

Expression and localization of members of the thrombospondin family during final follicle maturation and corpus luteum formation and function in the bovine ovary

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Abstract. The aim of this study was to characterize the expression patterns and localization of the thrombospondin family members (THBS1, THBS2) and their receptors (CD36 and CD47) in bovine ovaries. First, the antral follicles were classified into 5 groups based on the follicle size and estradiol-17beta (E2) concentration in the follicular fluid (< 0.5, 0.5–5, 5–40, 40–180 and >180 E2 ng/ml). Second, the corpus luteum (CL) was assigned to the following stages: days 1–2, 3–4, 5–7, 8–12, 13–16 and >18 of the estrous cycle and of pregnancy (month 1–2, 3–4, 6–7 and > 8). Third, the corpora lutea were collected by transvaginal ovariectomy before and 0.5, 2, 4, 12, 24, 48 and 64 h after inducing luteolysis by injecting a prostaglandin F2alpha analog. The mRNA expression of examined factors was measured by RT-qPCR, steroid hormone concentration by EIA, and localization by immunohistochemistry. The mRNA expression of THBS1, THBS2, CD36, and CD47 in the granulosa cells and theca interna was high in the small follicles and reduced in the preovulatory follicles. The mRNA expression of THBS1, THBS2, and CD47 in the CL during the estrous cycle was high, but decreased significantly during pregnancy. After induced luteolysis, thrombospondins increased significantly to reach the maximum level at 12 h for THBS1, 24 h for THBS2, and 48 h for CD36. The temporal expression and localization pattern of the thrombospondins and their specific receptors in the antral follicles and corpora lutea during the different physiological phases of the estrous cycle and induced luteolysis appear to be compatible with their inhibitory role in the control of ovarian angiogenesis.

Key words: Bovine, Corpus luteum, Luteolysis, Ovarian follicle, Thrombospondins

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The ovarian cycle in ruminants is characterized by repeated patterns of specific cellular proliferation, differentiation, and transformation that accompanies follicular development and the formation and function of the corpus luteum (CL) [1–4]. During these developments in the ovary, angiogenesis seems to be the most important regulatory event for the function of the follicle and CL in cows. Angiogenesis is regulated in large by the balance of various proangiogenic stimulators and a diverse group of antiangiogenic growth factors [5–8]. The main regulators of angiogenesis in ovarian follicles and corpora lutea include members of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), angiopoietin (ANPT), and hypoxia-inducible factor (HIF) families [6–10]. Factors within the extracellular matrix (ECM) have also been reported to be important in the control of ovarian cell development and function [11]. Members of the thrombospondin

(THBS) family also belong to this group of ECM proteins. This family of growth factors consists of five multidomain glycoproteins, of which THBS1 and THBS2 are grouped in the same class as they show high structural homology, whereas THBS3–THBS5 belong to a separate isoform class with significant structural differences and variation in the procollagen homology region compared to THBS1 and THBS2 [8, 12]. Considerable work has been done on the function of THBS1 and THBS2 in cell-matrix interactions and on their potential role as inhibitors of angiogenesis [13, 14]. The cellular effects of the THBS ligands are mediated through interactions with the cell-surface receptors CD36 and CD47 [15–17]; in particular, those of THBS1 and THBS2 are mediated by interactions with CD36, which leads to increased caspase-3 activity and finally to apoptosis [18]. THBS1 is the predominant form expressed in luteal endothelial cells (EC), whereas the major form in luteinized granulosa cells (GC) is THBS2 [19]. Some recent studies demonstrated the expression and localization of members of the THBS family in follicles and luteal cells (LC) of different species (humans, rats, and bovine), suggesting that THBS ligands and their receptors may contribute to the autocrine control of ovarian cell function during different physiological stages [8, 20–22].

Members of the THBS family have also been shown to regulate angiogenesis, but the expression and localization of THBS1, THBS2, and their receptors CD36 and CD47 under specific physiological

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phases, especially during pregnancy and induced luteolysis, in the bovine ovary has not been well described. We hypothesized that members of the THBS family are specifically involved in the function of the ovary. In the present study, we aimed at evaluating the expression pattern and localization of members of the THBS family (THBS1, THBS2) and their receptors (CD36 and CD47) in different timely defined follicular classes in the corpora lutea during different functional stages (estrous cycle and pregnancy) and after induced luteolysis in cows.

Materials and Methods

Entire reproductive tracts from cows were collected at a local slaughterhouse within 10–20 min after slaughter and were transported on ice to the laboratory. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (color, consistency, corpus luteum stage, number and size of follicles) and the uterus (color, consistency, and mucus) as described previously [23]. All the collected tissues (follicles and corpora lutea) after preparation were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction or were fixed for immunohistochemical evaluation.

Experiment 1: Collection of follicles during final follicular growth

Only the follicles that appeared healthy (well vascularized and with transparent follicular wall and fluid) and whose diameter was > 5 mm were used. Large follicles (> 14 mm) were collected only after CL regression, with signs of mucus production in the uterus and cervix and were assumed to be preovulatory. The surrounding tissue, theca externa (TE), was removed with forceps under a stereomicroscope as described previously [24, 25]. After aspiration of the follicular fluid (FF), the follicles were bisected, and their inner wall was gently scraped and flushed with Ringer's solution (Fresenius, Wendel, Germany) to remove the GC. The GC in the FF as well as the flushing solution was centrifuged at 2000 g for 10 min at 4°C . The theca interna (TI) and GC pellet were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. The FF was stored at -20°C until determination of progesterone (P4) and estradiol-17 β (E2) concentrations. Since healthy follicles have relatively constant P4 levels in the FF, only the follicles with P4 below 100 ng/ml in the FF were used for the evaluation to exclude atretic follicles. The follicles were classified according to the E2 content in the FF as follows: (i) <0.5 ; (ii) 0.5–5; (iii) 5–40; (iv) 40–180; and (v) >180 ng E2/ml FF. The corresponding size of the follicles were in the ranges of (i) 5–6 mm; (ii) 6–8 mm; (iii) 8–12 mm; (iv) 12–14 mm and (v) >14 mm ($n = 6$ –8 follicle/class). The GC and TI were investigated separately [24].

Experiment 2: Collection of bovine corpora lutea during the estrous cycle and pregnancy

The CL tissues of cows (German Fleckvieh) were collected at the local slaughterhouse within 10–20 min of slaughter. Corpora lutea were assigned to the following stages: days 1–2, 3–4, 5–7, 8–12, 13–16, >18 (after regression) of the estrous cycle and month 1–2, 3–5, 6–7, >8 of pregnancy ($n = 5$ –6 corpora lutea/class). The stage of the estrous cycle and pregnancy were determined by macroscopic

examination as described previously [23].

Experiment 3: Collection of bovine corpora lutea during induced luteolysis

Cows (Holstein Friesians and Brown Swiss) at the mid-luteal phase (day 8–12) were injected (intramuscular) with 500 μg of the prostaglandin F-2 α (PGF) analog Cloprostenol (Estrumate, Intervet, Germany). The corpora lutea were collected by transvaginal ovariectomy 0.5, 2, 4, 12, 24, 48, and 64 h ($n = 6$ /group) after PGF injection. The corpora lutea for the control group were collected from the cows at the mid-luteal phase (day 8–12). The luteal tissues were frozen in liquid nitrogen immediately after collection and stored at -80°C until RNA extraction. The experimental protocol was approved by the institutional care and use committee [26].

Determination of hormone concentrations

The measurement of the concentration of P4 and E2 in the FF of the follicles was performed as described by Berisha *et al.* [24]. The concentration of P4 in blood plasma after induced luteolysis was measured using enzyme-immuno-assay (EIA) [11].

Total RNA extraction and determination of RNA quality

Small slices of the deep-frozen (-80°C) corpora lutea and follicles were cut and weighed. Total RNA from the GC, TI, and CL tissues was extracted using peqGOLDTriFast (PqLab, Erlangen, Germany) according to the manufacturer's instructions. The DNA-free kit (Ambion, Austin, USA) was used for DNA digestion. RNA was dissolved in Rnase-free water and spectroscopically quantified at 260 nm. An optical density (OD) absorption ratio OD260 nm/OD280 nm in the range of 1.8–2.0 indicated the purity of the RNA. Degradation of the RNA was measured using 2100 Bioanalyzer (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) in conjunction with the RNA 6000 Nano Assay (Agilent Technologies) according to the manufacturer's instructions. The 2100 Bioanalyzer enables the measurement and standardization of RNA quality control [27]. The RNA samples are electrophoretically separated on a microfabricated chip and subsequently detected with laser-induced fluorescence induction. Each chip contains an interconnected set of microchannels that is used for the separation of nucleic acid fragments based on their size as they are driven through the chip electrophoretically. The RNA 6000 ladder standard is used as a reference for data analysis. The software compares the unknown samples to the ladder fragments to determine its concentration and to identify the ribosomal RNA peaks of the unknown sample. The 2100 Bioanalyzer electropherogram of the total RNA shows two distinct ribosomal peaks corresponding to either 18S or 28S ribosomal RNA (rRNA) and a relatively flat baseline between the 5S and 18S rRNA peaks. The automatically calculated RNA Integrity Number (RIN) allows classification of the total RNA based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact [28].

RNA reverse transcription and real-time PCR (qPCR)

Constant amounts of 1 μg of total RNA were reverse-transcribed to cDNA using the following master mix: 26 μl Rnase-free water, 12 μl 5 \times Buffer (Promega, Mannheim, Germany), 3 μl Random

Table 1. Primer sequences for ubiquitin (UBQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thrombospondin 1 and 2 (THBS1 and THBS2), and their receptors CD36 and CD47; respective RT-qPCR product length, and reference of the investigated factor or accession number in NCBI GenBank

Target	Sequence of nucleotide fragment*	size (bp)	NCBI/reference**
<i>UBQ</i>	For 5'-AGATCCAGGATAAGGAAGGCAT-3' Rev 5'-GCTCCACCTCCAGGGTGATT-3'	198	[26]
<i>GAPDH</i>	For 5'-GTCTTCACTACCATGGAGAAGG-3' Rev 5'-TCATGGATGACCTTGCCAG-3'	197	[63]
<i>THBS1</i>	For 5'-CTGCAACAACCCCAAACC-3' Rev 5'-TTGCACTCATCAACGCTTTG-3'	225	NM_174196
<i>THBS2</i>	For 5'-TAACAGGAAGAGCTGCCC-3' Rev 5'-GGGTTGGTGTGACACAGC-3'	216	NM_176872
<i>CD36</i>	For 5'-AAAGTTTCAATGGAAAGGACG-3' Rev 5'-TTATTTCAAGCTCCAAACACAGC-3'	214	NM_174010
<i>CD47</i>	For 5'-TTGAGCAGAGAAGGGGAAAC-3' Rev 5'-CAAGGACAGTGAGCAGTAGTCC-3'	214	NM_174708

* For: forward; Rev: reverse. ** NCBI GenBank accession number or reference of published sequence.

Primers (50 μ M) (Invitrogen, Carlsbad, Germany), 3 μ l dNTPs (10 mM) (Fermentas, St. Leon-Rot, Germany), and 200 U of M-MLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturer's instructions.

A master mix of the following reaction components was prepared: 6.4 μ l water, 1.2 μ l $MgCl_2$ (4 mM), 0.2 μ l forward primer (0.2 μ M), 0.2 μ l reverse primer (0.2 μ M), and 1.0 μ l LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The master mix (9 μ l) was then added to the strip tubes and 1 μ l PCR template containing 16.66 ng of reverse transcribed total RNA was added.

The following general real-time PCR protocol was employed using a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) for all the investigated targets (Table 1): denaturation for 10 min at 95°C, 40 cycles of a three-segmented amplification and quantification program (denaturation for 10 sec at 95°C, annealing for 10 sec at 60°C, elongation for 15 sec at 72°C), a melting step by slow heating from 60°C to 99°C with a rate of 0.5°C/sec and continuous fluorescence measurement, and final cooling down to 40°C. The qPCR data was analyzed using Rotor-Gene 3000 software (version 5.03, Corbett Research). The relative expressions of each target gene were calculated by the "comparative quantification" method using the "take off" points. The changes in the relative mRNA expression of the examined target genes were assessed by normalization to the constant ubiquitin (UBQ) expression, which served as a naturally expressed internal control. To obtain the Δ CT (cycle threshold) difference, the data were analyzed using the $\Delta\Delta$ CT method [29]. Thus, Δ CP was not subtracted from a control group value, but from the value 40, so that high "40- Δ CP" value indicated a high-gene expression level and vice versa [11].

Immunohistochemistry

Paraffin-embedded bovine follicles and corpora lutea (fixed in Bouin's fluid for 24 h) were cut into 5- μ m serial sections and collected on amino-propyltriethoxysilane (APES)-coated slides (SupraFrost Ultra Plus, Menzel-Gläser, Braunschweig, Germany).

The paraffin-embedded sections were dewaxed and then washed 3 times for 5 min with phosphate-buffered saline at pH 7.4. Endogenous peroxidase activity was blocked with 7.5% H_2O_2 (diluted in distilled water) at room temperature for 10 min. Non-specific antibody binding was blocked with Dakoprotein block serum free (Dako Deutschland, Hamburg, Germany) for 10 min. Next, the sections were incubated with polyclonal primary antibodies against THBS1 (Thrombospondin1 (N-20): sc12312, Santa Cruz Biotechnology, Heidelberg, Germany; diluted 1: 400, host goat) and then with secondary antibodies (Polyclonal Rabbit, code-number E 0466, DakoCytomation, Glostrup, Denmark; diluted 1:300, IgG (F(ab')₂), rabbit anti-goat) at 6°C overnight. Localization of the antigen was achieved using the avidin-biotin complex technique. Biotinylated secondary antibodies were then incubated with the sections for 16 h at room temperature. Subsequently, treatment with Strept-ABCComplex-HRP (Dako, Deutschland) was performed for 30 min at room temperature, and treatment with 1 mg/ml 3,3-diaminobenzidine tetrahydrochloride (DAB tablets (10 mg), BIOTREND Chemikalien, Cologne, Germany) was performed for 5 min. All the incubations were performed in a humidified chamber. The sections were then counterstained in hematoxylin (20 sec), dehydrated, and mounted with Eukitt quick-hardening mounting medium for microscopy (Fluka Analytical ©, Sigma-Aldrich Laborchemikalien, Seelze, Germany).

Negative controls were generated by incubating with the 3,3-diaminobenzidine reagent alone to exclude the possibility of detecting non-suppressed endogenous peroxidase activity. A lack of detectable staining in the negative controls demonstrated that the reactions were specific. For further negative control experiments, a non-specific antibody against lymphatic vessel endothelial hyaluron receptor (LYVE-1), diluted to the same final protein concentration as the used specific antibodies, was substituted for the primary antibodies. Images were then captured using a Leitz Laborlux microscope equipped with a Zeiss Axiocam camera (Zeiss, Munich, Germany). As positive controls, ovarian tissues from quails and cats of proven immunoreactivity were used.

Statistical analysis

The statistical significance of the differences in the mRNA expressions of the examined factors was assessed by one way ANOVA, followed by the Holm Sidak used as a multiple comparison test. Data that failed the normality or equal variance test were tested by one way ANOVA on ranks followed by the Kruskal-Wallis test (Sigma Stat 3.0). All the experimental data are shown as means \pm SEM (n = 5–8). Differences were considered significant if $P < 0.05$.

Results

Characterization of the follicle classes

For better characterization of the follicle classes during final growth and maturation, the concentrations of E2 and P4 were determined in the FF. The trends of the concentrations of E2 and P4 agreed with our experimental data and confirmed the validity of our method of follicle classification during final growth and maturation [24].

Confirmation of primer specificity and sequence analysis

The mRNA expression was analyzed by conventional and real-time RT-PCR (Rotor Gene 3000, Corbett Research). Initial RT-qPCR experiments verified specific transcripts for all the factors in the bovine follicles and corpora lutea. For the exact length verification, the RT-qPCR products were separated on 2% high-resolution agarose gel electrophoresis. Additionally, all the RT-qPCR products were verified by commercial DNA sequencing (TopLab, Munich, Germany). Each RT-qPCR product (Table 1) showed 100% homology to the known gene sequence published in NCBI GenBank.

Gene expression

To evaluate equal quantity and quality of the preceding RT reaction in each sample, the expression of the housekeeping genes UBQ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined in all the samples. We chose UBQ as a normalizer, as both the housekeeping genes were constantly expressed in all the samples. The results of mRNA expression of the examined factors (Figs. 1–3) are presented as changes ($40\text{-}\Delta\text{CT} \pm \text{SEM}$ from 6–8 follicles or corpora lutea per group) in the target gene expression, normalized to UBQ expression.

mRNA expression of THBS1, THBS2, CD36, and CD47 in the follicles during final follicular growth and development

The mRNA expression of THBS1, THBS2, CD36, and CD47 in the GC and TI tissues for the follicle classes is shown in Fig. 1. The mRNA expression of THBS1, THBS2, and their receptor CD36 (Fig. 1A, B, C) in the GC of the small follicle was high, but showed a continuous and significant down-regulation afterwards to a lower plateau in the preovulatory follicles (follicle size > 14 mm). The mRNA expression of the CD47 receptor in the GC did not show any significant difference between the follicle groups (Fig. 1D). In contrast, in the TI tissue, the mRNA expression of all the examined factors was high, which then showed a continuous and significant down-regulation afterwards to a lower plateau in the preovulatory follicles (Fig. 1E, F, G, H).

mRNA expression of THBS1, THBS2, CD36, and CD47 in the corpora lutea during the estrous cycle and pregnancy and during induced luteolysis

The mRNA expression of THBS1, THBS2, CD36, and CD47 in the corpora lutea during the estrous cycle and pregnancy is shown in Fig. 2. The mRNA expression of THBS1, THBS2, and CD47 in the CL groups during the estrous cycle was high but without apparent and clear changes, and significantly lower levels were observed during the whole period of pregnancy (Fig. 2A, B, D). In contrast, the mRNA expression of the CD36 receptor during the early luteal phase (days 1–7) was very weak, with significant and continuous increases afterwards to a maximum level in the corpora lutea during regression, followed by a significant down-regulation during the whole period of pregnancy (Fig. 2C).

After induced luteolysis, the mRNA expression of THBS1 and THBS2 in the CL groups was low, but increased continuously and significantly afterwards to a maximum level at 12 h for THBS1 and at 24 h for THBS2 (Fig. 3A, B). The mRNA expression of the CD36 receptor also showed the same tendency of significant upregulation in the CL group at 48 h after induced luteolysis (Fig. 3C). In contrast, the mRNA expression of the CD47 receptor did not show any significant difference between the CL groups after PGF injection (Fig. 3D).

Immunohistochemistry

The oocytes (OC) of all the follicles, from primordial follicles up to antral follicles, showed weak immunostaining for THBS1 in their cytoplasm (Fig. 4A). No expression of THBS1 was detected in the GC of any of the pre-antral follicles (Fig. 4A, B). Only in the mature antral follicles, the luminal layers of the GC were distinctly immunostained (Fig. 4C). In larger follicles, the myofibroblasts of the TE were always distinctly THBS-positive (Fig. 4A, B). The smooth muscles cells (SMC) of the blood vessels also showed strong immunostaining (Fig. 4B). In the forming corpora lutea (day 1–2), many large luteal cells (LLC) showed weak THBS1 expression in their cytoplasm (Fig. 4D). On day 5–7, many LLC showed an increased staining for THBS1, whereas the small luteal cells (SLC) remained negative or only weakly positive (Fig. 4E). In several of the SLC, the nuclei were also immunostained (Fig. 4E, thick arrow). Additionally, distinct immunostaining of the lymph vessel endothelia was frequently observed. On day 12–14, about half of the LC were positive for THBS1, and the rest showed no immunoreaction in their cytoplasm. The EC of larger veins and the SMC of smaller arteries were distinctly positive (Fig. 4F).

During the later stage of development of the corpora lutea, immunostaining for THBS1 decreased within the LC and was only very weak towards the end of the cycle. During pregnancy (Fig. 4G), the endothelium of the lymphatic vessels was distinctly positive, whereas the LC were negative or only moderately positive. In the corpora lutea during pregnancy, the LLC were moderately immunopositive. A distinct to strong immunostaining could be observed in the SMC of numerous small arteries and in the endothelium of larger lymph vessels (Fig. 4G).

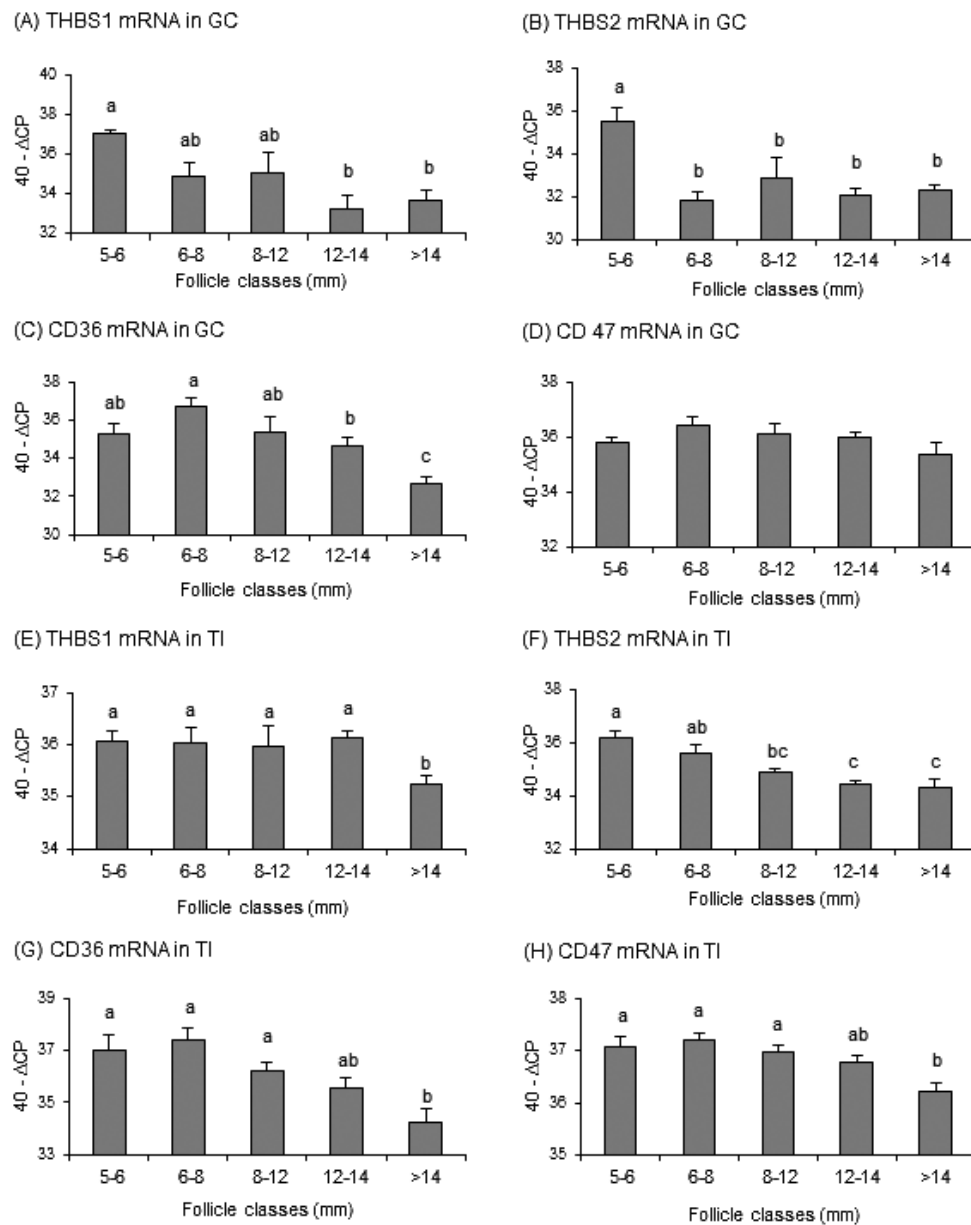


Fig. 1. mRNA expression of (A) THBS1, (B) THBS2, (C) CD36, and (D) CD47 in the granulosa cells (GC) and in the theca interna (TI): (E) THBS1, (F) THBS2, (G) CD36 and (H) CD47 at different stages of follicle development and maturation. Changes in the mRNA expression of the examined factors were assayed by normalization to the expression of UBQ used as the internal control. To obtain the CT (cycle threshold) difference, the data were analyzed using the $\Delta\Delta CT$ method [29]. Thus, ΔCP was not subtracted from the value of a control group, but from the value 40, so that high 40- ΔCP value indicated high-gene expression and vice versa. Results are presented as means \pm SEM (n = 6–8 follicles/class). Different superscripts denote statistically different values ($P < 0.05$). Classification of follicles was performed by follicle size and estradiol-17 β concentration in the follicular fluid.

Discussion

The present study reveals the expression pattern and localization of some members of the thrombospondin family (THBS1 and THBS2) and their receptors (CD36, CD47) in different timely defined follicle classes as well as in CL tissues during different functional stages (estrous cycle and pregnancy) and after induced luteolysis. During

these developmental changes in the ovary, angiogenesis seems to be the most important regulatory event for the formation and function of the follicle and CL [5–10, 30–32].

Although several angiogenic inhibitors have been reported, members of the THBS family have been shown to inhibit angiogenesis and cell migration through multiple mechanisms both *in vitro* and *in vivo* [19, 20, 33]. During cancer-treatment research, THBS expression

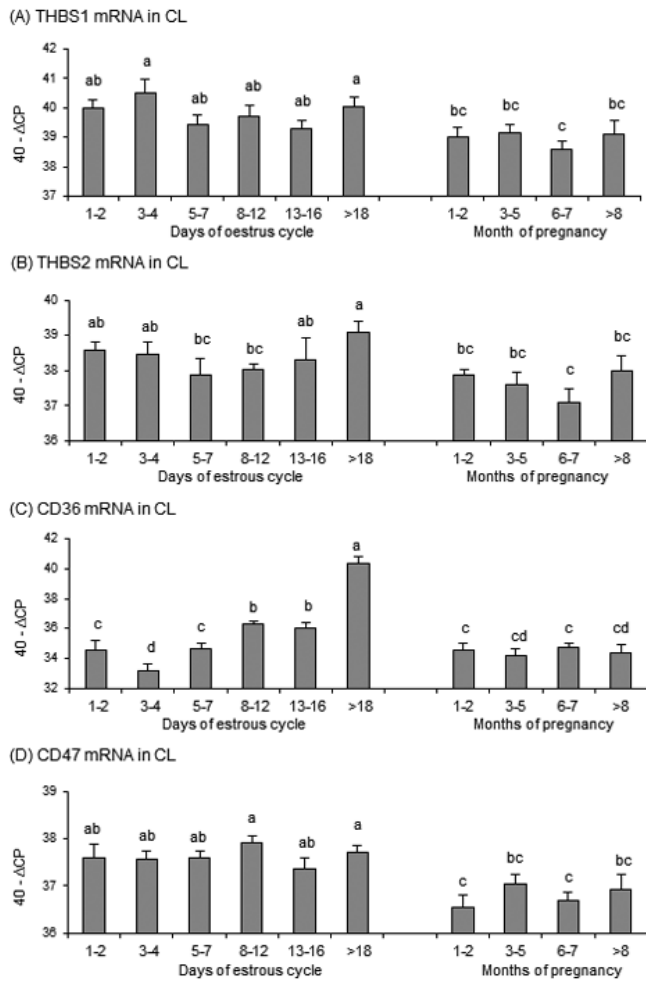


Fig. 2. mRNA expression of (A) THBS1, (B) THBS2, (C) CD36 and (D) CD47 in the corpus luteum (CL) during the estrous cycle and pregnancy. Changes in the mRNA expression of the examined factors were assayed by normalization to the expression of UBQ used as the internal control. Data are shown as $40-\Delta\text{CP} \pm \text{SEM}$ ($n = 5-6$ corpora lutea/class). Different superscripts denote statistically different values ($P < 0.05$).

was suggested to play a critical role in the process of angiogenesis, primarily by inhibition of VEGF-based angiogenesis [34, 35]. THBS inhibit angiogenesis by acting directly on endothelial cell migration, proliferation, survival, and apoptosis [13, 14].

In the present study, the mRNA expression of THBS1, THBS2, and their receptors CD36 and CD47 in the GC and TI tissue of small follicles was found to be high, which was followed by a continuous and a significant down-regulation in the preovulatory follicles (Fig. 1). Our panel of control experiments showed that the immunostaining for THBS1 can be considered specific.

Data from studies in rodent ovaries suggest that THBS contribute to the regulation of angiogenesis during follicular and luteal development [22]. It was reported that THBS1 has antiangiogenic effects on rat follicles and induces the apoptosis of GC *in vitro* [36]. THBS have also been found to be expressed and localized in human GC and

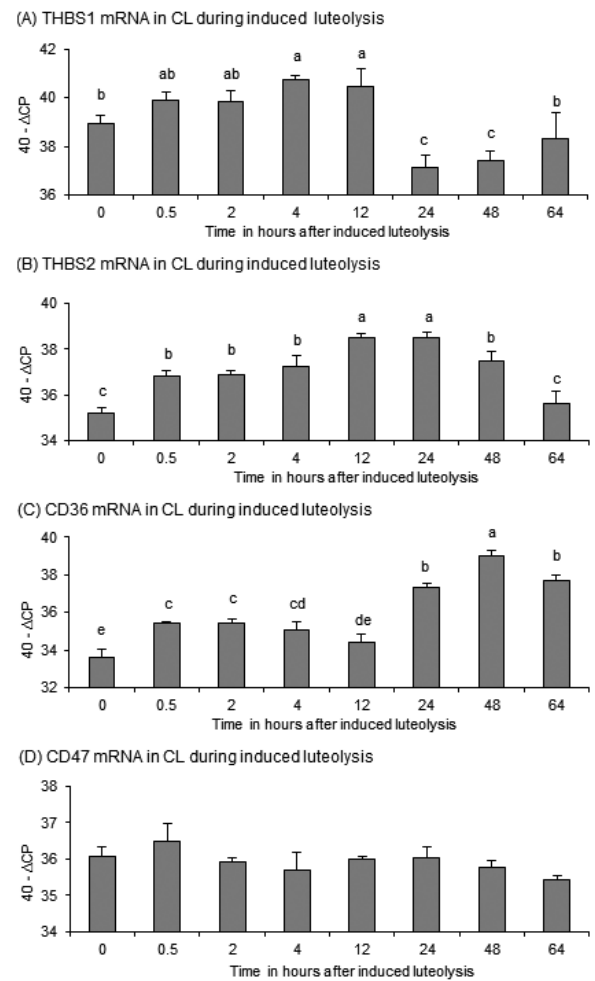


Fig. 3. mRNA expression of (A) THBS1, (B) THBS2, (C) CD36 and (D) CD47 in the corpus luteum (CL) during induced luteolysis. Changes in the mRNA expression of the examined factors were assayed by normalization to the expression of UBQ used as the internal control. Data are shown as $40-\Delta\text{CP} \pm \text{SEM}$ ($n = 5-6$ corpora lutea/class). Different superscripts denote statistically different values ($P < 0.05$).

large LC, suggesting that the ligand and receptor may contribute to the autocrine control of follicle cell function [21]. THBS1 binds CD47, and an increased expression of CD47 has been reported to correlate with increased EC apoptosis and suppression of angiogenesis [37]. The role of THBS in follicular function or atresia has been examined in the past using humans and various animal models, showing a decrease in both THBS1 and CD36 expression as follicular development progresses [8, 20, 38]. Although THBS1 and THBS2 were discovered as VEGF-regulated antiangiogenic factors [35, 39], they are now widely considered antiangiogenic factors with an inverse relationship reported between VEGF as the most important proangiogenic factor and members of the THBS family in different physiological and pathological conditions [40-42]. Greenaway *et al.* [8] suggested a similar inverse pattern in bovine ovaries during the estrous cycle. Previously, we characterized the expression and

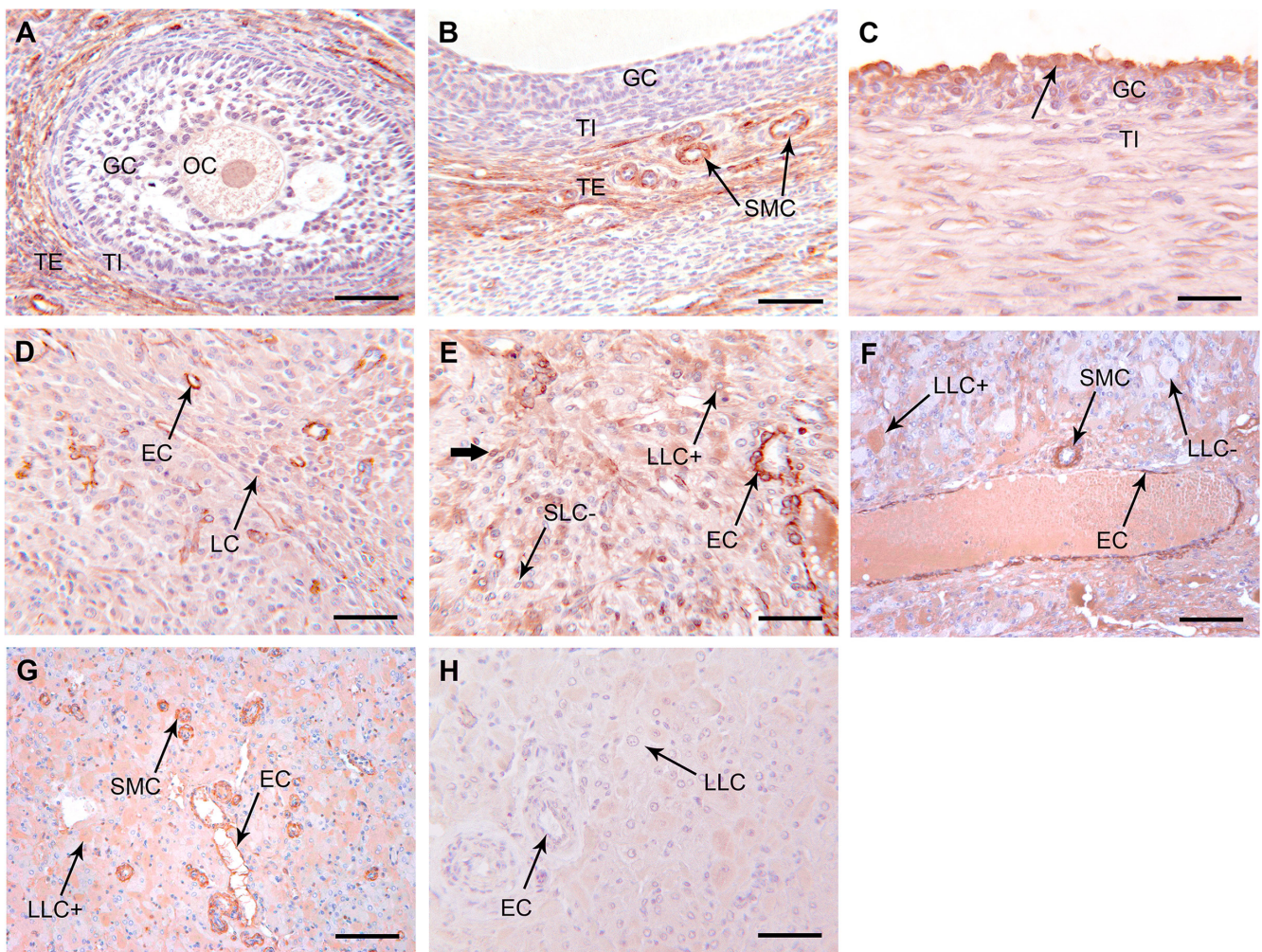


Fig. 4. (A) Secondary follicle. The oocytes (OC) of follicles show weak immunostaining for THBS1 in their cytoplasm. The granulosa cells (GC) and the cells of the theca interna (TI) are negative or only weakly positive, whereas the myofibroblast cells of the theca externa (TE) and smooth muscle cells (SMC) of the blood vessels are distinctly stained. Scale bar = 25 μ m. (B) Tertiary follicle. The GC and the TI are negative for THBS1. In the TE, the SMC of the blood vessels are strongly positive. Scale bar = 100 μ m. (C) Tertiary follicle. The GC and the TI remain negative. Only in the mature antral follicles, the luminal layer of the granulosa cells (arrow) appears to be distinctly positive for THBS1. Scale bar = 50 μ m. (D) Corpus luteum (d1–d2). In the corpus luteum (CL), the luteal cells (LC) show weak immunostaining for THBS1, whereas the endothelial cells (EC) of the capillaries are distinctly positive. Scale bar = 75 μ m. (E) Corpus luteum (d5–d7). On day 5–7, immunostaining for THBS1 increased in a subpopulation of the large luteal cells (LLC+), but the small luteal cells (SLC–) stained negative and can be differentiated. The EC of the veins are distinctly positive. Scale bar = 25 μ m. (F) Corpus luteum (d12–d14). The large luteal cells are negative (LLC–) or only moderately stained (LLC+), whereas the SLC remain negative. The SMC of the arterioles and the EC of the veins are distinctly positive. Scale bar = 100 μ m. (G) Corpus luteum, 5 months of pregnancy. Moderate immunostaining for THBS1 is observed in many large luteal cells (LLC+). The SMC of the blood vessels and the EC of the lymph vessels stain distinctly positive for THBS1. Scale bar = 100 μ m. (H) Negative control for the corpus luteum, 5 months of pregnancy. No immunostaining is visible, neither in the LLC nor in the EC. Scale bar = 75 μ m.

localization of members of the VEGF family in bovine ovaries during follicular development and luteal function [23, 24, 43].

Our previous results for VEGF in bovine ovarian tissue are consistent with the observations of others who used human, porcine, and rat ovarian tissues in their studies [20, 44, 45]. Our mRNA expression data are also in accordance with previous results from Greenaway *et al.* [8], confirming that THBS protein levels are higher in small follicles. In addition, Greenaway *et al.* [8], confirmed the GC as the primary area within the follicle involved in THBS

generation. These findings are supported by our previous reports, where the expression of VEGF in small follicles was found to be low, and that in preovulatory follicles just before ovulation was acutely increased [24]. Our previous data concerning VEGF expression [23] and the data on THBS expression from the current study reveal a clear inverse pattern of expression in the follicle during final development. In the present study, we also showed that expression of THBS1, THBS2, and their receptors CD36 and CD47 was the highest in both the GC and the TI of small follicle

classes before the development of an extensive vascular network surrounding the follicle (Fig. 1). Our previous results [23] indicate that increased expression and production of VEGF, which results in increased vascular permeability, appears to be an important, physiological aspect of healthy developing follicles in cows as well as in other species [20, 46, 47]. Therefore, we suggest that the inverse relationship between the expression pattern of members of the THBS family and VEGF in both the compartments, GC and TL, of preovulatory follicles contributes to a suitable microenvironment (angiogenesis control) for the preovulatory follicle, CL formation, and luteal function.

An interesting expression pattern of THBS and their receptors for luteal functions, especially during pregnancy and induced luteolysis, was observed in the current study. Previously, Farberov and Meidan [19] showed that THBS and their receptors CD36 and CD47 are expressed and localized in the LC and EC of bovine ovaries. In the CL groups in our study, during the estrous cycle, the mRNA expression of THBS1, THBS2, and CD47 was high, but without any significant changes, followed by a significant decrease during the whole period of pregnancy (Fig. 2A, B, D). In contrast, the mRNA expression of the CD36 receptor during the early luteal phase (period of angiogenesis, days 1–7) was very weak with significant and continuous increases afterwards (established capillary system) to a maximum level in the CL during regression (Fig. 2C). The low level of CD36 during luteal angiogenesis suggests lower inhibitory effects on angiogenesis. A major pathway by which THBS1 and THBS2 inhibit angiogenesis involves an interaction with CD36 [48–50]. The low level of CD36 during the early luteal phase (angiogenesis) correlates with the results of Petrik *et al.* [20] in rats, and these findings are also consistent with our previous report on the up-regulation of VEGF during luteal angiogenesis [23].

In our study, after induced luteolysis, the mRNA expression of THBS1 and THBS2 in the CL groups was low, but increased continuously and significantly afterwards to a maximum level at 12 h after PGF injection for THBS1 and at 24 h for THBS2 (Fig. 3A, B). CD36 mRNA expression showed the same tendency of significant up-regulation at 48 h after induced luteolysis (Fig. 3C). The increased CD36 expression, which is expected to promote the biological functions of THBS further, was observed at 0.5 h and reached the maximum level at 48 h after PGF injection. The inverse regulation between VEGF and THBS seems not to work during luteolysis, since VEGF is also down-regulated [51]. PGF administration elevates the expression of antiangiogenic genes including THBS (THBS1 and THBS2) and their receptor CD36 in the bovine ovary [52]. It was therefore suggested that the balance between pro- and anti-angiogenic factors might control the ability of the CL to either evade or undergo PGF-induced luteolysis. Farberov and Meidan [19] described THBS as antiangiogenic, proapoptotic compounds induced by PGF that affect the luteolytic process in the bovine ovary. THBS1 reduces luteal EC numbers and promotes apoptosis by activating caspase-3 [19], and it inhibits angiogenesis by acting directly on EC migration, proliferation, survival, and apoptosis [13, 14]. Few studies have shown that binding to CD36 causes intracellular signaling that initiates EC apoptosis and inhibition of neovascularization [18, 53]. This is consistent with observations indicating that targeted THBS2 inactivation *in vivo* results in a reduction of tumor cell apoptosis

and increases tumor vascularization [54]. In our previous study [51], continuous and significant down-regulation of proangiogenic VEGF and FGF factors was observed after induced luteolysis in the CL groups same as those of the current study. Consistent with our findings, an inverse expression profile for the most important proangiogenic factors VEGF and FGF2 was reported by Zalman *et al.* [52] in cows injected with PGF. In addition, Farberov and Meidan [19] suggested that the proapoptotic properties of THBS1, coupled with its ability to inhibit FGF2 expression and activity, might be critical for luteal regression. THBS1 was also found to be augmented in sheep CL during natural luteolysis [55].

As shown in our previous studies [56, 57], the increase in the mRNA and protein expression of angiopoietin-2 soon after PGF administration contributes to vessel destabilization and promotion of mature CL regression. The acute changes in local luteotropic (oxytocin, progesterone, IGF), angiogenic (VEGF, FGF and angiopoietins), and vasoactive factors (endothelin, angiotensin) suggest that modulation of vascular stability is a key component in the cascade of events leading to functional luteolysis [57–60]. This process is accompanied by regulation of proinflammatory cytokines, extracellular matrix-degrading proteases, and apoptosis-signaling cascades, leading to luteal regression [11, 61, 62]. The results of the current study provide additional evidence for the possible role of antiangiogenic THBS in different timely defined CL groups after induced luteolysis in cows. We suggest furthermore that the up-regulation of THBS and the CD36 receptor after induced luteolysis may cause intracellular signaling that initiates the apoptosis of EC and LC, thus inhibiting neovascularization during luteal regression [18, 53].

Taken together, the results of our study show a remarkable inverse and significantly regulated expression pattern of antiangiogenic THBS relative to the previously reported proangiogenic VEGF expression in ovarian follicles and corpora lutea [23, 24]. While the expression of THBS and their receptors decreases, that of VEGF, FGF2, and their receptors increases significantly along with follicle maturation and CL function. These results seem to be compatible with a role for these proteins in the control of ovarian angiogenesis. However, THBS were only weakly expressed during pregnancy (period of establishment of the capillary network), suggesting reduced vascular control during pregnancy. In conclusion, our results suggest that the examined members of the THBS family play the role of antiangiogenic factors in the local mechanisms regulating angiogenesis in bovine ovary function, namely during final follicle maturation, CL formation, and luteal function.

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