


1-1-2011

# Human trophoblast survival and invasion in the developing placenta: autocrine regulation by hbegf

Philip Jessmon  
*Wayne State University,*

Follow this and additional works at: [http://digitalcommons.wayne.edu/oa\\_dissertations](http://digitalcommons.wayne.edu/oa_dissertations)

 Part of the [Cell Biology Commons](#), [Molecular Biology Commons](#), and the [Obstetrics and Gynecology Commons](#)

---

## Recommended Citation

Jessmon, Philip, "Human trophoblast survival and invasion in the developing placenta: autocrine regulation by hbegf" (2011). *Wayne State University Dissertations*. Paper 353.

This Open Access Dissertation is brought to you for free and open access by DigitalCommons@WayneState. It has been accepted for inclusion in Wayne State University Dissertations by an authorized administrator of DigitalCommons@WayneState.

**HUMAN TROPHOBLAST SURVIVAL AND INVASION IN THE DEVELOPING  
PLACENTA: AUTOCRINE REGULATION BY HBEGF**

by

**PHILIP JESSMON**

**DISSERTATION**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**DOCTOR OF PHILOSOPHY**

2011

MAJOR: ANATOMY & CELLULAR BIOLOGY

Approved by:

\_\_\_\_\_  
Advisor

\_\_\_\_\_  
Date

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**COPYRIGHT BY  
PHILIP JESSMON  
2011  
All Rights Reserved**

## DEDICATION

Completion of this degree is dedicated to several people. First and foremost among them is my very beautiful and loving wife Julie, with whom I am honored to journey through life. Her support, encouragement, and devotion have brought me through my most difficult times, and mean more to me than mere words can express. I would like to dedicate this also to my parents who have provided a firm foundation upon which I could develop into the man I am today, and because of which I am equipped to earn this degree. My family, both old and new, deserve to be included in this dedication for their support in always inquiring about my work and offering words of encouragement. Last, but not least important, my friends have always provided much refreshment and prayer through an otherwise arduous process.

## ACKNOWLEDGMENTS

This dissertation is the capstone upon six years of work in Dr. Randall Armant's lab. There are many people who deserve credit for helping, guiding, and pushing me through this process. Foremost among those acknowledged is my advisor, Dr. Armant, who epitomizes mentorship and without whom I would never have completed this work or developed a great love for academic research. He has encouraged me towards greater scholarship and has taught me how to critically analyze data, question findings, and formulate thinking in the realm of science. Brian Kilburn has been both a friend and guide to me through almost all of my experimental techniques and has provided incredible support in finishing my projects. He has been an inspiration to me on both a personal and professional level, and I will greatly miss both his companionship and unsurpassed technical skill in the lab. Along the way I have had the pleasure of working with many people: Mike Kruger, Anelia Petkova, Po Jen Chiang, Sally Madsen-bouterse, Alex Harvey, Catherine Dupont, Tiffini Gibson, Katie Dennis, Ashok Kumar, Dr. Terri Woodard, Xiaoyu Jiang, and the staff of the Perinatology Research Branch headed by Dr. Roberto Romero. I am also indebted to my pre-doctoral Intramural Research Training Award fellowship and the wonderful individuals that I have met at the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), NIH, particularly Dr. Alan DeCherney. HTR-8/SVneo cells were generously provided by Dr. Charles Graham of Queens University.

This work is supported, in part, by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, DHHS and NIH Grants U54HD040093 and HD045966.

## TABLE OF CONTENTS

Dedication	ii
Acknowledgments	iii
List of Tables	x
List of Figures	xi
List of Abbreviations	xiii
Chapter 1. Background: Diverse Functions of HBEGF During Pregnancy	1
1. Introduction	1
1.1 Early Cyclical Regulation of HBEGF in the Endometrium	4
1.2. The Role of HBEGF at Implantation	8
1.3. The Role of HBEGF in Human Placentation	14
1.4. Looking Ahead	24
Chapter 2. Function-Specific Intracellular Signaling Pathways Downstream of Heparin-Binding EGF-like Growth Factor Utilized by Human Trophoblasts	26
Summary	26
Introduction	28
Materials and Methods	32
Cell Culture & Reoxygenation Injury	32

Cell Death Assay_____	34
Migration Assay_____	34
Western Blotting_____	35
Immunocytochemistry_____	36
ELISA_____	36
Statistics_____	36
Results_____	38
HBEGF activates multiple signaling pathways_____	38
HBEGF induces differentiation using multiple signaling pathways____	42
HBEGF prevents apoptosis using the MAPK14 pathway_____	42
Low O <sub>2</sub> increases synthesis of HBEGF through autocrine induction of MAPK14, ERK or JNK_____	45
Discussion_____	53
Chapter 3. Post-Transcriptional Regulation of Integrin Switching by Growth Factor Signaling in Human Extravillous Trophoblast Cells_____	59
Summary_____	59
Introduction_____	61
Materials and Methods_____	65
Cell Culture and Treatments_____	65

Western Blotting	65
Immunocytochemistry	66
ELISA	66
qPCR	66
Statistics	67
Results	68
Integrin Switching Induced by MG and HBEGF	68
Integrin Switching Induced by MG is not Dependent upon HBEGF Signaling	68
HBEGF Restores Integrin Switching On Growth Factor-reduced MG	70
HBEGF Induces Integrin Switching Through Multiple Signaling Pathways	74
Post-Transcriptional Regulation of Integrin Switching	74
Discussion	78
Chapter 4. MMP2 Controls Low Oxygen-Induced Autocrine HBEGF Signaling in Human First Trimester Trophoblast Cells	82
Summary	82
Introduction	84
Materials and Methods	87
Cell Culture and Treatments	87



Western Blotting	87
ELISA	87
qPCR	88
mRNA Microarray	88
Statistics	89
Results	90
O <sub>2</sub> regulates HBEGF through HIF1 $\alpha$ and HIF2 $\alpha$	90
New Transcription is Required to Increase HBEGF	90
O <sub>2</sub> Regulates Transcription of Metalloproteinases	95
MMP2 is Required to Increase HBEGF	98
Discussion	100
Chapter 5. Translational Regulation of HBEGF in Developing Human Trophoblast Cells	106
Summary	106
Introduction	108
Materials and Methods	112
Cell Culture and Treatments	112
qPCR	112

Western Blotting	113
ELISA	113
Generation of 3'UTR Luciferase Reporter Vectors	113
Bacterial Transformation	116
Transfection and Luciferase Assay	117
Custom TaqMan miRNA qPCR Plates	118
Bioinformatics	119
DGCR8 Knockdown	119
Statistics	120
Results	121
HBEGF mRNA is stable at 20% and 2% O <sub>2</sub>	121
HBEGF Protein Accumulation is Not Regulated by Turnover Rate	123
Translational Regulatory Activity of the HBEGF 3'UTR	123
Global Inhibition of miRNA Processing	125
Differential Expression of miRNAs Predicted to Target the HBEGF 3'UTR	127
Discussion	132
Chapter 6. Conclusion	138

References	141
Abstract	184
Autobiographical Statement	186

## LIST OF TABLES

Table 2.1. Inhibitors and inactive structural analogs used in this study_____	33
Table 2.2. ELISA quantification of HBEGF protein upregulation at 2% O <sub>2</sub> in the presence of signaling inhibitors_____	52
Table 4.1. Upregulation of HBEGF protein by CoCl <sub>2</sub> requires new transcription____	94
Table 4.2. Transcription of metalloproteinases at 2% O <sub>2</sub> in HTR-8/SVneo cells as detected by microarray_____	96
Table 4.3. Expression of metalloproteinases at 2% O <sub>2</sub> in HTR-8/SVneo cells measured by qPCR_____	97
Table 4.4. Inhibition of MMP2 prevents HBEGF upregulation at 2% O <sub>2</sub> _____	99
Table 5.1. Primer sequences for amplification of HBEGF 3'UTR and vector sequencing_____	115
Table 5.2. MiRNAs that target HBEGF_____	129

## LIST OF FIGURES

Figure 1.1. HBEGF Processing and Signaling_____	3
Figure 1.2. Juxtacrine Functions of HBEGF_____	12
Figure 1.3. HBEGF Stimulation of Trophoblast Invasion_____	19
Figure 1.4. HBEGF and Changing O <sub>2</sub> Levels in the Placenta_____	20
Figure 2.1. Identification of signaling pathways activated by HBEGF_____	39
Figure 2.2. Identification of signaling pathways activated by HBEGF using immunocytochemistry_____	40
Figure 2.3. Characterization of kinase inhibitors by western blotting_____	41
Figure 2.4. Differentiation stimulated by HBEGF_____	43
Figure 2.5. Signaling pathways required for HBEGF induction of extravillous differentiation_____	44
Figure 2.6. Signaling pathways required for HBEGF inhibition of apoptosis_____	46
Figure 2.7. Signaling pathways required for HBEGF inhibition of apoptosis_____	47
Figure 2.8. Signaling pathways required for inhibition of apoptosis at 2% O <sub>2</sub> _____	48
Figure 2.9. Signaling pathways required for increased synthesis of HBEGF during low O <sub>2</sub> _____	49
Figure 2.10. Signaling pathways required for increased synthesis of HBEGF during low O <sub>2</sub> _____	51
Figure 3.1. Integrin switching induced by HBEGF and MG_____	69
Figure 3.2. Inhibition of HBEGF signaling does not block integrin switching induced by MG_____	71

Figure 3.3. CTB cells do not invade GFR-MG	72
Figure 3.4. HBEGF induces integrin switching in CTB cells cultured on GFR-MG	73
Figure 3.5. Integrin switching induced by HBEGF is blocked by downstream signaling inhibitors	75
Figure 3.6. ITGA1 and ITGA6 are regulated post-transcriptionally	77
Figure 4.1. Stabilization of HIF1 $\alpha$ and HIF2 $\alpha$ by CoCl <sub>2</sub>	91
Figure 4.2. Upregulation of cellular and secreted HBEGF protein after treatment with CoCl <sub>2</sub>	92
Figure 4.3. Upregulation of HBEGF protein requires new transcription	93
Figure 5.1. HBEGF mRNA stability	122
Figure 5.2. HBEGF protein is stabilized by 2% O <sub>2</sub> but destabilized at 20% O <sub>2</sub>	124
Figure 5.3. Dual luciferase assay demonstrating repression of mRNA translation by the HBEGF 3'UTR	126
Figure 5.4. Western blot demonstrating siRNA knockdown of DGCR8	128
Figure 5.5. Differential expression of miRNAs in CTB cells	130
Figure 5.6. Map of HBEGF 3'UTR regions regulated by oxygen	131

## ABBREVIATIONS

EGF: epidermal growth factor

HBEGF: heparin-binding EGF-like growth factor

MG: Matrigel basement membrane

GFR-MG: growth factor-reduced Matrigel

CoCl<sub>2</sub>: cobalt chloride

HIF: hypoxia inducible factor

HRE: hypoxie responsive element

MMP: matrix metalloproteinase

ADAM: a disintegrin and metalloproteinase

HLA-G: major histocompatibility complex, class I, G

AKT: protein kinase B

PIK3: phosphoinositide-3-kinase

JNK: JUN N-terminal kinase; MAPK8

H/R: hypoxia/reoxygenation

hCG: human chorionic gonadotropin

SDS: sodium dodecyl sulfate

ITGA: integrin alpha ( $\alpha$ ) subunit

ITGB: integrin beta ( $\beta$ ) subunit

VEGF: vascular endothelial growth factor

BSG: basigin/CD147/EMMPRIN

TNFA: tumor necrosis factor alpha ( $\alpha$ )

TGFB: transforming growth factor beta ( $\beta$ )

HOXA10: homeobox A10

HGF: hepatocyte growth factor

PIGF: placental growth factor

PDGF: platelet-derived growth factor

bFGF: basic fibroblast growth factor

BTC: betacellulin

AREG: amphiregulin

TGFA: transforming growth factor- $\alpha$

LIF: leukemia inhibitory factor

IGF: insulin-like growth factor

IGFBP1: IGF binding protein 1



EVT: extravillous trophoblast

CTB: cytotrophoblast

STB: syncytiotrophoblast

E2: estrogen

P4: progesterone

ERBB: epidermal growth factor receptor/

HSPG: heparin sulfate proteoglycan

ELISA: enzyme-linked immunosorbent assay

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

MAPK: mitogen-activated protein kinase

uORF: upstream open reading frame

ARE: AU-rich element

miRNA: microRNA

UTR: untranslated region

RISC: RNA-induced silencing complex

miRNP: miRNA ribonucleoprotein complex

AGO: argonaute protein

FAK: focal adhesion kinase

PE: preeclampsia

PKC: protein kinase C

CRM197: cross-reactive material 197; non-toxic variant of diphtheria toxin

BCL2: B-cell CLL/lymphoma 2

uPA/uPAR: urokinase-type plasminogen activator/uPA receptor

## CHAPTER 1. Background: Diverse Functions of HBEGF During Pregnancy

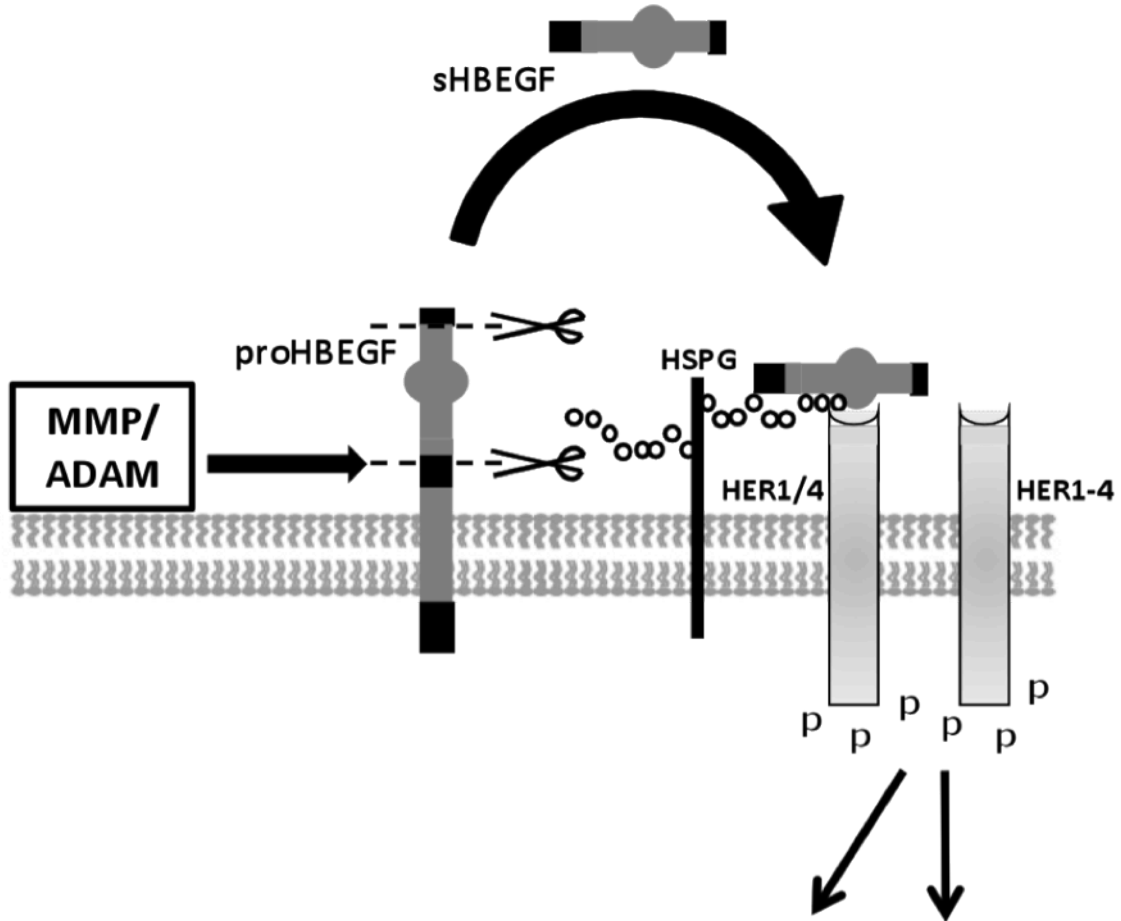
### 1. Introduction

Each month, the uterus prepares to receive a viable blastocyst under the direction of estrogen (E2) and progesterone (P4). The sex steroid hormones induce many changes in the endometrium that enhance its receptivity to an implanting blastocyst (Dey et al., 2004). Among these changes is the expression of secreted and membrane-bound signaling factors that influence embryo adhesion necessary for attachment to the luminal lining and later for invasion into the decidualized stroma. The embryo is also a source of several important factors, some of which are produced to augment the preparation of the endometrium. The concurrent developmental programs of the embryo and endometrium influence each other to synchronize their progression and produce an optimal environment for implantation. One critical factor that appears around the time of implantation is heparin-binding epidermal growth factor (EGF)-like growth factor (HBEGF). Evidence supporting a central role for HBEGF during implantation in mice is abundant and was recently reviewed (Lim and Dey, 2009). The cellular processes and regulatory factors that mediate the developmental program during blastocyst implantation have also been reviewed previously (Armant, 2005; Armant et al., 2000). The focus of this article is on the many timely influences of HBEGF and the EGF signaling system upon trophoblast cells in the peri- and post-implantation environment as pregnancy is established.

HBEGF and other members of the EGF family are synthesized as transmembrane proteins (e.g., proHBEGF) that can signal to their receptors on adjacent

cells (juxtacrine) or become secreted through the shedding activity of metalloproteinases that cleave the extracellular domain (Riese and Stern, 1998). The secreted form (e.g., sHBEGF) is free to bind receptors on the same cell (autocrine) or distant cells (paracrine). Processing of proHBEGF to sHBEGF and subsequent signaling through its receptors is illustrated in Fig. 1.1 EGF family growth factors, operating through four HER/erbB receptor tyrosine kinases, induce receptor dimerization and autophosphorylation, leading to downstream signaling that involves an extensive array of pathways (Holbro and Hynes, 2004a). HBEGF requires heparan sulfate proteoglycan (HSPG) as a cofactor for binding to its receptors, HER1 (a.k.a. EGF receptor) and HER4. Although HBEGF does not bind directly to HER2 or HER3, they can participate in downstream signaling through heterodimerization with other ligated HER proteins.

HBEGF performs a variety of functions in different cell systems (Raab and Klagsbrun, 1997). In its transmembrane form, proHBEGF appears to bind HER1 or HER4 of adjacent cells, participating in cell attachment or eliciting further signaling (Higashiyama et al., 1995; Raab et al., 1996). HBEGF induces chemotaxis and mitosis of NIH 3T3 cells through HER1 signaling (Elenius et al., 1997). Using a variety of carcinoma cell lines, it was shown that p53 expression induced by DNA damage subsequently upregulates HBEGF protein, which utilizes both MEK/ERK and PI3K/Akt pathways to inhibit cellular apoptosis (Fang et al., 2001). It also inhibits apoptosis in ovarian cancer, breast cancer, gastric cancer, melanoma and glioblastoma cells by activating HER1 and downstream ERK (Yotsumoto et al., 2008). Many of the diverse



**Figure 1.1 HBEGF Processing and Signaling.** In its transmembrane form, proHBEGF is proteolytically cleaved by metalloproteinases at the cell surface to remove (scissors) its pro-domain and secrete the extracellular domain (sHBEGF shedding). sHBEGF is then free to diffuse to nearby receptor tyrosine kinases where it can bind either HER1 (EGF receptor) or HER4. Receptor binding requires heparin sulfate proteoglycan (HSPG) as cofactor, and induces HER homo- or heterodimerization. Receptor autophosphorylation initiates downstream intracellular signaling. HSPG (heparin sulfate proteoglycan); HER (human epidermal growth factor receptor family member); MMP (matrix metalloproteinase); ADAM (a disintegrin and metalloproteinase)

cellular activities regulated by HBEGF transpire in the course of embryonic development as the placental tissues emerge.

### 1.1. Early Cyclical Regulation of HBEGF in the Endometrium

#### *Rodents*

It was first determined that HBEGF plays a central role in the establishment of pregnancy in experiments conducted using mice and rats. HBEGF expression is regulated in the mouse endometrium by leukemia inhibitory factor (LIF) (Song et al., 2000), a cytokine required for implantation (Stewart et al., 1992), as well as by P4 and E2 (Wang et al., 1994). LIF is necessary for the expression of HBEGF, amphiregulin (AREG), and epiregulin (EREG) mRNA in the luminal epithelium of the mouse uterus surrounding a newly implanted blastocyst, as evaluated in LIF<sup>-/-</sup> mice (Song et al., 2000). In both mice and rats, E2 upregulates HBEGF mRNA and protein levels in the luminal epithelium, whereas P4 and E2 together upregulate its expression in the stroma (Wang et al., 1994; Zhang et al., 1994a; Zhang et al., 1994b). This indicates that HBEGF mRNA is transcribed in vivo under conditions that normally stimulate cellular proliferation in the endometrium (Zhang et al., 1994b). It is important to note, however, that this hormonal regulation of HBEGF in the non-pregnant uterus does not necessarily trigger its expression during pregnancy, but may prepare the endometrium to respond to additional signals that induce HBEGF expression in specific cell populations.

The regulation of HBEGF in stromal cells suggests that it has a unique role in preparing the uterus for decidualization, in which stromal cells accumulate lipid and glycogen vacuoles and increase their secretion of a fibrous matrix. Near the implanting

blastocyst, there is an increase in vascular permeability and the expression of genes such as Bmp-2, Fgf-2, and Wnt-4 (Abrahamsohn and Zorn, 1993; Farrar and Carson, 1992; Paria et al., 2001). Beads coated with HBEGF can induce local increases in vascular permeability and upregulate expression of Bmp-2, suggesting that HBEGF participates in preparing the uterus to receive an implanting blastocyst (Paria et al., 2001). In cultured mouse stromal cells, HBEGF induces decidualization-like changes, including the upregulation of Cyclin D3 and the induction of stromal cell polyploidy (Tan et al., 2004).

#### *Humans & Non-human Primates*

HBEGF appears to regulate the endometrial cycle in other mammals, including humans and non-human primates. In contrast to rodents, in which sex steroids prepare the uterus for eventual HBEGF expression, E2 and P4 stimulate HBEGF expression in human endometrium prior to the appearance of a blastocyst (Lessey et al., 2002a), and, in baboons, it is under the control of P4 (Leach et al., 2001). As a result of its regulation by steroids, HBEGF accumulates in a cyclical fashion. During the late proliferative phase until the early secretory phase of the menstrual cycle in humans, HBEGF is predominantly localized in the stromal compartment of the endometrium (Chobotova et al., 2002a; Leach et al., 1999b; Yoo et al., 1997a). Although transcript levels are present in the luminal and glandular epithelia, protein levels do not appreciably accumulate at this time (Leach et al., 1999b; Yoo et al., 1997a). These findings are consistent with evidence from baboons where protein and message levels rise in the stroma during the proliferative phase and decline by day 5 post-ovulation (Leach et al., 2001). The correlation of HBEGF with E2 levels is strengthened by ex vivo evidence

demonstrating that HBEGF transcription is induced by E2 in stromal cells. Proliferative phase human stromal cells, when treated with either E2 or P4, upregulate transcription of HBEGF, though if treated with both steroids together, expression will appear in both stroma and the luminal epithelial cells (Lessey et al., 2002a). When interpreted in light of the menstrual cycle, this data suggests that the high E2 levels prior to ovulation induce HBEGF expression in stromal cells.

In the early secretory phase, HBEGF expression is induced in the luminal epithelium by the combination of E2 and P4 present at that time. Consistent with this idea, HBEGF expression decreases in the stromal compartment during the early secretory phase, coincident with decreasing E2 levels, but increases in both luminal and glandular epithelial cells (Leach et al., 1999b; Yoo et al., 1997a). HBEGF mRNA levels peak in the luminal and glandular epithelia during the mid secretory phase, just prior to the “window of implantation” (Yoo et al., 1997a). Protein levels follow shortly thereafter, rising in the early secretory phase and peaking during the window of implantation (days 19-22) when the uterus is optimally receptive to a blastocyst (Leach et al., 1999b). Baboons display a similar pattern in which HBEGF protein levels are highest 5 to 10 days post-ovulation (Leach et al., 2001). In rhesus monkeys, HBEGF increases during the proliferative phase, peaks during the window of implantation, and declines thereafter (Yue et al., 2000). Its maximal expression during the mid secretory phase supports the view that HBEGF is a key regulator of blastocyst implantation. Indeed, HBEGF is localized on the apical surface of human luminal epithelial cells and on the surface of pinopodes when it is at maximal levels in the human endometrium (Stavreus-Evers et al., 2002; Yoo et al., 1997a).



The decidualization reaction in humans is completely under the control of the sex steroids (Kodaman and Taylor, 2004), as opposed to rodents that have an estrous cycle that depends upon cues from the embryo (Dey et al., 2004). The human menstrual cycle prepares the uterus for implantation prior to arrival of an embryo, beginning in the early secretory phase, and reaching prominence by the window of implantation. In humans, several stimuli that induce stromal cell decidualization appear to do so through upregulation of HBEGF. If stromal cells are treated with 8-Br-cAMP, which is known to artificially induce decidualization, the soluble form of HBEGF is upregulated, as are its receptors, HER1 and HER4 (Chobotova et al., 2005). HBEGF then induces stromal cell production of prolactin and insulin-like growth factor binding protein-1, which naturally induce decidualization (Chobotova et al., 2005). Important for decidualization, HBEGF stimulates stromal cell growth via HER1, though not through HER4 (Chobotova et al., 2002a). The proliferative activity of HBEGF could be the result of its upregulation of IL-11 secretion (Karpovich et al., 2003). When stromal cells are treated with transforming growth factor- $\beta$  (TGFB) or tumor necrosis factor- $\alpha$  (TNFA), HBEGF expression is induced, which subsequently protects against apoptosis (Chobotova et al., 2005). Both the transmembrane and soluble forms of HBEGF are induced by these treatments, suggesting that HBEGF could be acting in an autocrine, paracrine or juxtacrine fashion as a survival factor (Chobotova et al., 2005). In addition, TNFA synergizes with HBEGF to stimulate cell growth by upregulating EGFR expression in stromal cell membranes (Chobotova et al., 2002a). More work is needed to determine which pathways are involved downstream of HBEGF signaling in stromal cells.

Decidualization and cytoprotection are only two of the important roles for HBEGF in the endometrium. HBEGF or conditioned medium from E2- and P4-treated stromal cells upregulates ITGB3, LIF and homeobox A10 (HOXA10) mRNA in primary human luminal epithelial cells (Liu et al., 2007) and  $\beta_3$  integrin mRNA in Ishikawa cells (Lessey et al., 2002a). Each of the corresponding proteins contributes to a uterine luminal epithelial surface that is receptive to an implanting blastocyst. ITGAV/ITGB3 serves as an attachment factor for osteopontin (secreted phosphoprotein 1, SPP1). SPP1 and ITGAV/ITGB3 are localized to both the glandular and luminal epithelia during the mid to late secretory phase, presumably to mediate embryo attachment (Apparao et al., 2001; Brown et al., 1992; Carson et al., 2002; Kao et al., 2002; Nardo et al., 2002; von Wolff et al., 2001). LIF stimulates human embryo development to the blastocyst stage (Sargent et al., 1998). In mice, LIF expression in the uterus is essential for embryo implantation (Stewart et al., 1992), as is uterine expression of HOXA10 (Satokata et al., 1995). HOXA10 message and protein are both found in the human endometrium most abundantly during the mid-secretory phase (Cermik et al., 2001; Gui et al., 1999; Li et al., 2002; Taylor et al., 1998). It has been suggested that HOXA10 induction by P4 during the window of implantation leads to a block in the stromal cell cycle, facilitating decidualization (Qian et al., 2005). Thus, the stimulatory effects of HBEGF upon stromal cells appear to be important not only in decidualization, but also in the preparation of the uterine luminal epithelium for an attaching blastocyst.

## 1.2. The Role of HBEGF at Implantation

### *Rodents*

Expression of HBEGF in the pregnant mouse uterus is necessary for the timely and successful implantation of a blastocyst (Xie et al., 2007). Evidence suggests that HBEGF expression during pregnancy is not induced by sex steroid, as in the non-pregnant uterus (Wang et al., 1994), but by the presence of a blastocyst (Das et al., 1994c). HBEGF mRNA is expressed in the uterine epithelium exclusively at the site of implantation 6-7 hr before attachment occurs, followed by an accumulation of the protein during attachment (Das et al., 1994c). Physiological interactions between the endometrium and blastocyst can be examined by experimentally-induced delayed implantation in ovariectomized mice treated with P4 (Yoshinaga and Adams, 1966). HBEGF expression does not occur during delayed implantation until the blastocyst is activated by administration of E2 (Das et al., 1994c), suggesting that the blastocyst induces HBEGF expression in the uterine luminal epithelium. Evidence from the hamster shows that HBEGF mRNA is expressed throughout the apical surface of the luminal epithelium prior to blastocyst hatching and implantation (Mishra and Seshagiri, 2000), but becomes localized to the epithelium surrounding an implanted blastocyst late on day 4 of gestation (Wang et al., 2002).

Several lines of investigation suggest that embryo-derived HBEGF is at least one of the signals that direct peri-implantation expression of HBEGF in the uterus. Indeed, mouse and hamster blastocysts express HBEGF (Hamatani et al., 2004; Leach et al., 2004a; Wang et al., 2002). Further confirmation comes from an experiment using beads coated with HBEGF that were placed in the uterus of pseudopregnant mice mated with vasectomized males (Paria et al., 2001). In this experiment, the HBEGF specifically induced endogenous uterine HBEGF gene expression, along with a local

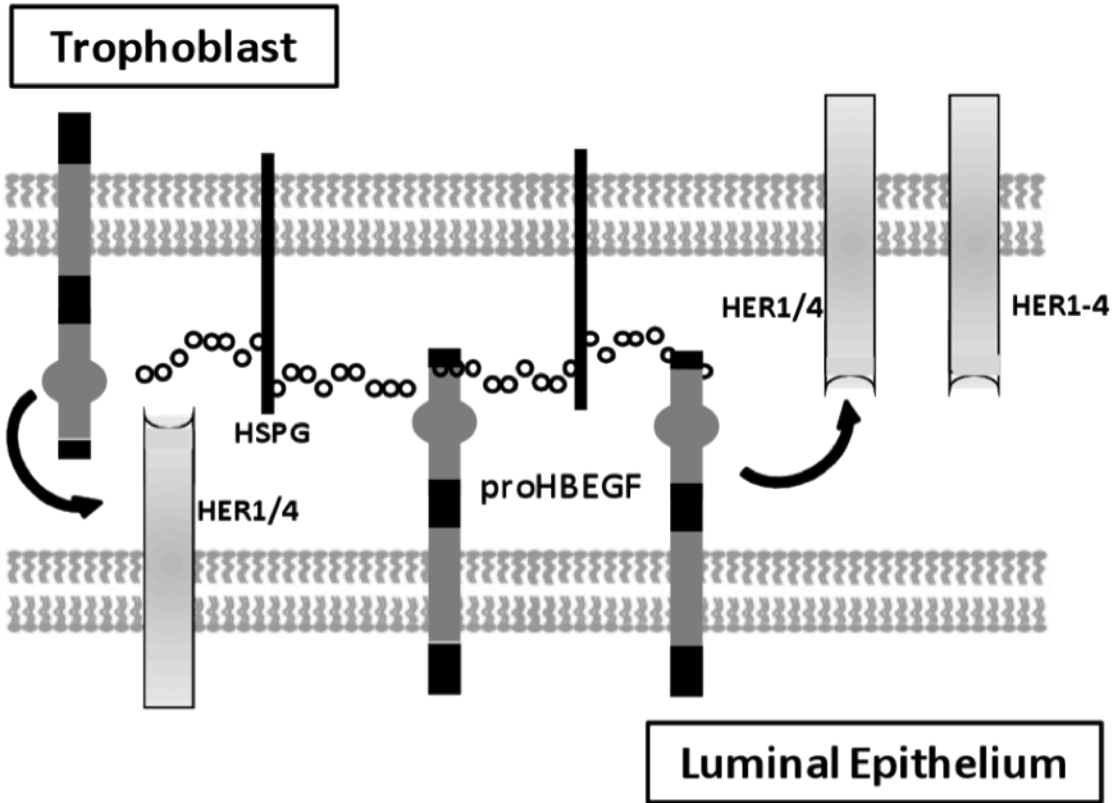
increase in vascular permeability and decidualization, comparable to changes arising from the presence of a blastocyst (Paria et al., 2001). Experiments using HBEGF-coated beads suggest that HBEGF produced by embryos induces the molecular and physiological changes in the uterus. These findings support the hypothesis that cross talk mediated temporally by HBEGF occurs between embryonic and maternal tissues in rodents. Its expression in the early preimplantation blastocyst (Leach et al., 2004a; Wang et al., 2002) implies that trophoctoderm-derived HBEGF could initially induce uterine HBEGF expression, along with other decidualization-related changes. Subsequently, uterine HBEGF secretion could advance trophoblast differentiation to an invasive phenotype and augment implantation. Additional work is required to distinguish the contribution of HBEGF from both of these sources and substantiate this hypothesis.

Evidence that HBEGF can advance the developmental program of the blastocyst is abundant and suggests that reciprocal signaling could exist between the embryo and uterus. Exogenous application of recombinant HBEGF matures rodent blastocysts in vitro, augmenting their ability to hatch from the zona pellucida, and accelerating trophoblast differentiation and adhesion competency (Das et al., 1994c; Wang et al., 2000). HBEGF increases the rate of hamster blastocyst hatching, adhesion competency, and trophoblast outgrowth (Mishra and Seshagiri, 2000; Seshagiri et al., 2002) and augments development of 8-cell rat embryos to the blastocyst stage (Tamada et al., 1999). Intraluminal injection of HBEGF induces implantation of rat embryos in a delayed implantation model (Tamada et al., 1999). Moreover, exposure of mouse embryos to HBEGF during culture before transferring blastocysts to the uterus

significantly increases the number of implantation sites that eventually form (Lim et al., 2006).

In mice, HER4 trafficks to the trophoctoderm surface late on day 4 and is a prerequisite for HBEGF signaling that accelerates trophoblast development (Wang et al., 2000). HBEGF can then bind HER1 and HER4 (preferentially to HER4) on the surface of mouse blastocysts in a HSPG-dependent fashion, leading to HER autophosphorylation (Das et al., 1994a; Lim et al., 2006; Paria et al., 1999; Wang et al., 2000). Lysophosphatidic acid also accelerates trophoblast outgrowth, and does so by transactivating HER1 and HER4 through  $Ca^{2+}$ -dependent HBEGF trafficking and shedding (Leach et al., 2004a). Cross-talk with other signaling pathways that advance blastocyst differentiation could similarly target HBEGF shedding to transactivate HER kinases. HBEGF signaling accelerates mouse blastocyst differentiation to an adhesive phenotype via  $Ca^{2+}$  influx through N-type voltage-gated channels and activation of protein kinase C and calmodulin (Wang et al., 2000). This signaling pathway induces trafficking of the integrin  $\alpha_5$  subunit to the surface of trophoctoderm cells where it mediates strong adhesion to fibronectin (Armant, 2005; Wang et al., 2000).

In addition to its ability to advance hatching and trophoblast differentiation, proHBEGF could directly mediate attachment of the trophoctoderm to the luminal surface of the endometrium (Fig. 1.2). Cells engineered to express proHBEGF bind to day 4 mouse blastocysts, but not to delayed blastocysts (Raab et al., 1996), suggesting that proHBEGF, by binding to its receptors, is capable of supporting the attachment reaction during implantation. Binding between these cells and normal day 4 blastocysts is blocked by treating the blastocysts with heparinase or a synthetic peptide



**Figure 1.2 Juxtacrine Functions of HBEGF.** Transmembrane proHBEGF is depicted mediating attachment between the trophoblast cell of a blastocyst and the surface of the uterine luminal epithelium. HBEGF can potentially bind HSPG, HER1 or HER4, to both mediate cell adhesion and elicit juxtacrine signaling downstream of HER kinases.

corresponding to the HSPG-binding domain of HBEGF (Raab et al., 1996). Furthermore, its potential binding partners HSPG, HER1 and HER4 are downregulated during delayed implantation, and are coordinately upregulated with HBEGF during activation with E2 (Paria et al., 1993; Paria et al., 1999; Smith et al., 1997). These reports provide intriguing information about the elusive mechanisms underlying diapause.

### *Humans*

In contrast to the rodent endometrium, which may require stimulation from an embryo for HBEGF expression (Paria et al., 2001), HBEGF expression in the human endometrium precedes the appearance of the blastocyst (Leach et al., 1999b). As in other species, HBEGF can simultaneously function during human implantation as an attachment factor and a growth factor. In humans, HBEGF, HER1 and HER4 are present in peri-implantation blastocysts with HER4 more prominent in the trophoctoderm (Chobotova et al., 2002b). This suggests that HBEGF present on the endometrium might bind its receptors on an apposed blastocyst, and vice versa. Indeed, mature human blastocysts adhere to immobilized proHBEGF, an interaction that is competitively inhibited by addition of sHBEGF (Chobotova et al., 2002b). Similar results were obtained when human blastocysts were applied to a fixed monolayer of CHO cells overexpressing proHBEGF. As a growth factor, HBEGF accelerates development of human embryos to the blastocyst stage and their subsequent hatching from the zona pellucida (Martin et al., 1998b; Sargent et al., 1998). While LIF can only induce development of embryos up to the blastocyst stage, HBEGF influences their maturation at least through the hatching stage (Sargent et al., 1998). Although these

studies are instructive, it remains to be demonstrated whether these diverse functions of HBEGF are imperative in vivo.

### **1.3. The Role of HBEGF in Human Placentation**

HBEGF is present throughout the course of human gestation. It is abundant in 1<sup>st</sup> trimester decidua, and in placental tissue during all three trimesters (Birdsall et al., 1996), though its presence in the syncytiotrophoblast (STB) layer is weak in first trimester placentas (Yoo et al., 1997a). HBEGF is also expressed in villous and extravillous cytotrophoblast (CTB) from Weeks 14-35 in normal placentas and those delivered pre-term (Leach et al., 1999b; Leach et al., 2002a). Several members of the EGF family and HER tyrosine kinases are expressed in placental trophoblast populations (Hofmann et al., 1992; Tanimura et al., 2004), indicating that the EGF signaling system is active during placental development.

#### *Structure of the Early Placenta*

As trophoblasts begin to invade into the endometrium, they differentiate along two paths (Hunkapiller and Fisher, 2008; Morrish et al., 1998). CTBs are highly proliferative, mononuclear cells that can fuse to form multinucleated STB cells. The STB initially invades into the endometrium. At the periphery of the conceptus, the chorionic villi emerge with a vascularized mesenchymal core and a stratified epithelium composed of CTB and an outer layer of STB. The STB transports nutrients, gases and waste products between the maternal blood and fetal blood in capillaries of the chorionic villi. A few weeks after implantation, anchoring villi appear at the surface of the endometrium (decidua basalis) as CTBs penetrate through the STB layer and



assemble into dense cellular columns. The most distal CTBs differentiate to an extravillous phenotype that invades interstitially. Upon reaching uterine blood vessels, the CTBs invade and remodel them, converting the spiral arteries into highly dilated vessels (Pijnenborg et al., 1983a). Endovascular trophoblast cells produce proteins characteristic of endothelial cells in a process known as *pseudovasculogenesis* (Blankenship and Enders, 1997; Zhou et al., 1997a; Zhou et al., 1997b). Invasive CTBs remodel the maternal spiral arteries into permanently dilated structures as far as the first third of the myometrium (Pijnenborg et al., 1983a). Maternal blood is thus directed to the intervillous space at increased flow rates (Hunkapiller and Fisher, 2008). As trophoblasts differentiate to extravillous and endovascular phenotypes, they undergo a process known as integrin-switching in which differentiation is accompanied by changes in expression of adhesion molecules (Damsky et al., 1994; Zhou et al., 1997a; Zhou et al., 1997b). Different integrins mediate attachment, invasion, and vascular remodeling. For example, ITGA6/ITGB4 is expressed by non-motile villous CTBs, while invasive interstitial trophoblast cells express ITGA1/ITGB1. CTBs of the columns in anchoring villi express  $\alpha_5\beta_1$  with differing levels of the other two integrins, depending on whether they have a proximal or distal position within the anchoring villi (Damsky et al., 1994). Primary CTB cultures from 1<sup>st</sup> trimester placentas undergo integrin switching during extravillous differentiation on Matrigel basement membrane, providing a useful experimental model.

### *Challenges of the Maternal Environment*

Blastocyst implantation takes place in a uterine environment low in O<sub>2</sub> (~18 mm Hg or 2%), a condition that appreciably changes (~60 mm Hg or 8%) only after the 10<sup>th</sup>

week of gestation (Burton and Jauniaux, 2004; Burton et al., 1999; Jauniaux et al., 2001b; Rodesch et al., 1992). Prior to the 10<sup>th</sup> week, invading endovascular trophoblasts occlude maternal blood vessels, leaving the intervillous space relatively hypoxic. Human trophoblast cells are programmed to function under these conditions, and, in fact, proliferate at higher rates when cultured at 2% O<sub>2</sub> (Genbacev et al., 1996b; Genbacev et al., 1997b). Furthermore, localized areas within the placenta are temporarily exposed to oxygenated maternal blood during this early developmental period, but they are able to survive these reoxygenation episodes (Hung and Burton, 2006). Perhaps more profound is the extensive introduction of fully oxygenated maternal blood that occurs around Week 10 as trophoblastic plugs become dislodged. The placental unit normally survives this insult and trophoblast invasion continues into the second trimester (Norwitz et al., 2001). Elevation of O<sub>2</sub> leads to free radical production, and evidence suggests that excessive oxidative stress may precipitate placental pathologies such as preeclampsia (Hung and Burton, 2006). Free radicals are normally deactivated in the placenta by xanthine dehydrogenase/xanthine oxidase, which is elevated in placentas during labor, when there is an abundance of local O<sub>2</sub> fluctuations (Many and Roberts, 1997). Clearly, mechanisms exist that protect trophoblasts from erratic O<sub>2</sub> levels.

#### *HBEGF Mediates Trophoblast Motility and Survival*

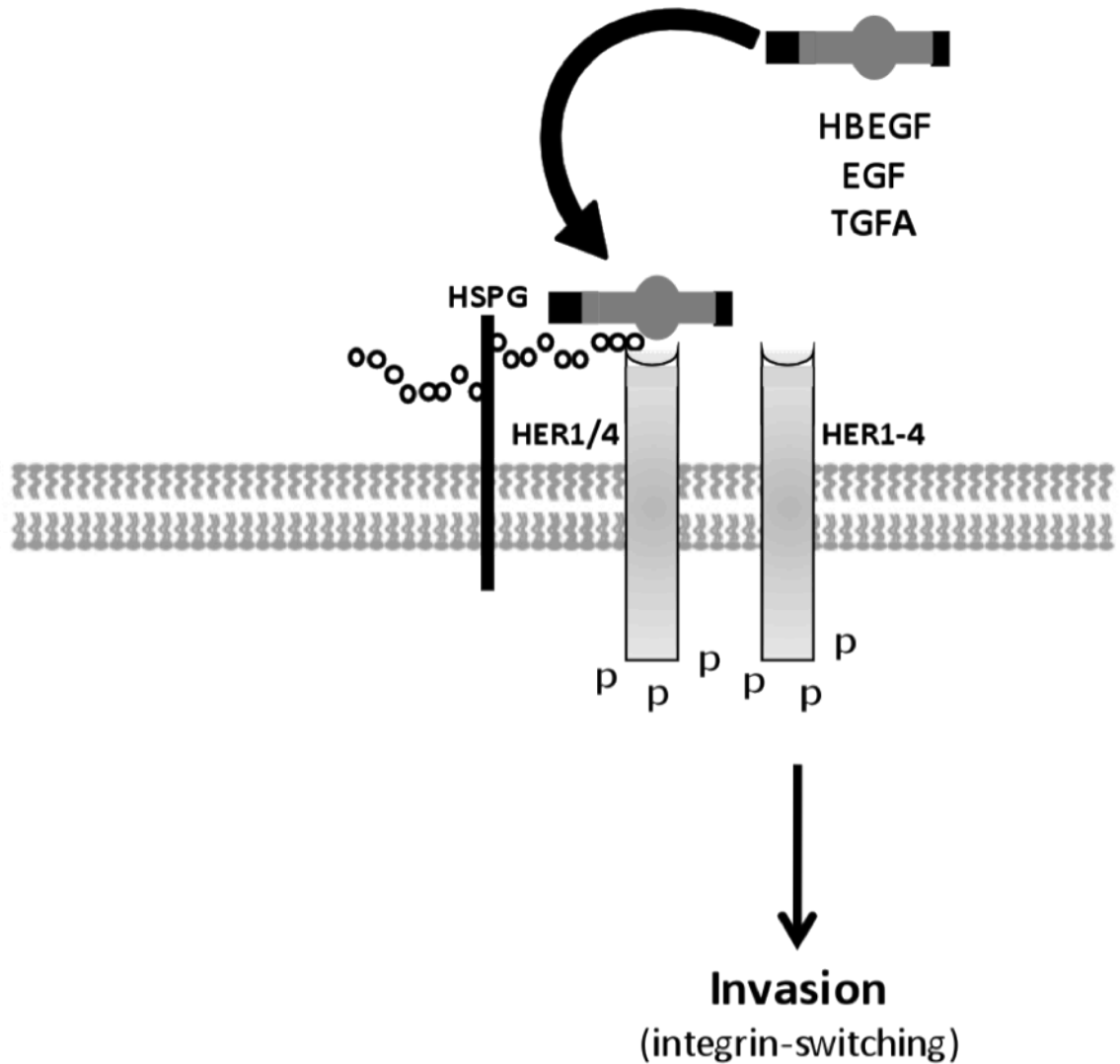
HBEGF stimulates trophoblast invasion from first trimester chorionic villous explants cultured on Matrigel basement membrane, but has little effect on CTB cell proliferation (Leach et al., 2004a). As gestation progresses, there is a decrease in trophoblast capacity to differentiate along the extravillous pathway (Damsky et al., 1994;

Librach et al., 1991). EGF augments the invasiveness of cultured first trimester CTBs, but with less effectiveness when CTBs are obtained later in gestation (Bass et al., 1994b). In HT-H cells (human embryonal carcinoma that spontaneously differentiate into trophoblasts), induction of HER1 phosphorylation by EGF or HBEGF requires derepression of the receptor by trophinin (Sugihara et al., 2007), a transmembrane protein expressed in both human trophoblasts and the uterine epithelium that is capable of mediating cell adhesion through homodimerization (Suzuki et al., 1999). When ligated, trophinin interaction with the cytoplasmic protein bystin is disrupted, releasing bystin inhibition of HER autophosphorylation (Sugihara et al., 2007). Therefore, HBEGF can activate HER downstream signaling only when opposing trophinin proteins are engaged by opposing cells. The relationship between the trophinin and EGF signaling systems could be particularly important for trophoblast invasive differentiation during blastocyst implantation.

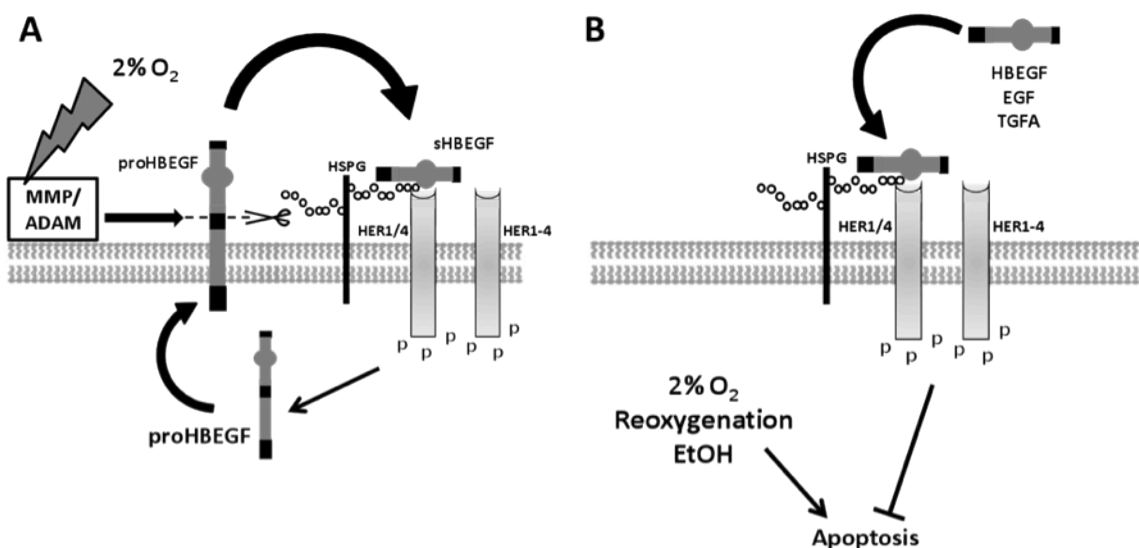
Cell lines generated from immortalized human cytotrophoblasts have proven to be highly useful for experimental investigation of implantation and early placentation. HTR-8/SVneo cells are an immortalized human CTB cell line originating from first trimester villous explants that characteristically secrete chorionic gonadotropin and become invasive in conjunction with appropriate integrin switching and upregulation of HLA-G (Graham et al., 1993b; Kilburn et al., 2000b). Therefore, HTR-8/SVneo cells closely resemble the primary CTB cells from which they are derived, providing a robust experimental model. HBEGF, EGF, and TGFA each induce integrin-switching in HTR-8/SVneo cells by signaling through HER1 or HER4 to increase the  $\alpha_1$  integrin subunit and decrease  $\alpha_6$  (Leach et al., 2004a). As a result, there is an increase in trophoblast

cell migration and invasive activity (Fig. 1.3). The extensive expression of HBEGF in trophoblasts, particularly within extravillous populations (Leach et al., 2002a), could be vital for their invasive activities during the establishment of pregnancy and physiological conversion of spiral arteries. As gestation continues and trophoblasts lose their capacity for extravillous differentiation (Damsky et al., 1994; Librach et al., 1991), the involvement of HBEGF and the EGF signaling system in trophoblast invasion wanes (Bass et al., 1994b). However, other roles for HBEGF could come into play late in gestation.

In vivo, placentation initiates in a relatively low  $O_2$  environment. Although CTBs proliferate faster at low  $O_2$  concentrations, invasion is more aggressive at higher  $O_2$  (Genbacev et al., 1996b; Genbacev et al., 1997b). Therefore, pseudovasculogenesis is initially moderate, and rapid CTB growth leads to occlusion of the spiral arteries. It has not been clear how trophoblast tissues survive during this hypoxic phase. We have observed that HBEGF, but not other EGF family members, is upregulated in the HTR-8/SVneo CTB cell line when exposed to 2%  $O_2$  (Armant et al., 2006). Using specific antagonists of HBEGF signaling, it can be demonstrated that HBEGF inhibits apoptosis due to low  $O_2$ , but has no effect on proliferation rates enhanced by low  $O_2$ . This study also established that cytoprotection requires metalloproteinase shedding of HBEGF and binding to either HER1 or HER4 (Fig. 1.4 A). Quantification of mRNA and protein levels indicated that HBEGF increases 100-fold through a post-transcriptional mechanism. Antagonizing HBEGF signaling prevents its upregulation, suggesting that low levels of resident proHBEGF are cleaved through activation of metalloproteinases in low  $O_2$  to



**Figure 1.3 HBEGF Stimulation of Trophoblast Invasion.** HBEGF activates HER kinases to enhance cytotrophoblast (CTB) motility and invasion. Other members of the EGF family, EGF (epidermal growth factor) and TGFA (transforming growth factor alpha) have similar capacities. Little is known about the responsible downstream signaling pathways, but there are associated changes in the expression of integrins ITGA6/ITGB4 and ITGA1/ITGB1 (integrin switching) by CTBs. Ligation of either HER1 or HER4 can induce integrin switching.



**Figure 1.4 HBEGF and Changing O<sub>2</sub> Levels in the Placenta.** In (A), HBEGF is secreted by first trimester CTBs cultured at low O<sub>2</sub>. Evidence suggests that low O<sub>2</sub> activates metalloproteinases that cleaves proHBEGF, releasing sHBEGF to signal through its receptors, HER1 or HER4. Downstream signaling activates a post-transcriptional mechanism to increase HBEGF synthesis. In (B), the cytoprotective activity of HBEGF in CTBs is depicted. Stress due to low O<sub>2</sub>, reoxygenation injury, or ethanol exposure increases CTB apoptosis. Accumulation of endogenous sHBEGF at low O<sub>2</sub> (as in A), or induction of the EGF signaling system by exogenous HBEGF, EGF or TGFA activates signaling downstream of HER kinases that inhibits apoptosis.

initiate its synthesis downstream of HER kinases. A recent study suggests that ADAM-17 may be responsible for initiating this signaling event *in vivo* (Hung et al., 2008). Further studies are needed to determine which metalloproteinases are directly responsible for HBEGF shedding in trophoblast cells. In contrast to 1<sup>st</sup> trimester CTBs, trophoblasts in villous explants from term placentas do not elevate HBEGF levels in response to low O<sub>2</sub> and their survival is compromised by low O<sub>2</sub> (Imudia et al., 2008). However, addition of HBEGF to term villous explants cultured at 2% O<sub>2</sub> inhibits trophoblast apoptosis, demonstrating its persistent role as a survival factor. These findings highlight the importance of HBEGF in the success of early placentation and in protecting trophoblast cells from the damaging effects of low O<sub>2</sub> encountered throughout gestation.

Using placental villous explants, Hung et al demonstrated that reactive oxygen species are generated in an *in vitro* reoxygenation injury model (Hung et al., 2001b), leading to apoptosis of the trophoblasts (Hung et al., 2002b). HBEGF can prevent apoptosis induced by reoxygenation injury in HTR-8/SVneo cells (Fig. 1.4 B) by signaling through its receptors, HER1 and HER4 (Leach et al., 2008a). Although HBEGF is downregulated within 30 min of elevating the O<sub>2</sub> concentration from 2% to 20%, accumulated sHBEGF generated by trophoblasts at low O<sub>2</sub> may protect against sudden exposure to oxygenated maternal blood in the uterine environment. Ethanol, a teratogenic substance with oxidative qualities, also induces apoptosis in HTR-8/SVneo cells (Wolff et al., 2007). When HTR-8/SVneo cells are exposed to ethanol and HBEGF together, apoptosis is inhibited in a HER1- or HER4-dependent fashion. During both reoxygenation injury and ethanol exposure, other members of the EGF family are

cytoprotective to varying degrees (Leach et al., 2008a; Wolff et al., 2007). In contrast, only HBEGF inhibits apoptosis in CTBs cultured at low O<sub>2</sub> (Armant et al., 2006). It has been proposed that insults such as reoxygenation injury are not always tolerated and can lead to placental pathologies (Hung and Burton, 2006). The activity of the EGF signaling system could be a critical variable that keeps oxidative stress in check.

### *Clinical Considerations*

Heparin has long been used to treat pregnant women with certain thrombophilias (Greer, 2003), but its impact on trophoblasts has only recently been investigated. Since heparan sulfate is an obligate cofactor for HBEGF, clinical application of heparin during pregnancy could influence the activity of this growth factor. Indeed, unfractionated heparin inhibits apoptosis induced by diverse signals in 1st trimester cytotrophoblast cells (Hills et al., 2006). The same downstream signaling pathways activated by heparin were also induced by treating the cells with HBEGF. It could be speculated that addition of its cofactor activates HBEGF in trophoblasts to enhance survival. On the other hand, fractionated heparin, also used as an anticoagulant, suppresses the invasive capacity of primary extravillous trophoblasts (Ganapathy et al., 2007). This suggests that there could be clinical advantages to using unfractionated heparin over fractionated heparin.

A pathological reduction of HBEGF expression is observed in placentas of patients with the hypertensive syndrome preeclampsia (Leach et al., 2002a), which is associated with decreased trophoblast invasion (Brosens et al., 1972b) and increased levels of trophoblast apoptosis (Allaire et al., 2000a; DiFederico et al., 1999b). This



finding led us to hypothesize that dysregulation of HBEGF and the resulting deficiencies in trophoblast function could contribute to preeclampsia, although it remains to be determined when during the course of gestation HBEGF levels are first reduced in preeclamptic pregnancies. Trophoblast invasion is shallow in preeclampsia possibly due to a lack of HBEGF-induced cell migration and a rise in apoptosis, exacerbated by reduced cytoprotection. The increased oxidative damage to trophoblast cells (Redman and Sargent, 2005) and the paucity of HBEGF in preeclamptic placentas is consistent with the hypothesis that HBEGF performs an important role as a survival factor throughout gestation. Patients delivering preterm without hypertensive disorder produce placentas with high levels of HBEGF (Leach et al., 2002a). However, there was an intermediate reduction of HBEGF in extravillous trophoblasts for those women who delivered small for gestational age infants. Intrauterine growth restriction, like preeclampsia, has been linked to aberrant trophoblast invasion (Khong et al., 1986) and elevated apoptosis (Ishihara et al., 2002), suggesting shared elements in the etiology of both disorders. Premature placental perfusion in preeclamptic pregnancies (Jauniaux et al., 2003) could induce apoptosis by stressing trophoblast cells before HBEGF has sufficiently accumulated during the early hypoxic period. Because there are varying levels of severity of placental reoxygenation, it has been hypothesized that preeclampsia may be the result of sub-lethal levels of reoxygenation, leading to abnormal placentation, whereas more pronounced reoxygenation will terminate the pregnancy (Leach et al., 2008a). More work is needed to determine if the etiology of this disease involves a lack of HBEGF production by trophoblasts or the activity of factors that suppress HBEGF signaling.

#### 1.4. Looking Ahead

HBEGF performs numerous functions during pregnancy that are conserved across mammalian species with divergent reproductive physiologies. In addition to preparing both the preimplantation embryo and uterus for their mutual interaction, HBEGF appears to directly facilitate the process of implantation. As development proceeds, HBEGF provides a stimulus for trophoblast invasion and serves as a critical survival factor in an environment subject to wide swings in oxygenation. Due to its roles in trophoblast cell differentiation and survival, dysregulation of HBEGF could have profound consequences, from complete pregnancy failure to the onset of obstetrical disorders. The association of its deficiency with preeclampsia is consistent with a critical role for HBEGF in human placentation. New insights into the contribution of HBEGF to placentation could be obtained with the mouse model, which has not been exploited. HBEGF-deficient transgenic embryos develop to term with heart defects (Iwamoto et al., 2003; Jackson et al., 2003), but this system has not been examined in depth for errors in placentation due to the absence of HBEGF. Additionally, the immune system undergoes unique changes during pregnancy that include tolerance of the fetal-maternal allograft and the recruitment of uterine natural killer cells, macrophages and dendritic cells to the implantation site. It is currently thought that these cells ensure proper implantation and that trophoblast cells help to orchestrate their immune function (Mor, 2008). However, the role of HBEGF in the trophoblast-immune relationship during pregnancy remains unexplored.

Tissue-specific differences in HBEGF function could stem from the microenvironment near targeted cells. Expression of its cofactor, HSPG, varies among

cells and with differentiation. Other growth factors, such as LIF, can profoundly affect HBEGF expression, as seen in the peri-implantation uterus. HBEGF forms complexes with other transmembrane proteins, including CD9 and ITGA3/ITGB1 (Raab and Klagsbrun, 1997), but the relevance of these interactions for implantation and placentation has not been investigated. There is a need for better understanding of the molecular interactions that regulate HBEGF expression, as well as the genes induced by HBEGF in reproductive tissues.

The ability of HBEGF to regulate diverse outcomes is not well understood. Autocrine regulation of HBEGF is post-transcriptional in CTBs cultured at reduced O<sub>2</sub> levels (Armant et al., 2006). One possibility is that HBEGF signaling releases its own message from translational repression. Maintenance of a dormant, stable HBEGF mRNA pool could provide a reserve for rapid mobilization during episodes of hypoxia without a high cost in energy. Although the exact nature of this post-transcriptional regulation is not yet understood, it would be useful to know whether HBEGF signaling similarly targets other proteins for synthesis. Finally, an examination of the intracellular circuitry downstream of the HER kinases activated by HBEGF will be important for clarifying the molecular basis of its varied functions.

## **CHAPTER 2. Function-Specific Intracellular Signaling Pathways Downstream of Heparin-Binding EGF-like Growth Factor Utilized by Human Trophoblasts**

### **SUMMARY**

Heparin-binding EGF-like growth factor (HBEGF) is expressed by trophoblast cells throughout gestation. First trimester cytotrophoblast cells are protected from hypoxia-induced apoptosis due to the accumulation of HBEGF through a post-transcriptional autocrine mechanism. Exogenous application of HBEGF is cytoprotective in a hypoxia/reoxygenation (H/R) injury model and initiates trophoblast extravillous differentiation to an invasive phenotype. The downstream signaling pathways induced by HBEGF that mediate these various cellular activities were identified using two human first-trimester cytotrophoblast cell lines, HTR-8/SVneo and SW.71, with similar results. Recombinant HBEGF (1 nM) induced transient phosphorylation of MAPK3/1 (ERK), MAPK14 (p38) and AKT within 15 minute, and JNK after 1-2 h. To determine which downstream pathways regulate the various functions of HBEGF, cells were treated with specific inhibitors of the ERK upstream regulator MEK (U0126), the AKT upstream regulator phosphoinositide-3 (PI3)-kinase (LY294002), MAPK14 (SB203580) and JNK (SP600125), as well as with inactive structural analogues. Only SB203580 specifically prevented HBEGF-mediated rescue during H/R, while each inhibitor attenuated HBEGF-stimulated cell migration. Accumulation of HBEGF at reduced O<sub>2</sub> was blocked only by a combination of U0126, SB203580 and SP600125. We conclude that HBEGF advances trophoblast extravillous differentiation through coordinate activation of PI3 kinase, ERK, MAPK14 and JNK, while only MAPK14 is required for its anti-apoptotic activity. Additionally, low O<sub>2</sub> induces an autocrine increase in HBEGF protein levels through MAPK14, JNK or ERK. These experiments reveal a complexity of the

intracellular signaling circuitry that regulates trophoblast functions critical for implantation and placentation.

## INTRODUCTION

Blastocyst implantation is a tightly regulated and dynamic process that establishes a pregnancy. Central to implantation are the trophoblast cells that populate the exterior of the blastocyst. These unique cells invade the endometrium interstitially and intravascularly (Pijnenborg et al., 1981), and can survive the changes in O<sub>2</sub> concentration that accompany early development of the placenta (Burton and Jauniaux, 2004). Early in this process, trophoblast cells function in a relatively hypoxic uterine environment, a condition that is drastically altered during the 10<sup>th</sup> week of pregnancy in humans when extravillous trophoblast cells occluding the maternal arteries dislodge, allowing highly oxygenated blood to enter the intervillous space within the developing placenta. Trophoblasts survive this oxidative challenge, and accelerate the pace of invasion (Norwitz et al., 2001). Oxygen fluctuations occur throughout pregnancy with great variation among individuals (Burton and Jauniaux, 2004). The elevation of O<sub>2</sub> after an ischemic episode can damage trophoblast cells due to the resulting oxidative stress (Hung et al., 2001a), possibly precipitating pathological outcomes (Hung et al., 2002a).

The epidermal growth factor (EGF) signaling system is capable of regulating diverse cellular activities, including survival, invasion and differentiation (Holbro and Hynes, 2004a; Riese and Stern, 1998). EGF-related growth factors are expressed abundantly in the receptive endometrium (Hofmann et al., 1991; Horowitz et al., 1993), with heparin-binding EGF-like growth factor (HBEGF) having a prominent role during peri-implantation development (Leach et al., 1999a; Lessey et al., 2002b; Yoo et al., 1997b). It is specifically expressed at the site of blastocyst attachment in mice, immediately prior to implantation (Das et al., 1994b), and appears cyclically in humans

at the apical surface of luminal epithelial cells during the period when the endometrium is most receptive for embryo implantation (Leach et al., 1999a). Conditional excision of HBEGF in the murine uterus delays blastocyst implantation and reduces litter sizes (Xie et al., 2007), suggesting that HBEGF is important not only for timely attachment of the blastocyst, but also for subsequent invasive events. Indeed, HBEGF accelerates the differentiation of mouse trophoblast cells to an adhesive phenotype (Wang et al., 2000), increasing the area over which they subsequently migrate (Das et al., 1994b). Similar stimulatory effects of HBEGF have been reported for human embryos (Martin et al., 1998a). HBEGF is implicated in both the successful invasion and survival of human cytotrophoblast cells (Armant et al., 2006; Leach et al., 2008a; Leach et al., 2004b). Members of the EGF family, including HBEGF, EGF and TGFA, are capable of inducing altered integrin expression and accelerating trophoblast migratory and invasive activity in first trimester human cytotrophoblast cells (Bass et al., 1994a; Leach et al., 2004b). EGF is capable of preventing cytokine-induced apoptosis in term cytotrophoblast and syncytiotrophoblast (Garcia-Lloret et al., 1996; Smith et al., 2002) and both EGF (Mackova et al., 2003) and HBEGF (Imudia et al., 2008) block apoptosis resulting from exposure to low O<sub>2</sub>. During the first trimester, trophoblast cells have the ability to survive and proliferate in the very low O<sub>2</sub> environment present at the implantation site (Genbacev et al., 1996a; Genbacev et al., 1997a). Investigation of their survival capacity revealed cytoprotective activity provided as HBEGF accumulates in cytotrophoblast cells when exposed to low O<sub>2</sub> tension (Armant et al., 2006).

Failure of trophoblast cells to survive and invade maternal tissues interferes with the remodeling of uterine spiral arteries required to increase blood flow to the growing

conceptus and is thought to contribute to preeclampsia (Allaire et al., 2000b; Brosens et al., 1972a; DiFederico et al., 1999a), intrauterine growth restriction (Ishihara et al., 2002; Khong et al., 1986) and spontaneous abortion (Burton and Jauniaux, 2004). The physiological interaction of trophoblast cells with O<sub>2</sub> during pregnancy is complex, with a preference for low levels during the first trimester. It has been hypothesized that fluctuations in O<sub>2</sub> during early pregnancy create hypoxia/reoxygenation (H/R) episodes that produces oxidative stress, which may compromise trophoblast survival (Hung and Burton, 2006; Hung et al., 2001a; Hung et al., 2002a). Indeed, activation of the EGF signaling system with HBEGF or related growth factors can prevent apoptosis due to oxidative stress caused by exposing first trimester cytotrophoblast cells to H/R (Leach et al., 2008a). Examination of placentas delivered by women with preeclampsia reveals a dramatic reduction in HBEGF expression (Leach et al., 2002b) and suggests the important role of this signaling system.

Though HBEGF appears to have many important functions in trophoblast cells, the underlying mechanisms have not yet been assessed. To this end, we have initiated experiments to identify intracellular signaling pathways that are responsible for the multiple outcomes of HBEGF signaling. Using two immortalized, human, first trimester cytotrophoblast cell lines, we have examined the downstream signaling circuitry that regulates the ability of HBEGF to autoregulate, induce migration and inhibit apoptosis. As with all EGF family ligands, HBEGF initially binds to and activates members of the HER/ERBB receptor tyrosine kinase family (Holbro and Hynes, 2004a; Riese and Stern, 1998). EGFR, ERBB2, ERBB4 (HERs 1, 2 and 4), but not ERBB3 (HER3), possess functional intracellular tyrosine kinase activities that, upon ligation and dimerization,



induce cross phosphorylation of their intracellular domains. Phosphorylated tyrosine residues then serve as docking sites for intracellular proteins that direct downstream signaling pathways, including phosphoinositide-3-kinase (PIK3) and MAPK cascades. Using inhibitors of the most common MAPK pathways and PIK3, we have examined their roles downstream of HBEGF in trophoblast cell extravillous differentiation, survival, and in the upregulation of HBEGF at low O<sub>2</sub>.

## MATERIALS & METHODS

### Cell Culture & Reoxygenation Injury

Two immortalized, first trimester, human cytotrophoblast cell lines, HTR-8/SVneo (Graham et al., 1993a) and SW.71 (Aplin et al., 2006), were cultured at 2% or 20% O<sub>2</sub>, as previously described (Leach et al., 2008a; Leach et al., 2004b). The HTR-8/SVneo cell line originates from first trimester villous explants and is immortalized by stably expressing the large T viral antigen (Graham et al., 1993a). The SW.71 cell line also originates from first trimester villous explants, but is immortalized by over expressing the telomerase enzyme (Aplin et al., 2006). SW.71 cells resemble extravillous trophoblasts, including their expression of human chorionic gonadotropin (hCG), vimentin, cytokeratin-7, and their invasion of Matrigel.

Exposure to H/R has been previously described (Leach et al., 2008a). Briefly, cells were cultured at 2% O<sub>2</sub> for 2 hours and then media was replaced with fresh media pre-equilibrated at 20% O<sub>2</sub> for an additional 6 hours of culture at 5% CO<sub>2</sub> and ambient O<sub>2</sub>. Cells cultured at 2% O<sub>2</sub> for 8 hours served as a control. Where indicated, cells were cultured in the presence of 1 nM recombinant human HBEGF (R&D Systems, Minneapolis, MN) with or without addition of inhibitors. Inhibitors and their inactive structural analogs (Table 2.1 (Bennett et al., 2001; Favata et al., 1998; Klutchko et al., 2006; Lee et al., 1994; Mitamura et al., 1995; Vlahos et al., 1994)) were purchased from Calbiochem (EMD, Carlsbad, CA) and were specific for JNK (JNK Inhibitor II and negative control), MAPK14 (p38) (SB203580 and SB202474), PIK3 (PI3K) (LY294002 and LY303511), and MEK (U0126 and U0124). Inhibitors of EGFR/ERBB2/ERBB4

Inhibitor	Target	Chemical Structure	IC <sub>50</sub>	Working Conc.
ERBB/HER Inhibitor	<b>ERBB1</b> <b>ERBB2</b> <b>ERBB4</b>	N-(4-((3-Chloro-4-fluorophenyl)amino)pyrido[3,4-d]pyrimidin-6-yl)2-butynamide	1 nM	10 nM
CRM197	<b>HBEGF</b>	CRM197 is a nontoxic diphtheria toxin with a G52E point mutation		10 µg/mL
U0126	<b>MEK</b>	1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene	75 nM	1 µM
U0124	Control	1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene	> 100 µM	1 µM
SP600125	<b>JNK</b>	1,9-pyrazoloanthrone	100 nM	1 µM
JNK Control	Control	N'-Methyl-1,9-pyrazoloanthrone	20 µM	1 µM
SB203580	<b>p38</b>	4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole, HCl	600 nM	1 µM
SB202474	Control	4-ethyl-2(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole		1 µM
LY 294002	<b>PI3K</b>	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	1.4 µM	10 µM
LY 303511	Control	2-Piperazinyl-8-phenyl-4H-1-benzopyran-4-one	> 100 µM	10 µM

**Table 2.1. Inhibitors and inactive structural analogs used in this study**

(ERBB/HER Inhibitor) tyrosine kinase activity (cat. #324840) and HBEGF signaling (cross-reacting material 197; CRM197) were also purchased from Calbiochem.

## Cell Death Assay

Cells fixed with 4% paraformaldehyde for 20 minutes were permabilized with 0.1% Triton-X100 for 15 minutes and terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end-labeled (TUNEL) using a kit from Roche Applied Science (Indianapolis IN), as previously described (Armant et al., 2006). Briefly, cell nuclei were counterstained with 1 mg/ml DAPI to obtain a “TUNEL Index” by calculating a ratio of TUNEL-positive nuclei to DAPI-positive nuclei. Previously, we determined that cell death in cytotrophoblast exposed to H/R or low O<sub>2</sub> alone in the absence of HBEGF signaling was due to apoptosis rather than necrosis (Armant et al., 2006; Leach et al., 2008a).

## Migration Assay

A modified Boyden chamber assay was conducted using sterile transwell inserts with polycarbonate membrane filters containing 8-mm pores (Corning) to examine the extravillous differentiation of trophoblast cells to a migratory phenotype. Transwell inserts were coated top and bottom with 10 µg/mL human plasma fibronectin (Invitrogen) in sterile PBS at 4°C overnight. Fibronectin was removed from each well and 500 µL of pre-warmed serum-free media was added to the lower chamber. Treatments were carried out prior to conducting the migration assays. For each treatment, cells were first serum-starved for 24 hours by culturing in DMEM/F-12 containing 5 mg/mL BSA. Media was then either exchanged for fresh serum-free media (vehicle control) or serum-free media containing 10 nM recombinant HBEGF without (positive control) or with (experimental groups) inhibitor. After 4 hours of culture, cells

were washed twice with 2 mL of serum-free media and culture was continued for an additional 20 hours. After their pretreatments, 50,000 cells were added to the upper chamber of triplicate transwell insert in a final volume of 200  $\mu$ L. Transwell plates were incubated at 37°C for 9 hours. Cells migrating to the underside of the membrane were trypsinized into the lower well, combining with cells that had detached from the underside of the membrane during culture. The cells were fixed with 10% formalin and mixed by pipetting. After allowing the cells to settle for 15 min, they were counted using a phase-contrast inverted light microscope at 100x, viewing 10 different fields in each well. From the average number of cells per field, the total number of cells in the lower well was calculated.

### **Western Blotting**

Western blots were performed as previously described (Kilburn et al., 2000a). Cellular lysates were diluted in SDS sample buffer containing 5%  $\beta$ -mercaptoethanol, run on precast 4-20% Tris-HCl gradient gels (BioRad, Hercules, CA) and blotted with primary antibodies. Antibodies against AKT1/2/3 (monoclonal rabbit), phospho-AKT1/2/3 (pAKT; Ser473; monoclonal rabbit), JNK1/2/3 (polyclonal rabbit), phospho-JNK1/2/3 (pJNK; Thr183/Tyr185; monoclonal mouse), MEK1/2 (polyclonal rabbit), and phospho-MEK1/2 (pMEK; Ser217/221; polyclonal rabbit) were purchased from Cell Signaling Technologies (Beverly MA). Antibodies against HBEGF (polyclonal goat), MAPK14 (polyclonal rabbit), phospho-MAPK14 (pMAPK14; Thr180/Tyr182; polyclonal rabbit), MAPK3/1 (ERK; monoclonal mouse), and phospho-ERK (pERK; pERK1 at Thr202/Tyr204 and pERK2 at Thr185/Tyr187; monoclonal rabbit) were purchased from R&D Systems (Minneapolis, MN). Secondary anti-rabbit, anti-goat and anti-mouse

antibodies purchased from Cell Signaling Technologies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Bands were observed for HBEGF, MEK/pMEK, ERK/pERK, MAPK14/pMAPK14, AKT/pAKT, and JNK/pJNK at 19-22, 45, 40/45, 40/45, 60, and 46/54 kDa, respectively.

### **Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and stained for the presence of HBEGF protein, as previously described (Kilburn et al., 2000a; Leach et al., 2004b). For secondary antibody labeling, a horseradish peroxidase-conjugated anti-mouse/anti-rabbit kit was used (Dako EnVision System-HRP), as described by Armant et al. (2006). Image analysis was performed according to published procedures (Leach et al., 2002b).

### **ELISA**

Enzyme-linked immunosorbent assays were carried out using the HBEGF DuoSet ELISA Development kit (R&D Systems), as previously described (Armant et al., 2006; Leach et al., 2008a). The optical density of the final reaction product was determined at 450 nm using a programmable multiplate spectrophotometer (Power Wave Workstation, Bio-Tek Instruments, Winooski, VT) with automatic wavelength correction.

### **Statistics**

All assays were performed in triplicate and all experiments were repeated at least 3 times and are reported as mean  $\pm$  s.e.m. Statistical significance was determined at

$p < 0.05$  by ANOVA with the Student-Newman-Keuls post hoc test, using SPSS version 12.0 statistics software (SPSS, Chicago, IL).

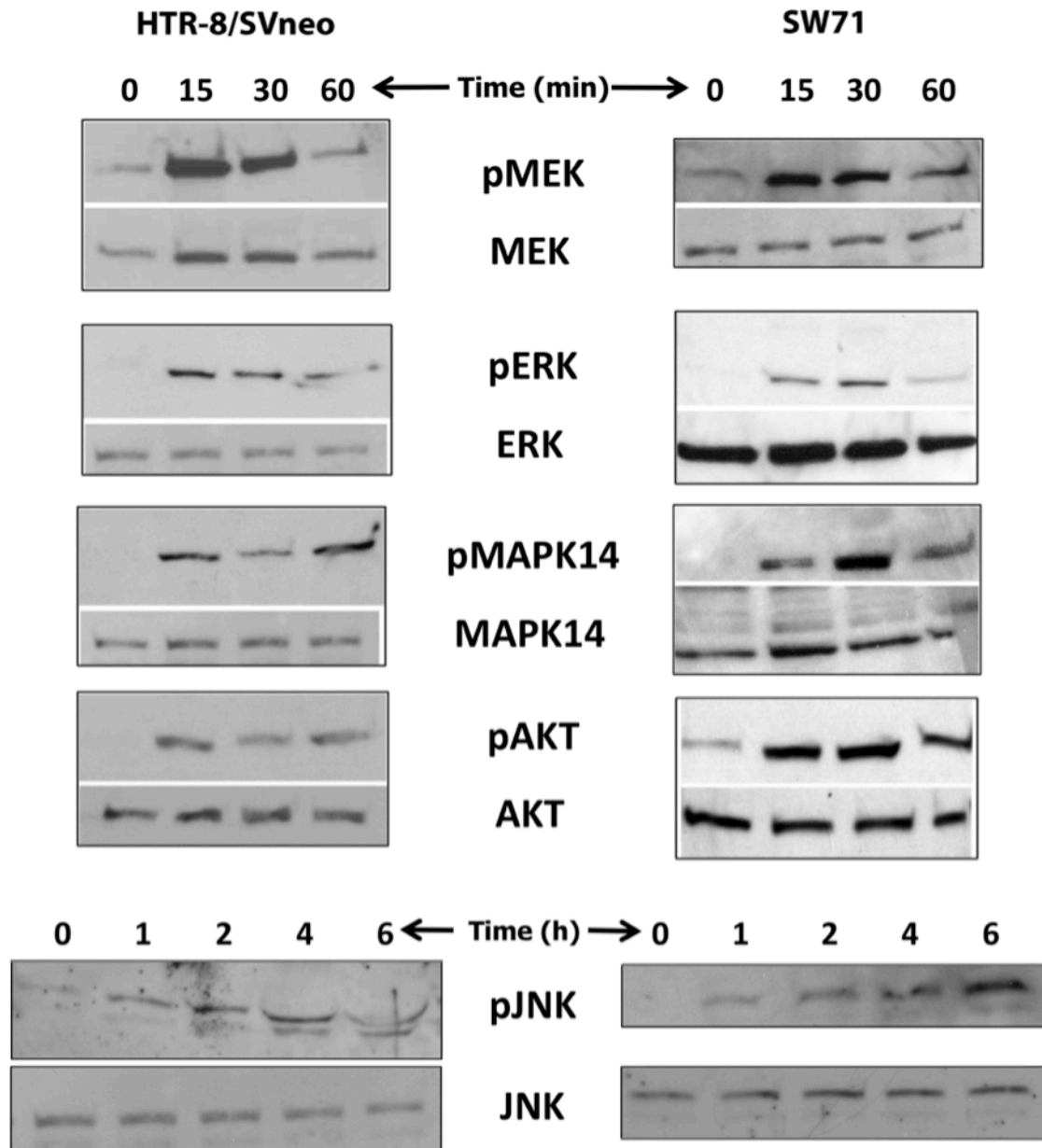
## RESULTS

### HBEGF activates multiple signaling pathways

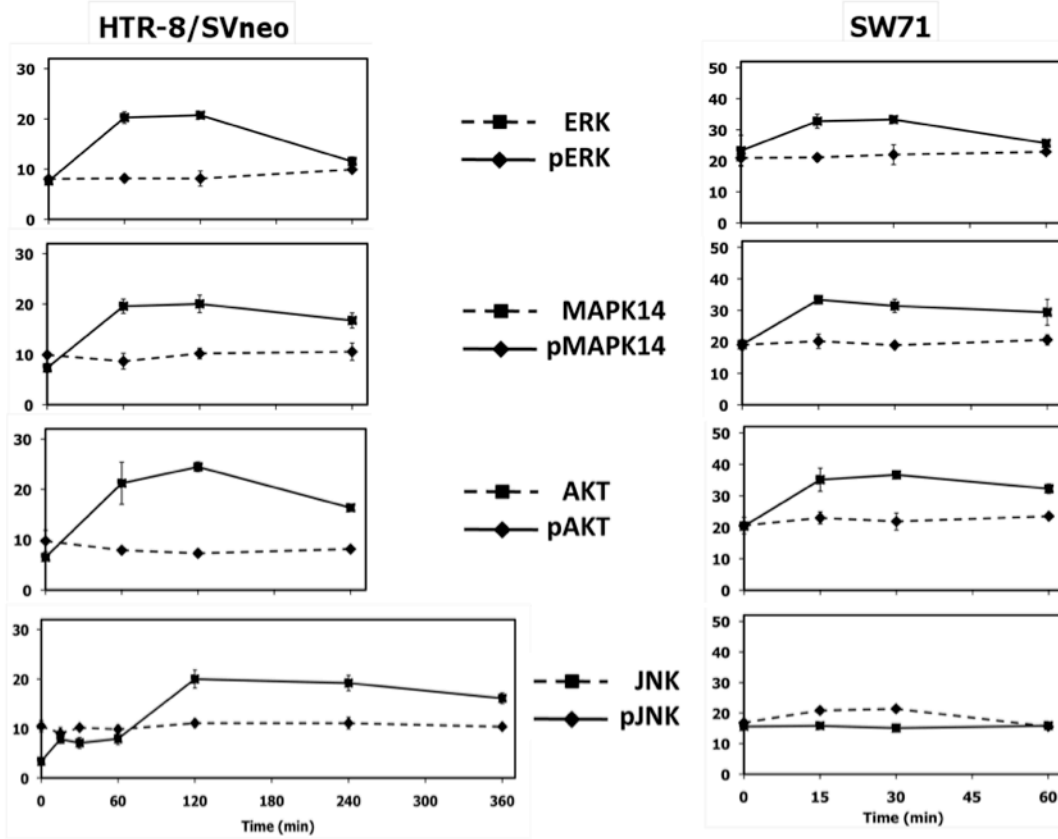
In order to identify signaling pathways targeted by HBEGF in human cytotrophoblast cell lines, cells were treated for 15 minutes to 1 hour with 10 nM recombinant HBEGF. After 15 minutes, western blotting and immunocytochemistry revealed a marked phosphorylation of MAPK14, ERK and AKT (Fig. 2.1 and Fig. 2.2, respectively). Phosphorylation was maintained for at least 45 minutes during treatment with HBEGF, then declined to levels observed before treatment. Phosphorylation of JNK, however, was not significant after 1 hour of HBEGF treatment, but occurred shortly thereafter and remained phosphorylated for up to 6 hours (Fig. 2.1 and Fig. 2.2). We conclude that HBEGF induces a rapid, transient activation of the MAPK14 and ERK and PIK3 pathways, but a slower or delayed activation of the JNK pathway, downstream of ERBB/HER receptor tyrosine kinases.

The rapid phosphorylations of ERK, MAPK14 and AKT were each attenuated by an inhibitor of ERBB/HER tyrosine kinase (Table 1) in a dose-dependent manner (Fig. 2.3 A), establishing that the activity of HBEGF was mediated through its cognate receptors. If individual pathways were blocked with their respective inhibitor, HBEGF-induced phosphorylation of the specific target kinase was blocked, but not the other kinases (Fig. 2.3 B-D). To validate JNK inhibition, an inhibitor of JNK blocked its phosphorylation when induced by H/R injury (Fig. 2.3 E). Treating with the inactive structural analogues of these inhibitors had no effect on the phosphorylation status of

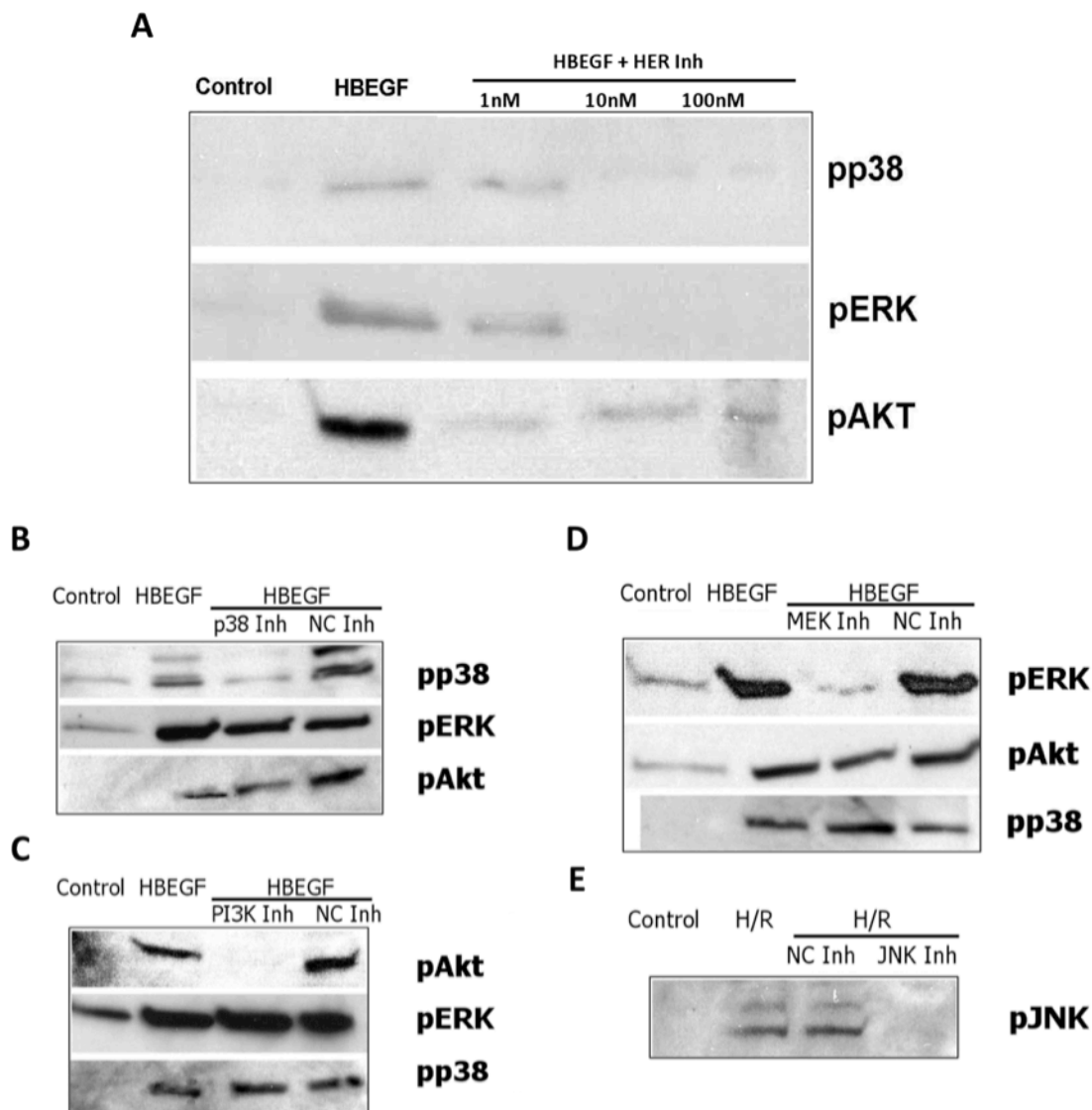




**Figure 2.1 Identification of signaling pathways activated by HBEGF.** Extracts were prepared from HTR-8/SVneo (left panels) or SW.71 (right panels) cell lines at the indicated times after treatment with 1 nM HBEGF and analyzed by western blotting. Each lane contained 30  $\mu$ g of protein extract and was labeled with antibodies against the indicated proteins (lower panels) or their phosphorylated forms (upper panels). Images shown are representative of at least three experiments.



**Figure 2.2 Identification of signaling pathways activated by HBEGF using immunocytochemistry.** Cultures of HTR-8/SVneo (left panels) or SW.71 (right panels) cells were treated with 1 nM HBEGF for the indicated times, fixed and labeled with antibodies against the indicated proteins or their phosphorylated forms. Image analysis was used to quantify the relative stain intensity, which is shown in arbitrary units on the y-axis. Values represent the average  $\pm$  SEM of triplicate samples.



**Figure 2.3 Characterization of kinase inhibitors by western blotting.** Extracts of HTR-8/SVneo cells were analyzed by western blotting after (A-D) culture for 30 minutes in the absence (Control) or presence of 1 nM HBEGF. Where indicated, cells were also treated with HBEGF plus (A) 1 – 100 nM ERBB/HER tyrosine kinase inhibitor (HER Inh), (B) 1  $\mu$ M SB203580 (MAPK14/p38 Inh), (C) 10  $\mu$ M LY294002 (PI3K Inh), or (D) 1  $\mu$ M U0126 (MEK Inh), or their inactive structural analogs, as indicated (NC Inh). In (E), cells were cultured for 8 hours at 20% O<sub>2</sub> (Control) or subjected to hypoxia/reoxygenation (H/R), as described in the Materials and Methods section, in the absence or presence of 1  $\mu$ M SP600125 (JNK Inh) or its inactive structural analog (NC Inh). All samples were labeled with antibody against the indicated phospho-proteins. Images shown are representative of at least three experiments.

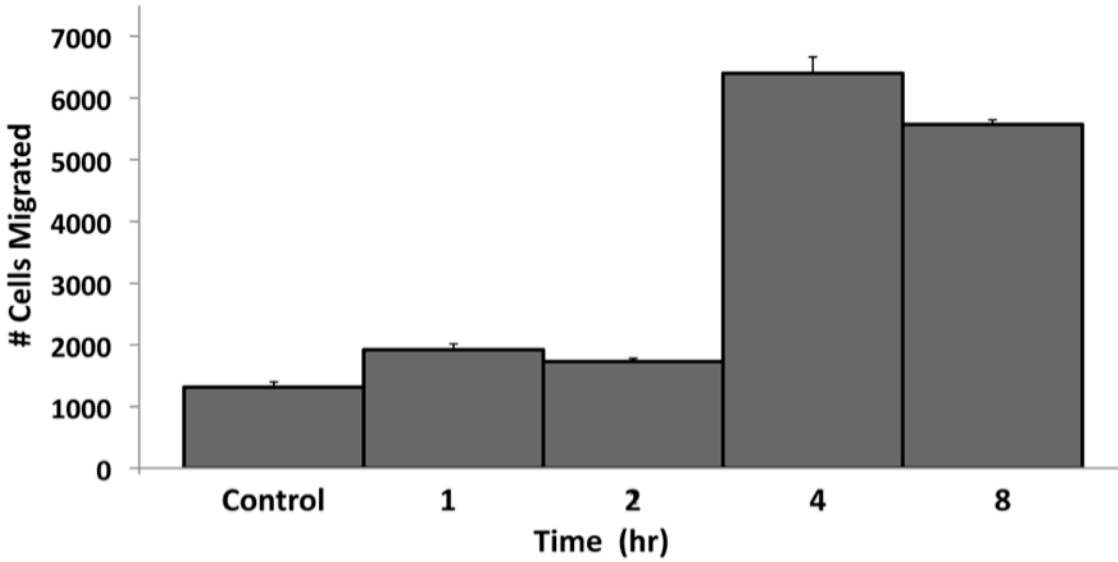
any of the target proteins. None of the treatments altered the total levels of any protein (data not shown).

### **HBEGF induces differentiation using multiple signaling pathways**

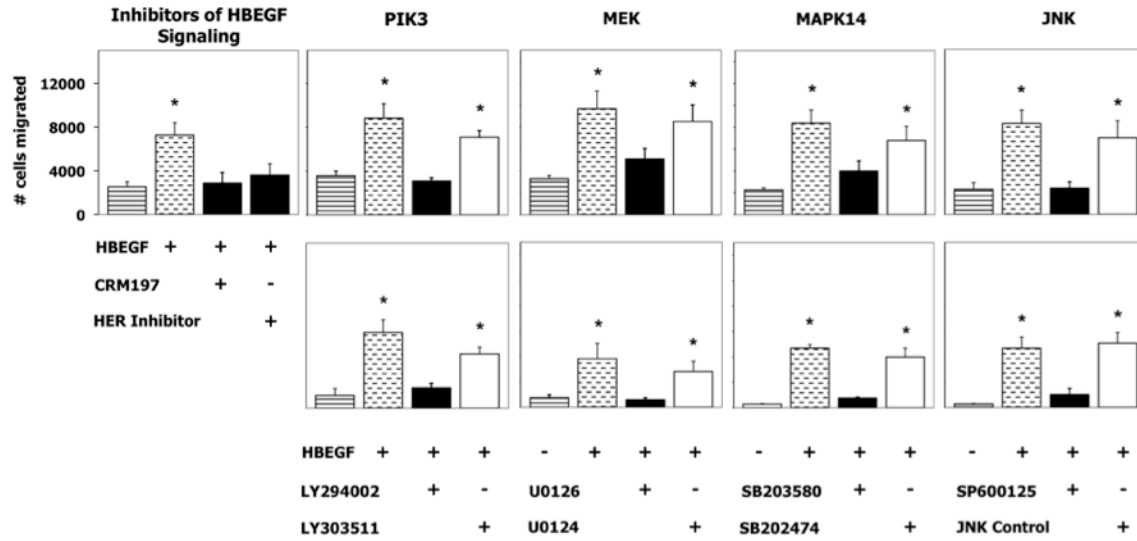
HBEGF has previously been shown to induce the extravillous differentiation of trophoblasts from first trimester villous explants as evidenced by an increase in cell migration (Leach et al., 2004b). Pharmacological inhibitors were used, with inactive structural analogues as controls (Table 2.1), to delineate the signaling pathways downstream of HBEGF that mediate this differentiation. A 4 h treatment with HBEGF 20 hours prior to assay was found in preliminary experiments (Fig. 2.4) to be optimal for stimulation of cell migration. As displayed in Fig. 2.5, HBEGF induced an increase ( $p < 0.05$ ) in migration that was blocked by inhibiting either ERBB/HER tyrosine kinase activity or HBEGF signaling. When cells were cultured in the presence of HBEGF and an inhibitor of any of the three MAPK pathways (MAPK14, MEK, or JNK) or the PIK3 inhibitor, the increase in migration was blocked (Fig. 2.5). The inactive structural analogues of each inhibitor were without effect. Therefore, all four pathways were utilized by HBEGF to initiate trophoblast extravillous differentiation.

### **HBEGF prevents apoptosis using the MAPK14 pathway**

It was recently reported that HBEGF prevents H/R-induced apoptosis in human cytotrophoblasts by signaling through its cognate receptors, EGFR and ERBB4 (Leach et al., 2008a). Cell death detected by TUNEL was found to be associated with several criteria for apoptosis. When trophoblast cells were exposed to H/R and monitored by TUNEL, there was a marked increase in apoptosis, as compared to cells cultured



**Figure 2.4 Differentiation stimulated by HBEGF.** The number of HTR-8/SVneo cells migrating through a fibronectin-coated transwell membrane insert and into the lower chamber were measured after culture in the absence (Control) or presence of 1 nM HBEGF for the indicated times. Values represent the average  $\pm$  SEM of triplicate samples.

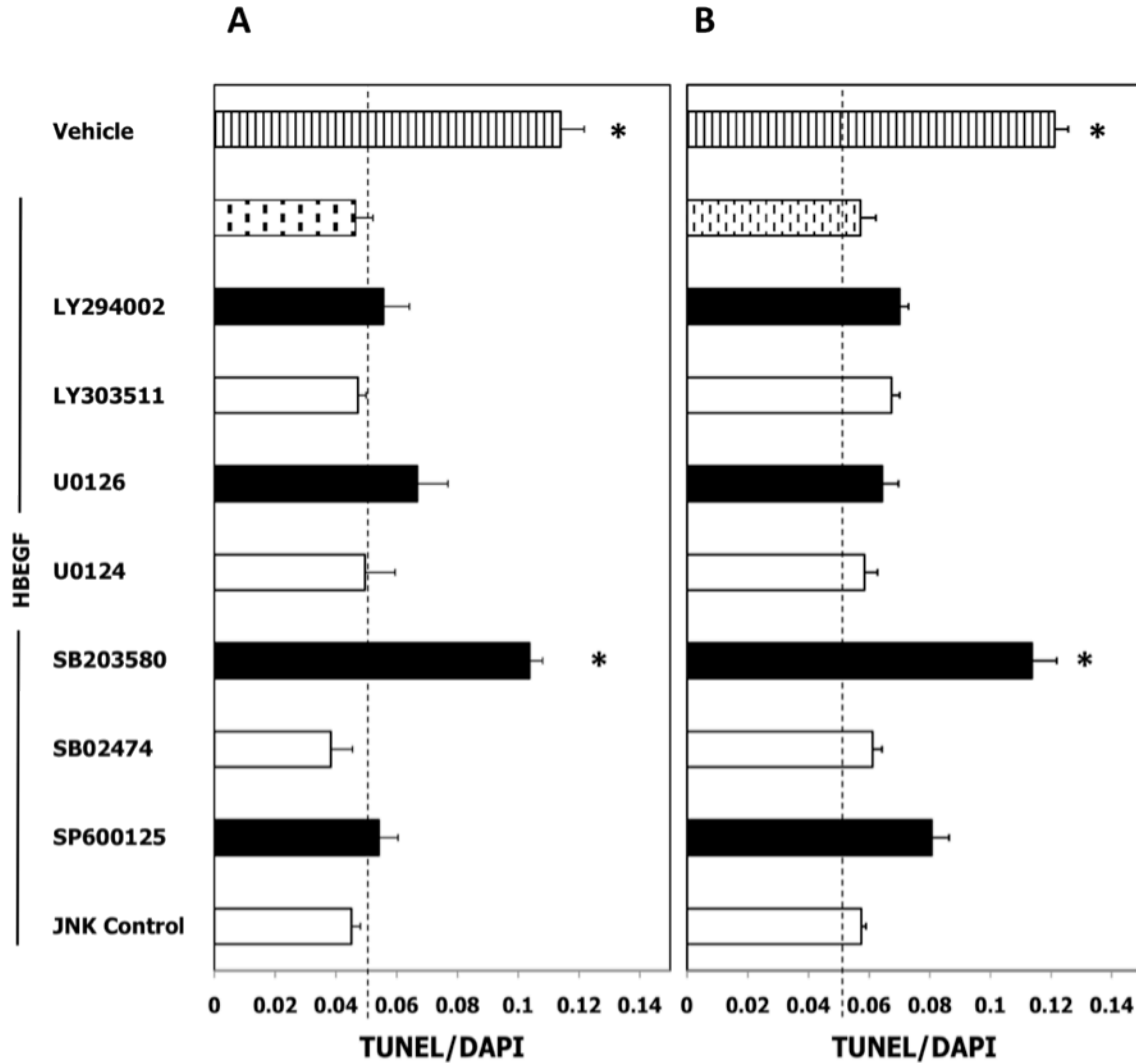


**Figure 2.5 Signaling pathways required for HBEGF induction of extravillous differentiation.** The number of SW.71 (upper panels) or HTR-8/SVneo (lower panels) cells migrating through a fibronectin-coated transwell membrane insert and into the lower chamber were measured after culture in the absence (striped bars) or presence (stippled bars) of 1 nM HBEGF, with kinase inhibitors (black bars) or the corresponding inactive structural analog (white bars), as indicated. Values represent the average  $\pm$  SEM of at least three experiments. \*,  $p < 0.05$

continuously at 2% O<sub>2</sub> (Fig. 2.6 and Fig. 2.7). Supplementation with recombinant HBEGF attenuated the increase in apoptosis. To identify the pathways utilized by HBEGF to inhibit apoptosis, cells were cultured with pharmacological inhibitors of PIK3, MEK, MAPK14, JNK or their inactive structural analogues (Table 2.1). Inhibition of MAPK14, but not the other kinases, blocked the cytoprotective effects of HBEGF during H/R injury (Fig. 2.6). The inactive structural analog of the MAPK14 inhibitor had no effect. We found using the same set of inhibitors that the MAPK14 pathway is also required for cytotrophoblast survival at 2% O<sub>2</sub> (Fig. 2.8), having previously found that apoptosis is specifically prevented by autocrine HBEGF signaling at low O<sub>2</sub> (Armant et al., 2006). Therefore, HBEGF signaling through the MAPK14 pathway appears to abrogate trophoblast apoptosis induced by H/R injury and low O<sub>2</sub>.

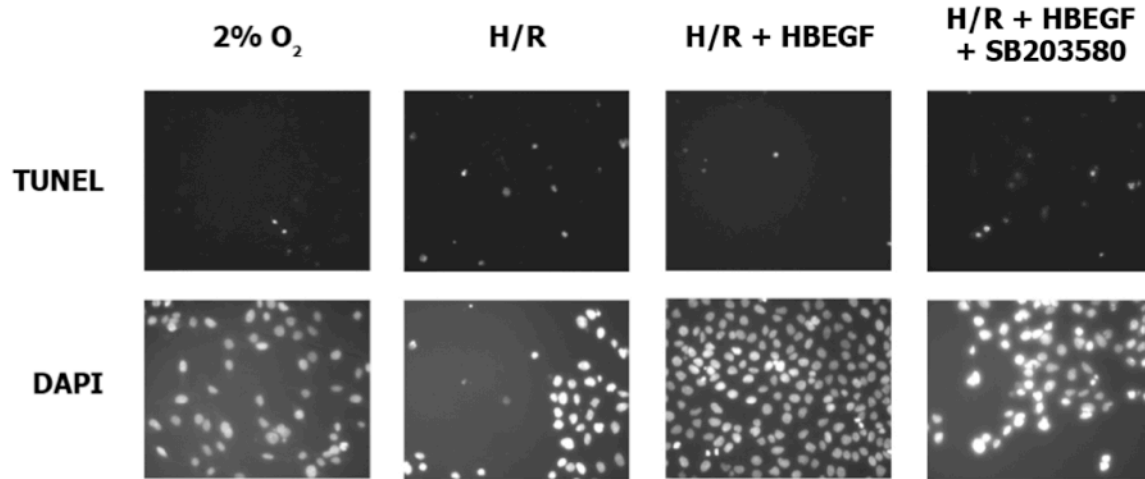
### **Low O<sub>2</sub> increases synthesis of HBEGF through autocrine induction of MAPK14, ERK or JNK**

HBEGF cellular and secreted protein levels are significantly increased in cytotrophoblast cells after 4 hours of culture at 2% O<sub>2</sub> (Armant et al., 2006), but the downstream pathways responsible for its upregulation have not been identified. Using the HBEGF-specific antagonist CRM197, it was confirmed by immunohistochemical staining of HBEGF that HBEGF signaling is required for its upregulation during exposure to low O<sub>2</sub> (Fig. 2.9). Individual inhibitors of downstream signaling pathways did not alter the increase in HBEGF observed at 2% O<sub>2</sub> (data not shown), so cytotrophoblast cells were treated with combinations of the inhibitors during hypoxic culture. By treating with all possible combinations of inhibitors or inactive structural analogues, it was determined that the three MAPK pathways, but not PIK3, were each



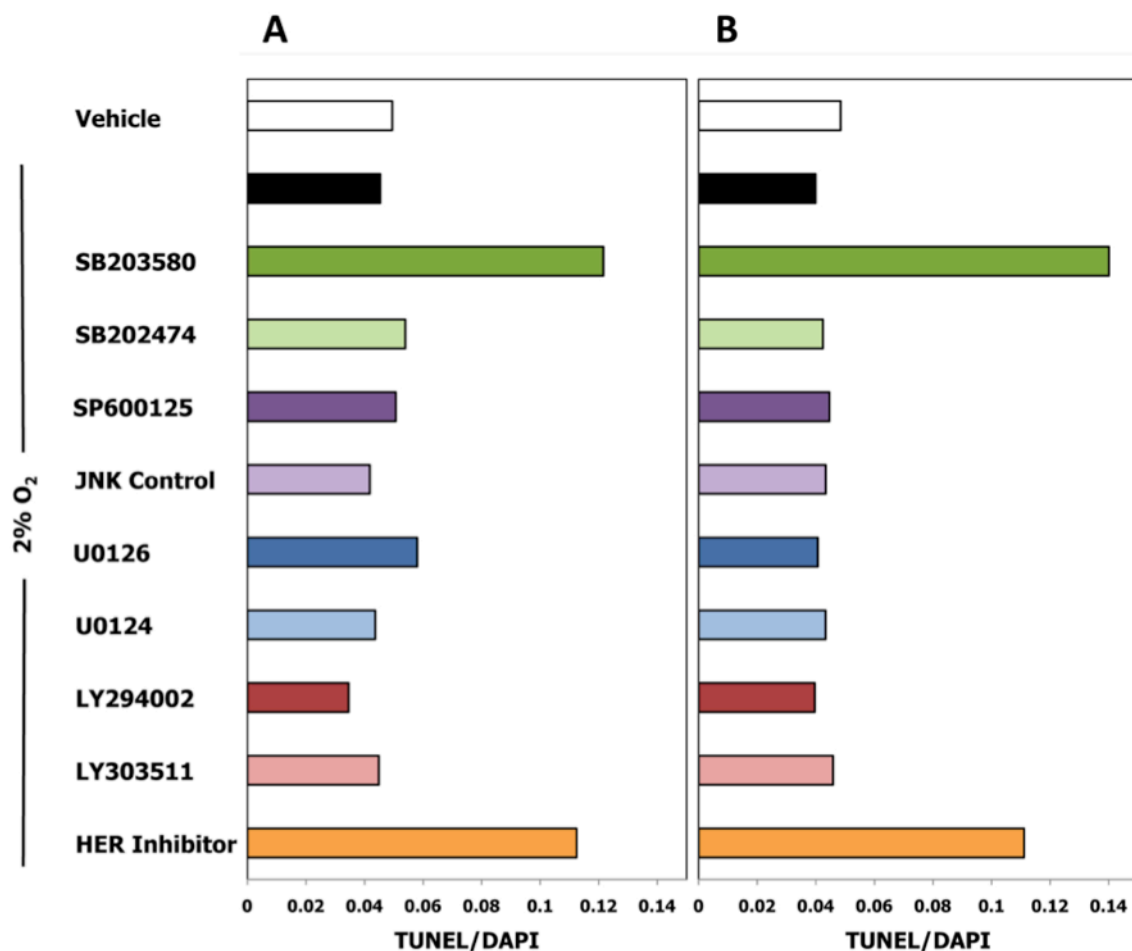
**Figure 2.6 Signaling pathways required for HBEGF inhibition of apoptosis.** The apoptotic indices were calculated in HTR-8/SVneo (A) or SW.71 (B) cell lines after exposure to H/R in the absence (striped bars) or presence (stippled bars) of 1 nM HBEGF, with kinase inhibitors (black bars) or the corresponding inactive structural analogues (white bars), as indicated. Significance was determined with reference to the apoptosis index observed in control cells cultured continuously at 2% O<sub>2</sub> (dotted line). Values represent the average  $\pm$  SEM of at least three experiments. \*,  $p < 0.05$





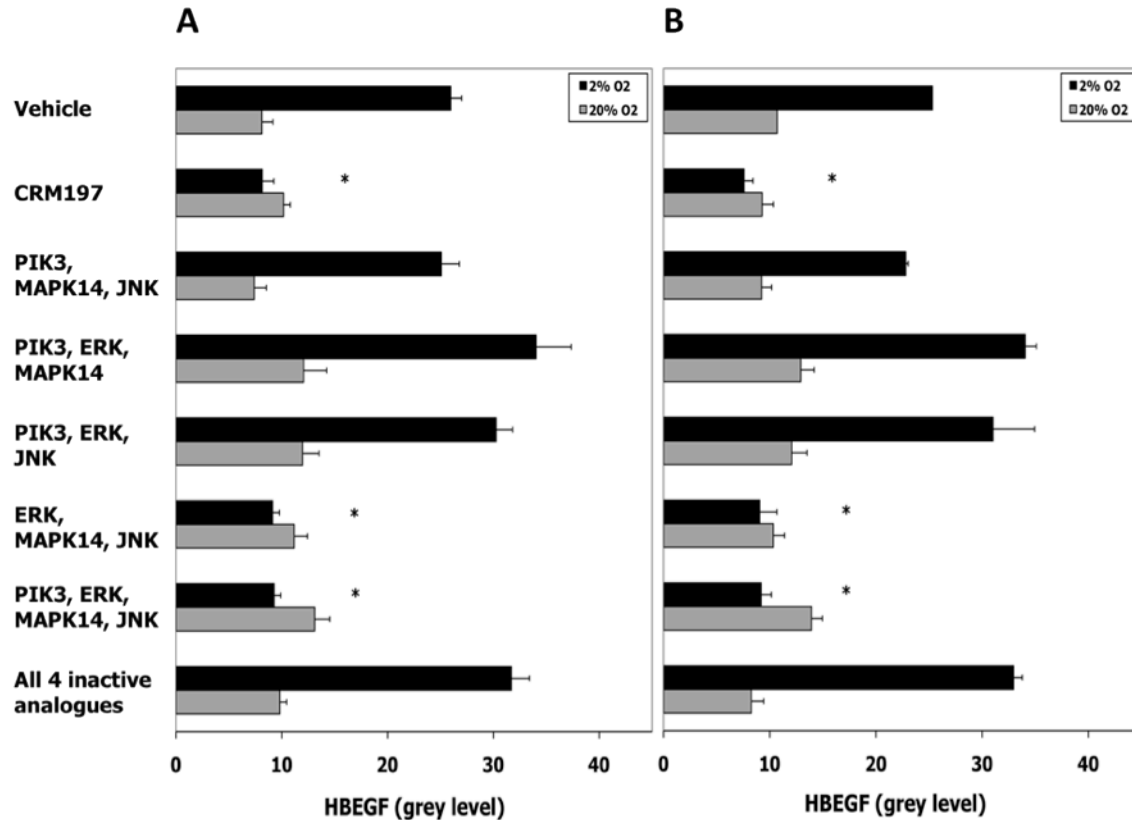
**Figure 2.7 Signaling pathways required for HBEGF inhibition of apoptosis.**

Representative images (200 x) show TUNEL-labeled cells after culture in 2% O<sub>2</sub>, or in H/R in the absence or presence of HBEGF, with and without the MAPK14 inhibitor.



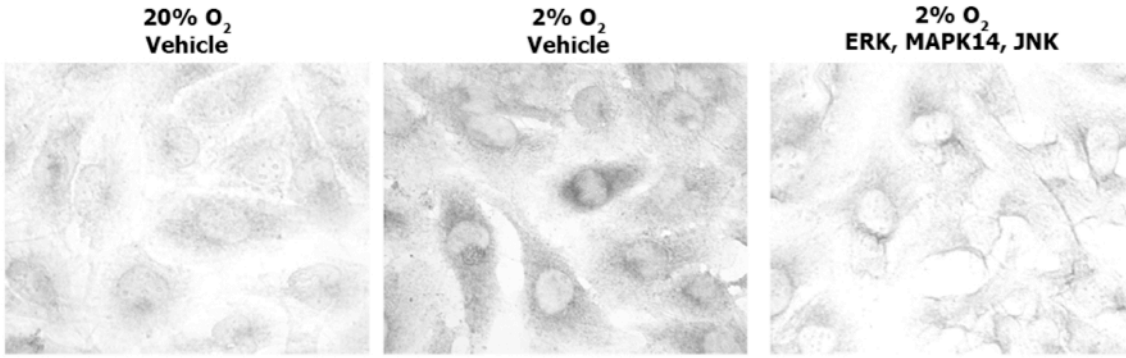
**Figure 2.8 Signaling pathways required for inhibition of apoptosis at 2% O<sub>2</sub>.**

The apoptotic indices were calculated in HTR-8/SVneo (A) or SW.71 (B) cell lines after culturing at 20% O<sub>2</sub> (white bar), 2% O<sub>2</sub> (black bar), or 2% O<sub>2</sub> in the presence of kinase inhibitors (dark colored bars) or the corresponding inactive structural analogues (light colored bars), as indicated. Cells were also cultured at 2% O<sub>2</sub> in the presence of 10 nM ERBB/HER kinase inhibitor (HER Inhibitor; orange bar).



**Figure 2.9 Signaling pathways required for increased synthesis of HBEGF during hypoxia.** HTR-8/SVneo (A) or SW.71 (B) cells were labeled with an antibody against HBEGF after culturing at 20% (grey bars) or 2% O<sub>2</sub> (black bars) in the presence of kinase inhibitors or their inactive structural analogues (inactive analogues), as indicated by their target pathway. The HBEGF-specific antagonist, CRM197, was also used to block HBEGF signaling. All possible combinations of inhibitors or their inactive structural analogs were assessed, but only the most relevant combinations are shown here. Image analysis was used to quantify the relative stain intensity, which is shown in arbitrary units on the horizontal axis. Values represent the average  $\pm$  SEM of at least three experiments. \*,  $p < 0.05$

capable of mediating the increase in HBEGF protein levels at 2% O<sub>2</sub> (Fig. 2.9 and Fig. 2.10). HBEGF accumulation was only prevented when all three MAPK inhibitors were simultaneously applied. These findings were confirmed by quantifying HBEGF concentrations in cell lysates and culture medium using a specific ELISA (Table 2.2).



**Figure 2.10 Signaling pathways required for increased synthesis of HBEGF during hypoxia.** Representative images (400 x) of cytotrophoblast cells depict the upregulation of HBEGF by hypoxia and inhibition of its upregulation in the presence of MAPK inhibitors.

**Table 2.2. ELISA quantification of HBEGF protein upregulation at 2% O<sub>2</sub> in the presence of signaling inhibitors.** Cellular HBEGF concentrations are reported in ng per mg of total protein.

<i>HTR-8/SVneo</i>			
	20% O <sub>2</sub>	2% O <sub>2</sub>	Fold-change
<b>Control</b>	3.08	68.78	22.37
<b>ERK, p38, JNK</b>	6.75	3.23	0.48
<b>3 Inactive Analogues</b>	2.34	79.02	33.75
<b>ERK, p38, JNK, PI3K</b>	1.65	2.40	1.46
<b>4 Inactive Analogues</b>	2.03	74.60	36.77

<i>SW71</i>			
	20% O <sub>2</sub>	2% O <sub>2</sub>	Fold-change
<b>Control</b>	1.04	88.20	84.99
<b>ERK, p38, JNK</b>	1.80	0.77	0.46
<b>3 Inactive Analogues</b>	1.09	60.91	55.72
<b>ERK, p38, JNK, PI3K</b>	1.88	0.87	0.46
<b>4 Inactive Analogues</b>	0.95	86.84	91.48

## DISCUSSION

The present investigation demonstrated that HBEGF transiently activates the MAPK14, JNK and ERK MAPK pathways, as well as the PIK3/AKT pathway, downstream of the ERBB/HER tyrosine kinases in human cytotrophoblast cells. Only the MAPK14 pathway appeared to be utilized to prevent apoptosis induced by O<sub>2</sub> fluctuations. However, it functioned in combination with the ERK, JNK, and PIK3 pathways to induce trophoblast extravillous differentiation. HBEGF signaling is required to increase HBEGF protein levels when O<sub>2</sub> is decreased to 2% (Armant et al., 2006), and both immunohistochemical and ELISA data indicated that HBEGF upregulation can be mediated by any one of the three MAPK pathways. These data confirm prior reports that HBEGF inhibits apoptosis (Armant et al., 2006; Imudia et al., 2008; Leach et al., 2008a; Wolff et al., 2007) and promotes differentiation of trophoblast cells towards a migratory, extravillous phenotype (Leach et al., 2004b; Lim et al., 2006; Wang et al., 2000). Moreover, the new findings identify separate downstream signaling pathways mediated by HBEGF-induced ERBB/HER activation that are responsible for each functional outcome.

Although HBEGF utilizes different pathways to mediate its diverse effects in trophoblasts, much remains to be learned about the downstream effectors that are involved. Several factors, in addition to HBEGF, that induce trophoblast migration include the ubiquitin-type plasminogen activator (uPA)/uPA receptor (uPAR) system, insulin-like growth factor (IGF), IGF-binding protein 1 (IGFBP1), hepatocyte growth factor (HGF) and endothelin 1 (Lala and Chakraborty, 2003). The uPA/uPAR system induces migration in two ways. It activates matrix metalloproteinases to initiate

extracellular matrix degradation, and induces intracellular  $\text{Ca}^{2+}$  signaling to activate phospholipase C (PLC), PIK3, and ERK (Liu et al., 2003). Metalloproteinases are necessary for the shedding and secretion of HBEGF (Holbro and Hynes, 2004a), which activated PIK3 and ERK in HTR-8SVneo cells, and can induce intracellular  $\text{Ca}^{2+}$  signaling (Jessmon and Armant, unpublished observation). IGF signaling also utilizes ERK to induce trophoblast migration, acting through the IGF type 2 receptor (McKinnon et al., 2001). IGFBP1 induces migration by binding the ITGA5/ITGB1 integrin, which leads to activation of focal adhesion kinase and ERK (Gleeson et al., 2001). HBEGF also affects integrin signaling, but through integrin switching rather than direct ligation (Leach et al., 2004b). TNF-alpha (TNFA) also can induce integrin switching, with upregulation of ITGA1 and downregulation of ITGA6 (Fukushima et al., 2003), as well as increased expression of vascular integrins (ITGAV and ITGB3) in an immortalized trophoblast cell line, TCL1 (Fukushima et al., 2005). Endothelin 1 activates two pathways: one involving PLC and intracellular  $\text{Ca}^{2+}$  signaling, and the other involving ERK (Chakraborty et al., 2003). EGF also increases migratory activity in both HTR-8/SVneo cells (Leach et al., 2004b; Qiu et al., 2004a; Qiu et al., 2004b) and freshly isolated first trimester human cytotrophoblasts (Bass et al., 1994a) through the coordinated activation of PIK3/AKT and ERK pathways (Qiu et al., 2004b). Signaling through the PIK3/AKT pathway requires p70S6K and MTOR activation, but only increases migration if the ERK pathway is simultaneously activated (Qiu et al., 2004b). It remains to be ascertained whether EGF and HBEGF operate through the same intracellular signaling pathways in first trimester cytotrophoblast cells. In the human extravillous cytotrophoblast cell line, SGHPL-4, EGF stimulates cell motility through the



PIK3/AKT, MAPK14, and ERK pathways (LaMarca et al., 2008). Interestingly, blocking the MAPK14 pathway with SB203580 also inhibited activation of AKT, suggesting that the pathways crosstalk (LaMarca et al., 2008). In contrast to EGF, the present study found that the PIK3, MAPK14, ERK, and JNK pathways mediated HBEGF induction of trophoblast migration without cross-talk. Useful insights would be gained by identifying potential downstream effectors common to these four pathways.

Several intermediates have been implicated in the regulation of trophoblast apoptosis. In contrast to our finding that MAPK14 mediates the cytoprotective activity of HBEGF, it has been reported that H/R induces apoptosis through MAPK14 activation of the JNK pathway in trophoblasts from term villous explants (Cindrova-Davies et al., 2007). It was recently discovered that H/R activates ASK1, leading to activation of both MAPK14 and JNK (Cindrova-Davies, 2009). This supports the notion that first and third trimester trophoblast cells engage different signaling mechanisms in response to oxidative stress. In another study, JNK was responsible for inducing apoptosis in human first trimester placental trophoblasts exposed to hyperosmolar stress (Zhong et al., 2007). In agreement, we have observed activation of JNK by H/R in the same cell line.

Currently, only a few anti-apoptotic factors are known in trophoblasts. When exposed to reactive O<sub>2</sub> species (H<sub>2</sub>O<sub>2</sub>), BeWo cells undergo apoptosis, concomitant with an increase in the tumor suppressor gene, *TP53*, and a decrease in its inhibitor, MDM2 (Heazell et al., 2009). Interestingly, MDM2 is expressed in trophoblasts throughout early gestation, but disappears from cytotrophoblast cells by the third trimester (Fulop et al., 1998; Heazell and Crocker, 2008). Cytotrophoblast cells also

express nuclear TP53 more strongly in the first trimester than at term (Marzusch et al., 1995; Roncalli et al., 1994). Taken together, it is likely that MDM2 suppresses the pro-apoptotic influence of TP53 in first trimester trophoblasts, while TP53 becomes prominent late in gestation. Indeed, trophoblast apoptosis is relatively low in the first trimester, even in the face of oxidative stress, but increases towards term as trophoblast cells become less tolerant to changes in O<sub>2</sub> (Imudia et al., 2008; Smith et al., 2002). Therefore, MDM2 is a potential intermediate in the anti-apoptotic pathway downstream of MAPK14 signaling.

In addition to the EGF signaling system component HBEGF, EGF is cytoprotective for trophoblasts. It can inhibit apoptosis in term placental explants (Moll et al., 2007) and isolated term cytotrophoblasts (Smith et al., 2002) through the PIK3/AKT pathway (Moll et al., 2007). Studies suggest that EGF does not utilize BCL2 to block cytokine-induced apoptosis in term cytotrophoblasts (Ho et al., 1999), but may work by decreasing the amount of ceramide produced during a pro-apoptotic signaling event (Payne et al., 1999). EGF can activate PIK3 ERK, JNK, and sphingosine kinase 1 (SPHK1) in these cells, all of which are required subsequently to inhibit apoptosis (Johnstone et al., 2005; Mackova et al., 2003). Interestingly, PIK3 and ERK are needed to block apoptosis (Mackova et al., 2003), and the activation of SPHK1 is partially downstream of PIK3 (Johnstone et al., 2005). EGF does activate MAPK14, but, in contrast to our findings, this pathway is not involved in the cytoprotective effects of EGF or the apoptotic pathway induced by cytokines (Johnstone et al., 2005). This could indicate a difference between pathways activated by EGF and HBEGF, but more likely reflects another difference between term and first trimester trophoblasts. However, EGF

increases proliferation of cytotrophoblasts in term villous explants, as assessed by MKI67 immunostaining (Moll et al., 2007), while HBEGF is a weak mitogen for first trimester cytotrophoblasts (Leach et al., 2004b) and term trophoblast cells (Imudia et al., 2008). Although EGF is cytoprotective, it is not upregulated with HBEGF in first trimester trophoblast cells in response to low O<sub>2</sub> and is, thus, less likely to be part of a cytoprotective mechanism during early gestation (Armant et al., 2006).

Previous work demonstrated that the upregulation of HBEGF in first trimester cytotrophoblast cells cultured at low O<sub>2</sub> is unique among the EGF ligand family (Armant et al., 2006). It should be noted that this increase in protein is not accompanied by any change in its mRNA, indicating that HBEGF is post-transcriptionally regulated by O<sub>2</sub>. The upregulation of HBEGF protein during low O<sub>2</sub> is downstream of ERBB/HER tyrosine kinase signaling and metalloproteolytic shedding of HBEGF from the cell surface, suggesting that newly secreted HBEGF activates its own translation through autocrine signaling. The present study confirmed that the HBEGF-specific antagonist CRM197 blocks upregulation of HBEGF and further indicated that this positive feedback loop can utilize any one of the three major MAPK pathways, but not PIK3. While this is the first report that HBEGF is post-transcriptionally regulated through MAPK signaling during low O<sub>2</sub>, other gene products are similarly regulated downstream of MAPK. For example, MAPK14 enhances translation of interleukin-8 in airway epithelial cells (Berube et al., 2009), activates translation of TNF in Kupffer cells and macrophages (Wang et al., 2009b; Yun et al., 2009), and stabilizes mRNA for interleukin 6 (Zhao et al., 2008) and CCAAT enhancer binding protein- $\delta$  (Li et al., 2008). The JNK pathway is involved in the post-transcriptional regulation of TNF (Wang et al., 2009a) and

angiopoietin 2 (Phelps et al., 2006). The ERK and PIK3 pathways are both involved in translation of the  $\text{Na}^+/\text{K}^+$  exchanger 1 (Chiang et al., 2008) in cervical cancer cells and cyclooxygenase 2 in ovarian cancer cells (Cao et al., 2007). In addition, microRNA (miRNA) is well known to regulate the translation and stability of mRNA in a gene-specific fashion (Valencia-Sanchez et al., 2006), and has been shown to vary in HTR-8/SVneo cells in response to changing  $\text{O}_2$  concentration (Donker et al., 2007). The potential role of miRNA in the translational regulation of HBEGF by  $\text{O}_2$  warrants future exploration.

This investigation has identified several intracellular signaling pathways activated by HBEGF in first trimester trophoblast cells and has linked them to its numerous physiological effects. Different pathways are utilized by HBEGF to induce extravillous trophoblast differentiation, block apoptosis, and autoregulate HBEGF protein levels. This information provides a foundation for delineating the intracellular circuitry and transcriptional activity linking HBEGF signaling through its cognate receptors to physiological outcomes necessary for trophoblast function during implantation and placentation in humans.

### **CHAPTER 3. Post-Transcriptional Regulation of Integrin Switching by Growth Factor Signaling in Human Extravillous Trophoblast Cells**

#### **SUMMARY**

As human cytotrophoblast (CTB) cells initiate extravillous differentiation, expression of invasion promoting integrins (e.g., ITGA1/ITGB1) increases and attachment-associated integrins (e.g., ITGA6/ITGB4) decreases. HBEGF is expressed in CTB cells and induces “integrin-switching” in vitro to the same extent as culture on Matrigel (MG) basement membrane. The human first trimester CTB cell line HTR-8/SVneo was cultured serum-free for 24 h on fibronectin or on MG to examine downstream mechanisms that regulate integrin expression. ITGA1 and ITGA6 visualized by immunofluorescence or immunoperoxidase labeling were altered as expected by culture on MG or with HBEGF, which was confirmed by quantitative image analysis. Inhibiting HBEGF signaling with a specific antagonist, CRM197, or with a pan-ERBB kinase inhibitor blocked integrin switching induced by HBEGF, but not by MG. Furthermore, CTB invasion and integrin switching failed on growth factor-reduced MG. Integrin switching, but not invasion, could be restored specifically with recombinant HBEGF. Inhibitors of MEK1/2 (U0126), PI3 kinase (LY294002), p38 (SB203580), and JNK (SP600125) were each able to specifically block integrin-switching induced by HBEGF. Integrin switching induced by treatment with HBEGF or culture on MG did not alter ITGA1 or ITGA6 mRNA expression, as quantified by real time PCR, indicating post-transcriptional regulation. In conclusion, HBEGF is sufficient to induce integrin-switching, although other factors in MG appear to have similar activity. The same downstream pathways necessary for HBEGF induction of integrin switching were

previously found to be required for HBEGF-induced CTB migration, supporting the view that integrin switching is a key inaugural step in invasive differentiation.

## INTRODUCTION

Embryo implantation in the uterine endometrium is mediated by trophoblast cells located on the surface of the blastocyst that, after invasion of the uterus, give rise to the placenta (Armant, 2005). This process requires trophoblast differentiation from an epithelial phenotype to an invasive phenotype (Knofler, 2010). Trophoblast invasion of the decidualized maternal stroma is both interstitial and intravascular, which results in the remodeling of the uterine spiral arteries to increase blood flow into the intervillous space of the developing placenta (Pijnenborg et al., 1983b). Alterations in the endometrial luminal epithelium, and signaling by hormones, cytokines, and growth factors are all associated with successful implantation (Norwitz et al., 2001). Paracrine and autocrine signaling drives developmental progression of the endometrium, as well as the differentiation of trophoblast cells. Trophoblast cells expressing divergent phenotypes are observed in close proximity during early development *in vivo*, suggesting that differentiation can occur rapidly under the direction of extrinsic cues in the microenvironment (Armant, 2005). This developmental plasticity suggests the hypothesis that the trophoblast utilizes signaling mechanisms that translate local cues into functional changes with high efficiency and speed.

Cytotrophoblast (CTB) stem cells of the placenta are a polarized epithelium attached to a basement membrane that strongly express E-cadherin and integrin  $\alpha 6\beta 4$  (ITGA6/ITGB4) (Damsky et al., 1992). Individual CTB cells proliferating within the anchoring villi differentiate to invasive extravillous trophoblast (EVT) cells that leave the cell column and invade the uterine decidua. As they do this, expression of ITGA6/ITGB4 diminishes and ITGA1/ITGB1 intensifies in a process termed “integrin

switching” (Damsky et al., 1994). Although the complete mechanism that regulates integrin switching *in vivo* is unknown, factors that induce trophoblast motility might initiate EVT differentiation through integrin switching. CTB cells undergo integrin switching while invading the decidua, suggesting that matrix components secreted by decidual fibroblasts are important mediators of this event (Damsky et al., 1992). Integrin switching can also be induced when CTB cells isolated from first trimester placentas are cultured on Matrigel (MG), a tumor-derived basement membrane (Damsky et al., 1994). An immortalized human first trimester CTB cell line, HTR-8/SVneo, similarly demonstrates integrin switching when cultured on MG (Kilburn et al., 2000b). When cultured on individual extracellular matrix components that are incapable of inducing integrin switching, integrin switching is restored in this cell line after treatment with EGF-like growth factors, including EGF, TGFA and HBEGF (Leach et al., 2004a).

HBEGF appears to act as a primary initiating factor in normal implantation. It is upregulated during the window of implantation in both mice (Das et al., 1994c) and humans (Leach et al., 1999b), and is specifically expressed juxtaposed to the implantation site in mice (Das et al., 1994c) and surrounding an implanted blastocyst in hamsters (Wang et al., 2002). In addition, intraluminal injection of HBEGF induces implantation of rat embryos in a delayed implantation model (Tamada et al., 1999). HBEGF expression is altered in the endometrium of women with unexplained fertility (Aghajanova et al., 2008) and conditional deletion of HBEGF in the mouse uterus will delay implantation of blastocysts without changing the preimplantation ovarian estrogen secretion (Xie et al., 2007). There is evidence that HBEGF is capable of stimulating



trophoblast invasion. Exogenous HBEGF accelerates blastocyst hatching from the zona pellucida and outgrowth of the trophoblast on fibronectin (Wang et al., 2000). HBEGF also increases blastocyst adhesion competency and trophoblast outgrowth in hamsters (Mishra and Seshagiri, 2000; Seshagiri et al., 2002). HBEGF induces trophoblast invasion from first trimester chorionic villi explants cultured on Matrigel (Leach et al., 2004a) and stimulates motility in an immortalized first trimester trophoblast cell line (Jessmon et al., 2010). However, the molecular regulation of integrin switching by either MG or growth factors remains to be established.

Integrin switching in trophoblast cells could be a key mechanism regulating normal implantation, and might also provide insights into pathologies stemming from inadequate trophoblast invasion, such as preeclampsia (PE). Integrin switching is highly restricted in placentas of women with PE, where expression of ITGA6 and other proteins associated with non-motile trophoblast persist (Zhou et al., 1993; Zhou et al., 1997a). Therefore, the shallow EVT invasion characteristic of PE could derive from this failure. Interestingly, HBEGF expression is greatly reduced in placentas of women with PE (Leach et al., 2002a), suggesting an association with the lack of integrin switching in PE, and a role in regulating integrin switching in healthy placentas.

The HTR-8/SVneo CTB cell line was used to evaluate integrin switching induced by MG or growth factors *in vitro*, examining extrinsic factors that could be responsible for integrin switching during implantation and early events of placentation. Since both MG and HBEGF induce integrin switching, it can be hypothesized that HBEGF and MG promote EVT differentiation through a common mechanism, perhaps due to presence of HBEGF bound to MG. Furthermore, it is important to identify the intracellular signaling

pathways that HBEGF utilizes to induce integrin switching in CTB cells. Previously, we found that signaling pathways downstream of HBEGF required to stimulate CTB motility include phosphoinositide-3-kinase (PIK3) and the three MAPK pathways mediated by mitogen-activated protein kinase 1/3 (MAPK1; ERK1/3), MAPK14 (p38) and c-Jun N-terminal kinase 1 (MAPK8/JNK) (Jessmon et al., 2010). Additionally, the role of transcription in regulating integrin switching was examined, since previous investigations have focused strictly on integrin protein levels and not reported whether the corresponding mRNAs were altered.

## MATERIALS & METHODS

### Cell Culture and Treatments

The first trimester human CTB cell line HTR-8/SVneo (Graham et al., 1993b) (provided by Dr. Charles Graham of Queens University) was cultured serum-free for 24 h on fibronectin- or MG-coated plastic, or on a gel formed by MG (BD Biosciences). Fibronectin was used to precoat plastic as previously described (Kilburn et al., 2000b). Plastic was coated with MG (Leach et al., 2004a; Liu et al., 2004) by diluting MG 1:5 in cold serum-free media, incubating in a culture flask for 1 h at 37°C, and washing residual MG away. Precoating with MG reduced background stain relative to cells cultured on a gel after subsequent immunocytochemical labeling of cells. MG was used to form gels by adding ice-cold, undiluted MG and incubating for 1 h at 37°C. The same procedures were followed for MG that had been depleted of its growth factors, (growth factor-reduced MG; GFR-MG; BD Biosciences). Recombinant HBEGF (R&D Systems), specific inhibitors of MEK1/2 (U0126), PIK3 (LY294002), p38 (SB203580), JNK (SP600125), and their corresponding inactive structural analogues (U0126, LY303511, SB202474, and JNK Inhibitor II negative control, respectively) were purchased from Calbiochem and were added during cell culture, as described by Jessmon et al. (Jessmon et al., 2010).

### Western Blotting

Western blots were performed, as previously described (Kilburn et al., 2000b). Briefly, cellular lysates were diluted in SDS sample buffer containing 5%  $\beta$ -mercaptoethanol, run on precast 4%–20% Tris-HCl gradient gels (BioRad), and blotted

with primary antibodies. Antibodies against ITGA1 (Upstate Biotechnology Inc.) and ITGA6 (Chemicon International) were diluted 1:1000 in Tris-Tween Buffered Saline (TTBS) containing 5% milk. Secondary anti-rabbit and anti-mouse antibodies (Cell Signaling Technologies) were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Bands were observed for ITGA1 and ITGA6 at 130-150 kDa and 120 kDa, respectively.

### **Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and stained for the presence of HBEGF protein, as previously described (Kilburn et al., 2000b; Leach et al., 2004a). ITGA1 and ITGA6 were visualized by immunocytochemistry using secondary antibodies conjugated to fluorescein and epifluorescence microscopy or a peroxidase-conjugated anti-mouse/anti-rabbit kit (Dako EnVision System-HRP), as described by Armant *et al.* (Armant et al., 2006). Image analysis was performed with an Orca (Hamamatsu) digital camera and quantified (peroxidase label only) according to published procedures (Leach et al., 2002a).

### **ELISA**

ELISA was conducted using the HBEGF DuoSet ELISA Development kit (R&D Systems), as previously described (Armant et al., 2006; Leach et al., 2008b). The optical density of the final reaction product was determined at 450 nm using a programmable multiplate spectrophotometer (Power Wave Workstation; Bio-Tek Instruments) with automatic wavelength correction.

### **qPCR**

ITGA1 and ITGA6 mRNAs were examined by endpoint and semi-quantitative real time RT-PCR and normalized to succinate dehydrogenase mRNA. According to the newly recommended MIQE guidelines, semi-quantitative real time RT-PCR will be referred to as qPCR (Bustin et al., 2009). RNA from HTR-8/SVneo cells was collected using the miRNeasy kit from Qiagen according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop spectrophotometer and purity was ascertained with an Agilent Bioanalyzer. Reverse transcription was performed using the Quantitect Reverse Transcription kit (Qiagen) and endpoint PCR was conducted using the HotStarTaq Master Mix Kit (Qiagen) according to the manufacturer's instructions. Thermal cycling for qPCR was conducted using the Quantitect SYBR Green PCR kit without UNG in a final volume of 25  $\mu$ L on an Applied Biosystems 7500 Fast real time thermal cycler. An appropriate housekeeping gene was chosen from a panel of genes: RRN18S, ACTB, GAPDH, RPL13A, RPLP0, and SDHA. SDHA (succinate dehydrogenase) had the least variability among all treatments. Semi-quantitative analysis was performed according to the  $\Delta\Delta$ Ct method (Pfaffl, 2001). Pre-validated primers for the housekeeping genes, ITGA1 and ITGA6 were obtained from Qiagen.

### **Statistics**

All assays were performed in triplicate, and all experiments were repeated at least three times and are reported as mean  $\pm$  SEM. Statistical significance was determined at  $P < 0.05$  by ANOVA with the Student-Newman-Keuls post hoc test, using SPSS version 12.0 statistics software (SPSS, Chicago, IL).

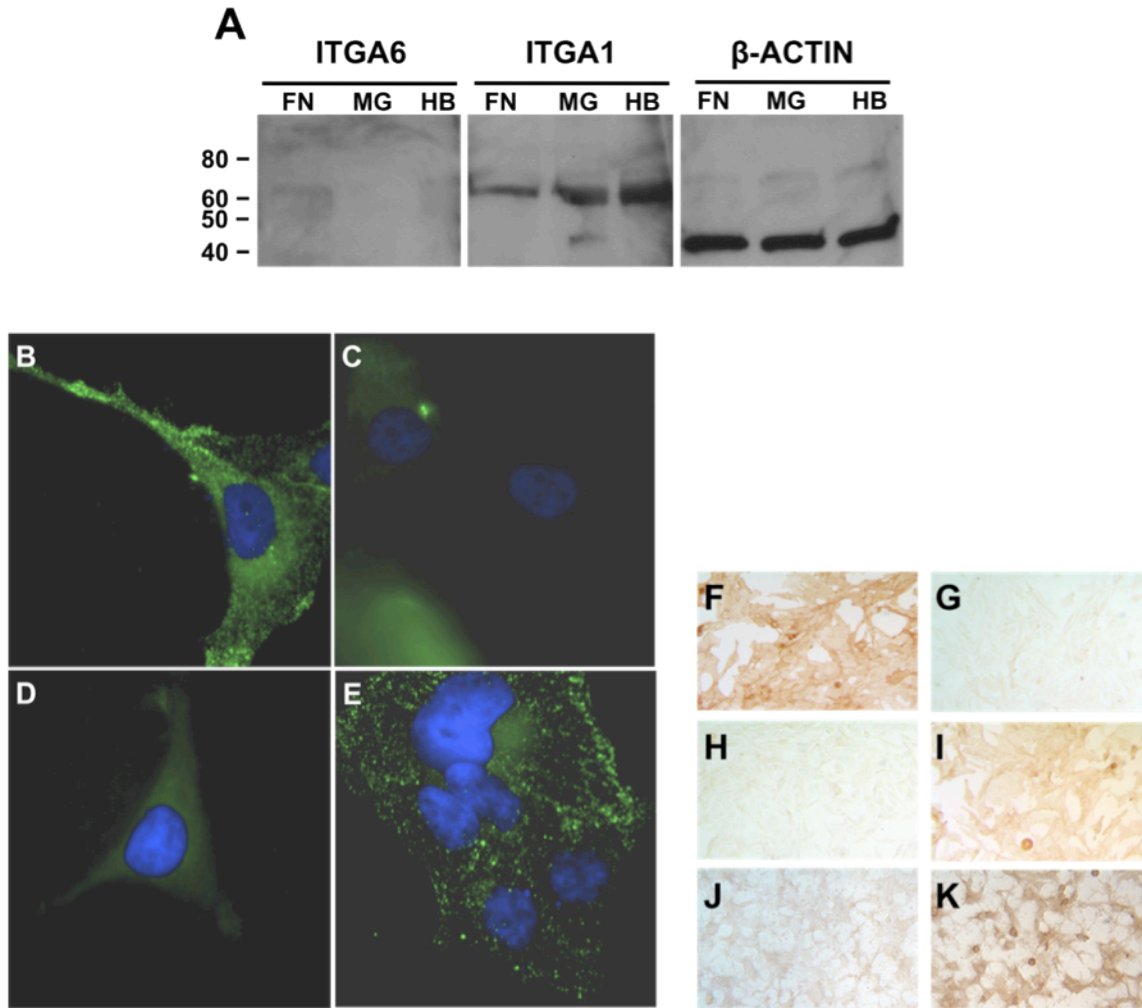
## RESULTS

### Integrin Switching Induced by MG and HBEGF

HTR-8/SVneo CTB cells were cultured on a MG gel or on fibronectin-coated plates with 1 nM HBEGF. Western blotting demonstrated that, as compared to controls (fibronectin-coated plates without HBEGF), culturing CTB cells in the presence of HBEGF or on MG upregulated ITGA1 and downregulated ITGA6 (Fig. 3.1 A). The same antibodies used for immunofluorescence microscopy demonstrated that treatment with HBEGF caused ITGA1 to localize within structures that appeared to be focal adhesions (Fig. 3.1 E compared to D), while ITGA6 disappeared from these structures (Fig. 3.1 C compared to B). Immunocytochemistry and quantitative image analysis (Fig. 3.1 F-K) established an equivalent effect of HBEGF supplementation (Fig. 3.1 H, I) or MG precoating (Fig. 3.1 J, K) on integrin switching as compared to culture on fibronectin alone (Fig. 3.1 F, G).

### Integrin Switching Induced by MG is not Dependent upon HBEGF Signaling

Since both HBEGF and MG could induce integrin switching, it is possible that HBEGF associated with the matrix could be responsible for the effects of MG. Therefore, the inhibition of HBEGF signaling during CTB culture on MG would be expected to prevent integrin switching. Previous investigation has demonstrated that HBEGF binds to ERBB1 or ERBB4 to activate downstream pathways (Armant et al., 2006). The abilities of a specific antagonist of HBEGF (CRM197) or a pan-ERBB kinase inhibitor (Ornskov et al., 2006) to block the effects of HBEGF on integrin switching were first examined. Both inhibitors were able to prevent integrin switching in



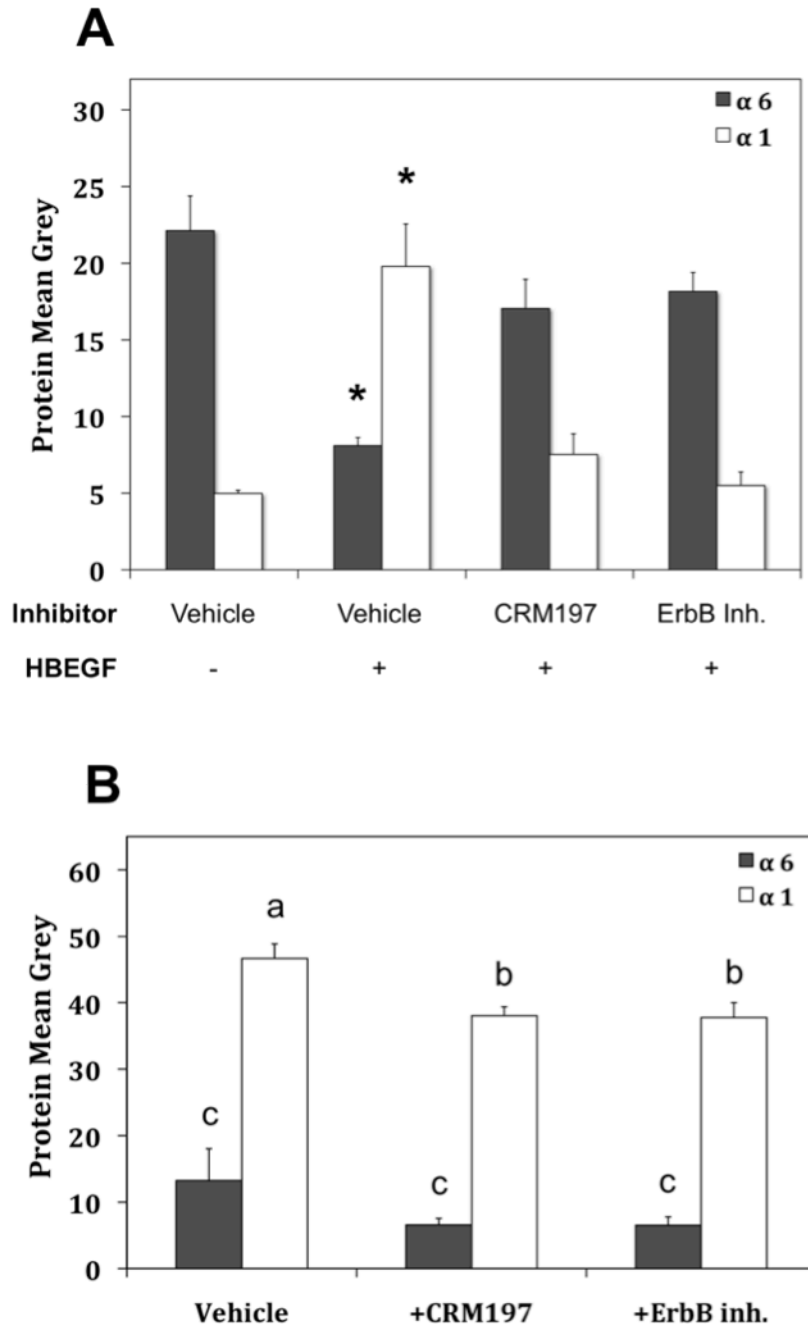
**Figure 3.1 Integrin switching induced by HBEGF and MG.** (A) Western blotting after culture on MG or on fibronectin (FN) in the presence of HBEGF (HB) for 24 h.  $\beta$ -actin was used as a loading control. (B-E) Immunofluorescence utilizing fluorescein-labeled secondary antibodies and DAPI counterstain to localize ITGA6 (B, C) and ITGA1 (D, E) protein with (C, E) or without (B, D) 1 nM HBEGF. (F-K) Immunocytochemistry demonstrating ITGA6 (F, H, J) and ITGA1 (G, I, K) protein in CTB cells cultured on FN (F, G) in the presence of 1 nM HBEGF (H, I) or on MG (J, K). (100x magnification)

CTB cells cultured on fibronectin in the presence of 1 nM HBEGF for 24 h (Fig. 3.2 A). However, when cells were cultured on a MG precoat in the presence of either inhibitor for 48 h, integrin switching was not abrogated (Fig. 3.2 B). In addition, HBEGF protein was not detectable by ELISA in solubilized MG (data not shown). These findings indicate that HBEGF and MG induce integrin switching independently, and that other components in MG are capable of inducing integrin switching.

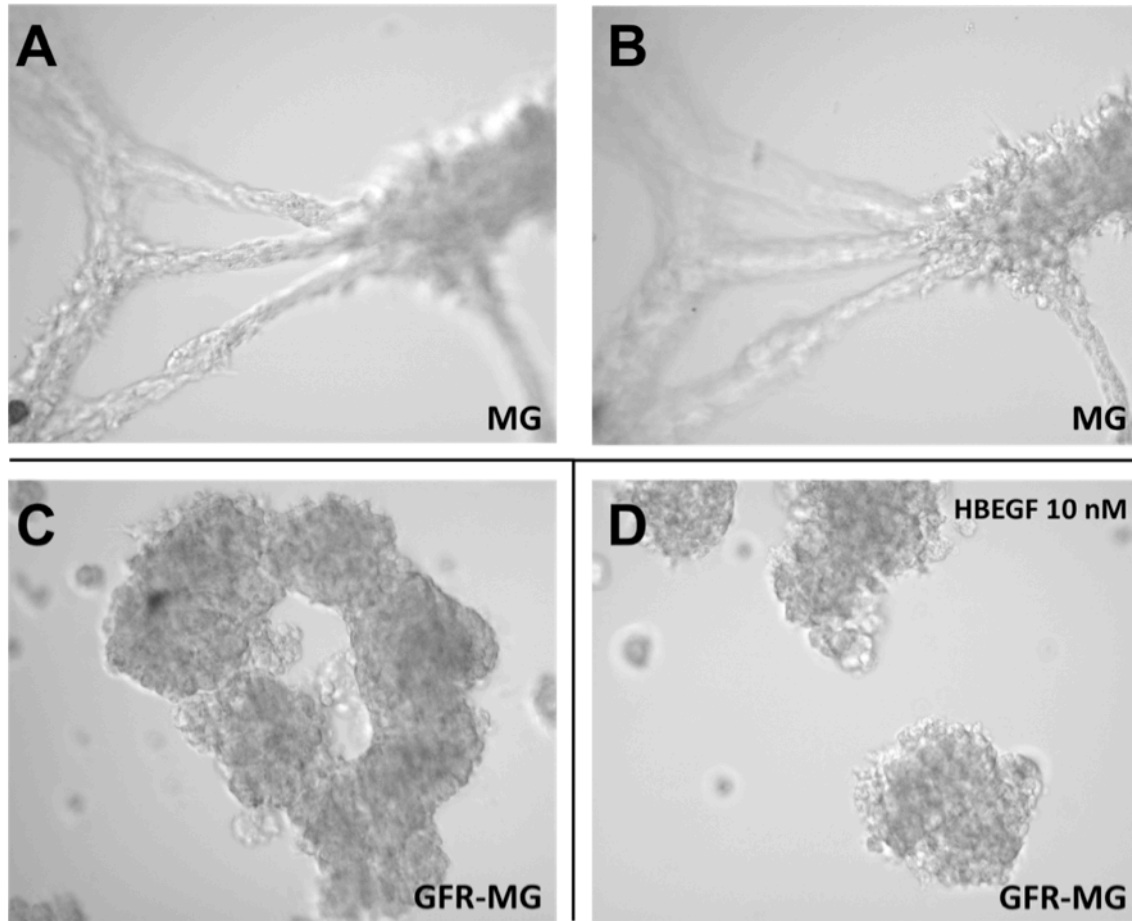
### **HBEGF Restores Integrin Switching On Growth Factor-reduced MG**

Although HBEGF does not appear to be responsible for the ability of MG to induce integrin switching, it is possible that other growth factors or matrix components of MG have that capacity. To examine the role of growth factors in MG, we first assessed invasion of CTB cells on gels composed of either MG or GFR-MG. Phase contrast light microscopy demonstrated that CTB cells failed to invade GFR- MG, whereas cells formed into invading streams on complete MG and penetrated deep into the gel (Fig. 3.3 A-C). Culture on GFR-MG in the presence of 10 nM HBEGF for 48 h was insufficient to rescue the invasive phenotype (Fig. 3.3 D), suggesting that while integrin switching is associated with invasion (Zhou et al., 1997b), other steps are required that cannot be restored by HBEGF signaling. Although there was no apparent change in cell invasion, we examined the potential of HBEGF and other growth factors thought to influence EVT differentiation (Knofler, 2010) to induce integrin switching on a GFR-MG precoat. After 48 h, immunocytochemistry and quantitative image analysis demonstrated that integrin switching occurred on MG, but not on GFR-MG, could be fully restored on GFR-MG by supplementation with 1 nM recombinant HBEGF, and was partially restored with EGF, TGFA, AREG, and VEGF (Fig. 3.4). More specifically, EGF

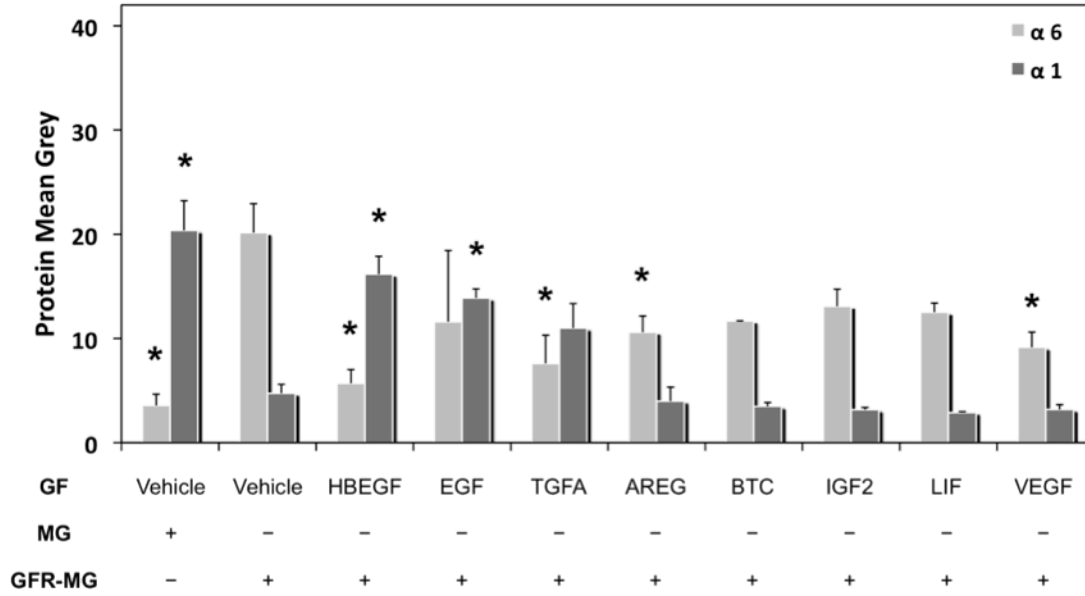




**Figure 3.2 Inhibition of HBEGF signaling does not block integrin-switching induced by MG.** Quantitative image analysis for ITGA1 (white bars) and ITGA6 (black bars) protein in cells cultured on fibronectin in the presence of HBEGF (A) or culturing on MG (B), an inhibitor of HBEGF (CRM197) or its receptors (ErbB Inh), as indicated. \*  $p < 0.05$  as determined by t-test (A). Lowercase letters designate significantly similar groups according to student-newman-keuls posthoc test (B).



**Figure 3.3 CTB cells do not invade GFR-MG.** Images acquired at two different focal depths of CTB cells cultured on a MG gel (A, B), or culture on GFR-MG gel (C) and in the presence of HBEGF (10 nM) for 48 h. Invasive streams of CTBs can be seen in A and B.



**Figure 3.4 HBEGF induces integrin-switching in CTB cells cultured on GFR-MG.**

Quantitative image analysis performed for ITGA6 (light bars) and ITGA1 (dark bars) protein in CTB cells cultured on MG or GFR-MG for 24 h and in the presence of recombinant HBEGF, epidermal growth factor (EGF), transforming growth factor- $\alpha$  (TGFA), amphiregulin (AREG), betacellulin (BTC), insulin-like growth factor 2 (IGF2), leukemia inhibitory factor (LIF), or vascular endothelial growth factor (VEGF), as indicated. \* indicates significantly different from GFR-MG vehicle control ( $p < 0.05$ ) according to one-tailed Student's t-test for each treatment. Analysis was run separately for ITGA6 and ITGA1.

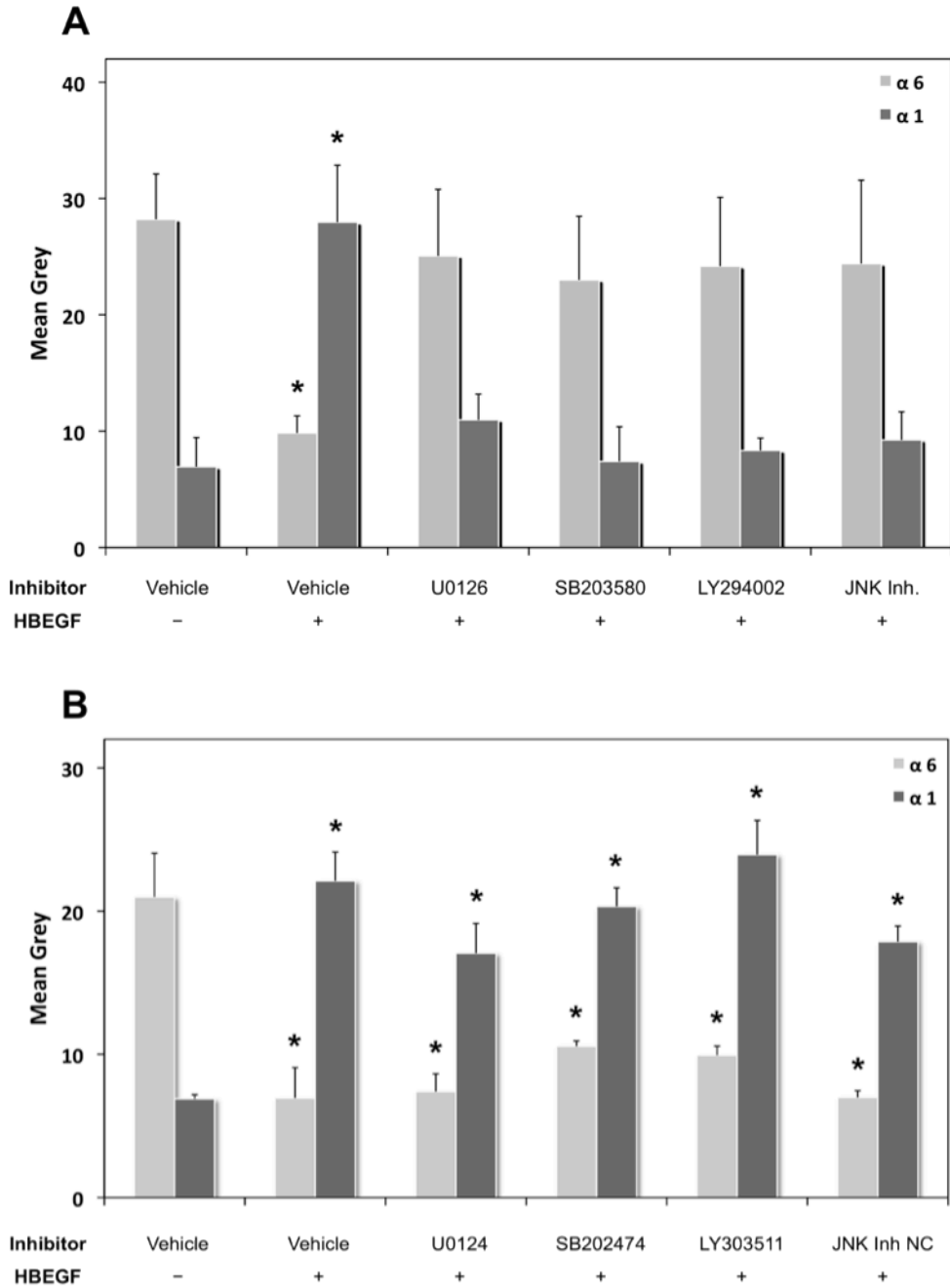
could increase the expression of ITGA1 compared to GFR-MG, but was unable to suppress production of ITGA6. Conversely, TGFA, AREG, and VEGF were able to decrease expression of ITGA6 compared to GFR-MG, but were unable to significantly upregulate ITGA1. BTC, another EGF-related growth factor, did not alter integrin expression. Surprisingly, IGF2 and LIF, previously found to promote trophoblast motility or invasion (Burton et al., 2007; Hamilton et al., 1998), had no effect on expression of ITGA1 or ITGA6.

### **HBEGF Induces Integrin Switching Through Multiple Signaling Pathways**

As HBEGF appears unique in its ability to fully induce integrin switching on GFR-MG, downstream intracellular signaling pathways utilized by HBEGF were examined. We previously reported that HBEGF induces CTB cell migration through the MEK1/2, PIK3, p38, and JNK pathways (Jessmon et al., 2010). Using inhibitors of each of these pathways, their involvement in HBEGF-induced integrin switching was examined. Inhibitors of MEK1/2 (U0126), PIK3 (LY294002), p38 (SB203580), and JNK (SP600125) were each able to block integrin switching induced by HBEGF, while less active structural analogues of each inhibitor were without effect (Fig. 3.5). These data indicate that HBEGF requires all four pathways to induce integrin switching.

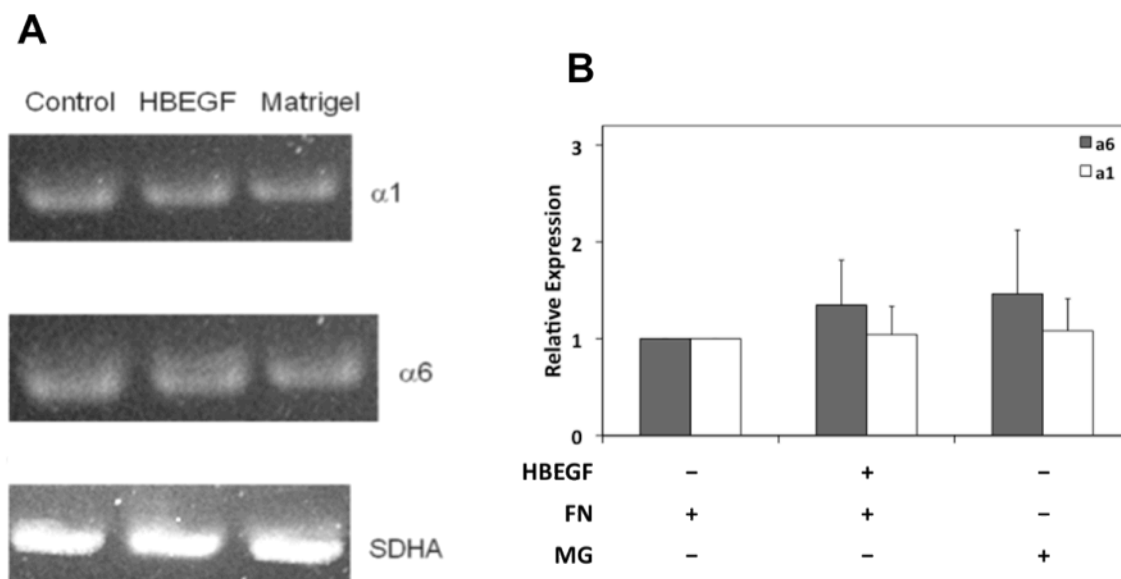
### **Post-Transcriptional Regulation of Integrin Switching**

To examine the effects of HBEGF and MG on integrin mRNA expression, RNA collected from CTB cells was reverse transcribed and both endpoint and qPCR were performed. As demonstrated by agarose gel electrophoresis after endpoint PCR, treatment with HBEGF or culture on MG did not appear to alter *ITGA1* or *ITGA6* mRNA



**Figure 3.5 Integrin-switching induced by HBEGF is blocked by downstream signaling inhibitors.** Quantitative image analysis performed for ITGA6 (light bars) and ITGA1 (dark bars) protein in CTB cells cultured on fibronectin for 24 h in the presence of (A) inhibitors of MEK1/2 (U0126), p38 (SB203580), PIK3 (LY294002), or JNK (JNK Inh), or (B) their respective inactive structural analogues, as indicated. One-tailed t-test as compared to vehicle control run separately for ITGA6 and ITGA1, \*  $p < 0.05$

expression (Fig. 3.6 A). Measurement of *ITGA1* and *ITGA6* transcripts by qPCR, normalized to *SDHA* expression, confirmed the absence of a change in the concentration of either mRNA due to either treatment (Fig. 3.6 B), suggesting that the proteins are regulated post-transcriptionally during integrin switching.



**Figure 3.6 ITGA1 and ITGA6 are regulated post-transcriptionally.** (A) Endpoint PCR for *ITGA1* and *ITGA6* in CTB cells cultured on fibronectin (control), in the presence of 1 nM HBEGF, or on a Matrigel gel for 24 h visualized by agarose gel electrophoresis. PCR for *SDHA* was used as a housekeeping control. (B) Averaged results from qPCR (normalized to *SDHA* expression) for *ITGA1* or *ITGA6* transcripts in CTB cells cultured on fibronectin (control), in the presence of 1 nM HBEGF, or on a Matrigel gel for 24 h.  $p > 0.05$  by 1-way ANOVA for both *ITGA1* ( $F = 0.026$ ) and *ITGA6* ( $F = 0.267$ ).

## DISCUSSION

CTB cells adhere to the maternal uterine endometrium and invade the underlying stromal tissue to establish a placenta. Integrin switching appears to fulfill an important role in the initiation of extravillous differentiation. Integrin ITGA6/ITGB4 is characteristic of non-motile CTB cells, while ITGA1/ITGB1 promotes invasion (Damsky et al., 1994), representative of several identified molecular modifications associated with EVT differentiation (19). Many cytokines, growth factors and hormones can regulate this change in protein expression (Knofler, 2010). Both HBEGF and MG induced integrin switching in an immortalized CTB cell line, and although the MG mechanism did not rely on HBEGF or ERBB signaling, both MG and HBEGF regulated expression through a post-transcriptional mechanism. It can be contemplated that CTB cells retain transcripts that are translated only when instructed by extrinsic cues encountered during development, which direct their differentiation and adaptation to local changes in the microenvironment. Therefore, specific proteins could be accumulated rapidly without the inefficiencies associated with transcription and its layers of regulation. Several mechanisms to repress or activate specific transcripts are known that involve ribonucleoprotein complexes and small inhibitory RNA or micro-RNA species (Filipowicz et al., 2008; Jopling et al., 2005; Orom et al., 2008; Pillai et al., 2007; Vasudevan and Steitz, 2007; Vasudevan et al., 2007).

The effects of MG were not dependent on HBEGF signaling or other growth factors of the EGF signaling system that utilize ERBB receptor tyrosine kinases, indicating that other growth factors associated with MG might induce extravillous differentiation of CTB cells. We were unable to detect HBEGF in MG by western



blotting or ELISA (data not shown); however, the effective activity of HBEGF (~1 nM) would be below detection limits. MG is a biologically active, basement membrane-like matrix secreted by the Engelbreth-Holm Swarm (EHS) tumor that is widely used in cell culture, cell differentiation and invasion assays (Biederer and Scheiffele, 2007; Kleinman and Martin, 2005; Terranova et al., 1986). CTB cells cultured on MG demonstrated integrin switching, but not when growth factors were removed. A previous analysis of growth factors known to be important in bone cell proliferation and differentiation determined that TGFB, EGF, IGF1, bFGF, and PDGF were present in MG (Vukicevic et al., 1992). The integrin switching observed on MG is also observed when first trimester CTB cells are cultured on a collagen IV/laminin matrix (Damsky et al., 1994). Disruption of interactions with laminin or collagen IV inhibits CTB invasion, which suggests that matrix components signal through adhesion molecules such as integrins to induce cellular invasion. This idea was not supported by the finding that matrix components of MG lacked the capacity to induce integrin switching. It is likely that signaling from both matrix components and growth factors can contribute to integrin switching. Indeed, CTBs grown on MG secrete PIGF and cells grown on laminin secrete both PIGF and VEGF-A, suggesting that these growth factors influence integrin switching (Zhou et al., 1997b; Zhou et al., 2002). Disruption of ligand binding to either VEGFR-1 or VEGFR-3 of CTBs cultured *in vitro* results in decreased ITGA1 expression, suggesting that VEGF signaling helps to mediate integrin switching. Our results suggest that VEGF, in combination with other signaling factors, may influence integrin switching in CTBs. It could be speculated that growth factors induce changes in CTB cells that allow matrix components to subsequently initiate integrin outside-in signaling

that induces invasion (Aplin et al., 1998; Armant, 2005; Burridge and Chrzanowska-Wodnicka, 1996; Giancotti, 2000; Hynes, 1992).

These are the first data to demonstrate that HBEGF regulates trophoblast invasion at the post-transcriptional level. A previous study that examined freshly isolated first and second trimester CTB cells detected an increase in *ITGA1* mRNA after culturing on MG for 24 hr (Damsky et al., 1994). It is important to note that cells were compared that were attached either to fibronectin (with or without HBEGF) or MG, while the previous study compared cells attached to MG with unattached, freshly isolated CTB cells. Therefore, differences in the findings could be due to cell attachment. Further analysis is required to delineate how HBEGF upregulates integrin protein post-transcriptionally. One possibility is that *ITGA1* mRNA is sequestered and translationally repressed. A change in microenvironment that initiates HBEGF signaling or other mechanisms that activate the appropriate downstream pathways could then derepress translation of the transcript. MicroRNAs (miRNAs) are widely studied for their role in translational repression. MiRNAs are ~22 nt endogenous small RNA species that target mRNA transcripts within complementary 3'UTR sequences to repress or activate translation (Kim and Nam, 2006; Pillai et al., 2007). A stable pool of *ITGA1* transcripts could be bound by miRNA and hence translationally repressed until derepressed by HBEGF signaling. HBEGF could simultaneously elevate expression of specific miRNAs that bind *ITGA6* mRNA to inhibit its translation. Through this mechanism, CTB cells would rapidly regulate integrins for invasion through extracellular matrices during embryo implantation without expending energy for transcription. Indeed, rapid mechanisms could be in place that allow CTB cells on the surface of a blastocyst to

adhere to the uterine epithelial surface and then invade the underlying basement membrane and decidual stroma. Changes in matrix components would be encountered with different integrin expression profiles. As the placenta forms, trophoblast cells in floating villi express predominantly ITGA4, CTB cells invading the decidua favor ITGA1 over ITGA6, and CTB cells that invade maternal blood vessels upregulate ITGAV, demonstrating that trophoblasts adapt to different extracellular matrix environments (Zhou et al., 1997b). Therefore, it is feasible that other rapid cellular responses are responsible for integrin-switching in trophoblasts during implantation.

Previously, a role in HBEGF-induced cell migration was established for the p38, ERK1/2 (p42/44), PIK3 and JNK pathways (Jessmon et al., 2010). These same downstream pathways were necessary for induction of integrin switching, supporting the view that integrin switching is a key inaugural step in EVT differentiation. It is likely that this switch is involved in HBEGF-induced cell migration. EGF can also induce trophoblast cell motility via similar downstream pathways. In SGHPL-4 cells, EGF stimulated motility through PIK3, p38 and ERK1/2 (p42/44) (LaMarca et al., 2008). In addition, EGF, as found in decidua conditioned media, promotes trophoblast migration, but through downregulation of the gap junction protein Cx40 in JAR cells (Oh et al., 1993; Wright et al., 2006). Integrin-mediated cell attachment likely initiates kinase signaling, either through constitutive localization of FAK to the integrin  $\alpha$  subunit, or growth factor-mediated recruitment of FAK to the integrin  $\beta$  subunit (Giancotti, 2000). It is possible that integrin attachment-mediated signaling integrates with HBEGF to regulate integrin switching.

## CHAPTER 4. MMP2 Controls Low Oxygen-Induced Autocrine HBEGF Signaling in Human First Trimester Trophoblast Cells

### SUMMARY

Heparin-binding EGF-like growth factor (HBEGF) is expressed by trophoblast cells throughout gestation, but is absent in placentas from women with preeclampsia. HBEGF is upregulated at low O<sub>2</sub> concentrations through a mechanism that requires metalloproteinase-mediated shedding of its extracellular domain. Signaling downstream of HBEGF receptors (ERBB1 and ERBB4) both increases HBEGF synthesis and prevents apoptosis caused by low O<sub>2</sub>. Transcriptional events and metalloproteinases involved in the upregulation of HBEGF protein were examined using a human first-trimester cytotrophoblast cell line, HTR-8/SVneo, exposed to 20% O<sub>2</sub>, 2% O<sub>2</sub> or 250 μM CoCl<sub>2</sub> that upregulates hypoxia inducible factor (HIF) at 20% O<sub>2</sub>. HBEGF protein, quantified by ELISA, increased in cells cultured at 2% O<sub>2</sub> for 6 h or in the presence of CoCl<sub>2</sub> for 2 h. The RNA polymerase II inhibitor, α-amanitin, inhibited HBEGF protein accumulation during either treatment, suggesting that factors regulating HBEGF upregulation require hypoxia-stimulated transcription. The accumulation of HIF1α and HIF2α at 2% O<sub>2</sub> or during CoCl<sub>2</sub> treatment was confirmed by western blotting. Microarray analysis of the transcriptome demonstrated that after 4 h at 2% O<sub>2</sub> expression of genes related to metalloproteinase function increased, including *BSG*, *MMP-2* and *MMP-15*. Inhibitors specific for MMP2 or both MMP2 and MMP9, but not MMP9 alone, prevented the increase in HBEGF at 2% O<sub>2</sub>. These findings indicate that the increased expression of HBEGF at 2% O<sub>2</sub> is dependent upon HIF and *de novo* transcription. Of several early response genes, experimental evidence implicates *MMP-*

2 as a critical member of a proteolytic cascade that mediates the shedding of HBEGF to initiate autocrine signaling required for its rapid synthesis.

## INTRODUCTION

A low O<sub>2</sub> environment is present at the human implantation site and persists for the first 10 weeks of pregnancy as invasive cytotrophoblast (CTB) cells penetrate the maternal decidua (Burton and Jauniaux, 2004; Burton et al., 1999; Jauniaux et al., 2001a; Rodesch et al., 1992). Whereas most cells do not survive well at low O<sub>2</sub> concentrations, trophoblast cells actually proliferate faster at 2% O<sub>2</sub> than at atmospheric levels (20%) (Genbacev et al., 1996b; Genbacev et al., 1997b). After 10 weeks of gestation, trophoblast plugs in the uterine spiral arteries are dislodged and maternal blood flows freely into the intervillous space of the placenta, raising O<sub>2</sub> from approximately 18 mm Hg to 60 mm Hg (Jauniaux et al., 2001a; Rodesch et al., 1992). As proliferation abates due to the rise in O<sub>2</sub>, trophoblast cells become more invasive (Norwitz et al., 2001). Survival factors must be in place that protect the trophoblast from hypoxia, particularly during early placentation. Several proteins have been identified that provide survival function to protect against apoptosis in CTB (Armant et al., 2006; Dash et al., 2005; Jaleel et al., 2004; Mackova et al., 2003; Singh et al., 2010).

A member of the epidermal growth factor (EGF) signaling system, heparin-binding EGF-like growth factor (HBEGF) is present at the implantation site in mice and is required for normal implantation (Leach et al., 1999b; Yoo et al., 1997a). Maternal HBEGF deficiency in mice defers on-time implantation, leading to reduced litter size (Xie et al., 2007), and suggesting its important role in implantation. HBEGF is also present in the human endometrium during the window of implantation (Leach et al., 1999b; Stavreus-Evers et al., 2002; Yoo et al., 1997a). HBEGF, but not other EGF family members, is upregulated in human CTB cells cultured at 2% O<sub>2</sub> and can

stimulate differentiation to the invasive phenotype at higher O<sub>2</sub> concentrations (Armant et al., 2006; Leach et al., 2004a). Inhibition of HBEGF signaling during CTB culture at low O<sub>2</sub> results in apoptosis, identifying HBEGF as a critical survival factor in that environment. During the first trimester, survival and invasive differentiation are key functions required for expansion of the placenta and remodeling of the spiral arteries (Hunkapiller and Fisher, 2008; Staun-Ram and Shalev, 2005; Zhou et al., 1997b). Previously, it was reported that HBEGF expression is deficient in placentas from women with preeclampsia, which could contribute to the reduced trophoblast invasion and increased apoptosis characteristic of this syndrome (Leach et al., 2002a).

HBEGF is synthesized intracellularly as transmembrane proHBEGF and is secreted through shedding from the cell membrane by proteolytic cleavage, allowing it to activate its cognate receptor tyrosine kinases (EGF receptor/ERBB1; ERBB4) and initiate intracellular signaling (Holbro and Hynes, 2004b). HBEGF specifically binds ERBB1 and ERBB4, but can transactivate ERBB2 or ERBB3 due to heterodimerization among the four ERBB receptor family members . Downstream of its receptors, HBEGF signaling induces an increase in trophoblast motility, prevents apoptosis, and upregulates its biosynthesis through an autocrine feedback mechanism (Armant et al., 2006; Jessmon et al., 2010; Leach et al., 2004a). Although HBEGF is upregulated (~100-fold) in CTB cells after exposure to 2% O<sub>2</sub> (Armant et al., 2006), HBEGF mRNA remains unchanged and abundant (2500 copies/cell) at both O<sub>2</sub> concentrations. Blocking HBEGF accumulation with a metalloproteinase inhibitor leads to increased apoptosis at low O<sub>2</sub> that is rescued with recombinant HBEGF, but not other EGF-like ligands. Autocrine HBEGF activity in CTB cells requires, in addition to

metalloproteinase-mediated shedding of HBEGF, binding to either ERBB1 or ERBB4. Blocking HBEGF signaling also prevents its upregulation at 2% O<sub>2</sub>, suggesting that low levels of resident proHBEGF are cleaved through activation of metalloproteinases to initiate its autocrine synthesis downstream of ERBB receptors in a positive feedback loop.

To further investigate the regulation of HBEGF expression by O<sub>2</sub>, we examined the roles of nacent transcription, O<sub>2</sub>-sensitive transcription factors and metalloproteinases, using an immortalized human CTB cell line.



## MATERIALS & METHODS

### Cell Culture and Treatments

The first trimester human CTB cell line HTR-8/SVneo (Graham et al., 1993b) was cultured at either 20% O<sub>2</sub> or 2% O<sub>2</sub>, as previously described (Leach et al., 2004a; Leach et al., 2008b). Cells were cultured in the presence of  $\alpha$ -amanitin (Sigma-Aldrich), CoCl<sub>2</sub> (250 $\mu$ M; Sigma-Aldrich) or inhibitors (EDM Chemicals, Inc., Gibbstown, NJ) specific for MMP2 and MMP9 ((2R)-[(4-Biphenylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide, BiPS; 100 nM), MMP2 only ((2-((isopropoxy)-(1,1'-biphenyl-4-ylsulfonyl)-amino))-N-hydroxyacetamide; 250 nM) or MMP9 only (MMP-9 Inhibitor I; 100 nM), prepared in sterile DMEM/F-12 media at the indicated times and concentrations.

### Western Blotting

Western blots were performed as previously described (Kilburn et al., 2000b). Briefly, cellular lysates were diluted in SDS sample buffer containing 5%  $\beta$ -mercaptoethanol, run on precast 4%–20% Tris-HCl gradient gels (BioRad), and blotted with antibodies against HIF1A (R&D Systems), HIF2A/EPAS1 (Novus Biologicals) or HIF3A (Novus Biologicals) diluted 1:1000 in TTBS and 5% milk. Densitometry was used to quantify grey levels over protein bands of interest using image analysis software (SimplePCI). Background grey levels determined in a blank lane were subtracted to obtain the specific grey level for each band.

### ELISA

ELISA was conducted using the HBEGF DuoSet ELISA Development kit (R&D Systems), as previously described (Armant et al., 2006; Leach et al., 2008b). The optical density of the final reaction product was determined at 450 nm using a programmable multiplate spectrophotometer (Power Wave Workstation; Bio-Tek Instruments) with automatic wavelength correction. Data are presented as picograms (pg) of HBEGF per microgram ( $\mu\text{g}$ ) of total protein.

### **qPCR**

RNA from HTR-8/SVneo cells was collected using the miRNeasy kit (Qiagen) according to the manufacturer's protocol. RNA concentration was determined using the NanoDrop spectrophotometer and purity was ascertained with an Agilent Bioanalyzer (Agilent). RNA was used either for microarray analysis, as described below, or in subsequent qPCR (Bustin et al., 2009) analysis. Reverse transcription was performed using the Quantitect Reverse Transcription kit (Qiagen) and qPCR was conducted with the Quantitect SYBR Green PCR kit without UNG (Qiagen) in a final volume of 25  $\mu\text{L}$ . An appropriate housekeeping gene was chosen from a panel of genes: RRN18S, ACTB, GAPDH, RPL13A, RPLP0, SDHA. For validating the microarray results, SDHA (succinate dehydrogenase) was chosen as an internal reference because it varied the least with all experimental treatments. Semi-quantitative analysis was performed according to the  $\Delta\Delta\text{Ct}$  method (Pfaffl, 2001). Primers for the housekeeping genes and for HBEGF were obtained from Qiagen.

### **mRNA Microarray**

The Agilent 60-mer Whole Human Genome G4112F Microarray (Agilent, Santa Clara, CA) having about 45,000 probes was used to obtain the global mRNA expression profiles (Agilent part number G4410-90040). Total RNA was processed, labeled, and hybridized onto the microarrays according to the manufacturer's protocols. Microarray slides were then scanned using the Agilent scanner and microarray data extracted using the Agilent Feature Extraction software v.9.5.3.1. Features altered at least 2-fold with good correlation between the duplicate time course patterns (Corr. Coeff > 0.7) and a signal above 100 were selected for further analysis.

### **Statistics**

All assays were performed in triplicate, and all experiments were repeated at least three times and are reported as mean  $\pm$  SEM. Statistical significance was determined at  $P < 0.05$  by ANOVA with post hoc students t-test, using SPSS version 12.0 statistics software (SPSS, Chicago, IL). Significance in the microarray analysis was determined at  $p < 0.1$ .

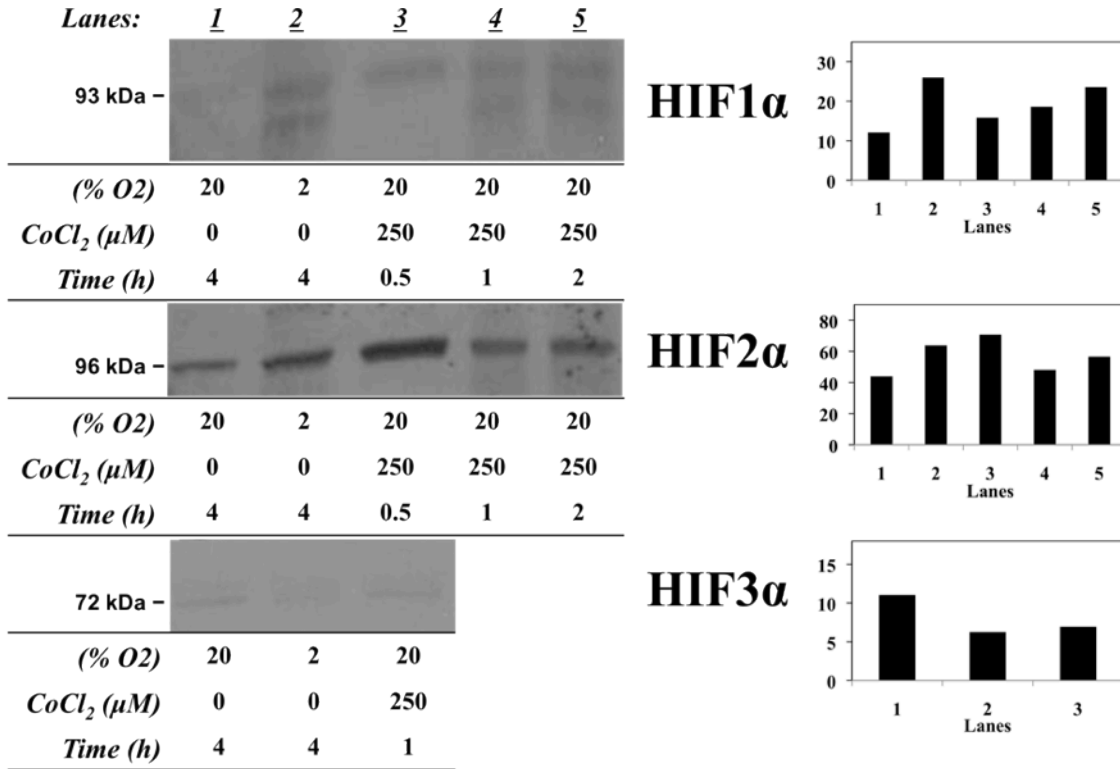
## RESULTS

### **O<sub>2</sub> regulates HBEGF through HIF1 $\alpha$ and HIF2 $\alpha$**

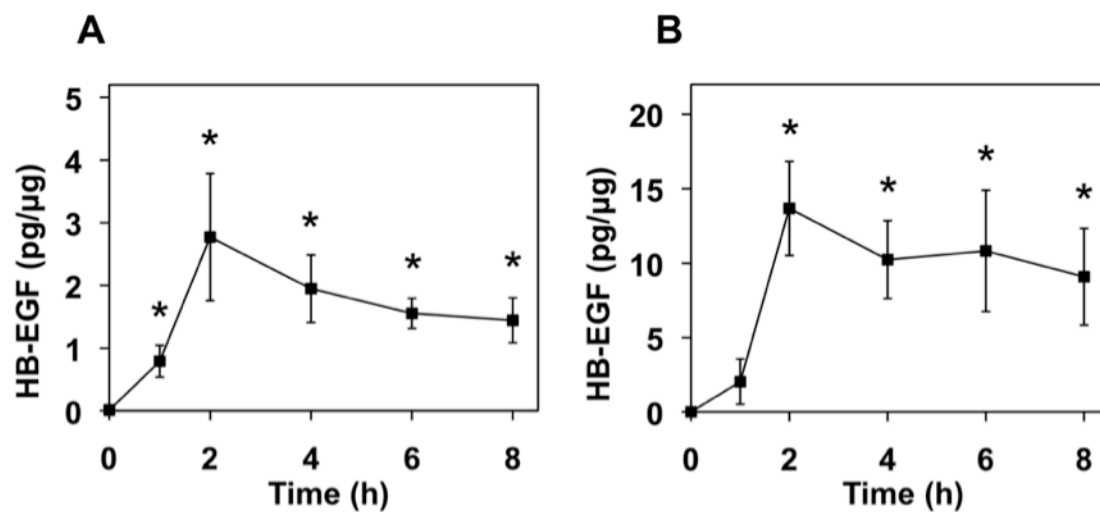
CoCl<sub>2</sub>, a chemical mimic of hypoxia that stabilizes HIF (Jiang et al., 1997), was used to examine the effect of 2% O<sub>2</sub> on HBEGF expression in the HTR-8/SVneo CTB cell line. Culturing CTB cells either for 4 h at 2% O<sub>2</sub> or in the presence of 250  $\mu$ M CoCl<sub>2</sub> for 30 min to 2 h stabilized the transcription factors, HIF1 $\alpha$  and HIF2 $\alpha$ , as indicated by their increased labeling on Western blots (Fig. 4.1). HIF2 $\alpha$  increased very rapidly during CoCl<sub>2</sub> treatment, peaking at 30 min, while HIF1 $\alpha$  took longer to accumulate. HIF3 $\alpha$  was either unaffected or decreased slightly in CTB cells culture at 2% O<sub>2</sub> or in the presence of CoCl<sub>2</sub>. In addition, treatment with CoCl<sub>2</sub> for 1 to 8 h increased both cellular and secreted levels of HBEGF protein (Fig. 4.2). These findings suggest that O<sub>2</sub> regulates HBEGF synthesis and secretion through its effect on the protein concentrations of HIF1 $\alpha$  and HIF2 $\alpha$ .

### **New Transcription is Required to Increase HBEGF**

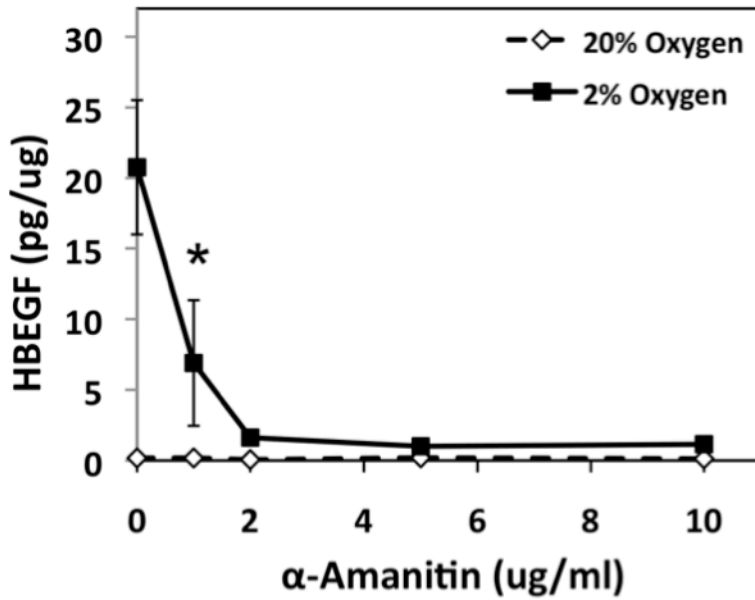
The involvement of HIF transcription factors in the regulation of HBEGF by O<sub>2</sub> suggested a mechanism based on differential gene expression. Therefore, CTB cells were cultured at 2% O<sub>2</sub> with or without  $\alpha$ -amanitin, an inhibitor of RNA polymerase-II (Lindell et al., 1970; Stirpe and Fiume, 1967), to examine the role of new transcription in the upregulation of HBEGF protein. Elevation of HBEGF protein after culture at 2% O<sub>2</sub> for 6 h was abrogated in a dose-dependent manner by  $\alpha$ -amanitin with maximal inhibition attained at 5  $\mu$ M (Fig. 4.3). The inhibitor also blocked the CoCl<sub>2</sub>-mediated increase in both cellular and secreted forms of HBEGF (Table 4.1). It was previously



**Figure 4.1 Stabilization of HIF1α and HIF2α by CoCl<sub>2</sub>.** Extracts of HTR-8/SVneo cells cultured at either 20% O<sub>2</sub>, 2% O<sub>2</sub> or at 20% O<sub>2</sub> in the presence of 250 μM CoCl<sub>2</sub> for 30 min - 2 h were examined by Western blotting for HIF1α (upper band), HIF2α, and HIF3α expression. Densitometry of bands after background subtraction is shown to the right with x-axis labels corresponding to respective Western blot lanes.



**Figure 4.2 Upregulation of cellular and secreted HBEGF protein after treatment with CoCl<sub>2</sub>.** ELISA for HBEGF in cellular extracts (A) and in media covering the cells (B) after treatment with 250 μM CoCl<sub>2</sub> for 30 min – 8 h. \* p < 0.05 according to Student's t-test following one-way ANOVA (F = 0.017 for lysate; F = 0.012 for media) comparing time points to zero hour time.



**Figure 4.3 Upregulation of HBEGF protein requires new transcription.** ELISA for HBEGF in extracts from cells cultured for 6 h at 20% (dashed line with open circles) or 2% (solid line with black squares) O<sub>2</sub> in the presence of varying doses of  $\alpha$ -amanitin, as indicated (1-tail students t-test, \* p < 0.05 compared to no treatment control).

**Table 4.1. Upregulation of HBEGF protein by CoCl<sub>2</sub> requires new transcription.**

$\alpha$ -Amanitin ( $\mu\text{g}/\text{mL}$ )	HBEGF (pg/ $\mu\text{g}$ )			
	Lysate		Media	
	CoCl <sub>2</sub> ( $\mu\text{M}$ )		CoCl <sub>2</sub> ( $\mu\text{M}$ )	
	0	250	0	250
0	0.012 $\pm$ 0.007	76.963 $\pm$ 37.262	0.00 $\pm$ 0.00	0.721 $\pm$ 0.62
5	0.035 $\pm$ 0.015	0.086 $\pm$ 0.051	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001
10	0.035 $\pm$ 0.016	0.043 $\pm$ 0.031	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001

HBEGF protein was quantified by ELISA in lysates and media covering CTB cells cultured with or without 250  $\mu\text{M}$  CoCl<sub>2</sub>. CoCl<sub>2</sub> treatment induced an increase in both cellular and secreted HBEGF that was abrogated by either 5 or 10  $\mu\text{g}/\text{mL}$   $\alpha$ -amanitin. Values are listed as averages  $\pm$  standard error.



demonstrated that HBEGF synthesis and secretion increase at 2% O<sub>2</sub> without a detectable change in levels of its transcript (Armant et al., 2006). Therefore, it is unlikely that HIF signaling directly targets *HBEGF* transcription. The effects of  $\alpha$ -amanitin on HBEGF suggest that transcription of other genes, perhaps those regulated by HIF1 $\alpha$  or HIF2 $\alpha$ , are required to initiate HBEGF protein synthesis.

### **O<sub>2</sub> Regulates Transcription of Metalloproteinases**

It was previously shown that HBEGF autocrine signaling through ERBB receptors upregulates HBEGF protein levels at 2% O<sub>2</sub> (Armant et al., 2006; Jessmon et al., 2010). Cell surface proHBEGF is proteolytically cleaved, releasing soluble HBEGF that can bind ERBB1 and ERBB4 to generate intracellular signaling (Holbro and Hynes, 2004). Therefore, low O<sub>2</sub> and HIF signaling could induce transcription of a metalloproteinase that sheds HBEGF to initiate autocrine signaling required for HBEGF accumulation. Microarray analysis of RNA from CTB cells cultured at 2% or 20% O<sub>2</sub> for 1, 2, or 4 h identified several transcripts that mediate metalloproteinase function that were differentially altered by low O<sub>2</sub>, including basigin (*BSG*), a disintegrin and metalloprotease 10 (*ADAM10*), *ADAM15*, *ADAM9*, matrix metalloproteinase 2 (*MMP2*), and *MMP15* (Table 4.2). *ADAM9* and *ADAM10* decreased during exposure to low O<sub>2</sub>, while the other transcripts increased. Most notably, *MMP2* increased significantly ( $p = 0.056$ ) by over 40-fold after 4 h. Subsequent qPCR analysis confirmed the increased expression and indicated that *ADAM9* and *ADAM10* actually were upregulated by low O<sub>2</sub> in CTB cells (Table 4.3). According to qPCR, *MMP15* and *MMP2* were maximally elevated 5.67 and 4.37 fold, respectively, at 4 h.

<b>Table 4.2. Transcription of metalloproteinases at 2% O<sub>2</sub> in HTR-8/SVneo cells as detected by microarray.</b>			
	<b>Fold Change in Signal</b>		
	<b>2% O<sub>2</sub> 1h</b>	<b>2% O<sub>2</sub> 2h</b>	<b>2% O<sub>2</sub> 4h</b>
<b>BSG</b>	1.56*	2.01*	2.61*
<b>ADAM10</b>	-1.22	-1.45*	-2.41
<b>ADAM15</b>	2.09*	2.42*	2.86*
<b>ADAM9</b>	-1.42*	-1.89*	-2.16*
<b>MMP2</b>	3.36	5.85	41.71*
<b>MMP15</b>	1.40*	1.85*	2.71*

Microarray analysis for metalloproteinase genes identified several transcripts upregulated in CTB cells cultured at 2% O<sub>2</sub> for 1 - 4 h as compared to 20% O<sub>2</sub>. Asterisks indicate significant values ( $p < 0.1$ ) according to microarray analysis compared to signal at 20% O<sub>2</sub>.

<b>Table 4.3. Expression of metalloproteinases at 2% O<sub>2</sub> in HTR-8/SVneo cells measured by qPCR.</b>			
	<b>Fold Change in Relative Expression</b>		
	<b>2% O<sub>2</sub> 1h</b>	<b>2% O<sub>2</sub> 2h</b>	<b>2% O<sub>2</sub> 4h</b>
<b>BSG</b>	2.13 ± 0.10	3.69 ± 2.55	3.74 ± 1.43
<b>ADAM10</b>	3.60 ± 2.73	4.03 ± 3.16	2.84 ± 1.00
<b>ADAM15</b>	3.17 ± 2.25	5.38 ± 4.31	2.89 ± 1.37
<b>ADAM9</b>	4.70 ± 3.88	3.86 ± 3.03	2.98 ± 1.05
<b>MMP2</b>	1.59 ± 0.41	5.56 ± 3.80	4.37 ± 2.03
<b>MMP15</b>	7.58 ± 6.76	3.75 ± 2.14	5.67 ± 3.25

QPCR was performed using the same RNA submitted for microarray analysis and two experimental repeats. Values for metalloproteinase genes represent averages (n = 3) ± standard error, normalized to SDHA expression. Several genes were upregulated in CTB cells cultured at 2% O<sub>2</sub> for 1 - 4 h as compared to 20% O<sub>2</sub>.

## **MMP2 is Required to Increase HBEGF**

To determine whether MMP2 has a functional role in HBEGF regulation by  $O_2$ , specific inhibitors of MMP2 and MMP9 were used to supplement culture medium during manipulation of  $O_2$  levels. Unlike MMP2, MMP9 expression did not change at 2%  $O_2$ , according to microarray analysis. CTB cells cultured at 2%  $O_2$  for 4 h failed to accumulate HBEGF in the media when MMP2 was inhibited (Table 4.4). However, MMP9 inhibition had no effect on the normal accumulation of HBEGF at low  $O_2$ . A third inhibitor that targets both MMP2 and MMP9 also inhibited HBEGF accumulation. We conclude that MMP2, but not MMP9, may directly or indirectly mediate the proteolytic cleavage of proHBEGF under control of  $O_2$  and  $O_2$ -sensitive HIF transcription factors. The initiation of HBEGF shedding, in turn, allows it to bind ERBB receptors and precipitate autocrine signaling, as previously demonstrated using a less specific inhibitor of metalloproteinase activity (Armant et al., 2006). MMP15, which is also upregulated at 2%  $O_2$  (Table 4.2 and 4.3), can activate MMP2 (Hitchon et al., 2002; Mohammad et al., 2010), suggesting that MMP15 could cleave proMMP2 in a proteinase cascade that initiates HBEGF shedding.

<b>Table 4.4. Inhibition of MMP2 prevents HBEGF upregulation at 2% O<sub>2</sub>.</b>		
<b>% O<sub>2</sub></b>	<b>Treatment</b>	<b>HBEGF (pg/μg)</b>
20	Vehicle	1.01 ± 0.54
2	Vehicle	46.41 ± 3.22
2	MMP2/9 Inh.	1.11 ± 0.53
2	MMP2 Inh.	1.07 ± 0.63
2	MMP9 Inh.	58.15 ± 8.74

HBEGF protein was measured by ELISA in the media covering CTB cells cultured for 4 h at either 20% or 2% O<sub>2</sub> in the presence of inhibitors of MMP2 and/or MMP9, as indicated. The upregulation of HBEGF seen after culturing CTB cells at 2% O<sub>2</sub> was abrogated by adding an inhibitor specific for MMP2 or an inhibitor of both MMP2 and MMP9. An inhibitor of MMP9 alone does not block the increase in HBEGF due to culture at 2% O<sub>2</sub>. Values are represented as average ± SEM.

## DISCUSSION

The regulation of HBEGF expression during implantation and placentation is critical for trophoblast survival and invasion. An autocrine, post-transcriptional mechanism induced at low  $O_2$  upregulates HBEGF synthesis and secretion, providing an important survival factor during early gestation (Armant et al., 2006; Jessmon et al., 2009). The present study provides evidence that a transcriptional event is required to initiate the increased HBEGF synthesis downstream of HIF signaling. It was hypothesized that HIF directs the transcription of a metalloproteinase that is directly or indirectly responsible for cleavage of transmembrane proHBEGF from the cell surface, initiating the autocrine feedback loop that increases HBEGF protein synthesis. Transcriptome analysis and qPCR validation implicated a protease cascade that includes MMP15 and MMP2 in HBEGF shedding. Finally, a functional role for MMP2 in the accumulation of HBEGF was established using a specific inhibitor. A mechanism is proposed wherein low  $O_2$  concentrations, as experienced by trophoblasts during the first 10 weeks of placentation, induce HIF-mediated transcription of MMP2 that participates in a protease cascade to shed HBEGF from the cell surface, possibly with its activation by MMP15 (Hitchon et al., 2002; Mohammad et al., 2010), to commence the autocrine HBEGF signaling that increases its biosynthesis using a constitutively-expressed pool of HBEGF mRNA.

HIF proteins are stabilized at low  $O_2$  (Wang and Semenza, 1993) and activate transcription of targeted genes by interacting with specific hypoxia response elements (HREs) in the gene promoters (Semenza, 2003). Although they often have similar effects, HIF1 $\alpha$  and HIF2 $\alpha$  also have distinct functions. In apoptosis, HIF2 $\alpha$  may be

more important in protecting against environmental stressors and HIF1 $\alpha$  may have a greater role in protecting against hypoxic damage (Brusselmans et al., 2001). HIF3 $\alpha$  appears to be a dominant negative inhibitor of HIF1 $\alpha$  that binds HIF1 $\alpha$  and prevents binding to HRE motifs (Makino et al., 2001). HIF3 $\alpha$  was not increased by low O<sub>2</sub> treatment of CTB cells. We suspected that either HIF1 $\alpha$  or HIF2 $\alpha$  may be involved in the response of CTB cells to low O<sub>2</sub>, based on their accumulation with CoCl<sub>2</sub> or 2% O<sub>2</sub> treatment. Hence, HIF proteins would be capable of regulating the shedding and subsequent accumulation of HBEGF protein during the first 10 weeks of gestation and in our experimental model. Others have examined the expression of HIFs in the human placenta and found that both HIF1 $\alpha$  and HIF2 $\alpha$  mRNA are expressed throughout gestation, though HIF2 $\alpha$  mRNA tends to increase as gestation progresses (Rajakumar and Conrad, 2000). Interestingly, protein expression for both, which is localized to trophoblast cells, is high in earlier stage placentae (5-11 weeks) when O<sub>2</sub> is low, but declines at later gestational ages (Caniggia et al., 2000; Rajakumar and Conrad, 2000). At 20% O<sub>2</sub>, HIF1 $\alpha$  mRNA and protein are upregulated in the EVT cell line TCL1 (Fukushima et al., 2008), suggesting a continued role after O<sub>2</sub> levels rise at the end of the first trimester. HIF1 $\alpha$  can be activated by growth factor signaling, independently of O<sub>2</sub> concentrations (Semenza, 2003). HIF1 $\alpha$  upregulates VEGF protein and causes integrin  $\alpha$ V $\beta$ 3 to aggregate in TCL1 cells, which leads to tube formation on Matrigel (Fukushima et al., 2008).

MMP2 appeared to be among the genes that were transcriptionally regulated at low O<sub>2</sub> and could be involved in a proteolytic cascade that culminates in HBEGF shedding. Additionally, studies of early placentation suggest that MMP2 is important in

trophoblast invasion (Staun-Ram et al., 2004). MMP2 protein and mRNA predominates in early first trimester trophoblasts and declines by the end of the first trimester, whereas MMP9 has the exact opposite pattern (Staun-Ram et al., 2004; Xu et al., 2000). MMP2 exists in a proteolytic cascade in  $\alpha$ T3-1 cells that leads to both proHBEGF shedding and EGFR transactivation (Roelle et al., 2003). In addition, its rapid release in CMTC9 cells after E2 stimulation is correlated with cleavage of proHBEGF (Torres et al., 2009). Bradykinin-induced proliferation of rabbit corneal cells is blocked by an inhibitor of both MMP2 and MMP9 or an inhibitor of HBEGF, suggesting that either MMP may be involved in the cleavage of proHBEGF (Cheng et al., 2011). In the present study, lack of a regulatory role for MMP9 in HBEGF expression at low O<sub>2</sub> was supported by inhibitor experiments that showed MMP9 not to be essential for HBEGF upregulation. Interestingly, low O<sub>2</sub> (0.1%) increases MMP2 mRNA in CTB cells isolated from first trimester placentae, but, in contrast to our findings, MMP2 secretion and enzymatic activity increase at 20% O<sub>2</sub> relative to CTB cells cultured at 5% O<sub>2</sub> (Onogi et al., 2011). Culture at 2% O<sub>2</sub> could differ significantly from 5% O<sub>2</sub>, and account for the different outcomes. Differential gene expression studies suggested that MMP15 could contribute to a proteolytic cascade that activates MMP2. Indeed, the MMP15 promoter contains two HREs (Zhu et al., 2011). MMP15 expression in psoriatic skin biopsies and bladder cancer tissue correlates with an increase in MMP2 proteolytic activity (Hitchon et al., 2002; Mohammad et al., 2010). MMP15 is a membrane-type matrix metalloproteinase (MT2-MMP) that can anchor MMP2 in the proximity of proHBEGF on the cell surface. Additionally, tissue inhibitors of metalloproteinases (TIMPs), which are also present during CTB invasion (Niu et al.,



2000; Onogi et al., 2011; Ruck et al., 1996), MT1-MMP and MMP2, facilitating their proteolytic activity (Butler et al., 1998). A similar mechanism (Strongin et al., 1995) should exist for MMP15 and MMP2. A specific inhibitor of MMP15 was not available to further explore its functional role; however, its expression pattern suggests that MMP15 operates upstream of MMP2 in a proteolytic cascade that leads to proHBEGF cleavage.

Other metalloproteinases, including members of the ADAM family, been implicated in the shedding of proHBEGF. Like MT-MMP subfamily members, ADAM metalloproteases are membrane anchored (Edwards et al., 2008) and could direct protease activity towards the cell surface where proHBEGF resides. Although an increase was detected in ADAM15 in CTB cells cultured at 2% O<sub>2</sub>, its function in implantation is currently unknown. Low O<sub>2</sub> upregulated ADAM9, ADAM10 and ADAM15 in human CTB cells, according to qPCR results. Activation of metalloproteinases with APMA leads to shedding of HBEGF, but not in TACE<sup>-/-</sup> cells, indicating that TACE/ADAM17 proteolytically cleaves proHBEGF (Merlos-Suarez et al., 2001). TACE/ADAM17 transfected into TACE-deficient cells increased shedding of HBEGF (Sunnarborg et al., 2002). ADAM17 was found to preferentially shed ERG, TGF $\alpha$ , AREG, and HBEGF. However, microarray results showed no significant change in its transcription at low O<sub>2</sub> (data not shown). ADAM9 can direct the TPA-induced shedding of proHBEGF (Izumi et al., 1998). In that study, PKC $\delta$  binds the intracellular domain of ADAM9 and brings it towards HBEGF to induce cleavage. In mouse embryonic fibroblasts generated from ADAM knockout mice, it was discovered that ADAM10 preferentially sheds EGF and BTC (Sahin et al., 2004). In human epithelial cells, lipoteichoic acid, a major polysaccharide in Gram-positive cell walls) induces ADAM10

to cleave proHBEGF, which subsequently binds ERBB1 and induces signaling (Lemjabbar and Basbaum, 2002). In COS7 cells transfected with mouse ADAM10, activation of GPCR leads to ADAM10-mediated shedding of HBEGF, which activates ERBB1 and the downstream Ras/Erk pathway (Yan et al., 2002). It is interesting that qPCR results demonstrated that ADAM10 was upregulated by low O<sub>2</sub>. ADAM12, another candidate sheddase for HBEGF, was not transcriptionally regulated, according to the microarray results (data not shown) (Asakura et al., 2002; Kurisaki et al., 2003). It is possible that ADAMs, particularly ADAM10, participate in the cascade that leads to shedding of HBEGF.

Basigin (BSG/CD147/EMMPRIN) is a transmembrane immunoglobulin superfamily member that is critical in embryo implantation and is expressed in the blastocyst and endometrium of mice and rats (Ding et al., 2002; Igakura et al., 1998; Xiao et al., 2002b). In the mouse, its expression is localized in the decidua surrounding an implanted blastocyst, and in the embryo (Xiao et al., 2002a), and BSG<sup>-/-</sup> mice fail to survive past implantation (Igakura et al., 1998). Recombinant BSG induces MMP3 and MMP9 transcription, secretion and activity in a mouse uterine stromal cell culture, but has no effect on MMP2 (Chen et al., 2009). It is possible that BSG expression by human trophoblasts targets metalloproteinases that participate in a proteolytic cascade to generate HBEGF shedding.

Mechanisms regulating HBEGF synthesis in CTB cells are critical in early pregnancy. HBEGF prevents apoptosis at low O<sub>2</sub> and stimulates motility at high O<sub>2</sub> in first trimester trophoblasts (Jessmon et al., 2010; Leach et al., 2004a). We have established a role for HIF1 $\alpha$  and HIF2 $\alpha$  in the regulation of HBEGF protein at low O<sub>2</sub>,

and suggest that HIFs may also have a role at high  $O_2$ , highlighting the fact that they are an important transcription factor in regulating early trophoblast development. In addition, evidence obtained using  $CoCl_2$  and mRNA expression analysis suggested that MMP2 is a HIF-regulated gene involved in HBEGF shedding. These findings suggest that low  $O_2$  concentrations during early pregnancy induce a HIF-mediated increase in metalloproteinase activity that sheds HBEGF from the cell surface of CTB cells. HBEGF then signals in an autocrine loop to increase its synthesis, stimulate trophoblast motility, and block apoptosis.

## CHAPTER 5. Translational Regulation of HBEGF in Developing Human

### Trophoblast Cells

#### SUMMARY

Heparin-binding EGF-like growth factor (HBEGF) prevents apoptosis of human trophoblast cells exposed to low oxygen, as occurs in early pregnancy, and is dramatically upregulated in vitro by 2% O<sub>2</sub> within 4 h without a change in HBEGF mRNA. Signaling through mitogen activated protein kinases (MAPKs) downstream of HBEGF is required for HBEGF protein accumulation, but the underlying post-transcriptional mechanism is not understood further. HBEGF mRNA was stable up to 6 h, suggesting that protein does not accumulate due to changes in mRNA degradation rates. Although HBEGF protein turnover was higher at 20% O<sub>2</sub>, stabilizing the protein with proteasome inhibitors was insufficient to increase cellular HBEGF, suggesting that HBEGF mRNA is translationally regulated by O<sub>2</sub>, perhaps through elements in its 3' untranslated region (UTR). A dual luciferase reporter construct containing the intact HBEGF 3'UTR or specific subregions was implemented to examine its translational regulatory potential. The intact 3'UTR reduced luciferase activity to approximately 30% (p<0.05). However, the isolated flanking regions increased reporter activity 4- to 5-fold (p<0.05), while two central domains modestly repressed reporter (~50%, p<0.05). The possible role of microRNA (miRNA) in the targeted regulation of HBEGF was examined by RNAi knockdown of the miRNA-processing protein, DGCR8. Knockdown of the DGCR8 protein correlated with reduced HBEGF labeling on western blots, suggesting that miRNA is required to maintain or stimulate HBEGF translation. Secondly, the abundance of 30 individual miRNAs predicted to target the HBEGF 3'UTR were

examined at both 20% and 2% O<sub>2</sub> by TaqMan qPCR, revealing no significant change in their expression. It is concluded that HBEGF is not transcriptionally or post-translationally regulated by O<sub>2</sub> in human trophoblast cells, but is translationally regulated through the interaction of miRNA with the HBEGF 3'UTR. It appears that the 3'UTR contains flanking elements that interact with miRNA to increase HBEGF translation, and that other 3'UTR domains attenuate the flanking region activity.

## INTRODUCTION

The epidermal growth factor (EGF) family member, heparin binding EGF-like growth factor, (HBEGF), is present in the uterus at the time of embryo implantation (Leach et al., 1999b; Yoo et al., 1997a), and its expression in cytotrophoblast (CTB) cells of the invading placenta indicate its central role in early implantation and subsequent placentation (Leach et al., 2002a). It is one of the earliest growth factors expressed in mice just prior to implantation, is localized in uterine cells surrounding an implanted embryo, and is found subsequently in the underlying uterine decidua of mice, human, and non-human primates (Das et al., 1994c; Leach et al., 2001; Leach et al., 1999b; Yue et al., 2000). In the human uterus, HBEGF is upregulated during the window of implantation. The implantation site in humans is a low O<sub>2</sub> environment, as the placenta is not fully oxygenated until after the 10<sup>th</sup> week of pregnancy when occluding CTB plugs within the uterine spiral arteries are dislodged and maternal blood flows freely into the intervillous space (Burton and Jauniaux, 2004; Burton et al., 1999; Jauniaux et al., 2001b; Rodesch et al., 1992). Previous *in vitro* work has demonstrated that HBEGF protein levels are dramatically upregulated in CTB cells cultured at low O<sub>2</sub> (2%), which simulates *in utero* O<sub>2</sub> concentrations during the first trimester (Armant et al., 2006). Other EGF family members are not altered by changes in O<sub>2</sub> concentration. Notably, trophoblast cells do not undergo apoptosis upon exposure to a sudden elevation of O<sub>2</sub> after the 10<sup>th</sup> week, but continue to invade into the uterine decidua. *In vitro*, HBEGF protects first trimester CTBs from apoptosis and promote their invasion (Armant et al., 2006; Leach et al., 2004a). Hence, its upregulation by low O<sub>2</sub> during the first trimester may protect trophoblast cells from oxygenation during increased maternal

blood flow, as well as induce invasion into the uterine decidua. The syndrome preeclampsia, in which trophoblast invasion is dramatically reduced and apoptosis is elevated, is characterized by a dramatic reduction in placental HBEGF protein levels (Leach et al., 2002a).

Previous work has established that HBEGF mRNA is not transcriptionally regulated by O<sub>2</sub> in human trophoblast cells (Armant et al., 2006). Even though protein translation is often globally downregulated by hypoxia (Koumenis et al., 2002), HBEGF protein levels increase dramatically at low O<sub>2</sub>. HBEGF upregulation occurs downstream of its autocrine signaling. ProHBEGF on the cell surface is proteolytically cleaved, allowing soluble HBEGF to bind its ERBB receptors and initiate intracellular signaling that, among other things, increases its own protein by a post-transcriptional mechanism. This autocrine mechanism is likely initiated by metalloproteinases upregulated at 2% O<sub>2</sub>, such as MMP2, that target proHBEGF for cleavage (Chapter 4).

The post-transcriptional regulation of HBEGF could be directed by mRNA stability, protein turnover, or a specific translational regulatory step. Examples exist where mRNA transcripts are destabilized in one condition, leading to a decrease in protein levels (Baudouin-Legros et al., 2005; Phelps et al., 2006), and stabilized in another condition (Zhao et al., 2008), leading to protein accumulation. A prior study has shown that HBEGF mRNA is stabilized in HeLa cells induced to undergo cellular stress by a chemotherapeutic agent (Sorensen et al., 2006). However, because HBEGF transcript levels are not altered by O<sub>2</sub> fluctuations (Armant et al., 2006), it is unlikely that mRNA stability is a factor in its regulation. Regulation of transcription and translation can be disparate processes, depending on the gene and cellular context (Koritzinsky et

al., 2005; Yukawa et al., 1999). It is possible that HBEGF proteolysis is inhibited by 2% O<sub>2</sub>, leading to its accumulation. This mechanism suggests that HBEGF is synthesized and constitutively degraded at 20% O<sub>2</sub>, but is stabilized at low oxygen. However, it can also be hypothesized that its accumulation is not simply due to an inhibition of its proteolysis, but to a regulation of its translation. Indeed, the maintenance of HBEGF transcripts in a pool stable at both 20% and 2% O<sub>2</sub> (Armant et al., 2006) suggests that its translation is initiated at low O<sub>2</sub>, and perhaps inhibited by high O<sub>2</sub>.

MicroRNAs (miRNAs) are ~22 nt endogenous small RNA species that primarily target the 3' untranslated region (3'UTR) of mRNA transcripts to suppress translation (Kim and Nam, 2006; Pillai et al., 2007). They are transcribed as primary miRNA transcripts in the nucleus by RNA Polymerase II and are subsequently processed by the nuclear proteins Drosha and DGCR8 into shorter pre-miRNA hairpin precursors. These pre-miRNAs are exported into the cytoplasm and processed into the mature ~22 nt form by Dicer (Bartel, 2004). A mature miRNA is incorporated into an RNA-induced silencing complex (RISC) or miRNP (miRNA ribonucleoprotein) where it binds with perfect complementarity to a conserved 6-8 nt seed region in the 3' UTR. MiRNAs have the unique capacity to bind a specific target transcript and, due to imperfect base pairing at nucleotides 10-11, inhibit translation without acting as siRNA and inducing degradation of the mRNA (Bartel, 2004; Filipowicz et al., 2008; Pillai et al., 2007). In effect, they mediate a very rapid and energetically efficient regulatory mechanism that occurs only at the translational level. Recent evidence demonstrating upregulation of TNFA translation during cell cycle arrest (Vasudevan et al., 2007) suggests that miRNAs may also upregulate translation. Hence, a miRNA-mediated mechanism could be



responsible for the translational suppression of HBEGF at higher O<sub>2</sub> concentrations or induction of its translation at low O<sub>2</sub>. In the placenta, miRNAs could rapidly regulate HBEGF in response to sudden changes in O<sub>2</sub> level, growth factor signaling or extracellular matrix composition experienced by developing trophoblast cells.

Specific miRNAs are expressed in placental tissues (Barad et al., 2004; Maccani and Marsit, 2009; Mouillet et al., 2011) and in trophoblast cells (Donker et al., 2007; Spruce et al., 2010) that target proteins important for trophoblast physiology. MiR-152 targets HLA-G in JEG3 cells (Zhu et al., 2010), miR-34a targets Notch1 and Jagged1 in HeLa and JAR cells (Pang et al., 2010), and miR-199b targets SET (protein phosphatase 2A inhibitor) in BeWo and JAR cells (Chao et al., 2010). Hence, it is feasible to hypothesize that miRNA that target HBEGF also exist in CTB cells. A recent study (Donker et al., 2007) demonstrated that, in first trimester trophoblast cells, the miRNP machinery is present in cells cultured at 20% and 2% O<sub>2</sub>, and differential regulation of miRNAs silence MED1 expression. Regulated expression of miRNAs that target HBEGF could likewise provide evidence that its translational suppression at 20% O<sub>2</sub> is mediated by miRNAs.

In this study, it was determined whether HBEGF protein is regulated by O<sub>2</sub> through a change in its stability, or if other post-transcriptional mechanisms are at work. We specifically examined the regulatory function of the 3'UTR of HBEGF and the role of miRNA species in the translational control of HBEGF.

## MATERIALS & METHODS

### Cell Culture and Treatments

The first trimester human cytotrophoblast cell line HTR-8/SVneo (Graham et al., 1993b) was cultured at either 20% O<sub>2</sub> or 2% O<sub>2</sub> as previously described (Leach et al., 2004a; Leach et al., 2008b). Cells were cultured for the indicated times in 1-5 µg/mL α-amanitin, an inhibitor of RNA Polymerase II that blocks *de novo* transcription (Sigma-Aldrich), in 250 µM CoCl<sub>2</sub> (Sigma-Aldrich) to increase HIF at 20% O<sub>2</sub>, in 10 µg/mL cyclohexamide to block *de novo* translation (Sigma-Aldrich), and in 1 µg/mL lactocystin or 100 µg/mL MG132 to inhibit the proteasome (EMD Biosciences).

### qPCR

RNA from HTR-8/SVneo cells was collected using the miRNeasy kit from Qiagen, according to the manufacturer's protocol. RNA concentration was determined using the NanoDrop spectrophotometer and purity was ascertained with an Agilent Bioanalyzer (Agilent). Reverse transcription was performed using the Quantitect Reverse Transcription kit (Qiagen) and qPCR (Bustin et al., 2009) was conducted with the Quantitect SYBR Green PCR kit without UNG in a final volume of 25 µL. An appropriate housekeeping gene was chosen from a panel of genes used for all samples (RRN18S, ACTB, GAPD, RPL13A, RPLP0, SDHA). For each experiment, the housekeeping gene with the least variability among all treatments was selected for normalization in subsequent qPCR experiments. For CoCl<sub>2</sub> experiments, RPL13a (ribosomal protein 13a) was chosen, and for all other experiments, SDHA (succinate dehydrogenase) had the least variability. Semi-quantitative analysis was performed

using the  $\Delta\Delta\text{Ct}$  method (Pfaffl, 2001). Primers for the housekeeping genes and for HBEGF were obtained from Qiagen.

### **Western Blotting**

Western blots were performed as previously described (Kilburn et al., 2000b). Cellular lysates were diluted in SDS sample buffer containing 5%  $\beta$ -mercaptoethanol, run on precast 4%–20% Tris-HCl gradient gels (BioRad), and transferred to nitrocellulose membranes. Monoclonal mouse antibodies against DGCR8 (0.3  $\mu\text{g}/\text{mL}$ , Proteintech) and GAPDH (1  $\mu\text{g}/\text{mL}$ , Ambion) were diluted in TTBS containing 5% milk. A polyclonal antibody against HBEGF (R&D Systems) was diluted to 0.2  $\mu\text{g}/\text{mL}$  in TTBS containing 5 mg/mL BSA.

### **ELISA**

ELISA was carried out using the HBEGF DuoSet ELISA Development kit (R&D Systems), as previously described (Armant et al., 2006; Leach et al., 2008b). The optical density of the final reaction product was determined at 450 nm using a programmable multiplate spectrophotometer (Power Wave Workstation; Bio-Tek Instruments) with automatic wavelength correction.

### **Generation of 3'UTR Luciferase Reporter Vectors**

Regions of the HBEGF 3'UTR were amplified by PCR and cloned into the PsiCheck-2 vector (Promega). This vector is unique in that two different luciferase genes (Firefly and Renilla) driven by two different promoters are present in the same vector. The *Renilla* gene serves as a reporter and the *Firefly* gene as an internal

control. HBEGF 3'UTR-derived inserts were cloned into a region immediately following the Renilla luciferase gene. Lyophilized primers (IDTDNA) used to amplify regions of the HBEGF 3'UTR were designed using Primer3 software ((Rozen and Skaletsky, 2000) in (Krawetz and Misener)) and aligned against the human transcriptome for specificity (NCBI BLASTN). Three adenosine nucleotides and restriction enzyme recognition sequences for XhoI and NotI were added to forward and reverse primers, respectively (Table 5.1). Secondary structure, primer dimers, and self-annealing were examined using OligoCalc (Kibbe, 2007). Regions amplified by these primers and expected amplicon sizes are listed in Table 5.1. After isolating RNA, cDNA was generated using the Omniscript Reverse Transcription kit (Qiagen). HBEGF 3'UTR fragments were amplified by endpoint PCR using the HotStarTaq Plus Master Mix kit (Qiagen) according to the manufacturer's instructions with the following formulation. In a total reaction volume of 50  $\mu$ L, 0.5  $\mu$ M of each primer and 50 ng of cDNA was used. Amplicon size was verified with 8  $\mu$ L of the final PCR reaction assessed by 0.8% agarose gel electrophoresis. If amplicons were of the expected size, the remaining 42  $\mu$ L of PCR products were processed using the Wizard SV Gel and PCR Clean-up Kit (Promega). In all restriction enzyme reactions, components were combined, gently mixed, and briefly centrifuged. Cleaned amplicon and the PsiCheck2 vector (1  $\mu$ g each) were digested in 0.2 mL thin-walled tubes (Molecular BioProducts) with both XhoI and NotI restriction enzymes (Promega) at the recommended concentration for 1 h at 37°C in Buffer D (supplied by Promega) in a thermal cycler, followed by a 15 min 70°C inactivation step. Cut PsiCheck2 vector was then treated with 1  $\mu$ L thermosensitive alkaline phosphatase (Promega) for 15 min, followed by a 15 min heat inactivation step.

**Table 5.1. Primer sequences for amplification of HBEGF 3'UTR and vector sequencing.**

<b>Forward Primers</b>	<b>Sequence</b>	<b>Target Site on HBEGF mRNA</b>
HBEGF 3'UTR	AAACTCGAGGGAGGTTATGATGTGGAAAATGA	844
HBEGF 3'UTR	AAACTCGAGCCTTTGCCACAAAGCTAGGA	1644
HBEGF 3'UTR	AAACTCGAGTTGCCTAGGCGATTTTGTCT	1829
HBEGF 3'UTR	AAACTCGAGAACAGGGAACATTGGAGCTG	2137
PsiCheck-2 vector	AGGACGCTCCAGATGAAATG	

<b>Reverse Primers</b>	<b>Sequence</b>	<b>Target Site on HBEGF mRNA</b>
HBEGF 3'UTR	AAAGCGGCCCGCAGATCCCTTGGTGGTACT	1363
HBEGF 3'UTR	AAAGCGGCCCGCCAGGAAATTGCCAAAGTA	1603
HBEGF 3'UTR	AAAGCGGCCCGCCCAAGTTAACCCCTACATCCTG	1778
HBEGF 3'UTR	AAAGCGGCCCGCATGAACCAGGTTTGGAAATACA	2315
PsiCheck-2 vector	CAAACCCTAACCACCGCTTA	

Primer sequences for amplification of the HBEGF 3'UTR and sequencing of the psiCheck2 vector are listed along with their binding locations.

Restricted amplicons and PsiCheck2 vector were combined in a 2:1, 4:1, or 6:1 ratio in a ligation reaction using Promega's LigaFast Rapid DNA Ligation System for 2 h at 37°C in a thermal cycler. To verify ligation, 1 µL of ligated product was then added to an endpoint PCR reaction mixture using primers that bind outside of the PsiCheck2 vector's multiple restriction site, as indicated in Table1. If the PCR reaction produced an appropriately-sized amplicon, the ligation reaction was used in subsequent bacterial transformations.

### **Bacterial Transformation**

One microliter of the ligation reaction mixture was added to a sterile 15 mL round bottom, thin-walled culture tube and placed on ice. 50 µL of JM109 bacteria (Promega) were added to each culture tube, directly on top of the ligation product. 1 µL of empty PsiCheck2 vector that had been cut with restriction enzyme and treated with alkaline phosphatase was also transformed into bacteria as a negative control. After a 2 min incubation on ice, bacteria were heat shocked by immersing in a 37°C water bath for 50-55 sec, and the incubating on ice for 10 min. Next, 950 µL of ice-cold SOCs media (Invitrogen) was added to each culture tube before incubation at 37°C with gentle agitation (200 rpm) for 90 min. After 200 µL of the bacterial mixture was added to an LB Agar plate containing AMP-100, X-GAL-80, and IPTG-50 (Teknova), it was incubated upright overnight at 37°C. Colonies were picked with sterile 10 µL pipette tips that were gently dabbed onto an agar plate and placed in 3 mL of LB broth (Becton, Dickinson & Co.) containing 100 µg/mL of Ampicillin. After culturing at 37°C overnight with gentle agitation (200 rpm), 100 µL of each bacterial mixture was drawn off, pelleted at 7500 rpm for 5 min in a 1.5 mL microcentrifuge tube, and resuspended in nuclease-free water

(IDTDNA). Resuspended bacteria were then heated to 100°C for 10 min, and 1 µL of the resulting lysate was added to an endpoint PCR reaction using the same primers utilized to verify ligation. PCR products were electrophoresed on 0.8% agarose gels, and those colonies that displayed the presence of a correctly-sized insert and no empty vector (as evidence by amplification of a ~220 bp fragment) were chosen for vector recovery using the Wizard Plus SV Miniprep Kits (Promega). Vectors were diluted to 100 ng/µL in nuclease-free H<sub>2</sub>O and 1 µL was used in subsequent transfection reactions. Vectors were sequenced using the same primers utilized to verify ligation and aligned to the HBEGF mRNA sequence (PUBMED sequence NM\_0001945.2) using Geneious (Drummond et al., 2011).

### **Transfection and Luciferase Assay**

HTR-8/SVneo cells were grown to 75% confluency in 6-well culture plates (Falcon, #353046). Media was replaced with serum-free media (DMEM/F-12 with 5 mg/mL BSA) containing transfection reagents and cells were cultured for 24 hr. To prepare transfection reagents, room temperature FuGene-6 transfection reagent (Roche Diagnostics) was added to unsupplemented DMEM/F-12 media in a 1.5 mL microcentrifuge, gently mixed, and incubated at room temperature for 5 min. Then, 100 ng of vector was added with gentle mixing and incubated at room temperature for 15 min. It was determined that 3 µL of FuGene-6 and 1 µL of vector DNA (100 ng) was optimal per 6-well transfection. The entire contents of each transfection mixture were then added dropwise to one well of the 6-well plate, and the plate was rocked to ensure proper mixing. After a 24 h incubation at 37°C, the media was removed, the cells were washed twice with 1X PBS and they were lysed in 500 µL 1X passive lysis buffer

(Promega) for 15 min on an orbital plate shaker at room temperature. Lysates were centrifuged at 5000 rpm for 5 min to remove cellular debris and 20  $\mu$ L supernatant was used in each dual luciferase reaction, conducted according to the manufacturer's protocol (Dual Luciferase Reporter Kit, Promega) in a 96-well plate with a clear, flat bottom. Negative controls consisting of 20  $\mu$ L passive lysis buffer and untransfected cells were also included. Each sample received 100  $\mu$ L of Luciferase Assay Reagent II reagent using a multichannel pipette. The plate was placed in a Veritas microplate luminometer (Turner BioSystems) and automated measurements were obtained after exactly 1 min, and five more times at regular intervals. Then, 100  $\mu$ L of Stop & Glo reagent (Promega) was added to each sample. Five automated measurements were then obtained at regular intervals beginning 1 min after addition of the second reagent. Renilla luciferase activities were normalized to corresponding Firefly luciferase activities at each of the five intervals, and an average ratio was calculated. Each transfection experiment was repeated at least three times.

### **Custom TaqMan miRNA qPCR Plates**

Custom 96-well plates containing lyophilized real time primers and probes for the 27 miRNA listed in Table 5.2 were obtained from Applied Biosystems. RNA samples collected using the miRNeasy kit were reverse transcribed using ABI miRNA-specific RT primers, mixed with Universal PCR Master Mix and transferred to the 96-well custom plates preloaded with real time primers (Applied Biosystems), as directed by ABI. Samples were amplified in an Applied Biosystems 7500 Fast real-time thermal cycler using a protocol provided by the custom plate manufacturer. Results were analyzed according to the  $\Delta\Delta$ Ct method (Pfaffl, 2001), using RNU48 as a housekeeping



control gene. Samples from five independent experiments (n=5) were used to determine the mean  $\pm$  SEM.

## **Bioinformatics**

A recent study suggested that TargetScan is among two of the best miRNA target site prediction programs as it considers site conservation more strictly (Baek et al., 2008). Both miRBase (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011) and TargetScan (Friedman et al., 2009; Grimson et al., 2007; Lewis et al., 2005) databases were searched for miRNAs that target HBEGF. Several miRNAs were listed in both databases, and results were combined to produce the candidates shown in Table 5.2. TESS (Schug and Overton, 1997), Tfsitescan, and TFSEARCH (Heinemeyer et al., 1998) were used to examine transcription factor-binding elements in the promoter of HBEGF. AREsite (Gruber et al., 2011) was used to examine possible AREs in the 3'UTR. EMBOSS GETORF was used to examine potential uORFs.

## **DGCR8 Knockdown**

Three siRNAs that target DGCR8 were obtained from Life Technologies (s29061, s29062, s29063). First, HTR-8/SVneo cells were tested for toxicity due to NeoFx reagent (Ambion) used to transfect the cells with siRNA. Toxicity was measured using the MultiTox-Fluor Multiplex Cytotoxicity Assay kit (Promega) and an optimal concentration of NeoFx (0.75  $\mu$ L per well of a 96-well plate) was determined (<10% toxicity after 48 hr). Cells were transfected in 96-well plates (5,000 cells per well) for 48 hr with various concentrations of each DGCR8 siRNA. Cells were fixed with ice-cold

methanol for 10 min, incubated with 0.1% Triton-X100 for 15 min, stained overnight with a primary antibody against DGCR8 (Santa Cruz Biotechnology, Inc.) at 4°C, incubated for 1 hr at room temperature with an anti-mouse/anti-rabbit labeled polymer (DAKO EnVision Dual Link) and bound antibody visualized by light microscopy using a DAB chromogen.

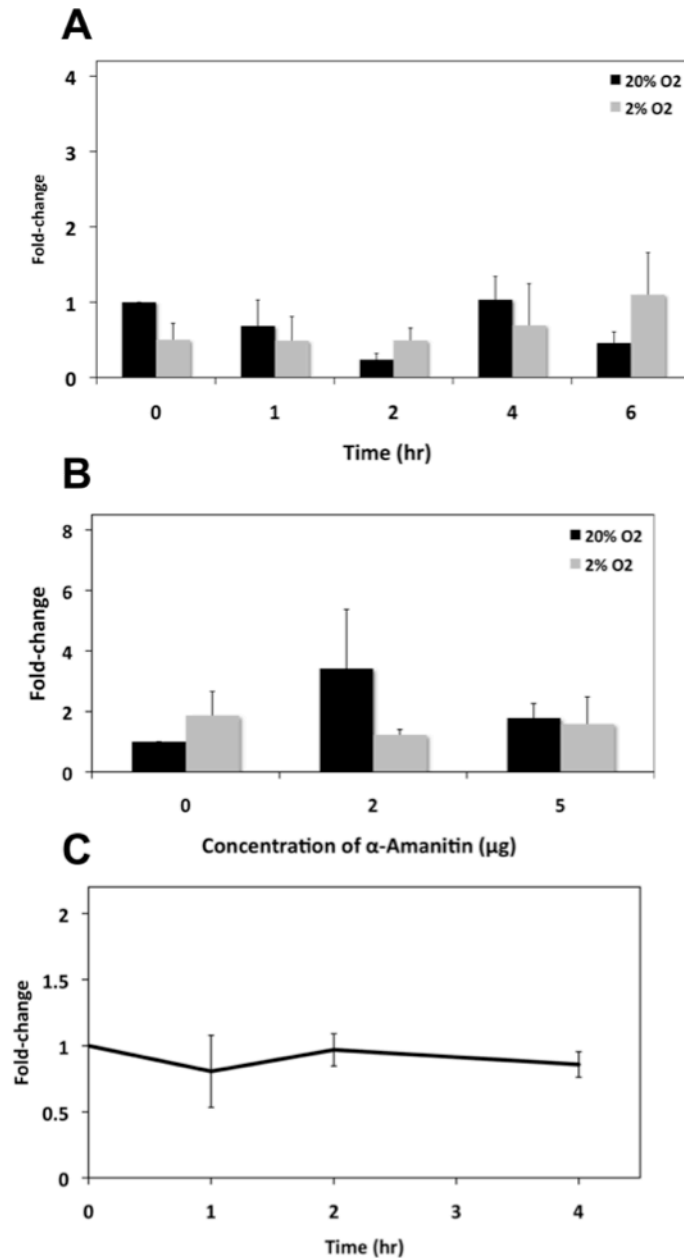
### **Statistics**

All experiments were repeated at least three times and are reported as mean  $\pm$  SEM. Statistical significance was determined at  $P < 0.05$  by 2-way ANOVA, followed by post-hoc analysis with Dunnett's t-tests, Student-Newman-Keuls and Student's t-tests using SPSS version 12.0 statistics software (SPSS) and StatPlus:mac (Wang et al., 2002).

## RESULTS

### HBEGF mRNA is stable at 20% and 2% O<sub>2</sub>

The presence of approximately 2500 copies of HBEGF mRNA at both 20% and 2% O<sub>2</sub> (Armant et al., 2006) suggests that the dramatic increase in HBEGF synthesis after 4 h of culture at 2% O<sub>2</sub> is controlled post-transcriptionally. To further examine the role of transcription, CTB cells were cultured at 20% or 2% O<sub>2</sub> in the presence or absence of  $\alpha$ -amanitin, an inhibitor of the RNA polymerase II enzyme. Control cells were cultured in either 20% or 2% O<sub>2</sub> for 6 h without inhibitor. Cells treated with  $\alpha$ -amanitin were first treated for 1 h before exposure to the inhibitor for the indicated times. The relative expression of HBEGF mRNA, normalized to SDHA mRNA, was determined by qPCR. As expected, HBEGF mRNA levels in the absence of inhibitor were similar at 2% and 20% O<sub>2</sub> (Fig. 5.1 A;  $p = 0.15$  by Student's t-test). The presence of 1  $\mu$ g/mL  $\alpha$ -amanitin did not significantly alter the abundance of HBEGF transcripts in CTB cells cultured at 2% or 20% O<sub>2</sub> for up to 6 h ( $p = 0.62$  by ANOVA). It was determined in previous experiments that HBEGF upregulation at 2% O<sub>2</sub> is maximally inhibited by 2-10  $\mu$ g/mL  $\alpha$ -amanitin. Therefore, higher concentrations of  $\alpha$ -amanitin were tested, producing a similar lack of effect (Fig. 5.1 B; ANOVA  $p = 0.58$ ). CoCl<sub>2</sub> treatment for up to 4 h, which stabilizes HIF2 $\alpha$  at 20% O<sub>2</sub> (Jiang et al., 1997) to mimic low O<sub>2</sub>, had no significant effect on HBEGF transcript levels (Fig. 5.1 C; ANOVA  $p = 0.45$ ), although HBEGF protein levels increased dramatically (data not shown). HBEGF mRNA levels were unaffected by the addition of  $\alpha$ -amanitin (1.39-fold difference from no treatment), suggesting that CoCl<sub>2</sub> also increases HBEGF protein levels post-transcriptionally. Therefore, transcription of HBEGF was not appreciably altered by O<sub>2</sub>



**Figure 5.1. HBEGF mRNA stability.** HBEGF mRNA levels were normalized to SDHA levels (A, B) or to RPL13a (C) and data is expressed as relative fold-change. (A) CTB cells were pretreated for 1 h with 1  $\mu$ g/mL  $\alpha$ -amanitin, then culture was continued for the indicated times at 20% or 2% O<sub>2</sub>, after which RNA was extracted. (B) Cells were also pretreated for 1 h with the indicated concentrations of  $\alpha$ -amanitin and cultured for an additional 6 h in the presence of the inhibitor, after which RNA was extracted. Cells without inhibitor (A, B) were cultured for 6 h in either 20% or 2% O<sub>2</sub>, as indicated. (C) HBEGF transcript levels measured in CTB cells for up to 4 h of culture in the presence of 250  $\mu$ M CoCl<sub>2</sub>. The x-axis represents time of culture in the presence of CoCl<sub>2</sub>. Experiments were repeated in triplicate and treatment data normalized to 20% O<sub>2</sub> control values.  $p > 0.05$  by two-way ANOVA for all experiments.

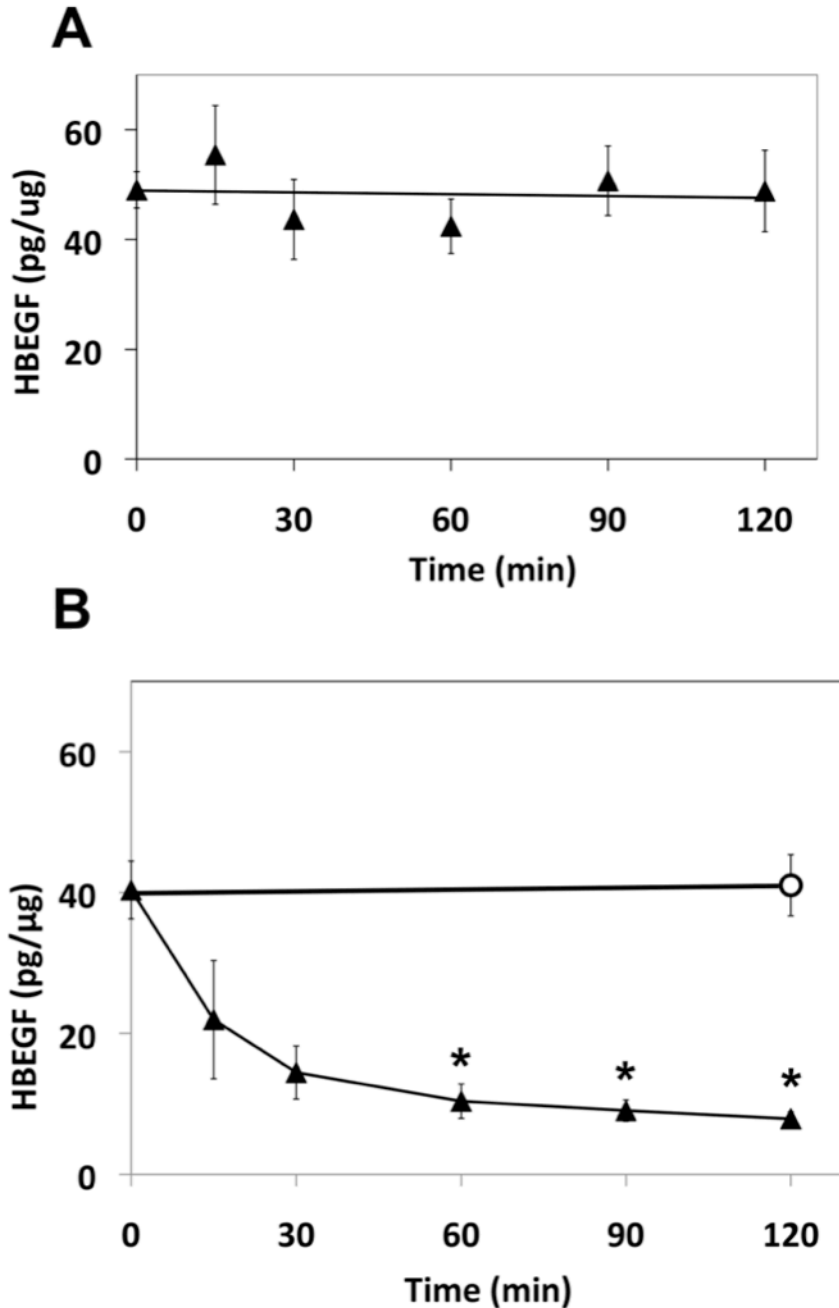
fluctuations or HIF, and remained stable in the absence of new transcription. Therefore, alterations in the transcription or stability of HBEGF mRNA does not account for the observed accumulation of HBEGF protein during hypoxia.

### **HBEGF Protein Accumulation is Not Regulated by Turnover Rate**

The observed increase in HBEGF protein levels at low O<sub>2</sub> could reflect a change in protein stability where it is degraded more slowly at 2% O<sub>2</sub>. To address this hypothesis, HBEGF protein levels were tracked after culture at 2% O<sub>2</sub> for 6 h to increase HBEGF. If translation was blocked with cyclohexamide while culture continued at 2% O<sub>2</sub> for an additional 2 hours (Fig. 5.2 A), HBEGF levels remained unchanged. In contrast, cells returned to 20% O<sub>2</sub> showed a marked destabilization of HBEGF protein which decreases significantly within 15 min (Fig. 5.2 B). HBEGF degradation was abrogated if cells were concomitantly treated with the proteasome inhibitors MG132 or lactocystin. Although differential stability of HBEGF was observed at 2% and 20% O<sub>2</sub>, HBEGF protein did not increase at 20% O<sub>2</sub> when cells were treated with proteasome inhibitors, indicating that stabilization of HBEGF degradation at 20% O<sub>2</sub> is insufficient for its accumulation. However, destabilization by proteolysis through the proteasome appears to have an important role in clearing HBEGF during reoxygenation.

### **Translational Regulatory Activity of the HBEGF 3'UTR**

The absence of evidence for regulation of HBEGF by O<sub>2</sub> through its transcription rate or the rate of mRNA or protein turnover suggested that it might accumulate due to an increased rate of translation. The differential regulation of HBEGF synthesis could occur by suppressing or activating translation of its mRNA under the direction of its

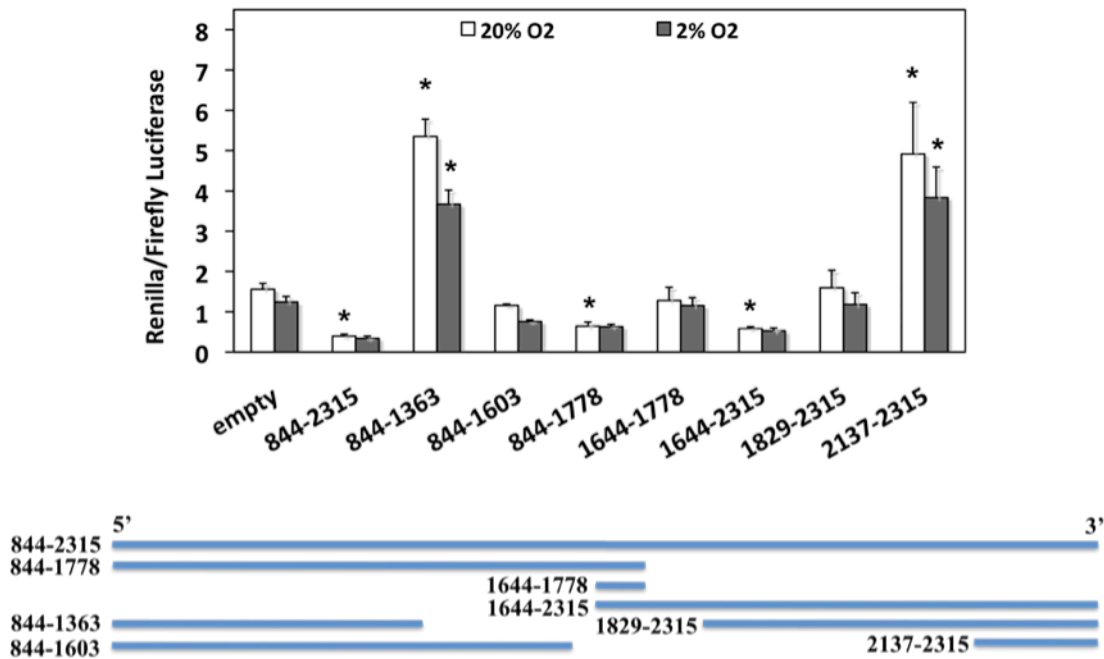


**Figure 5.2 HBEGF protein is stabilized by 2% O<sub>2</sub> but destabilized at 20% O<sub>2</sub>.** HTR cells were cultured for 6 h at 2% O<sub>2</sub>, allowing HBEGF protein to accumulate. Afterwards, cells were (A) exposed to cyclohexamide for up to 2 h (black diamond), or (B) cultured at 20% O<sub>2</sub> for up to 2 h (black triangles) and in the presence of either lactocystin or MG132 (white circle). Cell lysates were collected at the indicated times and HBEGF protein levels quantified by ELISA. \*  $p < 0.05$  according to pairwise comparisons.

large (1455 bp) 3'UTR, a region of mRNA known to be involved in transcript stability and translational control (Mignone et al., 2002). To determine whether the 3'UTR of HBEGF can control translation, a PsiCheck-2 luciferase reporter with inserts of the entire HBEGF 3'UTR or its subdomains was prepared and transfected into CTB cells cultured at 20% O<sub>2</sub>. The presence of certain portions of the HBEGF 3'UTR altered Renilla luciferase production, as compared to an empty vector control (Fig. 5.3). A vector containing the full-length 3'UTR (844-2315) significantly ( $p = 0.008$ ) repressed reporter activity, as did vectors containing the entire first half (844-1778;  $p = 0.031$ ) or second half (1644-2315;  $p = 0.019$ ) of the 3'UTR. In contrast, the 134 bp region overlapped by the two half 3'UTR vectors did not regulate reporter activity. Vectors containing regions 844-1603, 1644-1778, and 1829-2315 were also not active. Vectors containing either the 5' (844-1363) or 3' (2137-2315) flanking regions of the 3'UTR upregulated Renilla luciferase activity at both 20% O<sub>2</sub> and 2% O<sub>2</sub> ( $p < 0.001$  for both). Based on these observations, it appears that the two flanking domains of the 3'UTR increase translational activity when isolated from other 3'UTR sequences, while the presence of regions from 1363bp - 1644bp and 1778bp - 1829bp instigated translational suppression (Figure 5.6).

### **Global Inhibition of miRNA Processing**

As specific regions of the HBEGF 3'UTR were found to be responsible for the suppression or activation of luciferase production, it can be hypothesized that miRNAs that interact with the 3'UTR are required for this regulation. Therefore, the role of miRNA was examined by knocking down the miRNA-specific



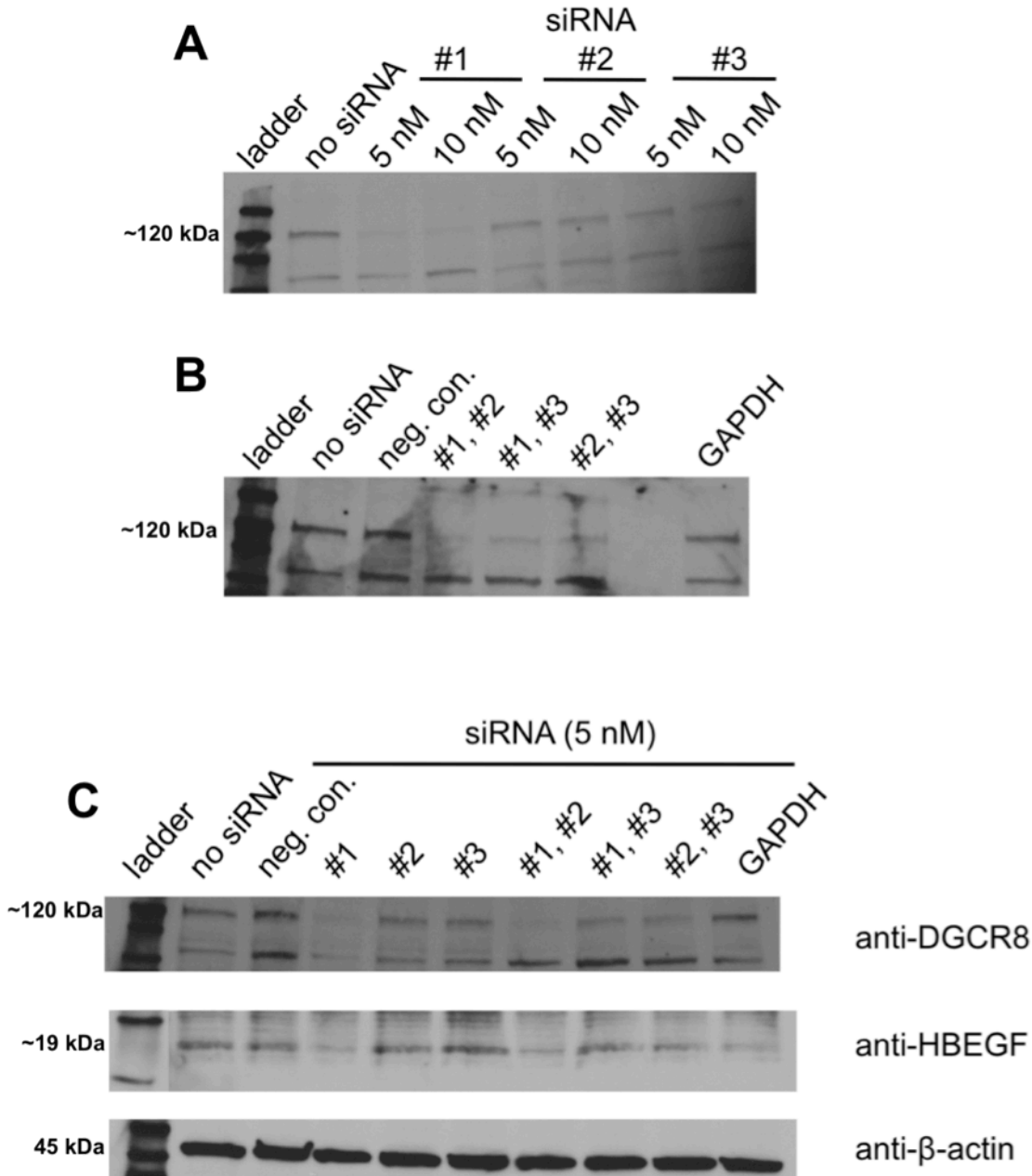
**Figure 5.3. Dual luciferase assay demonstrating repression of mRNA translation by the HBEGF 3'UTR.** Regions of the HBEGF 3'UTR cloned into the psiCheck-2 vector are labeled on the x-axis by their nucleotide position. Data is expressed as ratios of Renilla luciferase activity relative to Firefly luciferase. Luciferase ratios in cells cultured at 20% O<sub>2</sub> (light bars) and 2% O<sub>2</sub> (dark bars) for 24 h are shown. Asterisks for either 20% O<sub>2</sub> or 2% O<sub>2</sub> identify significant differences as compared to their respective empty vector control. The empty vector control (empty) designates vector without a cloned portion of the HBEGF 3'UTR. Values are represented as mean  $\pm$  SEM. Student's t-test, \*  $p < 0.05$



processing protein, DGCR8, using siRNA. Three different siRNAs designed to block DGCR8 expression were tested by western blotting for their ability to reduce DGCR8. It was found that siRNA #1 inhibited DGCR8 maximally and addition of the other two siRNAs did not augment the response (Fig. 5.4 A-B). However, knocking down DGCR8 in CTB cells did not lead to an increase in HBEGF protein levels as expected if miRNA repressed HBEGF translation (Fig. 5.4 C). Instead, the knockdown of DGCR8 with siRNA #1 led to a decrease in HBEGF protein levels, suggesting that miRNA could contribute to the upregulation of HBEGF synthesis at 2% O<sub>2</sub>.

### **Differential Expression of miRNAs Predicted to Target the HBEGF 3'UTR**

Having established a role for miRNA in regulating HBEGF accumulation, it was hypothesized that 3'UTR interacting miRNAs are differentially expressed in response to O<sub>2</sub> concentration in CTB cells. Bioinformatics were employed to identify miRNAs that could potentially bind the 3'UTR of HBEGF. Combining results from the miRBase database and TargetScan identified 27 miRNAs (Table 5.2). Custom qPCR plates designed to screen the candidate miRNAs were used to examine their differential expression in cells cultured for 4 h at either 20% O<sub>2</sub> or 2% O<sub>2</sub> (Fig. 5.5). All miRNAs except miR-581 and miR-623 were expressed in CTB cells. However, the remainder of miRNAs, with the exception of hsa-miR-376c ( $p = 0.03$ ), were expressed at levels that were not statistically different at high and low O<sub>2</sub>. These findings demonstrate that none of the miRNAs were differentially expressed at the two O<sub>2</sub> concentrations, suggesting that miRNAs targeting HBEGF are not transcriptionally regulated by O<sub>2</sub>. Therefore, the mechanism that increases HBEGF translation does not appear to involve an alteration in any of the selected candidate miRNAs in response to O<sub>2</sub> fluctuations.

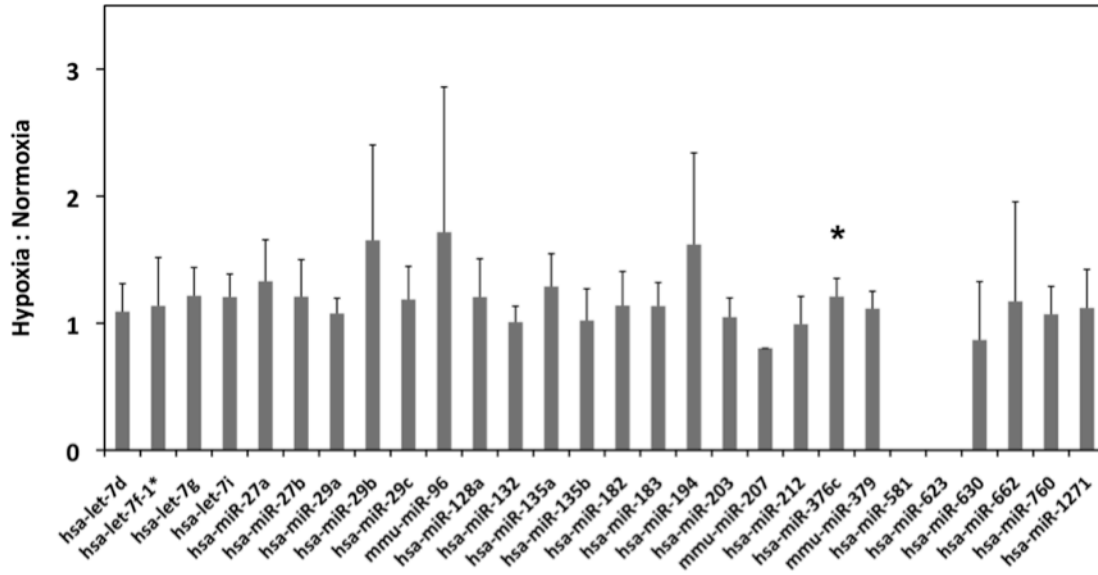


**Figure 5.4 Western blot demonstrating siRNA knockdown of DGCR8.** From the same experiment, Western blots were performed for DGCR8 protein (~120 kDa) in lysates of HTR cells treated for 48 h with (A) two different concentrations of each siRNA and (B) different pairings of the three siRNA (5 nM). Vehicle controls (no siRNA), scrambled siRNA controls (neg. con.), and an unrelated siRNA (GAPDH) were used to examine non-specific effects of the knockdown. In (C), lysates from HTR cells treated for 48 h with the 3 siRNA (5 nM) and their pairings were blotted for DGCR8. Membranes were stripped and reprobed for HBEGF (~19 kDa) and β-actin (~45 kDa), a loading control, to examine the effects of knocking down DGCR8 on the regulation of HBEGF protein.

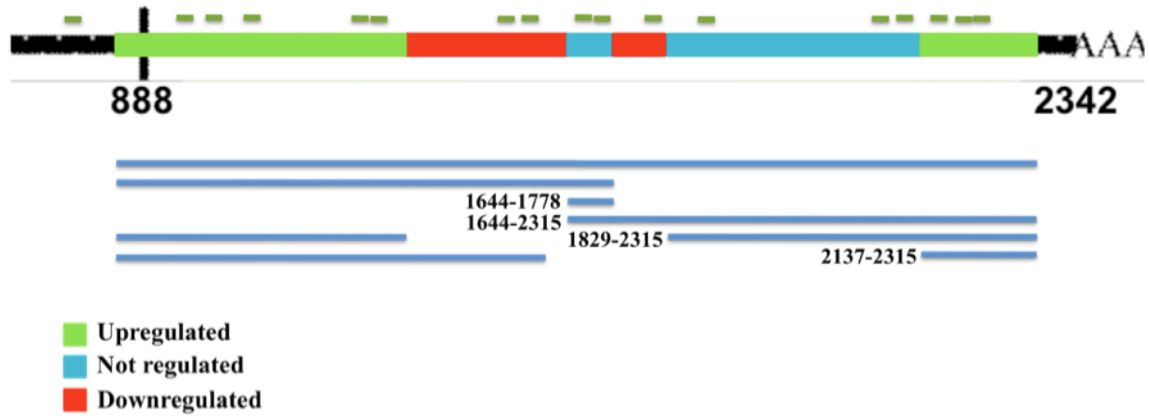
**Table 5.2. MiRNAs that target HBEGF.**

Name	Sequence	Target Site on HBEGF mRNA
hsa-let-7d	TCTCCATCATCCAACGTATCAA	911-932
hsa-let-7f-1*	GATATGTTAGATAACGGAAGGG	2173-2194
hsa-let-7g	ACTCCATCATCAAACATGTCAA	911-932
hsa-let-7i	ACTCCATCATCAAACACGACAA	910-932
hsa-miR-27a	AAGTGTCACCGATTCAAGGCG	1385-1405
hsa-miR-27b	AAGTGTCACCGATTCAAGACG	1385-1405
hsa-miR-29a	ATCGTGGTAGACTTTAGCCAAT	2026-2047
hsa-miR-29b	ATCGTGGTAAACTTTAGTCACAA	2025-2047
hsa-miR-29c	ATCGTGGTAAACTTTAGCCAAT	2026-2047
hsa-miR-96	AAACCGTGATCGTGTA AAAACGA	1050-1072
hsa-miR-128	AGTGTCACTTGGCCAGAGAAA	1399-1432
hsa-miR-132	ATTGTCAGATGTCCGGTACCAGC	1550-1571
hsa-miR-132	ATTGTCAGATGTCCGGTACCAGC	2110-2131
hsa-miR-135a	ATACCGAAAATAAGGATACTACT	1303-1324
hsa-miR-135b	ATACCGAAAAGTAAGGATACTACT	1303-1324
hsa-miR-182	AAACCGTTACCATCTTGAGTGTGA	1179-1202
hsa-miR-183	ATACCGTGACCATCTTAAGTGA	2242-2263
hsa-miR-194	ACATTGTCGTTGAGGTACACCT	1565-1587
hsa-miR-194	ACATTGTCGTTGAGGTACACCT	2269-2290
hsa-miR-203	CACTTTACAAATCCTGGTGATC	1548-1569
mmu-miR-207	CGAAGAGGACCGAGAGGAGGGAG	2055-2077
hsa-miR-212	ATTGTCAGAGGTCAGTGCCGG	1553-1571
hsa-miR-376c	TTGTATCTCCTTTAAGGTGCA	1492-1512
hsa-miR-379	ACCATCTGATACCTTGCATCC	1800-1820
hsa-miR-581	AGAACACAAGAGATCTAGTCA	816-836
hsa-miR-623	TAGGGAACGTCCCCGACAACCCA	1326-1346
hsa-miR-630	TCATAAGACATGGTCCCTTCCA	2221-2243
hsa-miR-662	AGGGTGCAACACCGGGTCGTC	1957-1977
hsa-miR-760	GCCGAGACCCAGACACCCCT	1755-1773
hsa-miR-1271	GAACCGTGGATCGTTCGTGAGT	1065-1085

Names, sequences and locations where the miRNAs are predicted to bind the HBEGF 3'UTR are listed.



**Figure 5.5 Differential expression of miRNAs in CTB cells.** Custom TaqMan qPCR assays identified miRNAs that are expressed in HTR cells, with the exception of miR-581 and miR-623. Values represent relative expression ratios of miRNAs in HTR cells cultured in 2% O<sub>2</sub> for 4 h as compared to cells cultured at 20% O<sub>2</sub>. \* p < 0.05 according to paired samples t-test.



**Figure 5.6 Map of HBEGF 3'UTR regions regulated by  $O_2$ .** Based upon the dual luciferase data, portions of the HBEGF 3'UTR demonstrated to repress (red), upregulate (green), or have no effect (blue) on Renilla luciferase production are indicated. Locations where primers were designed to bind the 3'UTR are labeled with arrows, and the resulting regions amplified by these primers are indicated by the solid lines below the 3'UTR map. MiRNAs predicted to bind the 3'UTR (Table 2) are indicated by small dashes above the 3'UTR map.

## DISCUSSION

Expression analysis and specific inhibition of HBEGF signaling established an autocrine, post-transcriptionally regulated increase in HBEGF synthesis and secretion in human trophoblast cells when the O<sub>2</sub> concentration is reduced to 2% (Armant et al., 2006). The upregulation of HBEGF at low O<sub>2</sub> requires activation of its cognate receptors (ERBB1 and ERBB4) and downstream signaling through any one of three MAPK pathways (MAPK1/3, MAPK14 or MAPK8) (Jessmon et al., 2010). Several post-transcriptional mechanisms were contemplated that could account for the regulation of HBEGF expression by O<sub>2</sub>. The stability of HBEGF mRNA could increase with reduced O<sub>2</sub>, as shown for the upregulation of NHE1 by EGF signaling through PI3K and MEK1/2 pathways (Chiang et al., 2008). IGF-1 induces stabilization of COX2 mRNA through sequences in its 3'UTR by activating the ERK1/2, p38, and PKC pathways (Cao et al., 2007). In G(0) growth arrested mammary epithelial cells, ultraviolet radiation activates p38 and translocation to the cytoplasm of the AU-rich element (ARE)-binding protein HuR (Brennan and Steitz, 2001), which stabilizes c/EBPdelta mRNA by binding AREs in its 3'UTR (Li et al., 2008). However, the steady state levels of HBEGF transcripts were similar at high and low O<sub>2</sub> concentrations (Armant et al., 2006) and the message persisted for 6 h at both O<sub>2</sub> levels when *de novo* transcription was inhibited by  $\alpha$ -amanitin. Alternatively, HBEGF protein could be degraded more rapidly at high O<sub>2</sub> concentrations and thus accumulate in low O<sub>2</sub>. Estrogen increases protein levels of CXCR4 without affecting a change in its mRNA (Sengupta et al., 2009). An agonist of the liver X receptor (LXR) induced TNFA protein production without altering mRNA levels, and appeared to do so through the p38 signaling pathway (Wang et al., 2009b).

However, blocking HBEGF degradation with proteasome inhibitors failed to instigate its accumulation in trophoblast cells cultured at 20% O<sub>2</sub>, suggesting that the upregulation of HBEGF at low O<sub>2</sub> is not due to decreased HBEGF turnover, but uses a mechanism that actively increases the translation of dormant HBEGF mRNA. HBEGF turnover was indeed strikingly faster at 20% than 2% O<sub>2</sub>, which could facilitate clearance of the protein during reoxygenation.

Given the lack of transcriptional regulation, it was hypothesized that HBEGF translation could be regulated by regions in its large 3'UTR. Luciferase reporter assays are a useful means to examine translational regulation by 3'UTR sequence (Pang et al., 2009) and provide evidence that specific regions of the HBEGF 3'UTR regulate its translation with stimulatory or inhibitory activities. Others have examined translational suppression by cotransfecting vectors containing cloned 3'UTR regions and miRNA in HEK293 cells (Jiao et al., 2010) and HTR-8/SVneo cells (Mouillet et al., 2010). Transfection with a 3'UTR reporter without addition of miRNA more closely recapitulates *in vivo* conditions by relying upon endogenous miRNA and translational machinery. Although there was no differential effect due to O<sub>2</sub> concentration, flanking regions of the 3'UTR (844-1363 and 2137-2315) strongly upregulated luciferase reporter, suggesting that these domains could have a role in stimulating HBEGF translation at 2% O<sub>2</sub>. In contrast, regions towards the center of the 3'UTR (1363-1603 and 1778-2315), when present with the flanking regions, repressed luciferase production and are likely responsible for the overall repressive effect of the complete 3'UTR sequence. Although a differential effect on reporter activity by oxygen was not observed, it could be hypothesized that when central and flanking regions of the 3'UTR are both present, the

suppressive influence could be attenuated by portions of the HBEGF mRNA not included in these reporter constructs, (e.g., the 5'UTR), resulting in accelerated translation of HBEGF at 2% O<sub>2</sub>. For example, the 5' terminal oligopyrimidine tract (5'TOP) motif in the 5'UTR of ribosomal proteins in neural U87 cells mediates translational upregulation (Orom et al., 2008). Also, the 5' noncoding region (5'NCR) of the hepatitis C viral genome (HCV) positively regulates accumulation of RNA (Jopling et al., 2005). Therefore, miRNA or other regulatory factors that bind regions outside the 3'UTR could interact with regions in the 3'UTR to induce translation at 2% O<sub>2</sub>.

Translational regulation is often effected through miRNAs that interact with the 3'UTR and induce formation of a protein complex (miRNA ribonucleoprotein; miRNP/RISC) that suppresses translation (Kim and Nam, 2006; Pillai et al., 2007). Therefore, we examined the role of miRNA in regulating HBEGF expression. When miRNA production was blocked by adding siRNA to knock down DGCR8, HBEGF protein declined, suggesting that miRNAs are required to positively regulate HBEGF translation. Hence, miRNA may either directly facilitate the upregulatory activity of the flanking regions of the 3'UTR (844-1363 and 2137-2315) or repress interactions of the interfering central domains with the flanking regions. Functional miRNA seed sequences are predominantly located near stop codons or near the Poly-A tail in 3'UTRs longer than 1.3 kb (Grimson et al., 2007) and translation can be increased due to potential miRNA-mediated action (Vasudevan and Steitz, 2007; Vasudevan et al., 2007), suggesting that flanking regions of the HBEGF 3'UTR are more likely to be target by a miRNA-mediated mechanism. Quantification of miRNAs that target HBEGF provided no evidence of their differential expression at 20% and 2% O<sub>2</sub>. Though



miRNA may be required for upregulating HBEGF translation, they may be expressed constitutively in CTB cells. These findings direct attention to proteins within the RNP complex that utilize HBEGF-targeting miRNA and may themselves be regulated by O<sub>2</sub>. Previous studies examining signaling downstream of HBEGF responsible for its autocrine upregulation (Jessmon et al., 2010) would suggest that the RNP proteins could be regulated downstream of MAPK signaling, either through phosphorylation or new transcription.

The suppressive influence of the central region, which may act independently of miRNA, could be mediated through interacting proteins. Regulatory proteins could be responsible for suppression of translation by interacting with the central regions of the 3'UTR. It has been demonstrated that RNA-binding proteins that bind the 3'UTR, including ARE-binding proteins, preferentially bind single-stranded RNA that can be provided by loops in the transcript (Hiller et al., 2006; Hudson et al., 2004). The protein Rnc1, a KH-type RNA-binding protein, binds UCAU tetranucleotide repeats in the 3'UTR of Pmp1 mRNA and stabilizes the transcript (Sugiura et al., 2003). Pumilio/FBF (PUF) proteins are known to mediate translational repression of target RNAs by binding a recognized motif in their 3'UTRs (Chritton and Wickens, 2010). Analysis of the secondary structure of the HBEGF 3'UTR indicates that there are more hairpins and loops in the flanking regions of the 3'UTR (clustered around nt 1100 and 2150, respectively) and in the central region (nt ~1300-2000). Proteins could bind AREs in the central region to mediate the repression observed at 20% O<sub>2</sub>. An examination of possible AREs in HBEGF using AREsite (Gruber et al., 2011) revealed that several different types of AREs potentially exist between 1503 and 1539, which is within one of

the downregulating regions of the 3'UTR. Since more AREs are present in regions of the 3'UTR that were repressive, proteins that bind to these sequences could be responsible for the repression at 20% O<sub>2</sub>. Site-directed mutagenesis would prevent binding of these proteins and could clarify the role of ARE-binding proteins in the regulation of HBEGF translation.

Specific genes are translationally regulated by O<sub>2</sub>. For example, low O<sub>2</sub> increases ATF4 protein, but doesn't affect its mRNA (Lu et al., 2004; Vatter and Wek, 2004). In addition, treatment with thapsigargin to induce ER stress shifts ATF4 mRNA from monoribosomes to polyribosomes, indicating that its preexistent mRNA pool is inefficiently translated until cells experience ER stress. This stress destabilizes eukaryotic initiation factor 2a (eIF2a), causing it to skip over the upstream open reading frame 2 (uORF2) in a process referred to as leaky scanning (Harding et al., 2000). In addition, eIF2-GTP is expressed in limited amounts at low O<sub>2</sub>, which increases the time required for initiation complexes to reinitiate translation after skipping over uORF2. This increased time allows the initiation complex to find the start codon, thus leading to preferential translation in hypoxia (Vatter and Wek, 2004). Prediction software (EMBOSS GETORF) recognizes nine potential uORFs in the HBEGF 5'UTR. UORFs are generally from 3-35 codons long and can mediate translational suppression or activation (Vilela and McCarthy, 2003). Thus, it is possible that uORFs in the 5'UTR of HBEGF contribute to preferential translation at 2% O<sub>2</sub>, perhaps overriding the suppressive central region of the 3'UTR.

The present study demonstrates that HBEGF is post-transcriptionally regulated by O<sub>2</sub> and suggests that regions of its 3'UTR direct translationally suppressing and

activating mechanisms. Evidence exists for a model in which flanking regions of the 3'UTR mediate translational upregulation of HBEGF that is suppressed by factors recruited to the central region of the 3'UTR at 20% O<sub>2</sub>. When O<sub>2</sub> levels are reduced (2%), other portions of the HBEGF mRNA, such as factors present at the 5'UTR, interfere with elements at the central region of the 3'UTR, allowing miRNAs already bound to the flanking regions to direct protein synthesis. While protein factors responsible for these mechanisms have yet to be identified, prior work suggests that the proteins are regulated downstream of HBEGF/ERBB and MAPK signaling (Jessmon et al., 2010). This study provides a basic understanding of the complex mechanisms that regulate HBEGF expression in CTBs during environmental changes in early pregnancy (Armant et al., 2006; Jessmon et al., 2010; Jessmon et al., 2009). Importantly, these mechanisms can rapidly up- or downregulate protein expression in response to changes in oxygen concentration without expending energy for de novo HBEGF transcription.

## CHAPTER 6. Conclusion

HBEGF is an important growth factor present during embryo implantation that regulates CTB physiology. Its protein levels are greatly increased in the low O<sub>2</sub> environment found in early implantation through an autocrine, post-transcriptional mechanism (Armant et al., 2006). Stabilizing HIF proteins at 20% O<sub>2</sub> with CoCl<sub>2</sub> suggested that a transcriptional event occurs downstream of these O<sub>2</sub>-sensitive factors that leads to HBEGF upregulation. Indeed, blocking nascent transcription at 2% O<sub>2</sub> with  $\alpha$ -amanitin confirmed that transcription is required. Furthermore, microarray analysis identified that MMP2 and MMP15 are highly upregulated at 2% O<sub>2</sub>, suggesting that they may be the targets of this low O<sub>2</sub>-induced transcriptional event. Prior studies established that matrix metalloproteinase (MMP) activity is necessary to induce autocrine HBEGF signaling that leads to an increase in its own translation (Armant et al., 2006). Blocking the activity of MMP2, but not MMP9, abrogated the increase in HBEGF at 2% O<sub>2</sub>, which led to the hypothesis that low O<sub>2</sub> induces transcription of MMP2, which is a regulated component of a proteolytic cascade responsible for proHBEGF cleavage from the cell surface and initiation of its signaling. Preventing HBEGF signaling at 2% O<sub>2</sub> with a specific antagonist of HBEGF or an inhibitor of its cognate receptors demonstrated that HBEGF then signals in an autocrine fashion to upregulate its own protein levels (Armant et al., 2006). Inhibitors of the downstream signaling pathways ERK, MAPK14 (p38), and JNK, when added together, were also able to block its increase, indicating that HBEGF utilizes any of these three pathways to induce its autocrine-induced translation. An examination of its protein and mRNA stability demonstrated that HBEGF protein is stable at 2% O<sub>2</sub> and its transcripts exist in

equivalent amounts at both high and low O<sub>2</sub>. Hence, its upregulation at low O<sub>2</sub> occurs through a specific translational activation event.

A post-transcriptional increase in HBEGF could be modulated by untranslated regions (UTRs) of its mRNA. An examination of the 3'UTR of *HBEGF* for potential regulatory elements demonstrated that distinct regions, when inserted into a luciferase reporter vector, modulate either up- or down-regulation of luciferase translation. Specifically, flanking regions of the 3'UTR mediated translational upregulation, and central regions mediated translational downregulation. The flanking regions are likely responsible for the increase in HBEGF translation at 2% O<sub>2</sub>. This regulation could be due to RNA-binding proteins or miRNA that binds to the 3'UTR. Knockdown of the miRNA-processing protein, DGCR8, decreased HBEGF protein levels, suggesting that miRNAs bind to the flanking regions of the 3'UTR and are responsible for the translation of HBEGF at 2% O<sub>2</sub>.

Once upregulated at 2% O<sub>2</sub>, HBEGF signaling blocks apoptosis in CTBs induced by low O<sub>2</sub> through the MAPK14 pathway (Jessmon et al., 2009). This same pathway is utilized by HBEGF to block apoptosis when cells cultured at 2% O<sub>2</sub> are reoxygenated. CTBs are likely exposed to reoxygenation in vivo during the 10<sup>th</sup> week of pregnancy when maternal arteries occluded by invading CTBs open and oxygen levels increase in the intervillous space of the placenta. Hence, HBEGF upregulation prior to 10 weeks is essential to CTB survival both at low O<sub>2</sub> and during subsequent reoxygenation.

After the first 10 weeks of development, HBEGF accumulated by CTBs and in uterine tissue could induce CTB differentiation to a highly motile phenotype. Inhibition

of signaling pathways downstream of the ERBB receptors demonstrated that HBEGF utilizes the ERK, JNK, MAPK14, and PI3K pathways to stimulate CTB motility. HBEGF induces upregulation of ITGA1 and downregulation of ITGA6 protein (integrin-switching) in CTB cells through these four pathways. This suggests that HBEGF could be responsible for inducing integrin expression patterns known to be characteristic of invading CTBs (Damsky et al., 1994; Zhou et al., 1997b). Interestingly, the switch of integrin expression induced by HBEGF occurs without an accompanying change in mRNA, suggesting that a mechanism exists to rapidly upregulate integrin proteins and conserve energy spent on nascent transcription. In the developmental program for implantation and placentation, such a mechanism would allow CTBs to quickly differentiate in response to changes encountered in growth factors, extracellular matrix or other extrinsic cues.

The syndrome preeclampsia, which is characterized by shallow CTB invasion into the maternal decidua and increased CTB apoptosis (Redman and Sargent, 2000), is associated with a dramatic reduction in placental HBEGF (Leach et al., 2002a). This correlation suggests that reduced HBEGF signaling during placentation contributes to reduced conversion of the spiral arteries in preeclamptic pregnancies. Although it remains to be discovered exactly how HBEGF is translationally regulated, the role of its 3'UTR and miRNAs has been established and will likely be an important foundation in future studies of preeclampsia and the regulation of early pregnancy. Future studies examining the mechanisms by which HBEGF regulates integrin-switching and cytoprotective mechanisms will likewise provide insights into trophoblast physiology during implantation.

## REFERENCES

- Abrahamsohn, P.A., and T.M. Zorn. 1993. Implantation and decidualization in rodents. *J Exp Zool.* 266:603-628.
- Aghajanova, L., K. Bjuresten, S. Altmae, B.M. Landgren, and A. Stavreus-Evers. 2008. HB-EGF but not amphiregulin or their receptors HER1 and HER4 is altered in endometrium of women with unexplained infertility. *Reprod Sci.* 15:484-492.
- Allaire, A.D., K.A. Ballenger, S.R. Wells, M.J. McMahon, and B.A. Lessey. 2000a. Placental apoptosis in preeclampsia. *Obstet Gynecol.* 96:271-276.
- Allaire, A.D., K.A. Ballenger, S.R. Wells, M.J. McMahon, and B.A. Lessey. 2000b. Placental apoptosis in preeclampsia. *Obstet. Gynecol.* 96:271-276.
- Aplin, A.E., A. Howe, S.K. Alahari, and R.L. Juliano. 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev.* 50:197-263.
- Aplin, J.D., S.L. Straszewski-Chavez, B. Kalionis, C. Dunk, D. Morrish, K. Forbes, D. Baczyk, N. Rote, A. Malassine, and M. Knofler. 2006. Trophoblast differentiation: progenitor cells, fusion and migration -- a workshop report. *Placenta.* 27 Suppl A:S141-143.
- Apparao, K.B., M.J. Murray, M.A. Fritz, W.R. Meyer, A.F. Chambers, P.R. Truong, and B.A. Lessey. 2001. Osteopontin and its receptor alphavbeta(3) integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *The Journal of clinical endocrinology and metabolism.* 86:4991-5000.

- Armant, D.R. 2005. Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. *Dev Biol.* 280:260-280.
- Armant, D.R., B.A. Kilburn, A. Petkova, S.S. Edwin, Z.M. Duniec-Dmuchowski, H.J. Edwards, R. Romero, and R.E. Leach. 2006. Human trophoblast survival at low oxygen concentrations requires metalloproteinase-mediated shedding of heparin-binding EGF-like growth factor. *Development.* 133:751-759.
- Armant, D.R., J. Wang, and Z. Liu. 2000. Intracellular signaling in the developing blastocyst as a consequence of the maternal-embryonic dialogue. *Semin Reprod Med.* 18:273-287.
- Asakura, M., M. Kitakaze, S. Takashima, Y. Liao, F. Ishikura, T. Yoshinaka, H. Ohmoto, K. Node, K. Yoshino, H. Ishiguro, H. Asanuma, S. Sanada, Y. Matsumura, H. Takeda, S. Beppu, M. Tada, M. Hori, and S. Higashiyama. 2002. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med.* 8:35-40.
- Baek, D., J. Villen, C. Shin, F.D. Camargo, S.P. Gygi, and D.P. Bartel. 2008. The impact of microRNAs on protein output. *Nature.* 455:64-71.
- Barad, O., E. Meiri, A. Avniel, R. Aharonov, A. Barzilai, I. Bentwich, U. Einav, S. Gilad, P. Hurban, Y. Karov, E.K. Lobenhofer, E. Sharon, Y.M. Shibolet, M. Shtutman, Z. Bentwich, and P. Einat. 2004. MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Res.* 14:2486-2494.
- Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 116:281-297.



- Bass, K.E., D. Morrish, I. Roth, D. Bhardwaj, R. Taylor, Y. Zhou, and S.J. Fisher. 1994a. Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. *Dev. Biol.* 164:550-561.
- Bass, K.E., D. Morrish, I. Roth, D. Bhardwaj, R. Taylor, Y. Zhou, and S.J. Fisher. 1994b. Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. *Dev Biol.* 164:550-561.
- Baudouin-Legros, M., A. Hinzpeter, A. Jaulmes, F. Brouillard, B. Costes, P. Fanen, and A. Edelman. 2005. Cell-specific posttranscriptional regulation of CFTR gene expression via influence of MAPK cascades on 3'UTR part of transcripts. *Am J Physiol Cell Physiol.* 289:C1240-1250.
- Bennett, B.L., D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, and D.W. Anderson. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U. S. A.* 98:13681-13686.
- Berube, J., C. Bourdon, Y. Yao, and S. Rousseau. 2009. Distinct intracellular signaling pathways control the synthesis of IL-8 and RANTES in TLR1/TLR2, TLR3 or NOD1 activated human airway epithelial cells. *Cell. Signal.* 21:448-456.
- Biederer, T., and P. Scheiffele. 2007. Mixed-culture assays for analyzing neuronal synapse formation. *Nat Protoc.* 2:670-676.
- Birdsall, M.A., J.F. Hopkisson, K.E. Grant, D.H. Barlow, and H.J. Mardon. 1996. Expression of heparin-binding epidermal growth factor messenger RNA in the human endometrium. *Mol Hum Reprod.* 2:31-34.

- Blankenship, T.N., and A.C. Enders. 1997. Expression of platelet-endothelial cell adhesion molecule-1 (PECAM) by macaque trophoblast cells during invasion of the spiral arteries. *Anat. Rec.* 247:413-419.
- Brennan, C.M., and J.A. Steitz. 2001. HuR and mRNA stability. *Cell Mol Life Sci.* 58:266-277.
- Brosens, I.A., W.B. Robertson, and H.G. Dixon. 1972a. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet.Gynecol.Annu.* 1:177-191.
- Brosens, I.A., W.B. Robertson, and H.G. Dixon. 1972b. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu.* 1:177-191.
- Brown, L.F., B. Berse, L. Van de Water, A. Papadopoulos-Sergiou, C.A. Perruzzi, E.J. Manseau, H.F. Dvorak, and D.R. Senger. 1992. Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. *Mol Biol Cell.* 3:1169-1180.
- Brusselmans, K., F. Bono, P. Maxwell, Y. Dor, M. Dewerchin, D. Collen, J.M. Herbert, and P. Carmeliet. 2001. Hypoxia-inducible factor-2alpha (HIF-2alpha) is involved in the apoptotic response to hypoglycemia but not to hypoxia. *J Biol Chem.* 276:39192-39196.
- Burridge, K., and M. Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol.* 12:463-518.
- Burton, G.J., and E. Jauniaux. 2004. Placental oxidative stress: from miscarriage to preeclampsia. *J. Soc. Gynecol. Investig.* 11:342-352.

- Burton, G.J., E. Jauniaux, and D.S. Charnock-Jones. 2007. Human early placental development: potential roles of the endometrial glands. *Placenta*. 28 Suppl A:S64-69.
- Burton, G.J., E. Jauniaux, and A.L. Watson. 1999. Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. *Am J Obstet Gynecol*. 181:718-724.
- Bustin, S.A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, and C.T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 55:611-622.
- Butler, G.S., M.J. Butler, S.J. Atkinson, H. Will, T. Tamura, S. Schade van Westrum, T. Crabbe, J. Clements, M.P. d'Ortho, and G. Murphy. 1998. The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. A kinetic study. *J Biol Chem*. 273:871-880.
- Caniggia, I., H. Mostachfi, J. Winter, M. Gassmann, S.J. Lye, M. Kuliszewski, and M. Post. 2000. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). *J Clin Invest*. 105:577-587.
- Cao, Z., L.Z. Liu, D.A. Dixon, J.Z. Zheng, B. Chandran, and B.H. Jiang. 2007. Insulin-like growth factor-I induces cyclooxygenase-2 expression via PI3K, MAPK and PKC signaling pathways in human ovarian cancer cells. *Cell. Signal*. 19:1542-1553.
- Carson, D.D., E. Lagow, A. Thathiah, R. Al-Shami, M.C. Farach-Carson, M. Vernon, L. Yuan, M.A. Fritz, and B. Lessey. 2002. Changes in gene expression during the

early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Molecular human reproduction*. 8:871-879.

Cermik, D., M. Karaca, and H.S. Taylor. 2001. HOXA10 expression is repressed by progesterone in the myometrium: differential tissue-specific regulation of HOX gene expression in the reproductive tract. *The Journal of clinical endocrinology and metabolism*. 86:3387-3392.

Chakraborty, C., Y.P. Barbin, S. Chakrabarti, P. Chidiac, S.J. Dixon, and P.K. Lala. 2003. Endothelin-1 promotes migration and induces elevation of  $[Ca^{2+}]_i$  and phosphorylation of MAP kinase of a human extravillous trophoblast cell line. *Mol. Cell. Endocrinol*. 201:63-73.

Chao, A., C.L. Tsai, P.C. Wei, S. Hsueh, A.S. Chao, C.J. Wang, C.N. Tsai, Y.S. Lee, T.H. Wang, and C.H. Lai. 2010. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. *Cancer Lett*. 291:99-107.

Chen, L., R.J. Belton, Jr., and R.A. Nowak. 2009. Basigin-mediated gene expression changes in mouse uterine stromal cells during implantation. *Endocrinology*. 150:966-976.

Cheng, C.Y., H.C. Tseng, and C.M. Yang. 2011. Bradykinin-mediated cell proliferation depends on transactivation of EGF receptor in corneal fibroblasts. *J Cell Physiol*.

- Chiang, Y., C.Y. Chou, K.F. Hsu, Y.F. Huang, and M.R. Shen. 2008. EGF upregulates Na<sup>+</sup>/H<sup>+</sup> exchanger NHE1 by post-translational regulation that is important for cervical cancer cell invasiveness. *J Cell Physiol.* 214:810-819.
- Chobotova, K., N. Karpovich, J. Carver, S. Manek, W.J. Gullick, D.H. Barlow, and H.J. Mardon. 2005. Heparin-binding epidermal growth factor and its receptors mediate decidualization and potentiate survival of human endometrial stromal cells. *J Clin Endocrinol Metab.* 90:913-919.
- Chobotova, K., M.E. Muchmore, J. Carver, H.J. Yoo, S. Manek, W.J. Gullick, D.H. Barlow, and H.J. Mardon. 2002a. The mitogenic potential of heparin-binding epidermal growth factor in the human endometrium is mediated by the epidermal growth factor receptor and is modulated by tumor necrosis factor-alpha. *J Clin Endocrinol Metab.* 87:5769-5777.
- Chobotova, K., I. Spyropoulou, J. Carver, S. Manek, J.K. Heath, W.J. Gullick, D.H. Barlow, I.L. Sargent, and H.J. Mardon. 2002b. Heparin-binding epidermal growth factor and its receptor ErbB4 mediate implantation of the human blastocyst. *Mech Dev.* 119:137-144.
- Chritton, J.J., and M. Wickens. 2010. Translational repression by PUF proteins in vitro. *RNA.* 16:1217-1225.
- Cindrova-Davies, T. 2009. Gabor Than Award Lecture 2008: pre-eclampsia - from placental oxidative stress to maternal endothelial dysfunction. *Placenta.* 30 Suppl A:S55-65.
- Cindrova-Davies, T., O. Spasic-Boskovic, E. Jauniaux, D.S. Charnock-Jones, and G.J. Burton. 2007. Nuclear factor-kappa B, p38, and stress-activated protein kinase

mitogen-activated protein kinase signaling pathways regulate proinflammatory cytokines and apoptosis in human placental explants in response to oxidative stress: effects of antioxidant vitamins. *Am. J. Pathol.* 170:1511-1520.

Damsky, C.H., M.L. Fitzgerald, and S.J. Fisher. 1992. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest.* 89:210-222.

Damsky, C.H., C. Librach, K.H. Lim, M.L. Fitzgerald, M.T. McMaster, M. Janatpour, Y. Zhou, S.K. Logan, and S.J. Fisher. 1994. Integrin switching regulates normal trophoblast invasion. *Development.* 120:3657-3666.

Das, S.K., H. Tsukamura, B.C. Paria, G.K. Andrews, and S.K. Dey. 1994a. Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. *Endocrinology.* 134:971-981.

Das, S.K., X.N. Wang, B.C. Paria, D. Damm, J.A. Abraham, M. Klagsbrun, G.K. Andrews, and S.K. Dey. 1994b. Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development.* 120:1071-1083.

Das, S.K., X.N. Wang, B.C. Paria, D. Damm, J.A. Abraham, M. Klagsbrun, G.K. Andrews, and S.K. Dey. 1994c. Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its

**apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development*. 120:1071-1083.**

**Dash, P.R., G.S. Whitley, L.J. Ayling, A.P. Johnstone, and J.E. Cartwright. 2005.**

**Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase. *Cell Signal*. 17:571-580.**

**Dey, S.K., H. Lim, S.K. Das, J. Reese, B.C. Paria, T. Daikoku, and H. Wang. 2004.**

**Molecular cues to implantation. *Endocr Rev*. 25:341-373.**

**DiFederico, E., O. Genbacev, and S.J. Fisher. 1999a. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am. J. Pathol*. 155:293-301.**

**DiFederico, E., O. Genbacev, and S.J. Fisher. 1999b. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol*. 155:293-301.**

**Ding, N.Z., C.Q. He, and Z.M. Yang. 2002. Quantification of basigin mRNA in mouse oocytes and preimplantation embryos by competitive RT-PCR. *Zygote*. 10:239-243.**

**Donker, R.B., J.F. Mouillet, D.M. Nelson, and Y. Sadovsky. 2007. The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. *Mol. Hum. Reprod*. 13:273-279.**

**Drummond, A.J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and W. A. 2011.**

- Edwards, D.R., M.M. Handsley, and C.J. Pennington. 2008. The ADAM metalloproteinases. *Mol Aspects Med.* 29:258-289.
- Elenius, K., S. Paul, G. Allison, J. Sun, and M. Klagsbrun. 1997. Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *Embo J.* 16:1268-1278.
- Fang, L., G. Li, G. Liu, S.W. Lee, and S.A. Aaronson. 2001. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. *Embo J.* 20:1931-1939.
- Farrar, J.D., and D.D. Carson. 1992. Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. *Biol Reprod.* 46:1095-1108.
- Favata, M.F., K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feeser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, R.A. Copeland, R.L. Magolda, P.A. Scherle, and J.M. Trzaskos. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273:18623-18632.
- Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet.* 9:102-114.
- Friedman, R.C., K.K. Farh, C.B. Burge, and D.P. Bartel. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 19:92-105.
- Fukushima, K., S. Miyamoto, H. Komatsu, K. Tsukimori, H. Kobayashi, H. Seki, S. Takeda, and H. Nakano. 2003. TNFalpha-induced apoptosis and integrin



switching in human extravillous trophoblast cell line. *Biol. Reprod.* 68:1771-1778.

- Fukushima, K., S. Miyamoto, K. Tsukimori, H. Kobayashi, H. Seki, S. Takeda, E. Kensuke, K. Ohtani, M. Shibuya, and H. Nakano. 2005. Tumor necrosis factor and vascular endothelial growth factor induce endothelial integrin repertoires, regulating endovascular differentiation and apoptosis in a human extravillous trophoblast cell line. *Biol. Reprod.* 73:172-179.
- Fukushima, K., M. Murata, M. Hachisuga, K. Tsukimori, H. Seki, S. Takeda, K. Asanoma, and N. Wake. 2008. Hypoxia inducible factor 1 alpha regulates matrigel-induced endovascular differentiation under normoxia in a human extravillous trophoblast cell line. *Placenta.* 29:324-331.
- Fulop, V., S.C. Mok, D.R. Genest, I. Gati, J. Doszpod, and R.S. Berkowitz. 1998. p53, p21, Rb and mdm2 oncoproteins. Expression in normal placenta, partial and complete mole, and choriocarcinoma. *J. Reprod. Med.* 43:119-127.
- Ganapathy, R., G.S. Whitley, J.E. Cartwright, P.R. Dash, and B. Thilaganathan. 2007. Effect of heparin and fractionated heparin on trophoblast invasion. *Hum Reprod.* 22:2523-2527.
- Garcia-Lloret, M.I., J. Yui, B. Winkler-Lowen, and L.J. Guilbert. 1996. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. *J. Cell. Physiol.* 167:324-332.
- Genbacev, O., R. Joslin, C.H. Damsky, B.M. Polliotti, and S.J. Fisher. 1996a. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro

- and models the placental defects that occur in preeclampsia. *J. Clin. Invest.* 97:540-550.
- Genbacev, O., R. Joslin, C.H. Damsky, B.M. Polliotti, and S.J. Fisher. 1996b. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J Clin Invest.* 97:540-550.
- Genbacev, O., Y. Zhou, J.W. Ludlow, and S.J. Fisher. 1997a. Regulation of human placental development by oxygen tension. *Science (Wash.DC)*. 277:1669-1672.
- Genbacev, O., Y. Zhou, J.W. Ludlow, and S.J. Fisher. 1997b. Regulation of human placental development by oxygen tension. *Science*. 277:1669-1672.
- Giancotti, F.G. 2000. Complexity and specificity of integrin signalling. *Nat Cell Biol.* 2:E13-14.
- Gleeson, L.M., C. Chakraborty, T. McKinnon, and P.K. Lala. 2001. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through alpha 5 beta 1 integrin via mitogen-activated protein Kinase pathway. *J. Clin. Endocrinol. Metab.* 86:2484-2493.
- Graham, C.H., T.S. Hawley, R.G. Hawley, J.R. MacDougall, R.S. Kerbel, N. Khoo, and P.K. Lala. 1993a. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp. Cell Res.* 206:204-211.
- Graham, C.H., T.S. Hawley, R.G. Hawley, J.R. MacDougall, R.S. Kerbel, N. Khoo, and P.K. Lala. 1993b. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res.* 206:204-211.

- Greer, I.A. 2003. Thrombophilia: implications for pregnancy outcome. *Thromb Res.* 109:73-81.
- Griffiths-Jones, S. 2004. The microRNA Registry. *Nucleic Acids Res.* 32:D109-111.
- Griffiths-Jones, S., R.J. Grocock, S. van Dongen, A. Bateman, and A.J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34:D140-144.
- Griffiths-Jones, S., H.K. Saini, S. van Dongen, and A.J. Enright. 2008. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* 36:D154-158.
- Grimson, A., K.K. Farh, W.K. Johnston, P. Garrett-Engle, L.P. Lim, and D.P. Bartel. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell.* 27:91-105.
- Gruber, A.R., J. Fallmann, F. Kratochvill, P. Kovarik, and I.L. Hofacker. 2011. AREsite: a database for the comprehensive investigation of AU-rich elements. *Nucleic Acids Res.* 39:D66-69.
- Gui, Y., J. Zhang, L. Yuan, and B.A. Lessey. 1999. Regulation of HOXA-10 and its expression in normal and abnormal endometrium. *Molecular human reproduction.* 5:866-873.
- Hamatani, T., T. Daikoku, H. Wang, H. Matsumoto, M.G. Carter, M.S. Ko, and S.K. Dey. 2004. Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. *Proc Natl Acad Sci U S A.* 101:10326-10331.

- Hamilton, G.S., J.J. Lysiak, V.K. Han, and P.K. Lala. 1998. Autocrine-paracrine regulation of human trophoblast invasiveness by insulin-like growth factor (IGF)-II and IGF-binding protein (IGFBP)-1. *Exp Cell Res.* 244:147-156.
- Harding, H.P., I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, and D. Ron. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell.* 6:1099-1108.
- Heazell, A.E., and I.P. Crocker. 2008. Live and let die - regulation of villous trophoblast apoptosis in normal and abnormal pregnancies. *Placenta.* 29:772-783.
- Heazell, A.E., N.N. Taylor, S.L. Greenwood, P.N. Baker, and I.P. Crocker. 2009. Does altered oxygenation or reactive oxygen species alter cell turnover of BeWo choriocarcinoma cells? *Reprod Biomed Online.* 18:111-119.
- Heinemeyer, T., E. Wingender, I. Reuter, H. Hermjakob, A.E. Kel, O.V. Kel, E.V. Ignatieva, E.A. Ananko, O.A. Podkolodnaya, F.A. Kolpakov, P.N. L., and N.A. Kolchanov. 1998. Databases on Transcriptional Regulation: TRANSFAC, TRRD, and COMPEL. . *Nucleic Acids Res.* 26:364-370.
- Higashiyama, S., R. Iwamoto, K. Goishi, G. Raab, N. Taniguchi, M. Klagsbrun, and E. Mekada. 1995. The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J Cell Biol.* 128:929-938.
- Hiller, M., R. Pudimat, A. Busch, and R. Backofen. 2006. Using RNA secondary structures to guide sequence motif finding towards single-stranded regions. *Nucleic Acids Res.* 34:e117.

- Hills, F.A., V.M. Abrahams, B. Gonzalez-Timon, J. Francis, B. Cloke, L. Hinkson, R. Rai, G. Mor, L. Regan, M. Sullivan, E.W. Lam, and J.J. Brosens. 2006. Heparin prevents programmed cell death in human trophoblast. *Mol Hum Reprod.* 12:237-243.
- Hitchon, C.A., C.L. Danning, G.G. Illei, H.S. El-Gabalawy, and D.T. Boumpas. 2002. Gelatinase expression and activity in the synovium and skin of patients with erosive psoriatic arthritis. *J Rheumatol.* 29:107-117.
- Ho, S., B. Winkler-Lowen, D.W. Morrish, J. Dakour, H. Li, and L.J. Guilbert. 1999. The role of Bcl-2 expression in EGF inhibition of TNF-alpha/IFN-gamma-induced villous trophoblast apoptosis. *Placenta.* 20:423-430.
- Hofmann, G.E., M.R. Drews, R.T. Scott, Jr., D. Navot, D. Heller, and L. Deligdisch. 1992. Epidermal growth factor and its receptor in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. *J Clin Endocrinol Metab.* 74:981-988.
- Hofmann, G.E., R.T. Scott, Jr., P.A. Bergh, and L. Deligdisch. 1991. Immunohistochemical localization of epidermal growth factor in human endometrium, decidua, and placenta. *J. Clin. Endocrinol. Metab.* 73:882-887.
- Holbro, T., and N.E. Hynes. 2004a. ErbB receptors: directing key signaling networks throughout life. *Annu.Rev.Pharmacol.Toxicol.* 44:195-217.
- Holbro, T., and N.E. Hynes. 2004b. ErbB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol.* 44:195-217.
- Horowitz, G.M., R.T. Scott, Jr., M.R. Drews, D. Navot, and G.E. Hofmann. 1993. Immunohistochemical localization of transforming growth factor- alpha in

- human endometrium, decidua, and trophoblast. *J. Clin. Endocrinol. Metab.* 76:786-792.
- Hudson, B.P., M.A. Martinez-Yamout, H.J. Dyson, and P.E. Wright. 2004. Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d. *Nat Struct Mol Biol.* 11:257-264.
- Hung, T.H., and G.J. Burton. 2006. Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia. *Taiwan J Obstet Gynecol.* 45:189-200.
- Hung, T.H., S.F. Chen, C.C. Hsieh, J.J. Hsu, M.J. Li, Y.L. Yeh, and T.T. Hsieh. 2008. Tumor necrosis factor-alpha converting enzyme in the human placenta throughout gestation. *Reprod Sci.* 15:195-209.
- Hung, T.H., J.N. Skepper, and G.J. Burton. 2001a. In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. *Am. J. Pathol.* 159:1031-1043.
- Hung, T.H., J.N. Skepper, and G.J. Burton. 2001b. In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. *Am J Pathol.* 159:1031-1043.
- Hung, T.H., J.N. Skepper, D.S. Charnock-Jones, and G.J. Burton. 2002a. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. *Circ. Res.* 90:1274-1281.
- Hung, T.H., J.N. Skepper, D.S. Charnock-Jones, and G.J. Burton. 2002b. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. *Circ Res.* 90:1274-1281.

- Hunkapiller, N.M., and S.J. Fisher. 2008. Chapter 12. Placental remodeling of the uterine vasculature. *Methods Enzymol.* 445:281-302.
- Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11-25.
- Igakura, T., K. Kadomatsu, T. Kaname, H. Muramatsu, Q.W. Fan, T. Miyauchi, Y. Toyama, N. Kuno, S. Yuasa, M. Takahashi, T. Senda, O. Taguchi, K. Yamamura, K. Arimura, and T. Muramatsu. 1998. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. *Dev Biol.* 194:152-165.
- Imudia, A.N., B.A. Kilburn, A. Petkova, S.S. Edwin, R. Romero, and D.R. Armant. 2008. Expression of Heparin-binding EGF-like Growth Factor in Term Chorionic Villous Explants and Its Role in Trophoblast Survival. *Placenta.* 29:784-789.
- Ishihara, N., H. Matsuo, H. Murakoshi, J.B. Laoag-Fernandez, T. Samoto, and T. Maruo. 2002. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am. J. Obstet. Gynecol.* 186:158-166.
- Iwamoto, R., S. Yamazaki, M. Asakura, S. Takashima, H. Hasuwa, K. Miyado, S. Adachi, M. Kitakaze, K. Hashimoto, G. Raab, D. Nanba, S. Higashiyama, M. Hori, M. Klagsbrun, and E. Mekada. 2003. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. U. S. A.* 100:3221-3226.
- Izumi, Y., M. Hirata, H. Hasuwa, R. Iwamoto, T. Umata, K. Miyado, Y. Tamai, T. Kurisaki, A. Sehara-Fujisawa, S. Ohno, and E. Mekada. 1998. A

metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* 17:7260-7272.

Jackson, L.F., T.H. Qiu, S.W. Sunnarborg, A. Chang, C. Zhang, C. Patterson, and D.C. Lee. 2003. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* 22:2704-2716.

Jaleel, M.A., A.C. Tsai, S. Sarkar, P.V. Freedman, and L.P. Rubin. 2004. Stromal cell-derived factor-1 (SDF-1) signalling regulates human placental trophoblast cell survival. *Mol Hum Reprod.* 10:901-909.

Jauniaux, E., V. Biernaux, E. Gerlo, and B. Gulbis. 2001a. Chronic maternal smoking and cord blood amino acid and enzyme levels at term. *Obstet Gynecol.* 97:57-61.

Jauniaux, E., J. Hempstock, N. Greenwold, and G.J. Burton. 2003. Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. *Am J Pathol.* 162:115-125.

Jauniaux, E., A. Watson, and G. Burton. 2001b. Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. *Am J Obstet Gynecol.* 184:998-1003.

Jessmon, P., B.A. Kilburn, R. Romero, R.E. Leach, and D.R. Armant. 2010. Function-specific intracellular signaling pathways downstream of heparin-binding EGF-like growth factor utilized by human trophoblasts. *Biol Reprod.* 82:921-929.



- Jessmon, P., R.E. Leach, and D.R. Armant. 2009. Diverse functions of HBEGF during pregnancy. *Mol Reprod Dev.* 76:1116-1127.
- Jiang, B.H., J.Z. Zheng, S.W. Leung, R. Roe, and G.L. Semenza. 1997. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem.* 272:19253-19260.
- Jiao, J., L.D. Herl, R.V. Farese, and F.B. Gao. 2010. MicroRNA-29b regulates the expression level of human progranulin, a secreted glycoprotein implicated in frontotemporal dementia. *PLoS One.* 5:e10551.
- Johnstone, E.D., M. Mackova, S. Das, S.G. Payne, B. Lowen, C.P. Sibley, G. Chan, and L.J. Guilbert. 2005. Multiple anti-apoptotic pathways stimulated by EGF in cytotrophoblasts. *Placenta.* 26:548-555.
- Jopling, C.L., M. Yi, A.M. Lancaster, S.M. Lemon, and P. Sarnow. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science.* 309:1577-1581.
- Kao, L.C., S. Tulac, S. Lobo, B. Imani, J.P. Yang, A. Germeyer, K. Osteen, R.N. Taylor, B.A. Lessey, and L.C. Giudice. 2002. Global gene profiling in human endometrium during the window of implantation. *Endocrinology.* 143:2119-2138.
- Karpovich, N., K. Chobotova, J. Carver, J.K. Heath, D.H. Barlow, and H.J. Mardon. 2003. Expression and function of interleukin-11 and its receptor alpha in the human endometrium. *Mol Hum Reprod.* 9:75-80.
- Khong, T.Y., F. De Wolf, W.B. Robertson, and I. Brosens. 1986. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-

eclampsia and by small-for-gestational age infants. *Br. J. Obstet. Gynaecol.* 93:1049-1059.

Kibbe, W.A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res.* 35:W43-46.

Kilburn, B.A., J. Wang, Z.M. Duniec-Dmuchowski, R.E. Leach, R. Romero, and D.R. Armant. 2000a. Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. *Biol. Reprod.* 62:739-747.

Kilburn, B.A., J. Wang, Z.M. Duniec-Dmuchowski, R.E. Leach, R. Romero, and D.R. Armant. 2000b. Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. *Biol. Reprod.* 62:739-747.

Kim, V.N., and J.W. Nam. 2006. Genomics of microRNA. *Trends Genet.* 22:165-173.

Kleinman, H.K., and G.R. Martin. 2005. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol.* 15:378-386.

Klutchko, S.R., H. Zhou, R.T. Winters, T.P. Tran, A.J. Bridges, I.W. Althaus, D.M. Amato, W.L. Elliott, P.A. Ellis, M.A. Meade, B.J. Roberts, D.W. Fry, A.J. Gonzales, P.J. Harvey, J.M. Nelson, V. Sherwood, H.K. Han, G. Pace, J.B. Smaill, W.A. Denny, and H.D. Showalter. 2006. Tyrosine kinase inhibitors. 19. 6-Alkynamides of 4-anilinoquinazolines and 4-anilinopyrido[3,4-d]pyrimidines as irreversible inhibitors of the erbB family of tyrosine kinase receptors. *J. Med. Chem.* 49:1475-1485.

- Knofler, M. 2010. Critical growth factors and signalling pathways controlling human trophoblast invasion. *Int J Dev Biol.* 54:269-280.
- Kodaman, P.H., and H.S. Taylor. 2004. Hormonal regulation of implantation. *Obstet Gynecol Clin North Am.* 31:745-766, ix.
- Koritzinsky, M., R. Seigneuric, M.G. Magagnin, T. van den Beucken, P. Lambin, and B.G. Wouters. 2005. The hypoxic proteome is influenced by gene-specific changes in mRNA translation. *Radiother Oncol.* 76:177-186.
- Koumenis, C., C. Naczki, M. Koritzinsky, S. Rastani, A. Diehl, N. Sonenberg, A. Koromilas, and B.G. Wouters. 2002. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. *Mol Cell Biol.* 22:7405-7416.
- Kozomara, A., and S. Griffiths-Jones. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39:D152-157.
- Krawetz, S., and S. Misener. *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ.
- Kurisaki, T., A. Masuda, K. Sudo, J. Sakagami, S. Higashiyama, Y. Matsuda, A. Nagabukuro, A. Tsuji, Y. Nabeshima, M. Asano, Y. Iwakura, and A. Sehara-Fujisawa. 2003. Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: involvement of Meltrin alpha in adipogenesis and myogenesis. *Mol Cell Biol.* 23:55-61.
- Lala, P.K., and C. Chakraborty. 2003. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. *Placenta.* 24:575-587.

- LaMarca, H.L., P.R. Dash, K. Vishnuthevan, E. Harvey, D.E. Sullivan, C.A. Morris, and G.S. Whitley. 2008. Epidermal growth factor-stimulated extravillous cytotrophoblast motility is mediated by the activation of PI3-K, Akt and both p38 and p42/44 mitogen-activated protein kinases. *Hum Reprod.* 23:1733-1741.
- Leach, R.E., R. Khalifa, D.R. Armant, A. Brudney, S.K. Das, S.K. Dey, and A.T. Fazleabas. 2001. Heparin-binding EGF-like growth factor modulation by antiprogestin and CG in the baboon (*Papio anubis*). *J Clin Endocrinol Metab.* 86:4520-4528.
- Leach, R.E., R. Khalifa, N.D. Ramirez, S.K. Das, J. Wang, S.K. Dey, R. Romero, and D.R. Armant. 1999a. Multiple roles for heparin-binding epidermal growth factor-like growth factor are suggested by its cell specific expression during the human endometrial cycle and early placentation. *J. Clin. Endocrinol. Metab.* 84:3355-3363.
- Leach, R.E., R. Khalifa, N.D. Ramirez, S.K. Das, J. Wang, S.K. Dey, R. Romero, and D.R. Armant. 1999b. Multiple roles for heparin-binding epidermal growth factor-like growth factor are suggested by its cell-specific expression during the human endometrial cycle and early placentation. *J Clin Endocrinol Metab.* 84:3355-3363.
- Leach, R.E., B. Kilburn, J. Wang, Z. Liu, R. Romero, and D.R. Armant. 2004a. Heparin-binding EGF-like growth factor regulates human extravillous cytotrophoblast development during conversion to the invasive phenotype. *Dev Biol.* 266:223-237.

- Leach, R.E., B.A. Kilburn, A. Petkova, R. Romero, and D.R. Armant. 2008a. Diminished survival of human cytotrophoblast cells exposed to hypoxia/reoxygenation injury and associated reduction of heparin-binding epidermal growth factor-like growth factor. *Am. J. Obstet. Gynecol.* 198:471.e471-471.e478.
- Leach, R.E., B.A. Kilburn, A. Petkova, R. Romero, and D.R. Armant. 2008b. Diminished survival of human cytotrophoblast cells exposed to hypoxia/reoxygenation injury and associated reduction of heparin-binding epidermal growth factor-like growth factor. *Am J Obstet Gynecol.* 198:471 e471-477; discussion 471 e477-478.
- Leach, R.E., B.A. Kilburn, J. Wang, Z. Liu, R. Romero, and D.R. Armant. 2004b. Heparin-binding EGF-like growth factor regulates human extravillous cytotrophoblast development during conversion to the invasive phenotype. *Dev. Biol.* 266:223-237.
- Leach, R.E., R. Romero, Y.M. Kim, T. Chaiworapongsa, B. Kilburn, S.K. Das, S.K. Dey, A. Johnson, F. Qureshi, S. Jacques, and D.R. Armant. 2002a. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. *Lancet.* 360:1215-1219.
- Leach, R.E., R. Romero, Y.M. Kim, T. Chaiworapongsa, B.A. Kilburn, S.K. Das, S.K. Dey, A. Johnson, F. Qureshi, S. Jacques, and D.R. Armant. 2002b. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. *Lancet.* 360:1215-1219.
- Lee, J.C., J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, and S.W. Landvatter. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (London).* 372:739-746.

- Lemjabbar, H., and C. Basbaum. 2002. Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. *Nat Med.* 8:41-46.
- Lessey, B.A., Y. Gui, K.B. Apparao, S.L. Young, and J. Mulholland. 2002a. Regulated expression of heparin-binding EGF-like growth factor (HB-EGF) in the human endometrium: a potential paracrine role during implantation. *Mol Reprod Dev.* 62:446-455.
- Lessey, B.A., Y. Gui, K.B. Apparao, S.L. Young, and J. Mulholland. 2002b. Regulated expression of heparin-binding EGF-like growth factor (HB-EGF) in the human endometrium: a potential paracrine role during implantation. *Mol. Reprod. Dev.* 62:446-455.
- Lewis, B.P., C.B. Burge, and D.P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 120:15-20.
- Li, B., J. Si, and J.W. DeWille. 2008. Ultraviolet radiation (UVR) activates p38 MAP kinase and induces post-transcriptional stabilization of the C/EBPdelta mRNA in G0 growth arrested mammary epithelial cells. *J Cell Biochem.* 103:1657-1669.
- Li, H., S. Chen, and F. Xing. 2002. [Expression of HOXA10 gene in human endometrium and its relationship with unexplained infertility]. *Zhonghua fu chan ke za zhi.* 37:30-32.

- Librach, C.L., Z. Werb, M.L. Fitzgerald, K. Chiu, N.M. Corwin, R.A. Esteves, D. Grobelny, R. Galaray, C.H. Damsky, and S.J. Fisher. 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J. Cell Biol.* 113:437-449.
- Lim, H.J., and S.K. Dey. 2009. HB-EGF: a unique mediator of embryo-uterine interactions during implantation. *Exp Cell Res.* 315:619-626.
- Lim, J.J., D.R. Lee, H.S. Song, K.S. Kim, T.K. Yoon, M.C. Gye, and M.K. Kim. 2006. Heparin-binding epidermal growth factor (HB-EGF) may improve embryonic development and implantation by increasing vitronectin receptor (integrin  $\alpha$ 5 $\beta$ 3) expression in peri-implantation mouse embryos. *J Assist Reprod Genet.* 23:111-119.
- Lindell, T.J., F. Weinberg, P.W. Morris, R.G. Roeder, and W.J. Rutter. 1970. Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. *Science.* 170:447-449.
- Liu, J., C. Chakraborty, C.H. Graham, Y.P. Barbin, S.J. Dixon, and P.K. Lala. 2003. Noncatalytic domain of uPA stimulates human extravillous trophoblast migration by using phospholipase C, phosphatidylinositol 3-kinase and mitogen-activated protein kinase. *Exp. Cell Res.* 286:138-151.
- Liu, X., G. Zhu, and G. Zhong. 2007. Regulatory effect of estrogen, progesterin and HB-EGF on the expression of HOXA10 gene in Ishikawa cells. *J Huazhong Univ Sci Technolog Med Sci.* 27:464-467.
- Liu, Z., B.A. Kilburn, R.E. Leach, R. Romero, B.C. Paria, and D.R. Armant. 2004. Histamine enhances cytotrophoblast invasion by inducing intracellular

- calcium transients through the histamine type-1 receptor. *Mol Reprod Dev.* 68:345-353.
- Lu, P.D., H.P. Harding, and D. Ron. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol.* 167:27-33.
- Maccani, M.A., and C.J. Marsit. 2009. Epigenetics in the placenta. *Am J Reprod Immunol.* 62:78-89.
- Mackova, M., R.T. Kilani, S.T. Davidge, and L.J. Guilbert. 2003. The effect of oxygen tension on intracellular survival signalling in primary villous trophoblasts. *Placenta.* 24:627-637.
- Makino, Y., R. Cao, K. Svensson, G. Bertilsson, M. Asman, H. Tanaka, Y. Cao, A. Berkenstam, and L. Poellinger. 2001. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature.* 414:550-554.
- Many, A., and J.M. Roberts. 1997. Increased xanthine oxidase during labour-- implications for oxidative stress. *Placenta.* 18:725-726.
- Martin, K.L., D.H. Barlow, and I.L. Sargent. 1998a. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum. Reprod.* 13:1645-1652.
- Martin, K.L., D.H. Barlow, and I.L. Sargent. 1998b. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod.* 13:1645-1652.



- Marzusch, K., P. Ruck, H.P. Horny, J. Dietl, and E. Kaiserling. 1995. Expression of the p53 tumour suppressor gene in human placenta: an immunohistochemical study. *Placenta*. 16:101-104.
- McKinnon, T., C. Chakraborty, L.M. Gleeson, P. Chidiac, and P.K. Lala. 2001. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. *J. Clin. Endocrinol. Metab.* 86:3665-3674.
- Merlos-Suarez, A., S. Ruiz-Paz, J. Baselga, and J. Arribas. 2001. Metalloprotease-dependent protransforming growth factor-alpha ectodomain shedding in the absence of tumor necrosis factor-alpha-converting enzyme. *J Biol Chem.* 276:48510-48517.
- Mignone, F., C. Gissi, S. Liuni, and G. Pesole. 2002. Untranslated regions of mRNAs. *Genome Biol.* 3:REVIEWS0004.
- Mishra, A., and P.B. Seshagiri. 2000. Heparin binding-epidermal growth factor improves blastocyst hatching and trophoblast outgrowth in the golden hamster. *Reprod Biomed Online.* 1:87-95.
- Mitamura, T., S. Higashiyama, N. Taniguchi, M. Klagsbrun, and E. Mekada. 1995. Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem.* 270:1015-1019.
- Mohammad, M.A., N.R. Ismael, S.M. Shaarawy, and M.M. El-Merzabani. 2010. Prognostic value of membrane type 1 and 2 matrix metalloproteinase

- expression and gelatinase A activity in bladder cancer. *Int J Biol Markers*. 25:69-74.
- Moll, S.J., C.J. Jones, I.P. Crocker, P.N. Baker, and A.E. Heazell. 2007. Epidermal growth factor rescues trophoblast apoptosis induced by reactive oxygen species. *Apoptosis*. 12:1611-1622.
- Mor, G. 2008. Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction. *Ann. N. Y. Acad. Sci.* 1127:121-128.
- Morrish, D.W., J. Dakour, and H. Li. 1998. Functional regulation of human trophoblast differentiation. *J Reprod Immunol*. 39:179-195.
- Mouillet, J.F., T. Chu, D.M. Nelson, T. Mishima, and Y. Sadovsky. 2010. MiR-205 silences MED1 in hypoxic primary human trophoblasts. *FASEB J*. 24:2030-2039.
- Mouillet, J.F., T. Chu, and Y. Sadovsky. 2011. Expression patterns of placental microRNAs. *Birth Defects Res A Clin Mol Teratol*.
- Nardo, L.G., G. Bartoloni, S. Di Mercurio, and F. Nardo. 2002. Expression of alpha(v)beta3 and alpha4beta1 integrins throughout the putative window of implantation in a cohort of healthy fertile women. *Acta obstetricia et gynecologica Scandinavica*. 81:753-758.
- Niu, R., T. Okamoto, K. Iwase, S. Nomura, and S. Mizutani. 2000. Quantitative analysis of matrix metalloproteinases-2 and -9, and their tissue inhibitors-1 and -2 in human placenta throughout gestation. *Life Sci*. 66:1127-1137.
- Norwitz, E.R., D.J. Schust, and S.J. Fisher. 2001. Implantation and the survival of early pregnancy. *N. Engl. J. Med*. 345:1400-1408.

- Oh, S.Y., S.A. Schmidt, and A.W. Murray. 1993. Epidermal growth factor inhibits gap junctional communication and stimulates serine-phosphorylation of connexin43 in WB cells by a protein kinase C-independent mechanism. *Cell Adhes Commun.* 1:143-149.
- Onogi, A., K. Naruse, T. Sado, T. Tsunemi, H. Shigetomi, T. Noguchi, Y. Yamada, M. Akasaki, H. Oi, and H. Kobayashi. 2011. Hypoxia inhibits invasion of extravillous trophoblast cells through reduction of matrix metalloproteinase (MMP)-2 activation in the early first trimester of human pregnancy. *Placenta.*
- Ornskov, D., E. Nexø, and B.S. Sørensen. 2006. Insulin-induced proliferation of bladder cancer cells is mediated through activation of the epidermal growth factor system. *FEBS J.* 273:5479-5489.
- Orom, U.A., F.C. Nielsen, and A.H. Lund. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell.* 30:460-471.
- Pang, A.L., S. Peacock, W. Johnson, D.H. Bear, O.M. Rennert, and W.Y. Chan. 2009. Cloning, characterization, and expression analysis of the novel acetyltransferase retrogene *Ard1b* in the mouse. *Biol Reprod.* 81:302-309.
- Pang, R.T., C.O. Leung, T.M. Ye, W. Liu, P.C. Chiu, K.K. Lam, K.F. Lee, and W.S. Yeung. 2010. MicroRNA-34a suppresses invasion through downregulation of Notch1 and Jagged1 in cervical carcinoma and choriocarcinoma cells. *Carcinogenesis.* 31:1037-1044.

- Paria, B.C., S.K. Das, G.K. Andrews, and S.K. Dey. 1993. Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. *Proc Natl Acad Sci U S A.* 90:55-59.
- Paria, B.C., K. Elenius, M. Klagsbrun, and S.K. Dey. 1999. Heparin-binding EGF-like growth factor interacts with mouse blastocysts independently of ErbB1: a possible role for heparan sulfate proteoglycans and ErbB4 in blastocyst implantation. *Development.* 126:1997-2005.
- Paria, B.C., W. Ma, J. Tan, S. Raja, S.K. Das, S.K. Dey, and B.L. Hogan. 2001. Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. *Proc Natl Acad Sci U S A.* 98:1047-1052.
- Payne, S.G., D.N. Brindley, and L.J. Guilbert. 1999. Epidermal growth factor inhibits ceramide-induced apoptosis and lowers ceramide levels in primary placental trophoblasts. *J. Cell. Physiol.* 180:263-270.
- Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
- Phelps, E.D., D.L. Updike, E.C. Bullen, P. Grammas, and E.W. Howard. 2006. Transcriptional and posttranscriptional regulation of angiopoietin-2 expression mediated by IGF and PDGF in vascular smooth muscle cells. *Am J Physiol Cell Physiol.* 290:C352-361.
- Pijnenborg, R., J.M. Bland, W.B. Robertson, and I. Brosens. 1983a. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta.* 4:397-413.

- Pijnenborg, R., J.M. Bland, W.B. Robertson, and I. Brosens. 1983b. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta*. 4:397-413.
- Pijnenborg, R., W.B. Robertson, I. Brosens, and G. Dixon. 1981. Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta*. 2:71-91.
- Pillai, R.S., S.N. Bhattacharyya, and W. Filipowicz. 2007. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol.* 17:118-126.
- Qian, K., H. Chen, Y. Wei, J. Hu, and G. Zhu. 2005. Differentiation of endometrial stromal cells in vitro: down-regulation of suppression of the cell cycle inhibitor p57 by HOXA10? *Molecular human reproduction*. 11:245-251.
- Qiu, Q., M. Yang, B.K. Tsang, and A. Gruslin. 2004a. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. *Mol. Hum. Reprod.* 10:677-684.
- Qiu, Q., M. Yang, B.K. Tsang, and A. Gruslin. 2004b. EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction*. 128:355-363.
- Raab, G., and M. Klagsbrun. 1997. Heparin-binding EGF-like growth factor. *Biochim Biophys Acta*. 1333:F179-199.
- Raab, G., K. Kover, B.C. Paria, S.K. Dey, R.M. Ezzell, and M. Klagsbrun. 1996. Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. *Development*. 122:637-645.

- Rajakumar, A., and K.P. Conrad. 2000. Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta. *Biol Reprod.* 63:559-569.
- Redman, C.W., and I.L. Sargent. 2000. Placental debris, oxidative stress and pre-eclampsia. *Placenta.* 21:597-602.
- Redman, C.W., and I.L. Sargent. 2005. Latest advances in understanding preeclampsia. *Science (Wash.DC).* 308:1592-1594.
- Riese, D.J., and D.F. Stern. 1998. Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays.* 20:41-48.
- Rodesch, F., P. Simon, C. Donner, and E. Jauniaux. 1992. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol.* 80:283-285.
- Roelle, S., R. Grosse, A. Aigner, H.W. Krell, F. Czubyko, and T. Gudermann. 2003. Matrix metalloproteinases 2 and 9 mediate epidermal growth factor receptor transactivation by gonadotropin-releasing hormone. *J Biol Chem.* 278:47307-47318.
- Roncalli, M., G. Bulfamante, G. Viale, D.R. Springall, R. Alfano, A. Comi, M. Maggioni, J.M. Polak, and G. Coggi. 1994. C-myc and tumour suppressor gene product expression in developing and term human trophoblast. *Placenta.* 15:399-409.
- Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol.* 132:365-386.

- Ruck, P., K. Marzusch, H.P. Horny, J. Dietl, and E. Kaiserling. 1996. The distribution of tissue inhibitor of metalloproteinases-2 (TIMP-2) in the human placenta. *Placenta*. 17:263-266.
- Sahin, U., G. Weskamp, K. Kelly, H.M. Zhou, S. Higashiyama, J. Peschon, D. Hartmann, P. Saftig, and C.P. Blobel. 2004. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol*. 164:769-779.
- Sargent, I.L., K.L. Martin, and D.H. Barlow. 1998. The use of recombinant growth factors to promote human embryo development in serum-free medium. *Hum Reprod*. 13 Suppl 4:239-248.
- Satokata, I., G. Benson, and R. Maas. 1995. Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature*. 374:460-463.
- Schug, J., and G.C. Overton. 1997. TESS: Transcription Element Search Software on the WWW. Computational Biology and Informatics Laboratory School of Medicine, University of Pennsylvania.
- Semenza, G.L. 2003. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*. 3:721-732.
- Sengupta, S., R. Schiff, and B.S. Katzenellenbogen. 2009. Post-transcriptional regulation of chemokine receptor CXCR4 by estrogen in HER2 overexpressing, estrogen receptor-positive breast cancer cells. *Breast Cancer Res Treat*. 117:243-251.
- Seshagiri, P.B., A. Mishra, G. Ramesh, and R.P. Rao. 2002. Regulation of peri-attachment embryo development in the golden hamster: role of growth factors. *J Reprod Immunol*. 53:203-213.

Singh, A.T., J.A. Keelan, and F. Sieg. 2010. Regulation of trophoblast migration and survival by a novel neural regeneration peptide. *Reprod Biomed Online*.

21:237-244.

Smith, S., R. Francis, L. Guilbert, and P.N. Baker. 2002. Growth factor rescue of cytokine mediated trophoblast apoptosis. *Placenta*. 23:322-330.

Smith, S.E., M.M. French, J. Julian, B.C. Paria, S.K. Dey, and D.D. Carson. 1997.

Expression of heparan sulfate proteoglycan (perlecan) in the mouse blastocyst is regulated during normal and delayed implantation. *Dev Biol*. 184:38-47.

Song, H., H. Lim, S.K. Das, B.C. Paria, and S.K. Dey. 2000. Dysregulation of EGF family of growth factors and COX-2 in the uterus during the preattachment and attachment reactions of the blastocyst with the luminal epithelium correlates with implantation failure in LIF-deficient mice. *Mol Endocrinol*. 14:1147-1161.

Sorensen, B.S., D. Ornskov, and E. Nexø. 2006. The chemotherapeutic agent VP16 increases the stability of HB-EGF mRNA by a mechanism involving the 3'-UTR. *Exp Cell Res*. 312:3651-3658.

Spruce, T., B. Pernaute, A. Di-Gregorio, B.S. Cobb, M. Merckenschlager, M. Manzanares, and T.A. Rodriguez. 2010. An early developmental role for miRNAs in the maintenance of extraembryonic stem cells in the mouse embryo. *Dev Cell*. 19:207-219.

Staun-Ram, E., S. Goldman, D. Gabarin, and E. Shalev. 2004. Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reprod Biol Endocrinol*. 2:59.



- Staun-Ram, E., and E. Shalev. 2005. Human trophoblast function during the implantation process. *Reprod Biol Endocrinol.* 3:56.
- Stavreus-Evers, A., L. Aghajanova, H. Brismar, H. Eriksson, B.M. Landgren, and O. Hovatta. 2002. Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. *Mol Hum Reprod.* 8:765-769.
- Stewart, C.L., P. Kaspar, L.J. Brunet, H. Bhatt, I. Gadi, F. Kontgen, and S.J. Abbondanzo. 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature.* 359:76-79.
- Stirpe, F., and L. Fiume. 1967. Effect of alpha-amanitin on ribonucleic acid synthesis and on ribonucleic acid polymerase in mouse liver. *Biochem J.* 103:67P-68P.
- Strongin, A.Y., I. Collier, G. Bannikov, B.L. Marmer, G.A. Grant, and G.I. Goldberg. 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem.* 270:5331-5338.
- Sugihara, K., D. Sugiyama, J. Byrne, D.P. Wolf, K.P. Lowitz, Y. Kobayashi, M. Kabir-Salmani, D. Nadano, D. Aoki, S. Nozawa, J. Nakayama, T. Mustelin, E. Ruoslahti, N. Yamaguchi, and M.N. Fukuda. 2007. Trophoblast cell activation by trophinin ligation is implicated in human embryo implantation. *Proc Natl Acad Sci U S A.* 104:3799-3804.
- Sugiura, R., A. Kita, Y. Shimizu, H. Shuntoh, S.O. Sio, and T. Kuno. 2003. Feedback regulation of MAPK signalling by an RNA-binding protein. *Nature.* 424:961-965.

- Sunnarborg, S.W., C.L. Hinkle, M. Stevenson, W.E. Russell, C.S. Raska, J.J. Peschon, B.J. Castner, M.J. Gerhart, R.J. Paxton, R.A. Black, and D.C. Lee. 2002. Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J Biol Chem.* 277:12838-12845.
- Suzuki, N., J. Nakayama, I.M. Shih, D. Aoki, S. Nozawa, and M.N. Fukuda. 1999. Expression of trophinin, tastin, and bystin by trophoblast and endometrial cells in human placenta. *Biol Reprod.* 60:621-627.
- Tamada, H., C. Higashiyama, H. Takano, N. Kawate, T. Inaba, and T. Sawada. 1999. The effects of heparin-binding epidermal growth factor-like growth factor on preimplantation-embryo development and implantation in the rat. *Life Sci.* 64:1967-1973.
- Tan, Y., M. Li, S. Cox, M.K. Davis, O. Tawfik, B.C. Paria, and S.K. Das. 2004. HB-EGF directs stromal cell polyploidy and decidualization via cyclin D3 during implantation. *Dev Biol.* 265:181-195.
- Tanimura, K., S. Nakago, H. Murakoshi, S. Takekida, T. Moriyama, H. Matsuo, K. Hashimoto, and T. Maruo. 2004. Changes in the expression and cytological localization of betacellulin and its receptors (ErbB-1 and ErbB-4) in the trophoblasts in human placenta over the course of pregnancy. *Eur J Endocrinol.* 151:93-101.
- Taylor, H.S., A. Arici, D. Olive, and P. Igarashi. 1998. HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. *J Clin Invest.* 101:1379-1384.

- Terranova, V.P., E.S. Hujanen, D.M. Loeb, G.R. Martin, L. Thornburg, and V. Glushko. 1986. Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. *Proc Natl Acad Sci U S A.* 83:465-469.
- Torres, C.G., A.M. Pino, and W.D. Sierralta. 2009. A cyclized peptide derived from alpha fetoprotein inhibits the proliferation of ER-positive canine mammary cancer cells. *Oncol Rep.* 21:1397-1404.
- Valencia-Sanchez, M.A., J. Liu, G.J. Hannon, and R. Parker. 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev.* 20:515-524.
- Vasudevan, S., and J.A. Steitz. 2007. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell.* 128:1105-1118.
- Vasudevan, S., Y. Tong, and J.A. Steitz. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science.* 318:1931-1934.
- Vattem, K.M., and R.C. Wek. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A.* 101:11269-11274.
- Vilela, C., and J.E. McCarthy. 2003. Regulation of fungal gene expression via short open reading frames in the mRNA 5'untranslated region. *Mol Microbiol.* 49:859-867.
- Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269:5241-5248.

- von Wolff, M., T. Strowitzki, V. Becker, C. Zepf, S. Tabibzadeh, and C.J. Thaler. 2001. Endometrial osteopontin, a ligand of beta3-integrin, is maximally expressed around the time of the "implantation window". *Fertility and sterility*. 76:775-781.
- Vukicevic, S., H.K. Kleinman, F.P. Luyten, A.B. Roberts, N.S. Roche, and A.H. Reddi. 1992. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp Cell Res*. 202:1-8.
- Wang, D., S. Zuehlsdorff, and A.C. Larson. 2009a. Rapid 3D radiofrequency field mapping using catalyzed double-angle method. *NMR Biomed*. 22:882-890.
- Wang, G.L., and G.L. Semenza. 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*. 90:4304-4308.
- Wang, J., L. Mayernik, J.F. Schultz, and D.R. Armant. 2000. Acceleration of trophoblast differentiation by heparin-binding EGF-like growth factor is dependent on the stage-specific activation of calcium influx by ErbB receptors in developing mouse blastocysts. *Development*. 127:33-44.
- Wang, X., H. Wang, H. Matsumoto, S.K. Roy, S.K. Das, and B.C. Paria. 2002. Dual source and target of heparin-binding EGF-like growth factor during the onset of implantation in the hamster. *Development*. 129:4125-4134.
- Wang, X.N., S.K. Das, D. Damm, M. Klagsbrun, J.A. Abraham, and S.K. Dey. 1994. Differential regulation of heparin-binding epidermal growth factor-like

- growth factor in the adult ovariectomized mouse uterus by progesterone and estrogen. *Endocrinology*. 135:1264-1271.
- Wang, Y.Y., M.K. Dahle, K.R. Steffensen, F.P. Reinholt, J.L. Collins, C. Thiemermann, A.O. Aasen, J.A. Gustafsson, and J.E. Wang. 2009b. Liver X receptor agonist GW3965 dose-dependently regulates lps-mediated liver injury and modulates posttranscriptional TNF-alpha production and p38 mitogen-activated protein kinase activation in liver macrophages. *Shock*. 32:548-553.
- Wolff, G.S., P.J. Chiang, S.M. Smith, R. Romero, and D.R. Armant. 2007. Epidermal growth factor-like growth factors prevent apoptosis of alcohol-exposed human placental cytotrophoblast cells. *Biol. Reprod*. 77:53-60.
- Wright, J.K., C.E. Dunk, J.E. Perkins, E. Winterhager, J.C. Kingdom, and S.J. Lye. 2006. EGF modulates trophoblast migration through regulation of Connexin 40. *Placenta*. 27 Suppl A:S114-121.
- Xiao, L.J., H. Chang, N.Z. Ding, H. Ni, K. Kadomatsu, and Z.M. Yang. 2002a. Basigin expression and hormonal regulation in mouse uterus during the peri-implantation period. *Mol Reprod Dev*. 63:47-54.
- Xiao, L.J., H.L. Diao, X.H. Ma, N.Z. Ding, K. Kadomatsu, T. Muramatsu, and Z.M. Yang. 2002b. Basigin expression and hormonal regulation in the rat uterus during the peri-implantation period. *Reproduction*. 124:219-225.
- Xie, H., H. Wang, S. Tranguch, R. Iwamoto, E. Mekada, F.J. Demayo, J.P. Lydon, S.K. Das, and S.K. Dey. 2007. Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. *Proc Natl Acad Sci U S A*. 104:18315-18320.

- Xu, P., Y.L. Wang, S.J. Zhu, S.Y. Luo, Y.S. Piao, and L.Z. Zhuang. 2000. Expression of matrix metalloproteinase-2, -9, and -14, tissue inhibitors of metalloproteinase-1, and matrix proteins in human placenta during the first trimester. *Biol Reprod.* 62:988-994.
- Yan, Y., K. Shirakabe, and Z. Werb. 2002. The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. *J Cell Biol.* 158:221-226.
- Yoo, H.J., D.H. Barlow, and H.J. Mardon. 1997a. Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. *Dev Genet.* 21:102-108.
- Yoo, H.J., D.H. Barlow, and H.J. Mardon. 1997b. Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. *Dev Genet.* 21:102-108.
- Yoshinaga, K., and C.E. Adams. 1966. Delayed implantation in the spayed, progesterone treated adult mouse. *J Reprod Fertil.* 12:593-595.
- Yotsumoto, F., H. Yagi, S.O. Suzuki, E. Oki, H. Tsujioka, T. Hachisuga, K. Sonoda, T. Kawarabayashi, E. Mekada, and S. Miyamoto. 2008. Validation of HB-EGF and amphiregulin as targets for human cancer therapy. *Biochem Biophys Res Commun.* 365:555-561.

- Yue, Z.P., Z.M. Yang, S.J. Li, H.B. Wang, and M.J. Harper. 2000. Epidermal growth factor family in rhesus monkey uterus during the menstrual cycle and early pregnancy. *Mol Reprod Dev.* 55:164-174.
- Yukawa, K., T. Tanaka, S. Tsuji, and S. Akira. 1999. Regulation of transcription factor C/ATF by the cAMP signal activation in hippocampal neurons, and molecular interaction of C/ATF with signal integrator CBP/p300. *Brain Res Mol Brain Res.* 69:124-134.
- Yun, J.H., J.E. Koo, and Y.S. Koh. 2009. Mitogen-activated protein kinases are involved in tumor necrosis factor alpha production in macrophages infected with *Orientia tsutsugamushi*. *Microbiol. Immunol.* 53:349-355.
- Zhang, Z., C. Funk, S.R. Glasser, and J. Mulholland. 1994a. Progesterone regulation of heparin-binding epidermal growth factor-like growth factor gene expression during sensitization and decidualization in the rat uterus: effects of the antiprogestin, ZK 98.299. *Endocrinology.* 135:1256-1263.
- Zhang, Z., C. Funk, D. Roy, S. Glasser, and J. Mulholland. 1994b. Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells. *Endocrinology.* 134:1089-1094.
- Zhao, W., M. Liu, and K.L. Kirkwood. 2008. p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. *J. Biol. Chem.* 283:1778-1785.
- Zhong, W., Y. Xie, Y. Wang, J. Lewis, A. Trostinskaia, F. Wang, E.E. Puscheck, and D.A. Rappolee. 2007. Use of hyperosmolar stress to measure stress-activated protein kinase activation and function in human HTR cells and mouse

trophoblast stem cells. *Reproductive sciences (Thousand Oaks, Calif.* 14:534-547.

Zhou, Y., C.H. Damsky, K. Chiu, J.M. Roberts, and S.J. Fisher. 1993. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. *J Clin Invest.* 91:950-960.

Zhou, Y., C.H. Damsky, and S.J. Fisher. 1997a. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest.* 99:2152-2164.

Zhou, Y., S.J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M. Wheelock, and C.H. Damsky. 1997b. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest.* 99:2139-2151.

Zhou, Y., M. McMaster, K. Woo, M. Janatpour, J. Perry, T. Karpanen, K. Alitalo, C. Damsky, and S.J. Fisher. 2002. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol.* 160:1405-1423.

Zhu, S., Y. Zhou, L. Wang, J. Zhang, H. Wu, J. Xiong, Y. Tian, and C. Wang. 2011. Transcriptional upregulation of MT2-MMP in response to hypoxia is promoted by HIF-1alpha in cancer cells. *Mol Carcinog.* 50:770-780.

Zhu, X.M., T. Han, X.H. Wang, Y.H. Li, H.G. Yang, Y.N. Luo, G.W. Yin, and Y.Q. Yao. 2010. Overexpression of miR-152 leads to reduced expression of human leukocyte



**antigen-G and increased natural killer cell mediated cytolysis in JEG-3 cells.**

***Am J Obstet Gynecol.* 202:592 e591-597.**

**ABSTRACT****HUMAN TROPHOBLAST SURVIVAL AND INVASION IN THE DEVELOPING PLACENTA: AUTOCRINE REGULATION BY HBEGF**

by

**PHILIP JESSMON****December 2011****Advisor:** Dr. David Randall Armant**Major:** Anatomy & Cellular Biology**Degree:** Doctor of Philosophy

HBEGF is a multifunctional protein in early pregnancy that induces cytotrophoblast (CTB) cell differentiation to an invasive phenotype, protects against apoptosis, and is involved in an autocrine signaling mechanism that leads to its own protein synthesis. CTBs exist in a low O<sub>2</sub> environment during the first 10 weeks of implantation, during which they invade the decidualized uterine stroma. Inhibitors of intracellular signaling pathways demonstrated that at 20% O<sub>2</sub> HBEGF induces an increase in cell migration through the ERK, MAPK14, JNK, or PIK3 pathways downstream of signaling through its ERBB receptors. Also downstream of these four pathways, HBEGF induces a post-transcriptional increase in ITGA1 and decrease in ITGA6 expression (i.e., integrin-switching). This phenotype is characteristic of invasive CTBs and is also induced by culture on Matrigel, but not on growth factor-reduced Matrigel. TUNEL assays indicate that HBEGF signals through the MAPK14 pathway to inhibit apoptosis in CTBs induced by 2% O<sub>2</sub> or reoxygenation injury. Previous studies

have identified that HBEGF is specifically translated in CTBs at low O<sub>2</sub> (2%), as compared to 20% O<sub>2</sub>. Utilizing a chemical mimic of low oxygen (CoCl<sub>2</sub>) that stabilizes HIF $\alpha$  at 20% O<sub>2</sub> and an inhibitor of nascent transcription, HBEGF protein was found to be upregulated downstream of HIF-mediated transcription of metalloproteinases. In addition, a cascade involving MMP2 exists that leads to HBEGF shedding and its autocrine signaling at 2% O<sub>2</sub>. It also signals through ERBB receptors to induce an autocrine post-transcriptional increase in its own protein levels through the ERK, MAPK14 or JNK pathways. HBEGF mRNA is stable at both 20% and 2% O<sub>2</sub> and its protein appears to be specifically regulated by O<sub>2</sub>. Dual luciferase vectors containing various fragments of the HBEGF 3'UTR identified regions that may post-transcriptionally regulate HBEGF mRNA. Regions flanking the 3'UTR may be bound by miRNA at 2% O<sub>2</sub>, leading to increased protein synthesis. At 20% O<sub>2</sub>, sequences in the central portion of the 3'UTR mediate translational suppression and may override the influence of the flanking regions, leading to translational suppression of HBEGF at high oxygen concentrations. These studies identify several mechanisms of HBEGF action in CTBs during early pregnancy. In addition, they begin to address the rapid post-transcriptional regulation of HBEGF by O<sub>2</sub>, a phenomena that is important in the survival and successful implantation of CTBs during implantation.

## AUTOBIOGRAPHICAL STATEMENT

My future as a PhD researcher began as a shy high school student who developed a great interest in human physiology and desired a career as a physical therapist. Undergraduate studies at the University of Michigan began to nurture that interest into a more general fascination with medical sciences. Not being accepted to medical school on my first round of applications queued me to temporarily pursue other educational avenues, namely, the Basic Medical Sciences masters program at Wayne State University in Detroit, MI. Through this masters program, my love for science and medicine was solidified. However, it also taught me something crucial about myself: it would be impossible for me to practice the quality of medical care I would find fulfilling given the current fiscal and political environments in medicine. I also realized that what I enjoyed most in my undergraduate studies was teaching my fellows students our classroom material as we studied for exams together. It thus seemed obvious that I should pursue a degree that prepared me to learn and teach cutting edge scientific theories. Having already begun to work in Dr. Armant's lab as a volunteer looking for insights into his future career path, I chose to stay and work on cellular and molecular signaling in human trophoblast cells. Over the years, working toward this degree has deeply challenged my resolve as a scientist and has forced me to grow as an individual. Dr. Armant's persistent dedication to quality science and sound thinking has undoubtedly prepared me for a fulfilling career as a research scientist—a path I would never have imagined possible without his example to follow. I look forward to a future in scientific inquiry and the constant intellectual growth that accompanies it.