NIH Public Access
Author Manuscript
*Placenta*. Author manuscript; available in PMC 2009 September 1.

Published in final edited form as:
*Placenta*. 2008 September ; 29(9): 784–789. doi:10.1016/j.placenta.2008.06.013.

# Expression of heparin-binding EGF-like growth factor in term chorionic villous explants and its role in trophoblast survival

**Anthony N. Imudia, MD**[1], **Brian A. Kilburn, BS**[1], **Anelia Petkova, MD**[1], **Samuel S. Edwin, PhD**[2], **Roberto Romero, MD**[2,3], and **D. Randall Armant, PhD**[1,4]

1*Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, USA*

2*Perinatology Research Branch, National Institute of Child Health and Human Development (NICHHD), National Institutes of Health (NIH), Bethesda, MD, USA*

3*Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI, USA*

4*Reproductive Biology and Medicine Branch, NICHHD, NIH, Bethesda, MD, USA*

## Abstract

Heparin-binding EGF-like growth factor (HBEGF) induces trophoblast extravillous differentiation and prevents apoptosis. These functions are compromised in preeclampsia. Because HBEGF is downregulated in placentas delivered by women with preeclampsia, we have examined its expression and cytoprotective activity in term villous explants. Chorionic villous explants prepared from non-pathological placentas collected by cesarean section at term were cultured at either 20% or 2% $O_2$ and treated with the HBEGF antagonist CRM197 or recombinant HBEGF. Paraffin sections were assayed for trophoblast death, proliferation and HBEGF expression using the TUNEL method, immunohistochemistry for nuclear Ki67 expression and semi-quantitative immunohistochemistry with image analysis, respectively. Trophoblast cell death was increased significantly after 8 h of culture with CRM197 or by culture for 2 h at 2% $O_2$. Exogenous HBEGF prevented cell death due to hypoxia. Proliferative capacity was not affected by culture at either 20% or 2% $O_2$. Contrary to first trimester placenta, term trophoblasts do not elevate HBEGF expression in response to hypoxia. However, low endogenous levels of HBEGF are required to maintain survival. Therefore, HBEGF-mediated signaling significantly reduces trophoblast cell death at term and its deficiency in preeclampsia could negatively impact trophoblast survival.

### Keywords

trophoblast; term villous explants; cell death; growth factors; hypoxia; preeclampsia

## 1. Introduction

Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality worldwide. Although the causes of this syndrome are poorly understood, strong evidence lends support to roles for abnormal trophoblast invasion, endothelial cell dysfunction and a systemic maternal

Address for Correspondence: D. Randall Armant, Ph.D, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, CS Mott Center, 275 East Hancock Avenue, Detroit, Michigan 48201-1405, USA, Tel: (313) 577-1748; (Fax): (313) 577-8554, Email: d.armant@wayne.edu.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

inflammatory response [1,2]. Increased rates of apoptosis have been reported in placentas from preeclamptic pregnancies [3,4]. A failure of physiological transformation of the spiral arteries by cytotrophoblast cells, which is associated with aberrant expression of adhesion molecules [5], has also been noted in preeclampsia. Additionally, restriction of trophoblast invasion [6] and survival [7] occurs in conjunction with the birth of small for gestational age (SGA) infants, suggesting shared elements in the etiology of both disorders.

An important ligand in the epidermal growth factor (EGF) signaling system, heparin-binding EGF-like growth factor (HBEGF), accumulates in both villous and extravillous trophoblasts and is expressed at high levels in the first trimester and throughout gestation in normal pregnancies [8,9]. HBEGF induces an invasive trophoblast phenotype in human [10] and mouse [11] blastocysts, and can initiate molecular and cellular changes characteristic of decidualization in a pseudopregnant mouse uterus [12]. Moreover, HBEGF is a survival factor that inhibits apoptosis [13]. Apoptosis induced by either transforming growth factor (TGF)-β or tumor necrosis factor (TNF)-α in human endometrial stromal cells is reduced by HBEGF [14]. These functions of HBEGF during early gestation could persist in trophoblast to sustain survival throughout pregnancy. Indeed, exogenous application of EGF has been shown to reduce cell death in human term trophoblast cells subjected to oxidative stress or pro-apoptotic cytokines [15-18] more effectively than other growth factor ligands of receptor tyrosine kinases [17,19]. We have shown in a first trimester human cytotrophoblast cell line that their survival at 2% $O_2$ is dependent on endogenous HBEGF signaling [20]. HBEGF, but not other EGF-like growth factors, increases within 4 h after exposure to 2% $O_2$ and interference with HBEGF signaling both prevents its upregulation and increases apoptosis. HBEGF is optimally cytoprotective at 1nM, which is approximately the concentration of medium conditioned by first trimester trophoblast cells cultured at 2% $O_2$. It is not known whether this survival mechanism is operative after the first trimester. Overall, these finding suggest that pathological interference with HBEGF signaling could jeopardize trophoblast survival and diminish invasive activity during placentation.

Although HBEGF can be detected in human placental tissues throughout gestation, its expression is reduced in pregnancies with SGA infants and is nearly undetectable in most pregnancies diagnosed with preeclampsia [21]. It is not clear how early during gestation the expression of this protein becomes dysregulated in women who are eventually diagnosed with preeclampsia or whether its loss contributes to the pathophysiology of disease. However, its putative roles in trophoblast invasion and survival, which are both insufficient in preeclampsia and intrauterine growth restriction, suggest a relationship. Furthermore, its function and regulation during mid to late gestation requires clarification, since that is the period when pathological downregulation has been documented. In this study, we aimed to establish the regulation of HBEGF by $O_2$ and its role in reducing cell death and preserving cell proliferation in term villous explants during in vitro culture and hypoxic stress. Our findings support its putative survival function and indicate the potential impact of its loss in pathologic placentas.

## 2. Materials and Methods

### 2.1. Participants

Placentas (n=5) were obtained by elective cesarean sections at term from patients who had non-pathologic pregnancies. Participants provided written informed consent, our protocol was approved by the Wayne State University Human Investigation Committee.

### 2.2. Villous Explant Culture

Several cotyledons from the central region of the placenta were removed and rinsed in cold sterile PBS to remove blood, then dissected to remove the decidua and blood vessels. The

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

chorionic villi were cut with scissors into pieces of approximately 5 mg wet weight, which were placed individually onto polycarbonate filter inserts in a 24-well culture plate (Costar, Corning, NY). The bottom chamber contained 500 μl DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin and streptomycin, while the upper chamber contained approximately 25 μl of this medium. Villous explants were cultured at 37°C in a humidified incubator containing 5% $CO_2$ and air (20% $O_2$) overnight before initiating experiments. Experiments commenced with addition of 150 μl of medium to the tissue in the upper chamber, with or without supplementation with 10 μg/ml CRM197 (EMD Chemicals, Inc., La Jolla, CA) or 1 nM recombinant HBEGF (R&D Systems, Minneapolis, MN), and culture was continued for 0, 2, 8 or 24 h. Another set of villous explants were cultured in plates placed into plastic bags fashioned from 4 mil tubular roll stock (Kapak Corp., Minneapolis, MN) that were flushed for 5 min with a mixture of 5% $CO_2$, 2% $O_2$, and 93% $N_2$ (Wilson Gases, Detroit, MI) before heat sealing. At the end of culture, tissue in each well was gently rinsed 3 times with PBS and fixed for 30 min in 10% neutral buffered formalin. Fixative was removed by rinsing 3 times with 150 μl of PBS. Tissues were embedded in paraffin and 5 m sections were cut and mounted on glass slides for analysis, as described below. Paraffin sections were deparaffinized with xylene and rehydrated into Tris-buffered saline before immunohistochemical or cell death assay.

### 2.3. Immunohistochemistry for HBEGF

Immunohistochemistry was performed using a DAKO (Carpinteria, CA) Autostainer Universal Staining System, as previously described [21]. Rehydrated sections of cultured explants were labeled for 1 h at 25°C with 5 μg/ml goat polyclonal antibody against human recombinant HBEGF (R&D Systems) that recognizes both membrane and secreted forms of the protein. Controls were incubated with 10 μg/ml non-immune goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Tissues were then incubated 1 h at 25°C with 0.1 μg/ml rabbit anti-goat IgG (Jackson Immunoresearch). To visualize and quantify antigen, an Envision System™ peroxidase anti-mouse/rabbit kit (DAKO) was used in conjunction with image analysis, according to our published procedure [21]. Slides were viewed at 400 × magnification using a Leica (Wetzlar, Germany) DM IRB inverted microscope and imaged with an ORCA digital camera (Hamamatsu, Hamamatsu City, Japan). Images were processed using Simple PCI (C-Imaging Corp., Cranberry Township, PA) to circumscribe individual villi and obtain the average grey level for semi-quantitative analysis, according to our published procedure [21]. Values obtained with IgG substituted for primary antibody were subtracted from each sample.

### 2.4. Cell Death and Proliferation Assays

Cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), using an alkaline phosphatase-based kit from Roche Applied Science (Indianapolis, IN) and counterstaining with hematoxylin. Slides were viewed at 200 × magnification and digital images were analyzed to determine the percentage of TUNEL/hematoxylin-labeled nuclei (TUNEL index) from triplicate fields. Sections labeled by immunohistochemistry with a mouse monoclonal antibody against Ki-67 (DAKO) were counterstained with hematoxylin and similarly assessed for the percentage of Ki-67/hematoxylin-labeled nuclei as an index of cell proliferation.

### 2.5. Statistical analysis

Assays were conducted using triplicate samples and all experiments were repeated using five different placentas. Data were analyzed using the SPSS Version 12.0 (SPSS, Chicago, IL) statistics program. For the effects of hypoxia and HBEGF on TUNEL and Ki67 expression, separate one-way ANOVAs were performed at each time point with inclusion of the control

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(0 h). Where significance was found, a Student-Newman-Keuls post hoc test was used to determine differences among the treatments. One-way ANOVAs followed by Student-Newman-Keuls post hoc tests were used to analyze the effects of CRM197 on cell death and proliferation, and hypoxia on HBEGF levels. Histograms present data as mean ± SE.

## 3. Results

Chorionic villi were collected after treatment for 0 to 24 h and evaluated for trophoblast cell death and proliferative capacity by determining TUNEL and nuclear Ki67 expression, respectively. Examples shown in Fig. 1 suggest low levels of TUNEL during culture at 20% $O_2$ (Panel A), but a strikingly higher number of TUNEL-positive trophoblast nuclei after only 2 h of culture at 2% $O_2$ (Panel B). TUNEL positive trophoblast cells were abundant after 24 h of hypoxic culture (Panel C), but not if the medium was supplemented with recombinant HBEGF (Panel D), suggesting a cytoprotective effect of the growth factor. Quantitative assessment of tissue from five placentas cultured 0-24 h confirmed these observations (Fig. 2A), demonstrating a significantly higher TUNEL index during culture at 2% than 20% $O_2$ and amelioration of cell death when hypoxic medium was supplemented with 1 nM HBEGF. Exogenous HBEGF had no effect on the low cell death levels during culture at 20% $O_2$.

Villous trophoblast cells from healthy, normotensive pregnancies contain endogenous HBEGF, but HBEGF is nearly absent in preeclampsia [21]. Therefore, we examined the effect of inhibiting endogenous HBEGF signaling during culture at 20% $O_2$ by exposure to the HBEGF antagonist CRM197, a non-toxic mutant of diphtheria toxin that specifically binds to HBEGF and prevents interaction with its receptors, HER1 and HER4 [22]. Significant elevation of the TUNEL index was observed at 8 and 24 h (Fig. 2B), suggesting that endogenous HBEGF signaling is required for trophoblast survival during in vitro culture.

Trophoblast cell proliferation was not strongly affected by hypoxia or HBEGF. Proliferation rates rose slightly, but not significantly, at 2% $O_2$ and were unchanged by supplementation with recombinant HBEGF (Fig. 3A). Inhibition of endogenous HBEGF signaling with CRM197 during culture at 20% $O_2$ inhibited proliferation transiently at 2 h, but nuclear Ki67 expression recovered by 8 h (Fig. 3B).

We previously demonstrated that first trimester human trophoblast cells rely on elevated HBEGF signaling to survive at 2% $O_2$ [20]. To determine whether this mechanism persists in trophoblast cells at term, HBEGF was localized by immunohistochemistry in villous explants. HBEGF antibody stained trophoblast and mesoderm at levels clearly above the background staining of non-immune IgG (Fig. 4A-D). Explants cultured for 24 h at either 20% or 2% $O_2$ appeared to express HBEGF at levels similar to the non-cultured control villi. Semi-quantitative analysis of HBEGF by immunohistochemistry revealed no difference in the levels of HBEGF during culture for 24 h at either 2% or 20% $O_2$ (Fig. 4E). Unlike first trimester human cytotrophoblast cells [20], trophoblast at term does not upregulate HBEGF in response to hypoxic stress.

## 4. Discussion

Our findings demonstrate that HBEGF contributes significantly to the survival of term trophoblast cells during stress induced by in vitro culture and that it can prevent apoptosis during exposure to hypoxia. Elimination of endogenous HBEGF signaling during villous explant culture using the antagonist CRM197 significantly increased cell death among villous trophoblast cells. Although HBEGF levels in the placenta were relatively low, they provided significant protection against cytological damage incurred during culture over a period of 8 h. More severe stress generated by culturing explants at 2% $O_2$ increased the TUNEL index within

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2 h from 15-20% to nearly 80%. Supplementation with a cytoprotective concentration of HBEGF [20] during hypoxic culture blocked the dramatic increase in cell death. Poor survival at 2% $O_2$ suggested that term trophoblast cells lack the ability found in first trimester cytotrophoblast cells to elevate HBEGF during exposure to low $O_2$ concentrations [20]. Indeed, HBEGF was not upregulated in term trophoblast during hypoxia, based on a semi-quantitative immunohistochemical approach previously shown to assess relative levels of HBEGF reliably in tissues [21] and cultured cells [20]. The inability of term trophoblast cells to engage the HBEGF-mediated hypoxia survival mechanism operative in the first trimester could contribute to the physiologic intolerance of term trophoblast tissue to low $O_2$ levels.

In vivo, stress upon the trophoblast mounts as gestation proceeds. Their success in surviving the challenges of increased demand by the growing fetus and maternal systemic changes brought about by pregnancy could determine pathologic outcomes, including preeclampsia and intrauterine growth restriction. HBEGF is a member of a family of growth factors related to EGF that activate ErbB/HER tyrosine kinase receptors [23]. HBEGF is upregulated in response to injury in kidney, muscle, and intestine [13,24,25]. Its exogenous application protects against apoptosis, as well as ischemia or reperfusion injury [26,27], as demonstrated here for term trophoblast exposed to hypoxia. Although HBEGF was not induced in term trophoblast by hypoxia, its basal level of activity was required for survival during explant culture. Therefore, endogenous expression of HBEGF could be expected to moderate stress encountered by placental tissues in the course of gestation.

The trophoblast cell invasion-promoting [28] and anti-apoptotic [20] activities of HBEGF place this molecule at a point of convergence of the pathophysiological abnormalities associated with preeclampsia that include inadequate trophoblast invasion and excessive cell death [1,2]. Apoptosis occurs normally in villous trophoblast during pregnancy [29] and is elevated in trophoblast populations of patients with preeclampsia [3,4]. We report here that a concentration of 1 nM HBEGF eliminated trophoblast cell death during villous explant culture at 2% $O_2$. This concentration of HBEGF was previously found to be secreted into medium by a first trimester trophoblast cell line exposed to 2% $O_2$ and to protect against apoptosis [20]. These cells were also protected by 1 nM HBEGF during oxidative stress caused by ethanol [30] or reperfusion injury [31]. Hypoxic induction of first trimester cytotrophoblast cell death in the absence of HBEGF signaling is mediated through the apoptotic pathway, based on observations of pyknotic nuclei, internucleosomal DNA cleavage, externalized phosphatidylserine and dependence on the caspase cascade [20]. A decline in HBEGF, as occurs dramatically in preeclampsia [21], could precipitate the failure of cellular systems that protect against oxidative stress and endothelial dysfunction. While HBEGF and the EGF signaling system may play a central role in trophoblast survival during early stages of placentation, there is presently no evidence of disruption to this pathway during the first trimester of pregnancies that later develop preeclampsia. However, HBEGF expression is clearly deficient in preeclamptic placentas at the time of termination or delivery [21]. We now present evidence using term villous explants that interruption of HBEGF signaling late in gestation could have profound effects on trophoblast survival that could contribute to placental insufficiency and secondary complications associated with the later stages of the disorder.

While the stress of subjecting term villous explants to in vitro culture at 2% $O_2$ increased apoptosis, it did not compromise the proliferative capacity of trophoblast cells. Although EGF family signaling is mitogenic for some cell types [23], this does not appear to be the case for human trophoblast cells [28] except in conjunction with its anti-apoptotic activity [30]. The observed levels of nuclear Ki67 expression were unperturbed during in vitro explant culture for 24 h at either 2% or 20% $O_2$. This contrasts sharply with reported stimulation of proliferation by low $O_2$ in first trimester human cytotrophoblast cells [32,33]. Moreover, inhibition of endogenous HBEGF signaling only transiently affected proliferation, prior to the time when

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

apoptosis increased. We suggest that this modest reduction in proliferation represents physiological stress preceding apoptosis rather than a requirement for HBEGF in cell replication.

First trimester trophoblast cells subjected to hypoxia specifically upregulate HBEGF protein by 100-fold within 4 h through an autocrine positive feedback loop that inhibits apoptosis [20]. The human trophoblast transforms between the first trimester and later stages in its ability to survive under hypoxic conditions. During the first trimester, hypoxia increases trophoblast proliferation [32,33], but it induces cell death in trophoblast cells obtained from midterm or term placentas [16,17,34]. Therefore, we have examined the effect of $O_2$ concentration on expression of HBEGF in term villous explants. Term villous trophoblast cells did not upregulate HBEGF at low $O_2$, as demonstrated by immunohistochemistry and semi-quantitative image analysis. Thus, term trophoblast appears to lack this HBEGF-mediated hypoxia survival mechanism. After the tenth week of gestation, $O_2$ levels rise considerably in the intervillous space and at sites of deep extravillous trophoblast invasion [35,36], greatly reducing trophoblast exposure to hypoxic conditions. Therefore, pressure to maintain the capacity to survive at low $O_2$ concentrations is minimal. Considering the numerous other sources of stress encountered by trophoblast cells during the final half of gestation and the ability of HBEGF to protect against cell death, the absence of HBEGF in placental tissues of women with preeclampsia could have significant pathophysiological consequences.

## Acknowledgements

We thank Mr. Michael Kruger for conducting statistical analyses. This work was supported by the intramural research program of the National Institute of Child Health and Human Development, National Institutes of Health, DHHS.

## References

[1]. Redman CW, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. Placenta 2000;21:597–602. [PubMed: 10985960]

[2]. Roberts JM. Endothelial dysfunction in preeclampsia. Semin Reprod Endocrinol 1998;16:5–15. [PubMed: 9654603]

[3]. Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. Obstet Gynecol 2000;96:271–276. [PubMed: 10908776]

[4]. DiFederico E, Genbacev O, Fisher SJ. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. Am J Pathol 1999;155:293–301. [PubMed: 10393861]

[5]. Zhou Y, Damsky CH, Fisher SJ. 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 1997;99:2152–2164. [PubMed: 9151787]

[6]. Khong TY, De WF, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br J Obstet Gynaecol 1986;93:1049–1059. [PubMed: 3790464]

[7]. Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol 2002;186:158–166. [PubMed: 11810103]

[8]. Leach RE, Khalifa R, Ramirez ND, Das SK, Wang J, Dey SK, Romero R, Armant DR. 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 1999;84:3355–3363. [PubMed: 10487711]

[9]. Yoo HJ, Barlow DH, Mardon HJ. 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 1997;21:102–108. [PubMed: 9291586]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

[10]. Martin KL, Barlow DH, Sargent IL. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. Hum Reprod 1998;13:1645–1652. [PubMed: 9688406]

[11]. Wang J, Mayernik L, Schultz JF, Armant DR. 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 2000;127:33–44. [PubMed: 10654598]

[12]. Paria BC, Ma W, Tan J, Raja S, Das SK, Dey SK, Hogan BL. 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 2001;98:1047–1052. [PubMed: 11158592]

[13]. Michalsky MP, Kuhn A, Mehta V, Besner GE. Heparin-binding EGF-like growth factor decreases apoptosis in intestinal epithelial cells in vitro. J Pediatr Surg 2001;36:1130–1135. [PubMed: 11479841]

[14]. Chobotova K, Karpovich N, Carver J, Manek S, Gullick WJ, Barlow DH, Mardon HJ. Heparin-binding epidermal growth factor and its receptors mediate decidualization and potentiate survival of human endometrial stromal cells. J Clin Endocrinol Metab 2005;90:913–919. [PubMed: 15562026]

[15]. Garcia-Lloret MI, Yui J, Winkler-Lowen B, Guilbert LJ. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. J Cell Physiol 1996;167:324–332. [PubMed: 8613474]

[16]. Levy R, Smith SD, Chandler K, Sadovsky Y, Nelson DM. Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor. Am J Physiol Cell Physiol 2000;278:C982–C988. [PubMed: 10794672]

[17]. Mackova M, Kilani RT, Davidge ST, Guilbert LJ. The effect of oxygen tension on intracellular survival signalling in primary villous trophoblasts. Placenta 2003;24:627–637. [PubMed: 12828921]

[18]. Moll SJ, Jones CJ, Crocker IP, Baker PN, Heazell AE. Epidermal growth factor rescues trophoblast apoptosis induced by reactive oxygen species. Apoptosis 2007;12:1611–1622. [PubMed: 17573555]

[19]. Smith S, Francis R, Guilbert L, Baker PN. Growth factor rescue of cytokine mediated trophoblast apoptosis. Placenta 2002;23:322–330. [PubMed: 11969343]

[20]. Armant DR, Kilburn BA, Petkova A, Edwin SS, Duniec-Dmuchowski ZM, Edwards HJ, Romero R, Leach RE. Human trophoblast survival at low oxygen concentrations requires metalloproteinase-mediated shedding of heparin-binding EGF-like growth factor. Development 2006;133:751–759. [PubMed: 16407398]

[21]. Leach RE, Romero R, Kim YM, Chaiworapongsa T, Kilburn B, Das SK, Dey SK, Johnson A, Qureshi F, Jacques S, Armant DR. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. Lancet 2002;360:1215–1219. [PubMed: 12401248]

[22]. Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E. 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 1995;270:1015–1019. [PubMed: 7836353]

[23]. Riese DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998;20:41–48. [PubMed: 9504046]

[24]. Horikawa M, Higashiyama S, Nomura S, Kitamura Y, Ishikawa M, Taniguchi N. Upregulation of endogenous heparin-binding EGF-like growth factor and its role as a survival factor in skeletal myotubes. FEBS Lett 1999;459:100–104. [PubMed: 10508925]

[25]. Nguyen HT, Bride SH, Badawy AB, Adam RM, Lin J, Orsola A, Guthrie PD, Freeman MR, Peters CA. Heparin-binding EGF-like growth factor is up-regulated in the obstructed kidney in a cell- and region-specific manner and acts to inhibit apoptosis. Am J Pathol 2000;156:889–898. [PubMed: 10702406]

[26]. Iwabu A, Murakami T, Kusachi S, Nakamura K, Takemoto S, Komatsubara I, Sezaki S, Hayashi J, Ninomiya Y, Tsuji T. Concomitant expression of heparin-binding epidermal growth factor-like

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

growth factor mRNA and basic fibroblast growth factor mRNA in myocardial infarction in rats. Basic Res Cardiol 2002;97:214–222. [PubMed: 12061391]

[27]. Xia G, Martin AE, Michalsky MP, Besner GE. Heparin-binding EGF-like growth factor preserves crypt cell proliferation and decreases bacterial translocation after intestinal ischemia/reperfusion injury. J Pediatr Surg 2002;37:1081–1087. [PubMed: 12077776]

[28]. Leach RE, Kilburn B, Wang J, Liu Z, Romero R, Armant DR. Heparin-binding EGF-like growth factor regulates human extravillous cytotrophoblast development during conversion to the invasive phenotype. Dev Biol 2004;266:223–237. [PubMed: 14738873]

[29]. Mayhew TM. Villous trophoblast of human placenta: a coherent view of its turnover, repair and contributions to villous development and maturation. Histol Histopathol 2001;16:1213–1224. [PubMed: 11642741]

[30]. Wolff GS, Chiang PJ, Smith SM, Romero R, Armant DR. Epidermal growth factor-like growth factors prevent apoptosis of alcohol-exposed human placental cytotrophoblast cells. Biol Reprod 2007;77:53–60. [PubMed: 17392498]

[31]. Leach RE, Kilburn BA, Petkova A, Romero R, Armant DR. 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 2008;198:471–477. [PubMed: 18395045]

[32]. Genbacev O, Joslin R, Damsky CH, Polliotti BM, Fisher SJ. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. J Clin Invest 1996;97:540–550. [PubMed: 8567979]

[33]. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science 1997;277:1669–1672. [PubMed: 9287221]

[34]. Kilani RT, Mackova M, Davidge ST, Guilbert LJ. Effect of oxygen levels in villous trophoblast apoptosis. Placenta 2003;24:826–834. [PubMed: 13129679]

[35]. Jauniaux E, Watson A, Burton G. 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 2001;184:998–1003. [PubMed: 11303211]

[36]. Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 1992;80:283–285. [PubMed: 1635745]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



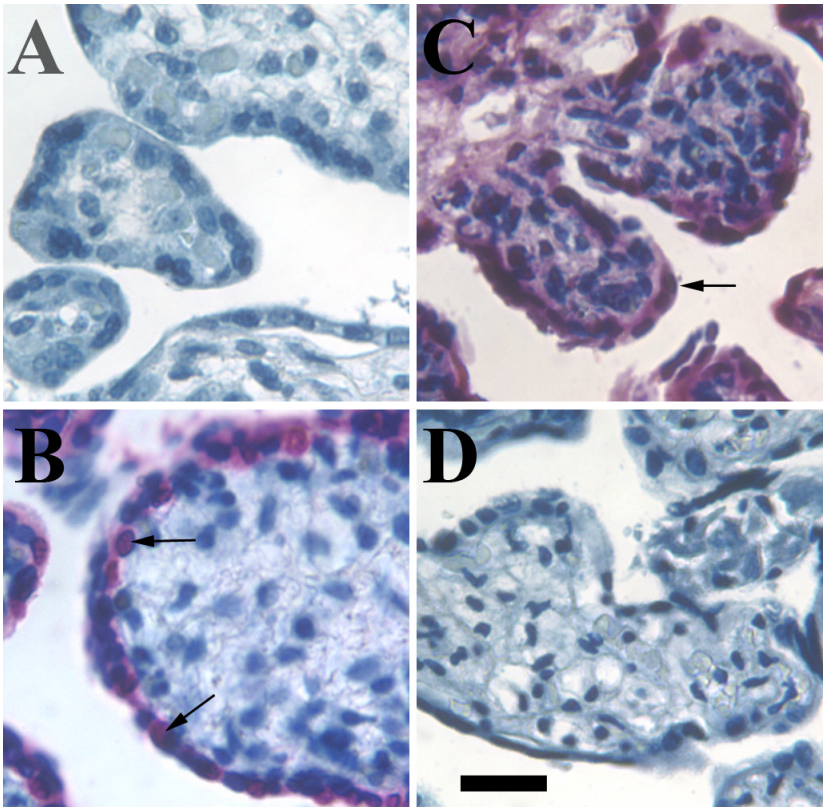


**Figure 1.**
Micrographs of chorionic villi cultured for 24 h at 20% (A) or 2% $O_2$ (C), 2 h at 2% $O_2$(B) and for 24 h at 2% $O_2$ in medium supplemented with 1 nM HBEGF (D). Arrows indicate nuclei of trophoblast cells stained red by TUNEL. Healthy nuclei counterstained with Hematoxylin appear blue. Bar = 50 μm.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

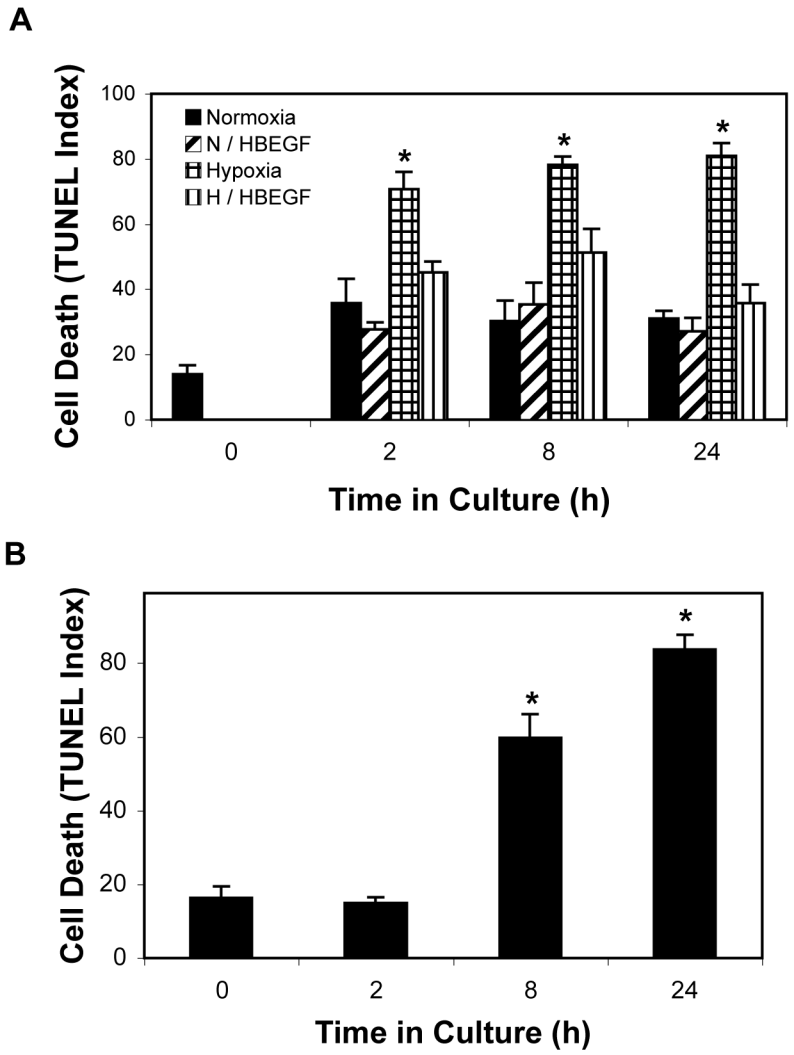


**Figure 2.**

The effect on trophoblast cell death of villous explant culture at 20% (Normoxia) or 2% (Hypoxia) $O_2$. In A, the TUNEL Index was determined during culture in medium alone or medium supplemented with 1 nM recombinant HBEGF at normoxia (N/HBEGF) or hypoxia (H/HBEGF). In B, 10 μg/ml CRM197 was added to the medium during culture at 20% $O_2$ to antagonize HBEGF signaling. *, $p<0.05$, compared to control (0 h).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

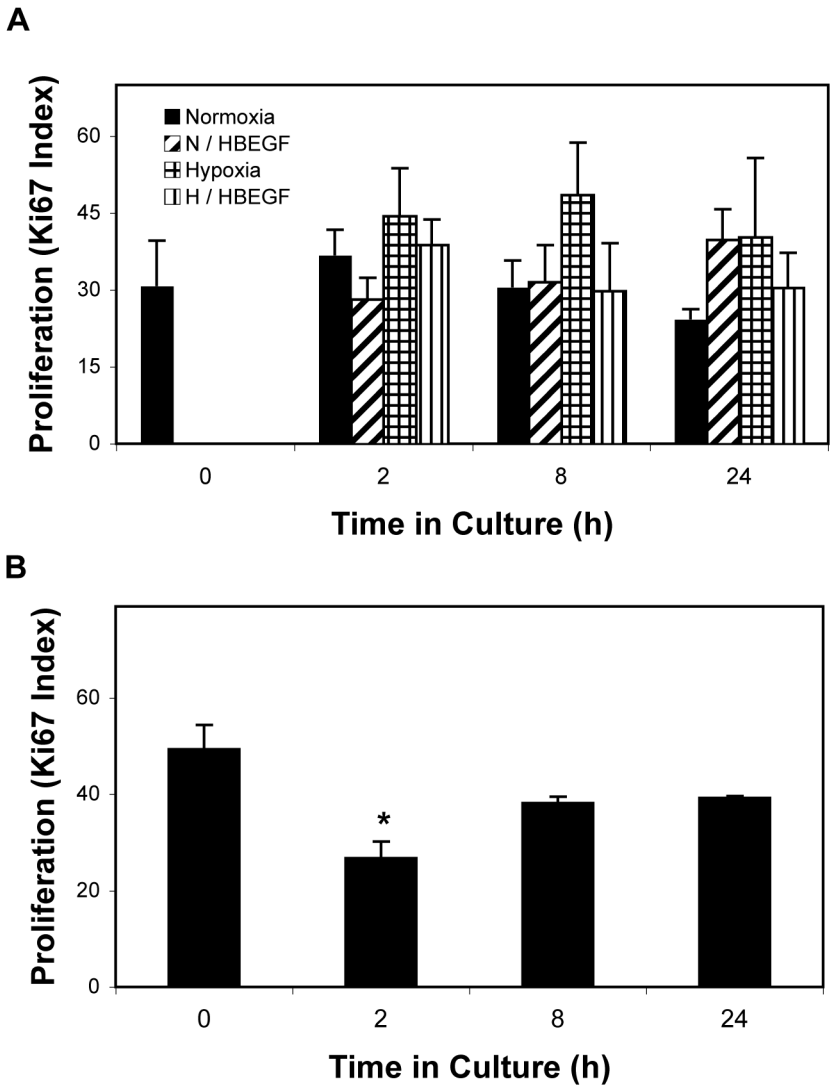


**Figure 3.**
The effect on trophoblast cell proliferation of villous explant culture under conditions identical to those described in Fig. 2. *, $p<0.05$, compared to control (0 h).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



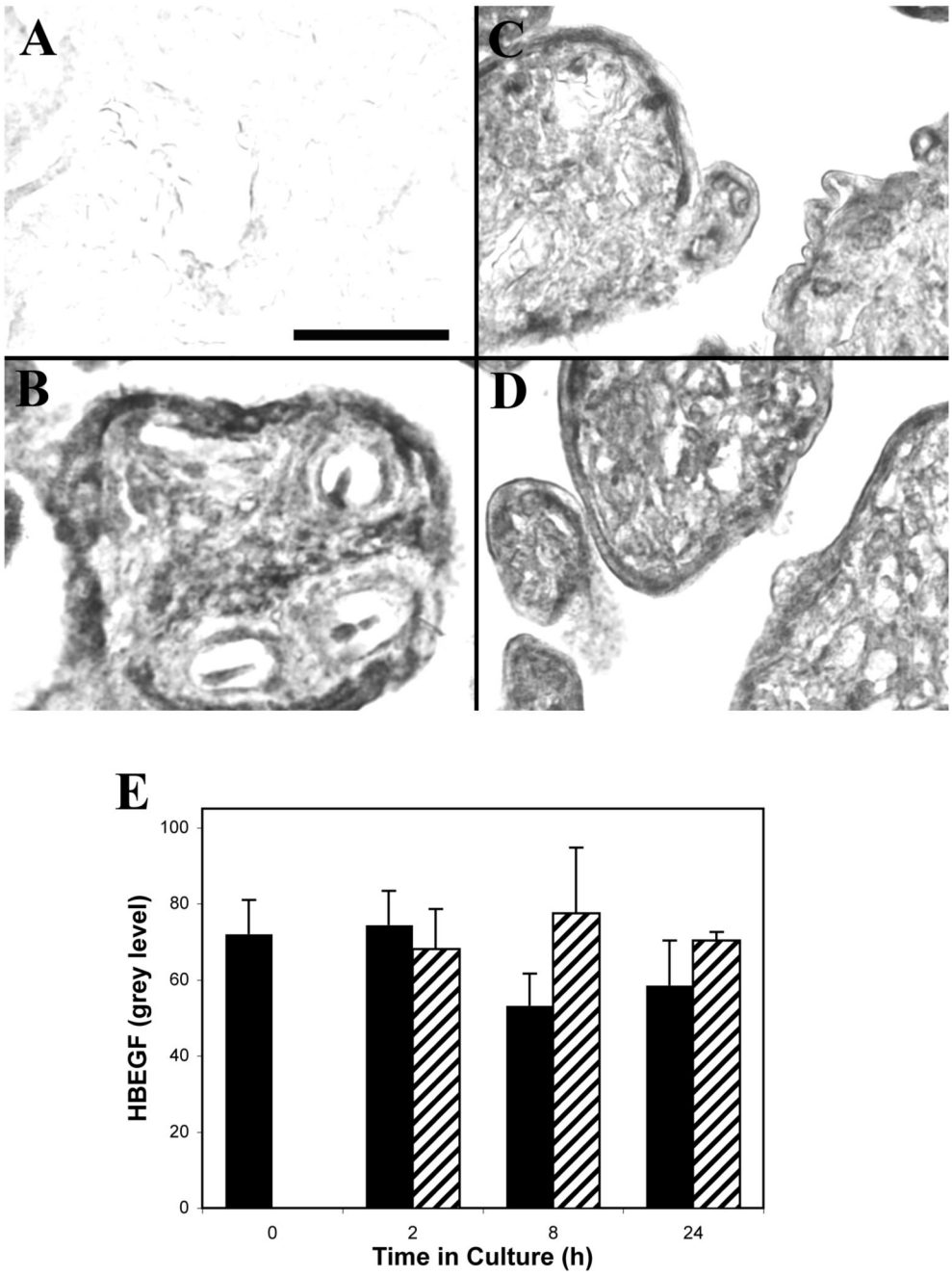


**Figure 4.**

HBEGF expression in villous explants during culture. Immunohistochemical staining of non-cultured (A,B) and in vitro cultured (C,D) villi that were labeled with non-immune IgG (A) or antibody to HBEGF (B-D). Explants were cultured for 24 h at 20% (C) or 2% (D) $O_2$. Bar = 50 μm. E. HBEGF immunohistochemical staining during culture at 20% (solid bars) or 2% (striped bars) $O_2$. The intensity of labeling (grey level) was quantified by image analysis in stained sections of villi cultured for the indicated times. A one-way ANOVA demonstrated no significant differences in HBEGF labeling among the treatments shown.