Thrombospondin and Vascular Endothelial Growth Factor Are Cyclically Expressed in an Inverse Pattern During Bovine Ovarian Follicle Development

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ABSTRACT

Angiogenesis does not normally occur in most adult tissues. However, in the ovary, there are cyclical vascular changes including angiogenesis that involve the interaction of numerous cytokines and growth factors. Angiogenic processes are regulated by a balance between pro- and antiangiogenic factors. The purpose of this study was to determine the expression of the antiangiogenic thrombospondin family and proangiogenic vascular endothelial growth factor (VEGF) in various sizes of healthy bovine follicles. Ovaries were collected from slaughterhouse animals and healthy follicles were sorted based on size (<0.5 cm, small; 0.5–1.0 cm, medium; >1.0 cm, large). Thrombospondin (TSP) protein levels were significantly higher in small follicles. Immunohistochemistry confirmed the granulosa layer as the primary area within the follicle involved in TSP generation and that small follicles had the highest proportion of immunopositive cells. TSP-1 and -2 mRNA levels were significantly higher in small follicles than either medium or large follicles. TSP colocalized with CD36 on granulosa cells (GC) in the follicle and in cultured cells. In contrast with TSP, VEGF expression increased during growth and development of the follicle. FSH stimulated GC expression of TSP, while LH had no effect. In summary, TSP-1 and -2 were coordinately expressed in the extracellular compartment of the ovary during early follicle development. VEGF was inversely expressed, with expression increasing as follicles developed. Regulated expression and localization of these proteins suggests that they may be involved in regulating growth and development of the follicle in a novel fashion.

follicle, follicular development, granulosa cells, growth factors, ovary

INTRODUCTION

Although organ vasculature is continuously developed and organized during the neonatal and early postnatal period, in the adult, these processes are normally rare, except in pathological situations such as wound healing and inflammation. The mammalian ovary, however, has special properties in that it undergoes rapid cyclical remodeling throughout the reproductive cycle, which involves numerous tightly controlled cellular and hormonal events [1]. Angiogenesis, the formation of new capillaries from existing blood vessels, is a complex process that occurs throughout follicular development and formation of the corpus luteum [2–4]. In the bovine ovarian cycle, a number of primordial follicles are selected to enter the cycle and develop into small antral follicles. The majority of these follicles will undergo atresia, with usually only one progressing to dominance and ovulation [5, 6]. After ovulation, theca and granulosa cells differentiate into steroidogenically active lutein cells to form the corpus luteum, which secretes progesterone to facilitate pregnancy (for review, see [7]). Research in several mammalian species indicates that, throughout this cycle, there are numerous vascular changes within the follicle that facilitate growth of the follicle, nutrient delivery, and hormone transport to support development of the oocyte [8, 9].

Follicular events are mediated by the cocomonard expression of both extracellular molecules [10] and intracellular factors produced in an autocrine and paracrine fashion by theca and granulosa cells. Peptide growth factors such as insulin-like growth factor (IGF)-I and -II [11], transforming growth factor-β (TGFβ) [12], epidermal growth factor (EGF) [13], and vascular endothelial growth factor (VEGF) [14–16], have been shown to participate in growth and differentiation of cells during folliculogenesis. Factors within the extracellular matrix (ECM) have also been reported to be important in the control of follicular development and atresia. Included in this group of ECM proteins are thrombospondin (TSP)-1 and -2 [17, 18]. The TSPs are a family of five multidomain glycoproteins, of which TSP-1 and -2 are grouped in the same class and have high structural homology. TSP-3 to -5 belong to a separate isofrom class, with significant structural differences and variation in the procollagen homology region compared with TSP-1 and -2. Considerable work has been done on the role of TSP-1 in vascular biology. TSP-1 and -2 function in cell-cell and cell-matrix interactions and are potent inhibitors of angiogenesis (for review, see [19]). The cellular effects of the TSPs are mediated through interaction with cell-surface receptors CD36 and Integrin Associated Protein (IAP, also known as CD47) [20–22].

In the ovary specifically, isolated granulosa cells have been shown to produce TSP-1, which in turn binds to the cell surface [23]. Using immunocytochemistry and reverse transcription-polymerase chain reaction, IAP and TSP-1 have been localized to human granulosa and large luteal cells, suggesting that the ligand and receptor may contribute to autocrine control of follicle cell function [24]. We have shown previously that TSP-1 and -2 are expressed in the rodent ovary and that their expression patterns are distinct [25]. For example, TSP-1 and CD36 are coordinately expressed in the early part of the rodent ovarian cycle, whereas TSP-2 is present in the late prevoluntary, early luteal phase of the rodent cycle. TSP is thought to contribute to...
the regulation of angiogenesis as well as follicular and lutal development in the rodent ovary [26]. VEGF is a potent proangiogenic factor and interacts with its receptors, Flk-1/KDR and Flt-1, to stimulate angiogenesis in the follicle and corpus luteum [27, 28]. VEGF is differentially expressed during follicular development, with reduced expression in early phases of the cycle and increased expression as the follicles grow and develop [29]. VEGF-induced angiogenesis is required for follicle growth, development, and viability [30, 31]. Although the biological roles of TSP have been investigated in a number of physiological systems and cell lines, the pattern of expression during bovine ovarian follicular development has not been characterized. TSP and VEGF are known to be inversely expressed in other normal and pathological situations [32–35]. However, this relationship has not been evaluated in the ovary until now. This study was therefore designed to test the hypothesis that TSP-1 and -2 are produced and secreted in the bovine follicle at various stages throughout the ovarian cycle and that they are expressed inversely to the proangiogenic factor VEGF. The results from this study will provide insight into some of the cellular mechanisms involved in regulating folliculogenesis during the bovine ovarian cycle.

MATERIALS AND METHODS

Animals and Tissue Collection

Ovaries were collected from Holstein cows from a local abattoir. The ovaries were collected at 37°C and transported to the slaughterhouse in an insulated container at approximately 4°C in 10 mM phosphate buffered saline (PBS) at a pH of 7.2 within 1 h of collection. The ovaries were rinsed in 70% (vol/vol) ethanol and were selected and separated based on follicle size in a similar fashion as has been reported previously [36]. Follicles of a diameter greater than 1.0 cm were classified as large follicles, those with a diameter of between 0.5 and 1.0 cm were considered to be medium size, and those follicles having a diameter of less than 0.5 cm were small. Follicular fluid was collected by aspiration with a 25-gauge needle and 1.0-ml tuberculin syringe. Following centrifugation, the supernatant was stored at −70°C for future protein determination. For analysis of TSP protein and mRNA, cells were isolated from the small, medium, and large follicles as follows. Follicles were stained with a scalpel and immersed in DNAse modified essential medium (DMEM) containing Ham F12 in a 1:1 ratio supplemented with 1% (vol/vol) fungizone, 2% (vol/vol) gentamycin, 1% (vol/vol) heparin, and 2% (vol/vol) penicillin/streptomycin. Granulosa cells (GCs) were pelleted with centrifugation and, for RNA experiments, were snap frozen in liquid nitrogen and stored at −70°C. For protein experiments, isolated cells were lysed with the addition of 500 μl lysis buffer (250 mM sodium deoxycholate, 5 mM phenylmethyl sulfon fluoride, 5 mg aprotinin, 500 μl Nonidet P-40, 500 μl 10% [wt/vol] SDS) and stored at −70°C. For the preparation of bovine platelet lysates, blood was collected from normal, mature Holstein cows maintained at the Porsonby Research Station, University of Guelph, under the guidelines of the Canadian Council on Animal Care. Blood was drawn by puncture of the external jugular vein with an 18-gauge needle and 60-ml syringe, to which 3.8% (wt/vol) citrate had been added as an anticoagulant. Platelets were isolated from whole blood with centrifugation and lysed with 500 μl lysis buffer (see above). Tissues to be used for immunohistochemistry were fixed in 10% neutral buffered formalin (Fisher Scientific, Nepean, ON, Canada) overnight at 4°C and were processed according to normal histological procedures. All procedures were approved by the Animal Care Committee of the University of Guelph, in accordance with the Canadian Council of Animal Care guidelines on the care and use of experimental animals.

In Vitro Culture

To determine the effect of gonadotropin stimulation on TSP expression, in vitro experiments were performed on freshly isolated bovine granulosa cells. Briefly, slaughterhouse ovaries were collected and rinsed in 70% ethyl hydroxide. Granulosa cells were isolated from small, medium, and large follicles and plated in DMEM/F12 supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin (Gibco BRL, Burlington, ON, Canada). At 70% confluence (approximately 24 h), cells were switched to serum-free DMEM/F12 (supplemented with 2% penicillin/ streptomycin) in the presence or absence of various concentrations of gonadotropins (FSH [Bioniche, Belleville, ON]: 10 ng/ml, 50 ng/ml, 100 ng/ml LH [Sigma, Oakville, ON]: 10 ng/ml, 50 ng/ml, 100 ng/ml) for 6 h. After treatment, GCs were lysed and total protein was isolated for Western blot analysis. TSP protein concentration was quantified using densitometry against bovine platelet lysate (bPL) controls. For immunofluorescence experiments, GCs were isolated from small bovine follicles and cultured on 22- × 22-mm coverslips in the bottom of six-well culture dishes (Gibco BRL) in DMEM/F12 with 10% until cells were attached (approximately 6 h). Cells were then rinsed in PBS, fixed in 10% (vol/vol) neutral buffered formalin, and processed for immunofluorescence experiments.

Immunohistochemistry/Immunofluorescence

Histological sections (5 μm) of small, medium, and large follicles from bovine ovaries collected at slaughter were cut from paraffin blocks with a rotary microtome and mounted on glass microscope slides (Superfrost Plus; Fisher Scientific). Immunohistochemistry was performed to localize TSP and VEGF within ovaries at various stages of the ovarian cycle using a modification of the avidin-biotin peroxidase method [37]. Briefly, slides were deparaffinized for 24 h at 4°C in a humidity chamber, rinsed in TSP (1:400 dilution; Medicorp Inc., Montreal, QC) or rabbit polyclonal anti-human VEGF (1:600 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Antiserum was diluted in 0.1 M PBS (pH 7.5) containing 2% (wt/vol) BSA and 0.01% (wt/vol) sodium azide (100 μl/slide). All subsequent incubations were at room temperature. Biotinylated anti-mouse or anti-rabbit IgGs (both 1:100 dilution; Vector Laboratories, Burlingame, CA) were diluted in the same buffer and applied for 2 h. The slides were then washed in PBS and incubated with avidin and biotinylated horseradish peroxidase (1:30 dilution) (Extravidin, Sigma Chemical Co., St Louis, MO). Peptide immunoreactivity was localized by incubation in fresh diazoniumbenzidine tetrahydrochloride (DAB tablets, 10 mg; Sigma) with 0.03% (vol/vol) hydrogen peroxide for 2 min. Tissue sections were counterstained with Carazzi hematoxylin for 1 min. Tissues were dehydrated and placed under a coverslip with Permount (Fisher Scientific). The percentage of immunopositive versus immunonegative follicular cells was calculated with integrated morphometry software (Metamorph; Universal Imaging Inc., Downingtown, PA). For analysis of colocalization of TSP and CD36, follicular tissue was collected and freshly isolated GCs from small follicles were allowed to attach to coverslips and were fixed in 10% neutral buffered formalin. Tissues and cells were incubated with a combination of rabbit polyclonal anti-TSP (1:600 dilution; Santa Cruz Biotechnology) and mouse monoclonal IgG raised against human CD36 (1:500 dilution; Santa Cruz Biotechnology) overnight at 4°C. Samples were then incubated with the appropriate secondary antibodies (all 1:100 dilution; Vector Laboratories) conjugated to fluorescein isothiocyanate (for TSP) or Texas Red (for CD36) for 2 h at room temperature. Nuclei were counterstained with DAPI (4′,6′-diamidino-2-phenylindole), antifade (Vectashield, Vector Labs) was applied, and the specimens were imaged with a fluorescent microscope. To establish specificity of staining in histology and fluorescence experiments, primary antisera was substituted with nonimmune serum and secondary antibody was omitted. In each case, staining was abolished.

Western Blot Analysis

TSP and VEGF proteins were detected and quantified using Western blot analysis. Protein samples (20 μg of total protein extracted from bovine follicular fluid from 10 μl of granulosa cell lysate, 10 μl of platelet lysates) were subjected to SDS-PAGE using an 8% separating gel. Pure human TSP (0.54 μg—a generous gift from Dr. Steven L. Gonias, University of Virginia), purified bovine platelet lysate (0.2 μg) and purified VEGF (5 μg; Santa Cruz Biotechnology) were used as positive controls. For VEGF Westerns, 5 μg VEGF (165aa) (Santa Cruz) was loaded as a positive control for each membrane. The separated proteins were electrotransferred to a polyvinylidine fluoride membrane (Millipore, Billerica, MA). Membranes were blocked overnight with 5% (wt/vol) skim milk at 4°C and then were incubated for 2 h at room temperature with mouse anti-human TSP (1:500 dilution; Medicorp Inc., PQ, Canada) or anti-VEGF (1:600 dilution; Santa Cruz Biotechnology) on a rocking platform. After washing with TTBS (TBS, 1% [vol/vol] Tween 20) and rebloking, peroxidase-conjugated sheep anti-mouse (1:800 dilution) or anti-rabbit (1:1000 dilution) was added for 1 h at room temperature on a rocking platform. Reactive protein was detected with ECL chemiluminescence (Boehringer
**RESULTS**

**Determination of TSP in Follicular Fluid and Granulosa Cell Lysates**

Western blot analysis was performed using a monoclonal antibody that did not distinguish between different isoforms of TSP. A representative Western blot for total TSP in follicular fluid is shown in Figure 1, and for granulosa cell lysates in Figure 2. For each of the Western experiments, equivalent amounts of total protein was loaded from follicular fluid and granulosa cell lysates of small, medium, and large follicles and compared with the human TSP standard and bovine TSP derived from activated platelets. Loading equivalency was verified with Coomassie Blue staining of all membranes. In all samples, TSP migrated as a single protein band of approximately 180 kDa under reducing conditions. It was clearly evident that TSP concentration was highest in both follicular fluid and granulosa cell lysates of small follicles, and this was confirmed with densitometric analysis of a minimum of three gels for each experiment. Using bovine platelet lysates as control, the relative amount of TSP protein in follicular fluid from small follicles was significantly \( P < 0.05 \) higher than in medium and large follicles (Fig. 1). TSP protein in follicular fluid from medium follicle was significantly \( P < 0.05 \) higher than in large follicles (Fig. 1). Again, using bovine platelet lysates as control, the TSP protein concentration in granulosa cell lysates from small follicles was higher \( P < 0.05 \) compared with medium and large follicles (Fig. 2). Granulosa cell lysate TSP protein levels were significantly \( P < 0.05 \) higher in medium follicles than large follicles (Fig. 2). Compared with follicular fluid, TSP protein was more highly concentrated in granulosa cell lysates at all stages of follicular development when assessed as a ratio with bovine platelet lysate TSP.

**Statistical Analysis**

Three replicates of the Western and Northern blots using different representative samples were used for statistical analysis. For Western and Northern blot analyses and culture experiments, data were subjected to one-way analysis of variance (ANOVA) followed by Tukey test when indicated by ANOVA (\( \alpha = 0.05 \)). For in vitro experiments, data were collected from three independent cultures for each treatment group, each in triplicate. For morphometric analysis of histological images, four separate follicles for each size category were used and the entire follicle was included in the analysis. One-way ANOVA, followed by Bonferroni test for differences between means, was used to assess statistical differences between the percentage of immunopositive cells at the various stages of follicle development.
Immunohistochemical Localization of TSP and CD36 in Bovine Follicles and Granulosa Cells

To characterize the cellular localization of TSP, immunohistochemistry was performed on tissue sections of isolated small, medium, and large follicles. TSP was localized primarily to the granulosa cell layer of small and medium follicles, with some immunopositive theca cells (Fig. 3). The majority of granulosa cells in small follicles stained for TSP, with fewer (P < 0.05) positive cells in medium follicles, and a further reduction (P < 0.05) in staining in large follicles (Fig. 3). Immunostaining of fluorescently labeled antibodies was performed to determine whether TSP and its receptor CD36 were colocalized in follicular tissue and in vitro in cultured granulosa cells. In follicular tissues, TSP and CD36 were colocalized to the granulosa cell layer (Fig. 4A). CD36 expression followed a similar pattern of expression to TSP in small and medium follicles, with abundant expression in small follicles and reduced immunoreactivity in medium follicles (Fig. 4A). In large follicles, TSP was absent, while a small percentage of GC remained positive for CD36 (Fig. 4A). In vitro, freshly isolated granulosa cells from small follicles also demonstrated colocalization of TSP and CD36 (Fig. 4B).

Evaluation of TSP-1 and -2 mRNA in Granulosa Cells

Northern blot analysis was used to determine whether granulosa cells of various sized bovine ovarian follicles were capable of synthesizing TSP and also to quantitate the expression of specific isoforms of the TSP gene. Although the antibody used for Western blot analysis and immunohistochemistry does not delineate between isoforms of TSP, specific cDNA probes for TSP-1 and -2 (provided by Dr. P. Bornstein, University of Washington [38, 39]) were used to determine the ontology of isoform expression in isolated granulosa cells from small, medium, and large bovine follicles. Representative Northern blots for TSP-1 and -2 are shown in Figure 5. As was seen with the analysis of TSP protein, mRNAs for TSP-1 and -2 were more abundant in small follicles compared with medium and large follicles. Statistical analysis showed that the abundance of TSP-1 and -2 mRNAs from isolated granulosa cells of small follicles was significantly greater (P < 0.05) than in either medium or large follicles. The abundance of mRNA for both isoforms in medium follicles was significantly less (P < 0.05) than small and significantly greater (P < 0.05) than in large follicles (Fig. 5). The pattern of expression for both isoforms was similar, and there were no significant differences between TSP-1 and -2 expression in small, medium, or large follicles.

Gonadotropin Regulation of TSP Expression in Isolated Granulosa Cells

Previous findings in the rat ovary indicated that TSP-1 and -2 expression was regulated, at least in part, by the gonadotropins [25]. We therefore evaluated the effect of gonadotropin stimulation on TSP expression in bovine GC. Freshly isolated bovine granulosa cells pooled from various sized follicles were cultured in DMEM/F12 in the presence or absence of FSH or LH at varying concentrations (10,
FIG. 5. Expression of TSP-1 and TSP-2 mRNA in granulosa cells of small (lane 1), medium (lane 2), and large (lane 3) bovine follicles detected using Northern blot analysis. Membranes were probed for 7S to detect loading consistency and TSP band intensity was quantified against 7S. Bars (mean ± SD) with different letters are statistically different (P < 0.05).

FIG. 6. Bovine granulosa cell production of TSP after treatment with FSH (A) and LH (B). Western blot analysis was performed after in vitro treatment with FSH (A) and LH (B). Densitometry was performed with bPL as control. Bars (mean ± SD) with different letters in the FSH treatment group are statistically different (P < 0.05). Protein concentrations in the LH-treatment groups were not statistically different.

Expression and Localization of VEGF During Follicle Development

Western blot analysis for VEGF was performed on 10 μg GC lysate protein separately isolated from small, medium, and large bovine follicles. In contrast with TSP, there was an apparent increase in the amount of VEGF protein during growth of the follicle (Fig. 7). The antibody used detects the 189, 165, and 121 amino acid splice variants of VEGF. The visible bands correspond to VEGF165 and VEGF121. Both subunit bands were used in calculating densitometry. Using relative densitometry of band intensity against purified VEGF-positive control, small follicles exhibited a band intensity of 112.3 ± 22.2 U. There was a significant (P < 0.05) increase in VEGF protein to 410.5 ± 106.3 U in medium and a further significant (P < 0.05) increase to 864.2 ± 306.4 U in large follicles (Fig. 7). Immunohistochemical experiments were performed to determine the localization of VEGF within the follicles during growth and development (Fig. 8). Morphometric analysis was performed on these immuno-sections to determine the percentage of VEGF-positive granulosa and theca cells in small, medium, and large follicles. In small follicles, there were only a few VEGF-positive granulosa (16% ± 9%) and theca (23% ± 11%) cells. In medium follicles, there was a significant (P < 0.05) increase in the percentage of immuno-positive granulosa (61% ± 12%) and theca (66% ± 14%) cells (Fig. 8). In large follicles, there was a further significant (P < 0.05) increase in granulosa (99% ± 14%) and theca (89% ± 8%) cells expressing VEGF (Fig. 8). VEGF immunoreactivity was restricted to granulosa (black arrows), theca (red arrows), and endothelial cells (green arrows) of adjacent blood vessels in the bovine ovary (Fig. 8).

DISCUSSION

The purpose of this study was to characterize the expression patterns of TSP-1 and -2 and their specific cell surface receptor CD36 as well as VEGF in the bovine ova-
ry during follicular development. We discovered a novel expression of members of the TSP family in the bovine ovary during follicle development. Based on relationships established in other systems, such as malignant tumors [32–35, 39–41] and our own studies with rats [25], we hypothesized that TSP and VEGF might display differential expression during the development of bovine ovarian follicles. We also hypothesized, based on our previous results in the rat [25], that TSP expression would be subject to regulation by gonadotropins.

It is well recognized in the field of cancer-treatment research that TSPs can be important inhibitors of angiogenesis, primarily through their inhibition of proangiogenic genes such as VEGF [42–49]. In the present study, the observed decrease in VEGF content and expression in medium and large bovine follicles compared with small follicles is associated with a concomitant increase in VEGF expression. TSP protein derived from granulosa cell lysates and immunohistochemical localization of protein in the granulosa cell layer revealed that a major source of the follicular-fluid TSP was from endogenous production and secretion within the granulosa cells lining the follicular antrum. As TSP is a large, inducible glycoprotein with an approximate size of 450 kDa and as we detected mRNA for TSP-1 and -2 in granulosa cells, it is very likely that the follicular TSP observed here was due primarily to endogenous production within granulosa cells. Although there were some immunopositive theca cells in developing follicles, the contribution of these cells to accumulation of TSP in follicular fluid remains to be determined. TSP appears to have an inverse relationship with tumor formation and vascularization, where deletion of the gene results in tumorigenesis and neovascularization [50], while its presence lowers tumor microvessel density and induces focal areas of necrosis and apoptosis within the tumor [47, 48].

Our present results using bovine ovarian tissue is in agreement with the observations of others using human and porcine ovarian tissues [29, 51], and our observations using rat ovarian tissue [25] demonstrates that VEGF is weakly expressed during early ovarian follicular development and becomes more pronounced in granulosa and theca cells as the dominant follicle(s) develop to the preovulatory stage. Further, in rats, exogenous stimulation with VEGF results in increased formation of small, preantral follicles [16]. Whether or not this phenomenon occurs in other species remains to be determined.

Studies with tissue from various species indicate that VEGF may have a number of functions in the ovary. For example, VEGF stimulates angiogenesis in follicles and corpora lutea of at least some mammals [27, 28]. Recently, we have shown that VEGF has a cytoprotective role in the extravascular granulosa cell compartment of the ovary [52]. In addition, VEGF increases vascular permeability [53] and changes in ovarian follicular vascular permeability are associated with increased expression of VEGF in response to stimulation by exogenous gonadotropins in vivo [54, 55]. While these observations have led to speculation regarding the role of VEGF in the reproductive disorder know as ovarian hyperstimulation syndrome [54, 55], our present results indicate that it is important to remember that increased expression and production of VEGF, and thus increased vascular permeability, appears to be an important, normal aspect of healthy developing follicles in cows as well as other species [25, 27, 28].

Previously, we demonstrated and characterized TSP and CD36 expression in the rat ovary during follicular and luteal development [25]. As in the present study, TSP-1, -2,
and CD36 are expressed predominantly by rat granulosa cells. However, in contrast with the indistinguishable pattern of expression of TSP-1 and -2 by bovine granulosa cells in the present study, TSP-1 and -2 are differentially regulated in the rat ovary, with TSP-1 expressed early in the development of rat antral follicles and TSP-2 expressed later during the ovarioly/early luteal phase [25]. The reason for this difference in isoform-specific expression patterns for bovine and rat ovarian follicle models is not yet understood. Recent studies in cattle have demonstrated, however, that TGFβ is highly expressed in early antral follicles and is significantly decreased in large follicles [56]. These observations regarding TGFβ appear to mirror the expression of TSP-1 and -2 that we have observed in bovine ovarian follicular tissue in the present study. In contrast, TGFβ is expressed in rodent ovarian tissue in a non-cyclic manner throughout the rat reproductive cycle [57]. Whether or not there is a correlation between the expression of ovarian TGFβ and the expression of TSPs in either bovine or rodent ovaries remains to be determined. Further, the observation that TGFβ can stimulate TSP-1 and -2 expression in bovine adrenal tissue [58] provides evidence to support further studies regarding the potential role of TGFβ in regulating TSP expression in bovine follicles.

Another interspecies difference appears to be the role of gonadotropins in the regulation of ovarian follicular TSP-1 and -2 expression. In the rat [25], LH appears to play the major role in regulating the positive expression of ovarian TSP-1 and -2. In distinct contrast, FSH appears to play the major role in stimulating bovine ovarian TSP-2 and -2 expression in small bovine follicles. In further contrast with rat follicles, TSP production by bovine granulosa cells did not increase in response to LH in vitro. This latter observation may be due to the fact that the bovine granulosa cells that were subjected to stimulation by LH in vitro were collected from follicles at various stages of development, including small- and medium-sized follicles. Such follicles are not mature and may not yet have undergone the critical point in granulosa cell differentiation where FSH and estradiol induce the expression of gonadotropin-responsive granulosa cell LH receptors. In contrast, gonadotropin-responsive LH receptors are present early in the development of rat ovarian antral follicles [59, 60]. Thus, species differences in the timing of LH receptors in granulosa cells during the rodent and bovine reproductive cycles may provide another partial explanation for the differences observed in rat and bovine granulosa cell expression of TSP isoforms in response to LH.

The significance of the apparent inverse relationship between the pattern of TSP isoform expression and VEGF by bovine ovarian granulosa cells during normal follicular development remains to be determined. Based on the results of this study, we propose that TSP-1 and -2 may act in autocrine and/or paracrine fashions to regulate cellular events that occur during the early phase of follicular development. It seems reasonable that the expression of TSP isoforms by bovine granulosa cells in the early stages of follicular development would serve to suppress vascularization of the granulosa cell compartment to optimize pre-ovulatory granulosa cell proliferation and follicular growth, which, in turn, would produce a postovulatory corpus luteum capable of maximal progesterone production to support the initial stages of a potential pregnancy. However, the significant differences in patterns of expression of TSP isoforms in developing follicles of rodents and cows and in the regulation of these patterns of expression by gonadotropins underscores the importance of further research regarding the expression and the roles of TSPs in higher order mammals of domestic importance as well as in primates, including women.

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