Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium

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ABSTRACT **Successful embryo development requires an extensive endometrial angiogenesis in proximity of implantation site. The glycoprotein hCG is produced even before implantation by trophoblast in normal pregnancy. In this manuscript, we demonstrate an angiogenic effect of hCG in several *in vivo* (chick chorioallantoïc membrane, matrigel plug assay, aortic ring assay) and *in vitro* experimental models. In contrast, human placental lactogen (hPL) did not display angiogenic properties. LH/hCG receptor was detected in endothelial cells by reverse-transcriptase polymerase chain reaction (RT-PCR) and by Western blotting. In mice aortic ring assay, angiostimulation by hCG was abrogated by deletion of LH/hCG receptor (LuRKO mice). Use of recombinant hCG and anti–hCG antibody (Ab) further confirmed the specificity of this angiogenic activity. By using dibutyryl cAMP, adenylate cyclase, or protein kinase A inhibitors, we demonstrate that hCG-mediated angiogenesis involves adenylyl-cyclase–protein kinase A activation. Addition of hCG to endometrial epithelial epithelial cells, but not to cultured endothelial cells, stimulated vascular endothelial growth factor (VEGF). VEGF and hCG also displayed additive activities. Altogether, these data demonstrate that peritrophoblastic angiostimulation may result from a paracrine dialogue between trophoblast, epithelial, and endothelial cells through hCG and VEGF.—Berndt, S., Perrier d'Hauterive, S., Blacher, S., Péqueux, C., Lorquet, S., Munaut, C., Applanat, M., Hervé, M. A., Lamandé, N., Corvol, P., van den Brûle, F., Frankenne, F., Poutanen, M., Huhtaniemi, I., Geenen, V., Noël, A., Foidart, J.-M. Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. *FASEB J.* 20, E2189–E2198 (2006)**

Key Words: angiogenesis · hCG

Successful embryo implantation requires a precise synchrony between the blastocyst and the uterine environment where a complex series of interactions occurs. Indeed implantation involves a complex interplay of growth factors, cytokines and prostaglandins resulting in inflammatory responses in the human endometrium [for a review see Kelly *et al.* (1)]. Although the precise mechanisms regulating implantation are still unknown, increasing evidence suggests that hCG, one of the major hormonal products of the first trimester human placental trophoblast cells plays an important role in these materno–embryonic interactions (2).

hCG is required to maintain pregnancy in the primate and exerts its effects in the ovary, through LH/hCG receptors, by stimulating progesterone secretion from the corpus luteum during the first trimester. Several studies indicate that hCG signals act also directly on endometrium (3) and modulate stromal and epithelial cell activities, already prior to and in preparation for blastocyst implantation [for a review, see Cameo *et al.* (4)]. For example, injection of hCG reduces apoptosis in human endometrium (5) and a local infusion of hCG to nonpregnant baboons functionally alters the major cell types present in baboon uterine endometrium, in a pattern that resembles that observed after implantation (6, 7).

hCG and LH activities are mediated by the same receptor (LH/hCG R). The expression of this receptor was previously thought to be restricted to gonadal tissue. However, recent studies have shown its presence in many other tissues throughout the reproductive and

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nonreproductive organs such as neural retina (8), adult rat spinal cord (9), human skin (10), mammary gland (11), and vascular tissue (12, 13). This G protein-coupled receptor activates mainly cAMP/PKA pathway (14, 15).

hCG increases uterine arterial blood flow (12) and also stimulates angiogenesis in the ovary, by stimulating vascular endothelial cell proliferation and VEGF expression (16). LH/hCG R expression has been documented in uterine endothelial cells by ligand binding of ^{125}I hCG and activity measurements (12).

A recent report has depicted urinary hCG as a proangiogenic factor (17). It remains possible, however, that such an activity could be the consequence of a contaminant present in the urinary extract, since anti HIV activity ascribed to hCG is considered in some studies to be the consequence of an urinary contaminant present in the preparation (18). Nevertheless, others provided evidence for induction of apoptosis in Kaposi's sarcoma spindle cells by the recombinant hCG β subunit (19).

One of the key local adaptations to pregnancy is the stimulation of maternal vessel network at the embryo implantation site. Normal fetal development requires extensive angiogenesis and important vascular remodeling that allows adequate supply of nutrients as well as gas and metabolite exchanges. Abnormal uterine blood supply is associated with higher perinatal morbidity and mortality caused by preterm delivery, preeclampsia, or intrauterine growth restriction. Stimulation of angiogenesis in many organs is mediated through enhanced expression of VEGF, an angiogenic cytokine produced by endometrial epithelial and stromal cells with the highest levels in the late secretory and premenstrual phases (20). Therefore, in this study, we aimed at (1) reevaluating the proangiogenic effect of hCG in different *in vivo* and *in vitro* models of angiogenesis, elucidating molecular mechanisms of this effect (2) and identifying the endometrial cellular target(s) triggered by hCG (3).

MATERIALS AND METHODS

Materials

Reagents tested were purified urinary hCG (Pregnyl®, Organon, Roseland, NJ, USA), human recombinant hCG (Ovitrelle® Serono, Geneva, Switzerland), hPL (Sigma, St. Louis, MO, USA), VEGF (PeproTech, London, UK), antihCG Ab (Sc-7821, Santa Cruz Biotechnology, Santa Cruz, CA, USA), dibutyryl-cAMP (D0260, Sigma), H89 dihydrochloride (Calbiochem, La Jolla, CA, USA), MDL-12 330A hydrochloride (Calbiochem), recombinant human FGF basic (R&D Systems, Abingdon, UK), polyclonal goat anti-mouse LHR Ab (Sc-26343, Santa Cruz Biotechnology).

LuRKO mice

LH/hCG receptor knockout mice (LuRKO) were generated by disruption of exon 11 of LHR gene (21). For aortic ring assay, thoracic aortas were removed from six male mice of 6 wk old.

Aortic ring assay

Rat aortic rings were cultured in three-dimensional collagen gels as described previously (22, 23). Effects of different compounds were tested by adding them into culture medium at day 0. In some studies, aortic explants resected from wild-type (WT) or LuR KO mice with the same genetic background (C57Bl) were used and maintained in the presence of 2% serum collected from WT or KO mice. For quantification, image analysis was performed on a Sun SPARC30 workstation with the software Visilog 5.0 from Noesis according to Blacher *et al.* (23). The following parameters were determined: number of microvessels (Nv); maximal microvessel length (Lmax); and total number of branching in microvessels (Nb).

In vivo mouse Matrigel plug assay

The mouse Matrigel plug assay was performed as described previously (24). Briefly, C57BL/6J mice were injected subcutaneously (s.c.) with 0.5 ml Matrigel containing heparin (10 U/ml) and the indicated amount of hCG or basic fibroblast growth factor (bFGF) (250 ng/ml). After 7 d, Matrigel plugs were carefully dissected and frozen. Plugs were lyophilized, weighed, and homogenized for 1 h in 400 μl of saponine 0.1%. Samples were spun at 10,000 rpm in a microcentrifuge for 6 min, and supernatants were collected for hemoglobin (Hb) measurement by using Total Hb kit (Sigma Diagnostics) (25). Results are expressed as Hb concentration normalized to plug wt.

Chicken chorioallantoic membrane (CAM) assay

Fertilized chicken eggs were incubated at 38°C in a humidified incubator and were prepared for implantation on day 3 of incubation. Incubation was performed with cell adhesion molecule (CAM) out of shells, in ventilated cell culture dishes (26). On day 8 of incubation, depending on CAM development, up to three silicone rings (with a thickness of 0.3 mm and an inner diameter of 9 mm) were placed on each membrane, hCG solutions (25 μl) containing 50, 200, or 500 IU were spotted into rings. Four days later, CAMs were injected intravenously (i.v.) with FITC-dextran to facilitate vasculature identification. To quantify angiogenesis (27), the following global parameters were determined: (a) the vessel area density defined as number of pixels forming the vascular network divided by total area, (b) the vessel length density defined as number of pixels forming the skeleton of vascular network divided by total area, (c) the first-order vessel density per area defined as number of vessel extremities divided by total area; and (d) the branching density defined as the number of crossing vessels divided by total area.

HUVEC culture

Human umbilical vein endothelial cells (HUVEC) isolated with collagenase I (Sigma) were grown on 0.2% gelatin-coated dishes in 10% FBS MCDB 131 supplemented with HEPES 25 mM, glutamin 2 mM, endothelial cell growth supplement (12 ng/ml), heparin (2.5 mg/ml), and antibiotics (penicillin–streptomycin 100 U/ml) as described previously (28). These cells were cultured in 5% CO_2 at 37°C, and media were replaced every 2 d. Cells at passage two to six were used for experiments.

Isolation of endometrial cells

Endometrial biopsies were collected from fertile women (one previous delivery) with normal cycles (n=18) undergoing surgery for voluntary sterilization or during hysteroscopy before assisted medical procreation (AMP) because of male infertility. The stage of menstrual cycle was established from women's menstrual history (based on the first day of last menstruation) and from histological dating performed by experienced pathologists, according to criteria of Noyes *et al.* (29). Nine patients were in proliferative phase, and nine were in secretory phase. The mean age was 33.5 yr. None of the patients received any hormonal treatment for three months prior to biopsy. Stromal and epithelial cells were isolated and cultured as described previously (30). The purity of the cell population was verified as described previously (2;30) by using appropriate antibodies to identify the epithelial cells (anti cytokeratin Ab), the stromal cells (anti vimentin and alpha smooth muscle actin), or the endothelial cells (CD34 Ab). All cultures had a purity higher than 97%.

HUVEC and endometrial epithelial cell (EEC) proliferation assay

HUVEC were allowed to adhere and spread on 96-well, gelatin-coated dishes (5×10^3 cells/well). Cells were treated with or without hCG (10–1000 U/ml) in MCDB131 containing 2% FBS for 3 d. Endothelial cell proliferation was evaluated using a 5-bromo-2′deoxyuridine (bromodeoxyuridine) colorimetric immunoassays applied according to manufacturer's instructions (Roche, Indianapolis, IN, USA). To study epithelial cell proliferation, EEC from three different endometria were cultured for 72h using bromodeoxyuridine (BrdU) cell proliferation colorimetric immunoassay applied according to manufacturer's instructions (Roche).

RT-PCR analysis

Total RNA was extracted from HUVEC cell monolayer supplemented or not with uhCG (100 IU/ml) or granulosa cells using an RNeasy Mini Kit® from Qiagen (Valencia, CA, USA), according to the manufacturer's instructions. Total RNA (250 ng) were subjected to reverse-transcriptase using the First Strand cDNA Synthesis Kit (Roche). In this method, RNA is reverse transcribed by avian myeloblastosis virus (AMV) into single-stranded cDNA. The cDNA was secondarily amplified by FastStart *Taq*DNA Polymerase (Roche) using the primers corresponding to human hCG/LHR cDNA sequence (upper primer 5′-TTCCTTAGGGTCCTGATTTGG −3′; and lower primer 5′- GTGAATAGCATAGGTGATGGTGTG −3′). PCR reaction mixture (50 μl) contained 20 pmol of each oligonucleotide primer and 2 IU of *Taq*DNA polymerase. The reaction was started at 94°C for 5 min and run for 40 cycles (45 s at 94°C, 1 min at 56°C, and 1 min at 72°C). The expected PCR product was 349 bp in length. 28S PCR primer pair (Eurogentec, Belgium) was used, resulting in a PCR product of 200 bp. PCR conditions for 28S were 95°C for 2 min, 17 cycles consisting of 94°C/15 s, 68°C/20 s, 72°C/10 s, and a final elongation step of 72°C/2 min. The amplification products were electrophoresed on a polyacrylamide gel, stained with Gelstar (Sanver Tech, Antwerpen, Belgium), and scanned with FluorSImager. Aliquots of 15 μl of amplified fragments were visualized in ethidium bromide-stained 2% agarose gel.

Western blot analysis

Total cell extracts were prepared by incubating cells in radioimmune precipitation assay buffer (50 mM Tris-HCl, ph 7.4; 150 mM NaCl; 1% Nonidet P40, 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 5 mM iodoacetamide; 2 mM phenylmethylsulfonyl fluoride). After centrifugation at 15,000 rpm for 30 min, at 4°C, the supernatant was stored frozen at –20°C. Protein concentration was determined by using DC protein assay Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to 5 mg/ml. HUVEC extracts were resolved by SDS-PAGE 10% concentrated under reducing conditions and transferred to nitrocellulose membranes (Hybond-enhanced chemiluminescence membrane; Amersham, Arlington Heights, IL). Membranes were exposed to polyclonal primary Ab raised against rat LH receptor (1/2500) (BP605 Ab, Acris antibodies, Hiddenhausen, Germany). After extensive washings, membranes were incubated with a secondary horseradish peroxidase-conjugated swine anti-rabbit Ab at 1/1000 (DAKO, Glostrup, Denmark). Signals were detected using an enhanced chemiluminescence (ECL) kit (PerkinElmer Life Sciences, Boston, MA, USA) according to manufacturer's instructions.

Cytokine and Db cAMP immunoassays

VEGF concentrations were measured by using ELISA kit (R&D Systems) with a sensitivity of 5 pg/ml and a range of 15.6–1000 pg/ml. Inter- and intra-assay coefficients of variation (CV) were <8.5% and <6.5%, respectively. To determine cAMP concentration, cyclic AMP enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) was used.

Statistical analysis

All experimental data are reported as mean ± SEM, and statistical analysis was performed by ANOVA test or Student's t test. $P < 0.05$ was considered as significant.

RESULTS

hCG stimulates *in vivo* angiogenesis

The angiogenic activity of hCG was first investigated by using rat aortic ring assay. Incubation with physiological concentrations of urinary hCG (uhCG) (8–800 IU/ml) or recombinant hCG (rhCG) (10–400 IU/ml) resulted in an important stimulation of vessel outgrowth (**Fig. 1*A***). A dose-dependent effect of both recombinant and uhCG was observed on the number of vessels (Nv), of branchings (Nb) as well as on the length of vessels (Lmax) (Fig. 1*B*). This dose-response curve displayed a bell-shape with maximal effect of uhCG and rhCG between 100 to 200 IU/ml. The specificity of the angiogenic effect of uhCG was further assessed by complete inhibition with a neutralizing anti-hCG Ab (Fig. 1*C*). The angiogenic effect of uhCG was also evaluated in two other *in vivo* models: the CAM and the Matrigel plug assays. Similarly, uhCG enhanced the complexity of vascular network in the CAM assay (**Fig. 2**). An original computer-assisted method of quantification based on image analysis (27) revealed that uhCG stimulated different morphological vessel parameters such as: end point density (2579 ± 879.10^{-7} in the absence of hCG *vs.* 3278 ± 811.10^{-7} in the presence of uhCG (50 IU) for 4 d; $P<0.05$), branching density

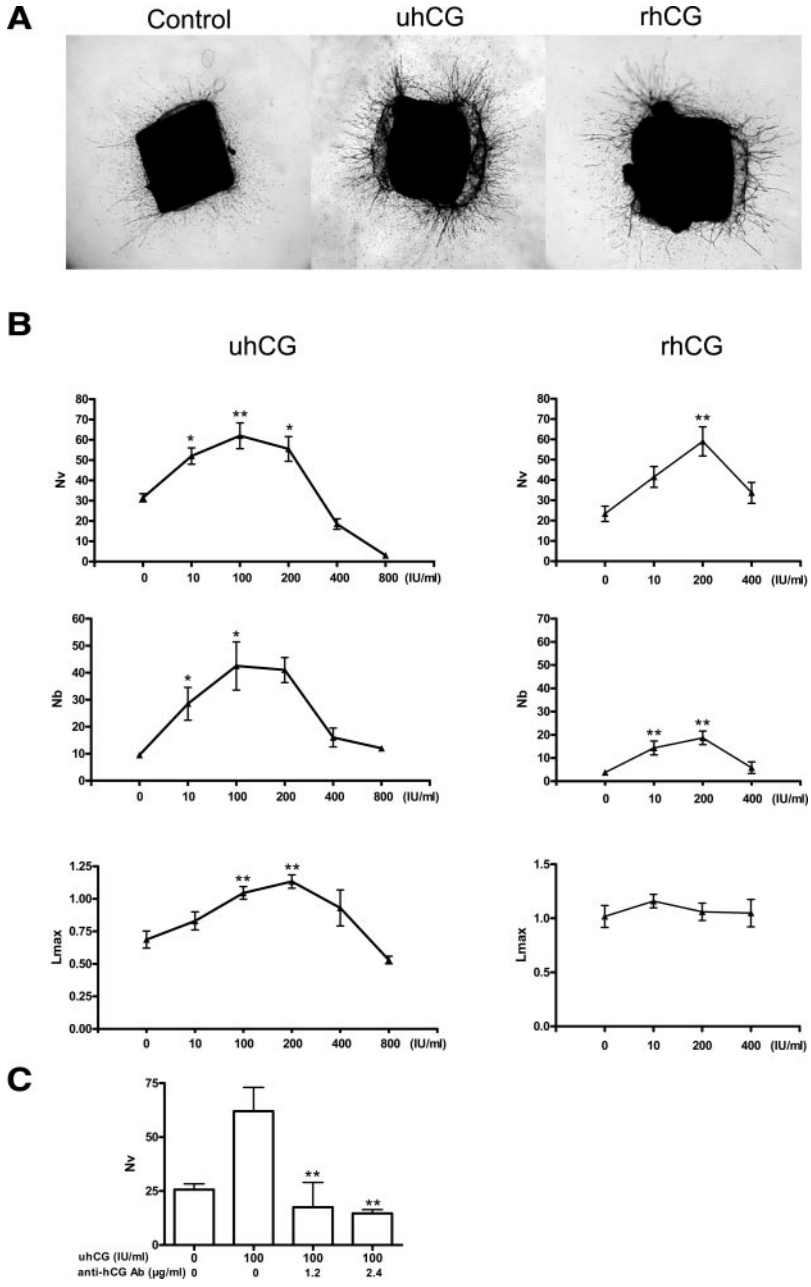


Figure 1. Angiogenic effect of urinary hCG (uhCG) and recombinant hCG (rhCG) in aortic ring assay. Rat aortic rings were cultured in a collagen gel for 10 d with various hCG concentrations. *A)* Photomicrographs (original magnification ×25) of aortic rings after 10 d of incubation with uhCG (Pregnyl, 100 IU/ml), with rhCG (100 IU/ml) or with vehicle (control). *B)* Quantification of microvessel outgrowth by computerized image analysis. Nv, vessel number; Nb, branching number; Lmax, maximal length (mm). $n = 4$; error bars = SE.; $^{*}P < 0.05$; $^{**}P < 0.01$. *C)* Histogram of vessel number (Nv) outgrowth from rat aortic rings incubated during 10 d with uhCG (100 IU/ml) supplemented with various concentrations (1.2 and 2.4 μg/ml) of anti-hCG Ab. $n = 4$, error bars = SE., $^{**}P < 0.01$. Experiments were repeated at least three times. Student's *t* test was used to evaluate whether differences among groups were significant. Statistical significance was set at $P < 0.05$.

(3465 ± 1194.10^{-7} in the absence of hCG *vs.* and 4615 ± 1265.10^{-7} in the presence of uhCG (50 IU) for 4 d; $P<0.05$), vessel area density (1596 ± 214.10^{-4} in the absence of hCG *vs.* and 1787 ± 229.10^{-4} in the presence of uhCG (50 IU) for 4 d; $P<0.05$) and length densities (1821 ± 374.10^{-5} in the absence of hCG *vs.* 2142 ± 4189.10^{-5} in the presence of uhCG (50 IU) for 4 d; $P>0.05$). Similar results were obtained at day 1 and day 4 post-uhCG supplementation (data not shown).

In matrigel plug assay, uhCG (500 IU/ml gel) stimulated the invasion of matrix by functional blood vessels to an extent comparable with that of 250 ng bFGF (**Fig. 3**). Higher doses (2500 IU) were less active. hPL (7.5 μg/ml) failed to stimulate blood vessel formation (data not shown).

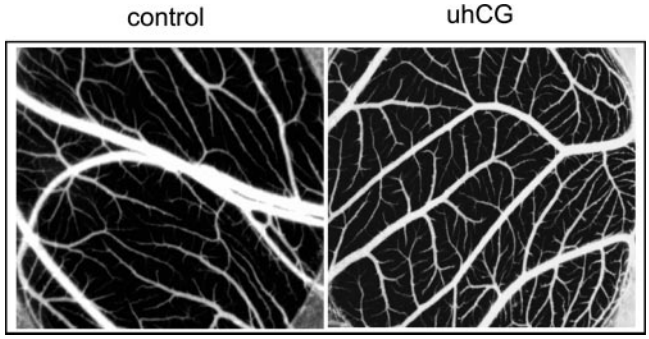


Figure 2. Angiogenic effect of urinary hCG (uhCG) in chicken chorioallantoic membranes (CAM) assay. Photomicrographs (original magnification ×12) of CAM at day 9 after culture supplemented during 24 h with uhCG (50 IU/ml) or with vehicle (control).

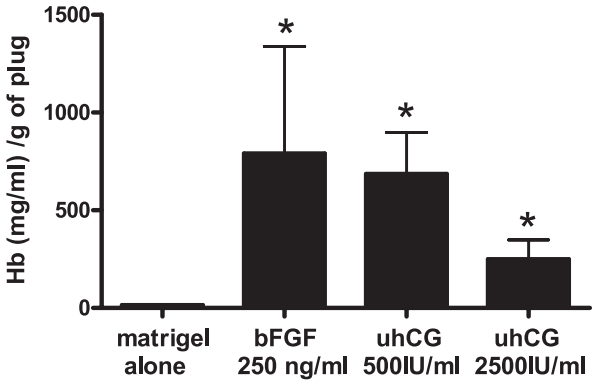
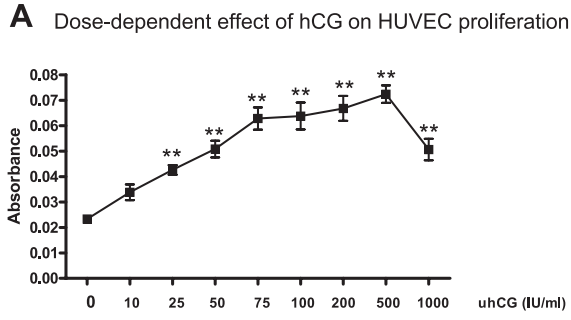


Figure 3. Angiogenic effect of urinary hCG (uhCG) in matrigel plug assay. Histogram of Hb concentration (mg/ml) normalized to 1g of matrigel plug (mean±SEM; *$P<0.05$, $n=7$). Matrigel was supplemented with vehicle (control), with uhCG (500, 2.500 IU/ml) or with bFGF (250 ng/ml) used as positive control.

hCG stimulates *in vitro* endothelial cell proliferation

As shown in **Fig. 4*A***, uhCG (10–200 U/ml) significantly increased HUVEC proliferation ($P<0.01$). hCG induced a three-fold increase in HUVEC proliferation rate at 200 U/ml, respectively (0.023±0.002 and 0.067±0.005; $P<0.01$). This effect is specific to endothelial cells, since hCG did not affect endometrial epithelial (Fig. 4*B*).



B Lack of effect of hCG on EEC proliferation

	EEC
Primary cultures N=3	Absorbance (mean+/- SD)
Basal	0.10 (± 0.02)
hCG 50IU/ml	0.10 (± 0.02)
EGF	0.22 (± 0.07) (p=0.02)
FCS 10%	0.25 (± 0.05)

Figure 4. Effect of uhCG on HUVEC and endometrial epithelial cell proliferation. *A)* Proliferation of HUVEC treated for 3 d with 10–1000 IU/ml hCG was evaluated by BrdU colorimetric immunoassay. Data are expressed as means ± SD, $n = 10$. **$P < 0.01$. *B)* Table of absorbance values (mean±SD) after BrdU colorimetric immunoassay.

The angiogenic effect of hCG on endothelial cells is mediated via activation of LH/hCGReceptor and cAMP/PKA pathway

hCG is known to exert its effect by interacting with its LH/hCG R leading to activation of the Gs/adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway (15). LH/hCG R mRNA was depicted by RT-PCR in HUVEC, stimulated or not with uhCG [granulosa cells extracted from large (>20 mm) antral follicles were used as positive control, while extracts from liver or dermal fibroblasts were constantly negative (data not shown) (**Fig. 5*A***)]. Western blot analysis also identified LH/hCG R in cellular extracts of HUVEC (Fig. 5*B*). Two bands corresponding to a partial processing of the full-length LHR (31) were detected in granulosa extracts used as positive control. Only one band was identified in endothelial cells. Treatment of HUVEC for 48 h with uhCG did not affect the expression of this receptor.

Dibutyryl cAMP stimulated vessel outgrowth from aortic ring (**Fig 6*Aa***, suggesting the importance of the PKA pathway during this angiogenic response. MDL12, an adenylate cyclase inhibitor could abolish the cAMP increase after uhCG (100 IU/ml) stimulation in HUVEC culture for 24 h (Fig. 6*A*b). However, H89, an inhibitor of PKA (32), reduced angiogenesis both in the presence or absence of hCG in the aortic ring assay (Fig. 6*B*), probably by reducing the endothelial cell proliferation that is stimulated by hCG (Fig. 6*C*).

In earlier studies, some of the extragonadal effects of

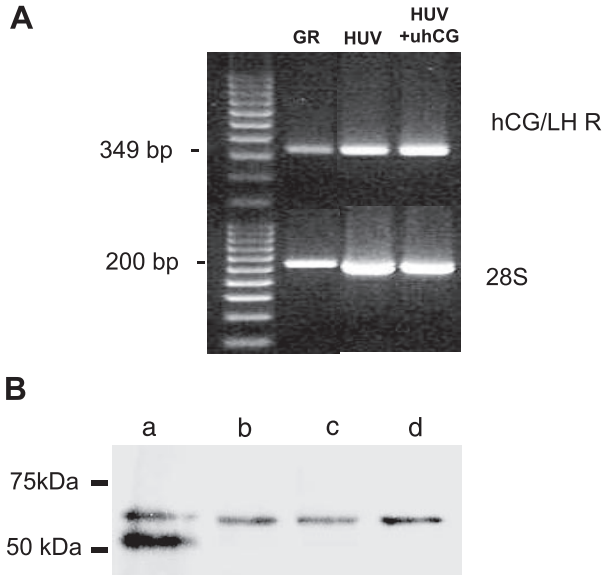


Figure 5. LH/hCG R expression in HUVEC. *A)* LH/hCG R RT-PCR product obtained from total RNA preparation of human granulosa (GR) used as positive control and of HUVEC incubated for 48 h without (–) or with uhCG (+ uhCG 100 IU/ml). The 28S RNA was used as loading control. *B)* Western immunoblotting for LH/hCG R in HUVEC after 48 h of culture (lane b), with 100 IU/ml of uhCG (lane c), and with 100 IU/ml of rhCG (lane d). Human granulosa cells (lane a) were used as positive control.

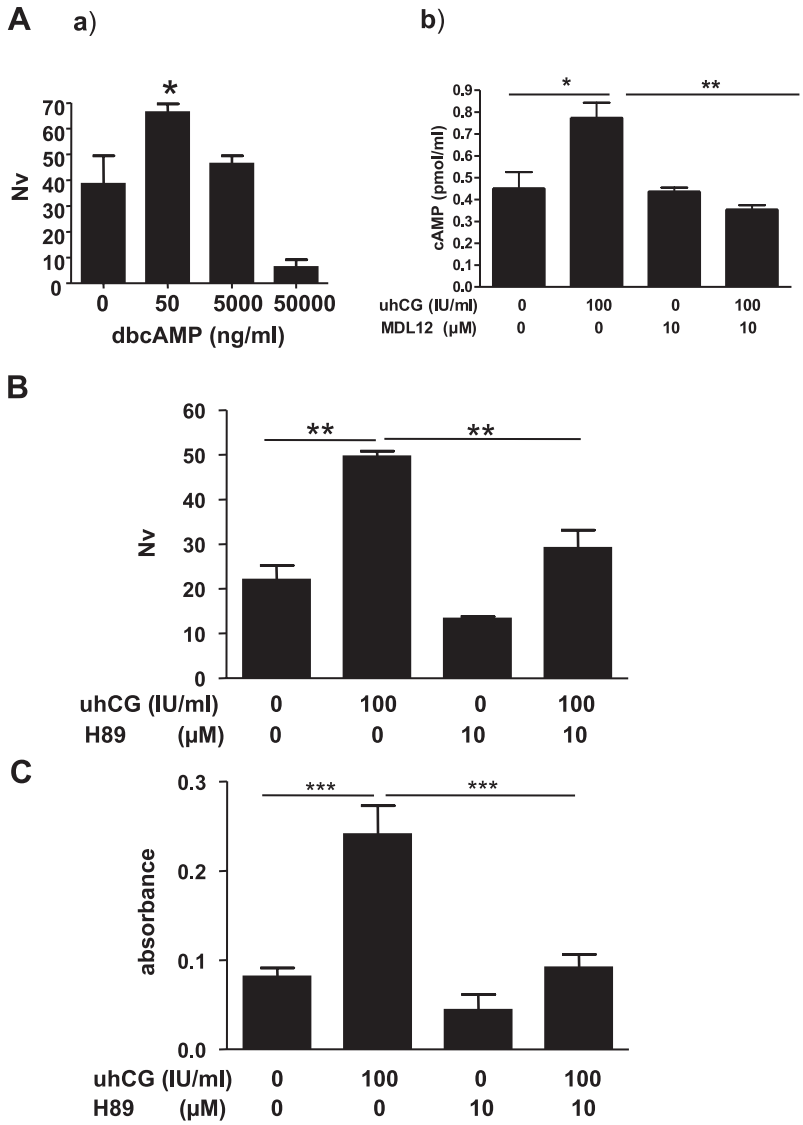


Figure 6. Adenylate cyclase/PKA implication in uhCG-mediated angiogenesis. *Aa*) Histogram of vessel number (Nv) outgrowth from rat aortic rings incubated during 9 d with dbcAMP concentrations ranging from 50 to 50000 (ng/ml). Data are expressed as means ± SD, $n = 4$. $^*P < 0.05$. *Ab*) cAMP concentrations (pmol/ml) in HUVEC after 48 h of culture without or with uhCG (100 IU/ml), in the presence or absence of an adenylate cyclase inhibitor, MDL12 (10 μM). *B)* Histogram of vessel number (Nv) outgrowth from rat aortic rings incubated during 9 d with uhCG (100 IU/ml), with a PKA inhibitor, H89 (10 μM), or both. Data are expressed as means ± SD, $n = 4$. *C)* HUVEC proliferation after 72 h incubation with uhCG (100 IU/ml), with H89 (10 μM), or both. Data are expressed as absorbance values after BrdU colorimetric immunoassay, means ± SD, $n = 15$. $^{***}P < 0.001$.

gonadotropins might have been caused by biologically active contaminants, for instance growth factors (33). It is, therefore, important to use also recombinant hormones, free from contaminants, and LuRKO tissues and cells for functional studies. We, therefore, evaluated the effect of uhCG and rhCG on vascular outgrowth from aortice rings from WT and LuRKO mice. Although hCG induced an angiogenic response in aortic rings of WT mice, no stimulation was achieved when aortic rings of LuRKO mice were used (**Fig. 7**). The presence of LHR in WT aorta and its absence in LuRKO were assessed by immunostaining using a polyclonal goat anti-mouse Ab raised against the C-terminal part of the receptor, which has been deleted in LuRKO mice (data not shown).

hCG exerts a paracrine effect on VEGF production by EEC

To determine whether hCG could modulate angiogenesis by affecting VEGF production, the effect of hCG on VEGF production was evaluated in endometrial epithelial cells (EEC) and HUVEC. Treatment with hCG failed to modulate VEGF production by HUVEC (data not shown) but significantly enhanced VEGF secretion by EEC. Indeed, a 1.5-fold increase of VEGF secretion was evidenced by ELISA in the medium of EEC conditioned for 48 h with uhCG (**Fig. 8**).

hCG and VEGF display additive activities

Knowing that hCG could enhance VEGF production by endometrial epithelial cells in culture, we hypothesize that a combination of these two factors could potentialize the angiogenic response. This was confirmed in the aortic ring assay (**Fig. 9**), where we could observe an additive effect between VEGF (10 ng/ml) and uhCG (100 IU/ml). This increased angiogenic response is significant regarding their stimulating effect when two factors are tested alone.

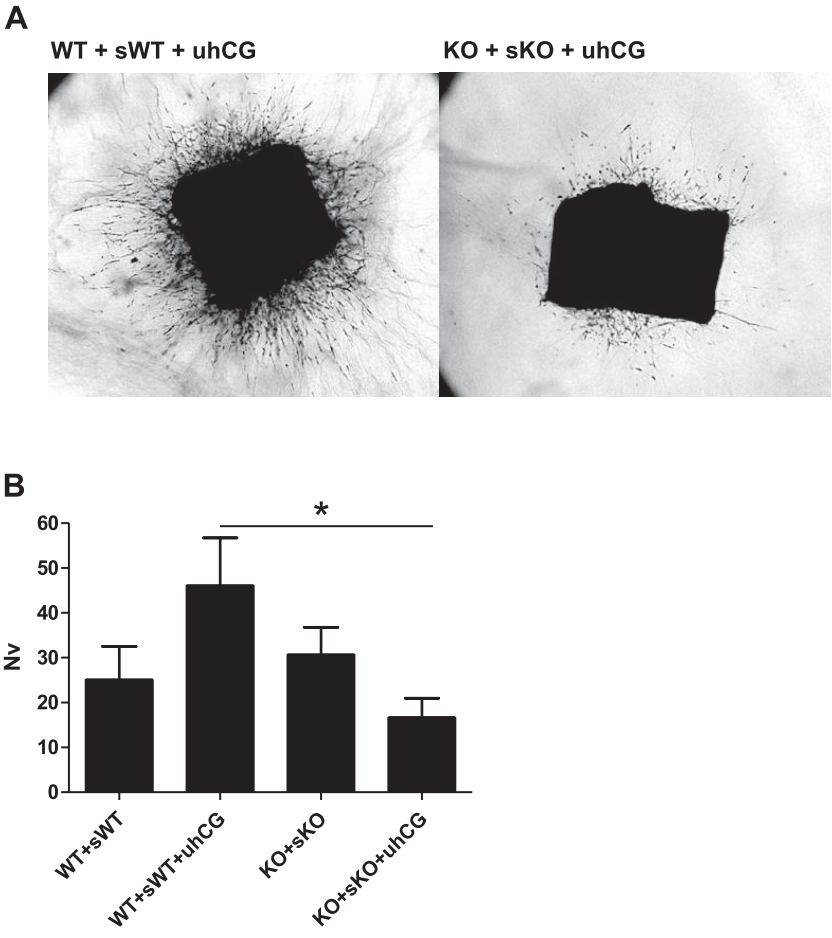


Figure 7. Comparison of uhCG-induced angiogenic effect between WT and LuRKO (KO) mice in the aortic ring assay. LuRKO mice aortic rings were cultured in a collagen gel during nine days in presence of autologous sera (2%) and/or urinary hCG (100 IU/ml). *A)* Photomicrographs (original magnification 25×) of aortic rings from WT mice (wild-type) and LuRKO mice after incubation with autologous sera and supplementation with uhCG (100 IU/ml). *B)* Quantification of microvessel outgrowth (Nv) by computerized image analysis of aortic ring assay after nine days of incubation with autologous sera supplemented or not with uhCG (100 IU/ml) in WT or KO mice aortics. sWT = WT mice serum. sKO = KO mice serum. Data are expressed as means ± SD, $n = 5$, $^*P < 0.05$

DISCUSSION

The effects of hCG are well-documented in gonadal tissue, for example, the luteotropic support for the corpus luteum (34). hCG prevents luteolysis process by maintaining luteal blood flow, limiting tissue remodeling through a reduction of matrix metalloproteinase-2 (MMP-2) activation and macrophage influx (35), and preventing apoptosis *via* an increase in *Bcl*II/Bax ratio (36). During gestation, hCG may play an autocrine/paracrine role in trophoblast invasion (37, 38).

In normal pregnancy, hCG expression is associated with endometrial angiostimulation occurring early in gestation. hCG increases blood supply and alters the uterine vasculature *via* vasodilatation, increasing permeability, development, and maturation of new vessel. (12, 39–41).

Our study demonstrates clearly a direct angiogenic effect of dimeric u and rhCG on endothelial cells in the aortic ring, CAM, matrigel plug, and endothelial cell proliferation assays. A dose-dependent effect was demonstrated on vessel sprouting in the aortic ring and in the matrigel plug assays, as well as on endothelial cell proliferation in HUVEC cultures, with optimal doses of 100–200 IU hCG/ml, while lower but also higher doses were less effective. Thus, our study is in line with the report by Zygmunt *et al.* (17). The blockade of angiogenesis in the aortic ring assay with anti-hCG Ab, the use of recombinant hCG, adenylcyclase activator/inhibitor (dbCAMP/MDL12), and PKA inhibitors demon-

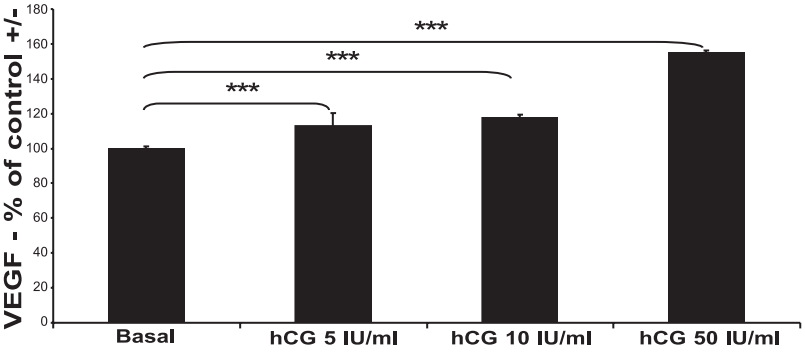


Figure 8. Paracrine effect of hCG on VEGF production by EEC. Production of VEGF by EEC (n=18) in basal condition and after treatment with hCG (1–50 IU/ml) expressed as mean ± SE of percentage of basal. $^{***}P < 0.002$ by Mann-Whitney test.

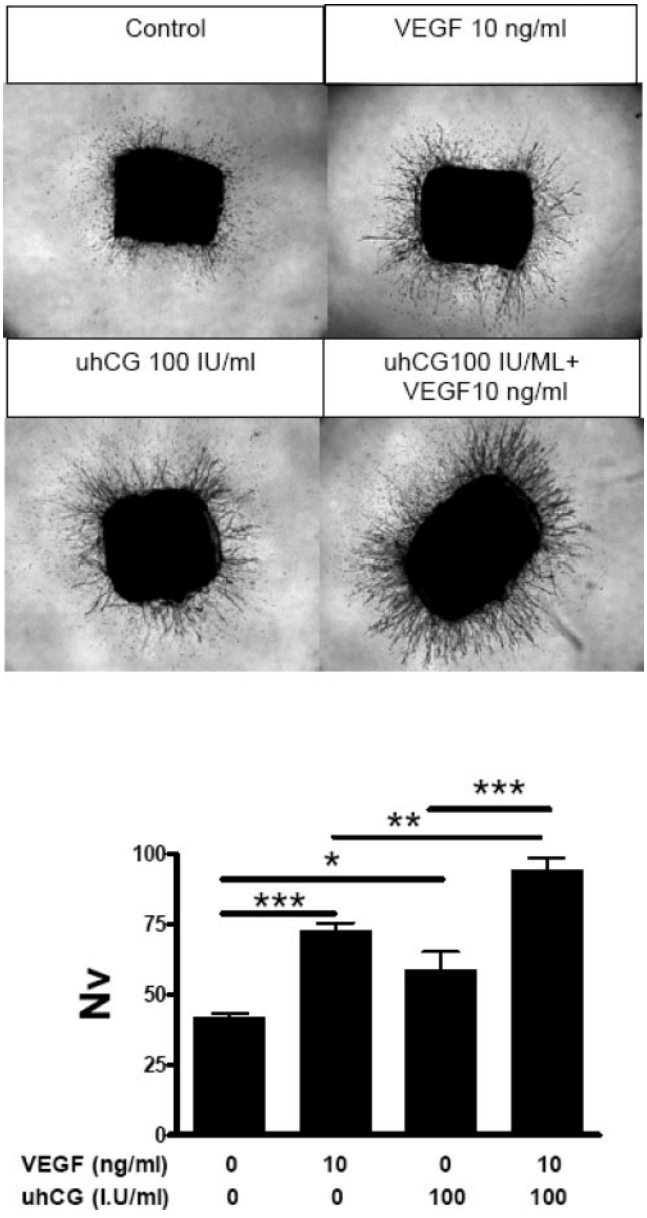


Figure 9. Additive angiogenic effects of hCG and VEGF in the aortic ring assay. Rat aortic rings were cultured in a collagen gel for 9 d with vehicle (control), VEGF (10 ng/ml), uhCG (100 IU/ml), or a combination of both factors. *A)* Photomicrographs (scale ×25) of aortic rings after 9 d of incubation with VEGF (10 ng/ml), uhCG (100 IU/ml), or a combination of both factors. *B)* Quantification of microvessel outgrowth by computerized image analysis of aortic ring assay after 9 d of incubation with VEGF (10 ng/ml), uhCG (100 IU/ml), or a combination of both factors (vascular endothelial growth factor, 10 ng/ml + uhCG 100 IU/ml). Nv, vessel number; number; $n = 4$, error bars = SE, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

strate that hCG acts through interaction with LH/hCG R. In addition, the abrogation of hCG proangiogenic action in LHR−/− aortic rings confirmed the authenticity of this effect. It could, therefore, not be ascribed to a contaminant present in the urinary extracts of hCG, as documented by others for the anti-HIV activities of hCG (18). In addition, we are providing evidence that hCG can exert angiogenic activity by a direct interaction with LH/hCG R expressed by endothelial cells.

LH/hCG R expression has been evidenced on different cell types. Several studies indicate that LH/hCG R is present in endothelium and smooth muscle of uterine blood vessels *in vivo* (12, 42). This observation is in accordance with previous reports showing that hCG elicits VEGF A mRNA and/or protein production in ovarian hyperstimulation syndrome (43–45) and in granulosa cells of various species (46). hCG was also depicted for stimulating markedly, in a dose-dependent manner, VEGF 165 secretion of cytotrophoblastic cells (47) and in human endometrium (38).

Furthermore, LH/hCG R has also been identified in endometrium with a maximal expression during the implantation window (48). We demonstrate here that addition of hCG to endometrial epithelial cells enhanced VEGF secretion.

Collectively, our data suggest that the trophoblast may stimulate the maternal endometrial angiogenesis via at least two mechanisms: the first one is related to a direct activation of endothelial cells mediated by hCG; and the second one implicates a paracrine loop involving an enhanced secretion of VEGF by EEC under the control of hCG. Finally, the additive effect of hCG and VEGF indicate that these two growth factors operate through different but converging signaling pathways. While VEGF acts through VEGFR-1 and R-2 tyrosine kinase receptors (49), hCG interacts with LH/hCG G-protein-coupled receptor and activates adenylate cyclase/cAMP pathway (15). Incubations of EEC with hCG were documented not to significantly increase intracellular cAMP, compared with Chinese hamster ovary cells stably transfected with the full-length hCG/LH R (50). hCG induced phosphorylation of extracellular signal regulated protein kinases 1 and 2 (ERK1/2). Phosphorylation of ERK1/2 in human endometrial epithelial cells was independent of protein kinase A, on the basis of the lack of cAMP production and lack of inhibition of phosphorylation in the presence of the protein kinase A inhibitor H-89 in hCG-stimulated EEC cells.

In our study, we provide evidence for a cAMP/PKA mediated pathway of angiostimulation in the aortic ring assay, since dbcAMP increased angiogenesis and the increased levels of cAMP in the presence of hCG could be abolished by MDL12, an adenylate cyclase inhibitor. Finally, H89, a PKA inhibitor, also reduced the *ex vivo* capillary sprouting stimulated by hCG. Others have previously shown in different models that the PKC transduction pathway plays an important role in capillary formation and survival phases of angiogenesis (51–54).

Although estrogens and progesterone have long been believed to be essential for developing an appropriate endometrial environment for blastocyst implantation, it is now evident that these effects are further modulated by peptide hormones and peptide growth factors secreted by a variety of cell types within the

uterine endometrium [for a review, see Filicori *et al.* (33)].

In conclusion, hCG appears to play a key role in the angiogenic synchrony between blastocyst and maternal endometrium through interactions that involve the LH/hCG R activation present at the surface of maternal endometrial endothelial and epithelial cells. This results in the induction of genes that might play a critical role in modulating the local angiogenesis, which ultimately results in an environment that is favorable to embryo implantation. FJ

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Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium

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SPECIFIC AIMS

Although hCG is known to play a key role in materno–embryonic interactions occurring during embryo implantation, its involvement during endometrial angiogenesis is not well documented. The present study aims at *1)* investigating the angiogenic activity of hCG in different *in vitro* and *in vivo* models; *2)* determining the cellular targets of hCG (endothelial, stromal and/or epithelial cells); and *3)* elucidating the cell surface receptor and signaling pathway involved in the angiogenic effects of hCG.

PRINCIPAL FINDINGS

1) hCG stimulates *in vivo* and *in vitro* angiogenesis

Incubation of rat aortic fragments with physiological concentrations of urinary hCG (uhCG) (8–800 IU/ml) or recombinant hCG (rhCG) resulted in a dose-dependent stimulation of vessel outgrowth (**Fig. 1**). The specificity of uhCG angiogenic effect was assessed by using two hormone preparations (rhCG and uhCG) (Fig. 1B) and neutralizing anti-hCG antibody (Ab) (Fig. 1*C*). In the chicken chorioallantoic membrane (CAM) assay, uhCG also enhanced the complexity of vascular network. In addition, *in vivo* matrix invasion by functional blood vessels was enhanced (matrigel plug assay) by uhCG. Further *in vitro* experiments demonstrated the capacity of uhCG to stimulate human vascular endothelial cells (HUVEC) growth rate. This effect is specific to endothelial cells, since hCG did not affect endometrial epithelial cell proliferation.

2) The angiogenic effect of hCG on endothelial cells is mediated via activation of LH/hCG receptor and cAMP/PKA pathway

The presence of LH/hCG receptor (LH/hCG R) at the surface of endothelial cells was evidenced by reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blotting analyses. Since interaction of hCG with its receptor could lead to the activation of Gs/adenyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway, we tested the effect of PKA inhibitors and dibutyryl cAMP on endothelial cell proliferation *in vitro* and on endothelial cells spreading from aortic rings. Interestingly, hCG significantly induced cAMP production in HUVEC and dibutyryl cAMP (dbcAMP) stimulated microvessel outgrowth. In contrast, an adenylate cyclase inhibitor (MDL12) and a PKA inhibitor (H89) both abolished hCG effects. To further document the implication of LH/hCG R in hCG-driven angiogenesis, we evaluated the effect of uhCG on aortic rings issued from LH/hCG R knockout (LuRKO) mice. Although hCG induced an angiogenic response in aortic rings of wild-type (WT) mice, no stimulation was achieved when aortic rings of LuRKO mice were used (**Fig. 2**).

These data demonstrate that hCG exerts its angiogenic effect by interacting with hCG/LH receptor and

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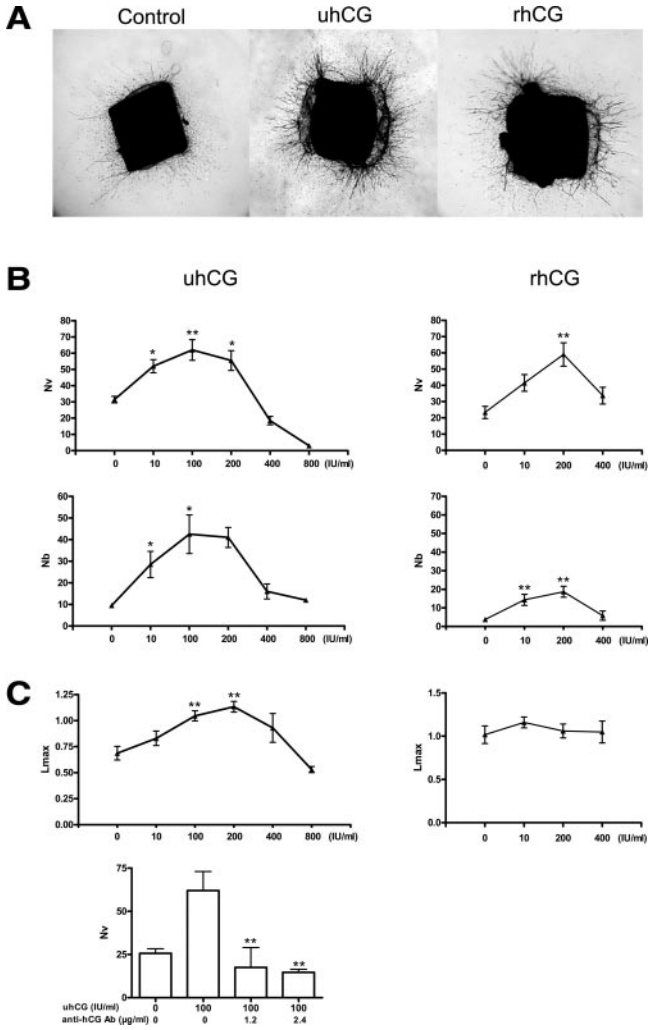

Figure 1. Angiogenic effect of urinary hCG (uhCG) and recombinant hCG (rhCG) in aortic ring assay. Rat aortic rings were cultured in a collagen gel for 10 d with various hCG concentrations. *A)* Photomicrographs (original magnification 25×) of aortic rings after 10 d of incubation with uhCG (Pregnyl®, 100 IU/ml), with rhCG (100 IU/ml), or with vehicle (control). *B)* Quantification of microvessel outgrowth by computerized image analysis. Nv, vessel number; Nb, branching number; Lmax, maximal length (mm). $n = 4$, error bars = SE., $^*P < 0.05$; $^{**}P < 0.01$. *C)* Histogram of vessel number (Nv) outgrowth from rat aortic rings incubated during 10 d with uhCG (100 IU/ml) supplemented with various concentrations (1.2 and 2.4 μg/ml) of antihCG Ab. $n = 4$, error bars = SE., $^{**}P < 0.01$. Experiments were repeated at least three times. Student's *t* test was used to evaluate whether differences among groups were significant. Statistical significance was set at $P < 0.05$.

through its main signaling pathway: adenylate cyclase/cAMP/PKA pathway.

3) hCG enhances vascular endothelial growth factor (VEGF) production through a paracrine effect on epithelial endometrial cells (EEC)

While hCG failed to modulate VEGF production by HUVEC, it significantly enhanced VEGF secretion by EEC. These results demonstrate that hCG can modulate endothelial cell proliferation and migration both directly and indirectly through a stimulation of VEGF production by EEC. In this context, of great interest is our finding that VEGF and uhCG displayed additive actions in the aortic ring assay. We therefore provide, for the first time, evidence for a functional link between hCG and VEGF in a paracrine loop involving trophoblasts, endothelial cells, and EEC.

CONCLUSION AND SIGNIFICANCE

In normal pregnancy, hCG expression is associated with an endometrial angiostimulation occurring early in gestation. hCG increases blood supply and alters the uterine vasculature *via* vasodilatation, increasing permeability, development, and maturation of new vessels.

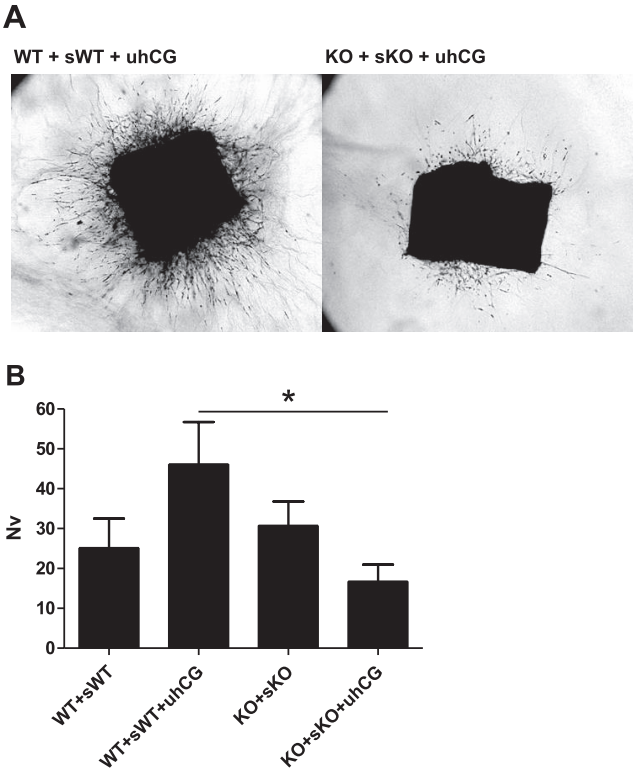

Figure 2. Comparison of uhCG-induced angiogenic effect between WT and LuRKO (KO) mice in the aortic ring assay. LuRKO mice aortic rings were cultured in a collagen gel during 9 d in presence of autologous sera (2%) and/or urinary hCG (100 IU/ml). *A)* Photomicrographs (original magnification 25×) of aortic rings from WT mice and LuRKO mice after incubation with autologous sera and supplementation with uhCG (100 IU/ml). *B)* Quantification of microvessel outgrowth (Nv) by computerized image analysis of aortic ring assay after 9 d of incubation with autologous sera supplemented or not with uhCG (100 IU/ml) in WT or KO mice aortics. sWT = WT mice serum. sKO = KO mice serum. Data are expressed as mean ± SD, $n = 5$, $^*P < 0.05$. Experiments were repeated at least three times. Differences between the experimental conditions were evaluated using the ANOVA analysis (*P* values <0.05 were considered significant).

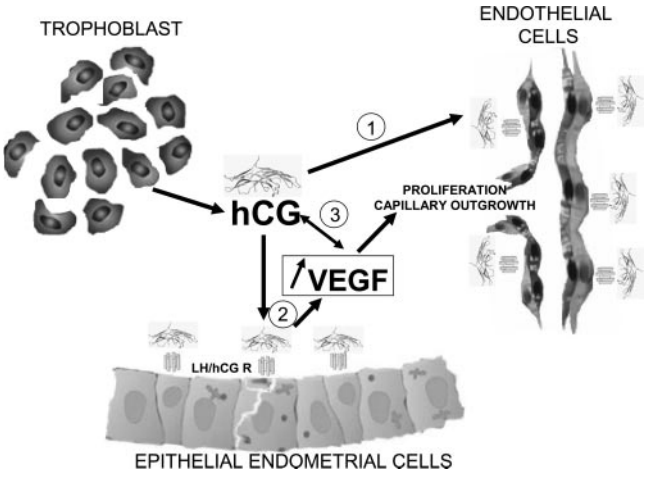

Figure 3. hCG plays a key role in the angiogenic synchrony between trophoblast, endothelial and EEC. Schematic diagram depicting the angiogenic activity of hCG during implantation regarding to our findings. *1)* Direct angiogenic effect of hCG via interaction with LH/hCG R at the surface of endothelial cells resulting in an increase of proliferation and capillary outgrowth. *2)* Indirect angiogenic effect via a paracrine loop involving an enhanced secretion of VEGF by EEC under the control of hCG. *3)* Additive effect of hCG and VEGF in stimulating capillary outgrowth.

LH/hCG R expression has been documented in uterine endothelial cells by ligand binding of ^{125}I hCG and activity measurements.

Our study demonstrates clearly a direct dose-dependent angiogenic effect of hCG on endothelial cells in the aortic ring, chicken chorioallantoic membrane (CAM), matrigel plug, and endothelial cell proliferation assays. Urinary and recombinant hCG exerted similar angiogenic effects, excluding the putative involvement of contaminating growth factors in urinary preparation. In addition, the hCG angiogenic stimulation in the aortic ring assay was abrogated by anti-hCG Ab. The use of recombinant hCG, adenylcyclase activator/inhibitor (dbAMPc/MDL12), and PKA inhibitor (H89) demonstrate that hCG acts through interaction with LH/hCG receptor, a G protein-coupled receptor that activates mainly the cAMP/PKA pathway. In addition, the abrogation of hCG proangiogenic action in LuRKO aortic rings confirmed the authenticity of this effect. Altogether these data demonstrate that the hCG effect could not be attributed to a contaminant present in the urinary extracts of hCG, as documented by others for the anti-HIV activities. Nevertheless, others provided evidence for induction of apoptosis in Kaposi's sarcoma spindle cells by the recombinant hCG β subunit.

Of particular interest is our finding that treatment of EEC with hCG enhanced VEGF secretion, leading thereby to an indirect effect on angiogenesis. Accordingly, others have reported the presence of LH/hCG R in endothelial cells and smooth muscle cells of uterine blood vessels. Although hCG was also shown to elicit VEGF mRNA and/or protein production in ovarian hyperstimulation syndrome and in granulosa cells of various species, our study is the first one reporting an effect of hCG on VEGF production by EEC.

Collectively, our data suggest that the trophoblast may stimulate the maternal endometrial angiogenesis via at least two mechanisms: (i) a direct activation of endothelial cells mediated by hCG and (ii) a paracrine loop involving an enhanced secretion of VEGF by EEC. Finally, the additive effect of hCG and VEGF indicates that these growth factors operate through different but converging signaling pathways. In conclusion, hCG appears thus to play a key role in the angiogenic synchrony between blastocyst and maternal endometrium through interactions involving activation of LH/hCG R present at the surface of maternal endothelial and epithelial cells.