Molecular Biology of the Cell
Vol. 21, 2832–2843, August 15, 2010

# Molecular Characterization of EG-VEGF-mediated Angiogenesis: Differential Effects on Microvascular and Macrovascular Endothelial Cells

**Sophie Brouillet,*†‡ Pascale Hoffmann,*†‡§ Mohamed Benharouga,†‡‖ Aude Salomon,*†‡ Jean-Patrick Schaal,§ Jean-Jacques Feige,*†‡ and Nadia Alfaidy*†‡**

*Institut National de la Santé et de la Recherche Médicale, Unité 878, Grenoble, France; †Commissariat à l'Energie Atomique, iRTSV, Grenoble, France; ‡Université Joseph Fourrier, Grenoble 1, France; §Centre Hospitalier Régional Universitaire de Grenoble, Département de Gynécologie, Obstétrique et Médecine de la Reproduction, Grenoble, France; and ‖Centre National de la Recherche Scientifique, UMR 5249, Grenoble, France

Submitted January 26, 2010; Revised May 27, 2010; Accepted June 21, 2010
Monitoring Editor: Asma Nusrat

**Endocrine gland derived vascular endothelial growth factor (EG-VEGF) also called prokineticin (PK1), has been identified and linked to several biological processes including angiogenesis. EG-VEGF is abundantly expressed in the highest vascularized organ, the human placenta. Here we characterized its angiogenic effect using different experimental procedures. Immunohistochemistry was used to localize EG-VEGF receptors (PROKR1 and PROKR2) in placental and umbilical cord tissue. Primary microvascular placental endothelial cell (HPEC) and umbilical vein-derived macrovascular EC (HUVEC) were used to assess its effects on proliferation, migration, cell survival, pseudovascular organization, spheroid sprouting, permeability and paracellular transport. siRNA and neutralizing antibody strategies were used to differentiate PROKR1- from PROKR2-mediated effects. Our results show that 1) HPEC and HUVEC express both types of receptors 2) EG-VEGF stimulates HPEC's proliferation, migration and survival, but increases only survival in HUVECs. and 3) EG-VEGF was more potent than VEGF in stimulating HPEC sprout formation, pseudovascular organization, and it significantly increases HPEC permeability and paracellular transport. More importantly, we demonstrated that PROKR1 mediates EG-VEGF angiogenic effects, whereas PROKR2 mediates cellular permeability. Altogether, these data characterized angiogenic processes mediated by EG-VEGF, depicted a new angiogenic factor in the placenta, and suggest a novel view of the regulation of angiogenesis in placental pathologies.**

## INTRODUCTION

The human placenta is a highly vascularized organ. By the end of gestation, it has developed a capillary network that is ~550 km in length and 15 $m^2$ in surface (Burton and Jauniaux, 1995). This network is essential for efficient materno-fetal exchange, but also plays a key mechanistic role in the elaboration of the placental villous tree. Vasculogenesis, and subsequent angiogenesis, are the pivotal processes for the enlargement of the placental vascular tree and placental development (Charnock-Jones and Burton, 2000; Leach *et al.*, 2002; Charnock-Jones *et al.*, 2004; Demir *et al.*, 2004, 2007). Failure in these processes can lead to preeclampia (PE), early pregnancy loss, and intrauterine growth restriction (IUGR). It is now well established that a close relationship exists between embryonic development and the degree of vascularization of the chorionic villi, and that normal chorionic villous vascularization is essential for undisturbed development of pregnancy (te Velde *et al.*, 1997).

Angiogenic growth factors are considered to be the main mediators of placental angiogenesis. Mouse models have demonstrated the importance of two families of ligands, namely vascular endothelial growth factors (VEGFs) and angiopoietins, and their respective tyrosine kinase receptors in fetal and placental angiogenesis (Risau, 1997; Neufeld *et al.*, 1999; Sibai *et al.*, 2005). Although VEGF-A and the angiopoietins are essentially selective for endothelial cells, they are widely expressed. Thus, it has been difficult to reconcile endothelial cell phenotypic diversity with the action of ubiquitous factors. Different studies have provided evidence for certain vascular bed-specific responses to VEGF (Palade *et al.*, 1979; Dellian *et al.*, 1996). In the placenta, VEGF acts differentially on the two types of endothelial cells that form this organ: the human placental microvascular endothelial cells (HPECs) that lie in the fetal capillaries of chorionic villi and the human umbilical vein macrovascular endothelial cells (HUVECs). Hence, it is likely that morphological and functional diversity among endothelia is achieved by several mechanisms, including vascular bed–specific response to ubiquitous mediators and the existence of unique mitogenic/differentiating factors with a tissue-restricted expression pattern.

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–01–0059) on June 29, 2010.

Address correspondence to: Nadia Alfaidy (nadia.alfaidy-benharouga@cea.fr).

Abbreviations used: EG-VEGF, endocrine gland derived endothelial growth factor; PROK1, prokineticin 1; PROK2, prokineticin 2; PROKR1, prokineticin receptor 1; PROKR2, prokineticin receptor 2.

**Table 1.** Primers used in this study

| Gene | Forward primer | Reverse primer | T (°C) for qPCR |
|---|---|---|---|
| PROKR1 | 5′-GTCCTCGTCATTGTCAAGAGCC-3′ | 5′-AAACACGGTGGGGAAGAAGTCG-3′ | 58 |
| PROKR2 | 5′-CATCCCATCGCCTTACTTTGC-3′ | 5′-CTTTTCCTTCACGAACACAGTGG-3′ | 58 |
| GAPDH | 5′-ACCCAGAAGACTGTGGATGG-3′ | 5′- TTCTAGACGGCAGGTCAGGT-3′ | 60 |
| Galpha12 | 5′- ATGGTCTCCTCCAGCGAGTA-3′ | 5′-CTTGATGCTCACGGTCTTCA-3′ | 60 |
| Galpha13 | 5′-GGAGATCGACAAATGCCTGT-3 | 5′-CAACCAGCACCCTCATACCT-3 | 60 |
| Galpha 11 | 5′-TGAGCGATGAGGTGAAGGAGTC-3′ | 5′-CGGTGAAGATGTTCTGGTAGACG-3′ | 60 |
| Galpha i1 | 5′-CGGTGCTGGTGAATCTGGTAAAAG-3′ | 5′-ACCTCCCCATAGCCCTAATGATAG-3′ | 60 |
| Galpha i2 | 5′-TGTCTACAGCAACACCATCCAGTC-3′ | 5′-CAATACGCTCCAGGTCGTTCAG-3′ | 60 |

qPCR, quantitative PCR.

The existence of organ-specific angiogenic factors has been postulated for many years (Stewart and Wiley, 1981; Roberts *et al.*, 1998), but only recently received confirmation when such a factor, named endocrine gland–derived vascular endothelial growth factor (EG-VEGF), was characterized and sequenced (LeCouter *et al.*, 2001). This new factor was found to be expressed in testis, adrenal gland, ovary, and placenta (LeCouter *et al.*, 2001). More importantly, its angiogenic action appeared to be restricted to endothelial cells derived from endocrine tissues (LeCouter *et al.*, 2001). In endothelial cells isolated from steroidogenic tissues, EG-VEGF was shown to promote proliferation, survival, and chemotaxis (LeCouter *et al.*, 2001; Lin *et al.*, 2002b). More importantly, in vivo delivery of adenoviruses encoding EG-VEGF resulted in endocrine tissue-specific angiogenesis (LeCouter *et al.*, 2001). EG-VEGF, also known as prokineticin-1 is a member of a class of proteins that includes Bv8, a frog peptide purified from the skin secretion of the yellow-bellied toad *Bombina variegata*, also known as prokineticin-2 (PROK2) in mammals. Human EG-VEGF and Bv8 proteins present 83% identity and share the same G protein–coupled receptors, termed PROKR1 and PROKR2 (Lin *et al.*, 2002a).

EG-VEGF expression in the placenta was briefly described in the initial report by LeCouter *et al.* (2001). In three recent publications from our group, we have shown that EG-VEGF and its receptors, but not PROK2, are highly abundant in human placenta during the first trimester of pregnancy, with the highest expression of EG-VEGF found in the syncytiotrophoblast layer; that their expression is up-regulated by hypoxia; that EG-VEGF controls trophoblast invasion; and that its circulating levels were significantly higher in PE patients (Hoffmann *et al.*, 2006, 2007, 2009). The specificity and the magnitude of EG-VEGF expression in endocrine glands, its similarity of action with VEGF, its up-regulation by hypoxia and deregulation in PE, suggested to us that this new factor might play important role in placental angiogenesis. Here, we sought to characterize the angiogenic processes mediated by EG-VEGF in a highly vascularized organ, the human placenta and compare its effects to the well studied angiogenic factor, VEGF. Both microvascular (HPEC) and macrovascular (HUVEC) cells were studied.

## MATERIALS AND METHODS

### *Tissues Collection*

Collection and processing of human placentas were approved by the district and local hospital ethical committees, and collection was performed according to the Grenoble Hospital and University Joseph Fourier code of practice. Informed patient consent was obtained in all cases. Human term placentas were obtained from uncomplicated pregnancies after elective caesarean section delivery between 38 and 40 wk of gestation (wg) of gestation. For each placenta both the placental disk and the attached umbilical cord were used for the isolation of HPEC and HUVEC cells, respectively.

### *Endothelial Cell Isolation*

***HPEC Cell Isolation.*** Primary HPECs were isolated from fetal vessels of human term placenta, as described elsewhere (Jinga *et al.*, 2000). Term trimester placentas were used for technical reasons, as the large size of the placenta at this gestational age allows the isolation of a sufficient number of endothelial cells. At this gestational age, EG-VEGF receptors are both expressed (Hoffmann *et al.*, 2007; Denison *et al.*, 2008). In brief, HPECs were isolated from term placental villi by enzymatic perfusion of the vasculature followed by separation of the resulting cells on a Percoll density gradient. The cells were further purified by a two-step selection procedure, based on differential trypsinization (Jinga *et al.*, 2000). HPECs were used in our experiments between passage 3 and 5. Cells were cultured at 37°C in 5% $CO_2$ in air (vol/vol) and relative humidity greater than 95%. HPECs were maintained in endothelial cell basal medium (MCDB131, Invitrogen, Cergy Pontoise, France) supplemented with fetal bovine serum (FBS, 15%, Invitrogen, Cergy Pontoise, France), EGM 2MV endothelial med single quotkit (Lonza, Basel, Switzerland), and antibiotics (1%) and were passaged upon reaching confluence, typically every 3–4 d.

***HPEC Characterization.*** Isolated HPEC cells were tested for their expression of endothelial cell markers CD31 (PECAM) and von Willebrand factor, for binding of *Ulex europaeus* lectin (UEA-I) and for their DiI-Ac-LDL uptake, and for smooth muscle cell contamination was assessed by immunostaining of smooth muscle actin, according to the following methods.

von Willebrand factor antigen, UEA-I lectin, and CD31. For the three antibodies the following protocol was used: HPECs were cultured on glass coverslips, rinsed three times with DMEM, fixed in cold acetone (20°C) for 5 min, and air-dried at room temperature (RT). Anti-human IgGs against von Willebrand factor antigen was used at 1/1000 dilution (rabbit IgG, Dako, France), UEA-I (Ulex lectin binding) was used at 1/10 (Sigma-Aldrich, St. Quentin Fallavier, France) and CD31 was used at 1/100 (mouse IgG, Dako). All antibodies were diluted in PBS. The glass coverslips with cultured cells (upside down) were exposed to antibodies in a moisture chamber, at 37°C, for 1 h. After extensive washing with PBS, the cells were incubated in the same conditions, with their specific secondary antibodies labeled with Cy2 (1/1000; Molecular Probes, Eugene, OR) for the vW*** or with FITC for CD31. Lectin UEA-1 was TRITC labeled. After 1 h, at 37°C, the coverslips were washed thoroughly with PBS (three times for 15 min), fixed in 2% paraformaldehyde for 10 min, and mounted in a drop of Vectashield (Dako), and placed under coverslips. Preparations were observed under a Leica confocal microscope (TCS-SP2; Deerfield, IL). Incubation in buffer without primary antibodies was used as negative control. The same protocol as for the staining for endothelial cell markers was also used to stain smooth muscle actin (1/70, mouse IgG, clone A14 from Dako).

Uptake of acetylated low-density lipoproteins (AcLDL). AcLDL coupled with fluorescent 1,1-dioctadecyl-3,3,3,3-tetramethyl-indocarbocyanide perchlorate (Molecular Probes, Eugene, OR; AcLDL-DiI) was prepared as described by Voyta *et al.* (1984). Confluent HPEC on glass coverslips were washed with PBS containing 1.2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Sigma-Aldrich), incubated with AcLDL-DiI (10 $\mu$g/ml) for 1 h, and examined with the fluorescence microscope. Controls consisted of similarly processed cultures, except that AcLDL-DiI was omitted from the incubation medium.

***HUVEC Isolation.*** HUVEC were isolated from human umbilical cord veins as described before (Hebert *et al.*, 1990). The isolated cells were cultured in EGM-2 endothelial medium bullet kit (Lonza) fetal calf serum at 37°C under 5% $CO_2$.

### *RNA Isolation and RT-PCR Analysis*

Total RNA was extracted from HPECs and HUVEC as previously described using a rapid RNA isolation system (Qiagen RNeasy, Courtaboeuf, France).

Reverse transcription was performed on 1 $\mu$g total RNA with Superscript II-RnaseH reverse transcriptase (Invitrogen).

### *Real-Time PCR Analysis*

GAPDH, and G proteins (G$\alpha$11, G$\alpha$12, G$\alpha$13, G$\alpha$i1, G$\alpha$i2) mRNA expressions were quantified by real-time RT-PCR using a Light Cycler apparatus (Roche Diagnostics, Meylan, France). The PCR was performed using the primers shown in Table 1 and SYBR green PCR core reagents (Light Cycler-FastStart Master SYBR Green I, Roche Diagnostics, Meylan, France). For negative controls, we used a complete DNA amplification mix, where the target cDNA template was replaced by water. PCR conditions were as follows: step 1, 94°C for 10 min; and step 2, 45 cycles consisting of 95°C for 15 s, temperature indicated in Table 1 for 5 s and 72°C for 10 s. The results were normalized to GAPDH mRNA expression level.

### *RNA Interference*

Expression of PROKR1 and PROKR2 mRNAs was inhibited by transfection of small interfering RNAs (siRNAs). Briefly, 1 d after plating, HPEC cells were transfected with or without 10–50 nM siRNA duplexes for PROKR1 or R2 genes, using RNA interference (RNAi) Max (Ambion, Austin, TX). siRNA duplexes (21-nucleotide) were purchased from Ambion. PROKR1 siRNA oligonucleotide templates were the following (5′-3′): antisense: GGCUUCUUACAAUGGCGGUtt; sense: ACCGCCAUUGUAAGAAGCCtt, and PROKR2 siRNA oligonucleotide templates were the following (5′-3′): antisense: GUUAUGGUGAUUAUGACCUtt; sense: AGGUCAUAAUCACCAUAACtg. Scrambled siRNA duplexes of these targeting sequences served as nonspecific control siRNA.

### *Immunohistochemistry*

Placental tissues were collected from first trimester termination of pregnancy between 9 and 10 wg. Umbilical cords were collected at term from caesarean deliveries. Immunohistochemistry was processed as described previously (Hoffmann *et al.*, 2009) .

### *Western Blotting Analysis*

Western blotting was used to demonstrate the effect of EG-VEGF on mitogen-activated protein (MAP) kinase and AKT activation, to verify the loss of PROKR1 and PROKR2 mRNAs in HPECs treated with specific siRNAs to these proteins and to demonstrate the blocking effect of neutralizing antibodies to PROKR1 and PROKR2. Western blotting was processed as previously described (Hoffmann *et al.*, 2009). The rabbit antibodies against MAP kinase was (1/40,000, Sigma-Aldrich), phospho-MAP-kinase (1/5000, Promega, Madison, WI), Akt (1/1000, Cell Signaling, Beverly, MA), phosphor-AKT (1/1000, Cell Signaling), PROKR1 (0.84 $\mu$g/ml, in house antibody), and PROKR2 (0.84 $\mu$g/ml, in house antibody). Mouse anti-human ß-actin anti-

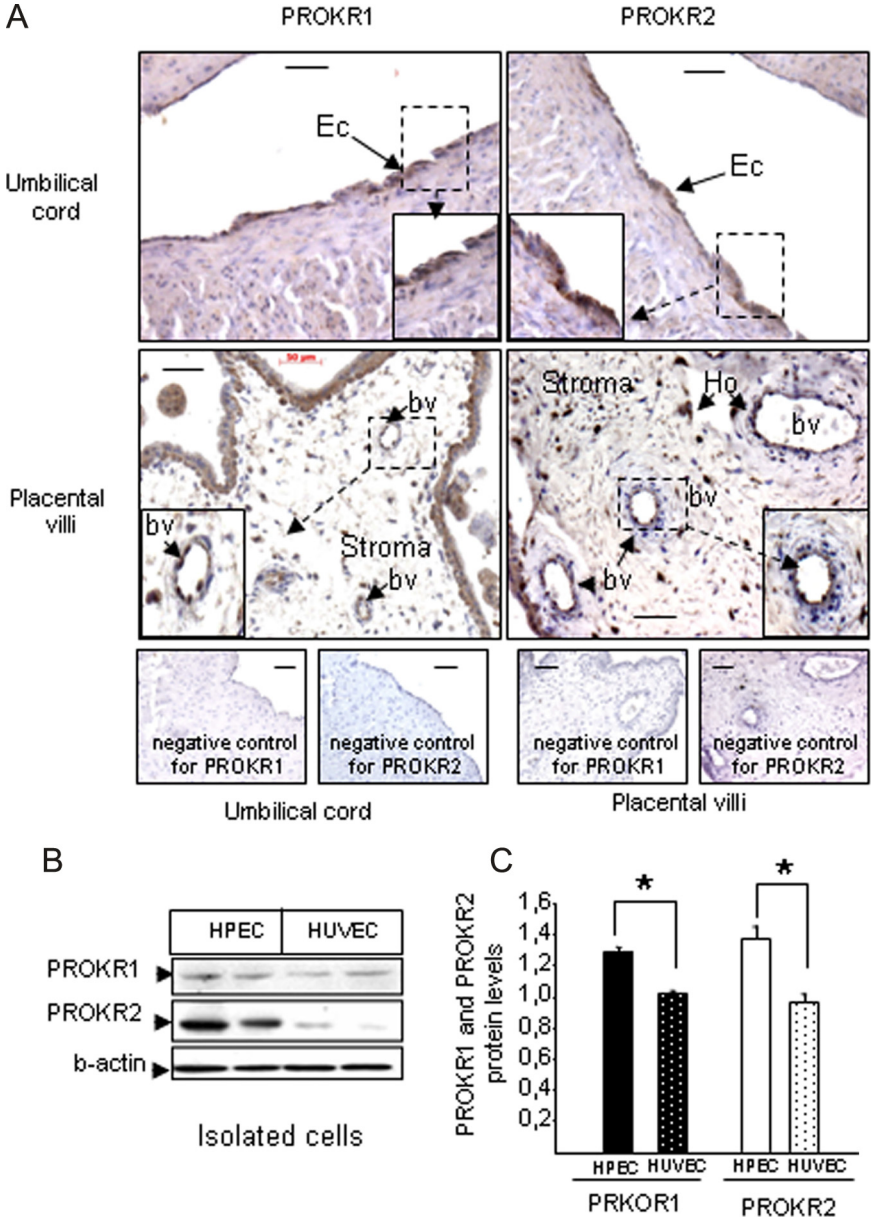


**Figure 1.** PROKR1 and PROKR2 protein expression in placental-tissue, umbilical cord and in isolated HPECs and HUVECs. (A) Chorionic villi and umbilical cord sections immunostained with anti-PROKR1 and anti-PROKR2 antibodies. The undersized photographs on the right show tissue sections incubated with the respective preimmune sera. Subset photographs in each panel represent higher magnifications for the staining in endothelial cells. CT, cytotrophoblast; Ho, Hofbauer cells; St, syncytiotrophoblast; Ec, endothelial cells; bv, blood vessels. Scale bar, 50 $\mu$m. (B) A representative Western blot analysis of PROKR1 and PROKR2 expression in HPECs and HUVECs. (C) A quantification of levels PROKR1 and PROKR2 protein expression in HPEC and HUVEC cells. *$p < 0.05$.

body (1/8000, Sigma-Aldrich) was used as an internal control for protein loading.

### *Assessment of HPEC and HUVEC Proliferation*

Both [$^3$H]thymidine incorporation and Ki67 staining were used. The effect of EG-VEGF on cellular proliferation was examined using recombinant human EG-VEGF (Tebu, Le Perray-en-Yvelines, France). Cells were placed in 48-well plates ($7 \times 10^4$ cell/well) and cultured overnight (37°C, 5% $CO_2$). The cells were serum-starved for 24 h and then incubated for 24 h in serum-free media containing 10–50 ng/ml EG-VEGF, which corresponds to 1–5 nM. The choice of these concentrations was based on the $IC_{50}$ of EG-VEGF for its receptors (2.7 nM; Lin *et al.*, 2002a; Maldonado-Perez *et al.*, 2007). For [$^3$H]thymidine, cells were labeled with 0.5 $\mu$Ci/ml [$^3$H]thymidine (Amersham, Les Ulis, France). The cells were subsequently washed in HBSS and incubated in 2 ml ice-cold 5% trichloroacetic acid for 20 min at RT. After washing, 0.4 ml of 0.1 M NaOH and 0.1% SDS was added; the lysates were transferred to Eppendorf tubes and counted in a liquid scintillation counter (Beckman, Krefeld, Germany; LS 6500). For Ki67 staining the cells were incubated for 24 h in serum-free media containing 10–50 ng/ml EG-VEGF, fixed in paraformaldehyde for 20 min, and then stained for Ki67 antibody (Dako).

### *Assessment of HPEC and HUVEC Migration*

Wound healing assay was performed to examine the effect of EG-VEGF on HPEC and HUVEC cell motility. Cells were seeded in equal number into six-well plates and processed as previously described (Hoffmann *et al.*, 2009). To further characterize the signaling pathway of EG-VEGF, inhibitors of MAP kinases and phosphatidylinositol 3′-kinase (PI3K) kinases (PD98059 and LY294002) have been used at 20 and 10 $\mu$M, respectively.

### *Apoptosis Detection*

HPEC cells were serum-starved for 24 h and then incubated for an extra 24 h in the absence or presence of EG-VEGF at different concentrations (10, 25, 50 ng/ml). The percentage of apoptotic HPEC cells was determined using caspase 3 staining. After a challenge with EG-VEGF the cells were fixed for 5 min in cold acetone and stained with anti-human caspase 3 IgGs at 1/2500 dilution (rabbit IgG, Cell Signaling). The same protocol used for HPEC characterization was applied for the caspase staining. For each slide, three randomly selected microscopic fields were observed, and ≥200 cells/field were evaluated.

### *Pseudovascular Morphogenesis Assay*

Approximately 150 $\mu$l of ice-cold Matrigel (BD Biosciences, Grenoble, France) was layered into each well of 24-well plates. The Matrigel was allowed to solidify completely at 37°C for 1 h. HPECs ($10^5$ cells per well) and HUVEC ($10^5$ cells per well) were added and incubated at 37°C in an atmosphere of humidified 95% air/5% $CO_2$ for 12 h. EG-VEGF treatment was applied at the same time as plating. Hourly observations were made under an inverted photomicroscope to document the developmental stages. Quantification of branching was performed after 10 h of culture by calculating the number of branching point formed in each well.

### *Generation of Endothelial Spheroids*

Confluent monolayers of HPECs and HUVECs were trypsinized. Cells were suspended in a mixture of 1 volume of 1.2% (wt/wt) methylcellulose and 4 volumes of culture medium containing 15% FCS and antibiotics. In these experiments, 3000 cells were suspended in culture medium/methylcellulose and seeded in nonadherent round-bottom 96-well plates (Greiner, Frickenhausen, Germany). Under these conditions all suspended cells contribute to the formation of a single endothelial cell spheroid. The spheroids were harvested within 24 h and transferred into a collagen gel (3.54 mg/ml, BD Biosciences) and different concentrations of EG-VEGF were added. Sprout formation was measured and compared with those induced by fibroblast growth factor 2 (FGF-2; 20 ng/ml) or VEGF (100 ng/ml). Sprouting of spheroids starts as early as 1 h of culture. Quantification of the sprouting was done using ImageJ software (http://rsb.info.nih.gov/ij/) after 12 h of culture by image analysis of microphotographs. At least three replicates were included within each experiment, and three different preparations of HPEC and HUVEC were analyzed. To differentiate between PROKR1- and PROKR2-mediated effects in HPEC cells, we performed two different sets of experiments. In the first one, the follow-up of sprouting was assessed in spheroids that had been formed from HPEC invalidated for PROKR1 or PROKR2 using siRNAs as described in this section. In the second set, the follow-up of sprouting was performed in spheroids that had been preincubated with PROKR1- or PROKR2-blocking antibodies (Covalab, Lyon, France) at 0.5 $\mu$g/ml final concentration and then challenged with EG-VEGF. Control rabbit IgGs were also used in the set of experiment where blocking antibodies were used. See Figure S3 for siRNA and blocking peptide strategies.

### *Measurement of Endothelial Monolayer Permeability*

***Transendothelial Electrical Resistance.*** HPEC were seeded onto Vitrogen-coated Transwell polystyrene filters (Corning, Dutsher, France) and grown to confluence for 10–11 d. Growth medium was replaced every other day. Twenty-four hours before the experiment, confluent endothelial monolayers were serum-starved. Experiments were initiated by washing the upper and lower compartments with warmed (37°C) 20 mM Tris-buffered saline, pH 7.4, supplemented with 25 mM glucose and 0.1% BSA (150 $\mu$l/upper and 600 $\mu$l/lower compartments). The inserts were allowed to equilibrate for 30 min at 37°C before initiating the experiments. After equilibration, the buffer was removed from the inserts, and fresh buffer containing various treatments was added to the upper compartment (70 U/ml thrombin, 25 ng/ml EG-VEGF). EG-VEGF and thrombin-induced changes in the resistance of the endothelial monolayers were measured using MilliCell ERS (Millipore, Bedford, MA) at 0, 5, 10, 15, 20, 25, and 35 min after adding the treatments. In this set of experiment, thrombin was used as a positive control for endothelial cell permeability (Rabiet *et al.*, 1996). Assays were performed in triplicate, and changes in resistance were normalized to their respective controls. In a different set of experiments, electrical resistance was determined in HPECs that were invalidated for PROKR1 or PROKR2 using specific siRNAs and in HPEC cells that were treated with PROKR1- or PROKR2-blocking antibodies (Covalab) before the challenge with EG-VEGF.

***[$^3$H]Mannitol Transport (Papp).*** HPEC were grown and treated as described in the previous section. After the addition of the treatments, the tracer

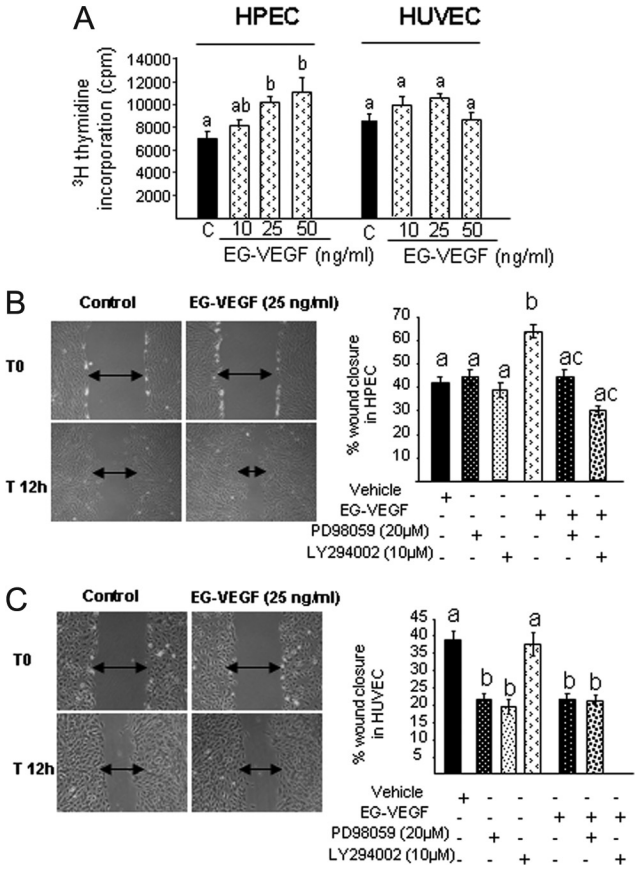


**Figure 2.** EG-VEGF increases HPEC but not HUVEC proliferation and migration. (A) [$^3$H]Thymidine incorporation into HPEC and HUVEC cells, in the absence or presence of EG-VEGF. A significant increase of HPEC proliferation was observed with 25 and 50 ng/ml EG-VEGF (*$p < 0.05$). No significant effect was observed on HUVEC cells. (B and C) Photographs of wounded HPEC and HUVEC monolayers, respectively, at 0, and 12 h after wounding. The plots show percentages of wound closure after 12 h of treatment with EG-VEGF in the absence or presence of PD98059 and the LY294002, the inhibitors of MAP kinases and PI3K, respectively. Bars with different letters are significantly different from each other ($p < 0.05$).

([$^3$H]mannitol, 125 nCi/well) was added. Samples (20 $\mu$l) of medium from the abluminal side were removed at 5, 10, 15, 20, 25 and 35 min and replaced with warm buffer. The assay was performed in triplicates. Radioactivity was measured using a scintillation counter. Papp (cm/s) was calculated using the following equation: Papp = dQ/dt × 1/Ax Co, where dQ/dt is the slope of the linear curve describing the cumulative amount of tracer transported (i.e., detected in the abluminal side) versus time [sec], A, the surface area of the filter (cm$^2$), and Co (units/ml) the initial concentration in the donor compartment (luminal side).

### *Statistical Analysis*

Statistical comparisons were made using one-way ANOVA analysis and tested for homogeneity of variance and normality ($p < 0.05$). Student's *t* test was also used when appropriate. Calculations were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

## RESULTS

### *Characterization of HPEC Cells*

After seeding, HPECs reached confluence in 10–12 d and presented an epithelial-like morphology as described before (Jinga *et al.*, 2000). As shown in Figure S1 of supplementary data, all cultured HPECs were strongly labeled for vWf, UEA lectin, and CD31 and were able to internalize LDL-DiI. Immunofluorescence staining for anti-smooth muscle actin was 98% negative, indicating that the HPEC culture was not contaminated with myofibroblastic cells. The cultured cells were highly positive for endothelial cell markers, suggesting the presence of mainly HPEC cells. Cell viability, assessed by trypan staining, was 95% before and after treatments.

### *Expression of EG-VEGF Receptors, PROKR1 and PROKR2, in Micro- and Macrovascular Placental Cells*

The first series of experiments were conducted to determine the types of prokineticin receptors expressed in HPEC and in HUVEC cells. PROKR expression was analyzed by immunohistochemistry on human placental tissue and human umbilical cord sections. Comparison of PROKR1 and PROKR2 expression levels was performed by Western blotting in isolated HPEC and HUVEC cells. As shown in Figure 1A, both PROKR1 and PROKR2 are expressed by endothelial cells within the placental villi. In the umbilical cord vein, both receptors were also detected in endothelial cells. Figure 1B illustrates a representative Western blot that shows differential expression of PROKR1 and PROKR2 in HPEC and HUVEC cells. Quantification of PROKR1 and PROKR2 protein levels in three independent experiments shows that both receptors are more abundant in HPEC than in HUVEC cells (Figure 1C).

### *EG-VEGF Effect on HPEC and HUVEC Proliferation*

In a previous report from the group of Ferrara (LeCouter *et al.*, 2001), it has been shown that EG-VEGF does not affect the proliferation of HUVECs. However, no data are available on its effect on HPEC proliferation. Here, we investigated the effect of EG-VEGF on the proliferation of both cell types. Proliferation was assessed using two different techniques: [$^3$H]thymidine incorporation (Figure 2A) and Ki67 staining (Figure S2). Our results show that EG-VEGF significantly increased HPEC cell proliferation in a dose-depen-

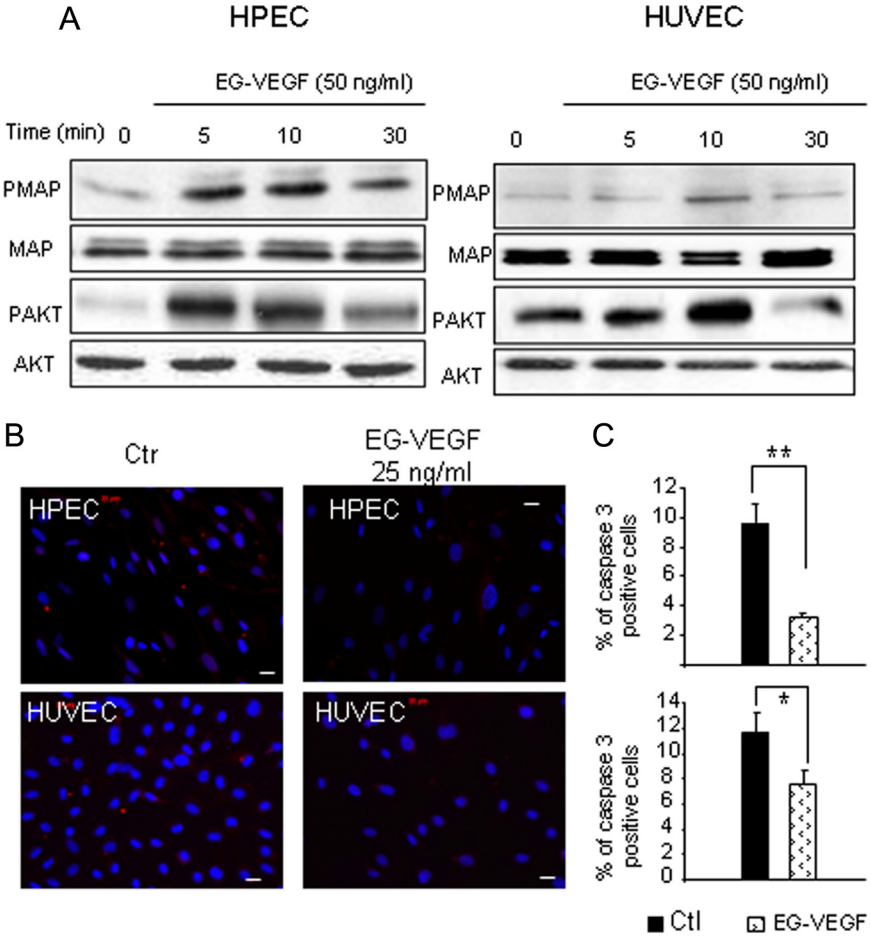


**Figure 3.** EG-VEGF is a survival factor for HPEC and HUVEC cells. (A) Representative Western blots of MAP kinase and AKT phosphorylations after treatment with EG-VEGF in HPEC and HUVEC cells. Standardization of the protein signals was done with antibodies against dephospho-MAP kinases and -AKT. (B) shows the effect of EG-VEGF on caspase 3 expression in HPEC and HUVEC cells after serum starvation and challenging with EG-VEGF (25 ng/ml). (C) The percentage of caspase 3–positive cells. Three randomly selected microscopic fields were observed, and ≥200 cells/field were evaluated. (**$p < 0.01$, *$p < 0.05$). Bar, 20 $\mu$m.

dent manner. However, no effect was observed on HUVEC proliferation. These results were confirmed by the observed increase in Ki67 staining in HPECs but not in HUVECs.

### *EG-VEGF Effect on HPEC and HUVEC Migration*

Because both HPEC and HUVEC cells expressed prokineticin receptors, we sought to investigate the effect of EG-VEGF on their migration using the monolayer wound assay. Figure 2, B and C, shows representative photographs of HPEC and HUVEC monolayers, at 0 and 6 h after their wounding with a pipette tip and subsequent incubation in the absence or presence of EG-VEGF. At 6 h of culture, the wound in HPEC was almost closed in the presence of EG-VEGF but not in the control condition. In HUVECs, EG-VEGF did not affect wound closure and therefore cell migration. Quantification of four independent experiments showed that treatment with EG-VEGF significantly increased the migration of HPECs. The closure of the wound reached 70% in the EG-VEGF condition versus only 40% in the control (Figure 2, D and E). These figures also show the effects of MAP kinases and PI3K inhibitors on the migratory process of HPECs and HUVECs in the absence or presence of EG-VEGF. Both inhibitors significantly decreased the response of HPEC to EG-VEGF without affecting their basal migration; however in HUVEC cells these inhibitors decreased the migratory process even at the basal state. These results suggest that HPEC and HUVEC respond differentially to promigratory stimuli, and this might in part explain their opposite response to EG-VEGF. To further investigate the differential response of HPECs and HUVECs to EG-VEGF, we compared the expression levels of the main G proteins reported to be associated to the activation of PROKR1 and PROKR2, i.e., G$\alpha$11, G$\alpha$12, G$\alpha$13, G$\alpha$i1, and G$\alpha$i2 (Chen *et al.*, 2005; Ngan *et al.*, 2008). Our data show that HPECs and HUVECs did not show any differences in their G$\alpha$12 and G$\alpha$13 levels; however, there was a slight difference in the expression level of G$\alpha$11, and threefold differences in those of G$\alpha$i1 and G$\alpha$i2 (Figure S4). G$\alpha$i1 was significantly more abundant in HUVEC compared with HPECs, and G$\alpha$i2 was significantly more abundant in HPECs than in HUVECs.

### *EG-VEGF Effect on HPEC and HUVEC Survival*

It is well documented that mitogenic and survival effects of EG-VEGF on endothelial cells involve the induction of MAPK and PI3K phosphorylation (Kisliouk *et al.*, 2003). We examined whether EG-VEGF activated these signaling pathways in HPECs and HUVECs. Cells were serum-starved for 24 h and then examined for the phosphorylation of p42/44 MAPKs and AKT in response to EG-VEGF treatment. Figure 3A shows the Western blot analysis of phospho-MAPKs and phospho-AKT after EG-VEGF treatment. Strong phosphorylation of p44/42 MAPKs and AKT proteins in response to EG-VEGF were observed after only 5 min in HPECs. In HUVECs, there was a slight increase in MAPK and AKT phosphorylation. EG-VEGF effect on AKT phosphorylation suggested to us that this factor might be a survival factor for both types of cells. This was confirmed by the measurement of HPEC and HUVEC survival after 24 h of serum starvation and a 24-h challenge with EG-VEGF (10, 25, 50, and 100 ng/ml). Caspase 3 staining was used to detect apoptotic cells. Figure 3B shows that serum starvation of HPEC and HUVEC resulted in 9.6 $\pm$ 1.23 and 11.7 $\pm$ 1.5% of cells becoming apoptotic in both cells types, respectively, and that the presence of EG-VEGF significantly decreased this proportion to 3.25 $\pm$ 0.23 and 7.6 $\pm$ 1.03%, respectively.

### *EG-VEGF Effect on Angiogenic Responses of HPEC and HUVEC Cells*

***Pseudovascular Organization.*** Endothelial cells are well known to self-organize as networks of vascular-like structures when grown on Matrigel (Murray, 2003). Here, we investigated the effect of EG-VEGF on pseudovascular organization of HPECs and HUVECs, with photographs taken every hour. Figure 4A shows representative photographs of HPEC and HUVEC cells at 0 and 10 h of culture on Matrigel and under different treatments. In the control condition, HPEC start to organize into tube-like structures by 3 h. By 10 h of culture only half of the plate was organized in a network of tubular structures. In the EG-VEGF treatment condition, this process was increased with an organization starting as early as after 1 h of culture, and the plate was completely organized by 10 h of culture. In contrast, EG-VEGF did not affect HUVEC organization. Quantification of four independent experiments shows that EG-VEGF significantly increased HPEC but not HUVEC organization as compared with the control condition, (Figure 4B).

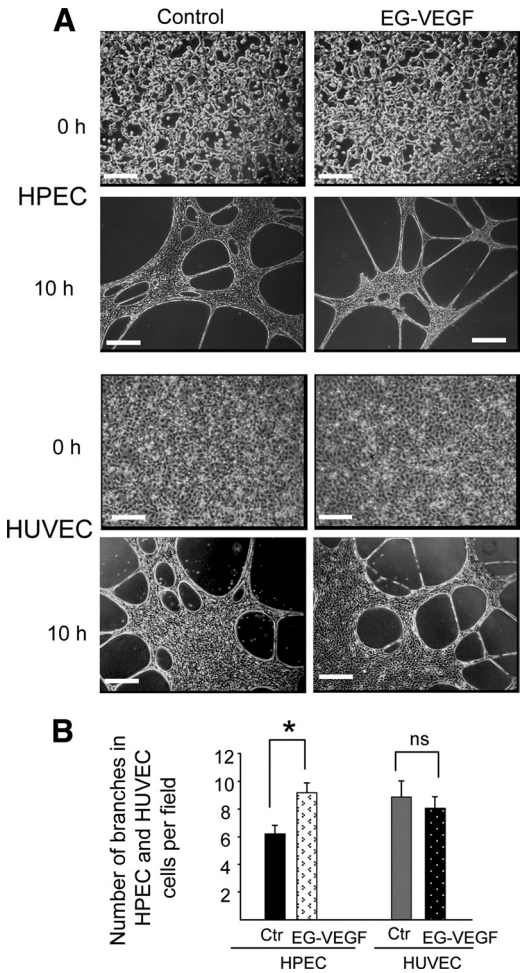


**Figure 4.** EG-VEGF increases HPEC but not HUVEC cord-like organization. (A) Photographs of HPEC and HUVEC cells cultured on Matrigel for 0 and 10 h in the absence or the presence of EG-VEGF (25 ng/ml). Note that EG-VEGF increased HPEC but not HUVEC organization into cord-like structures compared with the control condition. (B) Measurements of the number of branches formed by the cells after 10 h of culture in the absence or presence of EG-VEGF. *$p < 0.05$.

***Endothelial Cell Sprouting.*** A three-dimensional in vitro angiogenesis system was used to study the role of EG-VEGF on HPEC and HUVEC sprouting. A 12-h incubation of HPECs with EG-VEGF (25 ng/ml) resulted in a dose-dependent increase in sprout formation. No effect of EG-VEGF was observed on HUVEC sprouting. Figure 5, A and C, show representative photographs of HPEC and HUVEC spheroids at the time of their incubation with EG-VEGF and 12 h later. To control the response of the cells, we examined the effect of two potent angiogenic factors, FGF-2 and VEGF, on the sprouting. As expected, both FGF-2 and VEGF induced significant sprouting of the spheroids compared with the control conditions. Quantification of four independent experiments show that EG-VEGF treatment has a stronger effect than VEGF in HPEC cells. In HUVEC, no effect of EG-VEGF was observed (Figure 5, B and C).

In the aforementioned experiments, we have shown that HPEC express both type 1 and type 2 receptors for EG-VEGF (PROKR1 and PROKR2). To determine which type of receptor was involved in EG-VEGF effect on HPEC sprouting, we examined the effect of EG-VEGF on HPECs in which PROKR1 or PROKR2 mRNA expression was silenced by specific siRNAs, (see Figure S3 for siRNA strategy). In addition, the strategy of receptors blockade by specific neutralizing antibodies was also used (see Figure S3 for antibody strategy). Our results show that treatment with PROKR2 siRNA (Figure 6A) or antibody (Figure 6B) did not affect EG-VEGF stimulation of sprouting. However, PROKR1 siRNA or antibody reversed its effect. To measure the effect of EG-VEGF on HPEC sprouting, we quantified the number of sprouts formed under all conditions. The graphs in Figure 6, C and D, shows that EG-VEGF significantly increased the number of sprouts and that EG-VEGF effect was specifically reversed by the PROKR1 siRNA treatment and by PROKR1 antibody blockade.

### *EG-VEGF Effect on HPEC Permeability*

In the placenta, the microvascular endothelium is known to participate in angiogenesis and maintenance of blood fluidity (Rodgers, 1988; Murray, 2003) and is also a discriminating layer in materno-fetal transports of solutes and nutrients. Therefore, maintenance of a semipermeable barrier by the endothelium is critically important in endothelial cell function. Both permeability and paracellular transport of HPEC monolayers were assessed. HPEC permeability was measured in response to EG-VEGF and thrombin, an enhancer of electrolyte transport.

The endothelial barrier was evaluated by transendothelial electrical resistance (TEER). Figure 7A shows the percentage of increase in the permeability of a monolayer of HPEC in response to thrombin (70 U/ml), EG-VEGF (25 ng/ml), or VEGF (25 ng/ml). TEER was recorded every 5 min for 35 min. As expected, thrombin significantly increased HPEC permeability up to 35 min. Under EG-VEGF treatment, HPEC permeability increased as early as 5 min and was maintained for up to 20 min. VEGF showed a different profile from that of EG-VEGF with a significant increase at 5 min and a quick decline thereafter.

The effect of EG-VEGF on the paracellular transport was measured using [$^3$H]mannitol. The data summarizing the permeability coefficients for [$^3$H]mannitol transport (Papp) in response to thrombin and EG-VEGF are presented in Figure 7B. The basal permeability Coefficient (Papp) in HPEC was $1.7 \times 10^{-5}$ cm/s (Figure 7C). Thrombin almost doubled [$^3$H]mannitol transport through HPECs, and EG-

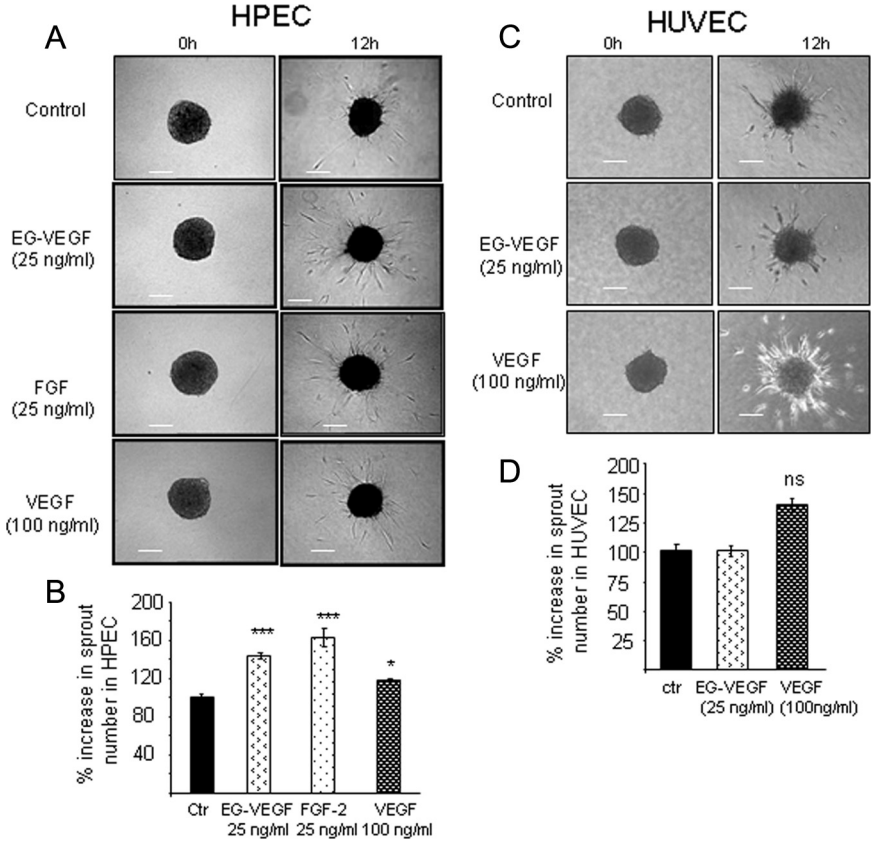


**Figure 5.** EG-VEGF, FGF, and VEGF effects on sprouting of HPEC spheroids. (A) Representative photographs of spheroids formed from HPEC cells and cultured in collagen gel for 0 or 12 h in the absence or presence of EG-VEGF (25 ng/ml), FGF-2 (25 ng/ml), and VEGF (100 ng/ml). Note that EG-VEGF increased HPEC spheroid sprouting compared with the control, FGF2, and VEGF conditions. (B) Quantification of the number of sprouts formed after 12 h in four independent experiments. (C) Representative photographs of spheroids formed from HUVEC cells and cultured in collagen gel for 0 and 12 h in the absence or the presence of EG-VEGF or VEGF (100 ng/ml). Note that EG-VEGF did not affect HUVEC spheroid sprouting compared with the control and VEGF conditions. Data represent the mean ± SEM (*$p < 0.05$, ***$p < 0.001$). Bar, 150 $\mu$m.

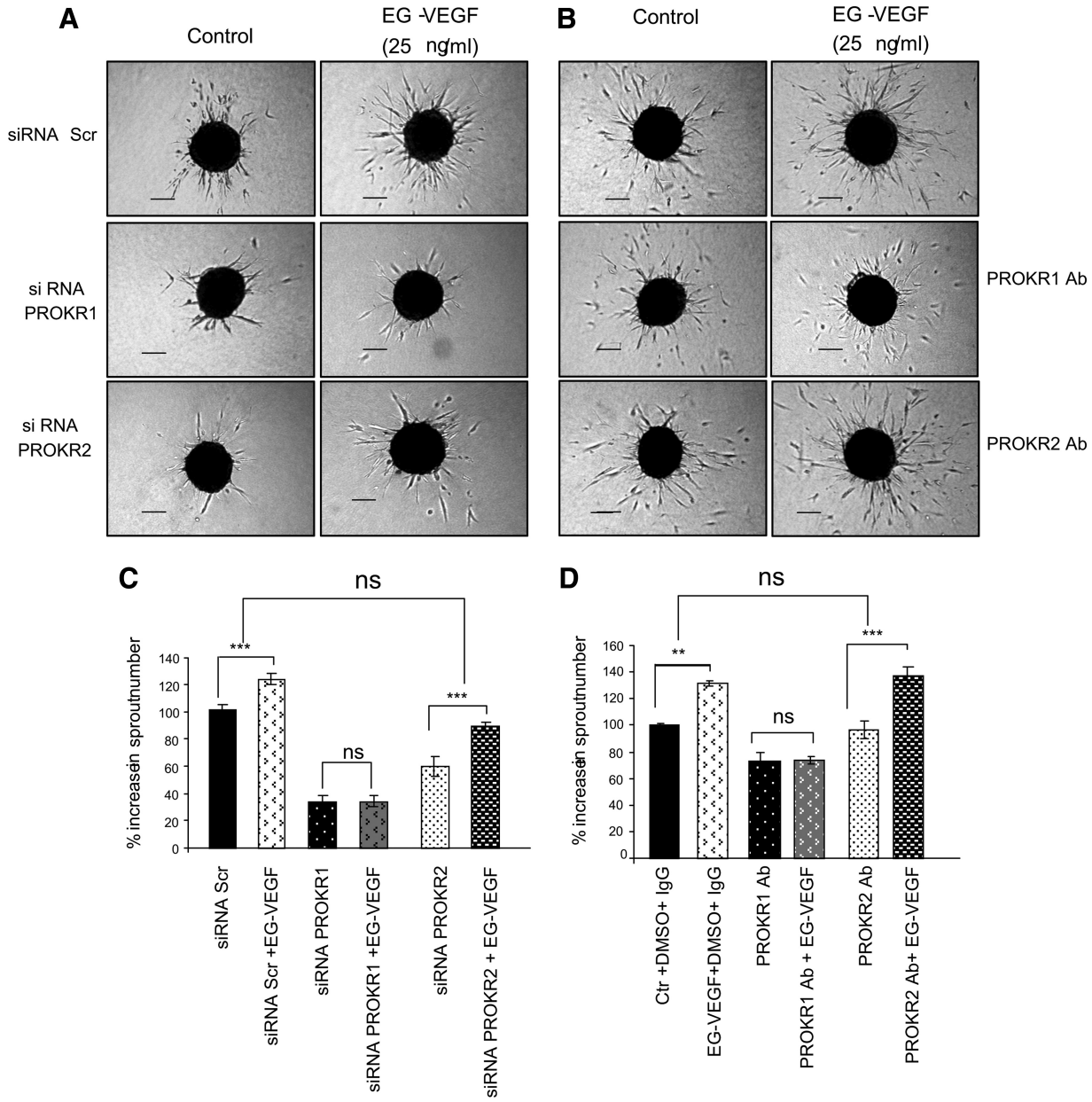


**Figure 6.** EG-VEGF angiogenic effects are mediated by PROKR1 and not PROKR2. (A and B) EG-VEGF (25 ng/ml) effect on spheroid sprouting of HPEC cells that had been silenced for PROKR1 and PROKR2 mRNA using siRNA (siRNA strategy), or treated with PROKR1 and R2 blocking antibodies (antibody strategy), respectively. (C and D) Quantifications of the number of sprouts in three independent experiments for both strategies. In the two sets of experiments, EG-VEGF significantly increased the number of sprouts. Both siRNA to PROKR1 and its blocking antibody inhibited EG-VEGF effect. However, nor siRNA to PROKR2, neither its blocking antibody did affect the spheroid sprouting. Data represent the mean ± SEM (***$p < 0.001$, **$p < 0.01$, ns, not significant). Bar, 100 $\mu$m.

VEGF increased this transport to almost the same levels achieved by thrombin.

As for the sprouting experiments, we also sought to determine the type of receptor that mediates EG-VEGF effects on permeability. Both, siRNA and antibody strategies were used to differentiate between the two receptor types. As expected, EG-VEGF significantly increased HPEC permeability in the absence of any other treatment. Scramble siRNA, siRNA PROKR1, or siRNA PROKR2 alone did not affect basal HPEC permeability. However, invalidation of PROKR2 significantly abolished the response to EG-VEGF. Under PROKR1 mRNA invalidation EG-VEGF effect was maintained (Figure 8A). These results were substantiated with the antibody strategy, showing that only PROKR2 blockade affects EG-VEGF effects on HPEC permeability. Altogether these results demonstrate that EG-VEGF mediates its effect on the permeability via the activation of PROKR2 and not PROKR1 receptor (Figure 8B).

## DISCUSSION

Using a multifaceted strategy that included molecular, immunochemical, and functional approaches, we have characterized the angiogenic processes mediated by the new factor, EG-VEGF, in a highly vascularized organ, the

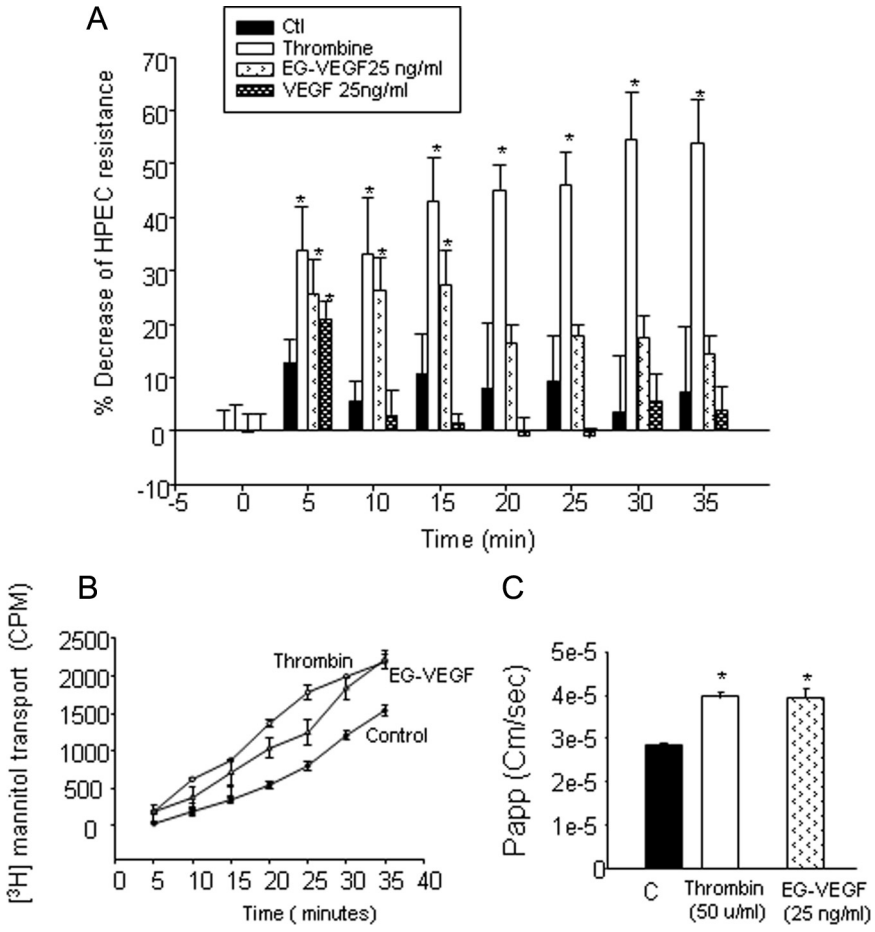


**Figure 7.** Effects of EG-VEGF, VEGF, and thrombin on the transendothelial electrical resistance (TEER) across HPEC monolayers. (A) The decrease in the TEER of HPEC cells after their incubation with EG-VEGF (25 ng/ml), VEGF (25 ng/ml), or thrombin (70 U/ml). Changes in resistance were measured at the time points 0, 5, 10, 15, 20, 25, and 35 min. Data represent the means ± SEM from three independent experiments. The results were normalized to the respective control. (*$p < 0.05$). (B). Effects of EG-VEGF and thrombin on the paracellular transport of [$^3$H]mannitol in HPEC cells. The graph represents the plot of [$^3$H]mannitol accumulation in the abluminal chamber of HPECs. (C) The permeability coefficient of EG-VEGF and thrombin that was calculated as described in *Materials and Methods*. Data represent the mean ± SEM from three independent experiments (*$p < 0.05$).

human placenta. We showed that EG-VEGF controls diverse angiogenic processes including proliferation, migration, tube organization, sprouting, endothelial permeability, and paracellular transport.

In previous reports, it has been shown that EG-VEGF induced phosphorylation of the mitogen-activated protein kinases, ERK1/2, and the Akt serine/threonine kinase of the PI3K cell survival pathway (Lin *et al.*, 2002b; Kisliouk *et al.*, 2003). Here, we showed that EG-VEGF activated both pathways in HPECs and HUVECs. These data suggest that EG-VEGF is a new mitogenic and prosurvival factor in the placenta.

In a previous report from the group of Ferrara, no effect of EG-VEGF was observed on HUVEC cell proliferation (LeCouter *et al.*, 2001). Here, we confirmed their findings by demonstrating that EG-VEGF has no effect on HUVEC proliferation and further showed its lack of effect on HUVEC migration, pseudovascular organization, and sprouting. Moreover, we observed that EG-VEGF is a potent angiogenic factor in the placenta by demonstrating its selective effect on HPEC, the microvascular cells that cover the fetal capillaries of chorionic villi and govern placental angiogenesis and growth.

Much of our knowledge on the control of placental angiogenesis comes from model systems, and particularly from HUVEC. These cells have been used as a model for endothelial cells in many studies describing the regulation of endothelial specific growth factors (Roberts *et al.*, 1998; Murthi *et al.*, 2007), and although they have proven to be a useful model, HUVECs are macrovascular endothelial cells exposed to oxygenated blood and thereby atypical endothelial cells in their physiological context. Nowadays, growing literature in this field shows that the placental microvascular endothelial cells differ in phenotype, gene expression, and physiology from macrovascular endothelial cells (Lang *et al.*, 2001, 2003; Lang *et al.*, 2003). Therefore, our findings add a new differentiating parameter between micro- and macrovascular cells in the placenta, and support a differential angiogenic potential for EG-VEGF in the two endothelial environments. The differential response of HUVEC and HPEC to EG-VEGF might be explained by their differential levels of G$\alpha$11, G$\alpha$i1, and G$\alpha$i2 expression. In fact, HPEC cells express three times more G$\alpha$i2 and three times less G$\alpha$i1 compared with HUVECs. The higher levels of G$\alpha$i1 over G$\alpha$i2 has been previously reported at the protein levels in HUVEC cells (Masri *et al.*, 2006). Moreover, our results are completely in line with studies that showed that the degree of inhibition of adenylyl cyclase was higher in cells expressing G$\alpha$i2 than in cells expressing G$\alpha$i1 (Massotte *et al.*, 2002; Masri *et al.*, 2006). In HPEC cells we have also demonstrated (data not shown) that EG-VEGF effect was abolished in the presence of pertussis toxin, indicating the direct involvement of Gi proteins in the signaling pathway of EG-VEGF in these cells.

In previous reports from our lab, we showed that PROKR1 and PROKR2 are strongly expressed in the placenta during the first trimester of pregnancy (Hoffmann *et al.*, 2006, 2007, 2009). Here, we substantiate these results and further show that PROKRs are significantly more abundant in HPEC than in HUVEC cells. This difference in EG-VEGF

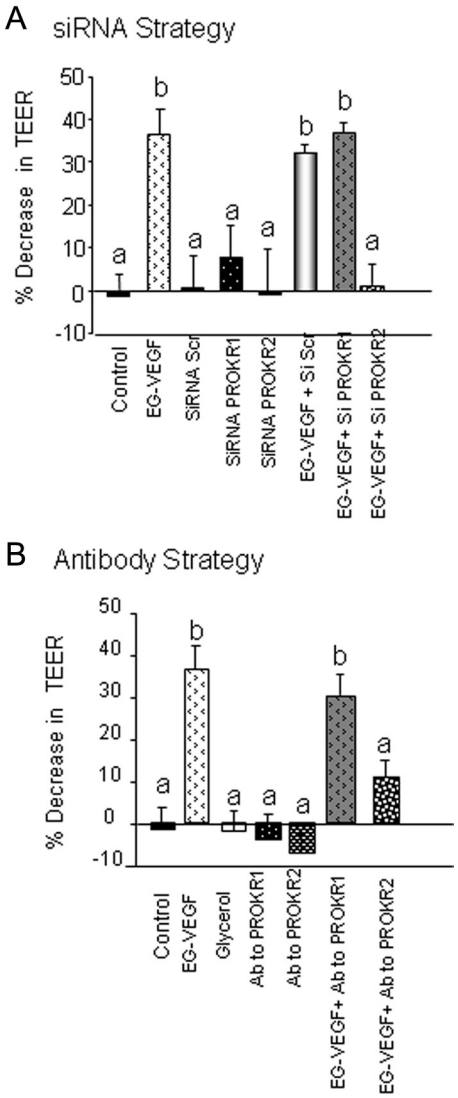


**Figure 8.** EG-VEGF effects on HPEC permeability are mediated by PROKR2 and not PROKR1: Panels (A) and (B) show EG-VEGF (25 ng/ml) effect on the permeability of HPEC cells that had been silenced for PROKR1 and PROKR2 mRNA using siRNA (siRNA strategy), or treated with PROKR1 and R2 blocking antibodies (antibody strategy), respectively. In the two sets of experiments, EG-VEGF significantly increased HPEC permeability. Both siRNA to PROKR2 and its blocking antibody inhibited EG-VEGF effect. However, nor siRNA to PROKR1, neither its blocking antibody did affect the permeability. Data represent the mean ± SEM. Bars with different letters are significantly different from each other ($p < 0.05$).

receptor levels might also explain the differential response of these cells to EG-VEGF.

Using siRNA and blocking antibody strategies in HPEC, we could differentiate the effects mediated by PROKR1 from those mediated by PROKR2. We showed that PROKR1 is associated with EG-VEGF angiogenic effects, whereas PROKR2 is rather associated with it effects on cell permeability, results that are fully in line with those recently published by the group of Nebigil who showed that the same differential role exists for these receptors in cardiomyocytes (Guilini *et al.*, 2010; Urayama *et al.*, 2008, 2009) and that PROKR1 overexpression in cardiomyocytes was associated with an increase in their survival (Urayama *et al.*, 2008). Therefore, one can speculate that EG-VEGF effects on HUVEC cell survival might well be mediated by PROKR1 and not PROKR2.

PROKR1 and PROKR2 receptors share 87% homology in their amino acid sequence, which may suppose similar activation mechanisms for the two receptors; however a difference in the final cellular response in a cell type that expresses both PROKRs, might also depend on the repertoires of G proteins present in each cell type. It is now well documented that the selectivity of coupling depends on the G-protein concentration in a given cell (Slessareva *et al.*, 2003). This suggests that in living cells the expression levels of specific G-protein subunits may regulate receptor-coupling preferences. In addition, we observed that HPEC and HUVEC cells express different Gαi proteins. This may allow these cells to perform different physiological functions in response to stimulation by the same ligand.

Transendothelial permeability is one of the specialized functions of the vascular endothelium. Here, we show that EG-VEGF increases the permeability and the paracellular transport of the placental microvascular endothelium, suggesting that this factor controls not only placental angiogenesis, but also some aspects of its physiological features such as, permeability and transport involved in the maternofetal exchanges. The endothelial cells of the human placenta are nonfenestrated, and adjacent cells are linked by junctional complexes comprising both tight and adherent junctions (Metz *et al.*, 1976; Palade *et al.*, 1979; Burton and Jauniaux, 1995). Recently, it has been shown that in hepatic sinusoidal cells (HHSECs), an endothelial cell that exclusively expresses PROKR2, which stimulates the internalization of ZO-1, the main protein involved in junctional complexes and cell–cell adhesion (Guilini *et al.*, 2010). These findings suggest that prokineticin effects on endothelial permeability may well involve a regulation of tight junction proteins. The mechanism by which EG-VEGF increases the permeability in HPEC, is, however, still to be investigated.

Various factors have been reported to stimulate placental angiogenesis and vasculogenesis in an autocrine or paracrine manner, as well as directly or indirectly by stimulating proliferation and differentiation of endothelial precursor cells (Gallery *et al.*, 1991). However, a specific factor for the placenta was still to be discovered. Here we show that EG-VEGF might be one of these tissue-specific factors that could control angiogenesis both during the first trimester and at term, as in term placentas, an increase in angiogenesis has been proposed to insure appropriate blood distribution for a successful delivery. One can therefore speculate that EG-VEGF might be involved in that process too.

The observation that EG-VEGF increases sprouting, vascular organization, and permeability of HPECs to an extent similar to those of VEGF and FGF-2, suggests that EG-VEGF is important in the development of the placental vascular bed. In the ovary, the angiogenic response induced by EG-VEGF is indistinguishable from that induced by VEGF (Lin *et al.*, 2002b), which indicates that EG-VEGF plays complementary or overlapping roles with VEGF in vascular development.

Recent data from our group have shown that EG-VEGF produced by the syncytiotrophoblast layer acts on the extravillous trophoblast cells outside the villi. However, an autocrine and/or paracrine effect of this cytokine is still to be investigated inside the villi. Here we bring evidence that EG-VEGF is a new factor that controls en-

dothelial growth within the placental villi and therefore participates to the development of the vascular tree, a process that drives the whole growth of the placenta.

In conclusion, our data characterized the molecular angiogenic processes mediated by EG-VEGF and suggest that this peptide should be ranked among the important regulators of human placental angiogenesis.

## ACKNOWLEDGMENTS

We acknowledge the following sources of funding: INSERM (U878), Commissariat à l'Energie Atomique (DSV/iRTSV/LAPV), the Région Rhône Alpes (CIBLE-2008), and ARC Equipement 2009. S.B. was supported by a Doctoral Scholarship from the French Ministry of Education.

## REFERENCES

Burton, G. J., and Jauniaux, E. (1995). Sonographic, stereological and Doppler flow velocimetric assessments of placental maturity. Br. J. Obstet. Gynaecol. *102*, 818–825.

Charnock-Jones, D. S., and Burton, G. J. (2000). Placental vascular morphogenesis. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol. *14*, 953–968.

Charnock-Jones, D. S., Kaufmann, P., and Mayhew, T. M. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation. Placenta *25*, 103–113.

Chen, J., Kuei, C., Sutton, S., Wilson, S., Yu, J., Kamme, F., Mazur, C., Lovenberg, T., and Liu, C. (2005). Identification and pharmacological characterization of prokineticin 2 beta as a selective ligand for prokineticin receptor 1. Mol. Pharmacol. *67*, 2070–2076.

Dellian, M., Witwer, B. P., Salehi, H. A., Yuan, F., and Jain, R. K. (1996). Quantitation and physiological characterization of angiogenic vessels in mice: effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. Am. J. Pathol. *149*, 59–71.

Demir, R., Kayisli, U. A., Seval, Y., Celik-Ozenci, C., Korgun, E. T., Demir-Weusten, A. Y., and Huppertz, B. (2004). Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: differences between placental vasculogenesis and angiogenesis. Placenta *25*, 560–572.

Demir, R., Seval, Y., and Huppertz, B. (2007). Vasculogenesis and angiogenesis in the early human placenta. Acta Histochem. *109*, 257–265.

Denison, F. C., Battersby, S., King, A. E., Szuber, M., and Jabbour, H. N. (2008). Prokineticin-1, a novel mediator of the inflammatory response in third-trimester human placenta. Endocrinology *149*, 3470–3477.

Gallery, E. D., Rowe, J., Schrieber, L., and Jackson, C. J. (1991). Isolation and purification of microvascular endothelium from human decidual tissue in the late phase of pregnancy. Am. J. Obstet. Gynecol. *165*, 191–196.

Guilini, C., Urayama, K., Turkeri, G., Dedeoglu, D. B., Kurose, H., Messaddeq, N., and Nebigil, C. G. (2010). Divergent roles of prokineticin receptors in the endothelial cells: angiogenesis and fenestration. Am. J. Physiol. Heart Circ. Physiol. *298*, H844–H852.

Hebert, C. A., Luscinskas, F. W., Kiely, J. M., Luis, E. A., Darbonne, W. C., Bennett, G. L., Liu, C. C., Obin, M. S., Gimbrone, M. A., Jr., and Baker, J. B. (1990). Endothelial and leukocyte forms of IL-8. Conversion by thrombin and interactions with neutrophils. J. Immunol. *145*, 3033–3040.

Hoffmann, P., Feige, J. J., and Alfaidy, N. (2006). Expression and oxygen regulation of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 and its receptors in human placenta during early pregnancy. Endocrinology *147*, 1675–1684.

Hoffmann, P., Feige, J. J., and Alfaidy, N. (2007). Placental expression of EG-VEGF and its receptors PKR1 (prokineticin receptor-1) and PKR2 throughout mouse gestation. Placenta *28*, 1049–1058.

Hoffmann, P., Saoudi, Y., Benharouga, M., Graham, C. H., Schaal, J. P., Mazouni, C., Feige, J. J., and Alfaidy, N. (2009). Role of EG-VEGF in human placentation: physiological and pathological implications. J. Cell Mol. Med. *13*, 2224–2235.

Jinga, V. V., Gafencu, A., Antohe, F., Constantinescu, E., Heltianu, C., Raicu, M., Manolescu, I., Hunziker, W., and Simionescu, M. (2000). Establishment of a pure vascular endothelial cell line from human placenta. Placenta *21*, 325–336.

Kisliouk, T., Levy, N., Hurwitz, A., and Meidan, R. (2003). Presence and regulation of endocrine gland vascular endothelial growth factor/prokineticin-1 and its receptors in ovarian cells. J. Clin. Endocrinol. Metab. *88*, 3700–3707.

Lang, I., Hoffmann, C., Olip, H., Pabst, M. A., Hahn, T., Dohr, G., and Desoye, G. (2001). Differential mitogenic responses of human macrovascular and microvascular endothelial cells to cytokines underline their phenotypic heterogeneity. Cell Prolif. *34*, 143–155.

Lang, I., Pabst, M. A., Hiden, U., Blaschitz, A., Dohr, G., Hahn, T., and Desoye, G. (2003). Heterogeneity of microvascular endothelial cells isolated from human term placenta and macrovascular umbilical vein endothelial cells. Eur. J. Cell Biol. *82*, 163–173.

Leach, L., Babawale, M. O., Anderson, M., and Lammiman, M. (2002). Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta. J. Vasc. Res. *39*, 246–259.

LeCouter, J., *et al.* (2001). Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature *412*, 877–884.

Lin, D. C., Bullock, C. M., Ehlert, F. J., Chen, J. L., Tian, H., and Zhou, Q. Y. (2002a). Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. J. Biol. Chem. *277*, 19276–19280.

Lin, R., LeCouter, J., Kowalski, J., and Ferrara, N. (2002b). Characterization of endocrine gland-derived vascular endothelial growth factor signaling in adrenal cortex capillary endothelial cells. J. Biol. Chem. *277*, 8724–8729.

Maldonado-Perez, D., Evans, J., Denison, F., Millar, R. P., and Jabbour, H. N. (2007). Potential roles of the prokineticins in reproduction. Trends Endocrinol. Metab. *18*, 66–72.

Masri, B., Morin, N., Pedebernade, L., Knibiehler, B., and Audigier, Y. (2006). The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. J. Biol. Chem. *281*, 18317–18326.

Massotte, D., Brillet, K., Kieffer, B., and Milligan, G. (2002). Agonists activate Gi1 alpha or Gi2 alpha fused to the human mu opioid receptor differently. J. Neurochem. *81*, 1372–1382.

Metz, J., Heinrich, D., and Forssmann, W. G. (1976). Gap junctions in hemodichorial and hemotrichorial placentae. Cell Tissue Res. *171*, 305–315.

Murray, J. D. (2003). On the mechanochemical theory of biological pattern formation with application to vasculogenesis. CR Biol. *326*, 239–252.

Murthi, P., So, M., Gude, N. M., Doherty, V. L., Brennecke, S. P., and Kalionis, B. (2007). Homeobox genes are differentially expressed in macrovascular human umbilical vein endothelial cells and microvascular placental endothelial cells. Placenta *28*, 219–223.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. *13*, 9–22.

Ngan, E. S., Shum, C. K., Poon, H. C., Sham, M. H., Garcia-Barcelo, M. M., Lui, V. C., and Tam, P. K. (2008). Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. Biochim. Biophys. Acta *1783*, 467–478.

Palade, G. E., Simionescu, M., and Simionescu, N. (1979). Structural aspects of the permeability of the microvascular endothelium. Acta Physiol. Scand. Suppl. *463*, 11–32.

Rabiet, M. J., Plantier, J. L., Rival, Y., Genoux, Y., Lampugnani, M. G., and Dejana, E. (1996). Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. Arterioscler. Thromb. Vasc. Biol. *16*, 488–496.

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671–674.

Roberts, W. G., Delaat, J., Nagane, M., Huang, S., Cavenee, W. K., and Palade, G. E. (1998). Host microvasculature influence on tumor vascular morphology and endothelial gene expression. Am. J. Pathol. *153*, 1239–1248.

Rodgers, G. M. (1988). Hemostatic properties of normal and perturbed vascular cells. FASEB J. *2*, 116–123.

Sibai, B., Dekker, G., and Kupferminc, M. (2005). Pre-eclampsia. Lancet *365*, 785–799.

Slessareva, J. E., Ma, H., Depree, K. M., Flood, L. A., Bae, H., Cabrera-Vera, T. M., Hamm, H. E., and Graber, S. G. (2003). Closely related G-protein-coupled receptors use multiple and distinct domains on G-protein alpha-subunits for selective coupling. J. Biol. Chem. *278*, 50530–50536.

Stewart, P. A., and Wiley, M. J. (1981). Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail-chick transplantation chimeras. Dev. Biol. *84*, 183–192.

te Velde, E. A., Exalto, N., Hesseling, P., and van der Linden, H. C. (1997). First trimester development of human chorionic villous vascularization studied with CD34 immunohistochemistry. Hum. Reprod. *12*, 1577–1581.

Urayama, K., Dedeoglu, D. B., Guilini, C., Frantz, S., Ertl, G., Messaddeq, N., and Nebigil, C. G. (2009). Transgenic myocardial overexpression of prokineticin receptor-2 (GPR73b) induces hypertrophy and capillary vessel leakage. Cardiovasc. Res. *81*, 28–37.

Urayama, K., Guilini, C., Turkeri, G., Takir, S., Kurose, H., Messaddeq, N., Dierich, A., and Nebigil, C. G. (2008). Prokineticin receptor-1 induces neovascularization and epicardial-derived progenitor cell differentiation. Arterioscler. Thromb. Vasc. Biol. *28*, 841–849.

Voyta, J. C., Via, D. P., Butterfield, C. E., and Zetter, B. R. (1984). Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J. Cell Biol. *99*, 2034–2040.