Regulation of gene expression in endothelial cells: the role of human follicular fluid

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Abstract

A precise regulation of angiogenesis is a prerequisite for an adequate maturation of ovarian follicles. Despite the production of vascular endothelial growth factor (VEGF) by granulosa cells in antral follicles, angiogenesis is restricted to the theca cell layer. The maturing follicle remains avascular before ovulation, implying regulatory mechanisms which prevent premature follicular vascularization. In order to investigate the role of follicular fluid and of granulosa cells in the regulation of endothelial gene expression, human umbilical vein endothelial cells (HUVECs) were incubated *in vitro* with media conditioned with human follicular fluid obtained from individual patients undergoing oocyte retrieval for *in vitro* fertilization procedures or with culture medium conditioned by human granulosa cells respectively. Using microarray technology, the gene expression pattern was compared between untreated monolayers of HUVECs and HUVECs treated either with follicular fluid or with granulosa cell conditioned media. We identified a total of 15 genes that were significantly up-regulated and 11 genes that were significantly down-regulated in endothelial cells treated with follicular fluid at least 2.5-fold in more than 70% of comparisons. Up-regulated genes involved in angiogenesis were the anti-angiogenic factors gro-beta (16.5-fold), angiopoietin-2 (3.9-fold), alpha-2-macroglobulin (24.3-fold) and the pro-angiogenic factors E-selectin (5.3-fold) and vascular cell adhesion molecule-1 (VCAM-1) (4.4-fold), whereas a significant down-regulation of the pro-angiogenic genes fibulin-5 (3.5-fold) and elastin (14.9-fold) could be observed. Culturing of HUVECs with conditioned medium from cultured human luteinized granulosa cells demonstrated a similar regulatory pattern of gene expression for fibulin-5, elastin, gro-beta, and E-selectin. The gene regulation in endothelial cells by follicular fluid could be confirmed by RT-PCR for gro-beta, angiopoietin-2, elastin, fibulin-5, and E-selectin. The present work reveals that compounds secreted by granulosa cells lead to the expression of anti-angiogenic factors on the transcript level in endothelial cells and thus could help to explain the temporal and spatial discrepancy between the high expression of VEGF and the restricted angiogenesis in the preovulatory follicle.

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Introduction

Angiogenesis is paramount in pathophysiological processes like wound healing and tumor growth. It is a rare event under physiological conditions in adults; it is restricted in the female reproductive tract to follicular development, corpus luteum formation, and uterine endometrial proliferation during the menstrual cycle (Findlay 1986, Reynolds *et al.* 1992). These reproductive tissues reveal tightly regulated cyclic growth including a number of programmed angiogenic processes especially during folliculogenesis and corpus luteum formation (Moor & Seamark 1986, Augustin 2000, Fraser & Wulff 2001). It is well known that with the development of the corpus luteum after ovulation vessels grow from the thecal vascular plexus into this developing endocrine gland and a number of factors promoting angiogenesis have been demonstrated in granulosa cells such as

vascular endothelial growth factor (VEGF), fibroblast growth factor (Yan *et al.* 1993, Kamat *et al.* 1995, Neulen *et al.* 1995, Redmer & Reynolds 1996, Laitinen *et al.* 1997, Ferrara *et al.* 1998, Berisha *et al.* 2000) as well as angiogenesis modulating factors such as angiopoietins (Ang)-1 and Ang-2 (Hazzard *et al.* 1999) and angiogenin (Koga *et al.* 2000). However, less is known about the inhibition of vessel formation, as despite the apparent production of VEGF by granulosa cells in antral follicles, angiogenesis is restricted to the theca cell layer before ovulation (Cavender & Murdoch 1988, Gordon *et al.* 1995, Goede *et al.* 1998). Thus, the maturing follicle remains avascular before ovulation, implying that regulatory mechanisms must be present to prevent premature follicular vascularization.

The role of the avascular granulosa layer in controlling angiogenesis in the developing follicles still appears to be controversial. Experimental evidence

suggests that granulosa cells are also the source of angiogenesis inhibitors, such as hyaluronic acid (Tempel *et al.* 2000) and 2-methoxyestradiol (Shang *et al.* 2001). However, detailed studies investigating the cascade of events controlling angiogenesis during folliculogenesis and corpus luteum formation in the human have not been reported. This is of special interest as defects in ovarian angiogenesis contribute to a variety of disorders including anovulation and infertility, pregnancy loss, ovarian hyperstimulation syndrome, and ovarian neoplasms (Neulen *et al.* 1995, Abulafia & Sherer 2000, Geva & Jaffe 2000a).

In order to investigate the role of follicular fluid constituents and of granulosa cells in the expression of angiogenesis-related endothelial genes, human endothelial cells were incubated *in vitro* with human follicular fluid or culture medium conditioned by human granulosa cells. Gene expression was evaluated by microarray technology, and transcriptomes of the differently treated endothelial cells were compared with regard to the regulation of angiogenic and antiangiogenic genes.

Materials and methods

Follicular fluid

Follicular fluid was obtained by follicular aspiration from 12 follicle-stimulating hormone (FSH)-treated women (mean age 34.5 years) undergoing oocyte retrieval for *in vitro* fertilization (IVF/ICSI) at the Department of Gynecological Endocrinology at the University Hospital, Aachen, Germany. IVF/ICSI was performed due to andrological reasons (5), tubal occlusion (4), endometriosis (1) or idiopathic reasons (2). Ovarian stimulation was induced with recombinant human FSH (Gonal F, Serono, Unterschleissheim, Germany; mean dosage per cycle: 2300 U) after pituitary desensitization with Nafarelin (Pharmacia, Freiburg, Germany; 400 µg daily intranasally). Oocyte retrieval was carried out 36–37 h after human chorionic gonadotropin (hCG) (Pregnesin, Serono) administration at a total dose of 5000 IU. After removal of oocytes, follicular fluid from each single patient was pooled (3 to 30 follicles/patient), centrifuged at 800 *g* for 5 min and the supernatant was frozen at -20°C until use.

The experimental design was approved by the local ethical committee of the University Clinic Aachen (# EK 2008). Written informed consent was obtained from patients individually.

Granulosa cell culture

Human granulosa cells were obtained by follicular aspiration from FSH-treated women undergoing oocyte retrieval for IVF/ICSI as described above. After centrifugation and removal of follicular fluid, the cell pellet

was dissolved in 1 ml PBS solution containing 20 IU hyaluronidase and incubated for 12 min at 37°C . Cells were washed with PBS, centrifuged, suspended in 1 ml PBS and separated from red blood cells by centrifuging through a Percoll gradient (50%; Biochrom, Berlin, Germany) at 2000 *g* for 10 min. Granulosa cells were carefully removed, suspended in 1 ml culture medium, centrifuged at 500 *g* for 5 min, resuspended in 1 ml culture medium and the cell number was evaluated. Cells were plated at a density of 5×10^5 cells/well in 6-well dishes (Costar, Bodenheim, Germany) and cultured in M199 Earle's Medium (Biochrom) supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin at 37°C in 95% air-5% CO_2 humidified environment. Cell culture medium was changed after 24 h and was then harvested after 4 days of culturing and stored at -20°C until use in further experiments.

Endothelial cell culture

For isolation of human umbilical vein endothelial cells (HUVECs), umbilical cords were obtained immediately after term deliveries and umbilical veins were flushed with PBS, filled with 10 ml phosphate buffered salt solution (PBS) containing 0.1% collagenase/dispase, and incubated for 30 min at 37°C . Veins were then flushed with 40 ml culture medium which was collected in a 50-ml tube, the cell-containing medium was centrifuged at 1200 r.p.m. for 8 min, the pellet was resuspended in culture medium (Endothelial Cell Growth Medium-2; Clonetics, Cambrex, Vervier, Belgium) and the cells were routinely cultured as monolayers through serial passaging in 25-cm² plastic cell culture flasks (Falcon, Heidelberg, Germany). Culture medium was changed at two-day intervals. When the cultures became confluent they were passaged by exposure to 0.05% trypsin/0.02% EDTA (Gibco BRL, Germany).

For each experiment, HUVECs of 3–5 umbilical veins were pooled and cells were seeded at a density of 0.5×10^6 cells/well in 6-well plates. After two days of culturing, cells were confluent and were incubated with 6-ml culture medium or with 4.5-ml culture medium supplemented with 1.5 ml follicular fluid or 1.5 ml granulosa cell conditioned medium respectively. After 4 days of incubation at 37°C endothelial cells were harvested and frozen at -20°C until RNA preparation.

For single comparisons of HUVECs treated with follicular fluid, endothelial cells were separately incubated with the follicular fluid of 9 different patients and compared with 2 different controls. For array analysis of HUVECs treated with granulosa cell conditioned medium, RNA of HUVECs treated separately with the granulosa cell conditioned medium of 3 different patients was pooled and compared with pooled RNA of 3 different controls.

Table 1 Oligonucleotide primers used for semiquantitative RT-PCR

Gene	Accession no.	Oligonucleotide	Sequence (5'→3')	PCR product size (bp)
Elastin	NM_00501	5'-primer 3'-primer	AAAGCAGCAGCAAAGTTCGG ACCTGGGACAACCTGGAATCC	288
Angiopoietin	NM_001147.1	5'-primer 3'-primer	AGCTGTGATCTTGTCTTGGC GTTCAAGTCTCGTGGTCTGA	445
Fibulin-5	NM_006329.1	5'-primer 3'-primer	GCATCAATACTGAAGGCGGG GCAGATGAAAGAGCCGTAGG	260
Gro-beta	M36820	5'-primer 3'-primer	CCGAAGTCATAGCCCACTC GGCCATTTTCTTGGATTCCCT	527
E-selectin	NM_000450	5'-primer 3'-primer	AGCCCAGAGCCTTCAGTGTA AACTGGGATTGCTGTGTCC	244
VCAM-1	NM_001078.1	5'-primer 3'-primer	TAAATGCCTGGGAAGATGG GGTGCTGCAAGTCAATGAGA	151
β-actin	NM_001101	5'-primer 3'-primer	ACCAACTGGGACGACATGGAGAAAA TACGCCAGAGGCGTACAGGGATAG	214
β-actin	NM_001101	5'-primer 3'-primer	ACCTTCAACACCCCAGCCATGTACG CTGATCCACATCTGCTGGAAGGTGG	698

Microarray analysis

Microarray analysis was performed for each of the 9 HUVECs probes incubated with follicular fluid from different patients and for the two controls. To evaluate the effect of granulosa cell conditioned medium, one array was carried out of the pooled RNA of 3 treated HUVECs probes and one array of the pooled RNA of 3 controls.

Total RNA was isolated from HUVECs using a Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (8–10 µg) was converted into double-stranded cDNA using a modified oligo-dT primer including a 5' T7 RNA polymerase promoter sequence (Dürig *et al.* 2003). The resulting cDNA was transcribed *in vitro* with T7 RNA polymerase in the presence of a mixture of ATP, CTP, UTP, GTP, biotin-11-CTP, and biotin-16-UTP as described previously (Dürig *et al.* 2003). Purified cRNAs were heat-fragmented in 40 mM Tris–acetate pH 8.1, 100 mM K⁺ acetate, and 30 mM Mg²⁺ acetate at 94 °C for 30 min. Human Genome HG-U133A microarrays (Affymetrix, High Wycombe, Herts, UK) with more than 22 000 probe sets were hybridized, washed, and stained according to the manufacturer's recommendations, involving a three-step staining and amplification procedure with streptavidin-phycoerythrin (SAPE), a biotinylated anti-streptavidin antibody, and a final SAPE step. The fluorescence intensities for the individual oligonucleotide probes on the arrays were determined with a confocal scanner from Affymetrix.

Affymetrix Microarray Suite 5.0 (MAS 5.0) software was used to determine the signals and detection calls of the individual probe sets. Scaling across all probe sets (global scaling) of a given array to a target intensity of 1000 was included to compensate for variations in the

amount and quality of the cRNA samples and other experimental variables. In a next step, pair wise comparison analyses of experimental and baseline arrays were performed by MAS 5.0 comparison analysis to determine expression changes as well as significance of change and number of comparisons which show a change, which are based on a statistical evaluation of the signals at the individual probe pair level. Filtering of the comparison results was performed using the Affymetrix Data Mining Tool 3.0. Individual cut-offs applied to the data sets are given in the legend to Table 2.

RT-PCR

RT-PCR was carried out with RNA of HUVECs treated with the follicular fluid of 10 different patients which had not been included in the microarray studies, and of 5 controls.

Total RNA (2 µg) of HUVECs was digested with DNase I (Invitrogen, Karlsruhe, Germany) for 15 min at room temperature and processed to cDNA by reverse transcription (RT) with M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a total volume of 50 µl. RT was performed at 37 °C for 60 min in a thermocycler (Biometra, Goettingen, Germany). Four microliters of the RT-reaction were used for PCR experiments. The target and the endogenous control (β-actin) amplifications were run in one tube (for primers see Table 1). PCR reaction was performed in a 50 µl volume using BioTherm Taq polymerase (Genecraft, Muenster, Germany) for 36 amplification cycles of 1 min denaturation at 94 °C, 1 min annealing at 59 °C and 1.5 min elongation at 72 °C. The PCR amplification was followed by a 10-min final extension at 72 °C. The generated PCR amplification products were

Table 2 Up- and down-regulated genes in HUVECs incubated with follicular fluid compared with controls incubated with medium only. Gene array data of 18 cross pair-wise comparisons of HUVECs treated with the follicular fluid of 9 different patients with 2 different controls. Genes were scored as differentially regulated when the following criteria were fulfilled: fold change >2.5 and change *P*-value <0.001 or fold change <−2.5 and change >0.999. Only those probe sets that fulfilled these criteria in more than 70% of the comparisons were selected for presentation. Change in gene expression is represented as mean fold change ± standard deviation (s)

Accession no.	Gene description	18 comparisons		Fold change	
		×/18	%	Mean	s
Up-regulated genes					
NM_020379.1	1,2-alpha-mannosidase IC (HMC)	18/18	100	5.3	1.35
W46388	superoxide dismutase 2, mitochondrial*	18/18	100	4.6	1.60
M36820	gro-beta	18/18	100	16.5	1.60
NM_001147.1	angiopoietin 2 (ANGPT2)*	17/18	94	3.9	1.38
M81104.1	CD34	16/18	88	4.2	1.26
NM_000201.1	ICAM1, CD54*	16/18	88	3.6	1.21
NM_001078.1	vascular cell adhesion molecule 1 (VCAM1)	15/18	83	4.4	1.33
NM_000014.3	alpha-2-macroglobulin (A2M)	14/18	77	24.3	3.33
AF030514.1	small inducible cytokine subfamily B (Cys-X-Cys), member 11*	14/18	77	4.2	1.31
NM_003524.1	H2B histone family member J (H2BFJ)	14/18	77	13.4	1.23
U40053	lanosterol 14-alpha-demethylase	13/18	72	3.2	1.17
A1817041	G protein-coupled receptor	13/18	72	3.3	1.18
N95226	G protein-coupled receptor 116	13/18	72	4.0	1.62
NM_000313.1	protein S (alpha) (PROS1)	13/18	72	3.2	1.15
NM_000450.1	selectin E (endothelial adhesion molecule 1) (SELE)	13/18	72	5.3	2.67
Down-regulated genes					
AF056085.1	GABA-B receptor, G protein-coupled receptor 51	18/18	100	4.8	1.41
NM_012252.1	transcription factor EC (TFEC)	17/18	94	4.7	1.34
NM_001394.2	dual specificity phosphatase 4 (DUSP4)*	17/18	94	4.7	1.35
NM_006329.1	fibulin 5 (FBLN5)	17/18	94	3.5	1.38
NM_000501	elastin	16/18	88	14.9	1.93
BC002700.1	keratin 7	16/18	88	4.0	1.28
NM_000903.1	diaphorase (NADH:NADPH) (cytochrome b-5 reductase) (DIA4)*	16/18	88	4.1	1.38
NM_014331.1	solute carrier family 7, member 11 (SLC7A11)*	15/18	83	3.3	1.19
NM_004915.2	ATP-binding cassette, sub-family G, member 1 (ABCG1)	15/18	83	4.7	1.25
NM_007361.1	nidogen 2 (NID2)	14/18	77	3.9	1.31
NM_005010.1	neuronal cell adhesion molecule (NRCAM)	13/18	72	2.8	1.05

*, Results for this gene could be confirmed by two different probe sets on the array.

electrophoresed on a 2% agarose gel and detected by ethidium bromide staining. With densitometric analysis using Gel imager (Intas, Goettingen, Germany) PCR products were normalized to β -actin and relatively quantified (Gelscan Professional V4.0, BioSciTec, Frankfurt, Germany).

The gene expression data were analyzed for statistical significance by Student's *t*-test. A *P* value less than 0.05 was considered to be significant.

Results

Up- and down-regulated genes in HUVECs treated with follicular fluid

Using gene-chip expression arrays we have analyzed more than 22 000 probe sets in untreated monolayers of HUVECs (*n*=2) and compared them with the gene

expression in HUVECs treated with follicular fluid (*n*=9), resulting in 18 comparisons. Those genes were evaluated which were significantly up- or down-regulated at least 2.5-fold and which were regulated in more than 70% of comparisons. Using these stringent selection criteria, we identified a total of 15 genes that were up-regulated (3.2– to 24.3-fold) and 11 genes that were down-regulated (3.3– to 14.9-fold) in HUVECs treated with follicular fluid (Table 2). The regulated genes include many genes involved in angiogenesis. Up-regulated genes known to be involved in angiogenesis were gro-beta (16.5-fold increase on average), Ang-2 (3.9-fold increase), E-selectin (5.3-fold increase), alpha-2-macroglobulin (24.3-fold increase), and vascular cell adhesion molecule-1 (VCAM-1) (4.4-fold increase), whereas a significant down-regulation of fibulin-5 (3.5-fold decrease) and elastin (14.9-fold decrease) could be observed.

Up- and down-regulated genes in HUVECs treated with granulosa cell conditioned medium

To analyze if the observed gene regulation induced by follicular fluid is mediated by factors produced by granulosa cells, HUVECs were treated with granulosa cell conditioned medium or with the corresponding follicular fluid of the same patients. Gene expression of those treated endothelial cells was compared with untreated controls by gene array analysis as described. In this experimental approach, HUVECs treated with granulosa cell conditioned medium ($n=4$) or with follicular fluid ($n=4$) as well as untreated controls ($n=4$) were pooled and for each experimental approach one array was performed. Genes which showed a significant up- or down-regulation are shown in Table 3. Most of the genes found to be regulated by follicular fluid were similarly regulated by incubation with medium supernatant of granulosa cells including up-regulation of gro-beta, E-selectin and down-regulation of fibulin-5 and elastin. VCAM-1, however, which was up-regulated in endothelial cells by incubation with follicular fluid in 15 out of 18 comparisons (see Table 2), was down-regulated by incubation with granulosa cell conditioned medium (Table 3). Also in this experiment, Ang-2 was up-regulated in endothelial cells by incubation with follicular fluid, but no regulation was observed after treatment with granulosa cell conditioned medium.

Analysis of expression of angiogenesis-related genes by RT-PCR

To confirm the finding of the array analysis, RT-PCR was performed in HUVECs treated with follicular fluid for those genes possibly involved in angiogenesis. A significant increase of gene expression was observed for gro-beta (43-fold), Ang-2 (1.43-fold), and E-selectin (2.15-fold) compared with untreated controls (Fig. 1). The increase in VCAM-1 expression which had been observed in microarray analysis (Table 1) could be confirmed by RT-PCR (1.23-fold increase), but was not significant here. Elastin gene expression as well as mRNA expression of fibulin-5 were significantly decreased (6.8-fold and 1.48-fold respectively) in HUVECs incubated with follicular fluid compared with untreated controls (Fig. 1).

Discussion

Our results indicate that human follicular fluid regulates the transcription of angiogenesis-related genes in endothelial cells. HUVECs incubated with medium conditioned with follicular fluid revealed a significant up-regulation of transcripts of the anti-angiogenic genes, gro-beta and Ang-2, and a significant down-regulation

of the pro-angiogenic genes, fibulin-5 and elastin, whereas E-selectin was up-regulated. Similar effects were achieved for fibulin-5, elastin, gro-beta, and E-selectin by culturing HUVECs with conditioned medium from cultured human luteinized granulosa cells. Thus, in endothelial cells exposed to follicular fluid from mature follicles or to granulosa cell conditioned medium anti-angiogenic effects prevailed on the transcript level.

The most prominent up-regulated anti-angiogenic gene detected was the chemokine gro-beta (MIP-2a). Gro-beta enhances monocyte adhesion to endothelial cells (Hagiwara *et al.* 1998), inhibits growth factor-stimulated proliferation of capillary endothelial cells, and the growth of Lewis lung tumors was curtailed by suppression of tumor-induced neovascularization (Cao *et al.* 1995).

A significant up-regulation could also be demonstrated for Ang-2 and for alpha-2-macroglobulin in HUVECs treated with follicular fluid; however, no significant regulation after treatment with granulosa cell conditioned medium could be observed. This could be due to too low a concentration of regulating factors secreted by granulosa cells into the culture medium. Moreover, factors contained in follicular fluid regulating these genes might originate from blood serum rather than from granulosa cells.

Ang-1 and Ang-2 are vascular endothelial cell-specific growth factors that play important roles during angiogenesis by binding to their tyrosine kinase receptor, Tie2 (Davis *et al.* 1996, Maisonpierre *et al.* 1997, Geva & Jaffe 2000b). Ang-2 was found to disrupt blood vessel formation by antagonizing the effect of Ang-1 and Tie2, and was thus considered to represent a natural Ang-1/Tie2 inhibitor (Maisonpierre *et al.* 1997, Asahara *et al.* 1998). The balance between the Ang-2:Ang-1 ratio and VEGF expression is important for the regulation of angiogenesis (Goede *et al.* 1998, Wulff *et al.* 2000) and the Ang/Tie system is supposed to be involved in the angiogenetic changes that occur during follicular development (Maisonpierre *et al.* 1997, Goede *et al.* 1998, Hazzard *et al.* 1999, Wulff *et al.* 2000, Hayashi *et al.* 2003) as well as in pathological angiogenesis in the ovary (Hata *et al.* 2002). The proteinase inhibitor alpha-2-macroglobulin has been shown to bind to VEGF (Bhattacharjee *et al.* 2000) and thus may have an influence on VEGF action. The up-regulation of alpha-2 macroglobulin gene expression also supports anti-angiogenic properties of follicular fluid.

In contrast to these results, transcription of the pro-angiogenic factors elastin and fibulin-5 was significantly down-regulated in endothelial cells treated with follicular fluid or granulosa cell conditioned medium. Fibulin-5 (also known as EVEC/DANCE) is an integrin-binding extracellular matrix protein abundantly expressed in vascular endothelial cells of vessels which contain elastic fibers and in developing arteries

Table 3 Up- and down-regulated genes in HUVECs incubated with granulosa cell (GC)-conditioned medium or follicular fluid compared with controls incubated with medium only (*n*=4 for each approach; pooled probes). The fold changes, change calls and *P*-values are given for those probe sets which were identified as being consistently regulated by follicular fluid in Table 2

Accession no.	Gene description	GC-conditioned medium		Follicular fluid	
		Fold change	Change <i>P</i> -value	Fold change	Change <i>P</i> -value
Up-regulated genes					
NM_020379.1	1,2-alpha-mannosidase IC (HMC)	3.15	↑	6.53	↑
W46388	superoxide dismutase 2, mitochondrial*	nc		2.27	↑
M36820	gro-beta	2.46	↑	3.01	↑
NM_001147.1	angiopoietin 2 (ANGPT2)*	nc		3.07	↑
M81104.1	CD34	1.95	↑	6.36	↑
NM_000201.1	ICAM1, CD54	1.78	↑	1.39	↑
NM_000014.3	alpha-2-macroglobulin (A2M)	nc		8.17	↑
AF030514.1	small inducible cytokine subfamily B (Cys-X-Cys), member 11*	2.62	↑	2.95	↑
NM_003524.1	H2B histone family member J (H2BFJ)	nc		7.73	↑
U40053	lanosterol 14-alpha-demethylase	1.75	↓	2.24	↑
A1817041	G protein-coupled receptor	1.44	↑	2.25	↑
N95226	G protein-coupled receptor 116	nc		4.26	↑
NM_000313.1	protein S (alpha) (PROS1)	1.88	↑	2.43	↑
NM_000450.1	selectin E (endothelial adhesion molecule 1) (SELE)	1.47	↑	nc	0.000027
Down-regulated genes					
AF056085.1	GABA-B receptor, G protein-coupled receptor 51	1.40	↓	2	↓
NM_012252.1	transcription factor EC (TFEC)	nc		nc	0.99998
NM_001394.2	dual specificity phosphatase 4 (DUSP4)*	nc		1.27	↓
NM_006329.1	fibulin 5 (FBLN5)	2.81	↓	6.36	↓
NM_000501	elastin	1.61	↓	18.51	↓
BC002700.1	keratin 7	nc		3.41	↓
NM_000903.1	diaphorase (NADH:NADPH) (cytochrome b-5 reductase) (DIA4)*	nc		1.80	↓
NM_014331.1	solute carrier family 7, member 11 (SLC7A11)	1.51	↓	1.63	↓
NM_004915.2	ATP-binding cassette, sub-family G, member 1 (ABCG1)	2.20	↑	11.00	↓
NM_007361.1	nidogen 2 (NID2)	2.10	↓	1.88	↓
NM_005010.1	neuronal cell adhesion molecule (NRCAM)	1.66	↓	0.99987	↓
NM_001078.1	vascular cell adhesion molecule 1 (VCAM1)	2.69	↓	2.28	↓
				nc	0.99998

↑ Up-regulation, ↓ down-regulation. *, Results for this gene could be confirmed by two different probe sets on the array.

Figure 1 RT-PCR (one representative gel for each experimental design) and densitometric analysis of expression of E-selectin, VCAM-1, gro-beta, angiopoietin-2 (Ang-2), elastin, and fibulin-5 in HUVECs incubated with medium containing follicular fluid (FF, $n=10$) or with culture medium only (C, $n=5$) for 4 days. While expression of E-selectin, gro-beta and angiopoietin-2 were significantly enhanced in endothelial cells treated with follicular fluid, expression of elastin and fibulin-5 were significantly suppressed compared with controls. Bars represent mean values+standard deviation. $*=P<0.05$. β -Actin was used as a standard for densitometric evaluation.

(Kowal *et al.* 1999, Nakamura *et al.* 1999). In addition, it provides anchorage of elastic fibers to cells by binding to elastin, thereby stabilizing and organizing elastic fibers in the vasculature (Midwood & Schwarzbauer 2002, Nakamura *et al.* 2002, Yanagisawa *et al.* 2002, Kuang *et al.* 2003).

The expression of fibulin-5 is up-regulated during vascular growth and maturation in lesions of injured

vessels (Kowal *et al.* 1999). It could be shown that the absence of fibulin-5 did not alter the proliferative effect of elastin, but rather perturbed elastic fiber architecture (Nakamura *et al.* 2002). In addition to its structural function, elastin provides an anti-proliferative effect on vascular smooth muscle cells, and elastin null mice died perinatally due to vascular occlusion (Li *et al.* 1998).

E-selectin is a calcium-dependent lectin that mediates adhesive interactions of circulating leucocytes with the vascular endothelium. It is synthesized and expressed on activated endothelial cells and can be induced by inflammatory cytokines (Bevilacqua *et al.* 1987, Carlos & Harlan 1994). Elevated levels of soluble E-selectin have been found in patients with vasculoproliferative disorders such as rheumatoid arthritis (Koch *et al.* 1993) and tumor growth (Benekli *et al.* 1998, Hebbar & Peyrat 2000). E-selectin is a potent angiogenic mediator as it could be shown to induce an increase in HUVEC tube formation in the Matrigel *in vitro* assay (Kumar *et al.* 2003). VCAM-1 has been correlated to angiogenesis and could be detected in human follicular fluid (Benifla *et al.* 2001). In the microarray analysis, the expression of VCAM-1 was enhanced in HUVECs incubated with follicular fluid; however, this regulation was not confirmed as significant using RT-PCR.

Recently, experimental evidence suggested that granulosa cells produce and follicular fluid contains pro-angiogenic cytokines such as VEGF (Ravindranath *et al.* 1992, Yan *et al.* 1993, Kamat *et al.* 1995, Neulen *et al.* 1995, 1998, Doldi *et al.* 1997, Laitinen *et al.* 1997, Otani *et al.* 1999, Berisha *et al.* 2000). In addition to pro-angiogenic molecules, negative regulators of angiogenesis were observed (Tempel *et al.* 2000, Shang *et al.* 2001, Grasselli *et al.* 2003). The present work demonstrates that factors contained in follicular fluid regulate the transcription of angiogenesis-modulating genes in endothelial cells and thus could help to explain the temporal and spatial discrepancy between the high expression of VEGF and the restricted angiogenesis in the preovulatory follicle. Follicular fluid may provide a molecular shield that prevents premature vascularization of the preovulatory follicle. The balance of these regulatory factors is presumed to change according to the stage of follicular development. Though follicular fluid and granulosa cells were isolated from IVF patients and thus had been exposed to hCG, they were still pre-ovulatory and can be helpful to evaluate molecular mechanisms involved in the regulation of ovarian angiogenesis.

HUVECs exposed to follicular fluid or luteinized human granulosa cell conditioned media exhibit both the expression of pro- and anti-angiogenic genes. Angiogenesis is strictly controlled by a balance of pro- and anti-angiogenic regulators (Hanahan & Folkman 1996) resulting in temporal and spatial tightly regulated angiogenesis and vessel function. Our data suggest the production of angiogenesis-modulating factors by granulosa cells of peri-ovulatory follicles in humans with regard to gene transcription. However, the regulation of protein level as well as the impact on the biological function of endothelial cells still has to be evaluated. Understanding the molecular mechanisms which regulate the complex process of angiogenesis is a major

challenge, with implications for the understanding of angiogenesis in reproductive biology as well as in pathological conditions.

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