Impaired Vascular Endothelial Growth Factor Expression and Secretion during *in vitro* Differentiation of Human Primary Term Cytotrophoblasts

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ABSTRACT

Introduction Vascular endothelial growth factor A (VEGF-A) is one of the main growth factors involved in placental vasculogenesis and angiogenesis, but its placental expression is still ambiguous. During *in vitro* cultures of primary term cytotrophoblasts, VEGF could not be detected in the supernatants by enzyme-linked immunosorbent assays (ELISA). One hypothesis is that VEGF is immediately and completely bound to its soluble receptor after secretion, and cannot be recognized by the antibodies used in the commercial ELISA kits. We decided to verify this hypothesis by measuring VEGF-A expression during *in vitro* cultures of primary term cytotrophoblasts. *Methods* Term cytotrophoblasts were cultured under 21% and 2.5% O_2 for 4 days. *VEGF-A* transcripts were quantified by real-time polymerase chain reaction. The proteins from cell lysates and concentrated media were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing and reducing conditions, and VEGF-A immunodetected by western blotting. *Results VEGF* mRNA expression did not increase during *in vitro* cell differentiation under 21% O_2, but slightly increased under 2.5% O_2 only at 24 hours. VEGF-A monomer was not detected in the cell lysates and in the concentrated supernatants, while a ~42 KDa band corresponding to the precursor L-VEGF was detected in all the cellular extracts. *Discussion* Isolated term villous cytotrophoblasts produce the L-VEGF precursor but they do not secrete VEGF-A even under low-oxygen tension. The question remains about the origin of VEGF in pregnancy but also about the biological role of L-VEGF, which can represent a form of storage for rapid VEGF secretion when needed.

Keywords: VEGF, Secretion, Human Trophoblasts, Differentiation, Placental Angiogenesis

I**NTRODUCTION**

During pregnancy, the placenta is the site of an intense vasculogenesis and angiogenesis to respond to the growing needs of the embryo and fetus at each step of its development. Vasculogenesis or formation of new blood vessels from endothelial precursor cells within the placental villous core starts as early as 5 weeks' gestation and is progressively followed by branching and non-branching angiogenesis [1].

The main angiogenic factors of the vascular endothelial growth factor (VEGF) family [2] that modulate placental vascular development are VEGF-A [3] and placental growth factor (PlGF) [4]. VEGF-A and PlGF are encoded by two different genes that give by alternative splicing several isoforms that differ by the length of their amino acid sequence. There are 5 major VEGF-A isoforms (121, 145, 165, 189, and 206) and 3 PlGF isoforms (131, 152, and 183). $VEGF_{165}$, the most abundantly secreted isoform, and PlGF form homodimers but also VEGF/PlGF heterodimers. VEGF binds to cell-surface tyrosine kinase receptors, VEGFR1 and VEGFR2 (previously known as *fms*-like tyrosine kinase receptor/FLT, and kinase-insert-containing receptor/KDR) [5], but also to the coreceptors neuropilins 1 and 2 (NRP1 and NRP2) [6], while PlGF binds only to VEGFR1 and NRP1. VEGFR2 binds to VEGF with a lower affinity than VEGFR1 (Kd ~100 to 125 pM, and Kd ~10 to 20 pM, respectively) but it has the strongest angiogenic activity. Interestingly, VEGFR1 also exists as a soluble form (sVEGFR1) [7] that binds with high affinity to VEGF and PlGF to modulate their angiogenic activity.

VEGF-A and PlGF are both expressed in the placenta throughout gestation. While PlGF expression was shown to be localized in the villous trophoblast layers of the placenta, VEGF expression in the placenta is still ambiguous. Most studies on placental localization of VEGF are based on i*n situ* hybridization experiments of placental tissue, northern blot analysis of isolated cytotrophoblasts, and immunohistochemistry. *VEGF* mRNA and protein were identified in extra villous and villous trophoblasts [8,9]. Others did not detect any *VEGF-A* mRNA in the trophoblasts but only in some cells of the villous stroma, in placental glands and macrophages of the placental bed [10,11]. The placental macrophages, called Hofbauer cells that are in close contact with the vasculogenic precursor cells in the tertiary villi in early pregnancy are also suspected of expressing VEGF-A [12].

Detection and quantification of VEGF-A by radioimmunoassay in the supernatants of isolated human primary term cytotrophoblasts was reported only by one group [13]. But we and others [14,15] have not been able to detect any secreted VEGF-A in the supernatants of these cells by ELISA, while PlGF and sVEGFR1 were easily detected and quantified. Several hypotheses may be advanced. First, VEGF-A is immediately and completely bound to its soluble receptor after secretion, and cannot be recognized by the antibodies used in the commercial ELISA

kits. Second, VEGF-A is produced and secreted at a low level under the detection limit of the ELISA kit. Last, it is also possible that these cells do not express VEGF-A protein at all.

To test these hypotheses, we decided to measure *VEGF-A* mRNA expression during *in vitro* cultures of primary term cytotrophoblasts under 21% and 2.5% O_2, and to detect VEGF protein in cell lysates and concentrated supernatants by western blotting under denaturing and reducing conditions.

MATERIAL AND METHODS

Placenta Collection

The placentas were collected from elective cesarean or vaginal full-term deliveries with the approval of the ethical committee at Université Catholique de Louvain, Brussels, Belgium. Written informed consent was received from all the patients.

Cytotrophoblast Isolation and Purification

Cytotrophoblasts were isolated and purified following a modified version of a protocol already published elsewhere [16]. In brief, the placenta was cut into 1-cm^2 pieces thoroughly washed with cold 0.9% NaCl solution. The fragments were digested at 37 °C with 2.4 U/ml dispase II (Life Technologies, New York, USA) and 100 U/ml DNase I, grade II (Roche, Mannheim, Germany). After sequential filtration through 200, 100 and 40-µm filters, the cells were separated by density gradient centrifugation in a 5–70% discontinuous Percoll™ gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cellular fraction corresponding to a density of 1.048-1.062 g/l was collected and washed with FBS-free Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Paisley, UK), containing 50 µg/ml gentamicin (Carl Roth, Karlsruhe, Germany). The cells were then counted with a hemocytometer. The cell viability was assessed by Trypan Blue exclusion (Sigma-Aldrich, Steinheim, Germany). The average yield was 107 x 10^6 cytotrophoblasts (±38 x 10^6) for 72 gr of placental tissue treated. We previously demonstrated that our cytotrophoblast preparations were ~98% pure [17].

Cell culture and treatments

The cells were cultured in IMDM complemented with 10% fetal bovine serum (Life Technologies), and 50 µg/ml gentamicin (Carl Roth) in 6-well plates with a total medium volume of 2 ml or in T75 flasks with a total volume of 15 ml at a density of 400,000 cells/cm^2. The mononucleated cytotrophoblasts were first allowed to adhere to the surface of the wells for the first 2 hours. The medium was then aspirated and the cells gently washed with 1X phosphate buffer saline (PBS) to remove syncytiotrophoblasts that potentially remained and unattached cells. Fresh medium was then added, and the cells were incubated for 4 days in two separate atmosphere-controlled incubators either at 21% O_2-5% CO_2 (normoxia) and 37°C or at 2.5% O_2-5% CO_2 (hypoxia) balanced with N_2 and 37°C.

The squamous cell carcinoma cell line, SiHa (ATCC, HTB-35) was cultured in Eagle's Minimum Essential Medium (EMEM) (Life Technologies) complemented with 10% FBS and 50 µg/ml gentamicin for 24 h under 21% O_2 or 2.5% O_2. Inhibition of VEGF secretion was achieved by adding 1X Protein Transport Inhibitor Cocktail (PTIC) (Life Technologies) to the medium of SiHa cells cultured for 24 hours under 2.5% O_2.

The medium was changed daily by removing the cells from the incubators and adding new medium previously equilibrated for 15 minutes in the respective incubators. The cells did not spend more than 5 minutes outside the incubators during daily medium change to prevent a possible hypoxia/reoxygenation effect.

Immunofluorescence

The cytotrophoblasts were cultured on Cellview™ cell culture slides (Greiner Bio-One, Frickenhausen, Germany) for 96 hours under 21% O_2. The cells were rinsed with cold 1X PBS and fixed for 10 min at 4 °C in 100% methanol, rinsed again with 1X PBS and stored at 4 °C in PBS for no longer than 4 days. The preparations were preincubated in 1X PBS containing 0.5% bovine serum albumin (BSA), 2.5% normal goat serum and 0.1% saponin for 15 min. at 4 °C, then incubated with the primary mouse monoclonal anti-Desmoplakin I+II (see table 1) diluted in the same solution overnight at 4 °C. The cells were rinsed six times for 5 min in PBS containing 0.1% saponin and then incubated for 1 h at room temperature with the same washing solution containing Alexa Fluor® 555-conjugated goat anti-rabbit IgG (H+L) $F(ab')_2$ fragment (Cell Signaling Technology, Danvers, MA, USA) diluted at 1:500. Cell nuclei were counter stained with Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) when slides were mounted using ProLong® Gold antifade reagent (Invitrogen, Eugene, OR, USA). The fluorescence was examined using cellSens image acquisition software v1.6 (Olympus Corporation, Tokyo, Japan).

RNA Extraction and Purification

The wells were washed three times with 1X PBS and total RNA was extracted and purified using Roti®-Prep RNA Mini kit (Carl Roth GmbH) following the manufacturer protocol. Total RNA concentration was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA). The ratios of absorbance at 260 and 280, and at 260 and 230 were used to estimate RNA quality, sufficient for subsequent qPCR experiments.

Reverse Transcription

Cellular RNA (0.5 µg) was used as a template for the reverse transcription using qScript™ cDNA superMix (Quanta Biosciences, Gaithersburg, MD, USA). cDNA was diluted 10 times with RNase-free water.

Plasmids for Copy Number Determination

Twenty nanograms of cDNA from undifferentiated cytotrophoblasts were used as template to amplify a 94-bp *HCGB* fragment, a 133-bp VEGF fragment, and a 222-bp *PlGF* fragment using primers designed for the real-time quantitative polymerase chain experiments (see table 1)

with Maxima Hotstart PCR Master Mix (Fermentas-Thermo Scientific, Foster City, CA, USA) in a GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA). The thermal protocol was: 95 °C, 2 min followed by 40 cycles of a denaturation step at 95 °C, 30 sec, a hybridization step at 60 °C, 30 sec and an extension step at 72 °C, 15 sec.

Amplified *HCGB, VEGF* and *PlGF* fragments were cloned into pCR2.1 or pCR4 vectors using TOPO-TA cloning kit (Life Technologies) and verified by sequencing (BaseClear, Leiden, Netherlands). The three plasmids were linearized by cutting with Xmn I enzyme (Promega, Madison, WI, USA).

Real-time quantitative polymerase chain reaction

Five nanograms of cDNA and 0.1 µmol/L specific primers for *HCGB*, *VEGF*, and *PlGF* (see table 1) were added to Takyon ROX SYBR 1X MasterMix dTTP blue (Eurogentec, Seraing, Belgium). The polymerase chain reaction was performed on a StepOne Real-Time PCR System (Applied Biosystem) following the recommended protocol (Tm at 60°C). A standard curve was done with linearized *HCGB, VEGF* or *PlGF standard* vectors. Five standard dilutions were used (300.000, 30.000, 3.000, 300 and 30 copies). The specificity of the amplification was verified by doing a melting curve, which generated a single sharp peak for each product.

Secreted Protein Quantification

Intracellular VEGF-A was quantified in whole cell lysates by ELISA using human VEGF-A (lysates) ELISA kit (Thermo Scientific Pierce, Frederick, MD, USA).

Secreted PlGF was quantified in the cell supernatants by ELISA using human PlGF Quantikine ELISA kit (R&D, Minneapolis, MN, USA).

Secreted HCGB was quantified on a B.R.A.H.M.S. Kryptor Compact Plus immune analyzer (Thermo Fisher Scientific) in the department of Laboratory Medicine, Cliniques Universitaires Saint-Luc (Brussels, Belgium).

Total Protein Extraction

Cells were detached from plates using Trypsine-like enzyme (TrypLE, Life Technologies, Carlsbad, CA, USA). Total protein extraction was performed using M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, Illinois, USA) following the manufacturer protocol. Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added to a final concentration of 1X.

Medium Concentration

High molecular weight proteins were removed from the supernatants using Pierce™ protein concentrator PES, 100 MWCO (Thermo Scientific, UK). The flow-through was then concentrated using Pierce™ Protein concentrator PES, 3K MWCO (Thermo Scientific). The medium was concentrated ~30 times, starting with 15 ml and obtaining a final volume of 500-600 µl after concentration.

Western blotting

Twenty micrograms of total protein extract or concentrated medium, and ten nanograms of recombinant human $VEGF_{165}$ (Cell Signaling, Danvers, MA, USA) were reduced with 50 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) (Thermo Scientific). The samples were loaded onto a 10% Acrylamide/Bisacrylamide Bolt™ Bis-Tris mini gel (Invitrogen by Thermo Fisher Scientific) and separated in 1X Bolt™ MES SDS running buffer for 22 minutes at 200 volts. Prestained Protein Ladder (ab116028, Abcam, Cambridge, UK) was used to check the transfer efficiency and to estimate the molecular weight of the proteins. Proteins were transferred onto an Invitrolon™ PVDF membrane (Invitrogen) for one hour at 20 volts in 1X Bolt™ transfer buffer with 10% methanol. After complete transfer, the membrane was blocked for one hour in 5% non-fat dry milk in 1X TBS-T (50 mM Tris/Tris-HCl, 138 mM NaCl, 20 mM KCl and 0.1% Tween-20). The membrane was then incubated overnight at 4 °C with the primary antibodies (see table 1) diluted in 1X TBS-T containing 5% non-fat dry milk. Specificity of the antibody against VEGF was determined by adding a blocking peptide (ab46160, Abcam) to the anti-VEGFA antibody solution for 30 minutes prior the overnight incubation at 4 °C. The membrane was washed three times with 1X TBS-T for 15 min each and incubated for one hour at room temperature with the HRP-conjugated secondary antibodies. Bound antibodies were detected using the SuperSignal™ West Pico PLUS chemiluminescent substrate (VEGF and PlGF detection) or Pierce ECL western blotting substrate (GAPDH detection) (Thermo Scientific, Rockford, IL, USA), and Amersham Hyperfilm™ ECL films (GE Healthcare Limited).

N-terminal anti L-VEGF antibody

A specific polyclonal antibody against L-VEGF was produced in rabbit using the services of Kaneka-Eurogentec (Seraing, Belgium) custom antibody services. The immunogen recommended by the manufacturer was a 15 amino-acid peptide fragment corresponding to the sequence Glu_{156}-Ala_{170} located in the 180–residue N-terminal L-fragment linked to the KLH carrier protein. The specificity of the antibody was tested with the purified peptide.

Statistical analysis

A minimum of 6 placentas was used to isolate and purify cytotrophoblasts. Gene and protein quantification measured by real-time qPCR and ELISA was done in duplicate for each sample. For the western blotting experiments, 3 placentas were used. All Results were expressed as the mean ± SEM, and were compared using unpaired sample t test followed by Mann-Whitney's post-test as indicated in the figure legends. Graphs and statistical analysis were performed using GraphPad Prism® version 8.0.1 for windows (GraphPad software, San Diego, CA, USA).

RESULTS

In vitro Cytotrophoblast differentiation into syncytiotrophoblasts

Since low-oxygen tension is known to upregulate VEGF expression, cytotrophoblasts were cultured for 96 hours (or 4 days) under 21% O_2, and 2.5% O_2. The cells and their supernatants were harvested every 24 hours for mRNA and protein quantification. Differentiation was assessed by quantification of *HCGB* mRNA expression by real-time PCR (Fig. 1a) and HCGB protein secretion by ELISA (Fig. 1b). When the cells were cultured under 21% O_2, *HCGB* mRNA copy number per µg total RNA increased from 0.26 x 10^6 (±0.09 SEM) copies at 0 h up to 4.75 x 10^7 (±2.67 x 10^7 SEM) at 96 h. To the contrary, under 2.5% O_2 which corresponds to hypoxia, the copy number increased only very slightly to a maximum of 0.76 x 10^6 (0.032 x 10^7 SEM) copies. The increase in *HCGB* mRNA expression under 21% O_2 was confirmed by quantification of HCGB protein secreted in the cell supernatants. After 96 hours in culture, HCGB protein secretion increased more under 21% O_2 (152.15 ng/ml) than under 2.5% O_2 (40 ng/ml). Finally, we also checked by immunofluorescence microscopy that the cells effectively formed multinucleated syncytiotrophoblasts under 21% O_2 (Fig. 1c).

VEGF and PlGF expression during in vitro syncytialization

Since our previous results indicated that the cytotrophoblasts efficiently differentiated into syncytiotrophoblast *in vitro*, we decided to quantify VEGF and PlGF expression in the same samples (Fig. 2). The primers to amplify *VEGF* hybridized in the 5' untranslated region (UTR) of the mRNA, therefore amplifying all the isoform transcripts. In undifferentiated cytotrophoblasts, the amount of *PlGF* mRNA transcripts was 810,000 copies per µg total RNA while the amount of *VEGF* mRNA transcripts was nearly twice less to ~354,000 copies per µg total RNA (Fig. 2a). Then, we quantified *VEGF* and *PlGF* mRNA expressions during the *in vitro* differentiation (Fig. 2b). When the cells were cultured under 21% O_2 for 96 hours, the *PlGF* copy number increased up to 4.21 x 10^6 (±0.5 SEM) copies, while the *VEGF* copy number decreased to 115,000 (±4,000 SEM) copies after 96 h. When the cells were cultured under 2.5% O_2, *PlGF* mRNA expression was inhibited, as expected. For *VEGF* transcripts, we observed a small increase only at 24 h under 2.5% O_2 but then the copy number decreased to reach 300,000 copies at 96 h, a copy number even lower than the copy number measured in undifferentiated cytotrophoblasts (0 h). Remarkably, *VEGF* mRNA expression did not increase significantly when the cells were cultured under low-oxygen tension.

Finally, we quantified PlGF protein in the cell supernatants by ELISA at 48 h and 96 h under both oxygen conditions (Fig. 2c). PlGF protein secretion increased under 21% O_2 from 242.7 pg/ml (±49.8 SEM) at 48 h to 524.97 pg/ml (±128.5 SEM) at 96 h. Under 2.5% O_2, there

was still some PlGF secreted in the supernatants but 4 to 6 times less than under 21% O_2, indicating that PlGF secretion was effectively inhibited by hypoxia.

The SiHa squamous carcinoma cell line as a positive control for VEGF expression

The SiHa cell line was used as a positive control for *VEGF* mRNA expression and protein secretion. We first verified the level of *VEGF* but also *PlGF* mRNA expression when the cells were cultured for 24 h under 21% O_2 and 2.5% O_2 (Fig. 3). The *PlGF* copy number was very low compared to the *VEGF* copy number in these cells. There was nearly ~1120 times less *PlGF* transcripts than *VEGF* transcripts ($1.6 \times 10^4 \pm 0.2 \times 10^4$ and $1.7 \times 10^7 \pm 0.5 \times 10^7$ SEM, respectively) under 21% O_2. When the cells were exposed to 2.5% oxygen for 24 hours, *PlGF* mRNA expression did not change but *VEGF* mRNA expression increased by 3.9-fold compared to 21% O_2. The SiHa cell line expressed VEGF, and its expression was upregulated by low-oxygen tension.

VEGF protein expression in cytotrophoblast total protein extracts

First, we decided to quantify VEGF-A in whole cell extracts using an ELISA kit design for cell lysate. Cell proteins were extracted from undifferentiated cells (0 h), from cells cultured for 24 hours under 21% O_2 and 2.5% O_2, and from cells cultured in the same oxygen conditions for 96 hours (Fig. 4). VEGF-A was detected in all the samples but there was no change in protein quantity. As previously seen with the *VEGF* mRNA expression, a low-oxygen concentration had no effect on VEGF protein expression.

Second, we decided to verify the expression of VEGF protein in total cellular extracts prepared from cells cultured for 96 hours under 21% O_2 and 2.5% O_2 by western blotting under denaturing and reducing conditions (Fig. 5, upper panel). Recombinant human VEGF-A_{165} (rec.hVEGF) and a total protein extract prepared from the SiHa cell line cultured for 24 h under 2.5% O_2 were used as a positive control. The rec.hVEGF monomer, which has an apparent molecular weight of ~24 kDa was strongly recognized by the rabbit polyclonal anti-VEGF antibody. Interestingly, in all our samples there was only a band at approximately 42 kDa, and absolutely no band at ~24 kDa. Since our samples were all treated with the strong reducing agent Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), the ~42 kDa band and the VEGF-A quantified by ELISA probably correspond to the monomer L-VEGF, the precursor form of VEGF, which has a predicted molecular weight of 47 kDa. The specificity of the immunoreaction was confirmed by using a blocking VEGFA peptide (supplemental data, s1).

Then, the identity of L-VEGF in cytotrophoblast was confirmed using a polyclonal antibody produced in rabbit specifically raised against a peptide located within the N terminal 180-aa fragment of the L-VEGF (Fig. 5, middle panel). As expected, rec.hVEGF was not recognized

by this anti-L-VEGF antibody while a 42-kDa band was observed in cytotrophoblast but also SiHa samples, confirming the identity of L-VEGF. Confirming the previous ELISA results, there were no differences in band intensities between 21% O_2 and 2.5% O_2. GAPDH was used as loading control, and appeared to be uniformly expressed, indicating that equivalent amounts of proteins were loaded onto the gel (Fig. 5, lower panel).

VEGF and PlGF in concentrated media

VEGF cannot be detected in cytotrophoblast supernatants by ELISA. This can be due to very low secreted VEGF amounts under the detection limit of the available commercial tests or to coupling of VEGF with another protein like its soluble receptor sVEGFR1. Therefore, we decided to concentrate the supernatants from the same cells used previously after removal of high molecular weight proteins. The media were concentrated approximately 30 times, and 20 µg of proteins were separated under denaturing and reducing conditions to allow the detection of monomers. We first decided to detect VEGF (Fig. 6a). The concentrated medium from the SiHa cell line cultured under 2.5% O_2 was used as a positive control. Once again, we did not observe any VEGF specific band in the media from cytotrophoblasts isolated and purified from three different placentas but a ~25 kDa band corresponding to the VEGF monomer was detected in the SiHa medium. The same results were obtained with a mouse monoclonal anti-VEGF-A antibody (supplemental data, s2). A 57 kDa band was also detected in all the samples but this band was non-specific since it was also detected in unconditioned concentrated complete medium (supplemental data, s3). The same samples were loaded on another gel for Coomassie blue staining and no loading difference was observed between samples (Fig. 6a, lower panel).

To verify that by concentrating the media, we did not lose the VEGF protein, we decided to immuno-detect PlGF in the same concentrated media (Fig. 6b). As a positive control, we used a cell lysate prepared from cytotrophoblasts cultured for 96 h under 21% O_2. A very intense band corresponding to the monomer form of PlGF was detected in the cell lysate in accordance with the high level of PlGF expression measured by real-time PCR and ELISA after 4 days in culture under 21% O_2. The same band was detected in the supernatant from cells cultured under 21% O_2, but was either not detected or its intensity was lower in the supernatants from cells cultured under 2.5% O_2. This result was in accordance with previous results showing that PlGF expression is inhibited by hypoxia. The non-specific 57 kDa band previously seen in the anti-VEGF western blotting was also observed in the concentrated media but not in the cell lysate. There were no differences in loading between samples as seen in the Coomassie blue staining of the gel (Fig. 6b, lower panel).

VEGF maturation is linked to secretion

To understand if VEGF monomer production from the premature L-VEGF was dependent on the process of secretion, the SiHa cells were cultured under 2.5% O_2 for 24 h with or without 1X protein transport inhibitor cocktail (PTIC). VEGF was then immunodetected in cell lysates and in concentrated media (Fig. 7). When secretion was inhibited, VEGF monomer was not detected anymore in the concentrated media, but it was also not observed in the cell lysates. VEGF monomer did not accumulate in the cells, indicating that production of VEGF from L-VEGF happens upon secretion.

DISCUSSION

In this study, we clearly demonstrated that VEGF-A is not produced and secreted by isolated term villous trophoblasts. While PlGF mRNA and protein expressions effectively increased during the *in vitro* differentiation of cytotrophoblasts in normoxia, *VEGF* mRNA expression continuously decreased. As expected, low-oxygen concentration inhibited PlGF expression, but it only slightly increased *VEGF* mRNA expression during the first 24 hours of the culture. Then, *VEGF* mRNA expression decreased continuously during the culture to reach the level measured in undifferentiated cytotrophoblasts after 96 hours. Numerous immunohistochemistry experiments of placental tissue from normal or pathological pregnancies showed a positive intracellular staining of VEGF in villous cytotrophoblasts and syncytiotrophoblasts, but these experiments could not give indications of the molecular weight of the detected protein. In our western blotting experiments using cell lysates from purified trophoblasts, the anti-VEGF antibody specifically detected a ~42 kDa protein. The use of a specific anti-L-VEGF antibody confirmed that this protein observed under reducing conditions is probably the L-VEGF (for long VEGF), which can be considered as the precursor form of VEGF. It was first described by Meiron *et al.* [18], and then by Tee and Jaffe [19], and by Huez *et al.* [20] in 2001. It was shown that this L-VEGF is exclusively intracellular, potentially localized in the cell nucleus, and is not secreted. It is translated from the same mRNA than VEGF from an alternative initiation CUG codon. VEGF and L-VEGF are therefore synthesized from the same messenger and VEGF may also be produced after protease cleavage of a 180 aa fragment of the L-VEGF protein. Interestingly, in the positive control SiHa cell line, the VEGF monomer was detected only in the conditioned medium but not in the cell lysate. A possible explanation for the absence of a positive band in the SiHa cell lysate may be that the maturation of VEGF from L-VEGF happens concomitantly to secretion. This happens very rapidly in these cancer cells. Therefore, the only protein that can be detected is the precursor form. However, in differentiated cytotrophoblast, this 20-25 kDa band could never been observed, even in the concentrated media. This result is corroborated by a single-cell RNA-seq analysis of trophoblast subtypes [21], which detected expression of PlGF only while the *VEGF* gene was not cited among the detected genes coding for secreted polypeptide hormones known to be produced by the placenta. It is therefore possible that in trophoblast cells in culture, VEGF secretion is inhibited by a factor present in the cell culture medium that needs to be identified.

The question remains about the origin of the secreted VEGF during pregnancy. A longitudinal study showed that serum concentrations of VEGF in normotensive (NT) pregnant women increased regularly during the pregnancy [22]. The concentrations at delivery were ~3 times higher than the concentrations measured at 12 weeks' gestation. The authors also found that the VEGF concentrations were much higher in preeclamptic patients, and that 24 h after the

delivery, they rapidly decreased to be equivalent to the concentrations measured in NT patients, indicating that the placenta was the major site of VEGF production. The placental macrophages called Hofbauer cells moderately express VEGF [23], but this secreted VEGF would mostly play a paracrine role in placental angiogenesis [24]. To the contrary, VEGF is highly expressed in the placental bed and the decidua parietalis [25], mostly in the extravillous trophoblasts (EVT) and trophoblast giant cells (TGC).

CONCLUSION

More experiments need to be done to understand why villous trophoblasts produce L-VEGF only but not VEGF. It is possible that L-VEGF has a different biological role than VEGF, or it may represent a form of storage that will allow rapid secretion of VEGF when needed. Since low-oxygen tension did not suffice to stimulate production and secretion of VEGF, it is logical to assume that another signal that still need to be identified may trigger its secretion.

Compliance with Ethical Standards

***Conflict of Interest**

Christophe Louis Depoix declares that he has no conflict of interest. Arthur Colson declares that he has no confict of interest. Corinne Hubinont declares that she has no conflict of interest. Frédéric Debième declares that he has no conflict of interest.

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***Ethical approval**

All procedures performed in studies involving our participants were in accordance with the ethical standards of the CUSL and Research Ethics Committee (approval # 2018-23OCT-397) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

***Informed consent**

Informed consent was obtained from all individual participants included in the study.

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TABLES AND FIGURES

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	
HCGB	GCT-ACT-GCC-CCA-CCA-TGA-CC	ATG-GAC-TCG-AAG-CGC-ACA-TC	
PIGF	CAG-AGG-TGG-AAG-TGG-TAC-CCT-TCC	CGG-ATC-TTT-AGG-AGC-TGC-ATG-GTG-AC	
VEGF	CCA-GCA-GAA-AGA-GGA-AAG-AGG-TAG	CCC-CAA-AAG-CAG-GTC-ACT-CAC	
Protein	**Dilution**	**Reference**	**Company**
Desmoplakin I+II	1/100	ab16434	abcam, Cambridge, UK
GAPDH	1/40000	VMA00046	Bio-Rad Laboratories, Oxfordshire, UK
VEGF-A	1 µg/ml	ab46154	abcam
	1/500	sc-7269	Santa Cruz Biotechnologies, Santa Cruz, CA, USA
L-VEGF	1 µg/ml		Eurogentec, Liège, Belgium
PlGF	2 µg/ml	AF-264-PB	R&D Systems, Minneapolis, MN, USA

TABLE 1 : List of primers and antibodies. (GAPDH, Glyceraldehyde-3-Phosphate DeHydrogenase; HCGB, Human Chorionic Gonadotropin Beta; PlGF, Placental Growth Factor; (L-)VEGF, (Long-)Vascular Endothelial Growth Factor).

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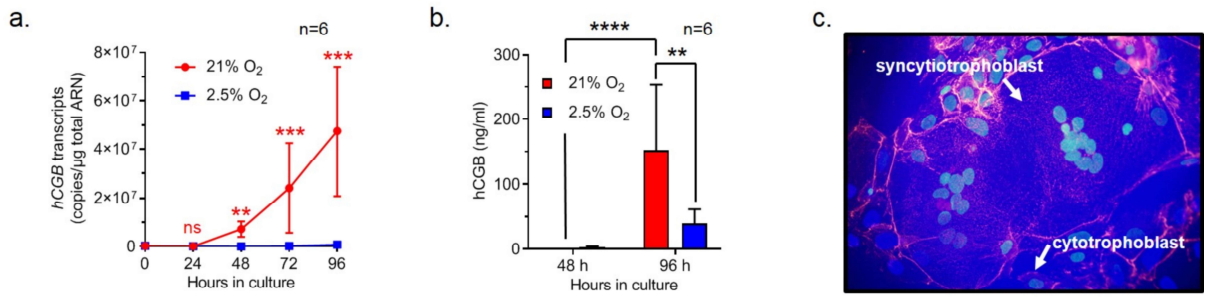


Fig. 1 Primary term cytotrophoblasts successfully differentiate into syncytiotrophoblasts *in vitro*. Cytotrophoblasts were isolated and purified from term placentas and cultured for 96 hours under 21% O_2 (red) and 2.5% O_2 (blue). The cells were harvested every 24 hours for total RNA extraction and cDNA synthesis, while supernatants were collected at 48 hours and 96 hours. Differentiation into syncytiotrophoblast was assessed by quantifying HCGB expression. A) *hCGB* copy number per micrograms of total RNA was quantified by real-time PCR in duplicate. The results were represented as the mean ± SEM of 6 independent experiments (n=6 placentas). Each time-point was compared to control 0 h using non-parametric t-test followed by Mann-Whitney's comparison test (*p<0.05, **p<0.01, ***p<0.001, ns= not significant). B) Secreted HCGB protein was measured by ELISA in the cell supernatants in duplicate. The values are the mean ± SEM of 6 independent experiments and were compared using t-test followed by Mann-Whitney's post-test (**p<0.01, 21% O_2 compared to 2.5% O_2 at 96 h; ****p<0.0001, 21% O_2 at 96 h compared to 21% O_2 at 48 h). C) Formation of multinucleated syncytiotrophoblasts at 96 hours under 21% O_2 was visualized by immunofluorescent microscopy after immunostaining of membrane desmoplakin (red) and counter staining of nuclei with Hoechst 33342 (blue). Original magnification X20.

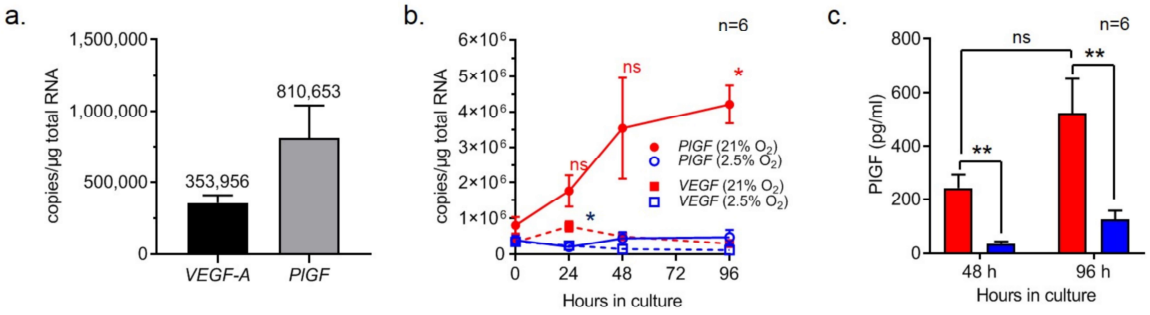


Fig. 2 PlGF and VEGF are differentially expressed during the *in vitro* differentiation of cytotrophoblasts. Isolated primary term cytotrophoblasts were cultured for 96 hours under 21% O_2 (red) and 2.5% O_2 (blue). A) *VEGF* and *PlGF* copy numbers per µg total RNA were quantified in duplicate in undifferentiated cytotrophoblasts. B) *VEGF* (squares) and *PlGF* (circles) transcripts were then quantified at 24, 48, and 96 hours. The results were represented as the mean ± SEM of 6 independent experiments (n=6 placentas). Each time-point was compared to control 0 h using non-parametric t-test followed by Mann-Whitney's comparison test (*p<0.05, ns= not significant). For simplification reason, when nothing is indicated, it means that the difference was not statistically significant. C) Secreted PlGF was quantified in duplicate by ELISA in the supernatant of the cells cultured under 21% O_2 (red bars) and 2.5% O_2 (blue bars) at 48 and 96 hours. The values are the mean ± SEM of 6 independent experiments and were compared using t-test followed by Mann-Whitney's post-test (**p<0.01, 21% O_2 compared to 2.5% O_2; ns= not significant, 96 hours versus 48 hours under 21% O_2).

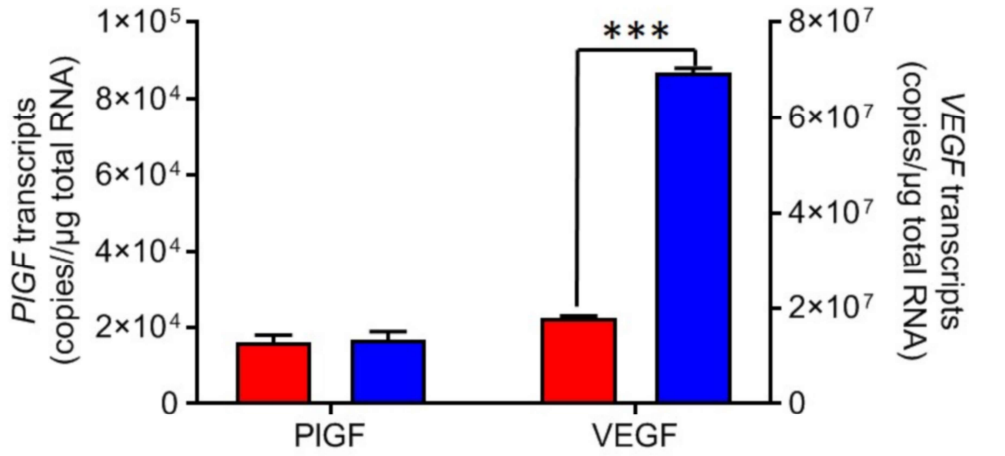


Fig. 3 The squamous cell carcinoma cell line, named SiHa, is a positive control for *VEGF* mRNA expression induced by low-oxygen tension. The cells were cultured for 24 hours under 21% O_2 (red bars) and 2.5% O_2 (blue bars). *PIGF* and *VEGF* copy number per micrograms total RNA was quantified by rt-qPCR in duplicate. The results are the mean ± SEM of three independent experiments. A 24h-exposure to low-oxygen tension induced a 4-fold increase of the number of VEGF transcripts in SiHa cells (***$p<0.001$, 2.5% O_2 compared to 21% O_2 using non-parametric t test followed by Mann-Whitney's post-test).

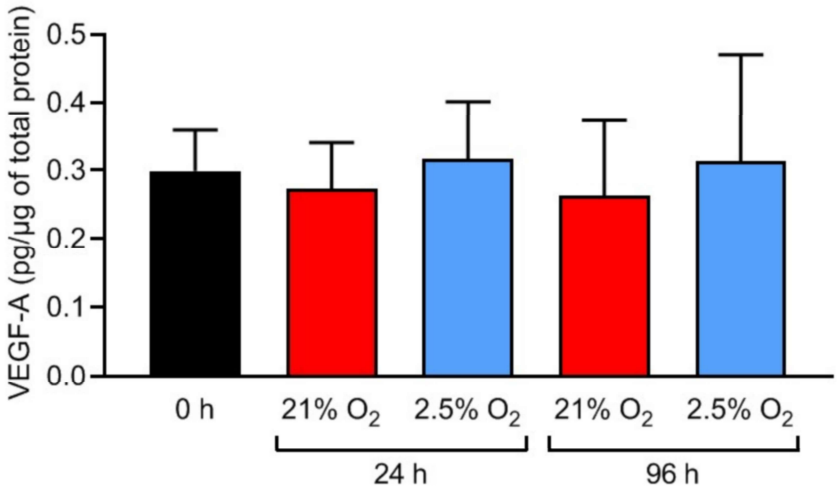


Fig. 4 Intracellular VEGF was detected in cell lysates by ELISA but its expression did not increase during cell culture and under low-oxygen tension. Cellular protein extracts from undifferentiated cytotrophoblasts (0 h, back bar), and from cells cultured for 24 hours and 96 hours, under 21% O_2 (red bars) and 2.5% O_2 (blue bars) were prepared from a minimum of three different placentas (n≥3). VEGF in cell lysates was then quantified in duplicate by ELISA, and each sample normalized to total protein content (pg/µg of total protein). The results were represented as the mean ± SEM.

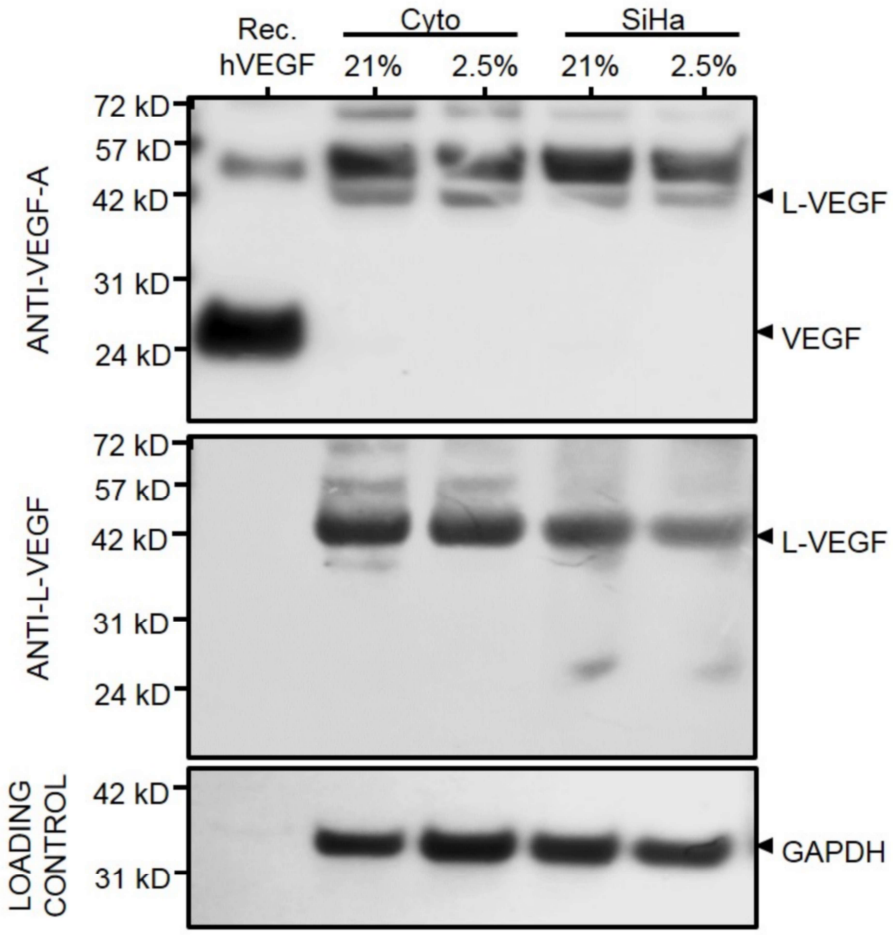


Fig. 5 L-VEGF only, the precursor form of VEGF was detected in trophoblast cell lysates. Twenty micrograms of proteins from cytotrophoblasts (Cyto) cultured for 96 hours under 21 and 2.5% O_2 were separated under denaturing/reducing conditions, and subjected to immunoblot. Cellular protein extracts from SiHa cells cultured for 24 hours under 21% O_2 and 2.5% O_2, and 10 ng recombinant human VEGF (Rec. hVEGF) were used as a VEGF-positive control. a) anti-VEGF immunoblot: no VEGF monomers were detected by the anti-VEGF antibody in cell lysates while Rec. hVEGF was strongly immunodetected. Only a ~42 KDa band corresponding to L-VEGF was observed. b) anti-LVEGF immunoblot: the 42 kDa band was detected in all the samples confirming the identity of the L-VEGF in cell lysates. There was no apparent changes in band intensity between the two oxygen conditions, confirming the previous ELISA results. c) anti-GAPDH immunoblotting was used as loading control. (kD, kiloDalton).

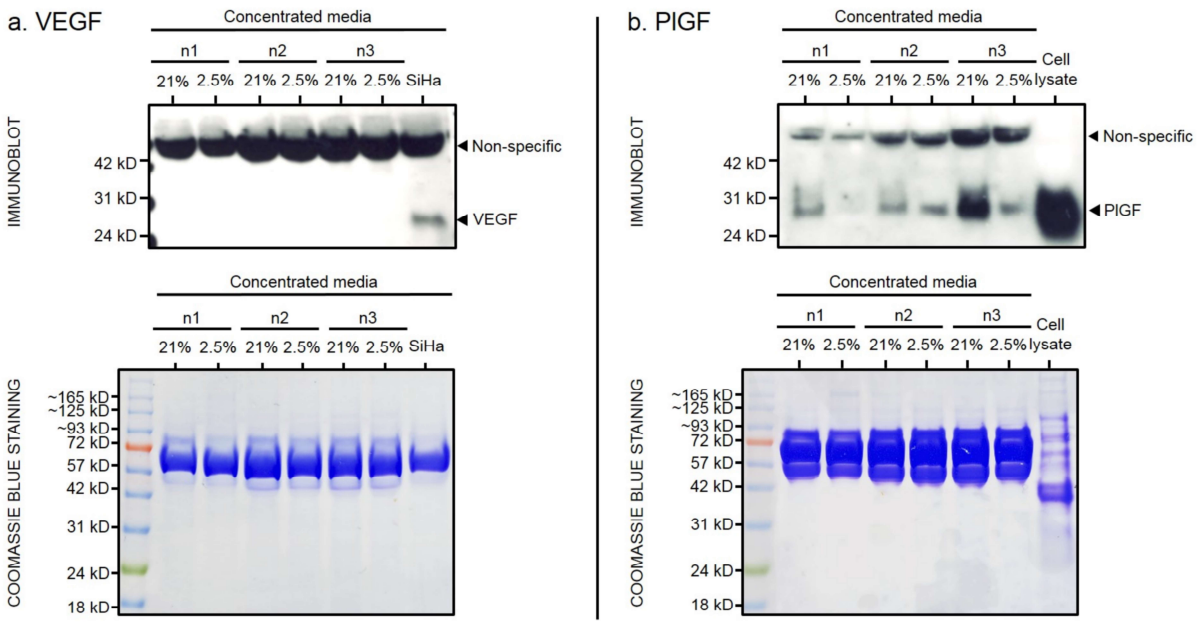
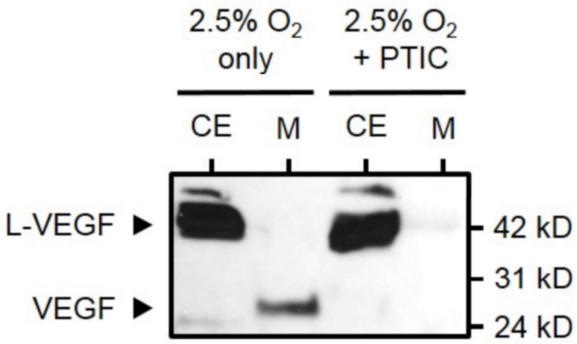


Fig. 6 PlGF but not VEGF monomers were efficiently detected in concentrated media from differentiated cytotrophoblasts. First, the high molecular weight proteins were removed from the culture medium of the three different cell preparations (n=3 placentas) previously used for cellular protein extracts with a 100K MCWO column. Then, the flow-through was concentrated ~30 times with a 3K MCWO column. Twenty micrograms of proteins were separated under denaturing and reducing conditions and immunoblotted with anti-VEGF (A) or anti-PlGF antibodies (B). For the anti-VEGF immunoblot, concentrated SiHa-conditioned medium was used as a positive control. For anti-PlGF immunoblot, a cell lysate prepared from cytotrophoblasts cultured for 96 hours under 21% O_2 was used as a positive control. A Coomassie blue staining of the gels was used to verify that there were no differences in loading between samples. (kD, kiloDalton; ns, nonspecific).



Supplemental data, s1 The anti-VEGFA antibody specifically recognized L-VEGF in cell lysates. Cell lysates from undifferentiated cytotrophoblasts (0 h), differentiated trophoblasts cultured for 4 days under 21% O2 and 2.5% O2, and SiHa cells cultured under 2.5% O2 for 24 hours were separated under denaturing/reducing conditions. The specificity of the immunodetection was verified by incubating the membranes with the anti-VEGFA antibody alone or in presence of a blocking peptide used for the immunization (left panel). GAPDH was used as a loading control and to verify the presence of proteins on both membranes (right panel). (Cyto, cytotrophoblasts; kD, kiloDalton).

Supplemental data, s2 the absence of secreted VEGF monomers in cytotrophoblast-conditioned medium was verified using two anti-VEGF antibodies with different clonality. Unconditioned complete medium, supernatants from cytotrophoblasts cultured for 96 hours under 21% O_2 and 2.5% O_2, and from SiHa cells cultured 24 hours under 2.5% O_2 were concentrated ~30 times. Forty micrograms of proteins were separated by PAGE under denaturing/reducing conditions and immunodetected with rabbit polyclonal or mouse monoclonal anti-VEGF antibodies (Ab). Rec.hVEGF (10 ng) was used as a positive control. Both antibodies recognized rec.hVEGF and VEGF monomer in SiHa-conditioned medium while there was no VEGF monomers detected in cytotrophoblast-conditioned media. A 50–57 kDa band was also detected by both antibodies. This band was also detected in the unconditioned medium and in the rechVEGF sample indicating that it was unspecific. (kD, kiloDalton).

Supplemental data, s3 The 50–57 kDa band is nonspecific. The 50–57 kDa band in the concentrated media was nonspecific since it was also detected in pure fetal bovine serum (FBS) and in the concentrated complete medium that was not conditioned by cells, and it was not detected in medium without FBS.