Slit2/Robo4 Signaling: Potential Role of a VEGF-Antagonist Pathway to Regulate Luteal Permeability

Slit2/Robo4 Signaling: Potenzielle Rolle eines VEGF-antagonistischen Systems in der Regulation der lutealen Permeabilität

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ABSTRACT

Introduction The corpus luteum (CL) is dependent on luteal vascular permeability, which is controlled by human chorionic gonadotropin (hCG) via vascular endothelial growth factor (VEGF). In this study we investigated the role of a potential VEGF antagonist pathway – Slit2/Robo4 – and its influence on endothelial cell adhesion.

Materials and Methods Luteinized granulosa cells (LGCs) were stimulated with hCG in the absence or presence of a VEGF inhibitor. The expression of VEGF and Slit2 were measured. Human umbilical vein endothelial cells (HUVECs) were stimulated with Slit2 or VEGF, and gene expressions of cadherin 5 (CDH5) and claudin 5 (CLDN5) were measured. Following Robo4 knockdown, CDH5, CLDN5 and endothelial permeability were measured.

Results Stimulation of human LGCs with hCG significantly increased VEGF while Slit2 expression was significantly suppressed. Inhibition of VEGF action after hCG stimulation did not change Slit2 suppression. Slit2 knockdown did not affect VEGF expression. While VEGF stimulation of HUVECs significantly suppressed CDH5 and CLDN5 gene expression, stimulation of HUVECs with Slit2 resulted in a significant increase in CDH5 and CLDN5. Robo4 knockdown was done, leading to downregulation of CDH5 and CLDN5 which resulted in significantly increased permeability.

Conclusions Our results indicate the existence of a VEGF-antagonist pathway in the CL that decreases vascular permeability. During the functional life of the CL the pathway is suppressed by hCG. It is possible that stimulation of this pathway could be used to treat ovarian hyperstimulation syndrome.

ZUSAMMENFASSUNG

Einleitung Voraussetzung für die regelrechte Funktion des Corpus luteum ist ein durchlässiges Gefäßsystem. Diese vaskuläre Durchlässigkeit wird durch die Einwirkung des humanen Choriogonadotropins (hCG) auf den Vascular Endothelial Growth Factor (VEGF) kontrolliert. In dieser Studie untersuchten wir ein potenzielles VEGF-antagonistisches System – das Slit2/Robo4-System – und dessen Auswirkung auf die endotheliale Zelladhäsion.

Material und Methoden Luteinisierte Granulosazellen (LGC) wurden mit hCG stimuliert mit oder ohne Beigabe eines VEGF-Hemmers. Es wurde die VEGF- und Slit2-Expression gemessen. Aus der menschlichen Nabelschnur gewonnene venöse Endothelzellen (HUVECs) wurden mit Slit2 oder VEGF stimuliert. Danach wurde die Genexpression von Cadherin 5 (CDH5) und Claudin 5 (CLDN5) gemessen. Es wurden ein Robo4-Knockdown durchgeführt und die nachfolgende CDH5- und CLDN5-Expression sowie die endotheliale Durchlässigkeit gemessen.

Ergebnisse Die Stimulation von menschlichen LGCs mit dem hCG führte zu einer wesentlichen Steigerung von VEGF, während die Slit2-Expression signifikant unterdrückt wurde. Die Hemmung der VEGF-Aktivität nach der hCG-Stimulation wirkte sich nicht auf die Unterdrückung der Slit2-Expression aus. Der Slit2-Knockdown hatte keine Auswirkungen auf die VEGF-Expression. Während die VEGF-Stimulation von HUVECs die Genexpression von CDH5 und CLDN5 signifikant unterdrückte, führte die Slit2-Stimulation von HUVECs zu einer signifikanten Steigerung der CDH5- und CLDN5-Expression. Es wurde ein Robo4-Knockdown durchgeführt, was zu einer Herabregulation von CDH5 und CLDN5 führte und die Durchlässigkeit signifikant steigerte.

Schlussfolgerung Unsere Ergebnisse weisen auf das Vorhandensein eines VEGF-antagonistischen Systems im Corpus luteum hin, das die vaskuläre Gefäßdurchlässigkeit mindert. Dieses System wird während der Funktionsdauer des Corpus luteum durch das hCG unterdrückt. Es kann daher angenommen werden, dass eine Stimulation dieses Systems zur Behandlung beispielsweise des ovariellen Hyperstimulationssyndroms eingesetzt werden könnte.

Introduction

The corpus luteum (CL) is a temporary active secretory gland that is essential for implantation and to maintain early pregnancy. The CL requires supplementary nutrients to synthesize steroidal hormones for release into the bloodstream. Thus, a permeable vasculature is necessary for normal luteal function, especially in early pregnancy [1].

It has been shown that under physiological conditions gonadotropins such as luteinizing hormone (LH) and human chorionic gonadotropin (hCG) regulate luteal permeability by influencing cell adhesion via vascular endothelial growth factor (VEGF) [2, 3]. Furthermore, it has been demonstrated that hCG increases VEGF in luteinized granulosa cells (LGCs), reducing endothelial adhesion. Endothelial cells form a barrier against fluids or molecules by the expression of intercellular adhesion molecules such as claudin 5 (CLDN5) and cadherin 5 (CDH5). These adhesion proteins seal the intercellular space [2, 3]. VEGF released by LGCs increases vascular permeability by down-regulating CLDN5 and CDH5 [2, 3]. Especially during early pregnancy – during the life span of the CL – VEGF is increased by hCG which results in further suppression of adhesion proteins and increased luteal permeability [2–5]. Overstimulation of such a pathway results in pathologically increased permeability such as that seen in ovarian hyperstimulation syndrome (OHSS). With this syndrome, the formation of multiple corpora lutea during in vitro fertilization (IVF) treatment results in pathologically increased VEGF levels, which in turn leads to a decrease in endothelial adhesion proteins [2, 6, 7]. This interaction leads to increased permeability – not just in the corpus luteum – but also in the peritoneal or pleural vasculature. The subsequent loss of fluid into the third space induces ascites, pleural infusion or edema, leading to hypotension, thrombosis or kidney failure. Thus, OHSS can be a life-threatening condition.

In view of the interactions of hCG and VEGF, it is reasonable to assume that antagonist factors and mechanisms are involved in the regulatory pathway of luteal permeability which, under normal conditions, could prevent hyperpermeability and could be used for therapeutic interventions. The magic roundabout Robo/Slit family, which was originally found to regulate axon guiding, might be a potential candidate for intervention [8, 9].

There are four different Robo genes (Robo1–4) in the human, of which Robo4 has the potential to regulate permeability [9–11]. Robo4 is widely expressed in endothelial cells. For a long time the presence of Robo4 was thought to be endothelium-specific. However, recent findings indicate that Robo4 might also be expressed in the ovine fetal ovary as well as in Skov-3 and Ovcar-3 cell lines and human ovarian cancer tissue [12, 13].

Slit proteins are secreted glycoproteins [14–16]. To date, there are three known members of the Slit family (Slit1, Slit2 and Slit3) [17]. Genetic and biochemical analyses have shown that they are ligands for the Robo receptors regulating axonal growth [18]. Although Robo4 lacks important binding domains, recent reports have clearly demonstrated Slit2/Robo4 interaction [19–25].

Slit/Robo signaling was found to affect cell adhesion via E-cadherin and β-catenin as well as regulating growth factors responsible for the regulation of cell adhesion as well as cell proliferation and survival [12, 26–28]. Robo4 knockdown experiments in HUVECs resulted in impaired endothelial migration. At the same time, administration of Slit2 reduced VEGF-induced migration of Robo4-positive cells (endothelial cells) in vitro [22–25, 29]. Likewise, VEGF-induced migration of endothelial cells in vitro could be prevented by Robo4 overexpression. In wildtype animals, Slit2 was able to inhibit certain effects of VEGF such as increased permeability and tube formation. This ability of Slit2 was lost in Robo4$^{\pm}$ mice. This suggests that Slit2/Robo4 signaling plays an important role in controlling vascular permeability [24].

In this study we investigated the potential role of Slit2/Robo4 signaling to regulate luteal permeability. Using stimulation assays and gene silencing techniques the interactions of hCG, VEGF and Slit2 were studied in LGC cultures. To further investigate the effects of exogenous Slit2 (compared to VEGF) HUVECs were incubated either with Slit2 protein or VEGF, and changes in the gene expression of CDH5 and CLDN5 were measured. After Robo4 knockdown, CDH5 and CLDN5 expression and endothelial permeability were measured.

Materials and Methods

The collection of human tissue (LGCs and HUVECs) was approved by the institutions involved following a favorable ethical review by the Ethics Committee of the University of Ulm. All patients had given their informed consent.

GLC isolation and culture

LGCs were extracted from follicular fluid obtained during oocyte collection from women undergoing IVF after ovarian stimulation using a standard protocol [30]. Six ml of F12 Dulbecco's modified Eagle's medium containing 1 % penicillin/streptomycin and Fungizone (Promocell, Heidelberg, Germany) were mixed with 6 ml follicular fluid. After centrifugation (5 minutes; 150 RCF [g]), the supernatant was discarded and the pellet resuspended in cell culture medium and centrifuged further. The cells were pooled and resuspended in 10 ml medium. Cell suspension was carefully layered over Biocoll separating solution (density 1100 g/ml, Biochrom, Berlin, Germany) followed by a centrifugation step (5 min, 1200 rpm without break). The LGC fraction was transferred to a fresh tube and mixed with an equivalent volume of PBS (Gibco by Life Technologies, Waltham, MA, USA) and centrifuged again. The supernatant was discarded. The pellet was resuspended in 5 ml medium containing 10 % FCS with 1 % penicillin/streptomycin and Fungizone and seeded in 6 cm culture dishes (Sarstedt, Newton, NC, USA).

The next day, the cells were rinsed with PBS, and the medium was renewed.

HCG stimulation and VEGF inhibition in LGCs

Pooled LGCs were seeded into Primaria 6-well plates (BD Falcon), ½ × 6 cm culture dishes for each well with 2 ml DMEM Ham's F12 cell culture medium, 10 % FBS (PAA Laboratories) and 1 % Fungizone (Promocell, Heidelberg, Germany). After 24 h the medium was completely removed. Each well was stimulated differently, with one control (only medium) or hCG 1000 IU/ml (Predalon, Essex Pharma, Munich, Germany) or 100 ng/ml VEGFA inhibitor [VEGF Recepto-1(Flt-1) Fc Chimera, Sigma-Aldrich, St. Louis, MO, USA], or combination of both. Stimulation was performed with 2 ml medium in each well. HCG was dissolved in medium immediately before stimulation and the residual solution was not used for later experiments. Flt-1 Fc was stored at −20 °C and defrosted just before use.

Small interfering RNA knockdown of Slit2 in LGCs and of Robo4 in HUVECs

One day before transfection, LGCs or HUVECs were seeded into T25 Primaria 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and cultured with growth medium as described above. On the second day, the medium was removed and 2 ml of fresh cell culture medium was added to each well. Transfection was performed as described below.

The transfection mix contained (per T25 flask) 400 µl of culture medium without serum, 6 µl of small interfering RNA mix (siRNA, see below) or negative control siRNA (Qiagen 1027281, 20 nmol, Qiagen, Hilden, Germany) and 12 (ml) µl transfection reagent (HiPerfect, Qiagen, Hilden, Germany).

The following siRNA probes were used:

- Slit2 (mixture of Slit2_1, SI00068432; Slit2_2, SI00068439; Slit2_4, SI00068453 and Slit2_5, SI03060008) (Qiagen, Hilden, Germany)
- Robo4 (mixture of Robo4_1, SI00123893; Robo4_2 SI00123900; Robo4_3, SI00123907 and Robo4_5, SI03066896) (Qiagen, Hilden, Germany)

The transfection mix was incubated for (at least) 5 minutes to allow transfection complexes to form. After incubation, the transfection mix was added to the cells. After 48 hours, transfection was repeated as described. After 48, 72 and 96 hours, RNA was isolated for confirmation of a successful knockdown.

Stimulation of HUVECs with VEGF or Slit2

HUVECs were stimulated with Slit2-N (MyBiosource, San Diego, CA, USA) protein (1000 ng/ml) for 48, 72 and 96 hours. The results were compared to effects of VEGF stimulation (2000 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA). An incubation time of 96 h has been shown to be optimal for our experimental setting (results are presented for this time and for the dosage described above).

Endothelial cell isolation and culture

Human umbilical cords were rinsed with water and disinfected with Isoseptol. The ends of the cords were cut under sterile conditions, and a flexible tube was inserted into each end and fixed with a cable tie. The umbilical veins were rinsed with phosphate buffered saline (PBS). One end was clamped, and the vein was filled from the other end with type I-A collagenase (1 mg/ml; Sigma-Aldrich, C 2674–100 mg, St. Louis, MO, USA) to detach the endothelial cells. Subsequently, the second end was clamped, and the cords were incubated in a water bath at 37 °C for 15 minutes. Human umbilical vein endothelial cells (HUVECs) were collected and mixed 1 : 1 with endothelial cell growth medium (C-22010, Promocell, Heidelberg, Germany) containing 10% fetal calf serum (FCS) with 1% penicillin/streptomycin (PAA). After centrifugation for 5 minutes at 1200 rpm, the supernatant was discarded and the pellet was resuspended in culture medium. The HUVECs were seeded in Primaria tissue flasks (25 cm^2) (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37 °C in 5% CO_2.

Image analysis of HUVECs culture

Photo documentation of the cell culture (VEGF ± Flt-1 Fc or Slit2 in HUVECs) was done with an Axiovert 25 microscope (Zeiss) using a cybershot DSC-S75 camera (Sony) at 10 × magnification.

RNA isolation

Total RNA from LGCs and HUVEC was extracted from cells with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified by absorbance at 260 nm, and total RNA (2.5 µg) was reverse transcribed into cDNA using cDNA High Capacity Reverse Transcription Kit in accordance with the manufacturer's protocol.

Real-time PCR

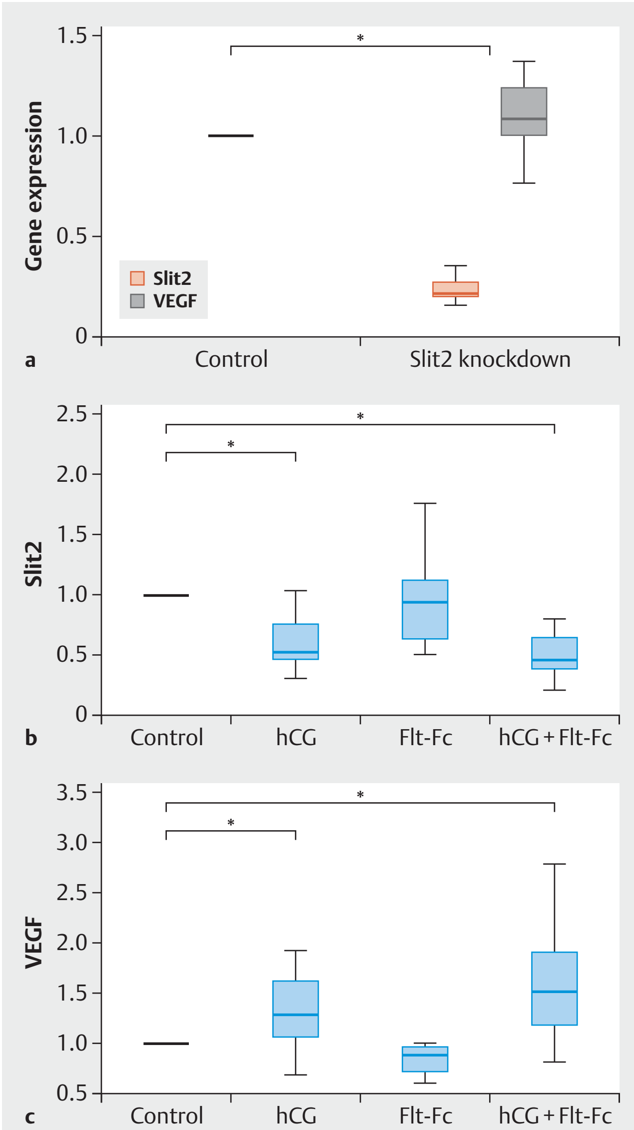
To quantify the expression of Slit2, Robo4, CDH5 and CLDN5, Taqman Gene Expression Assays (Robo4: Hs01058930_A1, Slit2: Hs00191193_m1, claudin 5: Hs00533949_s1, cadherin 5: Hs00174344_m1; Applied Biosystems, Carlsbad, CA, USA) were used according to the manufacturer's instructions using the Taqman Universal MasterMix (Applied Biosystems, Carlsbad, CA, USA). Amplification and detection of specific products was performed with the ABI Prism 7700 Sequence Detector system (Applied Biosystems, Carlsbad, CA, USA). The quantity of cDNA was normalized to the quantity of β2-microglobulin cDNA in each sample. The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression.

Permeability assay

To test the permeability of endothelial cells for macromolecules, HUVECs were seeded into an insert to allow the flow of a dye through the membrane. HUVECs were seeded on micropore inserts (pore size 0.4 µm) at a density of 2×10^5 cells per insert and cultured overnight. Bovine serum albumin (BSA) was labeled with trypan blue (66.7 mg trypan blue to 1.6 g BSA in 40 ml PBS), mixed 1 : 1 with culture medium and added to the insert, while unlabeled albumin was added to the lower compartment. Samples of the wells were taken after 10 and 30 minutes and measured for protein concentration at 607 nm. Each experiment was repeated on three separate occasions (at 48, 72 and 96 h) for knockdown experiments [2].

Statistics

In all gene expression experiments, results were normalized to results from untreated controls using the $2^{-\Delta\Delta CT}$ method [31]. This made it possible to determine the fold change in gene expression compared to controls and create linear data from exponential values. Samples were also normalized for permeability measurements, so that control values were 1. Statistical analysis was done using SPSS for Windows version 21.0. Data distribution of the variables was significantly different from normal distributions; thus, non-parametric statistical procedures were used for all analyses



▶ **Fig. 1** Slit2 and VEGF gene expression in LGCs after knockdown, stimulation and inhibition. Slit2 knockdown resulted in marked reduction of Slit2 gene expression (orange column) but did not result in any change in VEGF (red column) (**a**). Slit2 (**b**) and VEGF (**c**) gene expression after hCG stimulation and VEGF inhibition (Flt-1 Fc) in LGCs. The first column shows the control (normalized to 1), the second column shows gene expression after hCG treatment, the third column shows gene expression after VEGF inhibition (Flt-1 Fc) and the fourth column shows gene expression after simultaneous stimulation with hCG and VEGF inhibition (Flt-1 Fc). There was a significant decrease in Slit2 after hCG treatment which was not reversed when Flt-Fc was administered simultaneously. VEGF inhibition alone had no effect on Slit2 expression (**b**). VEGF gene expression was significantly elevated after hCG treatment and not affected by additional Flt-1 Fc administration (**c**).

presented here. Independent groups were compared using Kruskal-Wallis and Mann-Whitney U tests. $P < 0.05$ was taken as the level of significance. If the software identified only single outliers or extremes, they were excluded from statistical calculations. The same software was used to design the boxplots illustrating the results. The horizontal line inside the box represents the median and the box indicates the interquartile range (IQR; the middle 50% of scores). The ends of the whiskers denote the lowest and highest values within 1.5 IQR of the lower and upper quartile, respectively. Outliers and extremes are not presented in these charts.

Results

VEGF gene expression after Slit2 knockdown in LGCs

To investigate the direct interaction between Slit2 and VEGF we performed Slit2 knockdown in LGCs. Slit2 knockdown did not affect VEGF gene expression ($p = 0.2$) indicating independent pathways in LGCs (▶ **Fig. 1 a**).

Slit2 and VEGF gene expression in LGCs after stimulation with hCG and VEGF inhibition

Stimulation of LGC with hCG resulted in a significant ($p = 0.023$) reduction of Slit2 gene expression (▶ **Fig. 1 b**). At the same time, VEGF gene expression was significantly ($p = 0.004$) increased (▶ **Fig. 1 c**). HCG stimulation of LGC and simultaneous VEGF inhibition (Flt-1 Fc) did not prevent the suppression of Slit2 gene expression ($p < 0.001$) (▶ **Fig. 1 b**) indicating a VEGF-independent regulation of Slit2 signaling in LGCs by hCG.

Endothelial cell culture morphology of HUVECs after VEGF, Flt-1 Fc or Slit2 stimulation

In controls, HUVECs appeared to have contact to neighboring cells without a distinctive orientation (▶ **Fig. 2 a**). After VEGF stimulation, the endothelial cells appeared to have arrayed themselves in a cluster-like formation through which bands of other endothelial cells passed (▶ **Fig. 2 b**). Complete inhibition of VEGF in HUVECs by Flt-1 Fc (without VEGF stimulation) resulted in a complete lack of any cell arrangement (▶ **Fig. 2 c**). These cells appeared *not to rearrange themselves but to be fixed in place, probably by contact inhibition.* Compared to VEGF stimulation, the administration of Slit2 to HUVECs appeared to mimic the pattern of controls. An arrangement of endothelial cells in a distinct pattern was not observed (▶ **Fig. 2 d**).

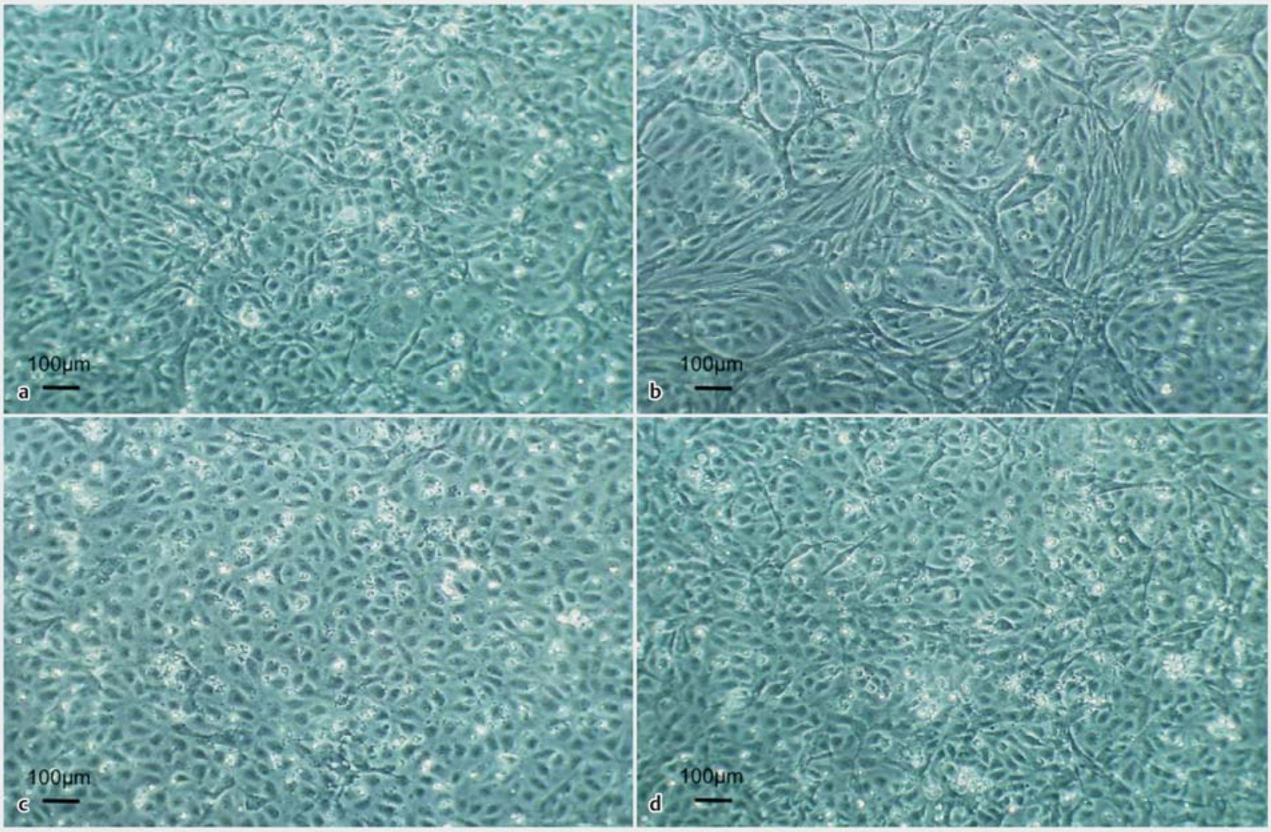
Gene expression of claudin 5 (CLDN5) and cadherin 5 (CDH5) in HUVECs after exogenous stimulation with Slit2 and VEGF

Stimulation of HUVECs with Slit2 significantly increased gene expression of CLDN5 ($p = 0.009$) and CDH5 ($p = 0.001$) (▶ **Fig. 3**).

At the same time, stimulation of HUVECs with VEGF revealed a significant decrease in gene expression of both adhesion proteins CLDN5 ($p = 0.014$) and CDH5 ($p = 0.016$) (▶ **Fig. 3**). These data indicate that exogenous Slit2 affects cell adhesion by increasing CLDN5 and CDH5 (antagonistic to VEGF).

Effects of Robo4 knockdown in HUVECs on CLDN5 and CDH5 expression and permeability (after 48, 72 and 96 h)

Successful gene silencing for Robo4 was confirmed for all incubation times ($p < 0.001$) (▶ **Fig. 4 a**). CLDN5 was significantly decreased at 48 h ($p = 0.004$), 72 h ($p = 0.14$) and 96 h ($p = 0.01$)



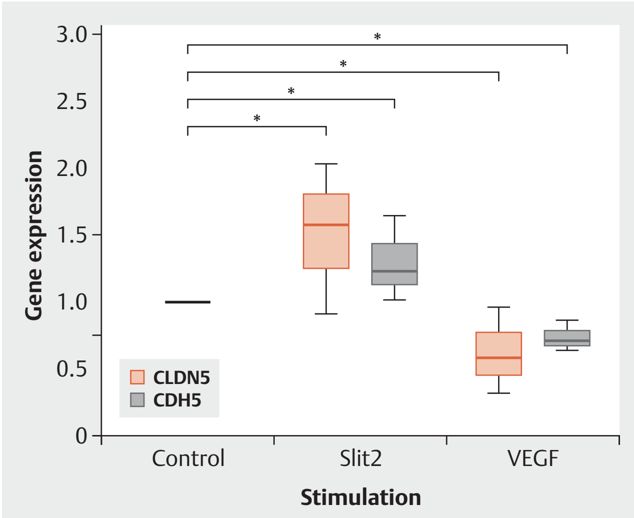
▶ **Fig. 2** Endothelial cell morphology of HUVECs after VEGF, Flt-1 Fc or Slit2 stimulation. Cell culture morphology of HUVECs (control) (**a**), HUVECS + VEGF (**b**), HUVECs + VEGF inhibition with Flt-1 Fc (**c**) and HUVECs + Slit2 (**d**) at 96 h of incubation (bar = 100 µm). VEGF stimulation resulted in an arrangement of endothelial cells (**b**), which was reversed after inhibition with Flt-1 Fc (**c**). Slit2 stimulation was comparable to controls; Slit2 did not affect the cells and did not lead to any kind of arrangement of cells (**d**).

(▶ **Fig. 4 b**). A significant reduction of CDH5 was observed after 72 h ($p = 0.013$) (▶ **Fig. 4 b**). Gene silencing for Robo4 was also associated with increased endothelial permeability, which was significant after 72 h ($p = 0.002$) and 96 h ($p < 0.001$) (▶ **Fig. 4 c**).

Discussion

The corpus luteum (CL) is one of the most highly vascularized tissues and one in which vascular permeability plays a critical role for normal luteal function [1]. VEGF is a crucial factor in the hormonal control of angiogenesis and permeability and mediates the effects of hCG in endothelial cells. VEGF is necessary for angiogenesis in the CL and for luteal function [32, 33]. Recently, our workgroup demonstrated functional interactions of junctional proteins acting as regulators of luteal permeability. It could be shown that hCG treatment of co-cultured LGCs with HUVECs decreased the production of CLDN5 and CDH5 in endothelial cells via LGC-derived VEGF. This was associated with an increase in endothelial permeability [2, 3]. Since permeability regulation is a highly sophisticated process, it is tempting to assume that there might be other factors involved in the regulatory pathway of luteal angiogenesis and permeability. In this study, we demonstrate for the first time the potential of Slit2/Robo4 to antagonize the action of hCG-induced VEGF on endothelial cell adhesion and luteal permeability which appears to be actively suppressed by hCG during the normal life span of the CL.

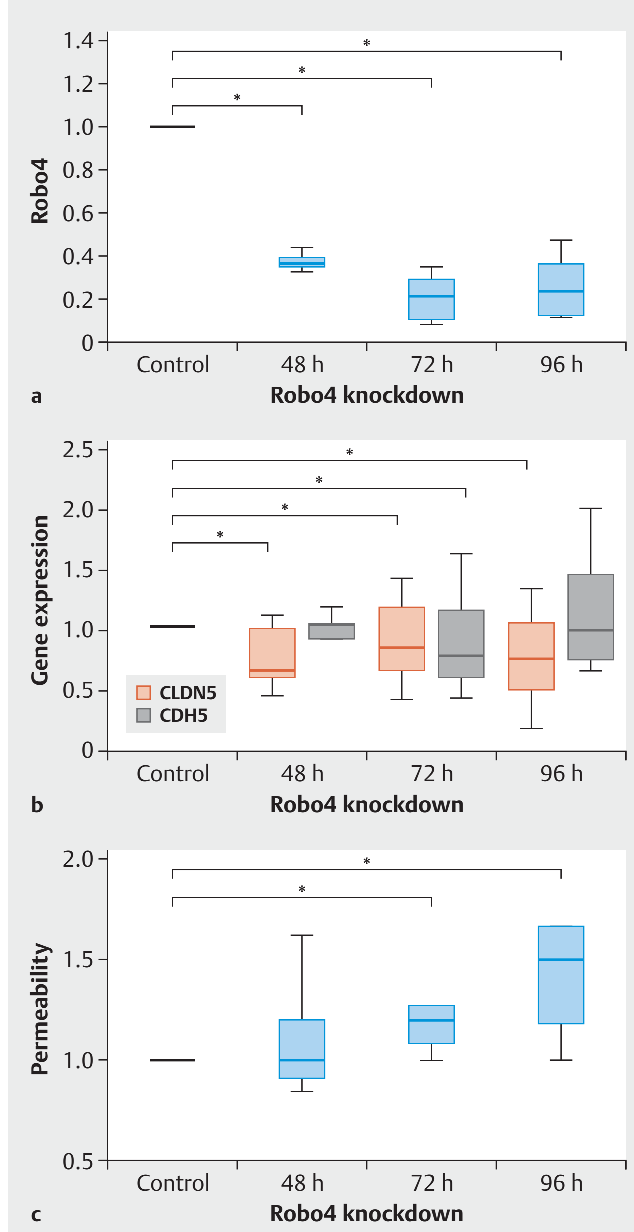
Previous studies have shown that the effect of the Slit2/Robo4 pathway is antagonistic to VEGF activity and its effect on angiogenesis and permeability [22–25]. VEGF can induce endothelial cell migration that can be prevented by Slit2 or Robo4 overexpression [24]. Our cell culture experiments showed that after VEGF stimulation HUVECs began to rearrange themselves to form plaques and cell clusters. It is probable that this is initiated by migration processes. However, nothing comparable happened after Slit2 stimulation. HUVEC migration appeared to be blocked and the culture was not distinguishable from controls. As the action of Slit2/Robo4 appears to be antagonistic to VEGF activity, it is likely that Slit2/Robo4 gene expression is regulated inversely to VEGF or negatively regulated by VEGF itself. In the current study, stimulation of LGCs with hCG resulted in the expected upregulation of VEGF [34, 35]. At the same time hCG treatment decreased Slit2 gene expression in LGCs. Since this effect could not be reversed by VEGF inhibition it has to be concluded that in LGCs



▸ **Fig. 3** Gene expression of CLDN5 and CDH5 in HUVECs after Slit2 or VEGF stimulation. Gene expression of CLDN5 (orange column) and CDH5 (red column) after exogenous stimulation of HUVECs with Slit2 and VEGF at 96 h. Both adhesion proteins (CLDN5 and CDH5) were found to be significantly increased after Slit2 stimulation. In contrast, VEGF stimulation resulted in a significant decrease in adhesion protein expression.

hCG acts directly on Slit2 gene expression and not via VEGF. Our data are consistent with the findings of Dickinson et al., who administered hCG to women to mimic early pregnancy [36]. This led to a downregulation of Slit2 in LGCs. When low dose stimulation with hCG was stopped, the expression of Slit2 increased significantly. The authors concluded that Slit/Robo signaling possibly promotes luteolysis. Indeed, after inhibition of Slit/Robo signaling they found a lower apoptosis rate. Our results together with the findings of Dickinson et al. showed that during the functional life span of the CL the Slit/Robo pathway in the CL is suppressed, indicating that hCG supports pro-permeability factors and suppresses permeability antagonists.

We showed previously that hCG-dependent upregulation of VEGF in HUVECs led to downregulation of the endothelial adhesion proteins CLDN5 and CDH5, which was associated with increased endothelial permeability [2, 3]. In this study, stimulation of endothelial cells (HUVECs) with Slit2 led to significantly increased gene expression of the adhesion proteins CLDN5 and CDH5, confirming the antagonistic action of Slit2 on endothelial cell adhesion compared to VEGF. Furthermore, we showed that Robo4 knockdown was followed by a significant decrease in CLDN5 and CDH5 and that this was associated with increased permeability. Interestingly, in our study a significant reduction of CDH5 was first observed after 72 h and expression was still reduced after 96 h, although not significantly, which may be due to the range of the data. CLDN5 was already significantly decreased at 48 h. This significant downregulation was still evident at 96 h. It has been demonstrated that downregulation of CLDN5 gene and protein expression in endothelial cells is followed by downregulation of the CDH5 gene and protein expression [37]. Probably, with manifest downregulation of CLDN5, CDH5 suppression is secondary to CLDN5. Since CLDN5 functions as a tight junction protein sealing the inter-endothelial space, it can be concluded that the observed downregulation of CLDN5 after Robo4 knockdown is associated with the measured increase in endothelial permeability at 72 h and 96 h. Thus, stimulation of the Robo4 receptor on endothelial cells can potentially be effective in increasing endothelial cell adhesion proteins and decreasing endothelial permeability.



▸ **Fig. 4** Gene expression of CLDN5 and CDH5 and permeability measurement in HUVECs after Robo4 knockdown. Gene expression of Robo4 (**a**), CLDN5 and CDH5 (**b**) and permeability measurement (**c**) at 48 h, 72 h and 96 h of incubation after Robo4 knockdown in HUVECs. Gene silencing of Robo4 was significant at all time-points (**a**). CLDN5 was significantly downregulated at 48 h, 72 h and 96 h of incubation (orange column), while CDH5 was first significantly reduced after 72 h (red column) (**b**). Endothelial permeability subsequently increased and was significant at 72 h and 96 h (**c**).

Our results led to the hypothesis that downregulation of Slit2/Robo4 is necessary to guarantee nutrient supply to and hormonal release of the CL. This potential has been shown in the regulation of embryonic vasculogenesis, angiogenesis as well as in the adult vasculature [24, 38–39]. The data in the literature is still controversial; however, results that point to the inhibitory role of Slit2/Robo4 in angiogenesis and permeability predominate [29, 39].

As a potential ligand for the Robo receptor Slit has been described as regulating axonal growth [18, 40]. Interestingly, Robo4 is said to lack important binding domains [19–21]. However, recent reports clearly showed Slit2/Robo4 interaction [22–25]. After generating Robo4 knockout mice, Jones et al. demonstrated that the ability of Slit2 to inhibit the effects of VEGF (such as increased permeability and tube formation) in wildtype animals was lost in Robo4 knockout mice [24]. The results together with the findings of our study show that both Slit2 and Robo4 influence endothelial permeability suggesting Slit2/Robo4 binding or a concordant regulatory mechanism.

Taken together, these results demonstrate that in the corpus luteum hCG acts on LGCs, reducing Slit2 gene expression and enhancing VEGF production. VEGF is secreted by LGCs and reaches the endothelial cells via diffusion. There, it decreases adhesion proteins (such as CDH5 and CLDN5), thereby increasing permeability [2, 24]. In our study, stimulation of luteal endothelial cells with Slit2 resulted in an upregulation of the adhesion proteins CDH5 and CLDN5 which opposed the effects of VEGF. Downregulation of CDH5 and CLDN5 – which has been attributed to VEGF – is probably further mediated by Slit2/Robo4 suppression. Our results suggest that Slit2/Robo4 signaling could have a potential role as a pathway antagonistic to VEGF for the regulation of endothelial permeability.

Conclusions

The CL is an interesting model to study hormonally regulated angiogenesis and permeability. Regulatory mechanisms via hCG, VEGF and adhesion proteins have been previously described, but it was not clear whether Slit2/Robo4 signaling also played a role. In our study we present for the first time Slit2/Robo4 as a potential signaling pathway antagonistic to VEGF that is suppressed by gonadotropin action during the normal life span of the CL and during pregnancy. Slit2/Robo4 has the potential to reverse the molecular regulatory effect of VEGF on endothelial cell adhesion, which would affect permeability in the CL, and could have a role in inducing luteolysis. It is tempting to speculate that Slit2/Robo4 could be a potential target to treat conditions associated with hyperpermeability such as OHSS.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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