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ORIGINAL PAPER

Effects of corticotropin-releasing hormone and stresscopin on vascular endothelial growth factor mRNA expression in cultured early human extravillous trophoblasts

Senn Wakahashi · Koji Nakabayashi · Nobuyuki Maruo · Ai Yata · Noriyuki Ohara · Takeshi Maruo

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Abstract Corticotropin-releasing hormone (CRH) takes a role in the regulation of the onset of parturition. Stresscopin (SCP) is a high affinity ligand for CRH receptor (CRHR)-2. CRHR-2 inhibits VEGF-induced neovascularization. In the present study, we investigated the effects of CRH and SCP on VEGF expression in early placental extravillous trophoblasts (EVTs). Isolation and culture of trophoblasts differentiating into EVTs were performed by the enzymatic digestion of anchoring early placental villi. The presence of CRH, SCP, CRHR-1, and CRHR-2 in cultured EVTs was examined by RT-PCR and immunocytochemistry. The effects of CRH and SCP on *VEGF* mRNA levels in cultured EVTs were assessed by real-time RT-PCR. CRH, SCP, CRHR-1, and CRHR-2 were expressed in cultured EVTs at mRNA and protein levels. Treatment with either 100 nM CRH or 100 nM SCP for 24 h decreased *VEGF* mRNA levels in cultured EVTs. The CRH- and SCP-induced decrease in *VEGF* mRNA levels was counteracted by the concomitant treatment with CRHR-2 antagonist antisauvagine-30, but not with CRHR-1 antagonist antalarmin. We demonstrated that CRH and SCP inhibited *VEGF* mRNA expression in cultured EVTs through the interaction with CRHR-2, suggesting that CRH and SCP may inhibit angiogenesis during early placentation.

Keywords Corticotropin-releasing hormone · Extravillous trophoblast · Stresscopin · Vascular endothelial growth factor

S. Wakahashi · K. Nakabayashi · N. Maruo · A. Yata · N. Ohara · T. Maruo (✉)
Department of Obstetrics and Gynecology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
e-mail: maruo@kobe-u.ac.jp

Introduction

In female reproductive system, stress is known to affect menstrual disorders and pregnancy maintenance [1]. During stress, hypothalamic neuropeptide corticotropin-releasing hormone (CRH) stimulates pituitary ACTH and adrenal cortisol secretion, leading to the classic "fight or flight" responses that have short-term protective and adaptive effects and are characterized by the activation of the CRH–adrenocorticotropin–glucocorticoid axis, mediated by CRH receptor (CRHR)-1. In contrast, the CRHR-2 mediates the stress-coping responses during the recovery phase of stress [2].

Recently, new functional CRH family members urocortin (UCN), stresscopin-related peptide (SRP), and stresscopin (SCP, urocortin 3) have been identified [2]. The biological actions of CRH and UCN are mediated via binding to two G protein-coupled receptors, CRHR-1and CRHR-2 [3, 4]. These receptors share 70% identity at the amino acid level, but have different binding properties for the members of the CRH family [5–7]. CRH and UCN bind to both CRHR-1 and CRHR-2, while SRP and SCP bind exclusively to CRHR-2 [2]. These two receptor subtypes have different physiological functions and are distributed in the brain, pituitary, and various peripheral tissues [3, 4, 8–11]. CRHR-1 has four splice variants (1α, 1β, 2α, and C) and CRHR-2 has three splice variants (α, β, and γ), and these receptor subtypes are thought to have different actions [12, 13]. CRHR-2 is important for stress-coping responses, including dearousal, sustained hypophagia, and reduced blood pressure during the recovery phase of stress response, and for avoiding damage incurred by excessive exposure to the "fight or flight" responses initiated by the activation of pituitary CRHR-1 [14, 15]. Elucidation of the biological roles of these endocrine or paracrine stress

regulators is important for the understanding of normal and pathological status in female reproductive system. Moreover, Bale et al. demonstrated in their study using CRHR-2 deficient mice that CRHR-2 was a critical component in the pathway necessary for the inhibition of neovascularization [16].

Neovascularization is crucial for successful early placentation. Hemochorial placentation is dependent on the establishment and maintenance of a competent fetoplacental vascular network formed by branching (first and second trimesters) and non-branching (third trimester) angiogenesis. In human placentas, branching angiogenesis is important for both the development of the villous vasculature and the formation of terminal villi [17]. Chorionic tissues contain heterogeneous populations of trophoblasts including villous cytotrophoblasts and syncytiotrophoblasts, and extravillous trophoblasts (EVTs) [18]. The development of the human fetus depends on the ability of EVTs to invade the maternal uterine tissues to anchor the placenta and the fetus to the maternal endometrium, enabling the fetus to gain access to the maternal circulation [19–21]. For successful hemochorial placentation, vascular development and reduction in maternal blood flow resistance are necessary, and vascular endothelial growth factor (VEGF), angiopoietins, and their receptors seem to be involved in the regulation of this molecular process [22]. Besides its mitogenic action on vascular endothelial cells, VEGF has been reported to stimulate the proliferation of the choriocarcinoma cell line BeWo and human EVT cell line HTR-8 [23–25]. On the other hand, a recent study of Kaufmann et al. has identified that the failure in the development of functional maternal vasculature during branching angiogenesis leads to severe conditions such as intrauterine growth restriction of the fetus and preeclampsia of the pregnant woman [26]. Low VEGF levels or increased production of VEGF antagonists such as a soluble form of VEGF receptor-1 (flt-1) have been proposed as possible mediators of preeclampsia [17, 27, 28]. A failure in neovascularization at the site of EVT invasion into the endometrium is thought to be a primary cause of abnormal placentation, which eventually leads to miscarriage in the first trimester of pregnancy and preeclampsia in the second or third trimester of pregnancy [29, 30].

Human EVTs express and release CRH, UCN and express CRHR-1 [31, 32]. Little is known about the effects of CRH family peptides on VEGF expression in early placental EVTs. Thus, in the present study, we investigated the presence of CRH, SCP, and VEGF in cultured early placental EVTs, along with the effects of these peptides on *VEGF* mRNA expression in those cells. This study demonstrates that CRH and SCP may take an inhibitory role in an autocrine/paracrine manner in the regulation of VEGF expression in early placental EVTs.

Results

RT-PCR analysis of CRH, SCP, CRHR-1, CRHR-2β, and VEGF mRNA in cultured EVTs

The mRNA expression of CRH, SCP, CRHR-1, CRHR-2β, and VEGF in cultured EVTs after 24 h subsequent cultures was examined by RT-PCR analysis. The 122-bp band corresponding to CRH, 237-bp band to SCP, 164-bp band to CRHR-1, and 126-bp band to CRHR-2β were observed in cultured EVTs and chorionic villi (Fig. 1). All bands were sequenced to be correct. For *VEGF* mRNA expression, the 98-bp and 228-bp bands corresponding to VEGF isoform 121 and 165, respectively, were observed in cultured EVTs and chorionic villi (Fig. 1).

Immunocytochemical analysis of CRH, SCP, CRHR-1, CRHR-2, and VEGF in cultured EVTs

To confirm the presence of CRH, SCP, CRHR-1, CRHR-2, and VEGF proteins in cultured EVTs after 24 h subsequent culture, immunocytochemical analyses were performed using their specific antibodies (Table 2). CRH, SCP, CRHR-1, CRHR-2, and VEGF were all positively immunostained in cultured EVTs. The replacement of the primary antibody with blocking peptide resulted in a lack of positive immunostaining (Fig. 2).

Effect of CRH treatment on VEGF mRNA levels in cultured EVTs

Real-time RT-PCR analysis revealed that the treatment with CRH of 10 nM, 30 nM, and 100 nM significantly ($P < 0.05$) decreased *VEGF* mRNA levels in cultured EVTs, to 72%, 62%, and 48%, respectively, compared with untreated control (Fig. 3). Treatment with CRH significantly ($P < 0.05$) inhibited *VEGF* mRNA levels in cultured EVTs in a dose-dependent manner. To examine whether the CRH-induced decrease in *VEGF* mRNA levels was mediated through interaction with its receptors, the EVTs cultured with CRH were concomitantly treated with either CRHR-1 specific antagonist antalarmin or CRHR-2 specific antagonist antisauvagine-30, because CRH could bind both two receptors. The treatment with either 1 M antalarmin alone or 1 M antisauvagine-30 alone did not affect *VEGF* mRNA levels in cultured EVTs compared with untreated control cultures. While 100 nM CRH-induced decrease in *VEGF* mRNA levels was not recovered by the concomitant treatment with 1 M antalarmin, the 100 nM CRH-induced decrease in *VEGF* mRNA in cultured EVTs was significantly ($P < 0.05$) recovered by the concomitant treatment with 1 M antisauvagine-30 compared with treatment with 100 nM CRH alone (Fig. 3). This revealed that CRHR-2

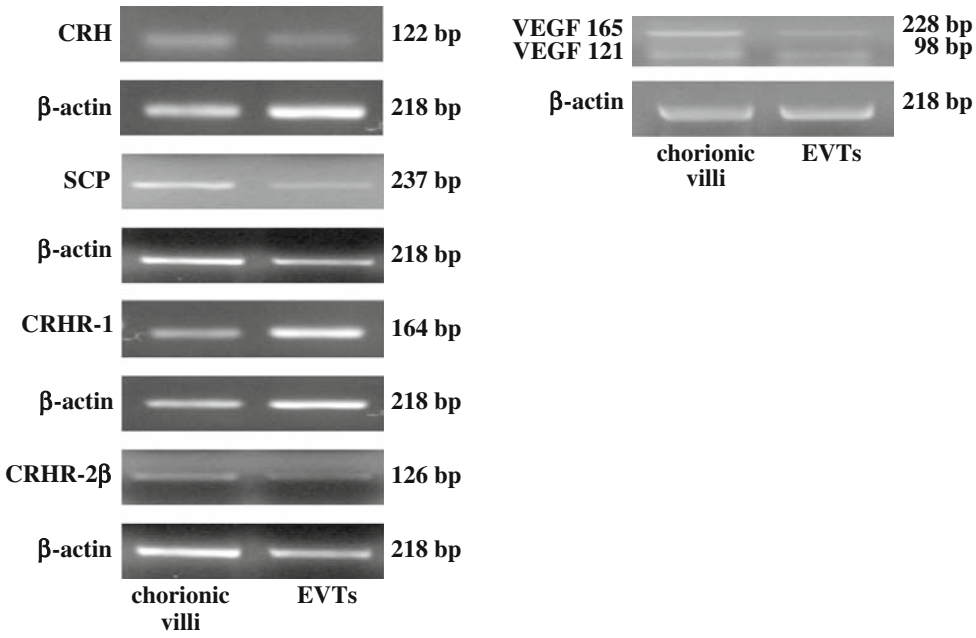

Fig. 1 The mRNA expression of CRH, SCP, CRHR-1, CRHR-2β, and VEGF in early placental EVTs assessed by RT-PCR. The 122-bp PCR product specific for CRH, the 237-bp PCR product specific for SCP, the 164-bp PCR product specific for CRHR-1, the 126-bp PCR product specific for CRHR-2β and the 98-bp and 228-bp PCR product specific for VEGF 121 and 165 were detected

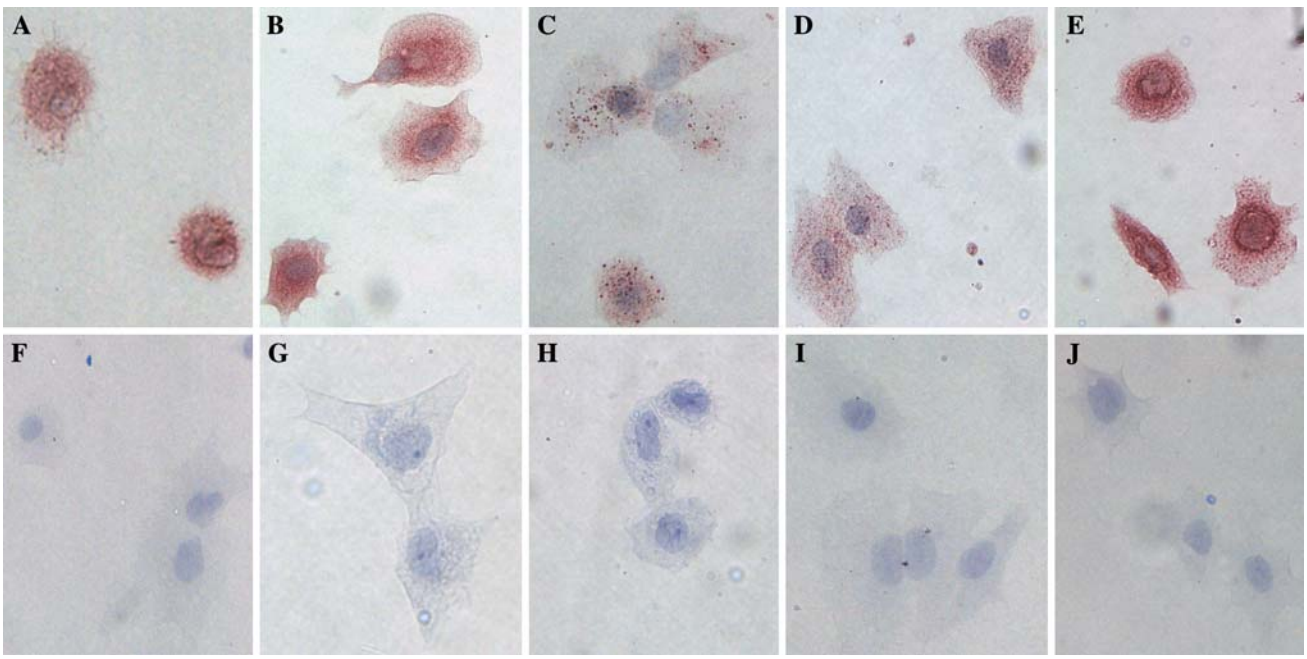

Fig. 2 Immunocytochemical staining of CRH, SCP, CRHR-1, CRHR-2, and VEGF in early placental EVTs attached to FN-precoated dishes after 48-h subculture. Immunocytochemical analysis of the cells after 48-h subculture demonstrated that CRH (**A**), SCP (**B**), CRHR-1 (**C**), CRHR-2 (**D**), and VEGF (**E**). The replacement of the primary antibody with peptide resulted in a lack of positive immunostaining in the cells CRH (**F**), SCP (**G**), CRHR-1 (**H**), CRHR-2 (**F**), and VEGF (**J**). Original magnification, ×400

antagonist antisauvagine-30 could antagonize the effect of CRH on *VEGF* mRNA levels in cultured EVTs.

Effect of SCP treatment on VEGF mRNA levels in cultured EVTs

The treatment with SCP 10 nM, 30 nM, and 100 nM attenuated *VEGF* mRNA levels to 65%, 61%, and 31%, respectively, compared with untreated control cultures, indicating that SCP treatment significantly ($P < 0.05$) decreased *VEGF* mRNA levels in cultured EVTs in a dose-dependent manner compared with untreated control cultures (Fig. 4). To explore whether the SCP-induced decrease in *VEGF* mRNA levels was mediated through interaction with its specific receptors, the EVTs cultured with SCP were concomitantly treated with CRHR-2

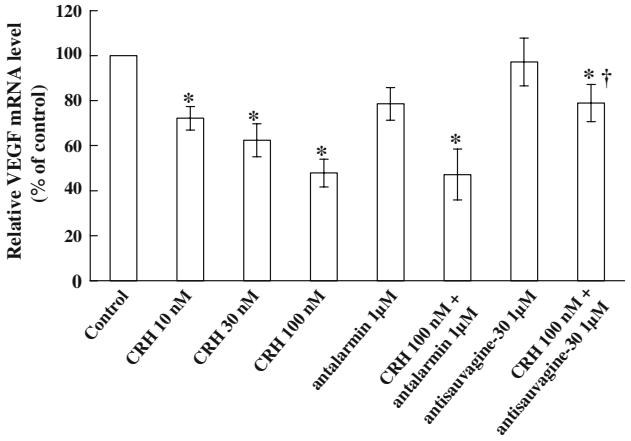

Fig. 3 Effect of CRH on the *VEGF* mRNA levels in cultured early placental EVTs concomitantly treated with either CRHR-1 antagonist, antalarmin or CRHR-2 antagonist, antisauvagine-30. The amount of *VEGF* mRNA was extrapolated from a standard curve by quantitative RT-PCR, and the results are expressed as the relative *VEGF* mRNA levels in EVTs compared to the untreated controls. Data represent the mean ± SEM of three independent experiments. * $P < 0.05$ compared with untreated control, † $P < 0.05$ compared with treatment with 100 nM CRH

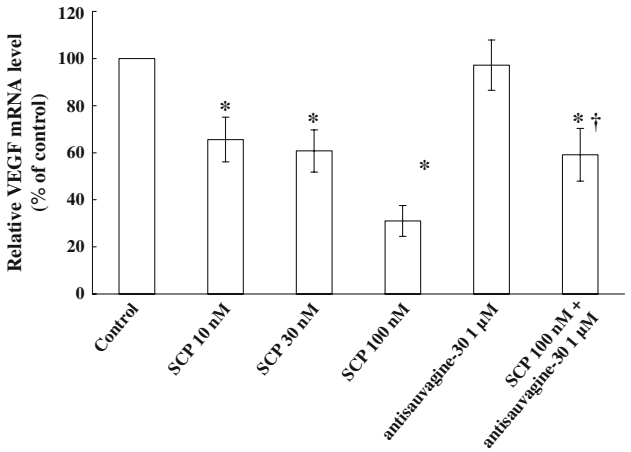

Fig. 4 Effect of SCP on the *VEGF* mRNA levels in cultured early placental EVTs concomitantly treated with either CRHR-2 antagonist, antisauvagine-30. The amount of *VEGF* mRNA was extrapolated from a standard curve by quantitative RT-PCR, and the results are expressed as the relative *VEGF* mRNA levels in EVTs compared to the untreated controls. Data represent the mean ± SEM of three independent experiments. * $P < 0.05$ compared with untreated control, † $P < 0.05$ compared with treatment with 100 nM SCP

specific antagonist antisauvagine-30. Treatment with 1 M antisauvagine-30 alone did not affect *VEGF* mRNA levels compared with untreated control cultures. However, the 100 nM SCP-induced decrease in *VEGF* mRNA in cultured EVTs was significantly ($P < 0.05$) recovered by the concomitant treatment with 1 M antisauvagine-30 compared with treatment with 100 nM SCP alone (Fig. 4). This demonstrates that antisauvagine-30 could antagonize the effect of SCP on *VEGF* mRNA levels in cultured EVTs.

Discussion

CRH has been implicated in playing a role in the regulation of the onset of parturition [33]. Although CRH is a hypothalamic neuropeptide, CRH is produced in several human reproductive organs, including the endometrial glands and decidualized stroma, as well as villous trophoblasts and placental deciduas [34]. Human EVTs also express and release CRH, UCN and express CRHR-1 [32, 35]. Thus far, locally produced CRH has been reported to promote implantation and maintenance of early pregnancy by inducing apoptosis of the surrounding activated T lymphocytes at the fetal-maternal interface through CRHR-1 [32]. On the other hand, CRH has been described to inhibit trophoblast invasion by decreasing the expression of carcinoembryonic antigen-related cell adhesion molecule 1 through CRHR-1 [35]. The exact functions of placental CRH during early pregnancy still remain to be elucidated.

CRH, a 41-amino acid peptide, can activate both CRHR-1 and CRHR-2. CRHR-1 expression in human EVTs has been confirmed, whereas CRHR-2 expression in human EVTs has not been shown yet [32]. In the present study, RT-PCR analysis based on the sequence analysis of the cDNAs obtained from human EVTs indicated that CRHR-1 and CRHR-2, especially CRHR-2β had all correct sequence. Immunocytochemical analysis also demonstrated the presence of CRHR-1 and CRHR-2 proteins in human EVTs. It is, therefore, now evident that both CRHR-1 and CRHR-2 are present in human EVTs. CRHR-2 is a receptor, which can be bound by the newly identified SCP and SRP [2]. Although SCP is a newly identified member of CRH family peptides in the central nervous system, SCP is expressed in many organs ubiquitously. In the pancreas, SCP secreted from pancreas beta cells regulates glucagon and insulin secretion by its binding to the receptor, CRHR-2 in the pancreas beta cells in a paracrine manner [36].

In the present study, we evaluated the effects of the CRH family peptides on the expression of VEGF in cultured early placental EVTs. This is because CRHR-2 knockout mouse indicates that CRHR-2 suppresses vascularization through inhibiting the expression of VEGF [16]. We found that SCP and its receptor, CRHR-2 were identified in cultured early placental EVTs and that both SCP and CRH inhibited VEGF expression through their binding to CRHR-2 in those cells. Since both CRH-induced decrease in *VEGF* mRNA and SCP-induced decrease in *VEGF* mRNA in cultured EVTs were reversed by the concomitant treatment with CRHR-2 antagonist, antisauvagine-30, but not with CRHR-1 antagonist antalarmin, the inhibitory effects of CRH and SCP on the VEGF expression in those cells were mediated through CRHR-2, but not through CRHR-1. It is, therefore, likely that CRH and SCP may affect vascularization in early placentation

through the inhibition of VEGF expression in early placental EVTs.

During early placentation, differentiation and developmental processes such as trophoblast invasion and complete remodeling of maternal vasculature are regulated by angiogenic growth factors and their receptors. EVTs have a unique ability to invade maternal blood vessels to replace the maternal vascular endothelium within the spiral artery segments in the myometrium, and to remodel the tunica media of these arteries [26]. Angiogenic growth factors are considered to be the main mediators of these processes. Actually the importance of VEGF, angiopoietins and their tyrosine kinase receptors in placental angiogenesis has been demonstrated in the mouse [17, 37]. The discrete phenotypes of the null animals suggest the distinct roles for individual families with VEGF family members being important for the initial stages of vasculogenesis and angiogenesis and with angiopoietins being involved in the latter stages of fetal blood vessel maturation [17, 38]. On the other hand, in human EVT cell line HTR-8, VEGF was shown to stimulate proliferation but not migration or invasiveness [24].

The present study is, to our knowledge, the first to reveal that both CRH and SCP are present in cultured early placental EVTs, and that CRH and SCP, through their binding to CRHR-2, inhibit *VEGF* mRNA expression in cultured early placental EVTs. These findings suggest that CRH and SCP produced in early placental EVTs may take an inhibitory role in the autocrine/paracrine regulation of placental vascular development through attenuating *VEGF* mRNA expression in those cells. In this context, it is noteworthy that CRH is increased in most samples including myometrium, fetal membranes, and chorionic villi in women with spontaneous abortions when compared with the levels in elective abortions [39].

Materials and methods

Clinical material

Normal early placental tissues were obtained from 32 patients who underwent elective abortion at 4 to 8 weeks of gestation for psychosocial reasons. The gestational age of the placenta was determined by estimating the duration of pregnancy from the date of the patient's last menstrual period and by ultrasound examination. The use of placental tissues in the present study was approved by the institutional review board. Informed consent was obtained from each patient.

Cell culture

Trophoblasts differentiating into EVTs was isolated and purified according to the techniques using the enzymatic digestion of anchoring chorionic villi, as described previously (Oki et al., 2004). Trophoblasts from the cell column were directly accessible to enzymatic digestion and were released from the tissues in aggregates, whereas the isolation of villous cytotrophoblasts required more intensive enzymatic digestion of chorionic villi [40]. Purified human fibronectin (FN); 10 g/ml (ICN Biomedicals, Aurora, OH) was incubated at 37°C for 1 h in 5% CO_2, and then 0.5 ml FN was placed into the dishes. Chorionic villi were incubated in PBS containing 0.125% trypsin (Sigma–Aldrich Corp., Tokyo, Japan), 4.2 mM $MgSO_4$, 25 mM HEPES, and 50 Kunitz unit/ml deoxyribonuclease type IV (Sigma-Chemie, Saint-Quentin, Fallavier, France) at 37°C for 15 min without agitation. After tissue sedimentation, the supernatant was filtered (100 m pore size). The collected cells were sedimented twice with PBS. Trypsin digestion was stopped with 5% FBS (BioWhittaker, Walkersville, MD). The cells obtained were centrifuged at 300*g* for 10 min, diluted to a concentration of 5–6 $\times$ 10^5 cells/2 ml, and then plated on FN-precoated, six-well, 35-mm culture dishes (BD Biosciences, Oxnard, CA) and FN-precoated, two-well chamber plastic slides (Nalge, Nunc International, Naperville, IL). The cells were maintained in bicarbonate-buffered RPMI Medium 1640 (Invitrogen Life Technologies, Grand Island, NY), supplemented with 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 g/ml streptomycin, and incubated in 5% CO_2 at 37°C. For 3 h, the cells were washed three times and were cultured for 48 h at 37°C in 5% CO_2 in bicarbonate-buffered RPMI Medium 1640 supplemented with 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 g/ml streptomycin. The cells attached to FN-precoated dishes were characterized by immunocytochemical analyses of cytokeratin-7 (CK7), human placental lactogen (hPL), ErbB1, and ErbB2 to identify the trophoblastic origin of the cells as we reported previously [40]. The immunocytochemical features of the cells attached to FN-precoated dishes after 48-h subculture were positive for immunostaining of CK7, hPL, and ErB2 and negative for immunostatining of ErB1, indicating the characteristic features of EVTs [41]. The cells were also characterized by RT-PCR analyses of ErbB1 and ErbB2 after 48-h subcultures (date not shown). Thereafter, cultured cells were stepped down to a condition by incubating in RPMI Medium 1640 without FBS supplemented with 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 g/ml streptomycin. After 48 h of subsequent cultures, the expression of CRH, SCP, CRHR-1, CRHR-2, and VEGF was examined by RT-PCR and immunocytochemistry. The cultured cells were treated for the subsequent 24 h with the different doses (10–100 nM) of CRH and SCP, which were dissolved in PBS and distilled water, respectively, according to the manufacture's protocol

(Phoenix Pharmaceuticals Inc., Belmond, CA) in the presence or absence of 1 M antalarmin (42) (Sigma–Aldrich Corp., Tokyo, Japan), or 1 M antisauvagine-30 [43] (Phoenix Pharmaceuticals Inc., Belmond. CA). After 24 h of subsequent cultures, VEGF expression in cultured EVTs was monitored by real-time quantitative RT-PCR.

Total RNA extraction and cDNA synthesis

After 24 h of subsequent cultures, total RNA was isolated from EVTs using RNeasy Micro Kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized from 4 μg total RNA using a Transcriptor 1st Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany).

RT-PCR

PCR was performed using 0.1 μg cDNA as a template in a 25-μl reaction buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100 containing 1.0 pM of each primer, 2.5 mM deoxy-NTPs, and 2.5 Taq DNA polymerase (Promega Corp., Madison, WI)]. Reactions were amplified by a Gene Amp PCR System 9600-R (PerkinElmer, Norwalk, CT) using the following thermal profile. The specific oligonucleotide primers designed to amplify the sequences of CRH, SCP, CRHR-1, CRHR-2, and VEGF are listed in Table 1. The 10 microliters reaction mixture was electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide. Distilled water was used as a negative control for all of the reactions in place of the cDNA. The resultant PCR products were cloned into TA cloning vector (Invitrogen, San Diego, CA) and sequenced (Bio Matrix Research, Inc, Chiba, Japan). The sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information.

Immunocytochemistry for cultured early human EVTs

Immunocytochemical staining was performed using the avidin-biotin immunoperoxidase method with the use of Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Briefly, cultured cells attached to the FN-precoated chamber slides were washed three times with PBS at room temperature and were fixed in 1.0% formaldehyde for 10 min. The fixed cells were preincubated with 3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity, and then blocked with 5% blocking serum in PBS for 30 min to saturate nonspecific binding sites. The primary antibodies against CRH, SCP, CRHR-1, CRHR-2, and VEGF were diluted with PBS containing 5% blocking serum. The fixed cells were incubated overnight at 4°C and washed three times for 20 min with PBS. The fixed cells were incubated with biotinylated polyvalent antibody, and then were carried out with avidin-biotinylated enzyme complex. Thereafter, chromogenic reaction was developed by incubation with prepared solution of DAB substrate Kit (Vector Laboratories, Inc., Burlingame, CA). Negative controls were performed by substituting the primary antibody with peptides. The cells were counterstained with Harris hematoxylin, and mounted in 65% methylbenzene toluol phenylmethane. The primary antibodies used are indicated in Table 2.

Real-time quantitative RT-PCR

After 24 h of subsequent cultures with either CRH or SCP, total RNA was obtained from cultured EVTs. For *VEGF*

Table 1 Primer sequences used in RT-PCR

Sense primer, antisense primer	Sense primer, antisense primer	Annealing temperature/cycle	PCR product (bp)
CRH	5′-TCCGAGGAGCCTCCCATC-3′	65°C/32 cycle	122
	5′- AATCTCCATGAGTTTCCTGTTGC-3′		
SCP	5′-TACCTGCGCAGCAGAGACGCCTCTTC-3′	65°C/32 cycle	237
	5′-CTTGGCGATGTTGAAGAGGAGGTTCAT-3′		
CRHR-1	5′-TGGTGTCCGCTACAATACCA-3′	65°C/35 cycle	164
	5′-AGTGGCCAGGTAGTTGATG-3′		
CRHR-2β	5′-TCAGCCGTGAGGAAGAGGTG-3′	66°C/35 cycle	126
	5′-GGCCGTCTGCTTGATGCTGT-3′		
VEGF	5′-CACATAGGAGAGATGAGCTTC-3′	57°C/33 cycle	*
	5′-CCGCCTCGGCTTGTCACAT-3′		
β-actin	5′-AAGAGAGGCATCCTCACCCT-3′	52°C/35 cycle	218
	5′-TACATGGCTGGGGTGTTGAA-3′		

*The VEGF primers correspond to exon 4 and exon 8 of the VEGF gene. These amplified fragments of 98, 168, 228, 300, and 350 base pairs (bp) correspond to human VEGF isoforms 121, 145, 165, 189, and 206, respectively

Table 2 Antibodies used for immunocytochemistry

Antibodies	Species	Dilution	Source
CRH	Rabbit	1:100	Phoenix Pharmaceuticals, Inc. Belmont, CA
SCP	Rabbit	1:1500	Phoenix Pharmaceuticals, Inc. Belmont, CA
CRHR-1	Goat	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
CRHR-2	Goat	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
VEGF	Mouse	1:200	Sigma-Aldrich Corp., Tokyo, Japan

mRNA levels, the ratio of *VEGF* to *GAPDH* mRNA copy number was measured with real-time quantitative RT-PCR using a LightCycler system and SYBR green I dye (Roche Molecular Systems, Indianapolis, IN). The relative *VEGF* mRNA levels in EVTs treated with either CRH or SCP were calculated compared to untreated controls. The VEGF and GAPDH primers and their standard cDNAs were synthesized (Search-LC, Heidelberg, Germany). The reaction mixture contained 2 μl LC DNA Master SYBR Green I, 2 μl of GAPDH or VEGF LightCycler-Primer Set (Search-LC, Heidelberg, Germany), and 10 μl cDNA with dilution at 1:500. The final volume was adjusted to 20 μl with H_2O. The PCR conditions were programmed according to the primer supplier's instructions. Fluorescent products were measured by a single acquisition mode after each cycle. To distinguish specific products from non-specific products, and primer dimers, a melting curve was obtained.

Statistical analysis

Results were expressed as the mean ± SEM of three independent experiments. Statistical analyses were carried out by one-way ANOVA and posthoc Student's *t*-test. *P* value of <0.05 was considered to be significant.

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References

1. S.N. Kalantaridou, A. Makrigiannakis, E. Zoumakis, G.P. Chrousos, J. Reprod. Immunol. **62**, 61–68 (2004)
2. S.Y. Hsu, A.J. Hsueh, Nat. Med. **7**, 605–611 (2001)
3. M.H. Perrin, W.W. Vale, Ann. N. Y. Acad. Sci. **885**, 312–328 (1999)
4. S.C. Weninger, A.J. Dunn, L.J. Muglia et al., Proc. Natl. Acad. Sci. U.S.A. **96**, 8283–8288 (1999)
5. A. Slominski, J. Wortsman, A. Pisarchik et al., FASEB J. **15**, 1678–1693 (2001)
6. D. Richard, Q. Lin, E. Timofeeva, Eur. J. Pharmacol. **440**, 189–197 (2002)
7. D.K. Grammatopoulos, G.P. Chrousos, Trends Endocrinol. Metab. **13**, 436–444 (2002)
8. J. Vaughan, C. Donaldson, J. Bittencourt et al., Nature **378**, 287–292 (1995)
9. Y. Muramatsu, K. Fukushima, K. Iino et al., Peptides **21**, 1799–1809 (2000)
10. Y. Kimura, K. Takahashi, K. Totsune et al., J. Clin. Endocrinol. Metab. **87**, 340–346 (2002)
11. M. Saruta, K. Takahashi, T. Suzuki, A. Torii, M. Kawakami, H. Sasano, J. Clin. Endocrinol. Metab. **89**, 5352–5361 (2004)
12. D. Grammatopoulos, Y. Dai, J. Chen et al., J. Clin. Endocrinol. Metab. **83**, 2539–2544 (1998)
13. W.A. Kostich, A. Chen, K. Sperle, B.L. Largent, Mol. Endocrinol. **12**, 1077–1085 (1998)
14. G.W. Smith, J.M. Aubry, F. Dellu et al., Neuron **20**, 1093–1102 (1998)
15. T. Kishimoto, J. Radulovic, M. Radulovic et al., Nat. Genet. **24**, 415–419 (2000)
16. T.L. Bale, F.J. Giordano, R.P. Hickey et al., Proc. Natl. Acad. Sci. U.S.A **99**, 7734–7739 (2002)
17. P. Hoffmann, J.J. Feige, N. Alfaidy, Endocrinology **147**, 1675–1684 (2006)
18. P. Kaufmann, M. Castellucci, Trophoblast. Res. **10**, 21–65 (1997)
19. O. Genbacev, S.A. Schubach, R.K. Miller, Placenta **13**, 439–461 (1992)
20. J.A. Irving, J.J. Lysiak, C.H. Graham, S. Hearn, V.K. Han, P.K. Lala, Placenta **16**, 413–433 (1995)
21. G. Aboagye-Mathiesen, J. Laugesen, M. Zdravkovic, P. Ebbesen, Clin. Diagn. Lab. Immunol. **3**, 14–22 (1996)
22. C. Wulff, H. Wilson, S.E. Dickson, S.J. Wiegand, H.M. Fraser, Biol. Reprod. **66**, 802–812 (2002)
23. D.S. Charnock-Jones, A.M. Sharkey, C.A. Boocock et al., Biol. Reprod. **51**, 524–530 (1994)
24. A. Athanassiades, G.S. Hamilton, P.K. Lala, Biol. Reprod. **59**, 643–654 (1998)
25. A. Athanassiades, P.K. Lala, Placenta **19**, 465–473 (1998)
26. P. Kaufmann, S. Black, B. Huppertz, Biol. Reprod. **69**, 1–7 (2003)
27. Y. Zhou, M. McMaster, K. Woo et al., Am. J. Pathol. **160**, 1405–1423 (2002)
28. R.J. Levine, S.E. Maynard, C. Qian et al., N. Engl. J. Med. **350**, 672–683 (2004)
29. R. Thadhani, W.P. Mutter, M. Wolf et al., J. Clin. Endocrinol. Metab. **89**, 770–775 (2004)
30. Y. Bdolah, S.A. Karumanchi, B.P. Sachs, Croat. Med. J. **46**, 728–736 (2005)
31. C.M. Bamberger, V. Minas, A.M. Bamberger, I. Charalampopoulos, Y. Fragouli, H.M. Schulte, A. Makrigiannakis, Placenta **28**, 127–132 (2007)
32. A. Makrigiannakis, E. Zoumakis, S. Kalantaridou et al., Nat. Immunol. **2**, 1018–1024 (2001)
33. M. McLean, A. Bisits, J. Davies, R. Woods, P. Lowry, R. Smith, Nat. Med. **1**, 460–463 (1995)
34. P. Florio, W. Vale, F. Petraglia, Peptides **25**, 1751–1757 (2004)
35. A.M. Bamberger, V. Minas, S.N. Kalantaridou et al., Am. J. Pathol. **168**, 141–150 (2006)
36. C. Li, P. Chen, J. Vaughan et al., Endocrinology **144**, 3216–3224 (2003)
37. S.E. Maynard, J.Y. Min, J. Merchan et al., J. Clin. Invest. **111**, 649–658 (2003)
38. S.K. Smith, Hum. Reprod. **15**(Suppl 6), 59–66 (2000)

39. B. Madhappan, D. Kempuraj, S. Christodoulou et al., Endocrinology **144**, 2285–2290 (2003)
40. N. Oki, H. Matsuo, S. Nakago, H. Murakoshi, J.B Laoag-Fernandez, T. Maruo, J. Clin. Endocrinol. Metab. **89**, 5213–5221 (2004)
41. A. Tarrade, R. Lai-Kuen, A. Malassine, V. Tricottet, P. Blain, M. Vidaud, D. Evain-Brion, Lab. Invest. **81**, 1199–1211 (2001)
42. Webster, Lewis, Torpy, Zachman, Rice, Chrousos, Endocrinology 137, 5747–5750 (1996)
43. Higelin, Neuropharmacology 40, 114–122 (2001)