis....................................................................................Page 10

1.4 Trophoblast invasion and trophoblast populations.................................Page 10-12
  1.4.1 Factors affecting trophoblast invasion................................................Page 12
      1.4.1.1 Oxygen tension .............................................................................Page 12-13
      1.4.1.2 Protease expression........................................................................Page 13-15
      1.4.1.3 Placental Hormones ....................................................................Page 15
      1.4.1.4 Angiogenic factors.........................................................................Page 15-17
      1.4.1.5 Kiss1 gene, kisspeptins and GPR-54 .............................................Page 17-19
      1.4.1.6 Kiss1 gene, kisspeptins and GPR-54 in pregnancy......................Page 19-21

1.5 Transformation of the Spiral Arteries .........................................................Page 21-23

1.6 Failure of Transformation of the Spiral Arteries:

  Clinical Sequelae ............................................................................................Page 23
      1.6.1 Preeclampsia ......................................................................................Page 23
      1.6.2 Intrauterine Growth Restriction .......................................................Page 23
      1.6.3 Aims of Study ....................................................................................Page 23-24
Chapter 1 General Introduction

1.1 Introduction

Pregnancy is a cherished event in most communities across the world. Although for the most part pregnancies progress uneventfully, pregnancy-related complications occur in the form of miscarriage, preeclampsia, intrauterine growth restriction (IUGR), preterm birth and even intrauterine death. These complications contribute significantly to global maternal and perinatal morbidity and mortality particularly in the developing world (Moodley, 2011, Sanders, 1999).

Hypertensive disorders of pregnancy include gestational hypertension, chronic hypertension with superimposed preeclampsia and preeclampsia. Preeclampsia is the leading cause of direct maternal deaths in South Africa and contributes significantly to adverse perinatal outcomes. This disorder is typified by development of new-onset proteinuria and hypertension after 20 weeks of pregnancy which is part of a systemic multi-organ endothelial dysfunction in the mother, with involvement in the brain (eclampsia, intracerebrovascular haemorrhage and posterior reversible encephalopathy syndrome), lungs (pulmonary oedema), heart (left ventricular dysfunction), kidneys (renal impairment and failure) (Cornelis et al., 2011), liver (dysfunction or capsular rupture) and coagulation system (thrombocytopenia and disseminated intravascular coagulopathy). The fetal sequelae include intrauterine growth restriction secondary to placental insufficiency, placental abruption, preterm birth and intrauterine demise. Despite extensive research focusing on this condition, the pathophysiology of preeclampsia still remains poorly understood. The global prevalence of preeclampsia ranges between two and eight percent (Duley, 2009).

Despite imprecise knowledge about the pathophysiology of pregnancy-related morbidity, it is generally acknowledged that abnormal placentation remains the foundation upon which pregnancy-related pathology develops (Khong and Brosens,
Effective trophoblast invasion is influenced by molecular pathways involving cellular proliferation, invasion, expression of particular adhesion molecule profiles (Aplin, 1993, Arimoto-Ishida et al., 2009), evasion of maternal immune detection, elusion of apoptotic signals and stimulation of angiogenesis. Successful trophoblast cell populations constituting a healthy pregnancy manifest most of the above traits which allow deep invasion of maternal tissues and transformation of myometrial spiral arteries. Failure of transformation of spiral arteries is the definitive mutual pathway underlying most pregnancy-related pathologies as untransformed vessels (from high resistance low capacity to high capacity low resistance) are incapable of adequately providing for the enormous metabolic demands of pregnancy (Brosens, 2011, Espinoza et al., 2006).

It is widely accepted that normal placental development (placentation), which is the growth and development of the placenta and the accompanying development of the uterine capacity to supply blood required by the fetus, translates to healthy and successful pregnancy. Hence understanding the molecular mechanisms involved in normal placentation and what is perturbed in abnormal placental development is likely to result in appropriate prophylactic and therapeutic interventions. Such interventions would have a significant impact on global maternal and perinatal mortality in keeping with the objectives of the United Nations Millennium Development Goals (MDG) 4 and 5, namely reduction in maternal mortality and improvement in childhood survival (Lalonde and McMullen, 2009).

Numerous factors impede our precise understanding of the aetiology of pregnancy-related pathology such as preeclampsia, despite considerable financial and human resource investment in research. One such factor is our lack of precise knowledge about the molecular details involved in normal pregnancy, which makes comprehension of
pregnancy-related pathology more challenging. The other factor is that over the years the placenta rather than the placental bed has been the focus of investigation. The placental bed is a site of immense cross-talk between fetal trophoblast and maternal decidua (Herrler et al., 2003), and ultimately the location where transformation of the maternal spiral arteries occurs.

This chapter introduces the placenta and the placental bed which are components of the core maternal-fetal interface, the various trophoblast cell populations and their respective roles in maternal invasion and spiral arterial transformation. In addition the ligands and receptors that are the subject of this thesis are also introduced, with kisspeptin and its receptor GPR-54 (also known as Kiss1R) primarily involved in the invasion pathway while VEGF-A, Prokineticin-1 or PROK-1 (also known as EG-VEGF) and their respective receptors VEGFR-1, VEGFR-2 and PROK1-R are involved in angiogenesis.

Chapter Two is a detailed description of the materials and methods utilized for gene and protein expression studies. Chapter Three focuses on the expression of the above-mentioned invasion and angiogenic factors across the maternal-fetal interface of healthy human pregnancies. Chapter Four compares the maternal-fetal expression of these ligands and receptors in healthy pregnancy and preeclampsia. Chapter Five explores the effect of placental kisspeptin stimulation on angiogenic ligand and receptor transcript expression in healthy and preeclamptic pregnancies. The final chapter is a general discussion with concluding remarks and proposed future prospective work.

1.2 The Placenta

The embryonic origin of the placenta is the trophectoderm layer of the blastocyst. It is of paramount importance for gaseous exchange and nutrient transport from the mother
across to the fetus (Gude et al., 2004, Burton et al., 2001). In addition the placenta is crucial for anchoring the developing embryo to the maternal tissues. Healthy pregnancy and fetal development are therefore dependent upon normal placental formation and development (placentation). The placenta consists of primary, secondary and tertiary villous structures lined by the outermost multinucleated syncytiotrophoblast layer which is bathed in maternal blood (Figure 1.1).

![Figure 1.1 Coronal section of the placental villous tree bathed in the intervillous space (IVS), illustrating stem, primary, secondary and terminal villi. Insert shows magnification of a terminal villus lined by outermost syncytiotrophoblast layer (shown as green), inner cytotrophoblast cell layer (pink) and villous capillary vessels (orange and blue).](image-url)
Directly underlying the syncytiotrophoblast layer which is a true syncytium devoid of cellular boundaries, is the mononuclear cytotrophoblast cell layer with distinct cellular walls (Figure 1.2). The cytotrophoblast is the trophoblast stem cell with ability to differentiate into the overlying proliferative syncytiotrophoblast layer which is responsible for gaseous exchange and nutrient transport or to cytotrophoblast columns which enter the invasive extravillous pathway.

**Figure 1.2** Sagittal section of the placental villus showing cytotrophoblast cells (pink) with distinct cellular walls. The villous cytotrophoblast can either differentiate into the outermost multinucleated syncytiotrophoblast cell layer (green) bathed in the intervillous space (IVS) or form cytotrophoblast columns (CC) which enter the invasive pathway. Within the villous mesenchyme (light yellow) lie villous capillary vessels (blue and orange), villous macrophages (Hofbauer cells) and other villous stromal cells. The nuclei are depicted in dark blue.
Chapter 1 General Introduction

The extravillous trophoblast (EVT) is key to invading the maternal uterine wall (decidua) resulting in anchorage of the conceptus to the mother and transformation of the maternal spiral arteries (Kam et al., 1999). Much research has focused on understanding factors responsible for which differentiation pathway cytotrophoblast cells ultimately take; proliferative or invasive (Sasagawa et al., 1996, Wright et al., 2010, Xu et al., 2002, Caniggia et al., 2000, Genbacev et al., 1996).

Due to the crucial role the placenta plays in determining fetal health and pregnancy outcome, the study of the placenta (also known as placentology) is becoming increasingly important in addressing problems relating to maternal and child health. Furthermore, the association between abnormal placentation and development of diseases of adulthood such as late-onset diabetes and hypertension (placental programming), has made the detailed study of the placenta even more compelling than ever before. Placental programming, also known as developmental origins of health and disease (DOHaD) tenders an immense opportunity for the possible realisation of true adult preventive medicine in the coming decades. (Barker, 2012, Barker and Thornburg, 2013, Hanson and Gluckman, 2011).

1.3 Maternal Decidua

The term decidua originates from “deciduous” trees which akin to menstrual shedding, loose their leaves in a seasonal manner. Decidualisation of the endometrium commences in the mid-luteal phase of pregnancy secondary to post-ovulatory progesterone production. A rise in progesterone triggers morphological changes initially in the endometrial glandular epithelium with appearance of basal subnuclear vacuoles followed by an increase in stromal cell size and glycogen production in the endometrial stroma. In addition to glycogen, the decidual stromal cells secrete prolactin, growth factors (e.g fibroblast and epidermal growth factor), extracellular
matrix proteins (collagen, laminin and fibronectin) and insulin-like growth factor binding protein-1 (IGFBP-1) which are thought to enhance implantation. (Loke and King., 1995)

The glandular epithelial changes result in production of factors such as, mucin, glycogen and glycoproteins which traditionally have been thought to support the developing blastocyst prior to accessing maternal spiral arteries. The decidua however has also been thought to modulate invasion of EVT and thus prevent potentially catastrophic over-invasion of trophoblast cells. The main objective of decidualisation is to optimise embryo implantation and is thus accompanied by secretion of a whole host of factors including production of VEGF-A and expression of matrix proteases. (Pijnenborg et al., 2010)

The endometrial lining in pregnancy is known as the decidua. The decidua consists of the decidua basalis and capsularis which underlie the placenta (and are part of the placental bed) as well as the decidua parietalis which has no involvement in placentation (Figure 1.3)
Coronal section of the uterus with placenta in situ

**Figure 1.3** Coronal section of the uterus with the placenta in situ illustrating the umbilical cord (UC) with placenta. The placenta rests on the placental bed (PB) constituted by the decidua basalis (DB-green) and junctional zone myometrium (black). The junctional zone myometrium is part of the inner third of the myometrium (pink). The decidua parietalis (DP-yellow) has no involvement with the placentation.

### 1.3.1 The Placental Bed

The placental bed consists of the decidua basalis, interstitial trophoblast and underlying myometrium with spiral arteries (Lyall, 2002). As extravillous trophoblast invades the decidua basalis, it interacts with collagen, as well as maternal stromal and immune cell components. The immune cell component of the maternal-fetal interface consists of decidual leukocytes, macrophages and decidual natural killer cells (dNKs) also known as uterine natural killer cells (uNKs). The maternal stroma consists of amongst others decidual fibroblasts, stromal cells, glandular tissue, vessels with vascular endothelial cells and collagen matrix. Although initially thought to limit trophoblast invasion by
eradicating invading EVT, uNK cells, unlike their peripheral counterparts (peripheral blood natural killer cells) exhibit no cytotoxic activity. Recent evidence suggests that uNK cells may be responsible for modulating maternal immune tolerance and spiral arterial transformation in conjunction with interstitial trophoblast (Williams et al., 2009). Additionally, uNK cells play a key role in trophoblast independent remodelling of spiral arteries (Gerretsen et al., 1983, Whitley and Cartwright, 2010).

1.3.2 Decidua Parietalis
The decidua parietalis is the innermost uterine layer (endometrial layer) in pregnancy, has no overlying placenta and is devoid of trophoblast cells. It has no invading trophoblast nor transformed spiral arteries (Figure 1.3).

1.4 Trophoblast invasion and trophoblast populations
The progenitor of all trophoblast cells is the villous cytotrophoblast (vCT). The vCT can either differentiate into proliferative syncitiotrophoblast (sTB) or give rise to invasive cytotrophoblast columns (Figure 1.2). The sTB is responsible for gaseous exchange and nutrient transport from the maternal intervillous space to the placental villous capillaries. On the other hand the cytotrophoblast columns represent the early stages of the invasive pathway. These columns subsequently give rise to the invasive extravillous trophoblast (EVT) cells (Huppertz, 2003). The EVT may either invade the maternal decidua and are then known as interstitial trophoblast or colonize the decidual and myometrial components of spiral arteries as endovascular trophoblasts (Kaufmann et al., 2003). It is endovascular trophoblasts that are thought to transform the maternal spiral arteries from high resistance low capacity structures to low resistance high capacity vessels capable of supporting a healthy pregnancy. It is, however, unclear whether the origin of endovascular trophoblast is from direct endovascular invasion of spiral arteries by trophoblast emanating from cytotrophoblast columns (inside out) or
from interstitial invasion of surrounding perivascular trophoblast (outside in).

Invasion of trophoblast cells is highest in the first trimester of pregnancy when placentation is being established and only extends as far as the inner third of the myometrium (also known as the junctional zone myometrium). What stops invasion of trophoblast cells beyond the junctional zone myometrium remains largely unknown however both under-invasion and over-invasion have the potential for severe maternal as well as fetal morbidity and mortality. While the former is associated with intrauterine growth restriction and preeclampsia, the latter is thought to result in morbid placental adherence- placenta accreta, increta and percreta. The placenta cretas often result in poor placental separation at parturition leading to massive postpartum haemorrhage requiring hysterectomy (Belfort., 2010, Hannon et al., 2011, Laban et al., 2014). As morbid placental adherence was rare prior to the advent of caesarean deliveries, part of the pathophysiology likely involves dysfunctional decidual components along the uterine scar (Jauniaux and Jurkovic., 2012).

As early as 1958, Robertson and Dixon studied the vascular changes in the placental beds of both normotensive and hypertensive pregnancies (Brosens et al., 1967, Robertson et al., 1967 a). They found that hypertensive pregnancies were characterized by lack of transformation of the maternal spiral arteries (Dixon and Robertson, 1958). This lack of spiral artery transformation was initially thought to be secondary to defective trophoblast invasion of maternal tissues in preeclamptic pregnancies. However, early spiral arteriolar changes in the junctional zone myometrium occur prior to the onset of trophoblast invasion (Brosens et al., 2002) and it is likely that immune cells (particularly uNK cells) within the maternal decidua play pivotal roles in spiral arterial transformation. Early vascular changes in the spiral arteries possibly commence with decidualisation of the endometrium in response to luteal phase hormonal changes.
A healthy pregnancy is therefore dependant upon successful invasion of the maternal tissues by fetal trophoblast cells in the early stages of pregnancy. Failure of this trophoblast invasion leads to poor remodelling of the maternal spiral arterial and development of conditions of placental malperfusion such as pre-eclampsia, intrauterine growth restriction, miscarriage (Ball et al., 2006) and preterm delivery.

The molecular mechanisms governing trophoblast invasion remain poorly understood and part of the difficulty is in the relative inaccessibility of the trophoblast-decidual interface during pregnancy. The use of in-vitro models and animal studies have however facilitated some understanding of maternal-fetal dialogue.

1.4.1 Factors affecting trophoblast invasion

A whole host of factors have been implicated in the differentiation, proliferation and invasion of trophoblast cells. How deeply the trophoblast cells invade is a function of the balance between pro-invasive and anti-invasive factors present in the fetal and the maternal tissues at one time (Knofler, 2010). Amongst some of the key factors studied are oxygen tension, expression of proteases, angiogenic factors and genes involved in trophoblast proliferation and motility.

1.4.1.1 Oxygen tension

Oxygen tension is likely the most important factor determining invasive capacity and affects cytotrophoblast differentiation and trophoblast invasion (Robins et al., 2005, Genbacev et al., 1996, James et al., 2006). Some studies have shown that under hypoxic conditions, cytotrophoblast stem cells favour a more invasive extravillous pathway with less expression of syncytin and human placental lactogen typically expressed by syncytiotrophoblasts (Robins et al. 2005). Others however have demonstrated elevated
expression of both Hypoxia Inducible Factor (HIF) and Transformation Growth Factor Beta 3 (TGF-β3) in early first trimester. HIF-1 is a heterodimer molecule constituted by HIF-1 alpha (HIF-1α) and HIF-1 beta (HIF-1β). HIF-1α is the constitutively expressed transcriptional factor responsible for cellular responses to low oxygen tension (Gultice et al., 2009). Under high oxygen conditions HIF-1α is inactivated by degradation and is thus unable to form the heterodimeric HIF (in conjunction with HIF-1β) responsible for binding to hypoxia response elements affecting cellular transcription. Transformation Growth Factor Beta 3 (TGF-β3) inhibits EVT differentiation and invasion (Caniggia et al., 2000) likely via suppression of metalloproteinase activity (Lash et al., 2005). Hence under hypoxic conditions there is increased expression of heterodimeric HIF-1 and TGF-β3 favoring the proliferative rather than invasive pathway. A high oxygen environment is detrimental to early embryogenesis due to the generation of reactive oxygen species (ROS) to which the embryo is poorly adapted. Prior to 9 weeks gestation the embryo is protected from ROS by the plugging of spiral arteries by endovascular trophoblast. This vascular plugging results in reduced blood flow to the intervillous space which provides a lower oxygen environment for the growing fetus.

1.4.1.2 Protease expression

For trophoblast cells to invade maternal decidua they must first attach to extracellular matrix and then migrate by traction. Cells interact with extracellular matrix via expression of cell surface adhesion molecules, in particular intergrins. The latter are transmembrane glycoproteins with extracellular and cytoplasmic domains and are constituted by various α and β subunits. The combination of the various α and β subunits determine which extracellular matrix (ECM) protein the cell will bind to. ECM proteins include amongst others, fibronectin, type I and IV collagen as well as laminin which forms an integral part of the villous basement membrane. (Loke and King., 1995). Villous trophoblast cells are known to express the α6β4 intergrin
(laminin receptor), which they progressively lose as they enter the invasive extravillous pathway. In contrast invasive interstitial trophoblast mostly express α₅β₁ integrin, the fibronectin receptor which seems to be associated with cellular motility.

There are two families of proteases that degrade extracellular matrix, the plasminogen activators and the metalloproteinases. The plasminogen activators, tissue Plasminogen Activator (tPA) and urokinase Plasminogen Activator (uPA) convert inactive plasminogen to active plasmin and are controlled by Plasminogen Activator Inhibitors-1, -2 and -3 (PAI-1, -2 and -3). Similarly the metalloproteinases are secreted as inactive pro-enzymes and are classified into three sub-families depending on which substrates they mainly degrade. The Collagenases (MMP-1 and MMP-5) act on fibrillar collagens and cleave α-chains of type I, II and III collagens. The Gelatinases (MMP-2 and MMP-9) as their name suggests cleave gelatin which is denatured collagen, type IV (basement membrane) collagen and type V collagen. Lastly the Stromelysins (MMP-3, MMP-7 and MMP-10) act on a broad range of substrates including proteoglycans, glycoproteins such as fibronectin and laminin as well as type IV collagen. The action of metalloproteinases is controlled by tissue inhibitors of metalloproteinases (TIMPs). Of the two broad families of matrix-degrading proteinases (i.e the plasminogen activators and the metalloproteinases), the latter have been shown to play a more significant role in trophoblast migration and invasion.

The ability of trophoblast cells to express proteases that digest extracellular matrix greatly enhances their invasiveness (Moll and Lane, 1990). In particular the invasion-enhancing expression of gelatinases (MMPs) 2 and 9, and their membrane-type activators (membrane-type metalloproteinase-1; MT-MMP-1 (also known as MMP-14)) have been extensively studied in the placenta (Bischof et al., 1995, Hurskainen et al., 1998, Isaka et al., 2003, Bjorn et al., 1997) and demonstrate a gestational age
dependent differential pattern of expression (Staun-Ram et al., 2004). On the other hand the expression of tissue inhibitors of metalloproteinases (TIMPs) by the maternal decidua down-regulates this invasive capacity (Xu et al., 2000, Anacker et al., 2011).

1.4.1.3 Placental Hormones
Gonadotrophin Releasing Hormone (GnRH) I and II are expressed in vCT and EVT and upregulate the expression of MMP-9 and MMP-2 in vitro (Liu et al., 2010). Similarly human chorionic gonadotrophin (hCG) upregulates MMP-2 expression via ERK and AKT signaling pathways (Prast et al., 2008). Placental growth factor (PIGF) and Insulin-like Growth Factor II (IGF-II) likewise promote the invasive capacity of these cells. Insulin-like Growth Factor Binding Protein-4 (IGFBP-4) is exclusively expressed by the maternal decidua and is a potent inhibitor of IGF-II action by rendering it less bioavailable thus providing maternal inhibition to trophoblast invasion. In contrast placentally expressed IGFBP-4 protease (also known as PAPP-A) cleaves IGFBP-4, increases bioavailable IGF-II and results in promotion of trophoblast invasion (Giudice et al., 2002).

1.4.1.4 Angiogenic factors
Vasculogenesis is the de novo formation of new blood vessels from haemangiogenic progenitor cells while angiogenesis is the development of new microvessels from existing vessels (Demir et al., 2007). In humans, placentation involves vasculogenesis from day 21 post-fertilization during formation of tertiary chorionic villi and angiogenesis from day 32 during continued chorionic growth and neo-vascularization of the decidua (Kaufmann et al., 2004). The placenta develops by branching angiogenesis from day 32 post-fertilisation to 24 weeks and by non-branching angiogenesis from 24 weeks to term (Charnock-Jones et al., 2004).
Vascular Endothelial Growth Factor (VEGF), previously known as vascular permeability factor (Senger et al., 1983) plays a pivotal role in angiogenesis. VEGF is a homodimeric glycoprotein of 45kDa which stimulates growth of vascular endothelial cells, promotes vascular permeability, induces a powerful angiogenic response in vivo and is a potent mitogen. VEGF has various isoforms (VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E) of which VEGF-A in particular is key in angiogenesis of placentation and pregnancy (Clark et al., 1998b). VEGF-A mediates its mitogenic and angiogenic effects by binding to three receptor tyrosine kinases (Ferrara et al., 2003). VEGF Receptor-2 (VEGFR-2) also known as the Kinase Domain Receptor (KDR) is the main mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF. VEGF Receptor-1 (VEGFR-1) on the other hand is known as the “decoy receptor” as it scavenges off and reduces the amount of VEGF-A available to bind to VEGFR-2. VEGFR-1 is therefore anti-angiogenic. Lastly VEGFR-3 is involved in lymphangiogenesis (Ferrara et al., 2003). VEGF mRNA has been localized to cytотrophoblast columns and invading EVT (Charnock-Jones et al., 1994) while VEGFR-1 and VEGFR-2 mRNA expression has been localized to EVT and endothelial cells respectively (Clark et al., 1996). VEGF promotes trophoblast invasion by stimulating the activity of proteases such as urokinase plasminogen activator (uPA) and MMP-9 (Anteby et al., 2004).

Recently, Endocrine Gland Specific VEGF (EG-VEGF) also known as Prokineticin-1 (PROK-1) and its cognate receptor PROK1R have been characterized (LeCouter et al., 2001, Soga et al., 2002). Similar to VEGF, EG-VEGF induces proliferation, migration and fenestration in capillary endothelial cells. Furthermore it also possesses a HIF binding site in its promoter and like VEGF its expression is induced by hypoxia. However unlike VEGF, its expression is restricted to steroidogenic organs like the ovary, testis, adrenal and the placenta and its action is specific to capillary endothelial cells derived from these endocrine glands. While VEGF mediates its action via receptor
tyrosine kinases, EG-VEGF does so through G-protein-coupled receptors (PROKR1 and PROKR2). In the placenta the action of EG-VEGF is thought to be complementary to that of VEGF because, while VEGF is expressed in cytotrophoblast and EVT, EG-VEGF is mainly expressed by syncytiotrophoblast (Hoffmann et al., 2006). In vitro, EG-VEGF expression is regulated by hypoxia (Hoffmann et al., 2006) and interestingly seems to suppress EVT migration, invasion and tube-like formation (Hoffmann et al., 2009). Other important angiogenic role players affecting trophoblast invasion include placental growth factor, fibroblast growth factor and endoglin.

1.4.1.5 Kiss1 gene, kisspeptins and GPR-54

RF (R-Arginine, F-Phenylalanine) amides are a group of peptide hormones that have a common Arg-Phe-NH₂ motif at their C-terminus and all bind to G protein-coupled receptors. RFamides are involved in many processes throughout the body including inflammatory responses, control of food intake and development. One major area where RFamides play a role is reproduction, involving two RFamides: Gonadotrophin Inhibitory Hormone (GnIH) and Kisspeptin. Of the two, kisspeptin has been more widely characterized and it is this peptide that is the focus of this research project.

Kisspeptin was originally isolated as an anti-metastatic peptide from melanoma cells and was initially termed metastin (Lee et al., 1996). Kisspeptin is encoded by the Kiss1 gene and is synthesized as a precursor protein of a 145 amino acid polypeptide (Ohtaki et al., 2001). Posttranslational modification of the transcript results in four kisspeptins with 54 (metastin), 14, 13 and 10 amino acids which all include the essential carboxyl terminal 10 amino acids (Fig 1.4).

The 10 amino-acid peptide (kisspeptin-10), YNWNSFGLRF-NH₂ which ends with an RF-amide is sufficient to activate the receptor.
Kisspeptins are the natural ligands of an orphan G-protein coupled receptor, GPR54 (also known as Kiss1R or AXOR12)(Muir et al., 2001). The GPR54 receptor has a 396 amino-acid open reading frame and is related to the galanin receptor family, although it does not bind galanin.

![Prepro-Kisspeptin](image)

**Figure 1.4** The structure of the human kisspeptin pre-pro-protein (Kp-145) and its posttranslational products, kisspeptin 54, 14, 13 and 10. (Adapted from Tena-Sempere, Hum Reprod Update 2006)

The GPR54 receptor identified in the human is highly conserved among mammals with an 81% homology to the rat receptor. The receptor signals via phosphatidyl inositol biphosphate hydrolysis which results in accumulation of inositol triphosphates (IP$_3$) and diacylglycerol (DAG), as well as, activation of extracellular regulated kinase 1/2 (ERK1/2) and p38 kinase (Kotani et al., 2001).

Kisspeptin and its cognate receptor GPR54 are expressed mainly in the brain and placenta and they have been implicated in the initiation of puberty, regulation of adult fertility and in placentation.

Mutations in the GPR 54 gene, as well as in the KiSS-1 gene are associated with isolated hypogonadotrophic hypogonadism in both humans and rodents (Seminara...
et al., 2003, de Roux et al., 2003, Semple et al., 2005). Hence kisspeptins and their native receptors seem to play a vital role in pubertal maturation as well as normal reproductive development (Funes et al., 2003, Seminara and Kaiser, 2005, Lanfranco et al., 2005, Tenenbaum-Rakover et al., 2007). In contrast a recent publication has demonstrated that pubertal development occurs in the absence of kisspeptin/GPR 54 signalling in mice with genetic ablation of the ligand and receptor neurons (Mayer and Boehm, 2011).

1.4.1.6 Kiss1 gene, kisspeptins and GPR-54 in pregnancy

Horikoshi et al demonstrated elevation of plasma metastin in human pregnancy and localized its mRNA and protein expression to the syncitiotrophoblast layer of the placenta (Horikoshi, 2003). Kiss1 and its receptor GPR54 were identified among candidate genes to play an important role in the invasive and migratory properties of trophoblast cells (Janneau, 2002).

By comparing mRNA signatures of first trimester placentae with those from the third trimester, one group demonstrated that Kiss1 and GPR-54 transcripts were abundant in the former, suggesting an important role in invasion (Bilban et al., 2004). They also showed that the smallest cleavage product (kisspeptin-10) inhibited trophoblast cell invasion. The expression of both ligand and receptor was demonstrated in villous trophoblast whereas only the receptor was expressed by extravillous trophoblast. They suggested an autocrine/paracrine control over invasion and furthermore demonstrated that this process was mediated via intracellular calcium mobilization. In the same study both Kiss-1 and Kiss-1R mRNAs were highest in the first trimester in comparison to term, further suggesting a role of invasion inhibition for kisspeptin at a time when trophoblast invasion is at its highest. In contrast to tissue expression levels, circulatory kisspeptin concentrations increase with advancing gestation and
dramatically fall post-delivery (Horikoshi, 2003).

Our understanding of trophoblast invasion is largely based on studies done on various villous and extravillous trophoblast cell lines, as well as rodent in-vivo and in-vitro experimental models (King et al., 2000). There are limited studies conducted on kisspeptins and placentation in humans. Recently, a group studied Kiss1 and MMP9 expression in primary trophoblast cultures from 40 women with pre-eclampsia and compared them with those from 20 normal term pregnancies (Qiao et al., 2005b).

Expression levels were measured using real-time PCR and Western blot analyses from the primary trophoblast cells. They analyzed the difference in Kiss1 and MMP9 expression between normal and preeclamptic pregnancies and correlated their findings with perinatal outcome. The expression of Kiss1 was significantly higher in the trophoblasts of preeclamptic women compared to normal pregnancy whereas that of MMP9 was significantly lower in preeclamptic than normal pregnancies. Furthermore there was significant positive correlation between MMP9 expression in preeclamptic women and neonatal birth weight. The opposite (significant negative correlation) was found for Kiss1 gene expression and neonatal birthweight.

The same group found a positive correlation between invasive capacity and MMP9 expression and a negative correlation for invasive capacity and Kiss1 expression by comparing mRNA expression from human placental tissue of molar, preeclamptic and normal pregnancies(Qiao et al., 2005a). Interestingly, a prospective study utilizing chorionic villous sampling of human first trimester placental tissue demonstrated no difference in MMP-2 or MMP-9 expression between pregnancies destined for health or preeclampsia (Huisman et al., 2004). One study (Yan et al., 2001) demonstrated
suppression of MMP-9 expression by Kiss1 via reduced NF-κβ binding to the MMP-9 promoter while the other, using a gastric cancer cell-line showed that Kiss1 suppressed MMP-9 expression via p38 MAP kinase signalling pathway (Lee and Kim, 2009). Furthermore kisspeptin inhibits tumour angiogenesis by suppressing Sp1-mediated VEGF expression and FAK/Rho GTPase activation (Cho et al., 2009). In vitro, kisspeptin suppresses the sprouting of new vessels from placental arteries and tube-like structure formation in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner (Ramaesh et al., 2010).

Kisspeptin therefore seems to be play some role in regulating the expression of genes involved in matrix degradation and angiogenesis both of which remain crucial pathways in placentation.

As discussed earlier there are permissive and inhibitory factors involved in trophoblast invasion on mutual sides of the maternal-fetal interface. Very few human studies have examined gene and protein expression on both sides of this interface, in a cohort of patients. In order to understand the molecular dynamics of maternal-fetal dialogue, it is important to examine all components of this interface in both healthy and pathological pregnancies.

There is adequate evidence that kisspeptin and its cognate receptor, GPR54 play key roles in the control of trophoblast invasion and placental development.

1.5 Transformation of the Spiral Arteries
Effective placentation is required for a successful pregnancy outcome. During placentation, transformation of the placental bed spiral arteries from high resistance low capacity vessels to low resistance high capacity ones is crucial to support the
developing fetus. The lack of transformation of these spiral arteries is associated with poor pregnancy outcomes such as preeclampsia and intrauterine growth restriction (IUGR) (Brosens, Robertson et al. 1967; Robertson, Brosens et al. 1967). Transformation of spiral arteries involves the replacement of vascular endothelial cells with endovascular trophoblast as well as vascular smooth muscle and elastin with trophoblast-embedded fibrinoid matrix. The result of loss of vascular smooth muscle and elastin is vessel independence from vasomotor activity and absent vascular recoil respectively. The effect of these changes are flaccid, dilated spiral arterial vessels with considerable volume citation.

The spiral arteries are branches of the basal arteries and have decidual and myometrial components. The initiation of spiral arterial transformation is thought to occur as early as the late luteal phase with progesterone-mediated changes to the basal decidua and junctional zone myometrium. Some of these early decidualisation events include vacuolisation of the vascular endothelial and medial layers, vessel wall oedema likely driven by angiogenic factors like VEGF, disruption of the elastica and disorganization of the vascular smooth muscle. This is sometimes referred to as trophoblast-independent or decidua-associated remodeling and uNK cells likely play a crucial role in this phase prior to invasion of trophoblast cells.

The trophoblast dependent phase involves invasion of interstitial trophoblast stemming from cytotrophoblast columns of anchoring villi. These invading mononuclear interstitial trophoblast cells cluster around the decidual segments of spiral arteries as perivascular trophoblast and are thought to enter the already disrupted spiral arterial walls by intravasation. Having entered the vessel wall they likely emit apoptotic signals responsible for further vascular smooth muscle and elastin disruption and eradication, secrete fibrinoid material and replace vascular endothelial cells to
become endovascular trophoblast. Due to paucity of perivascular trophoblast around myometrial spiral arteries, endovascular trophoblast in these vessels is likely the result of intravascular migration of endovascular trophoblast from decidual spiral arteries rather than by intravasation of perivascular citation. It is more the scarcity of endovascular trophoblast in the myometrial rather than decidual spiral arteries that seems to be the initial insult in pregnancy-related disorders.

1.6 Failure of Transformation of the Spiral Arteries: Clinical Sequelae

1.6.1 Preeclampsia
Preeclampsia is the leading cause of direct maternal deaths and contributes significantly to perinatal morbidity and mortality in South Africa and the rest of the world. This disorder is typified by new-onset proteinuria and hypertension after 20 weeks of pregnancy which is part of a systemic multi-organ endothelial dysfunction in the mother and is often complicated by intrauterine growth restriction (IUGR) in the fetus. Despite extensive research focusing on this condition, the pathophysiology of preeclampsia still remains poorly understood however it is largely acknowledged that lack of deep spiral arterial transformation is at the core of its development.

1.6.2 Intrauterine Growth Restriction
Intrauterine growth restriction defines a clinical entity where the fetus fails to reach its maximum genetic growth potential. It has various causes including chromosomal abnormalities, congenital infections, inherited metabolic disorders and preeclampsia. It can however develop without any clinically apparent cause and is then known as isolated intrauterine growth restriction. The aetiology of the isolated form is likely related to poor placentation resulting in inadequate transformation of the spiral arteries.
1.6.3 Aims of Study

The hypothesis of this study was that the expression of kisspeptin and/or its receptor as well as target gene regulation would be different across the maternal-fetal interface of normal and abnormal placentation.

The aims of this study were therefore to

1. Investigate the expression of kisspeptin and its receptor GPR-54 in the maternal-fetal tissues, namely the placenta, placental bed and decidua parietalis of healthy pregnancies.

2. Investigate the expression of kisspeptin-mediated genes, namely MMP-9 and angiogenic regulators (VEGF-A, PROK-1 and their receptors) in the maternal-fetal tissues of healthy pregnancies.

3. Explore differences in the maternal-fetal tissue expression of Kiss1, GPR-54 and angiogenic ligands (VEGF-A and PROK-1) between healthy and preeclamptic pregnancies.

4. Investigate differences in maternal-fetal tissue expression and circulatory levels of kisspeptin, VEGF-A and PROK-1 in healthy and preeclamptic pregnancies.
CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Tissue Sampling and Collection ......................................................... Page 26

2.2 Gene Expression Studies ................................................................. Page 26-27
  2.2.1 RNA Extraction ................................................................. Page 26-27
  2.2.2 Complimentary DNA Synthesis ........................................ Page 27
  2.2.3 Real-Time Reverse Transcriptase Polymerase Chain
      Reaction ................................................................................ Page 27-28

2.3 Protein Expression Studies ............................................................... Page 30
  2.3.1 Protein Extraction ............................................................... Page 30
  2.3.2 Protein Quantification ........................................................ Page 30
  2.3.3 SDS-PAGE and Western Blotting ....................................... Page 31
  2.3.4 Immunohistochemistry .......................................................... Page 32
    2.3.4.1 Kisspeptin Immunostaining ........................................ Page 32-33
    2.3.4.2 Kisspeptin Receptor (GPR-54) Immunostaining .... Page 33
    2.3.4.3 MMP-9 Immunostaining ............................................. Page 33
    2.3.4.4 VEGF-A Immunostaining ............................................ Page 34
    2.3.4.5 VEGF-A and Pancytokeratin
          Co-immunostaining .......................................................... Page 34
    2.3.4.6 VEGF Receptor-1 (VEGF-R1) and VEGF
          Receptor-2 (VEGF-R2) Immunostaining ............................ Page 34-35
    2.3.4.7 EG-VEGF (Prokineticin-1) Immunostaining .............. Page 35

2.4 Enzyme-linked Immunosorbent Assays (ELISAs) ......................... Page 35
  2.4.1 Maternal and Cord Serum Kisspeptin-10
      Quantification ...................................................................... Page 35-36
  2.4.2 Maternal and Cord Serum VEGF-A Quantification.......... Page 36-37
  2.4.3 Maternal and Cord Serum EG-VEGF Quantification .... Page 37-38

2.5 Statistical Analysis ........................................................................ Page 38
2.1 Tissue Sampling and Specimen Collection

At caesarean section the placenta and placental bed were sampled using a technique previously employed by Pijnenborg and colleagues (Pijnenborg et al., 1991). In addition the decidua parietalis was sampled as a negative control, being a neutral site that has had no involvement in placentation. Each of the tissue samples collected was divided into four parts and put into four different collection tubes. One tissue part was collected in RNA stabilisation solution (RNA Later®, Ambion Cat # AM 7021) and snap frozen in liquid nitrogen for RNA extraction. The second and third parts of the tissue samples were collected in empty screw–top Bijou tubes for protein extraction and kisspeptin stimulation studies. The fourth part of the sample was collected in paraformaldehyde in order to make wax-embedded tissue blocks for immunohistochemical studies.

2.2 Gene Expression Studies

2.2.1 RNA Extraction

One millilitre of TRIzol® reagent (Invitrogen™ Cat no 15596-018) was used per 50mg of tissue. The tissue was homogenised with a Tissue Ruptor® (TR 12520437) Qiagen Instruments Hombrechikon, Switzerland) on dry ice to minimise RNA degradation and the homogenate was kept on ice for 10 minutes. Then the homogenate mixture was centrifuged (12000rpm at 4°C for 15min) to get rid of cellular debris. The supernatant was collected and added to 200μl of ice-cold BCP (1-Bromo-3-Chloro-Propane from Sigma Cat # B-96373) per 1ml of TRIzol utilised. The mixture was vigorously shaken for 15 seconds and incubated on ice for 10 minutes. The solution was centrifuged (12000rpm at 4°C for 15min) to separate the protein from the RNA. The clear supernatant (RNA) was decanted into a new tube and 500μl of 100% propanol was added to precipitate the RNA. The tube was again centrifuged, propanol decanted
and the sample air dried. Subsequently the RNA pellets were washed in 75% Ethanol and briefly air dried. The resultant pellets were resuspended in 10-100\,\mu l of DEPC (Diethylpyrocarbonate) water depending on the size of the pellet and the RNA solution was stored at -70°C for cDNA synthesis. RNA concentration was determined by measuring absorbance at 260\,nm and 280\,nm employing a NanoDrop® ND1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

### 2.2.2 Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesised from the RNA extracted from the tissue samples. The RNA was reverse transcribed with Multiscribe Reverse Transcription reagents from Applied Biosystems (Part number N808-0234). The reverse transcription reaction had a total volume of 20\,\mu l and consisted of DEPC H₂O (3.7\,\mu l), 10x RT Buffer (2\,\mu l), MgCl₂ (4.4\,\mu l), dNTPs (4\,\mu l), Random Hexamers(1\,\mu l), RNAse Inhibitor (0.4\,\mu l), Reverse Transcription Enzyme (0.5\,\mu l) and (2\,\mu l) of RNA. The thermal cycling parameters of the reaction were 25°C for 10 minutes, 4°C for 10 minutes, 48°C for 45 minutes and 95°C for 5 minutes. This was carried out using the ABI GeneAmp® PCR System 2700 (Applied Biosystems).

### 2.2.3 Real-Time Polymerase Chain Reaction

Gene expression studies were conducted using the ABI 7900 Real-Time PCR machines from Applied Biosystems. In addition Taqman PCR reagents, mastermix and 96-well plates from Applied Biosystems were utilized.

For a single sample 50\,\mu l reaction (in duplicate) the following were added: 19.05\,\mu l H₂O, 25\,\mu l PCR mastermix, 0.75\,\mu l 18s ribosomal RNA mix (forward primer, reverse primer and probe), 0.6\,\mu l each of target gene forward and reverse primers and 2\,\mu l each of target gene probe and cDNA sample. After mixing, each sample including
a no reverse transcriptase (negative control) and reference cDNA (positive control) were added to 96-well MicroAmp® PCR plates (Applied Biosystems, Warrington, UK) and sealed with nuclease-free MicroAmp® optical adhesion film. The standard thermal cycling protocol was conducted as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 95 seconds and 60°C for 1 min. The following primer/probe pairs supplied by Sigma® pharmaceuticals were utilised (Table 2.1) in conjunction with the Taqman® mastermix (Applied Biosystems). 18s ribosomal RNA was used as an internal gene expression control. The ΔΔCt method was employed to analyse relative gene expression.
### Table 2.1 Primers and Probe Sequences used for Real-Time (RT) PCR Reactions

<table>
<thead>
<tr>
<th>Primer/Name</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
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<td>GPR54 Forward Primer</td>
<td>5'-GGTGCTGGCAGCCTTCAT-3'</td>
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<tr>
<td>GPR54 Reverse Primer</td>
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<td>5'-CACACTCATGGCGTCAGAGT-3'</td>
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<td>Kiss1 Reverse Primer</td>
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<td>Kiss1 Probe</td>
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<tr>
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<td>5'-AGC AAG GAC AGG TGT GGT GC-3'</td>
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<tr>
<td>PROKR1 Reverse Primer</td>
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<td>5'-TAC CTC CAC CAT GCC AAG TG-3'</td>
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<td>5'-GGCCACTACTGTGCCGCTTGG-3'</td>
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<td>5'-GGCCACTACTGTGCCGCTTGG-3'</td>
<td>5'-GGCCACTACTGTGCCGCTTGG-3'</td>
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<td>MMP9 Probe</td>
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<td>5'-[FAM]-TGCAAGTTGCTCAACTACATCCAGCAGG-[TAMRA]-3'</td>
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<tr>
<td>VEGFR2 Reverse Primer</td>
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<td>5'-TTA CAG CTT CCA AGT GGC TAA GG-3'</td>
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<td>VEGFR2 Probe</td>
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<tr>
<td>VEGFR1 Reverse Primer</td>
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<td>5'-ATG TGC CAA ATG GTG TAC ATG T-3'</td>
<td>5'-ATG TGC CAA ATG GTG TAC ATG T-3'</td>
</tr>
<tr>
<td>VEGFR1 Probe</td>
<td>5'-[FAM]-CCATGACCAAGCTATGATGA[3]-[TAMRA]-3'</td>
<td>5'-[FAM]-CCATGACCAAGCTATGATGA[3]-[TAMRA]-3'</td>
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<td>5'-CGG CTA CCA CAT CCA AGG AA-3'</td>
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<td>18S Reverse Primer</td>
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<td>18S Probe</td>
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<td>5'-[VIC]-TGCAAGTTGCTCAACTACATCCAGCAGG-[TAMRA]-3'</td>
<td>5'-[VIC]-TGCAAGTTGCTCAACTACATCCAGCAGG-[TAMRA]-3'</td>
</tr>
</tbody>
</table>


Chapter 2  General Materials and Methods

2.3   Protein Expression Studies

2.3.1   Protein Extraction

Tissue was lysed with cold RIPA lysis buffer containing (25mM Tris HCl pH 7.6, 150mM NaCl, 1% Triton X, 1% sodium deoxycholate and 0.1% SDS) that was supplemented with Urea to a final concentration of 4M and Complete® EDTA-free protease inhibitor tablets (ref 11 873 580 001) (Roche®). One millilitre of the supplemented RIPA was used per 50mg of tissue. The tissue was left in the buffer on ice for 30 minutes. The mixture was poured into a mortar and snap-frozen with liquid nitrogen. The solid material was crushed into a fine powder using a pestle with intermittent use of liquid nitrogen as required. The powder was collected in 2ml epindorff tubes and centrifuged at 12000rpm for 15 minutes @ 4°C. The supernatant was collected and the protein concentration quantified using the BCA (Bicinchoninic acid) method.

2.3.2   Protein Quantification

The Pierce BCA Protein Assay Kit (Prod # 23225) from Thermoscientific was used to quantify protein concentration according to the manufacturer’s instructions. Serial dilutions of bovine serum albumin (BSA) at 2mg/ml in 0.9% saline and 0.05% sodium azide were made for the standard curve. Ten microliters (10μl) of each standard solution and unknown samples were pipetted in triplicate onto a 96-well microplate. Using a multi-channel pipette, 200μl of working reagent 1:50 dilution of Reagent B (4% cupric sulphate): Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) was added to each sample well and the microplate incubated for 30 min at 37°C. The samples were then placed in an ELISA microplate reader and absorbance read at 595nm. Protein concentrations were calculated from the BSA standard curve.
2.3.3 SDS-PAGE and Western Blotting

A total of 20μg of protein in 20μl was loaded per gel well on a stacking gel after denaturing in Laemmli buffer (125mM Tris HCL pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue) at 95°C for 5 minutes. The protein sample was separated on a 15 % SDS-PAGE resolving gel (Appendix). The gels were run at 110V for 80 minutes. Spectra® Multicolor Broad Range Protein Ladder (Product no 26634) from Thermoscientific was used to mark molecular weights of proteins in Kilodaltons (kDa). The gel was then blotted onto a 0.45μm PVDF membrane (Amersham Hybond-P). The protein transfer was done using a semi-dry technique (Novex® Semi-dry Blotter (Cat No SD1000) Invitrogen) at a constant voltage of 14V for 90 min.

The membranes were blocked on a shaker with 5% milk in TBST at room temperature for 1 hour. They were then incubated at 4°C overnight with the primary antibody (Rabbit anti-VEGF A antibody (sc-152) Santa Cruz Biotechnology) at a dilution of 1:50 in blocking solution. Two gels were simultaneously run for each sample resulting in two membranes, one for detection and the other for control. The twin membrane (negative control) was incubated with a pre-incubated mixture of anti-VEGF A and VEGF A blocking peptide. For MMP9 protein detection rabbit anti-human MMP9 antibody (Product no ab38898, abcam®) and MMP9 blocking peptide (Product no ab41067, abcam®) were used. The membranes were washed 3x with TBST for 5 min. and subsequently incubated with secondary antibody Goat Anti-Rabbit Peroxidase (GARP) at a dilution of 1: 5000 at room temperature for 1 hour and thereafter washed again with TBST for 5min. Visualisation was done by application of a peroxidase based chemiluminescent solution (Supersignal® West Pico Chemiluminescent Substrate –Thermoscientific (Prod no 34080) and development of the photographic film (Amersham Hyperfilm® ECL- GE Healthcare Limited (Product code 28906836)) in a dark room.
2.3.4 Immunohistochemistry

Wax-embedded tissue blocks were sectioned and fixed to slides (Histobond®) with the three sections of placenta, placental bed and decidua parietalis fixed across one slide. This arrangement ensured standardised treatment of sections across the maternal-fetal interface on one slide. In addition all slides were batch-stained in order to minimise “inter-slide” staining variability.

2.3.4.1 Kisspeptin Immunostaining

The slides were de-waxed and rehydrated in xylene and decreasing concentrations of ethanol. Antigen retrieval was done by pressure cooking the slides in 0.01M Citrate Buffer. The slides were then blocked in 10% Hydrogen Peroxide in Methanol for 30 mins in order to quench endogenous peroxide activity. Thereafter the slides were first washed in tap water and in Tris-Buffered Saline (TBS), (25mM Tris, 150mM NaCl, 2mM KCl, pH 7.4). A second blocking step was done with normal donkey serum (NDS) to reduce non-specific binding of the primary antibody.

The slides were incubated at 4°C overnight with the kisspeptin primary antibody (GQ2) diluted 1: 1500 in blocking serum (NDS). (The primary antibody was raised in the sheep against kisspeptin 54 and was obtained as a gift from Professor Bloom’s laboratory). The next morning the slides were washed in TBS (2 x 5mins) and secondary antibody Donkey Anti-Sheep Peroxidase (DAShP) IgG (Cat no 713-005-003, Jackson Immunoresearch) diluted 1: 750 in blocking serum was added. The slides were washed again in TBS (3 x 5mins). Then Tyramide Cyanine 5 Signal Amplification (Cat no NEL 745, Perkin Elmer LAS, Inc) diluted (1:50) was applied to the slides and incubated at room temperature for 10minutes. The slides were then washed in TBS (2 x 5mins) and DAPI 405 used as a nuclear counterstain at dilution of 1: 1000 in PBS was applied for 10mins at room temperature. The DAPI was washed off in TBS (2 x 5mins) and
Chapter 2 General Materials and Methods

the slides mounted in Permaflour® mounting medium. Visualisation was done on the Zeiss LSM 710 META confocal laser microscope.

2.3.4.2 Kisspeptin Receptor (GPR-54) Immunostaining

The slides were de-waxed, rehydrated and then blocked with 10% hydrogen peroxide (H₂O₂) in methanol. They were then washed in tap water and TBS and subsequently blocked with normal goat serum. Incubation with primary antibody against GPR54 (R2 1213 custom manufactured by EZ Biolabs raised in the rabbit) at a dilution of 1: 1600 was done at 4°C overnight. A similar incubation with pre-immune rabbit serum at the same dilution was conducted as a negative control. The next morning the slides were washed in TBS and secondary antibody Goat Anti-Rabbit Peroxidase (1: 200) (Dako Cat no P0448) was applied. The slides were again washed in TBS and Tyramide 546 (Cyanine 3) Signal Amplification (Perkin Elmer LAS, Inc) was applied. The Tyramide was washed off in TBS and Sytox Green (1: 1000 in TBS) was used as a nuclear stain. After washing with TBS the slides were mounted in Permaflour®. The Zeiss LSM 710 META confocal laser microscope was employed for visualisation.

2.3.4.3 MMP-9 Immunostaining

The slides were de-waxed and rehydrated. Heat-mediated antigen retrieval in 0.01 M citrate buffer was followed by quenching endogenous peroxide with 10% H₂O₂. Blocking to prevent non-specific antibody binding was done with NDS for 1 hour and primary rabbit anti-MMP9 antibody (ab38898 (1 mg/ml)) (1:200) from Abcam® was applied overnight. The slides were washed in TBS (3x 5min) and Donkey Anti-Rabbit-555 (DAR-555) (1:1000) (A31572 from Invitrogen) was used as secondary antibody. The slides were again washed with TBS as before followed by staining with DAPI for nuclei. To visualize the slides, the Zeiss LSM 710 META confocal laser microscope was used.
Chapter 2 General Materials and Methods

2.3.4.4 VEGF-A Immunostaining
The slides were dewaxed, rehydrated, and antigen retrieved and blocked with 10% hydrogen peroxide in methanol. Normal Donkey Serum (NDS) was used for blocking to prevent non-specific binding. Rabbit Anti-VEGF A antibody (VEGF (A-20) (sc-152)-Santa Cruz Biotechnology) at a dilution of 1:50 was applied overnight followed by three 5 min TBS washes in the morning. Donkey Anti-Rabbit 555 (Invitrogen) at (1:1000) dilution was used as secondary antibody and thereafter washed (3x 5 min) with TBS. DAPI (1: 1000) nuclear stain was subsequently applied followed by visualization on the confocal laser microscope as above.

2.3.4.5 VEGF-A and Pancytokeratin Co-immunostaining
The slides were dewaxed, rehydrated, antigen retrieved and blocked with methanol hydrogen peroxide for half an hour. NDS was used as blocking serum and primary rabbit anti-VEGF A and mouse anti-human Pancytokeratin (AbID 0774/Cat no C2562, Sigma®) antibodies were mixed, applied to the slides and incubated overnight. The former was diluted at 1: 50 and the latter was used at a dilution of 1: 15 000. To wash off the primary antibodies, the slides were washed three times (5 mins) with TBS and secondary antibodies DAR-555 (1:1000) and Goat Anti-Mouse 488 (GAM-488) (1:500) were simultaneously applied. Again three times TBS washes were done and then the nuclei were stained with DAPI. The Zeiss LSM 710 META confocal laser microscope was used for visualisation.

2.3.4.6 VEGF Receptor-1 (VEGF-R1) and VEGF Receptor-2 (VEGF-R2) Immunostaining
The slides were de-waxed and rehydrated. Heat-mediated antigen retrieval was done in 0.01 M Citrate buffer followed by quenching of endogenous tissue peroxidase activity with 10% methanol hydrogen peroxide for 30 minutes. NDS was employed to
block non-specific antibody binding. The slides were incubated simultaneously with primary mouse anti-VEGFR1 (ab9540) and rabbit anti-VEGFR2 antibodies (ab2349) from Abcam® overnight. In the morning the slides were washed thrice with TBS for 5 min and secondary GAM Alexa-488 and DAR-555 antibodies were applied for an hour. This was followed by TBS washes (3x 5min) and thereafter DAPI (1: 1000) was utilised for nuclei staining. The slides were visualised using the LSM 710 microscope.

2.3.4.7 EG-VEGF (Prokineticin-1) Immunostaining

The slides were de-waxed, rehydrated and antigen retrieved in citrate buffer. 10% hydrogen peroxide in methanol was used to quench endogenous peroxide activity for 30 min. After washing with distilled water and then TBS, the slides were blocked with NDS for 1 hour. Primary anti-PROK1 rabbit polyclonal antibody (ab42802, abcam®), diluted 1:50 in NDS was applied and incubated overnight at 4°C. After washing with TBS three times (5mins each wash), secondary DAR-555 (A31572, Lot 819572-Invitrogen) antibody at a dilution of 1:1000 was applied on a shaker for 30 minutes. The slides were subsequently washed in TBS (3x 5mins) and DAPI (1:1000) was used as nuclear stain. The slides were visualised using the LSM 710 confocal microscope.

2.4 Enzyme-linked Immunosorbent Assays (ELISAs)

2.4.1 Maternal and Cord Serum Kisspeptin-10 Quantification

The maternal and fetal circulatory kisspeptin-10 levels in the serum were measured using a human kisspeptin-10 sandwich enzyme immunoassay (EIA) kit from Phoenix Pharmaceuticals (EK-048-56). The assay detects human kisspeptin 10 (Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH₂) from a minimum concentration of 0.06ng/ml to 100ng/ml (Linear range 0.06-0.8ng/ml) with an inter- and intra-assay variability of <15% and <10% respectively.
Briefly, 50µl of kisspeptin standards and 50µl of patient samples were pipetted into a 96-well immunoplate. To this, 25µl of primary kisspeptin antibody and 25µl of biotinylated kisspeptin peptide per well were added and incubated at room temperature for 2 hours. The immunoplate was then washed 4 times with 350µl per well of 1X assay buffer. A 100µl of Streptavidine-Horse Radish Peroxidase (SA-HRP) solution was added per well and incubated at room temperature for 1 hour.

The plate was again washed four times with assay buffer (350µl/well) and 100µl of tetramethyl-benzidine (TMB) substrate solution was added to each well and the plate incubated at room temperature for 1 hour. The reaction was terminated with 100µl of 2N HCl per well and absorbance read on a microplate reader at 450nm. A standard curve of relative absorbance versus concentration of standard solutions was plotted and the kisspeptin concentrations of the unknown samples were interpolated from the standard curve.

### 2.4.2 Maternal and Cord Serum VEGF-A Quantification

The concentration of VEGF-A protein in the maternal and cord sera was analysed using a human VEGF-A Human ELISA kit from abcam® (ab 119566) with an intra-assay coefficient of variability (CV) of 6.2%. The microwells were washed with 400µl of wash buffer (PBS with 1% Tween 20) and thereafter 100 µl of VEGF-A protein standards and serum samples were added to a pre-coated 96-well plate. The plate was covered with an adhesive film and incubated at room temperature on a shaker for 2 hours to allow binding to pre-coated antibody. The plate was then washed six times with wash buffer followed by addition of a 100 µl of biotinylated anti-human VEGF-A antibody into each well and incubation of the plate at 37°C on a shaker for 1 hour.
The plate was again washed six times and 100µl of Streptavidin-HRP was added to each well followed by incubation of the plate on a shaker for 30 minutes at room temperature. The plate was then washed again six times with wash buffer and a 100µl of TMB colour developing agent was added to each well and incubated at room temperature in the dark for 10 minutes.

Colour development was monitored until the highest VEGF-A standard had developed a dark blue colour at which point a 100µl of Stop Solution (1M Phosphoric acid) was added into each well and the absorbance immediately read at 450nm on a microplate reader. A standard curve of relative absorbance versus concentration of standard solutions was plotted and the concentration of VEGF-A in the samples was interpolated from the standard curve.

### 2.4.3 Maternal and Cord Serum EG-VEGF Quantification

The concentration of EG-VEGF (Prokineticin-1) protein in the maternal and cord sera was analysed using a human EG-VEGF ELISA kit from abcam® (ab 119591). Briefly, 100µl of EG-VEGF protein standards and serum samples were added to a pre-coated 96-well plate. The plate was incubated at 37°C for 90 minutes to allow binding to pre-coated antibody. A 100 µl of biotinylated anti-human EG-VEGF antibody was added into each well and the plate was incubated at 37°C for 60 minutes. The plate was then washed three times with 0.01M TBS and a 100 µl of Avitin-Biotin-Peroxidase Complex (ABC) at a dilution of 1:100 was added into each well and the plate incubated at 37°C for 30 minutes.

The plate was then washed five times with TBS and 90 µl of 3,3’5,5’-tetramethylbenzidine (TMB) colour developing agent was added to each well and the plate was incubated in the dark for 30 minutes at 37°C. Finally, a 100 µl of TMB
stop solution was added into each well and the O.D. absorbance immediately read at 450nm on a microplate reader. A standard curve of relative absorbance versus concentration of standard solutions was plotted and the EG-VEGF concentrations of the samples were calculated from the standard curve.

2.5 Statistical Analysis

One Way Analysis Of Variance (One Way ANOVA) followed by Dunn’s Multiple Comparison’s Test was utilized to compare gene expression in different maternal-fetal tissues. The Mann-Whitney test was employed to compare tissue mRNA transcript expression as well as differences in circulating protein levels between healthy and preeclamptic pregnancies. The statistical package utilized was GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at P < 0.05 with (***) (***) and (*) signifying p<0.0001, p<0.001 and p<0.05 respectively.
3.4.4 VEGF-A gene and protein expression are highest in the placental bed .................................................................Page 55-58

3.4.5 VEGFR-1 and VEGFR-2 transcripts and protein are differentially expressed across the maternal-fetal interface .....Page 58

3.4.6 PROK-1 and PROK-1R transcript and protein expression are highest in the placenta ........................................Page 59-61

3.5 Discussion ........................................................................................................................................Page 62-69
Chapter 3  EXPRESSION OF KISS-1, MMP-9 AND ANGIOGENIC REGULATORS

3.1 Abstract

Genes involved in invasion of trophoblast cells and angiogenesis are crucial in determining pregnancy outcome. The expression profiles of candidate genes was examined in both fetal and maternal tissues to enhance our understanding of maternal-fetal dialogue. The expression of genes involved in trophoblast invasion, namely Kiss1, Kiss1 Receptor (GPR-54) and MMP9 as well as the expression of angiogenic ligands Vascular Endothelial Growth Factor-A (VEGF-A) and Prokineticin-1 (PROK1) was investigated and their respective receptors (VEGFR1, VEGFR2 and PROK1R) across the maternal-fetal interface of healthy human pregnancies. The placenta, placental bed and decidua parietalis were sampled at elective caesarean delivery. Real-time PCR (RT-PCR) was used to investigate transcription, while immunohistochemistry and Western blot analyses were utilized to study protein expression.

The expression of Kiss1 (p<0.001), GPR-54 (p<0.05) and MMP9 (p<0.01) were higher in the placenta compared to the placental bed and decidua parietalis. In contrast, the expression of VEGF-A was highest in the placental bed (p<0.001). VEGF receptor mRNA expression studies revealed differential expression of VEGFR1 and VEGFR2 with VEGFR1 expression highest in the placenta (p<0.01) and VEGFR2 was most expressed in the placental bed (p<0.001). Lastly, both PROK1 (p<0.001) and its receptor PROK1R (p<0.001) had highest expression in the placenta.

The findings that genes associated with trophoblast invasion were highly expressed in the placenta suggest that the influence on invasion capacity may largely be exercised at the fetal level. Whereas the findings on angiogenic gene expression profiles suggest that angiogenesis may be regulated by two distinct pathways with the PROK1/PROK1R system specifically mediating angiogenesis in the fetus and VEGFA/VEGFR2 ligand-receptor pair predominantly mediating maternal angiogenesis.
3.2 Introduction

Effective placentation is required for successful pregnancy outcome. During placentation, transformation of the spiral arteries from high resistance low capacity vessels to low resistance high capacity structures is crucial for successful support of the conceptus. The lack of transformation of these spiral arteries is associated with poor pregnancy outcomes such as preeclampsia and intrauterine growth restriction (IUGR) (Brosens et al., 1967, Robertson et al., 1967). The transformation of spiral arteries is determined by adequate endovascular invasion of extravillous trophoblast (EVT) cells. The ability of EVT cells to express gelatinases like MMP9 aids extracellular matrix degradation and enhances invasiveness. The expression of MMP9 in particular, has been associated with high invasiveness, while that of Tissue Inhibitors of Metalloproteases (TIMPs) markedly reduces this invasive potential (Xu et al., 2000, Luo et al., 2011). Kiss1 gene was initially discovered as a tumour metastasis suppressor gene of melanoma cells but this has subsequently been demonstrated in various cancer cell-lines, animal models and human cancer tissue (Lee and Welch, 1997, Li et al., 2009, Liang and Yang, 2007). The Kiss1 gene encodes peptides known appropriately as kisspeptins (kp) and posttranslational processing of the original Kiss1 transcript results in kisspeptins of various lengths (kp-54,-14,-13 and -10) of which the smallest cleavage product, kp-10, has particular relevance to the placenta (Bilban et al., 2004). Kisspeptins mediate their functions via G-Protein Coupled Receptor 54 (GPR54) also known as Kiss1 Receptor (Kiss-1R) (Ohtaki et al., 2001, Muir et al., 2001, Kotani et al., 2001).

The molecular machinery and pathways involved in tumour metastasis and invasion of extravillous trophoblast (EVT) cells into the maternal decidua are similar (Ferretti et al., 2007). However the invasiveness of trophoblast cells is highly regulated temporally and spatially. The highest invasion is observed in the first trimester of pregnancy.
and geographically only extends as deep as the inner third of the myometrium. The clinical repercussions of “overinvasion” and “underinvasion” are placenta accreta as well as preeclampsia and IUGR, respectively. The former leads to massive obstetric haemorrhage secondary to retained placenta at the time of delivery while the latter (a consequence of maternal vascular maladaptation), contributes significantly to maternal and perinatal mortality, particularly in the developing world (Moodley, 2011).

Kisspeptins limit the invasion of primary trophoblast cells and EVT cell-lines in vitro (Bilban et al., 2004). Furthermore up-regulation of Kiss1 expression and down-regulation of MMP9 gene expression have been associated with pregnancies complicated by preeclampsia and IUGR (Qiao et al., 2005b, Qiao et al., 2005a). Interplay between diverse factors affecting EVT invasion comprises a delicate equilibrium between pro-invasive and anti-invasive factors which is required for adequate invasion and a healthy gestation. Genes involved in angiogenic pathways appear to play a central role in achieving this equilibrium because ultimately the final common pathway to trophoblast invasion is spiral arteriolar transformation. In addition it has been demonstrated that the transformation of EVT cells from expressing an epithelial-type cell adhesion profile to an endothelial-type one enhances their invasiveness and subsequent transformation of spiral arteries (Zhou et al., 1997).

Angiogenesis, the development of new vessels from existing vessels plays an important role in placentation (Reynolds et al., 2001, Kaufmann et al., 2004). This process is driven by a host of angiogenic factors and their respective receptors and under physiological conditions only occurs during ovarian folliculogenesis, embryogenesis and placentation (Carmeliet, 2005). Among other angiogenic factors during embryogenesis and placentation, Vascular Endothelial Growth Factor A (VEGF-A) is key. The endometrial mRNA transcript of VEGF-A increases in the luteal
phase in preparation for implantation (Demir et al., 2007). Furthermore VEGF-A ligand and receptor knockouts result in embryonic demise, underpinning the importance of this angiogenic factor in the early stages of human placentation and development (Carmeliet et al., 1996). The expression of VEGF-A in the placenta has been extensively studied (Sharkey et al., 1993, Charnock-Jones et al., 1994, Clark et al., 1996, Clark et al., 1998b).

More recently endocrine gland specific VEGF (EG-VEGF), also known as Prokineticin-1 (*PROK1*) has been described (Le Couter et al., 2001). EG-VEGF, similar to VEGF, induces proliferation, migration and fenestration in capillary endothelial cells but is only expressed in steroidogenic organs such as the ovary and the placenta (Maldonado-Perez et al., 2007). The role of EG-VEGF is thought to be complementary to that of VEGF in these tissues.

The majority of factors involved in trophoblast invasion have mainly been studied in the fetal compartment of the maternal-fetal interface i.e. the placenta. Very few studies have examined both sides of this interface concomitantly. To understand the molecular and cellular interactions involved in maternal-fetal dialogue, both components of this interface should be studied in healthy pregnancies before exploring pathological pregnancies. Furthermore, the expression of invasion-limiting *Kiss1* and that of angiogenic factor *EG-VEGF* have never been investigated across the maternal-fetal interface of pregnancy. In addition the expression of key angiogenic factor *VEGF* and its complementary associate *EG-VEGF* have never been compared across this interface.

Hence the aim of this study was to investigate the expression of genes involved in trophoblast invasion, (*Kiss-1* and *MMP-9*) along with that of angiogenic ligands
Chapter 3 EXPRESSION OF KISS-1, MMP-9 AND ANGIOGENIC REGULATORS

(VEGF-A, PROK1) and their respective receptors (VEGFR1, VEGFR2 and PROK1R) across healthy maternal-fetal tissues, namely the placenta, placental bed and decidua parietalis.

3.3 Materials and Methods

3.3.1 Ethics Statement

The study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (REF: 080/2008). Written informed consent was obtained from all patients and the research was conducted according to the ethical principles of the Helsinki Declaration (2001). Patients were counselled in their language of choice prior to the day of their elective procedure. They were presented with Patient Information Leaflets which provided the details of the study. The capacity of the patients to comprehend this information was always assessed and where there was doubt about comprehension, consent for study participation was withheld. Patients decision to participate in the study did not compromise their management and care. The patients were at liberty to make this choice without coercion. The clinicians responsible for the patient’s care were not involved in the recruitment for these studies.

3.3.2 Study Participants

All recruited patients had elective caesarean delivery in the absence of labour. The inclusion criteria for the study were healthy patients with singleton pregnancies undergoing elective caesarean section for the indication of previous caesarean section or fetal malpresentation. Patients in labour, with a background of two previous caesarean sections, multiple pregnancies, with pregnancy-related complications such as gestational diabetes, gestational hypertention, placenta praevia, preeclampsia,
intrauterine growth restriction as well as patients with underlying medical disorders were excluded from the study. Patients were recruited from Groote Schuur and Mowbray Maternity Hospitals in Cape Town, South Africa.

3.3.3 Tissue Sampling and Specimen Collection

The placenta and placental bed were sampled at elective caesarean section using a previously described technique (Pijnenborg et al., 1991). Briefly, prior to delivery of the placenta, apposing sections of the placenta and placental bed were marked by placing a needle and suture from the placenta and traversing the uterine wall. A core of the placental tissue around the suture in apposition to the maternal surface (i.e. which contains the maternal end of the placenta) was then sampled and the rest of the placenta delivered. The section of the placental bed where the sampled placenta was resident could then be identified with the aid of the remaining suture and was subsequently biopsied. The adequacy of the sampled placental bed was confirmed by an independent histopathologist. In addition, the decidua parietalis which has no involvement in placentation was sampled. Collected tissue samples were divided into three portions. One portion was collected for RNA extraction, while the second and third portions were utilised for protein extraction and preparation of wax-embedded tissue blocks respectively.

3.3.4 RNA Extraction

1 ml of Trizol reagent (Invitrogen®) was used per 50 mg of tissue. Tissue was homogenised on ice with a Tissue Ruptor® (Qiagen). The homogenate was centrifuged using the Eppendorff 5804 R machine (15294 g at 4 degrees celcius for 15 minutes (mins)) and the supernatant was collected and added to 200 ml of ice-cold BCP (1-Bromo-3-Chloro-Propane (Sigma). The mixture was shaken for 15 seconds (secs) and kept on ice for 10 mins. The solution was centrifuged again and 500 ml of propanol
was added to precipitate the RNA. The RNA pellets were washed in 75% ethanol, air dried and re-suspended in DEPC-treated water.

### 3.3.5 cDNA Synthesis

RNA was reverse transcribed with Multiscribe Reverse Transcription reagents (Applied Biosystems). For a 20 ml reverse transcription reaction, the following were used; DEPC-treated H2O (3.7 ml), 10x RT Buffer (2 ml), MgCl2 (4.4 ml), dNTPs (4 ml), Random Hexamers (1 ml), RNase Inhibitor (0.4 ml), Reverse Transcriptase (0.5 ml) and (2 ml) of RNA. The ABI GeneAmp® 2700 Thermal Cycler was used and the cycling parameters were 25°C (10 mins), 4°C (10 mins), 48°C (45 mins) and 95°C (5 mins). Real-Time PCR Gene expression studies were conducted using the ABI 7900 RT-PCR instrument (Applied Biosystems). The standard thermal cycling protocol was conducted as follows: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of (95°C for 95 secs and 60°C for 1 min).

The Genebank accession numbers of the genes investigated are as follows: KiSS1 (NM_002256), GPR-54 (NM_032551), MMP9 (NM_004994), VEGFA (NM_001171623), VEGFR1 (FLT) (NM_002019), VEGFR2 (KDR) (NM_002253), PROK1 (NM_032414), PROKR1 (NM_138964). Primer/Probe pairs corresponding to these genes (Sigma Pharmaceuticals) were utilised (Table 1) in conjunction with the Taqman® Mastermix. All RT-PCR gene expression data is presented as Means ± SEM and gene expression was relative to 18s ribosomal RNA (internal control) and reference cDNA.

### 3.3.6 Immunohistochemistry

Tissue blocks were sectioned and fixed to slides (Histobond®) with the placenta, placental bed and decidua parietalis fixed across one slide and stained together.
3.3.6.1 Kisspeptin immunostaining.

Slides were de-waxed in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was done by pressure cooking the slides in 0.01 M Citrate Buffer. The slides were then blocked in 10% Methanol Hydrogen Peroxide for 30 mins, washed in water and then TBS (Tris-Buffered Saline). Normal Donkey Serum (NDS) was used for blocking followed by incubation with Sheep anti-kisspeptin-54 (GQ2) antibody (1:1500) (provided by Professor Bloom’s laboratory) overnight at 4°C. Thereafter slides were washed in TBS (3x5 mins) and incubated for 30 mins with secondary Donkey anti-Sheep Peroxidase antibody (Jackson Immunoresearch) (1:750) (1.8 mg/ml). The slides were washed again in TBS (365 mins) and incubated for 10 mins with Tyramide Cyanine-5 (Perkin Elmer) (1:50). Thereafter slides were washed in TBS (2x5 mins) and microwaved in citrate buffer for 2.5 mins and incubated in hot buffer for 30 mins. They were then washed in TBS and incubated with Mouse anti-α-Smooth Muscle Actin antibody (Sigma Aldrich) (1:5000) for 1 hour. After washing in TBS, secondary Donkey anti-Mouse-555 (DAM-555) (1:200) (2 mg/ml) (Invitrogen) antibody was applied for 30 mins and washed off with TBS. Nuclear stain DAPI 405, (1:1000 in PBS), was applied for 10 mins at room temperature and washed in TBS. Slides were then mounted with Permaflour® and viewed with Zeiss LSM 710 META confocal microscope.

3.3.6.2 GPR54 immunostaining.

The same protocol as above was followed except no antigen retrieval was required and Normal Goat Serum was used for blocking. Primary Rabbit anti-GPR54 (R2 1213) custom manufactured by EZ Biolabs (1:1600) was used. Pre-immune serum was utilised as negative control. Goat anti-Rabbit Peroxidase (1:200) (Dako) was used as secondary antibody followed by Tyramide-546 (Cyanine-3) (Perkin Elmer). Sytox Green (1:1000 in TBS) was used to stain the nuclei.
3.3.6.3 MMP9 immunostaining.
The slides were de-waxed and rehydrated. Heat-mediated antigen retrieval in 0.01 M citrate buffer was followed by quenching endogenous peroxide with 30% hydrogen peroxide in methanol. Blocking to prevent non-specific antibody binding was done with NDS and primary rabbit anti-MMP9 antibody (ab38898 (1 mg/ml)) (1:200) from Abcam® was applied overnight. Donkey Anti-Rabbit-555 (1:1000) (A31572 from Invitrogen) was used as secondary antibody followed by DAPI staining for nuclei.

3.3.6.4 PROK1 immunostaining.
The slides were prepared as above with antigen retrieval, blocked with NDS and incubated with primary Rabbit anti-PROK1 antibody (Abcam®) (1:50) in NDS. Donkey Anti-Rabbit-555 (DAR-555) (1:1000) (2 mg/ml) from Invitrogen was used as secondary antibody and the nuclei were stained with DAPI (1:1000 in PBS).

3.3.6.5 VEGF-A immunostaining.
Slides were de-waxed, rehydrated, antigen retrieved and bathed in hydrogen peroxide for 30 mins. Blocking was done with NDS. Rabbit Anti-VEGF A antibody (VEGF (sc-152) (1:50) (200 mg/ml) (Santa Cruz) and DAR-555 (1:1000) were used as primary and secondary antibody respectively. The nuclei were stained with DAPI.

3.3.6.6 VEGF-A and Pancytokeratin Co-Immunostaining.
Slides were de-waxed, rehydrated, antigen retrieved and blocked with hydrogen peroxide in methanol. NDS was used for blocking. Rabbit anti-VEGF-A (1:50) and Mouse anti-Pancytokeratin (1:15000) (Sigma) were applied to the slides and incubated overnight. DAR-555 (1:1000) and Goat Anti-Mouse Alexa-488 (1:500) in NDS were used as secondary antibodies. DAPI was used to stain the nuclei.
Chapter 3  EXPRESSION OF KISS-1, MMP-9 AND ANGIOGENIC REGULATORS

3.3.6.7 VEGFR1 and VEGFR2 Co-Immunostaining.

The slides were de-waxed and rehydrated. Heat-mediated antigen retrieval was done in 0.01 M Citrate buffer followed by quenching of endogenous tissue peroxidase activity with 30% methanol hydrogen peroxide for 30 minutes. NDS was employed to block non-specific antibody binding. The slides were incubated simultaneously with mouse anti-VEGFR1 (ab9540) and rabbit anti-VEGFR2 antibody (ab2349) from Abcam®. Goat Anti-Mouse Alexa-488 and Donkey Anti-Rabbit-555 were used concurrently as secondary antibodies. DAPI was utilised for nuclei staining.

3.3.7 Protein Extraction and Quantification

RIPA lysis buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% Triton X, 1% sodium deoxycholate and 0.1% SDS) was used for tissue lysis. 4M Urea and protease inhibitor tablets (Roche®) were added to the buffer. The tissue was incubated on ice for 30 mins, then poured into a mortar, snap-frozen with liquid nitrogen and crushed into fine powder using a pestle. The powder was collected in 2 ml epindorff tubes and centrifuged at 12000 rpm for 15 mins @ 4°C. The supernatant was collected and protein concentration quantified using the Pierce BCA Protein Assay Kit (Thermoscientific).

3.3.8 Western Blot Analysis

20 mg of protein/well was loaded after denaturing in loading buffer at 95°C for 5 mins. The proteins were separated on a 15% SDS-PAGE gel run at 110 V for 80 mins. The gel was then blotted onto a 0.45 mm PVDF membrane (Amersham Hybond-P) using Novex® Semi-dry Blotter (Invitrogen). The membranes were blocked at room temperature for 1 hour in 3% BSA and then incubated at 4°C overnight with Rabbit anti-VEGF-A antibody (sc-152) (1:50). For negative control the membrane was pre-incubated with a mixture of anti-VEGF-A and VEGF-A blocking peptide (Abcam®). After washing, Goat anti-Rabbit Peroxidase (1:5000) was applied and the blot was
visualised using a film (Amersham HyperfilmTM ECL- GE Healthcare Limited). Beta-
Actin (β-Actin) was used as loading control.

3.3.9 Statistical Analysis
Graphpad 5 Prism software (version 5.00 for Windows, GraphPad Software, San
Diego California USA, www.graphpad.com) was utilised for statistical analysis with
One-Way ANOVA followed by Dunn’s Multiple Comparison’s Test. P<0.05 conferred
statistical significance.

3.4 Results

3.4.1 Clinical Data
Twenty-eight patients with healthy pregnancies were included in the study. The
indications for caesarean section were a previous caesarean section in 23 patients
(82.14%) and malpresentations in five (17.86%) patients. The clinical parameters are
presented as means with standard error of the means (Mean ± SEM) (Table 3.1).
Table 3.1 Patient Data

<table>
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<th>Age (years)</th>
<th>Gravidity</th>
<th>Parity</th>
<th>Gestation (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>28.47 ± 0.87</td>
<td>2.433 ± 0.18</td>
<td>1.1 ± 0.16</td>
<td>38.03 ± 0.06</td>
</tr>
</tbody>
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Table depicting patients clinical data presented as means with standard error of the means (SEM) showing Age (in years), Gravidity (number of pregnancies irrespective of viability) Parity (number of pregnancies that have reached viability) and Gestation (duration of pregnancy in weeks)

3.4.2 Kiss1 and GPR54 Expression are Highest in the Placenta

The expression of Kiss1 and GPR54 genes in the placenta, placental bed and decidua parietalis was determined by RT-PCR (Fig. 3.1A). There was seven times more Kiss1 gene expressed in the placenta compared to the placental bed and eighteen times more Kiss1 gene expression in the placenta compared to the decidua parietalis (p<0.001). However there was no significant difference in Kiss1 gene expression between the placental bed and the decidua parietalis. The gene expression level of GPR-54 was only modestly higher (1.6 times) in the placenta compared the placental bed and decidua parietalis (Fig. 3.1B) and its expression in the placenta was much lower compared to that of Kiss1.

Immunohistochemical studies detected kisspeptin protein expression only in the syncytiotrophoblast layer of the placenta (Fig. 3.1C). GPR-54 protein was expressed in the placenta and specifically localized to the villous cytotrophoblast as well as extravillous trophoblast cell populations (Fig. 3.2A-C). No GPR54 immunostaining was observed in the decidua basalis and parietalis.
Figure 3.1 Kiss1 and GPR54 (Kiss1 R) expression in the Placenta, Placental bed and Decidua parietalis. Real-time PCR (Means ± SEM) showing Kiss1 (A) and GPR54 (B) expression in the three maternal-fetal tissues. (*** ) and (*) signify p<0.001 and P<0.05 respectively. (C) shows kisspeptin immunostaining in the Placenta, Placental Bed and Decidua Parietalis, with kisspeptin protein expression (green fluorescent staining) in the placental syncitiotrophoblast (sTB) layer. Smooth Muscle Actin (yellow) was used as a positive control for slide staining and can be seen staining vascular smooth muscle (vsm) and smooth muscle (sm). The decidua basalis (db) and decidua parietalis (dp) can be seen in the placental bed and decidual sections respectively. Kisspeptin staining is negative in the placental bed and decidua parietalis. The nuclei were stained with Dapi (blue)
Figure 3.2  GPR54 (Kiss1R) immunostaining in the Placenta.

Fig 3.2 demonstrates GPR54 immunohistochemistry with individual villous cytotrophoblast cell staining for GPR54 (red) in the placental villus (pv) (A). (B) shows GPR54 (red) immunostaining of villous cytotrophoblasts (vCT), cytotrophoblast columns (CC) and extravillous trophoblast cells (EVTs). The placenta is stained with GPR54 pre-immune serum (negative control) in (C). The nuclei are stained with Sytox Green (green) in all images.
Chapter 3  EXPRESSION OF KISS-1, MMP-9 AND ANGIOGENIC REGULATORS

3.4.3 MMP9 Transcript Expression is Highest in the Placenta

Next the expression of MMP9 mRNA in the three maternal-fetal tissues was determined. The expression of MMP9 mRNA was at least two-fold higher in the placenta compared to the placental bed and four-fold higher in the placenta compared to the decidua parietalis (Figure 3.3A). There was no difference in MMP-9 mRNA expression between the placental bed and decidua parietalis. Immunohistochemistry was used to localize MMP9 protein in the placenta, placental bed and decidua parietalis (Figure 3.3B). MMP9 protein was localized in the villous trophoblast cells and mesenchyme of the placenta while in the placental bed the epithelial border of the decidua basalis stained positive for MMP9. There was minimal MMP9 staining in the decidua parietalis.

3.4.4 VEGF-A Gene and Protein Expression are Highest in the Placental Bed

The expression of major angiogenic factor VEGF-A gene and protein were investigated in the placenta, placental bed and decidua parietalis. VEGF-A gene expression was 7-fold higher in the placental bed compared to the placenta (p<0.001) and 1.6-fold higher in the placental bed compared to the decidua parietalis (Figure 3.4A). After protein extraction and quantification from the three maternal-fetal tissues, Western Blot analysis confirmed RT-PCR findings showing more VEGF-A protein expressed in the placental bed and decidua parietalis compared to the placenta (Figure 3.4B). Immunostaining of placental sections showed mild VEGF-A staining of the syncytiotrophoblast, cytotrophoblast as well as staining of villous capillary endothelial cells (Figure 3.4C). On the other hand, strong VEGF-A immunostaining was observed in the placental bed sections relative to the placenta and decidua parietalis. Immunoco-localization of VEGF-A and Pancytokeratin (a marker for trophoblast cells) revealed that placental bed VEGF-A expression was predominantly from extravillous trophoblast cells and trophoblast giant cells (Figure 3.4D).
**Figure 3.3 MMP9 expression in the Placenta, Placental Bed and Decidua Parietalis.**

(A) shows relative MMP9 mRNA expression based on RT-PCR in the Placenta, Placental Bed and Decidua Parietalis. (*** and **) signify $p<0.001$ and $p<0.01$ respectively. (B) depicts localization of MMP-9 protein at the three maternal-fetal sites. In the placenta MMP9 immunostaining (red) was observed in the villous mesenchyme (vM) as well as the villous trophoblast cells (vT) of the placental villus (pv). In the placental bed MMP9 staining was mainly located on the decidual border of the decidua basalis (Db). In comparison, there was minimal MMP9 staining in the border of the decidua parietalis (Dp).
Figure 3.4 The expression of VEGF-A in the Placenta, Placental Bed and Decidua Parietalis. Real-time PCR (Means ± SEM) showing mRNA expression of VEGF-A, in the three maternal-fetal tissues (A). (*** and **) signify p<0.001 and p<0.01 respectively. (B) illustrates VEGF-A Western Blot Analysis of the Placenta, Placental Bed and Decidua Parietalis obtained from different patients. Bands are present in both the maternal Placental Bed (PB) and Decidua Parietalis (DP). Bands between 20 and 40 Kd represent the various VEGF-A isoforms. The molecular weight of VEGF is 20 Kd (Kilodaltons) while that of the homodimer is 40 Kd. (C) demonstrates VEGF-A immunostaining in the placenta with VEGF-A protein (red) expressed in both the syncytiotrophoblast (sTB) and cytotrophoblast (cTB) cells of the placental villus (pv). VEGF-A staining is also observed in the endothelial cells of villous capillary vessels.
(vCv). DAPI (blue) is used to stain the nuclei. (D) shows co-localisation of VEGF-A (red) and Pancytokeratin (PCK) (green) in a cluster of Extravillous Trophoblast (EVT) cells staining yellowish-orange. Multinucleated Trophoblast Giant Cells (TGCs) can be seen amongst the EVTs. The lumen of a spiral artery (SA) containing red blood cells can be noticed on the left. DAPI (blue) was used to stain nuclei. The nuclei of the TGCs are different in morphology and size in comparison to those of the surrounding cells.

3.4.5 VEGFR1 and VEGFR2 Transcripts and Protein are Differentially Expressed Across the Maternal-fetal Interface

VEGF-A mediates its effects via interaction with a number of its receptors. The mRNA and protein expression of angiogenic receptors, VEGF Receptor 1 (VEGFR1) and VEGF Receptor 2 (VEGFR2) was examined in the three maternal-fetal tissues. Using RT-PCR, VEGFR1 also known as FLT (fms-like tyrosine kinase) transcript expression was highest in the placenta (3-fold higher expression in the placenta compared to the placental bed and 7-fold higher expression in the placenta compared to the decidua parietalis) (Figure 3.5A). In contrast, the mRNA expression of VEGFR2 also known as Kinase Domain Receptor (KDR) was highest in the placental bed (almost 4-fold more compared to the placenta) (Figure 3.5B).

Using immunohistochemical co-localisation of VEGFR1 and VEGFR2, it was found that in the placental sections, VEGFR1 protein was expressed by the cytotrophoblast columns (CC) and extravillous cytotrophoblast (EVT) while in the placental bed it was expressed by interstitial trophoblast (Figure 3.5C). On the other hand, VEGFR2 protein was mostly immunolocalised to the placental bed with minimal expression in the placenta. VEGFR2 staining in the placental bed was neither from interstitial trophoblast nor decidual macrophages as verified with CD68 staining but was limited to the decidual stroma.
3.4.6 **PROK1 Transcript and Protein and PROK1R Transcript Expression are Highest in the Placenta**

The mRNA expression of both Prokineticin 1 (PROK1) and its receptor PROK1R were highest in the placenta compared to the placental bed and decidua parietalis (Figure 3.6A and 3.6B). Immunohistochemical staining of the placental sections localized PROK1 protein to the microvillous border of the placental syncytiotrophoblast layer (Fig 3.6C) with minimal PROK1 immunostaining of extravillous trophoblast cells (EVTs) in placental bed sections.
Figure 3.5  The Expression of VEGF Receptors in the three maternal-fetal compartments. Panel A demonstrates VEGFR1 and B illustrates VEGFR2 mRNA expression in the placenta, placental bed and decidua parietalis. Panel C demonstrates VEGFR1 and VEGFR2 co-immunostaining in the placenta and placental bed and decidua parietalis. In the placental section VEGFR1 (green) staining can be seen in the cytotrophoblast columns (CC) and extravillous trophoblast (EVT). No VEGFR2 staining (red) was observed in the placental sections. Red blood cells (RBCs) auto-fluoresce with both green and red dyes and can be seen (yellow) in the image. In the placental bed section both VEGFR1 (green) and VEGFR2 (red) staining were detected. VEGFR1 staining was observed in the interstitial extravillous trophoblast (EVT) of the decidua basalis while VEGFR2 staining was limited to the decidual stroma. In comparison to the placenta and placental bed sections, there was minimal VEGFR1 and VEGFR2 staining in the decidua parietalis (Dp).
Figure 3.6 PROK1 and PROK1R expression in the Placenta, Placental Bed and Decidua Parietalis. Real-time PCR (Means ± SEM) showing PROK1 (A) and PROK1R (B) mRNA expression in the three maternal-fetal tissues. (***), (**), and (*) signify p<0.001, p<0.01 and p<0.05 respectively. Panel (C) and (D) illustrate PROK1 immunostaining in placental tissue. PROK1 protein (red) can be seen most abundantly expressed in the microvillous brush border (BB) of the syncytiotrophoblast layer(C). Morphological detail of the villous cytotrophoblast (cTB) and syncytiotrophoblast (sTB) cells are indicated. The PROK1 primary antibody was omitted in (D) (negative control) and no PROK1 immunostaining is demonstrable. The nuclei were stained with DAPI (blue).
3.5 Discussion

Understanding normal maternal-fetal dialogue is the first step in deciphering pregnancy-related complications and can be valuable in optimizing assisted reproductive technology. In this chapter the expression profiles of genes involved in trophoblast invasion and angiogenesis which are both essential for placentation was investigated. The expression of Kiss1, VEGF-A, PROK1 and their respective receptors GPR54, VEGFR1, VEGFR2 and PROK1R as well as the expression of MMP9 in the placenta, placental bed and decidua parietalis was examined. This study was conducted in a population of young healthy patients mostly in their second pregnancies with no pregnancy-related complications nor chronic medical conditions.

Kisspeptin via its receptor, GPR54 has previously been shown to be an important role player in tumour metastasis suppression and trophoblast invasion. Kiss1, kisspeptin and GPR54 have never been investigated across maternal-fetal tissues, in particular the maternal placental bed and decidua parietalis. Strikingly high Kiss1 gene expression in the placenta and virtually no kisspeptin (protein) expression in the maternal tissues (placental bed and decidua parietalis) was observed. In contrast, GPR54 gene expression in the maternal tissues was only 50% less than that expressed in the placenta. Kisspeptin and GPR54 have previously been shown to inhibit trophoblast invasion in an autocrine/paracrine manner in primary human trophoblasts (Bilban et al., 2004). The maternal expression of GPR54 may therefore play a role in limiting excessive invasion of trophoblast cells in maternal tissues while high kisspeptin and GPR54 expression in the placenta may be involved in fetal autoregulation of invasion in maternal tissues (Fig. 3.7). This may suggest that the control of trophoblast invasion via the kisspeptin/GPR54 pathway is primarily exercised at the fetal level and is not only restricted to the first trimester but maintained to term. Kisspeptin could therefore have a role in the maintenance of placental invasive homeostasis until term. Another
Chapter 3  EXPRESSION OF KISS-1, MMP-9 AND ANGIogenic REGULATORS

possibility is that invasive capacity may also be controlled at the maternal level via GPR54 paracrine signalling. Recently Taylor et al., 2014 demonstrated that kisspeptin inhibits trophoblast invasion via a mechanism of increased EVT adhesiveness. High \textit{MMP9} expression in the placenta was demonstrated and this likely increases the fetal ability to degrade extracellular matrix, in particular type IV collagen and enhances maternal tissue invasion. Kisspeptin downregulates MMP9 expression in various cancers (Lee and Kim, 2009, Yan et al., 2001) and this may be the mechanism by which kisspeptin inhibits trophoblast invasion. Furthermore, Kiss1 has been shown to reduce \textit{MMP9} transcription by interfering with binding of NFkb to the \textit{MMP9} promoter in a cell line model (Yan et al., 2001). Downregulation of MMP9 expression is associated with poor fetal growth and maternal preeclampsia (Zhang et al., 2006, Qiao et al., 2005a, Zhang et al., 2011) most likely by a mechanism involving limited trophoblast invasion and poor transformation of maternal spiral arteries. These data demonstrate the importance of the expression of gelatinases (among other proteases) by the fetal compartment in sustaining healthy pregnancies.

\textit{VEGF-A} mRNA expression was highest in the maternal compartment (placental bed), yet numerous studies have focused on \textit{VEGF} expression in the placenta and more so in pathological pregnancies (Sgambati et al., 2004, Padavala et al., 2006, Chung, 2004, Akercan et al., 2008, Shiraishi et al., 1996, Gurel et al., 2003). Consistent with the RT-PCR findings, the VEGF-A protein expression was highest in the placental bed and decidua parietalis. Furthermore this study demonstrated that extravillous trophoblast (EVT) and Trophoblast Giant Cells (TGCs) express VEGF-A protein in the placental bed. A study using in-situ hybridization showed that macrophages in the maternal decidua (Sharkey et al., 1993) expressed the highest VEGF-A mRNA however our studies detecting VEGF-A protein clearly showed localization to EVTs and minimal VEGF-A localization to macrophages.
Future studies investigating pregnancy pathologies should therefore focus on changes in VEGF-A expression the placental bed rather than in the placenta. When examining VEGF receptor mRNA expression, the expression of *VEGFR1* mRNA was found to be highest in the placenta while that of *VEGFR2* was highest in the maternal placental bed and decidua. VEGFR1 also known as the "decoy" receptor, is thought to bind to VEGF-A and thus scavenge and reduce the amount of bioavailable VEGF-A to bind to VEGFR-2 (which mediates its angiogenic and mitogenic effects) (Kendall and Thomas., 1993, Park et al., 1994). Low VEGF-A mRNA and protein and high *VEGFR1* mRNA levels were observed in the placenta. VEGF-A may (via interaction with VEGFR1) be suppressed in the placenta while VEGFR2 mediates the angiogenic and mitogenic effects of VEGF-A in the maternal placental bed. Interestingly immunohistochemical staining for VEGFR1 protein was strong in extravillous trophoblast (cytotrophoblast columns in the placenta and interstitial trophoblast in the placental bed).

Could this possibly be a mechanism by which these invading "fetal" cells suppress maternal angiogenesis in favour of their own angiogenic enhancement? Previously high expression of VEGFR1 both in the placenta and circulation (sFLT1) of pregnant women has been reported and was thought to regulate the action of VEGF-A in successful pregnancies (Clark et al., 1998). On the other hand, both VEGF-A and VEGFR2 protein expression are reduced in the placentae of first trimester miscarriages compared to healthy controls (Vuorela et al., 2000). Furthermore both VEGF as well as VEGF receptor knockout mice result in embryonic demises (Carmeliet et al., 1996, Fong et al., 1995, Shalaby et al., 1995). This indicates the importance of VEGF-mediated vasculogenesis and angiogenesis in early pregnancy. The findings from this study suggest that VEGF-A may be more important in the placental bed than the placenta in healthy human pregnancies at term. This would imply the potential role of a different angiogenic factor other than VEGF in maintaining placental angiogenesis.
after de novo vasculogenesis is mediated by VEGF in early pregnancy.

Employing RT-PCR, this study has demonstrated that both the mRNA expression of PROK1 and PROK1R were highest in the placenta while that of its corresponding angiogenic factor, VEGF-A was highest in the placental bed. PROK1 and its cognate receptor PROK1R have recently been shown to be important tissue-specific role players in the angiogenesis of steroidogenic organs like the ovary, testis, adrenal and placenta (LeCouter et al., 2001). The action of PROK1 is thought to be complementary to that of VEGF in these tissues. PROK1 has also been shown to be important in enhancing maternal-fetal dialogue in receptive endometrium (Evans et al., 2009) and thus likely to be important in early pregnancy health.

By employing immunohistochemical analysis on first trimester placental tissue another study demonstrated the differential expression of VEGF and PROK1 in the placenta, with the former being expressed in the cytotrophoblast and EVT cells whilst the latter was distinctly expressed in the syncytiotrophoblast (Hoffmann et al., 2006). Of importance, this study found no PROK1 immunostaining in extravillous trophoblast. The same group also demonstrated differential placental expression of these angiogenic factors depending on gestation, with PROK1 and PROK1R increasing between 8 and 10 weeks of gestation while VEGF mRNA remained unaltered throughout the first trimester. They concluded that this suggested complementary roles of PROK1 and VEGF in early pregnancy.

Taken together with the findings of this study, these data are suggestive of distinct but complementary roles of these angiogenic factors where VEGF-A and its mitogenic receptor VEGFR2 may be mainly, but not exclusively, responsible for mediating maternal angiogenesis and spiral arteriolar transformation in the placental bed while PROK1
and PROK1R exclusively mediate placental villous angiogenesis in the fetus. VEGF-A likely plays a critical role in the initial stages of villous capillary vasculogenesis and early angiogenesis (as evidenced by lethality in VEGF receptor and ligand knockout embryos) and may facilitate integration of invasive EVT cells into the maternal vascular system. PROK1 on the other hand is potentially involved in maintaining angiogenesis after VEGF-A has initiated vascular events in early pregnancy.

Kisspeptin could in addition to inhibition of metalloprotease activity, accomplish its anti-metastatic effects through the suppression of angiogenesis via factors such as VEGF and PROK1. Kisspeptin has been shown to reduce Human Umbilical Vein Endothelial Cells (HUVEC) migration, invasion and tube formation via Specificity Protein-1 (SP1) mediated suppression of VEGF expression (Cho et al., 2009). In an in vitro study, kisspeptin was shown to inhibit both new vessel sprouting and tube structure formation in HUVECs in a dose dependant manner (Ramaesh et al., 2010). Kisspeptin could therefore limit fetal invasion both by suppressing placental MMP-9 expression as well as inhibiting placental bed angiogenesis via the suppression of VEGF-A expression.

It should however be borne in mind that all the above findings are a description of the maternal-fetal interface at term. This is an acknowledged limitation of the study as a description of the molecular details of this interface in early pregnancy would provide more information on maternal-fetal dialogue. Nonetheless attempting such a study in early pregnancy could prove challenging firstly because true placental bed biopsies comprising decidua, extravillous trophoblast and myometrium might well prove difficult to attain in early pregnancy and secondly studies of such a nature (ie involving placental bed sampling) might be limited by ethical considerations.
In summary this study has examined differential gene expression in both the fetal and maternal tissues of the maternal-fetal interface. Figure 3.7 is a depiction of the schematic representation of the anatomy of the maternal-fetal interface (Fig 3.7A) as well as the putative interactions of factors examined in this study at this interface (Fig 3.7B). The high fetal expression of Kiss1, GPR54 as well as MMP-9 which are all genes involved in invasion suggest that the control of invasive capacity (at least involving these genes) may predominantly be exercised at the fetal level.

Examination of genes involved in angiogenesis revealed high expression of PROK1 and PROK1R ligand-receptor pair in the fetus and high expression of VEGF-A and VEGFR2 ligand-receptor pair on the maternal side. VEGFR1, which is anti-VEGF, was highly expressed in the fetus. These findings may be suggestive of angiogenesis being prokineticin-maintained in the placenta while maternal angiogenesis in the placental bed is mainly mediated by VEGF-A. Further studies will be required to explore the potential of this dual angiogenesis hypothesis. The findings could unveil the possible prophylactic use of VEGFR2 agonists in women with a predisposition for preeclampsia and the potential use of PROK1 agonists in the treatment of fetuses with growth restriction. Having defined the differential gene expression profiles across the maternal-fetal interface of healthy pregnancies, the next chapter compares these profiles with those from pathological pregnancies.
Figure 3.7A The anatomy of the maternal-fetal interface (sagittal section)

Fig 3.7A represents a sagittal anatomy of the maternal-fetal interphase illustrating the amniotic cavity with amniotic fluid (light grey), umbilical cord and placenta (white) and underlying muscular layers consisting of inner 1/3 of the myometrium (darkest grey) and outer 2/3 of the myometrium (white).
Figure 3.7B The schematic of putative interaction at the maternal-fetal interphase

Fig 3.8 Figure 3.7B represents potential putative interactions between kisspeptin (kp), GPR54, angiogenic ligands (VEGF-A, PROK1) and their receptors (VEGFR1, VEGFR2, PROK1R) as well as MMP9 at the maternal-fetal interphase. (-) signifies inhibit, hence in addition, kisspeptin inhibits MMP9 and VEGF-A expression.
CHAPTER FOUR

Reciprocal Expression of Angiogenic Regulators and Kiss1 across the Maternal-Fetal Interface of Preeclamptic Pregnancies.

4.1 Abstract..................................................................................................................Page 71-72
4.2 Introduction..............................................................................................................Page 72-74
4.3 Methods ..................................................................................................................Page 74
   4.3.1 Study Participants ..................................................................................Page 75-76
   4.3.2 Tissue Sampling......................................................................................Page 75
   4.3.3 RNA Extraction, cDNA Synthesis and RT-PCR ......................................Page 76
   4.3.4 Protein Expression Studies.................................................................Page 77
   4.3.4.1 Semi-quantitative analysis of kisspeptin
           immunostaining..................................................................................Page 77-78
   4.3.5 Statistical Analysis...............................................................................Page 78
4.4 Results......................................................................................................................Page 78
   4.4.1 Clinical Parameters ...............................................................................Page 78
   4.4.2 KISS1 gene and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies...........................................................................Page 79-80
   4.4.3 GPR-54 mRNA transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies...........................................................Page 80-86
   4.4.4 VEGF-A gene and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies...........................................................Page 87-91
   4.4.5 EG-VEGF (PROK1) transcript and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies...............................Page 91-94
4.5 Discussion.................................................................................................................Page 94-103
4.1 ABSTRACT

Preeclampsia is a common pregnancy-associated disorder and is one of the leading causes of maternal and perinatal morbidity and mortality worldwide. The precise molecular mechanisms underlying the development of preeclampsia still remain unknown. Decreased trophoblast invasion and compromised maternal spiral arterial transformation have been implicated in its pathophysiology. In this chapter the expression of genes involved in trophoblast invasion ($KISS1$ and $GPR-54$ as well as angiogenic ligands ($VEGF-A$, $PROK1$) were investigated in the placenta, placental bed and decidua parietalis of normal and preeclamptic pregnancies. RT-PCR was utilized to examine gene expression while immunohistochemistry and Western Blot were employed to investigate protein expression. Circulatory protein concentrations of kisspeptin, VEGF-A and PROK-1 were determined using ELISA. High kisspeptin protein expression was found in the placentae of pregnancies complicated by preeclampsia in comparison to healthy pregnancies. There was minimal $Kiss-1$ mRNA and kisspeptin expression in the placental bed and decidua parietalis of both healthy and preeclamptic pregnancies. No difference was found in $GPR-54$ mRNA expression across the maternal-fetal tissues of healthy and preeclamptic pregnancies. $Prokineticin-1$ mRNA expression was suppressed in the placentae and $VEGF-A$ mRNA in the placental beds of preeclamptic pregnancies respectively. In addition, there was less VEGF-A protein expressed in the maternal tissues (placental bed and decidua parietalis) and cord sera of preeclamptic pregnancies in comparison to healthy controls. No significant difference was found in mean maternal or cord serum EG-VEGF levels between healthy and preeclamptic pregnancies. Increased kisspeptin expression in the placenta along with suppressed pro-angiogenic gene ($VEGF-A$ and $Prokineticin-1$) expression in maternal (placental bed) and placental components of the maternal-fetal interface respectively, is consistent with reduced trophoblast invasiveness as well as compromised angiogenesis and may represent a
molecular mechanism that explains the development of preeclampsia.

4.2 INTRODUCTION

Preeclampsia is a pregnancy-specific condition that is typified by the development of new onset hypertension and proteinuria after 20 weeks of pregnancy. The International Society for the Study of Hypertension in Pregnancy (ISSHP) defines hypertension as a systolic blood pressure ≥ 140mmHG and a diastolic blood pressure of ≥ 90mmHG. Significant proteinuria is defined as the presence of ≥ 300mg of urinary protein over a 24-hour period. More recently the ISSHP has revised the diagnostic criteria for preeclampsia. (Tranquilli., 2013) Preeclampsia is one of the more severe hypertensive disorders to complicate pregnancy (Davey and Macgillivray, 1986).

Preeclampsia is commonly the leading cause of maternal and perinatal morbidity and mortality worldwide (Duley, 2009). The maternal and fetal complications associated with this disorder are a reflection of a generalised endothelial cell dysfunction with manifestations in various organs. The maternal complications related to preeclampsia are eclampsia (seizure disorder), pulmonary oedema, renal impairment, liver and coagulation defects not uncommonly presenting as the syndrome of Haemolysis Elevated Liver enzymes and Low Platelets - HELLP syndrome (Weinstein, 2005). The fetal complications which are an indication of placental dysfunction include IUGR, placental abruption and intrauterine death (Nisell et al., 2000).

The precise molecular pathophysiological mechanisms underlying this disorder still remain unclear. It is however largely acknowledged that there is inadequate spiral arterial transformation in the maternal placental beds of pregnancies complicated by preeclampsia (Brosens, 1977, Robertson et al., 1967b, Kim et al., 2003, Brosens et al., 2002, Moodley and Ramsaroop, 1989). It is also recognised that invasion of
Chapter 4 Reciprocal Expression of Angiogenic Regulators

the maternal decidua by extravillous trophoblast (EVT) cells as well as angiogenesis play crucial roles in the transformation of maternal spiral arteries from vessels of high resistance low capacity, to low resistance high capacity ones capable of supporting a healthy pregnancy (Kam et al., 1999).

Hence understanding the expression of genes involved in trophoblast invasion and angiogenesis across the maternal-fetal interface is critical in providing further insights about pregnancy-related pathologies such as preeclampsia.

Kisspeptin inhibits trophoblast invasion in vitro (Bilban et al., 2004, Roseweir et al., 2012). Kisspeptin serum concentrations significantly increase as pregnancy progresses and considerably fall post-delivery (Horikoshi, 2003) whereas the placental expression of both Kiss-1 and GPR-54 are highest in the first trimester and lowest at term (Janneau et al., 2002, Bilban et al., 2004). Several studies have reported elevated Kiss-1 mRNA and protein expression in trophoblasts (Qiao et al., 2005b) and placentae of pregnancies complicated by preeclampsia (Cartwright and Williams, 2012, Qiao et al., 2012, Zhang et al., 2006, Zhang et al., 2011). While one study reported increased GPR-54 expression in preeclampsia (Cartwright and Williams, 2012), the other found no difference in placental GPR-54 mRNA and protein expression between healthy and preeclamptic pregnancies (Qiao et al., 2012). Also, decreased maternal serum kisspeptin levels in early pregnancy have been associated with development of preeclampsia (Armstrong et al., 2009, Cetkovic et al., 2012). Nonetheless no study has thus far examined the expression of kisspeptin and GPR-54 across maternal-fetal tissues and circulation more so in the context of preeclampsia.

Recent data suggests that kisspeptin may also inhibit metastasis via restriction of angiogenesis (Ramaesh et al., 2010, Cho et al., 2009). The simultaneous maternal-
fetal expression of angiogenic ligands \((\text{VEGF-A} \text{ and } \text{PROK-1})\) and kisspeptin in both tissue and circulation have never been investigated in pregnancies complicated by preeclampsia. The study hypothesis was that the expression of these factors would be different across the maternal-fetal interphase as well as maternal-fetal circulations of healthy and preeclamptic pregnancies.

In this chapter the gene and protein expression of \(\text{KISS1, GPR-54}\) and angiogenic ligands, \(\text{VEGF-A} \text{ and } \text{PROK1}\) were examined across the maternal-fetal tissues of both healthy and preeclamptic pregnancies. In addition, both maternal and cord circulatory levels of these proteins were concurrently examined in healthy as well as preeclamptic pregnancies.

4.3 METHODS

4.3.1 Study Participants

All patients recruited had elective caesarean section deliveries in the absence of labour. The inclusion criteria for the control arm of the study were healthy patients with singleton pregnancies undergoing elective caesarean section. Patients were excluded from the control arm of the study if they were in labour, had multiple pregnancies, had more than one previous caesarean section, had pregnancy-specific complications such as gestational diabetes, gestational hypertension, placenta praevia, preeclampsia, and intrauterine growth restriction. In addition, underlying chronic medical disorders such as preexisting diabetes or chronic hypertension were also exclusion criteria for the study. Patients in the control arm of the study were the same cohort as those described in Chapter 3.
The inclusion criteria for the experimental arm were patients with singleton pregnancies and a diagnosis of preeclampsia who were having elective caesarean delivery. Preeclampsia was defined as new-onset hypertension (diastolic blood pressures of \( \geq 90\text{mmHg} \) on two separate occasions, 6 hours apart or \( \geq 100\text{mmHg} \) on one occasion) and significant proteinuria (24 hour protein collection \( \geq 300\text{mg} \)). Patients who were in labour, had multiple pregnancies, had more than one previous caesarean section, gestational diabetes, gestational hypertension, placenta praevia, as well as patients with pre-existing medical disorders were excluded from the experimental arm of the study. In addition, patients who had chronic hypertension with superimposed preeclampsia were excluded from the study.

### 4.3.2 Tissue Sampling

The placenta and placental bed were sampled at elective caesarean section using a technique previously described (Pijnenborg et al., 1991). Briefly, prior to delivery of the placenta, juxtaposed sections of the placenta and placental bed were marked by placing a needle and suture from the placenta and traversing the uterine wall. The central zones of the placenta (rather than peripheral) were marked. A core of the placental tissue around the suture (which contains the fetal surface) was then sampled and the rest of the placenta delivered. The section of the placental bed where the sampled placenta was resident was identified with the aid of the remaining suture and biopsied. The adequacy of the sampled placental bed was confirmed for presence of trophoblast, myometrium and spiral arteries by an independent histopathologist. In addition, the decidua parietalis which has no involvement in placentation (negative control) was sampled. (See Fig 1.3)

Collected tissue samples were first homogenized using a fine scalpel and equitably divided into three portions. The first portion was collected for RNA extraction while
the second and third portions were utilised for protein extraction and preparation of wax-embedded tissue blocks respectively. In addition maternal and cord blood were simultaneously taken at the time of delivery. The blood samples were centrifuged at 10 000 rpm for 10 minutes to obtain serum which was subsequently frozen.

### 4.3.3 RNA Extraction, cDNA Synthesis and RT-PCR

For gene expression studies, RNA extraction, cDNA synthesis and RT-PCR were conducted as previously described in Chapter Two. The Genebank accession numbers of the genes investigated are as follows: *KISS1* (NM_002256), *GPR-54* (NM_032551), *VEGF-A* (NM_001171623) and *PROK1* (NM_032414). Primer/Probe pairs corresponding to these genes (Sigma® Pharmaceuticals) were utilised (Table 4.1) in the presence of the Taqman® Mastermix. 18S ribosomal RNA was utilized as internal control and gene expression was quantified relative to the same reference cDNA used for all RT-PCR studies.
Table 4.1 Primers and Probe Sequences used for Real-Time (RT) PCR Reactions II

<table>
<thead>
<tr>
<th>Primer or Probe</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>10GPR54 Forward Primer</td>
<td>5’-GGTGCTGGGGCGACTTCAT-3’</td>
</tr>
<tr>
<td>GPR54 Reverse Primer</td>
<td>5’-CACACTCATGGCCGGTCAGAGT-3’</td>
</tr>
<tr>
<td>GPR54 Probe</td>
<td>5’-[FAM]-TGCAAGTTCGTAACCTACACACGAGG-[TAM-RA]-3’</td>
</tr>
<tr>
<td>11Kiss1 Forward Primer</td>
<td>5’-GGCAAGCCTCAAAGGCACCTT-3’</td>
</tr>
<tr>
<td>Kiss1 Reverse Primer</td>
<td>5’-GGAAAGGACTGCTGCCAAGA-3’</td>
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<tr>
<td>Kiss1 Probe</td>
<td>5’-[FAM]-TGCCCTTTCACCAAGATGAACTCAGGG-[TAM-RA]-3’</td>
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<tr>
<td>12PROK1 Forward Primer</td>
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<td>PROK1 Probe</td>
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<tr>
<td>13VEGF Forward Primer</td>
<td>5’-TAC CTC CAC CAT GCC AAG TG-3’</td>
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<td>VEGF Reverse Primer</td>
<td>5’-TAG CTG CGC TGA TAG ACA TCC A-3’</td>
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<tr>
<td>VEGF Probe</td>
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<td>18S Reverse Primer</td>
<td>5’-GCT GGA ATT ACC GCG GCT -3’</td>
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<tr>
<td>18S Probe</td>
<td>5’-[VIC]-TGC TGG CAC CAG ACT TGC CCTC -[TAMRA]-3’</td>
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4.3.4 Protein Expression Studies

Western blot analysis was performed as previously detailed for VEGF-A (Chapter 2). Immunohistochemical studies were conducted for kisspeptin protein content and ELISA was utilized to determine circulatory protein levels for kisspeptin-10, VEGF-A and PROK-1 as previously described.

4.3.4.1 Semi-quantitative analysis of kisspeptin immunostaining

The slides were evaluated for areas of maximum kisspeptin staining and tile scans of these areas were captured. The extent of slide staining was semi-quantitatively assessed by employing the H-score (Akercan et al., 2008). A modified version of the H-score was utilised according to the equation, Mod H-score = Σ (I x PPV),
where I represents (Intensity of staining), ∑ (the sum of) and PPV the (Percentage of Placental Villi that stain that intensity). The intensity of staining (I) was quantified as 0 (no staining), +1 (weak staining), +2 (moderate staining), +3 (strong but patchy or incomplete villous staining) and 4+ (very strong and complete villous staining). The modified H-score therefore represents the sum of (∑) the percentage of placental villi staining a particular intensity.

4.3.5 Statistical Analysis

The Mann-Whitney test was employed to compare tissue mRNA transcript expression as well as differences in circulating protein levels between healthy and preeclamptic pregnancies. The statistical package utilized was GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at p < 0.05.

4.4 RESULTS

4.4.1 Clinical Parameters

Thirty healthy control patients and nineteen patients with preeclampsia were recruited. Patients with preeclampsia were of a younger age, had lower gravidity and had lower gestational ages in comparison to healthy controls. The gravidity was higher in control patients in comparison to preeclamptic pregnancies. The parity was comparable between the two groups. All the demographic data are summarized in Table 4.2
Table 4.2 Patient Data

<table>
<thead>
<tr>
<th></th>
<th>Healthy (N=30)</th>
<th>Preeclampsia (N=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>28.47 ± 0.87</td>
<td>25.00 ± 1.31</td>
<td>0.0378*</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.43 ± 0.18</td>
<td>1.90 ± 0.20</td>
<td>0.0464*</td>
</tr>
<tr>
<td>Parity</td>
<td>1.10 ± 0.16</td>
<td>0.68 ± 0.15</td>
<td>0.0934</td>
</tr>
<tr>
<td>Gestation (wks)</td>
<td>38.03 ± 0.06</td>
<td>32.95 ± 0.53</td>
<td>&lt; 0.0001***</td>
</tr>
</tbody>
</table>

(*** and (*) signify p<0.001 and P<0.05 respectively)

4.4.2 KISS1 gene and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies.

The expression of KISS1 mRNA transcript in the placenta, placental bed and decidua parietalis obtained from both healthy pregnancies and pregnancies complicated by preeclampsia was determined. There was higher KISS1 mRNA expression in the placentae of both healthy and preeclamptic pregnancies in comparison to the placental bed and decidua parietalis (Figure 4.1). There was no difference in KISS1 mRNA expression in the placenta, placental bed and decidua parietalis of preeclamptic in comparison to healthy pregnancies.

Immunohistochemical methods were employed to examine kisspeptin expression in the placentae, placental beds and decidua parietalis of healthy and preeclamptic pregnancies (Figure 4.2). There was no kisspeptin immunoreactivity in the placental bed and decidua parietalis of both healthy and preeclamptic pregnancies. In the placenta, kisspeptin immunostaining was localized to the villous syncitiotrophoblast and cytotrophoblast cell layers. There was individual variability in percentage and
intensity of villous staining in both healthy (Figure 4.2A) and preeclamptic (Figure 4.2B) pregnancies. A more intense and complete kisspeptin immunostaining was observed in the syncitio- and cytotrophoblast layers of preeclamptic in comparison to healthy pregnancies (Figure 4.2B). The semi-quantitative immunohistochemical scores (modified H-scores) were significantly higher in preeclamptic when compared to healthy placentae (Figure 4.2C), indicating higher placental kisspeptin content in preeclampsia.

4.4.3 GPR-54 mRNA transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies.

Next the level of GPR-54 mRNA across maternal-fetal tissues was determined using RT-PCR. There was no difference in GPR-54 mRNA expression between the placenta, placental bed and decidua parietalis of healthy and preeclamptic pregnancies (Figure 4.4).
Figure 4.1  Relative Kiss1 transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies. Relative Kiss1 mRNA expression in the Placenta, Placental Bed and Decidua Parietalis of Healthy (blue bars) and Preeclamptic (red bars) Pregnancies. mRNA expression was quantified relative to 18S RNA by RT-PCR.
A. The immunohistochemical expression of kisspeptin protein in healthy placentae.
B. The immunohistochemical expression of kisspeptin protein in preeclamptic placentae.
Immunohistochemical Analysis of Kisspeptin Expression in the Placentae of Healthy and Preeclamptic Pregnancies

**Figure 4.2 (A-C)** represents immunohistochemical expression of kisspeptin protein in the placentae of both Healthy (A) and Preeclamptic Pregnancies (PET) (B). Each image in the figures depicts a tile scan of the placenta obtained from a single patient. Patients were denoted numbers preceded by letters “C” signifying (Control) and “P” (Preeclampsia). Each tile scan is representative of the strongest immunohistochemical staining on the slide. The tile scans show kisspeptin staining (green fluorescence) of villous syncytiotrophoblasts (vST). Alpha smooth muscle actin (α-SMA) was used as positive control for slide staining and can be seen staining vascular smooth muscle within the placental villi (yellow). The nuclei were stained with DAPI (blue). **Panel C** is a graphical representation of H-scores between healthy (blue) and preeclamptic (red) pregnancies.
Chapter 4  Reciprocal Expression of Angiogenic Regulators

An ELISA was used to quantify circulatory kisspeptin-10 levels in the maternal and cord sera of healthy and preeclamptic pregnancies (Figure 4.3). The mean circulatory kisspeptin-10 levels in the maternal and cord sera of healthy pregnancies were 1.66 ± 0.59 ng/ml and 2.03 ± 0.44 ng/ml respectively (mean ± SEM). In preeclamptic pregnancies, the corresponding levels were 0.58 ± 0.39 ng/ml in the maternal serum and 1.78 ± 0.38 ng/ml in the cord serum. There were no differences between the mean maternal and cord serum kisspeptin-10 concentrations in healthy pregnancies however in preeclamptic pregnancies there were significantly lower circulating maternal kisspeptin-10 levels when compared to cord levels. In addition, the mean maternal circulatory kisspeptin-10 level in preeclamptic pregnancies was much lower in comparison to the maternal circulatory levels in healthy pregnancies.

![Figure 4.3 Circulatory kisspeptin-10 levels in the Maternal and Cord Sera of Healthy and Preeclamptic Pregnancies. Kisspeptin-10 levels (ng/ml) in the maternal (plain bars) and cord sera (pixelated bars) of healthy (blue) and preeclamptic (red) pregnancies as determined by ELISA. The data are presented as means ± SEM.](image_url)

* p = 0.0394
Figure 4.4 Relative GPR-54 transcript expression across the maternal-fetal interface of healthy and preeclamptic pregnancies. Relative GPR-54 mRNA expression in the Placenta, Placental Bed and Decidua Parietalis of Healthy (blue bars) and Preeclamptic (red bars) Pregnancies. mRNA expression was quantified relative to 18S RNA by RT-PCR.

Western blot analysis was utilised to examine VEGF-A protein expression in the placenta, placental bed and decidua parietalis of healthy and preeclamptic pregnancies. There was less VEGF-A protein expressed in the maternal tissues (placental bed and decidua parietalis) of preeclamptic pregnancies when compared to healthy controls (Figure 4.5B), while expression levels in the placenta were similar.
4.4.4 VEGF-A gene and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies.

RT-PCR was utilized to study VEGF-A mRNA transcript expression in the placenta, placental bed and decidua parietalis of healthy and preeclamptic pregnancies. The mean relative VEGF-A mRNA expression in the placenta, placental bed and decidua parietalis of healthy pregnancies were 0.15 ± 0.06, 0.71 ± 0.22 and 0.47 ± 0.13, respectively (Figure 4.5A). However in pregnancies complicated by preeclampsia, the mean relative VEGF-A mRNA expression in the same tissues were significantly lower. The VEGF-A mRNA transcript levels in preeclamptic pregnancies were 7-fold less in the placenta 0.02 ± 0.01, 11-fold less in the placental bed 0.06 ± 0.01 and 5-fold less in the decidua parietalis 0.09 ± 0.04 when compared to healthy pregnancies (Figure 4.5A).
Figure 4.5. The Expression of VEGF-A transcript and protein in the Placenta, Placental bed and Decidua parietalis in Healthy and Preeclamptic Pregnancies. (A) depicts the expression of VEGF-A mRNA transcript in the Placenta, Placental bed and Decidua Parietalis of Healthy and Preeclamptic Pregnancies. mRNA expression was determined using RT-PCR relative to 18S ribosomal RNA. Data are presented as
means ± SEM. (B) depicts a representative western blot analysis of VEGF-A protein content in the Placenta (P), Placental Bed (B) and Decidua Parietalis (D) of healthy (C013) and preeclamptic (P018) pregnancies. VEGF-A bands are visible at 20kD and 40kD (homo-dimer). The red squares demarcate the maternal tissues (Placental Bed and Decidua Parietalis).

Next, circulatory VEGF-A levels in the maternal and cord sera of healthy and preeclamptic pregnancies were determined using a human VEGF-A sandwich ELISA. VEGF-A concentrations were interpolated from a standard curve of known VEGF-A concentrations as a function of optical density at 450nm (Figure 4.6). The ‘goodness of fit’ $r^2$ value for the standard curve was 0.9874.

![Figure 4.6 Human VEGF-A ELISA Standard Curve](image)

**Figure 4.6 Human VEGF-A ELISA Standard Curve** shows a standard curve of known VEGF-A concentrations (dots) in (pg/ml) as a function of optical density at 450nm (OD 450nm) determined using a human VEGF-A sandwich ELISA. The ‘goodness of fit’ (straight line) $r^2$ value for the standard curve was 0.9874.
Figure 4.7 Serum VEGF-A concentrations in maternal and cord sera of healthy and preeclamptic pregnancies. This figure depicts the mean maternal (plain bars) and cord (pixelated bars) serum VEGF-A concentrations in healthy (blue) and preeclamptic (red) pregnancies. (A) shows the mean VEGF-A concentrations in the cord and maternal serum of healthy pregnancies (B) depicts the mean maternal and cord serum VEGF-A
concentrations in pregnancies complicated by preeclampsia. (C) illustrates differences in cord serum VEGF-A concentrations of healthy and preeclamptic pregnancies. The mean cord serum VEGF-A concentrations in preeclamptic pregnancies were significantly lower in comparison to the mean cord serum concentrations of healthy pregnancies (p=0.0115).

In healthy pregnancies the mean maternal serum VEGF-A concentrations (mean ± SEM) were 43 ± 14 fg/ml, while the cord levels were significantly higher (852 ± 294 fg/ml) whereas in preeclamptic pregnancies there was no significant difference between mean maternal (74 ± 44 fg/ml) and cord serum VEGF-A concentrations 240 ± 132 fg/ml (Figure 4.7A and B). More importantly, the mean cord serum VEGF-A concentrations in preeclamptic pregnancies 240 ± 132 fg/ml were significantly lower when compared to mean cord serum levels in healthy pregnancies 852 ± 294 fg/ml (Figure 4.7C).

4.4.5 PROK-1 (EG-VEGF) transcript and protein expression across the maternal-fetal interface of healthy and preeclamptic pregnancies.

RT-PCR was utilized to study PROK-1 mRNA transcript in the placenta, placental bed and decidua parietalis of healthy and preeclamptic pregnancies. The mean PROK-1 mRNA expression levels in the placenta, placental bed and decidua parietalis of healthy pregnancies were 3.851 ± 0.861, 1.132 ± 0.272 and 1.329 ± 0.786, respectively (Figure 4.8A). In pregnancies complicated by preeclampsia, the mean PROK-1 mRNA levels in the placenta, placental bed and decidua parietalis were much lower and were 0.037 ± 0.015, 0.063 ± 0.013 and 0.058 ± 0.029, respectively. There were significantly lower mean PROK-1 mRNA expression levels in the placenta (100-fold less), placental bed (17-fold less) and decidua parietalis (22-fold less) of pregnancies complicated by preeclampsia in comparison to those in healthy pregnancies (Figure 4.8A). The largest difference in PROK-1 mRNA expression between preeclamptic and healthy pregnancies was observed in the placenta.
A human immunosorbent assay was utilised to determine maternal and cord serum PROK1 concentrations in both healthy and preeclamptic pregnancies. The ‘goodness of fit’ $r^2$ value for the standard curve was 0.9787 (Figure 4.8B). In healthy pregnancies, the EG-VEGF protein concentrations (mean ± SEM) in the cord sera (20.96 ± 5.04) pg/ml were significantly less than those observed in the maternal serum (54.80 ± 10.95) pg/ml (Figure 4.8B). There was no difference in the mean maternal (43.12 ± 9.25) pg/ml and cord serum (46.58 ± 29.92) pg/ml PROK-1 protein concentrations in pregnancies complicated by preeclampsia. When comparing healthy and preeclamptic pregnancies, no difference in mean maternal or cord serum PROK-1 levels was observed (Figure 4.8C).
**Figure 4.8** Relative PROK-1 transcript and protein expression across the Maternal-Fetal Interface of Healthy and Preeclamptic Pregnancies. (A) represents the expression of PROK1 mRNA in the Placenta, Placental bed and Decidua Parietalis of Healthy (blue) and Preeclamptic (red) Pregnancies. mRNA expression was determined
using RT-PCR relative to 18S ribosomal RNA. Data are presented as means ± SEM. (**) and (***) signify p<0.01 and p<0.001 respectively. B) shows standard curve of known PROK-1 concentrations (dots) in (pg/ml) as a function of optical density at 450nm (OD 450nm) determined using a human PROK-1 sandwich ELISA. The ‘goodness of fit’ (straight line) $r^2$ value for the standard curve was 0.9787. (C) depicts mean maternal (plain bars) and cord (pixelated bars) serum PROK-1 concentrations in healthy (blue) and preeclamptic pregnancies (red). (D) shows mean cord serum (pixelated) PROK1 levels in healthy (blue) and preeclamptic pregnancies (red).

4.5 DISCUSSION

*KISS1* and its gene product (kisspeptins) have previously been demonstrated to inhibit trophoblast cell invasion (Bilban et al., 2004, Roseweir et al., 2012). Post-translational processing of the *KISS1* gene results in kisspeptins of various lengths, ranging from the longest kisspeptin-145, kisspeptin-54 also known as metastin, kisspeptin-14, kisspeptin-13, to the shortest cleavage product kisspeptin-10. Kisspeptins have been of particular interest in the study of preeclampsia because limited trophoblast invasion is thought to result in inadequate transformation of the spiral arteries which is the fundamental pathophysiology of the disease (Kam et al., 1999). *KISS1* and kisspeptins have thus been studied both in the placenta and maternal circulation of pregnancies complicated by preeclampsia. Although studies have focused on different forms of kisspeptins, one study (Bilban et al., 2004) demonstrated kisspetin-10 to be the most physiologically relevant kisspeptin in the placenta.

This study revealed significantly elevated kisspeptin-10 protein and a trend towards increased *KISS1* mRNA expression in the placentae of preeclamptic pregnancies. This is in keeping with a previous study which investigated *KISS1* mRNA expression in trophoblasts isolated from patients with preeclampsia and healthy term pregnancies
(Qiao et al., 2005b). In that study, increased KISS1 mRNA and metastin (kisspeptin-54) expressed in trophoblasts isolated from placentae of patients with preeclampsia was observed and there was a correlation between expression levels and severity of disease. Recently, others have confirmed increased KISS1 mRNA expression in the placentae of preeclamptic pregnancies (Qiao et al., 2012, Zhang et al., 2011). In contrast, a few studies have reported decreased placental KISS1 mRNA expression in pregnancies complicated by preeclampsia (Qiao et al., 2005a, Cartwright and Williams, 2012). Nonetheless from a pathophysiological perspective, it is much more intuitive to envisage elevated placental KISS1 gene or protein expression in preeclamptic pregnancies as it explains the currently accepted aetiological mechanism of decreased trophoblast invasion. To the knowledge of the writer, no study has thus far investigated KISS1 mRNA expression in the maternal tissues (placental bed or decidua parietalis) of pregnancies complicated by preeclampsia. This study reports increased kisspeptin expression in preeclamptic placentae in comparison to placentae of normal pregnancies and no difference in placental bed or parietal decidua KISS1 mRNA expression between healthy and preeclamptic pregnancies.

Furthermore no difference in GPR-54 mRNA expression in the placenta, placental bed or decidua parietalis of healthy and preeclamptic pregnancies was observed. One study (Cartwright and Williams, 2012) recently reported increased GPR-54 mRNA and protein expression in the placentae of preeclamptic patients. There is a discrepancy in the literature of the correlation between kisspeptin tissue expression levels and circulating kisspeptin concentrations. Therefore in this study the maternal-fetal tissue KISS1 mRNA expression as well as circulatory kisspeptin concentrations were simultaneously examined in healthy and preeclamptic pregnancies. Despite the finding of increased kisspeptin expression in the placentae of pregnancies complicated by preeclampsia, there were decreased circulating kisspeptin levels in
the maternal serum of preeclamptic when compared to healthy pregnancies. Two other studies have previously reported decreased circulating kisspeptin levels in pregnancies complicated by preeclampsia. The one was a prospective study which described a positive correlation between low plasma kisspeptin levels in the second and third trimester of pregnancy and subsequent development of preeclampsia and gestational diabetes (Cetkovic et al., 2012). In this study, low kisspeptin levels were thought to correlate well with placental dysfunction and adverse perinatal outcomes. The other was a retrospective case-control study which reported low serum kisspeptin-10 concentrations at 16-20 weeks gestation in pregnancies complicated by preeclampsia and intrauterine growth restriction (Armstrong et al., 2009). In fact low circulating kisspeptin levels have not only been associated with preeclampsia but also with early pregnancy bleeding (Kavvasoglu et al., 2012), intrauterine growth restriction (Armstrong et al., 2009, Smets et al., 2008) and gestational diabetes (Cetkovic et al., 2012) such that low circulating kisspeptin levels could potentially be utilised as a surrogate marker for placental dysfunction.

Diminished circulating kisspeptin levels in preeclampsia could be explained by compromised endovascular invasion resulting in a reduced amount of kisspeptin-secreting trophoblast cells in the maternal spiral arteries. In support of this hypothesis is the detection of decreased circulating KISS1 mRNA in the maternal whole blood of pregnancies complicated by preeclampsia (Farina et al., 2006). Against this postulate is the reported increased incidence of fetal trafficking in pregnancies complicated by preeclampsia (Hahn., 2002). With increased trophoblast debris in circulation which is a feature of fetal trafficking, one would expect elevated levels of kisspeptins in circulation in preeclampsia.
Failure of adequate transformation of maternal spiral arteries is thought to be the fundamental pathophysiological mechanism underlying the development not only of preeclampsia but also of a range of pregnancy-related complications including intrauterine growth restriction, miscarriage and preterm labour (Brosens, 2011). Central to maternal vascular transformation is the expression of angiogenic ligands, in particular VEGF in the maternal placental bed. It is therefore not surprising that VEGF, specifically VEGF-A has been extensively studied in pregnancies complicated by preeclampsia. Many studies have examined the expression of VEGF in the placenta while others have investigated its expression in circulation, however very few studies have explored its expression both in circulation and maternal-fetal tissues simultaneously.

This study focused on the maternal-fetal expression of VEGF-A transcript at tissue level (placenta, placental bed and decidua parietalis) as well as circulating protein concentrations in the maternal and cord sera of preeclamptic and healthy pregnancies. At tissue level, decreased VEGF-A mRNA and protein expression in the placenta, placental bed and decidua parietalis of preeclamptic pregnancies were found. Of the three maternal-fetal tissues investigated, the most significant difference in VEGF-A mRNA expression between healthy and preeclamptic pregnancies was in the maternal placental bed. There are at least eleven studies that have investigated VEGF mRNA expression in the placentae of preeclamptic pregnancies. Of these studies, five (Andraweera et al., 2012, Cooper et al., 1996, Govender et al., 2012, Park et al., 2010, Sgambati et al., 2004) reported decreased mRNA expression in placenta, three (Tsatsaris et al., 2003, Zhou et al., 2010, Chung, 2004) increased expression while the other three studies (Han et al., 2012, Ranheim et al., 2001, Toft et al., 2008) reported no difference in placental VEGF mRNA expression between preeclamptic and normal pregnancies (Table 4.3). Studies examining placental VEGF-A protein expression in
preeclampsia are also conflicting. Seven studies have investigated differences in VEGF protein expression in the placentae of healthy and preeclamptic pregnancies. Three of these studies (Lyall et al., 1997, Milovanov et al., 2008, Wang et al., 2010) found decreased placental VEGF protein expression in preeclamptic pregnancies, three (Akercan et al., 2008, Bosco et al., 2010, Toft et al., 2008) reported increased expression and one study (Chung, 2004) found no difference in placental VEGF-A protein expression between healthy and preeclamptic pregnancies purely for the purpose of comparison.

Invasion of trophoblast cells into maternal tissues and transformation of the spiral arteries occur in the placental bed. Thus, the molecular processes transpiring in the placental bed are pivotal to the understanding of pregnancy physiology and pathophysiology. In this study markedly suppressed placental bed VEGF mRNA expression was found in preeclamptic pregnancies. In concordance with the current study, one study (Kim et al., 2012) recently reported decreased VEGF mRNA in the placental beds of preeclamptic pregnancies while the other studies (Ranheim et al., 2001, Tsatsaris et al., 2003) reported no difference in placental bed mRNA expression between preeclamptic and healthy pregnancies. Of the four studies that examined differences in VEGF protein expression in the placental bed of healthy and preeclamptic pregnancies, three studies (Cirpan et al., 2007, Kim et al., 2012, Milovanov et al., 2008) found decreased VEGF protein expression in preeclampsia while the third study (Vuorela and Halmesmaki, 2006) reported no difference in expression (Table 4.3). Hence when evaluating VEGF-A tissue expression in the placentae of preeclamptic pregnancies, most studies reported decreased VEGF-A mRNA expression and there was minimal concordance between studies examining VEGF-A protein expression. In addition, most studies examining placental bed expression in preeclamptic pregnancies reported decreased VEGF-A protein expression and only one study found decreased
VEGF-A mRNA expression. It is very tempting to postulate decreased VEGF-A protein expression in the placental bed as the primary trigger of preeclampsia but this could equally be secondary to poorly vascularised maternal tissues.

This study found lower circulating maternal VEGF-A protein concentrations in comparison to cord levels in both healthy and preeclamptic pregnancies. However, the cord VEGF-A concentrations in preeclampsia were significantly lower in comparison to those in healthy pregnancies. Quite the opposite to the present findings, one similar study (Salama et al., 2011) reported higher maternal VEGF-A concentrations when compared to cord levels in both healthy and preeclamptic pregnancies and significantly higher cord VEGF concentrations in preeclamptic pregnancies.

No difference in circulating maternal VEGF-A protein concentrations between healthy and preeclamptic pregnancies was observed. There are at least eleven studies that examined circulating maternal VEGF concentrations in preeclamptic pregnancies. Five of these studies (Laskowska et al., 2008, Livingston et al., 2000, Purwosunu et al., 2009, Semczuk-Sikora et al., 2007, Zhou et al., 2010) reported decreased maternal VEGF concentrations in preeclamptic pregnancies, four (Bussen et al., 2003, Salama et al., 2011, Sharkey et al., 1996, Tsatsaris et al., 2003) reported increased concentrations and two (Han et al., 2012, Sezer et al., 2012) reported no difference in circulating maternal VEGF concentrations between preeclamptic and healthy pregnancies. There seems to be no clear agreement amongst the studies reporting on circulating maternal VEGF levels in preeclamptic pregnancies.

Lastly, PROK-1 has recently been shown to be an important placental angiogenic factor crucial for early placental villous development facilitated by stimulation of cytotrophoblast cells and proliferation of endothelial cells (Brouillet et al., 2013). PROK-
Chapter 4  
Reciprocal Expression of Angiogenic Regulators

1 is also important in maintaining early fetal-maternal interactions (Evans et al., 2009). The current study found significantly decreased PROK-1 mRNA expression levels in the placenta, placental bed and decidua parietalis of pregnancies complicated by preeclampsia. Furthermore, the greatest difference in mRNA expression between preeclamptic and healthy pregnancies was in the placenta and not in the placental bed as was observed with VEGF mRNA expression. Previously no difference in PROK1 expression was found between the placentae of preeclamptic and healthy pregnancies (Chung, 2004). Interestingly, in healthy pregnancies maternal-cord circulatory studies showed lower maternal VEGF-A protein levels in comparison to cord levels and higher maternal PROK1 protein concentrations when compared to cord levels. This suggests different and possibly complementary roles of these angiogenic factors as previously reported (Hoffmann et al., 2007), in pregnancy health even at term.

This difference in topographical expression between two angiogenic factors within the context of pathological pregnancies may shed more light about their relative physiological importance across the maternal-fetal interface. Based on the tissue expression studies comparing health and preeclampsia conducted in this study, it seems tempting to consider VEGF-A as the maternally significant angiogenic factor chiefly responsible for transformation of the spiral arteries within the placental bed, while EG-VEGF is the placentally vital angiogenic factor mainly responsible for villous angiogenesis.

This study has a few limitations, firstly it was conducted in later pregnancy and events such as trophoblast invasion and angiogenesis are best studied in early pregnancy when placentation is most critical in determining pregnancy outcome. However, due to ethical considerations, true placental bed tissue biopsies (with representative interstitial trophoblast, myometrium and spiral arteries) would be difficult to obtain.
Chapter 4  
Reciprocal Expression of Angiogenic Regulators

in early pregnancy and the prediction of later development of preeclampsia during this period still remains a challenge. Secondly, due to risk of serious maternal and fetal complications often associated with preeclampsia, delivery prior to term is often recommended. It would therefore be ethically imprudent to obtain gestationally matched tissue from otherwise uncomplicated healthy pregnancies (Hahn., 2002). The advantage of studying late gestation is that the effects of kisspeptin on placentation, invasion and angiogenesis may be different in late in comparison to early gestation. In addition studying kisspeptin expression and circulatory levels in late pregnancy may be important in indicating villous.

Further studies need to investigate the role of other angiogenic factors like placental growth factor (PGF), endoglin, angiopoetins as well as angiogenic receptors such as VEGFR1, VEGFR2 and angiopoetin receptors across the maternal-fetal interface of health and disease. A better understanding of their role and the interplay among them may potentially reveal targeted opportunities for the development of drugs aimed at alleviation or prophylaxis of preeclampsia and/or intrauterine growth restriction.
### Table 4.3  Studies Investigating Maternal and Fetal Tissue VEGF expression in Preeclamptic Pregnancies

<table>
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<tr>
<th>Study</th>
<th>mRNA</th>
<th>Protein</th>
<th>Method</th>
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<tr>
<td><strong>Placenta</strong></td>
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<tr>
<td>Akercan 2008</td>
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<td>Andraneera 2012</td>
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<td>RT-PCR</td>
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<td>Chung 2004</td>
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<td>RT-PCR/WB</td>
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<td>Cooper 1996</td>
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<td>Govender 2012</td>
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<td>Han 2012</td>
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<td>RT-PCR</td>
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<td>Toft 2008</td>
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<td>Tsatsaris 2003</td>
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<td>Zhou 2010</td>
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<td>This study</td>
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<td>RT-PCR</td>
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<td><strong>Placental Bed</strong></td>
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<td>Kim 2012</td>
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<td>RT-PCR/IH/WB</td>
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Chapter 4  Reciprocal Expression of Angiogenic Regulators

Abbreviations: IH (Immunohistochemistry)  
RT-PCR (Real-time Polymerase Chain Reaction)  
WB (Western Blot)  
PET (Preeclampsia)  
HELLP (Haemolysis, Elevated Liver Enzymes and Low Platelets)  
↑ Increased Expression  
↓ Decreased Expression  
↔ No Difference in Expression
CHAPTER FIVE

The Effect of Kisspeptin Stimulation on the transcript expression of VEGF-A and its receptors (VEGFR-1 and VEGFR-2) in healthy and preeclamptic placentae.

5.1 Abstract.................................................................................................................Page 105
5.2 Introduction..............................................................................................................Page 106
5.3 Material and Methods ............................................................................................Page 107
  5.3.1 Study Participants ..............................................................................................Page 107-108
  5.3.2 Tissue Sampling and Specimen Collection .......................................................Page 108
  5.3.3 Tissue Explant (Kisspeptin Stimulation) Experiments ....................................Page 108
  5.3.4 RNA Extraction ...............................................................................................Page 108-109
  5.3.5 cDNA Synthesis .............................................................................................Page 109
  5.3.6 Real-Time PCR ...............................................................................................Page 109
5.4 Statistical Analysis .................................................................................................Page 109-110
5.5 Results ....................................................................................................................Page 110
  5.5.1 The effect of kisspeptin-10 stimulation on placental VEGF-A transcript expression ..................................................Page 110
  5.5.2 The effect of kisspeptin-10 stimulation on placental VEGFR-1 transcript expression ..................................................Page 111
  5.5.3 The effect of kisspeptin-10 stimulation on placental VEGFR-2 transcript expression ..................................................Page 112
5.6 Discussion ..............................................................................................................Page 112-115
5.1 Abstract

Normal placental development in early pregnancy is critical in determining a healthy pregnancy outcome. Angiogenesis is an indispensable component of normal placentation. VEGF-A plays a fundamental role in angiogenesis and mainly mediates its angiogenic effects via VEGF receptor -1 and -2 (VEGFR-1 and VEGFR-2). In order to study the role of kisspeptin on the mRNA expression of VEGF-A, VEGFR-1 and VEGFR-2 in normal and abnormal placentation, kisspeptin stimulation experiments were conducted on placental explants from healthy and preeclamptic pregnancies. RT-PCR was utilized to study placental VEGF-A, VEGFR-1 and VEGFR-2 mRNA expression following tissue explant incubation with kisspeptin for various time-points over a 24-hour period. The stimulation results showed no effect of kisspeptin-10 on the expression of VEGF-A, VEGFR-1 or VEGFR-2 mRNA. However in healthy pregnancies, kisspeptin stimulation resulted in non-significant suppression of placental mRNA expression of VEGF-A and VEGFR-2 at 8 hours and VEGFR-1 at all time-points. Whereas in preeclamptic pregnancies placental stimulation with kisspeptin resulted in a non-significant up-regulation of VEGF-A mRNA expression at 8 hours, VEGFR-2 at all time-points. In summary kisspeptin stimulation had no effect on the expression of VEGF-A mRNA or its receptors. However observed trends towards contrasting angiogenic factor suppression in healthy placentae and up-regulation in preeclampsia may suggest different angiogenic regulatory roles by kisspeptin in pregnancy pathology and health.
5.2 Introduction

Angiogenesis is a process involving the development of new vessels from existing vessels (Carmeliet, 2005). The placenta is a highly vascular organ and mainly develops by branching angiogenesis (Reynolds and Redmer, 2001). Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis (Hoeben et al., 2004). VEGF has various subtypes (A-E) of which VEGF-A is the most predominant in the placenta (Clark et al., 1998b), in particular the VEGF-A \textsubscript{165} isoform. VEGF-A mediates its angiogenic effects principally via threonine kinase receptors VEGFR-1 and VEGFR-2 (Ferrara et al., 2003).

\textit{Kiss1} metastasis suppressor gene encodes kisspeptins (Kps) of various lengths (Kp-154, -54, -13 and -10). Kisspeptin-10 is the most biologically active in the placenta and inhibits migration and invasion in primary trophoblast cultures (Bilban et al., 2004). Furthermore kisspeptin administration results in phenotypical suppression of vessel development in first trimester explants (Ramaesh et al., 2010). Our research group has recently demonstrated that kisspeptin-10 suppresses VEGF-A mRNA expression in first trimester primary trophoblast cultures (Francis et al., 2014).

Various studies including the work described in Chapter 4 have demonstrated elevated kisspeptin expression in preeclamptic placentae (Qiao et al., 2012, Qiao et al., 2005b, Zhang et al., 2011). The effects of kisspeptin stimulation however have never been compared between healthy and preeclamptic placental tissue. The objective of this study was to investigate the effects of kisspeptin-10 administration on transcript expression of key angiogenic factors namely VEGF-A, VEGFR-1 and VEGFR-2 in placental explants from healthy and preeclamptic pregnancies.
5.3 Materials and Methods

The study was approved by the Human Ethics Committee of the Faculty of Health Sciences, University of Cape Town (REF: 080/2008). Written informed consent was obtained from all study participants and the research was conducted according to the ethical principles of the Declaration of Helsinki (Bruce-Chwatt, 1965, 2001). The patients were counselled in their preferred language and were presented with Patient Information Leaflets (PILs) providing the details of the study. The comprehension of this information was always assessed and in cases of uncertainty, consent was withdrawn. Study participation remained the patient’s prerogative without any coercion and their decision not to participate did not compromise their further management and care. Furthermore recruitment to the study did not involve the patient’s clinical carers but members of the research team.

5.3.1 Study Participants

All patients recruited had elective caesarean section deliveries in the absence of labour. The inclusion criteria for the control arm of the study were healthy patients undergoing elective caesarean section for the indications of either previous caesarean section or fetal malpresentation. Patients were excluded from the control arm of the study if they were in labour, had more than one previous caesarean section, had multiple pregnancies, had pregnancy-specific complications such as gestational diabetes, gestational hypertension, placenta praevia, preeclampsia, intrauterine growth restriction. In addition, underlying chronic medical disorders such as preexisting diabetes or chronic hypertension were also an exclusion from the study. Patients were recruited from Groote Schuur Hospital which is a tertiary referral centre and Mowbray Maternity Hospital which is a secondary referral obstetric hospital in Cape Town, South Africa. The inclusion criteria for the experimental arm were patients with a diagnosis of preeclampsia who were having elective caesarean sections. Patients in labour,
patients who had two previous caesarean sections, gestational diabetes, gestational hypertension, placenta praevia, as well as patients with preexisting medical disorders were excluded from the experimental arm of the study. In addition, patients who had chronic hypertension with superimposed preeclampsia were excluded from the study.

5.3.2 Tissue Sampling and Specimen Collection
Placental samples from healthy and preeclamptic pregnancies were collected at elective caesarean delivery.

5.3.3 Tissue Explant (Kisspeptin Stimulation) Experiments
Fresh placental tissue samples were washed with PBS and collected on ice. The placental tissue (50mg) was equally apportioned for various time points in tissue culture 9-well plates and kept in a humidified incubator at 37°C and 5% CO₂. Placental tissue explants were still viable after 24hrs of incubation. The samples were treated with either vehicle (DMEM F12 medium) or vehicle plus kisspeptin (100nM) at each time point (8, 12 and 24hours). After each time point had elapsed, the media was suctioned off from the 9-well plates and 1ml TRIzol® reagent was added to the tissue for RNA extraction purposes. For the zero (0) hour time point, TRIzol® reagent was immediately added instead of medium.

5.3.4 RNA Extraction
1ml Trizol was used per 50mg of placental tissue for lysis. Tissue was homogenised on ice with a Tissue Ruptor® (Qiagen). The homogenate was centrifuged at 15000g at 4°C for 15minutes and 200μl of ice-cold BCP (Sigma) was added to the supernatant. The mixture was shaken for 15seconds and kept on ice for 10mins. The solution was again centrifuged and the top aqueous phase was removed and transferred to a new tube. This was followed by addition of 500μl of propanol and the tube was again
centrifuged to precipitate RNA. RNA pellets were washed in 75% ethanol, air dried and re-suspended in DEPC-treated water.

5.3.5 cDNA Synthesis

RNA was reverse transcribed with Multiscribe Reverse Transcription reagents (Applied Biosystems). DEPC-treated H$_2$O (3.7 μl), 10x RT Buffer (2 μl), MgCl$_2$ (4.4 μl), dNTPs (4 μl), Random Hexamers (1 μl), RNase Inhibitor (0.4 μl), Reverse Transcriptase (0.5 μl) and (2 μl) of RNA were utilized for a 20 μl reverse transcription reaction. The ABI GeneAmp® 2700 Thermal Cycler was programmed for the following reaction steps: 25°C (10 mins), 45°C (10 mins), 48°C (45 mins) and 95°C (5 mins).

5.3.6 Real-Time PCR

The ABI 7900 RT-qPCR instrument (Applied Biosystems) was utilized to perform gene expression studies. The standard thermal cycling protocol was conducted as follows: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of (95°C for 95 secs and 60°C for 1 min). The Genebank accession numbers of the genes investigated are as follows: VEGF-A (NM_001171623), VEGFR-1 (NM_002019) and VEGFR-2 (NM_002253). Primer/Probe pairs corresponding to these genes (Sigma® Pharmaceuticals) were utilised (Table I) in the presence of the Taqman® Mastermix. All RT-qPCR gene expression data is presented as Means ± SEM and expression is relative to 18S ribosomal RNA (internal control) and reference cDNA.

5.4 Statistical Analysis

Repeated measures two-way ANOVA was employed to compare placental mRNA transcript expression at various time points between healthy and preeclamptic pregnancies. The statistical package utilized was GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
5.5 Results

5.5.1 The effect of kisspeptin-10 stimulation on placental \(\text{VEGF-A}\) transcript expression.

In healthy pregnancies an increase in the basal placental \(\text{VEGF-A}\) mRNA expression at 8 and 12 hours was observed which reverted to baseline levels at 24 hours. Kisspeptin stimulation resulted in a modest reduction in placental \(\text{VEGF-A}\) mRNA expression at 8 and 12 hours but this did not reach statistical significance. In preeclamptic pregnancies the basal placental \(\text{VEGF-A}\) mRNA expression levels were comparable through all time-points with a non-significant increase at 8 hours following kisspeptin stimulation (Figure 5.1).

**Figure 5.1.** The effect of kisspeptin-10 stimulation on \(\text{VEGF-A}\) mRNA expression in healthy and preeclamptic placental tissue. Relative \(\text{VEGF-A}\) mRNA expression following healthy (A) and preeclamptic (B) placental explant incubation with 100nM kisspeptin-10 for 8, 12 and 24 hour durations. \(\text{Pv}\) (blue and red bars) represents placental stimulation with vehicle (DMEM-F12 medium) while \(\text{Pkp}\) (yellow) signifies placental stimulation with 100nM kisspeptin-10. mRNA expression was quantified relative to 18S RNA and control cDNA by RT-PCR.
5.5.2 The effect of kisspeptin-10 stimulation on placental VEGFR-1 transcript expression

In healthy pregnancies, basal VEGFR-1 mRNA expression was comparable at all time points with a consistent non-significant suppression following kisspeptin stimulation. Similarly in preeclamptic placentae, the basal VEGFR-1 mRNA expression was consistent and kisspeptin stimulation had a non-significant increase in VEGFR-1 transcript expression over time (Figure 5.2). Thus, kisspeptin had no effect on VEGFR-1 mRNA expression in both healthy and preeclamptic pregnancies.

Figure 5.2. The effect of kisspeptin-10 stimulation on VEGFR-1 mRNA expression in healthy and preeclamptic placental tissue. Relative VEGFR-1 mRNA expression following incubation of healthy (A) and preeclamptic (B) placental explants with 100nM kisspeptin-10 for 8, 12 and 24 hour periods. Pv (blue and red bars) represents placental stimulation with vehicle (DMEM-F12 medium) while Pkp (yellow) signifies placental stimulation with 100nM kisspeptin-10. mRNA expression was quantified relative to 18S RNA and control cDNA by RT-PCR.
5.5.3 The effect of kisspeptin-10 stimulation on placental VEGFR-2 transcript expression.

Kisspeptin had no effect on VEGFR-2 expression in both healthy and preeclamptic pregnancies (Figure 5.3)

Figure 5.3 The effect of kisspeptin-10 stimulation on VEGFR-2 mRNA expression in healthy and preeclamptic placental tissue. Figure 5.3 represents relative VEGFR-2 mRNA expression following incubation of healthy (A) and preeclamptic (B) placental explants with 100nM kisspeptin-10 for 8, 12 and 24 hour periods. Pv (blue and red bars) represents placental stimulation with vehicle (DMEM-F12 medium) while Pkp (yellow) signifies placental stimulation with 100nM kisspeptin-10. mRNA expression was quantified relative to 18S RNA and control cDNA by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

5.6 Discussion

This study examined the response of healthy and preeclamptic placental tissue to stimulation with kisspeptin-10 over a 24 hour time course. In particular we investigated the effect of kisspeptin-10 on mRNA expression of angiogenic ligand VEGF-A and its
Chapter 5  Effect of Kisspeptin Stimulation

receptors (VEGFR-1 and VEGFR-2) in these tissues. Kisspeptin had no effect on the mRNA expression of VEGF-A, VEGFR-1 or VEGFR-2.

Suppression of VEGF-A mRNA (Francis et al., 2014) and angiogenesis (Ramaesh et al., 2010) by kisspeptin-10 in first trimester placental tissue has been reported and is likely one mechanism involved in inhibition of trophoblast invasion and tumour metastasis. In Chapter three we postulated that VEGF-A is likely more involved in maternal rather than placental angiogenesis in healthy pregnancies while in Chapter 4 placental VEGF-A mRNA expression was suppressed in pregnancies complicated by preeclampsia (Figure 4.5). Furthermore, placental kisspeptin-10 expression was elevated in preeclampsia when compared to healthy pregnancies (Figure 4.2). Hence the failure of kisspeptin-10 to have any effect on the mRNA expression of VEGF-A and its receptors might be explained by the already elevated placental expression of kisspeptin in healthy and more so in preeclamptic placentae. If this is the case then suppression of endogenous kisspeptin expression in the placenta with recently described kisspeptin antagonists (Roseweir et al., 2009) prior to stimulation with kisspeptin could be attempted in the future.

It still remains of interest what the effect of kisspeptin on angiogenic events in the maternal placental bed and decidua parietalis would be. There is always sufficient placental tissue available to conduct stimulation experiments however the amount of placental bed and decidua parietalis is usually limited. This restricted the ability to conduct these experiments on maternal tissue.

VEGFR-1 antagonises VEGF-A and is normally produced in large quantities by the placenta (Clark et al., 1998a). The soluble form of VEGFR-1 (sFLT-1) has been consistently found to be elevated in the circulation and placentae of preeclamptic
patients (Levine et al., 2004, Luttun and Carmeliet, 2003, Maynard et al., 2003, Sitras et al., 2009, Munaut et al., 2012). Furthermore, introduction of the sFLT-1 gene induces a preeclampsia-like phenotype in pregnant Sprague-Dawley rats (Maynard et al., 2003). Kisspeptin-10 stimulation resulted in a trend towards suppression of placental VEGFR-1 expression in healthy pregnancies and an elevated expression in preeclampsia. This raises the question of whether the elevated expression of VEGFR-1 in preeclamptic placentae could be kisspeptin driven.

Unlike VEGFR-1 which antagonises VEGF-A, VEGFR-2 mediates the angiogenic and mitogenic effects of VEGF-A, and is the key angiogenic receptor. Kisspeptin stimulation had no effect on placental VEGFR-2 mRNA expression. Recently, a study examining placental and circulatory VEGFR-1 and VEGFR-2 mRNA in preeclampsia reported lower VEGFR-2 in the circulation of preeclamptic patients (Munaut et al., 2012). Furthermore the levels of soluble VEGFR-1 in circulation decreased post-delivery in keeping with its placental origin whereas that of VEGFR-2 persisted albeit at lower levels, suggesting a maternal endothelial origin of the peptide.

In the previous chapter we demonstrated suppressed angiogenic ligand expression (EG-VEGF in the placentae and decreased VEGF-A in the placental beds) of pregnancies complicated by preeclampsia. The observed trend of elevated angiogenic VEGF-A expression in preeclamptic placentae in response to kisspeptin stimulation may be a compensatory mechanism to suppressed placental (EG-VEGF mediated) and placental bed (VEGF-A mediated) angiogenesis. The suppressed VEGF-A expression in the placental beds of preeclamptic pregnancies reported in most studies (Table 4.3) may be secondary to elevated placental VEGFR-1 expression which may in part be kisspeptin driven.
A concession has to be made that these are observed trends rather than statistically significant effects. As kisspetin is abundantly expressed in pregnancy (Horikoshi, 2003), future work should involve suppression of endogenous kisspeptin with kisspeptin antagonists prior to stimulation experiments as this may further elaborate the tissue responses.

Chronic exposure to kisspeptin triggers the desensitization of GPR-54. In the placenta, there is high concentrations of kisspeptin that increase during trimester the first trimester, therefore chronic exposure to GPR-54 actually reflects a physiological situation. The net outcome is an integration between receptor de-sensitization and re-sensitization.
CHAPTER SIX – GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion ................................................................. Page 117
   6.1.1 The role of kisspeptin in pregnancy health .................. Page 117-118
   6.1.2 The role of kisspeptin in preeclampsia......................... Page 118-121

6.2 Conclusions ........................................................................ Page 121-122

6.3 Future Studies..................................................................... Page 122-123
6.1 DISCUSSION

Pregnancy-related disorders contribute significantly to global maternal and perinatal morbidity and mortality. To have an impact on pregnancy-related pathology requires a comprehensive understanding of the molecular processes involved in placentation and subsequent maternal-fetal dialogue in pregnancy health. In this dissertation the role that kisspeptin, encoded by the *Kiss-1* gene and signalling via its receptor *GPR-54*, plays in placentation is investigated. In particular its expression along with that of angiogenic ligand and receptors at gene and protein levels is explored across the maternal-fetal interface of pregnancy health and pathology. Lastly, its role on the transcript expression of angiogenic ligand *VEGF-A* and its receptors is examined.

6.1.1 The role of kisspeptin in pregnancy health

The expression of kisspeptin and *GPR-54* had previously been reported in human first trimester trophoblasts (Bilban et al., 2004, Park et al., 2012, Qiao et al., 2005a), placenta (Cartwright and Williams, 2012, Qiao et al., 2012, Qiao et al., 2005b, Ramaesh et al., 2010, Zhang et al., 2006, Zhang et al., 2011) and trophoblast cell-lines (Roseweir et al., 2012, Zhang et al., 2011). Their expression had never been investigated across maternal-fetal tissues, in particular the maternal placental bed and decidua parietalis. This study found high *Kiss-1* and *GPR-54* mRNA expression in the placenta in comparison to the placental bed and decidua parietalis. Immunohistochemistry detected neither protein in the maternal tissues (Matjila et al., 2013). Similar to others (Bilban et al., 2004), it was found that the expression of kisspeptin was restricted to the syncytiotrophoblast while that of its receptor was localized to the villous syncytio- and cytotrophoblasts as well as extravillous cytotrophoblast columns. Kisspeptins inhibit trophoblast migration and invasion and likely play an invasive trophoblast auto-regulatory role across the maternal-fetal interface from the first trimester to term in healthy pregnancies.
In healthy pregnancies decreased VEGF-A mRNA and protein in the placenta in comparison to the placental bed which had highest expression across the maternal-fetal interface was observed. At the same time, the mRNA expression of both PROK-1 and its receptor (PROK-1R) were expressed highest in the placenta when compared to the placental bed and decidua parietalis. Altogether these findings suggest that VEGF-A is unlikely to be the dominant factor mediating angiogenesis in the placenta and that kisspeptin may play a role in its placental suppression in favour of prokineticin-mediated angiogenesis. It is now known that prokineticin-1 plays a key role in endometrial receptivity, regulation of implantation-related genes and placental angiogenesis (Evans et al., 2008, Hoffmann et al., 2006, Brouillet et al., 2010, Salker et al., 2010).

6.1.2 The role of kisspeptin in the preeclampsia

Due to its effects on tumour metastasis suppression and inhibition of trophoblast migration and invasion, the role of kisspeptin has naturally generated interest in preeclampsia research, whose underlying pathophysiology involves limited invasion of trophoblast and inadequate transformation of spiral arteries. The expression of Kiss-1 transcript and protein was investigated across the maternal-fetal tissues (placenta, placental bed and decidua parietalis) of healthy and preeclamptic pregnancies. In addition, circulatory kisspeptin-10 levels in maternal and cord sera of healthy and preeclamptic pregnancies were explored. Elevated expression of kisspeptin-10 was found in the placentae of preeclamptic pregnancies along with suppressed mean maternal kisspeptin-10 levels in circulation. Although Kiss-1 mRNA was modestly higher in the preeclamptic placentae, there was no difference in placental bed and decidua parietalis. Kiss-1 mRNA expression between healthy pregnancies and preeclampsia. Most studies have reported elevated expression of Kiss-1 mRNA and protein in trophoblast (Qiao et al., 2005b) and placentae of preeclamptic pregnancies.
(Qiao et al., 2012, Zhang et al., 2006, Zhang et al., 2011). Only one study recently found decreased placental Kiss-1 mRNA and protein expression along with increased placental Kiss-1R (GPR-54) in preeclampsia (Cartwright and Williams, 2012). The current study and others (Qiao et al., 2012) found no difference in placental GPR-54 expression between healthy and preeclamptic pregnancies, in addition this research reports no difference in placental bed and decidua parietalis GPR-54 receptor expression between pregnancy health and preeclampsia. The elevated placental Kiss-1 expression in preeclampsia is in keeping with the currently accepted pathophysiology of the condition.

This study shows that kisspeptin stimulation resulted in a trend towards suppression of placental VEGF-A expression in healthy pregnancies. In a separate study our group recently demonstrated that kisspeptin suppresses VEGF-A mRNA expression in primary human trophoblast cells isolated from first trimester terminations (Francis et al., 2014). The current study also demonstrated significant suppression of angiogenic factor transcript expression across the maternal-fetal interface of pregnancies complicated by preeclampsia, with VEGF-A suppressed in the placental bed and PROK-1 in the placenta. (Fig 6.1). As others have demonstrated that kisspeptin suppresses both VEGF-A expression (Cho et al., 2009) and angiogenesis (Ramaesh et al., 2010), it is likely that elevated placental kisspeptin expression results in suppressed placental bed VEGF-A expression and compromised angiogenesis in the maternal spiral arteries of pregnancies complicated by preeclampsia.
Figure 6.1 The schematic of putative interaction at the maternal-fetal interface of healthy and preeclamptic pregnancies. This figure illustrates increased kisspeptin expression (green) in the syncytiotrophoblast layer of preeclamptic placenta. This leads to reduced cytotrophoblast column formation and EVT populations (pink cells). The consequence is less interstitial and endovascular trophoblast invasion and transformation of decidual (DSA) and myometrial (MSA) components of spiral arteries in the decidua basalis and junctional zone myometrium. Reduced interstitial trophoblast populations in preeclampsia also translates to less interaction between trophoblast and immune cellular components in the decidua basalis such as decidual lymphocytes and UNK cells. This may limit production of cytokines and factors that favour immune tolerance leading to compromised trophoblast-independent transformation of maternal spiral arteries. Increased kisspeptin likely results in suppressed VEGF-A expression in
In healthy pregnancies the maternal and cord serum kisspeptin-10 levels were comparable but in preeclamptic pregnancies maternal concentrations were significantly lower than cord levels. Furthermore circulatory kisspeptin-10 levels in preeclampsia were significantly lower than those in healthy pregnancies. Hence despite elevated tissue kisspeptin-10 expression levels in preeclampsia, mean circulatory concentrations were much lower when compared to healthy pregnancies. Several studies have demonstrated the association between low circulating kisspeptin levels and other pregnancy-related pathology including preeclampsia (Armstrong et al., 2009, Cetkovic et al., 2012, Kavvasoglu et al., 2012, Smets et al., 2008). This is just another instance exemplifying that peripheral circulatory measurements are not necessarily reflective of tissue expression. It can be postulated that decreased circulating kisspeptin levels in preeclampsia or pregnancy-related pathology may be a consequence of compromised endovascular invasion resulting in a reduced amount of kisspeptin-secreting trophoblast cells in the maternal spiral arteries. There is therefore a potential to utilize diminished kisspeptin levels in the maternal circulation as a surrogate for placental dysfunction (Cetkovic et al., 2012).

6.2 Conclusion

Placentation is a key process to understand as it directly determines pregnancy outcome. More crucial to comprehend are molecular events in the maternal-fetal interface. This work describes the expression of Kiss-1, kisspeptin, its receptor GPR-54 and angiogenic factors (which are crucial in placentation) across the maternal-fetal tissues, first in pregnancy health, and their subsequent derangement in preeclampsia. The study defines for the first time the expression of Kiss-1 and GPR-54 as well as that of recently described angiogenic factor PROK-1 and its receptor PROK-1R
across maternal-fetal tissues. In particular their expression in the maternal placental bed which is the primary site of maternal-fetal interaction and transformation of spiral arteries is described. Basal angiogenic factor expression across maternal-fetal tissues was suggestive of PROK-1/PROK-1R ligand-receptor pair as key role players in placental angiogenesis while VEGF-A/VEGFR-2 likely mediated angiogenesis in the maternal placental bed. Preeclamptic pregnancies were characterised by significant angiogenic factor suppression with decreased PROK-1 and VEGF-A expression in the placenta and placental bed respectively. The most remarkable suppression was that of VEGF-A in the placental bed and this has been a consistent finding of the few studies examining placental bed VEGF-A expression in preeclampsia. Stimulation of placental tissue from healthy and preeclamptic pregnancies showed no effect of kisspeptin-10 on VEGF-A, VEGFR-1 or VEGFR-2 mRNA expression however this could have been due to high endogenous kisspeptin expression in placental tissue. Lastly this study shows for the first time that while placental kisspeptin-10 expression is elevated in preeclampsia, peripheral circulatory concentrations are suppressed. This finding, along with supportive data from other studies demonstrating low circulating kisspeptin levels in pregnancy-related pathology, introduces the possibility of utilizing diminished circulatory kisspeptin concentrations as a surrogate marker of placental dysfunction.

6.3 Future Studies

The differential role of kisspeptin in healthy and preeclamptic pregnancies needs further exploration. Pre-treatment with kisspeptin antagonists to suppress endogenous kisspeptin levels will be crucial in these experiments. The effects of kisspeptin on placental and decidual components from healthy and other pathological pregnancies (e.g. intrauterine growth restriction and recurrent miscarriage) sharing the same pathophysiology as preeclampsia should be investigated. The effect of kisspeptin
on the placental angiogenic factor PROK-1 and its signalling pathways needs further study together with kisspeptin-mediated epigenetic changes in pregnancy.

Lastly, in vivo studies conducted in an animal model reflective of deep placentation as is the case in human pregnancy, needs to be utilized to further explore the role of kisspeptin in maternal-fetal dialogue and angiogenesis.
APPENDIX

BUFFERS AND SOLUTIONS

**RIPA Buffer**
25mM Tris HCl pH 7.6
150mM NaCl
1% Triton X
1% Sodium deoxycholate
0.1% SDS

**Laemmli Buffer**
125mM Tris-HCl pH 6.8
4% SDS
20% Glycerol
5% 2-mercaptoethanol
0.05% bromophenol blue

**Lysis Buffer**
150mM NaCl
10% Glycerol
0.6% (v/v) NP-40
50mM Tris HCl pH 7.4
10mM EDTA (add protease cocktail inhibitor tablet before use)

**10x Phosphate-Buffered Saline (PBS)**
80g NaCl
2g KCl
14.4g Na$_2$HPO$_4$
2.4g KH$_2$PO$_4$
800ml distilled H$_2$O, pH adjusted to 7.4 to a final volume of 1L.
**SDS-PAGE Stacking and Resolving Gels**

30% Acrylamide

1.5M Tris (pH 6.8) Stacking Gel / (pH 8.8) Resolving Gel

10% SDS

Distilled H$_2$O

TEMED

10% APS

**10x Tris-Buffered Saline (TBS)**

80g NaCl

2g KCl

30g Tris Base

800ml distilled H$_2$O, pH adjusted to 7.4 to a final volume of 1L.

**1x TBST (1L)**

100ml (10x) TBS

900ml distilled H$_2$O

1ml Tween 20®

**Transfer Buffer**

25mM Tris HCl

0.192M Glycine

20% Methanol
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