Role of Regulatory and Angiogenic Cytokines in Invasion of Trophoblastic Cells

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Problem
Trophoblast invasion is a temporally and locally restricted process, which regulates implantation and oxygen arrival to the embryo through the dialog with spiral artery endothelium. Trophoblast factors with angiogenic potential are activated by hypoxia. Their capacities to induce proliferation, migration, and invasion of trophoblastic cells have been investigated.

Method of study
The expression of interleukin (IL)-6, CD126, CD130, vascular endothelial growth factor (VEGF), and hypoxia inducible factor-1α (HIF-1α) has been silenced in JEG-3 choriocarcinoma cells by using siRNA. Silencing efficacy has been assessed by ELISA, PCR or Western blotting. Proliferation has been measured by flow cytometry, migration by a transwell assay, and invasion by a Matrigel assay.

Results
Proliferation was significantly reduced by silencing of CD126 or CD130, migration by silencing of IL-6, VEGF, or HIF-1α, and invasion by silencing of IL-6 and HIF-1α.

Conclusion
The expression of IL-6, VEGF, and HIF-1α in trophoblastic cells is involved in the control of trophoblast invasion and migration.

Introduction

The success of embryo implantation depends on achieving the orchestration of trophoblast proliferation, migration, and invasion into the endometrium to establish not only the anchoring to the uterine wall but also a blood supply for the conceptus.[1–3] This angiogenic process depends on spiral arteries invasion, occlusion, and endothelial remodeling by a highly invasive and migratory sub-population known as extravillous trophoblast (EVT). They invade the uterus and remodel its vasculature to establish an adequate exchange of key molecules between maternal and fetal circulation. Studies conducted to elucidate mechanisms that modulate EVT cell proliferation, migration, and invasion have revealed that this regulation is provided by a variety of factors in the EVT cell microenvironment.[4,5]

On the other hand, feto-maternal interface is constituted by a complex net of cytokines, which regulate immunomodulation as well as the vascularization process.[6] It seems to be crucial to identify which molecules are implicated in the transition from an inflammatory process prior to implantation to an anti-inflammatory response necessary for placental vascularization. In this sense, interleukin-6 (IL-6) was

recently postulated as a regulatory factor implicated in the transition from inflammatory to anti-inflammatory prevalence and from innate to acquired immunological response.[7] We previously demonstrated that recombinant IL-6 prevents abortion in the CBA/J × DBA/2 murine model. Furthermore, recombinant IL-6 increases placental IL-6[8] and vascular endothelial growth factor (VEGF) levels.[9] Hypoxia inducible factor-1α (HIF-1α) is a common transcription molecule for VEGF and IL-6 and is implicated in placental and endometrial angiogenesis.[10] CD126 is the specific receptor chain for IL-6 and CD130 is the common gamma-chain for several receptors for cytokines of the IL-6 family.

Considering these data, we aimed to investigate the possible role of IL-6, VEGF, and HIF-1α in the regulation of proliferation, migration, and invasion of trophoblastic cells. To achieve this goal, we silenced the expression of the aforementioned factors by use of RNA interference (RNAi).

Materials and methods

Cell Line

JEG-3 is an adherent human choriocarcionoma cell line preserving several trophoblast-like capacities including production of pregnancy related cytokines. For use in Argentina, this cell line was a kind gift from 'Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina', and for experiments in Germany JEG-3 cells have been purchased at the German Collection of Cell Lines and Microorganisms (DSMZ; Braunschweig, Germany),

Cell Culture

JEG-3 cell cultures were performed at 10^6 cells/175 cm^2 flask and maintained under standardized conditions (37°C, 5% CO_2 humidified atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco) and 1% penicillin/streptomycin antibiotic solution ('AAS', Gibco).

RNA Interference

Small interfering RNA (siRNA) oligonucleotides were designed to interfere exclusively with IL-6, its receptor chains CD126 and CD130, VEGF or HIF-1α mRNAs. As a negative control, a non-genomic combination was used. All oligos were purchased from Ambion Inc. (Huntingdon, UK; Table I). A 10-μM stock solution was prepared and aliquots were stored at −70°C. The following RNA interference procedure has been performed as previously described:[11] In brief, 5×10^5 JEG-3 cells were seeded in six well plates and cultured in F12 medium as described above until 50% confluence. At this stage, cells were washed twice with phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.4) and once with Optimem medium (Invitrogen, Karlsruhe, Germany). Ten microliters of annealed oligos (10 μM) were mixed with 175 μL Optimem medium. In a separate tube, 4 μL Oligofectamine (Invitrogen) were added to 11 μL Optimem medium. Both solutions were mixed and incubated for 20 min at room temperature (RT). Culture medium from growing JEG-3 cells was aspirated and 800 μL of fresh Optimem medium was added, followed by the addition of 200 μL of annealed oligos in Oligofectamine. Culture plates were gently shook to mix the annealed oligos followed by incubation at 37°C in humidified atmosphere at 5% CO_2. After 4 hr, 500 μL of Ham F12 containing 30% FCS was added. Supernatants at 24–48 hr were collected, centrifuged at 3000 × ***g*** and kept at −70°C for further analyses. Gene silencing efficacy has been tested by enzyme-linked immunosorbent assay (ELISA) (for IL-6 and VEGF), Western blotting (HIF-1α) or PCR (CD126 and CD130).

Enzyme-Linked Immunosorbent Assay

Determination of IL-6 and VEGF expression was performed by a double antibody-sandwich ELISA[12] using a commercially available kit (R&D, Minneapolis, MN, USA). Briefly, 96-well plates were coated

Table I siRNA Oligonucleotid Sequences

Non-genomic control siRNA (ctrl)	GCC ACU UAU AAA UUC GUU Ctt
HIF-1α siRNA	GGC AGC AGA AAC CUA CUG C Ctt
IL-6 siRNA	GGA CAU GAC AAC UCA UCU C Ctt
CD 126 siRNA	CGA CUC UGG AAA CUA UUC A Ctt
CD 130 siRNA	GGC AUG CCU AAA AGU UAC U Ctt
VEGF siRNA	CGA UCG AUA CAG AAA CCA C Ctt

HIF-1α, hypoxia inducible factor-1α; siRNA, small interfering RNA; IL-6, interleukin-6; VEGF, vascular endothelial growth factor.

with anti-cytokine monoclonal antibody over night. After successive washes with PBS, the free sites were blocked with 1% of bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in PBS for 1 hr at room temperature. Microtitre plates were washed twice with PBS containing 0.05% Tween-20 (PBS-t, Anedra, San Fernando, Argentina). Various test culture supernatants along with appropriate controls as standards (100 μL/well) were added and plates were further incubated for 2 hr at RT. After successive washings with PBS-t, the biotinilated detection antibody was added and plates were incubated for 1 hr at RT.

After further washings, horseradish peroxidase (HRP) with streptoavidine were added and incubated for further 20 min at RT. Color reaction was induced by 3,3′, 5,5′- tetrametilbencidine (TMB; MP Biomedicals, Cleveland, OH, USA) and stopped with 1 M H_2SO_4 solution. The samples were analyzed in an automatic plate reader (Metertech, Taipei, Taiwan) at a wave length of 450 nm, with wavelength correction at 540 nm. Concentrations were calculated as pg/mL.

Western Blots

Cells were suspended in 100 μL lysis buffer (20 mM HEPES, 2 mM EGTA, 0.2 mM EDTA, 0.12 M NaCl, 1% Triton) and supplemented with protease inhibitors (5 mM beta-glycerophosphate, 3 mM $MgCl_2$, 20 μg/mL aprotinine (trasylol), 20 μg/mL leupeptine, 5 μg/mL pepstatine A, 647 ng/mL antipain, 10 μg/mL bestatine, 0.1 mM PMSF, 1 mM sodium orthovanadate). Protein concentration of cell lysates was determinated using a kit based on the Bradford method (Sigma). Briefly, 20 μg of whole cell extract was solublized in gel loading buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% β-mercaptoethanol), denatured for 10 min by boiling and separated on 10% acrylamide SDS gels. After protein transfer, nitrocellulose membranes were blocked in TRIS-buffer supplemented with 2% milk powder. Protein transfer during Western blots was controlled by Ponceau S (Roth, Germany) staining of the nitrocellulose membrane. Goat anti-HIF-1α antibodies (R&D systems) were applied for 12 hr at 1:1000 dilution. For detection, HRP-conjugated anti-goat IgG (Sigma) was employed at a dilution of 1:10,000 for 1 hr. Visualization was performed using a LumiGlo Chemiluminescence reagent (New England Biolabs Frankfurt am Main, Germany).

Reverse Transcriptase-Polymerase Chain Reaction

To isolate total RNA, JEG-3 cells were harvested and processed using Tri reagent (Sigma-Aldrich) followed by chloroform-isopropanol-ethanol method as previously described.[13] The isolated RNA (5 μg) was subjected to first strand cDNA synthesis using oligo-dT primers, dNTP mixture, RT buffer, and Thermoscript reverse transcriptase following the manufacturer's protocol (Thermoscript RT PCR System; Invitrogen, Carlsbad, CA, USA). Extent of silencing of transcripts for CD 130 and CD 126 in control and experimental samples was determined by PCR using the above synthesized cDNA as template and gene specific primers for CD 130 (Forward primer: 5′-AATGCCCTTGGGAAGGTTACA-3′, Reverse primer: 5′-ACAGTTCTCTGAGTTGATCACTGAT-3′), CD 126 (Forward primer: 5′-CCCTGAGCTTTCTGCTATCAAAG-3′; Reverse primer: 5′-ATTTAGTCCTGCTTCAATTGCA-3′) and Actin (Forward primer: 5′-AGATGACCCAGATCATGTTTGAGA-3′, Reverse primer: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′). PCR was performed in a 50 μL reaction mixture consisting of ~30 ng cDNA, 10 mM dNTP, 50 pmol each of forward and reverse primers and Platinum Taq DNA polymerase following manufacturer's instructions (Invitrogen). Amplification was carried out in a thermal cycler (MJ Research PTC-200 DNA Engine System; GMI Inc., Ramsey, MN, USA) using the following protocol: initial denaturation for 10 min at 94°C followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s and extension at 72°C for 30 s with a final extension of 10 min at 72°C.

Proliferation, Migration, and Matrigel Invasion Assays

Matrigel (BD Biosciences, Heidelberg, Germany) was prepared as described previously, applied to Nucleopore filters (for 24-well plates) and allowed to polymerize at 37°C for 4 hr.[14] Forty-eight hours after siRNA transfection, JEG-3 cells were trypsinized with 5% Trypsin-EDTA 1X (Gibco, Canada) and 1.5×10^5 cells/well were incubated at 37°C 5% CO_2. After 24 hr of culture, absolute number of trespassed (invasive) cells was determined by using a flow cytometer (PARTEC, PAS III, Argentine). Results were expressed as a ratio. For analysis of migration, the procedure was the same, but Nucleopore membranes were not coated with Matrigel. For analysis

of proliferation, cells were cultured for 24 hr, trypsinized, and a defined volume of this suspension was counted by a flow cytometer.

Statistical Analysis

Data on proliferation, migration, and invasion were analyzed by a Student's *t*-test or one-way ANOVA followed by the Tukey multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant. Experiments were repeated seven times (for each single experiment independent transfections have been performed).

Results

Efficacy of RNAi for HIF-1α

HIF-1α production has been significantly silenced by the use of the respective siRNA as assessed by Western blotting 48 hr after transfection (Fig. 1a).

Efficacy of RNAi for CD126 and CD130

JEG-3 cells were transfected with siRNA against CD 130 and CD 126, which led to more than 70% silencing of their expression at the transcript level

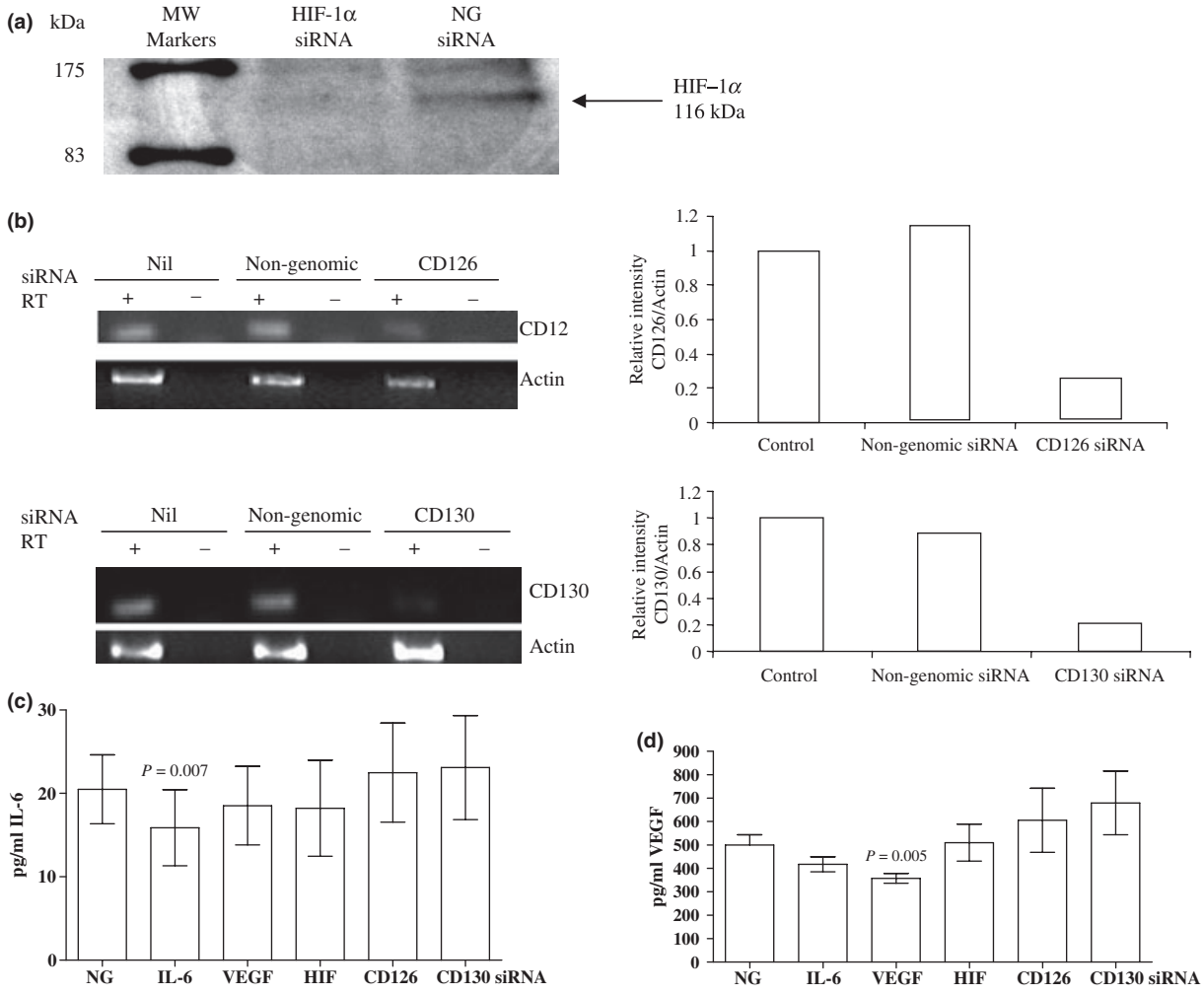

Fig. 1 Control of silencing efficiency in JEG-3 cells after transfection with different specific siRNA and non-genomic controls. Cultures have been performed with 5×10^5 cells in 1.5 mL medium. (a) Western blot for HIF-1α 48 hr after transfection. (b) RT-PCR for CD126 and CD130 by using specific primers 24 hr after transfection. Actin was used as an internal control. Bars show respective density of bands. (c) IL-6 and (d) VEGF concentration in supernatants 48 hr after transfection with different siRNA. $n = 7$ (independent transfections for each experiment). NG, non-genomic; MW, molecular weight. Error bars indicate standard deviation.

after 24 hr of transfection (Fig. 1b). RT-PCR for actin was used as an internal control.

Effects of RNAi on IL-6 and VEGF Production

After siRNA transfection, JEG-3 culture supernatants were analyzed by ELISA for IL-6 production. Transfection with siRNA for IL-6 receptors, HIF-1α or VEGF did not affect IL-6 synthesis. However, a significant decrease was observed after 48 hr of silencing with IL-6 siRNA (Fig. 1c). This effect was not evident in 24 hr culture supernatants (data not shown).

Same effect was found for VEGF production, with a significantly decreased VEGF expression only in 48 hr culture supernatants after transfection with VEGF siRNA (Fig. 1d).

Effect of RNAi on Trophoblast Proliferation, Migration, and Invasion

Only silencing of the IL-6 receptors (CD126 and CD130) was able to significantly decrease trophoblast proliferation (Fig. 2a). In contrast, transwell migration was affected by RNAi of IL-6, VEGF, and HIF-1α production ($P < 0.02$), but not by silencing CD126 or CD130 (Fig. 2b).

JEG-3 invasion through Matrigel was significantly reduced when cells were transfected with IL-6 or HIF-1α siRNA, but not with VEGF or IL-6 receptor siRNA (Fig. 2c).

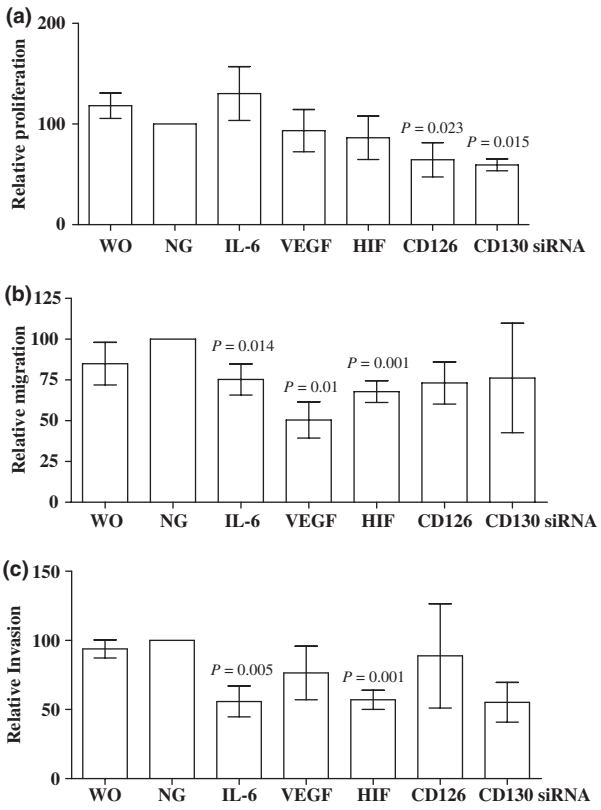

Fig. 2 Functional analyses of JEG-3 cells 48 hr after transfection with different specific siRNA and non-genomic controls. All results have been normalized to the values for non-genomic siRNA control transfections, which were defined as 100. (a) Proliferation has been assessed after another 24 hr by counting total cell number using a flow cytometer. (b) After 24 hr, cells passed through transwell into the lower chamber have been counted by flow cytometry. (c) Same as (b), but transwell membranes have been coated with Matrigel. w/o, control without siRNA and without transfection reagent; NG, non-genomic. Error bars indicate standard deviation. $n = 7$.

Discussion

The invasive capacity of cells of embryonic origin seems to be a crucial factor in the establishment of feto-maternal circulation in the placenta, as trophoblast cells are required to modulate endometrial spiral arteries. This invasion needs to be strictly controlled both spatially and temporally. In this sense, IL-6 was suggested to be evolved in the control of invasion and proliferation of villous trophoblast cells.[15] In addition, angiogenesis is required for the invasion process and *vice versa*.[16] This mechanism can be stimulated by lowered physiological oxygen or hypoxia, which is the major inducer of HIF-1α. Furthermore, it has been proposed that hypoxia plays an important role in the upregulation of VEGF receptor gene expression,[17] which is an essential regulator of developmental vascularization.[18] Taking into account these data, we decided to investigate the effect of post-transcriptional gene silencing of IL-6, its receptor chains, VEGF and HIF-1α on trophoblast proliferation, migration, and invasion.

Data obtained showed that only the silencing of IL-6 receptors (CD126 and CD130) was able to significantly decrease trophoblast proliferation. Similar effects have been described in other tissues and cell types, such as in glioma cells[19] or synovia, where antiproliferative effects have been induced by application of a blocking IL-6 receptor antibody.[20] It may be surprising that silencing IL-6 receptors did not induce the same effect as silencing of IL-6 itself. However, we considered as a possible explanation

that the remaining activity of IL-6 in IL-6 silenced cells may be sufficient to maintain a constant level of proliferation.

Migration of JEG-3 cells through a transwell membrane was significantly reduced by silencing of IL-6, HIF-1α, and VEGF, which induced the strongest effects. Migration inducing capacities of this angiogenic cytokine have been reviewed in detail elsewhere.[21] In trophoblastic cells, VEGF acts via autocrine stimulation loop.[22]

Invasion of JEG-3 cells through Matrigel has been significantly reduced by transfection with IL-6 or HIF-1α siRNA, but only tendentiously with VEGF siRNA. Silencing of CD130 had similar effects to silencing of IL-6, but which due to higher standard deviation was non-significant, while the effects of CD126 silencing were slighter. This discrepancy may be caused by the shared use of CD130 and also by other IL-6 family cytokines, whereof some may be also produced by JEG-3 cells and work in an autocrine manner. Our results are congruent with several previous studies, when the factors of interest have not been silenced, but instead been added.[23,24]

Considering these results, we suggest that deficient levels of IL-6 and VEGF in the feto-maternal interface may lead to impaired establishment of feto-maternal circulation in the placenta. HIF-1α could be the factor which modulates the expression of these regulatory and angiogenic cytokines. This might be one mechanism implicated in the immunomodulation during the vascularization process, in which IL-6 regulates the termination of the inflammatory predominance during the implantation window toward placental angiogenesis. Deficient placental IL-6 levels may cause an inappropriate regulation which ends in an exacerbated inflammation. In severe cases, this effect may lead to a poor angiogenesis followed by ischemic fetal death.

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