Reproductive Biology and Endocrinology

Research

Temporal and spatial expression of tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and -2) in the bovine corpus luteum

Bo Zhang[1,2], Marsha A Moses[1,2] and Paul CW Tsang*[3]

Address: [1]Department of Surgery, Harvard Medical School and Children's Hospital, Boston, MA 02115, USA, [2]The Vascular Biology Program, Children's Hospital, Boston, MA 02115, USA and [3]Department of Animal and Nutritional Sciences, University of New Hampshire, Durham, NH 03824, USA

Email: Bo Zhang - bo.zhang@tch.harvard.edu; Marsha A Moses - marsha.moses@tch.harvard.edu; Paul CW Tsang* - pct@cisunix.unh.edu

* Corresponding author

Published: 07 November 2003

Reproductive Biology and Endocrinology 2003, **1**:85

Received: 03 October 2003
Accepted: 07 November 2003

This article is available from: http://www.rbej.com/content/1/1/85

Abstract

The matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), may mediate the dramatic structural and functional changes in the corpus luteum (CL) over the course of its life span. In addition to regulating MMP activity, TIMPs are also involved in a variety of cellular processes, including cell proliferation and steroidogenesis. In a series of initial studies, we determined that matrix metalloproteinase inhibitory activity was present in protein extracts from early (4 days old, estrus = day 0), mid (10–12 days old) and late (16 days old) CL (n = 3 for each stage). Reverse zymography revealed four metalloproteinase inhibitory protein bands with relative molecular masses that are consistent with those reported for TIMP-1 to -4. In order to gain a better understanding of TIMPs and their role in luteal function, we further characterized this inhibitory activity with a particular focus on the temporal and spatial expression of TIMP-1 and TIMP-2 in the bovine CL. Northern blotting revealed that the TIMP-1 transcript (0.9 kb) was expressed at a higher ($p < 0.05$) level in early and mid cycle CL than in the late stage. In contrast, two TIMP-2 mRNA species, one major 1 kb species and one minor 3.5 kb species, were significantly ($p < 0.05$) increased in the mid and late cycle CL than in the early. Western blotting analyses demonstrated no differences in TIMP-1 (29 kDa) protein levels between early and mid stages, while its levels decreased ($p < 0.05$) from the mid to late stage CL. Conversely, TIMP-2 (22 kDa) protein was detected at a low level in the early CL, but significantly ($p < 0.05$) increased in the mid and late stages. Immunohistochemistry revealed that both TIMP-1 and -2 were localized to large luteal cells from all three ages of CL. TIMP-1 was also localized in capillary smooth muscle cells, while TIMP-2 was restricted to the endothelial cells in the capillary compartment. In conclusion, the different temporal expression patterns of TIMP-1 and TIMP-2 suggest that TIMP-1 may be important for luteal formation and development, while TIMP-2 may play significant roles during luteal development and maintenance. Furthermore, the distinct localization of these two inhibitors in the vascular compartment indicates that they may serve diverse physiological functions during different stages of luteal angiogenesis.

Background

The corpus luteum (CL) is a transient, dynamic endocrine gland, which develops from the postovulatory follicle [1]. Dramatic structural and functional changes are associated with the development, maintenance and regression of the CL [1]. These remodeling events require the participation of matrix metalloproteinases (MMPs), a growing family of zinc and calcium dependent proteolytic enzymes that collectively digest all known macromolecules constituting the extracellular matrix [2,3]. Generally, the catalytic activity of the MMPs is highly regulated at three levels, gene expression, proteolytic activation of latent proenzymes, and inhibition of activity by binding of endogenous tissue inhibitors of metalloproteinases (TIMPs) to the catalytic domain [2,4].

Four TIMPs, TIMP-1 [5,6], TIMP-2 [7,8], TIMP-3 [9], and TIMP-4 [10], have been identified. Among them, TIMP-1 and TIMP-2 are the two most studied inhibitors. TIMP-1, a glycoprotein with a molecular mass of approximately 29 kDa, was the first member of this family to be cloned [5]. TIMP-1 can bind the active forms of all known MMPs [4] and the latent form of MMP-9 [11]. In addition to suppressing the activity of MMPs, TIMP-1 also possesses mitogenic activity for a variety of cell types, such as gingival fibroblasts and erythroid precursor cells [12]. TIMP-1 may regulate steroidogenesis in that it stimulates progesterone production by rat granulosa cells [13]. In CL from a variety of species, the expression of TIMP-1 and TIMP-1-like proteins and messenger RNA has been determined, including cow [14,15], sheep [16], rat [17], mouse [18], monkey [19], and human [20].

TIMP-2 is an unglycosylated protein with an approximate molecular mass of 22 kDa [7,21]. TIMP-2 is also able to bind most active MMPs and inhibit their proteolytic activity [4]. Among the members of the MMP family, TIMP-2 preferentially binds to MMP-2 [21,22]. Paradoxically, however, TIMP-2 may also be involved in pro-MMP-2 activation by participating in the formation of a membrane type 1-MMP (MT1-MMP)/TIMP-2/pro-MMP-2 trimolecular complex on the cell membrane [23–25]. Similar to TIMP-1, TIMP-2 stimulates proliferation of a variety of cell types [26]. TIMP-2 mRNA expression has been determined in the sheep [27], cow [28], human [29], rat [30], and mouse [18] CL, while a TIMP-2-like protein was detected in the cow CL [15].

The variety and intensity of events that occur over the CL life span suggest a tightly regulated temporal and spatial interplay between MMPs and TIMPs. Our previous studies demonstrated that remodeling of the bovine CL involves a variety of MMP species, for example, MMP-2, MMP-9, and MT1-MMP. In the present study, we determined TIMP inhibitory activity and profiled the coordinate mRNA and protein expression patterns and cellular distribution of TIMP-1 and TIMP-2 in early, mid, and late stages of the bovine CL obtained over the estrous cycle.

Methods

Animal Model and Tissue Collection

Corpora lutea were collected from cyclic, nonlactating dairy cows, which were housed at the University of New Hampshire Dairy Teaching and Research Center. CL were removed by colpotomy on day 4, 10, and 16 of the estrous cycle (day 0 = estrus; n = 3 per day). For day 4 CL, the ovary was removed by an ecraseur after the cow received an epidural anesthetic [2% (w/v) mepivacaine hydrochloride; 0.01 mL/kg BW; Upjohn, Kalamazoo, MI], and the CL was then dissected from ovarian stroma. The day 10 and 16 CL were enucleated from the ovarian stroma. Based on progesterone concentrations determined by radioimmunoassay, the CL collected on day 16 of the estrous cycle were functionally active (8.99 ± 1.84; ng/ml ± SEM). The luteal tissue was subsequently used for total RNA and protein extraction. All animal experimentation protocols in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Hampshire.

Radiometric MMP Assay

Total protein was extracted from luteal tissues in a lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 0.02% (w/v) sodium azide, 10 mM EDTA, 1% (v/v) Triton X-100, 10 μg/ml aprotinin (Sigma, St Louis, MO), 1 μg/ml aminoethyl benzenesulphonyl fluoride (Sigma, St Louis, MO)] which we previously described [31]. Equivalent amounts of luteal protein extracts were added to wells of 96-well microtiter plates containing polymerized ^{14}C-acetylated collagen in the presence of a known amount of MMP-1 activity from the conditioned medium of bovine corneal explants [32]. The plates were then incubated at 37°C for 2.5 hours, after which the supernatant containing degraded ^{14}C-acetylated collagen fragments was collected and counted in a scintillation counter. A unit is defined as the amount of protein required to inhibit one unit of corneal collagenase by 50%.

Reverse Zymography

In order to simultaneously distinguish the activity of TIMP proteins in bovine CL, reverse zymographic analysis was performed. Equivalent aliquots of CL tissue extracts were mixed with loading buffer [2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 50 mM Tris-HCl, pH 6.8] and applied to 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.5 mg/ml gelatin. After electrophoresis, gels were rinsed twice with 2.5% (v/v) Triton X-100, followed by incubation with conditioned medium of human fibrosarcoma HT1080 cells, which is a rich source of various MMPs, for 4 hours to degrade

gelatin. After rinsing, gels were incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, pH 8.0). Subsequently, gels were stained for 30 minutes with 0.1% (w/v) Coomassie Blue G-250, and destained with a solution of 30% (v/v) methanol and 10% (v/v) glacial acetic acid. Whereas the MMPs present in the HT-1080 conditioned medium digest the gelatin within the gel, the TIMPs in the luteal samples inhibit MMP action, and were visualized as dark bands on a clear background. Prestained SDS-PAGE protein standards (Bio-Rad Laboratories, Hercules, CA) were run in an adjacent lane.

Northern Blotting

TIMP-1 and TIMP-2 mRNA expression in the CL was studied by Northern blotting as described previously [31]. Briefly, total RNA was extracted from luteal tissues using TRIZOL (GIBCO-BRL, Carlsbad, CA) according to the manufacturer's instructions. Twenty micrograms of total RNA were fractionated on 1.0% (w/v) agarose gels containing formaldehyde and were transferred onto Hybond™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were first incubated in pre-hybridization buffer [50% (v/v) formamide, 5× SSC, 0.2% (w/v) SDS, and 2% (w/v) blocking reagent] for two hours at 42°C. Hybridization was carried out in the hybridization buffer containing TIMP-1 [33], TIMP-2 [7], or cyclophilin (a generous gift from Dr. Robert Thompson, University of Michigan) cDNA probes at 55°C overnight. These cDNA probes were labeled with digoxigenin (DIG)-dUTP using the DIG DNA Random-primed Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN). The blots were then washed twice at room temperature in 2× SSC with 0.1% (w/v) SDS, followed by higher stringency washes in 0.2× SSC with 0.1% SDS at 65°C. Hybridized probes were detected using an anti-DIG antibody conjugated to alkaline phosphatase (used at 1:5000; Roche Molecular Biochemicals, Indianapolis, IN) in 1% (w/v) blocking reagent. The signals were detected by CSPD (Roche Molecular Biochemicals, Indianapolis, IN), a chemiluminescent substrate for alkaline phosphatase. The blots were visualized by developing Kodak XAR-5 films with a Konica Medical Film Processor (Tokyo, Japan).

Western Blotting Analysis

Equivalent amounts of tissue protein extracts were subjected to SDS gel electrophoresis on 12% (w/v) polyacrylamide gels under reducing conditions before transfer onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Prestained SDS-PAGE protein standards (Bio-Rad Laboratories, Hercules, CA) were also loaded in an adjacent lane. Nonspecific binding sites were blocked with 5% (w/v) nonfat powdered milk in TBST [0.01 M Tris-HCl, 0.15 M NaCl, and 0.05% (v/v) Tween-20, pH 8.0] for 2 hours. Two primary antibodies, mouse anti-human TIMP-1 monoclonal antibody (Oncogene Research Products, Cambridge, MA) and mouse anti-human TIMP-2 monoclonal antibody (Oncogene Research Products, Cambridge, MA), were used at 1 μg/ml and 2 μg/ml, respectively. After an overnight incubation at 4°C, the membranes were washed five times with TBST (each time for 15 minutes), followed by a 1-hour incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (1:15,000, Pierce, Rockford, IL) at room temperature. The blots were then washed with TBST, and developed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), according to the manufacturer's instructions.

Immunohistochemistry

Frozen tissue sections (6 μm) were cut onto Superfrost®/Plus slides (Fisher Scientific, Pittsburg, PA). Sections were air-dried at room temperature for 5 min before a 10-min fixation in cold acetone. Endogenous peroxidase activity was quenched by incubating with 0.3% (v/v) hydrogen peroxide for 30 min. Nonspecific binding was blocked by 5% (w/v) BSA for 30 min. Sections were subsequently incubated at room temperature for 1 hour with a 1:40 dilution of mouse anti-human TIMP-1 monoclonal antibody (Oncogene Research Products, Cambridge, CA) or with a 1:50 dilution of mouse anti-human TIMP-2 monoclonal antibody (Oncogene Research Products, Cambridge, CA). After washing, slides were incubated with goat anti-mouse IgG followed by VECTASTAIN® Elite ABC Kit (Vector Laboratories, CA), according to the manufacturer's instructions.

To further determine the presence of vascular smooth muscle cells (VSMC) and endothelial cells in the luteal tissue, consecutive sections were also stained with an antibody against α-actin and von Willebrand Factor (VWF), cellular markers for VSMC and endothelial cells, respectively [34]. For every tissue, an adjacent section placed on the same slide was used as a negative control, where BSA substituted for the primary antibody. For each tissue (3 corpora lutea for each age), 10 to 20 sections were stained. A minimum of 20 areas was examined.

Data Analysis

Intensities of Northern and Western blots, in which each sample was run in triplicate, were determined by UN-SCAN-IT™ digitizing software system (Silk Scientific, Orem, UT). The data were analyzed by ANOVA, followed by Tukey's test for multiple comparisons.

Results

Metalloproteinase Inhibitor Activities in the Bovine CL

The radiometric MMP assay for TIMP activity revealed that the protein extract from all three ages of CL possessed

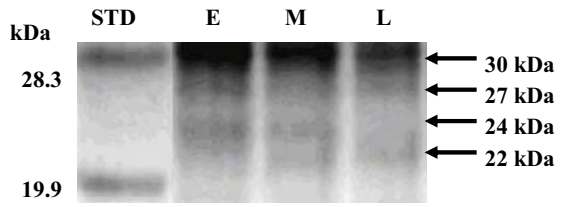

Figure 1
Reverse zymographic analysis of tissue inhibitors of metalloproteinases (TIMPs). Inhibitor activities in the early (E), mid (M), and late (L) stages of bovine CL are shown. Lane 1 indicates the prestained SDS-PAGE standards (STD; Bio-Rad Laboratories, Hercules, CA). Their corresponding molecular masses (kDa) are indicated on the left. Four bands possessing MMP inhibitory activities (indicated by arrows on the right) were observed in luteal samples. These bands correspond to TIMP-1 (~30 kDa), TIMP-3 (~27 kDa), TIMP-4 (~24 kDa), and TIMP-2 (~22 kDa), respectively.

significant metalloproteinase inhibitory activity (inhibitory units per gram total protein ± SEM; day 4, 22,933 ± 1703; day 10, 24,066 ± 33; day 16, 22,166 ± 2938). However, there was no significant difference ($P > 0.05$) among the three stages of CL studied. Based on these observations, reverse zymography was then used to distinguish metalloproteinase inhibitory activities in luteal tissues. Four protein bands were identified, and they migrated at ~30 kDa, ~27 kDa, ~24 kDa, and ~22 kDa, corresponding to the molecular masses reported for TIMP-1, -3, -4, and -2, respectively (Figure 1). TIMP-1 appeared to be the predominant TIMP present in the bovine CL.

TIMP-1 and TIMP-2 mRNA Expression in the Bovine CL during the Estrous Cycle

Northern blotting demonstrated that a single TIMP-1 transcript (0.9 kb) was present in all three ages of CL (Figure 2A). The 18S rRNA was used to normalize sample loading. The densitometric ratio of TIMP-1 mRNA to 18S rRNA was high in the early and mid cycle CL, but decreased significantly ($p < 0.05$) in the late stage (Figure 2B). The same pattern was observed when cyclophilin was used to normalize sample loading.

Two TIMP-2 mRNA species, a major one at 1 kb and a minor one at 3.5 kb, were present in all ages of CL (Figure 3A). In contrast to the expression pattern of TIMP-1 mRNA, both species of TIMP-2 mRNA, shown as the densitometric ratio of TIMP-2 mRNA to a house-keeping gene cyclophilin, were low in the early stage, but increased significantly ($p < 0.05$) in the mid and late cycle CL (Figure 3B). The same pattern was observed when 18S RNA was used to normalize sample loading.

TIMP-1 and TIMP-2 Protein Levels in the Bovine CL

Western blotting revealed an approximate 30 kDa immunoreactive TIMP-1 protein band in all ages of CL examined (Figure 2C). The level of TIMP-1 protein was not different between the early and mid cycle CL ($p = 0.324$), but it was significantly decreased ($p < 0.05$) in the late stage CL (Figure 2D). In addition, a 22 kDa TIMP-2 immunoreactive protein was also observed in all three ages of CL (Figure 3C). The TIMP-2 protein level in the mid and late cycle CL was significantly greater ($p < 0.05$) than in the early stage (Figure 3D).

Cellular Localization of TIMP-1 and TIMP-2 Proteins in the Bovine CL

Immunohistochemistry using TIMP-1 and TIMP-2 specific antibodies demonstrated that TIMP-1 was present in both large luteal cells and vascular smooth muscle cells (Figure 4A,4C, and 4E). The localization of TIMP-1 in the VSMC compartment was validated by staining for α-actin, a cellular marker for VSMC (Figure 4G). Although large luteal cells from all three ages of CL expressed TIMP-1, visual observations revealed that the highest level of expression was in cells of the mid-cycle CL (Figure 4C). TIMP-1 was expressed in VSMC of the early (Figure 4A) and late (Figure 4E), but not the mid cycle CL (Figure 4C). In all ages of CL, endothelial cells, as validated by staining with an endothelial cell marker VWF (Figure 4H), and large luteal cells showed positive staining for TIMP-2 protein (Figure 4B,4D and 4F). No positive signals were observed in the sections devoid of primary antibodies against TIMP-1 or TIMP-2 (data not shown).

Discussion

In order to eventually elucidate the physiological roles of TIMP-1 and TIMP-2 in the CL, we first determined the temporal and spatial expression patterns of these two inhibitors during the estrous cycle.

In the present study, TIMP-1 mRNA was determined to be highly expressed in the early and mid cycle bovine CL, but decreased in the late stage. In the porcine CL, the TIMP-1 transcript was also highly expressed in the early stage, and is slightly reduced as the estrous cycle progresses before it decreases significantly in the regressing stages [35]. In contrast, TIMP-1 mRNA expression in the ovine [16] and human [29] CL does not change throughout the estrous or menstrual cycle, respectively. In the pseudopregnant rat, a different pattern emerges whereby the strongest TIMP-1 mRNA expression is observed during CL formation and regression [36]. Clearly, there are species differences with

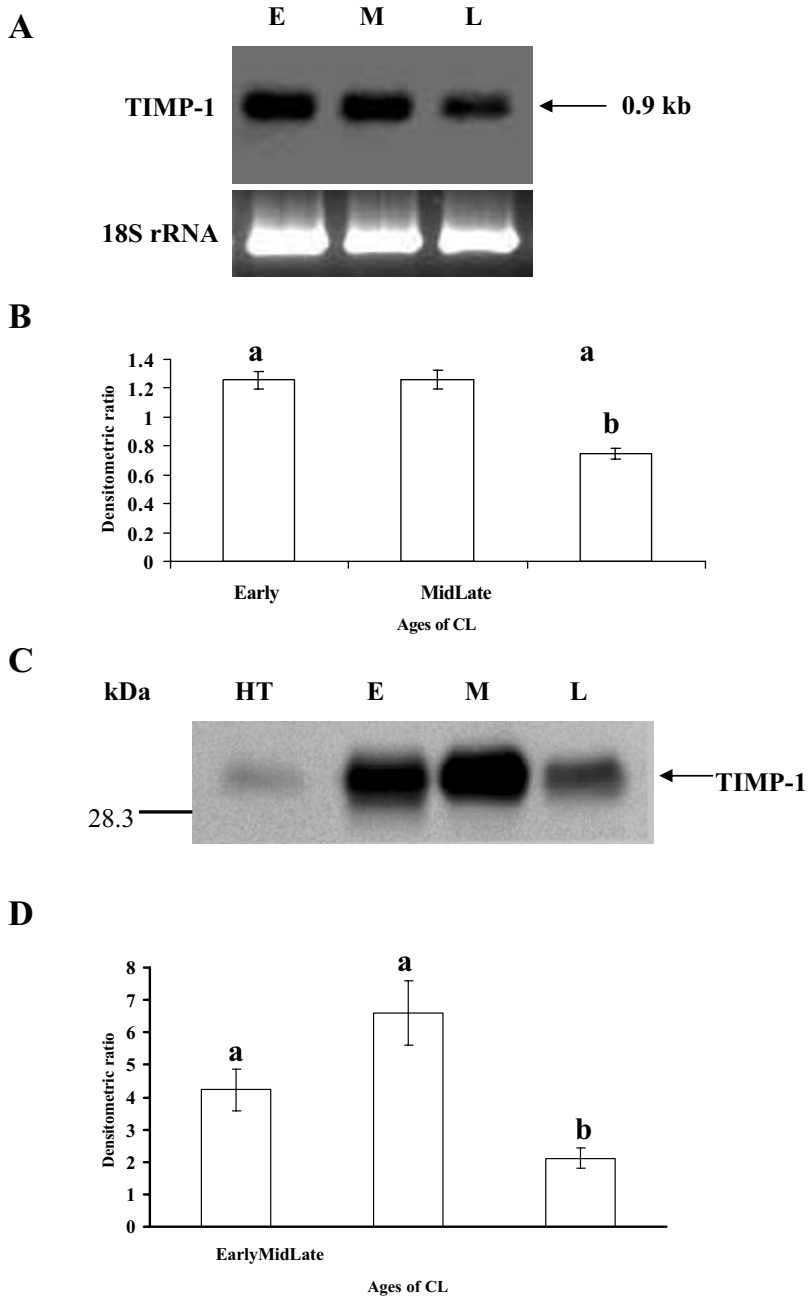

Figure 2
TIMP-1 mRNA and protein expression in the bovine CL. **A**) Northern blotting of TIMP-1 mRNA in early (E), mid (M), and late (L) stage CL is shown in the upper panel. The arrow indicates the 0.9 kb TIMP-1 transcript. The ethidium bromide stained 18S rRNA in corresponding luteal samples is shown in the lower panel. **B**) Changes in TIMP-1 mRNA, expressed as a densitometric ratio of TIMP-1 mRNA to 18S rRNA, are shown in the lower panel. Dissimilar letters denote significant difference at $p < 0.05$. **C**) A representative Western blot of TIMP-1 in early (E), mid (M), and late (L) stage CL. Conditioned medium of HT1080 cells (HT) was loaded in the first lane and was used as a positive control. **D**) TIMP-1 protein levels in different stages are presented as a ratio of band intensity in luteal samples to that in HT1080 conditioned medium. Dissimilar letters denote significant difference at $p < 0.05$.

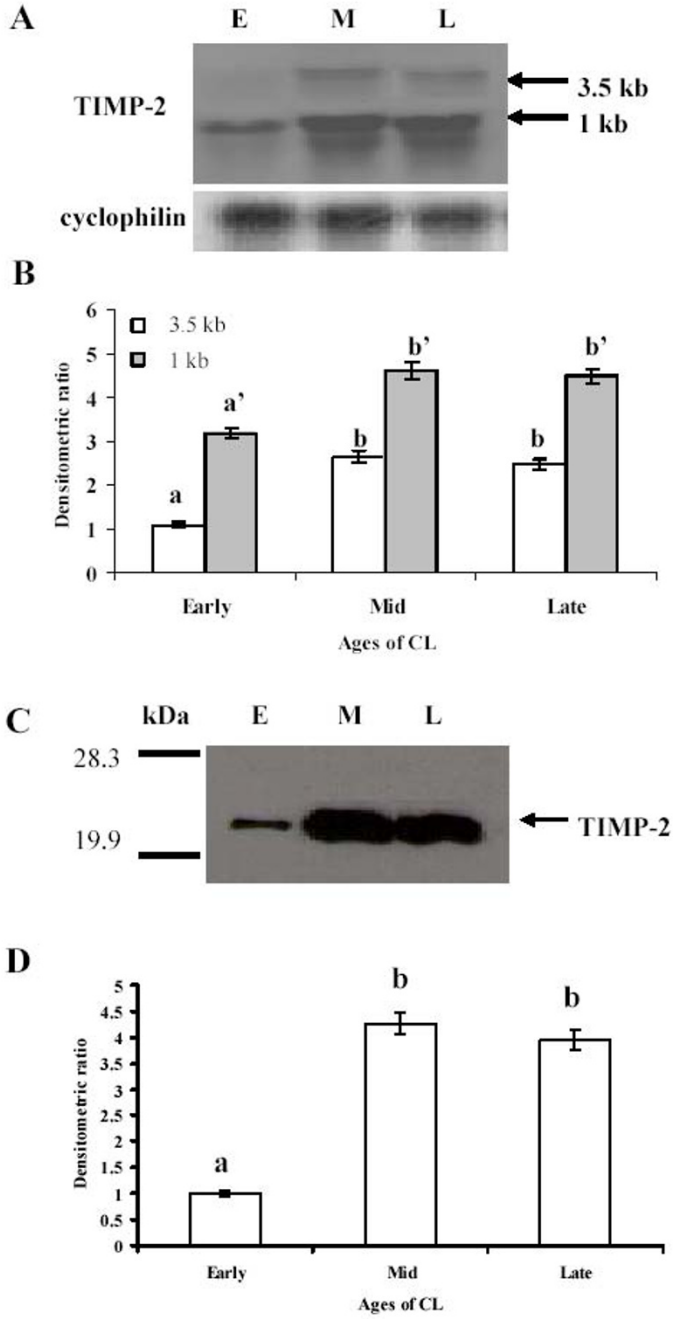

Figure 3
TIMP-2 mRNA and protein expression in the bovine CL. **A**) In the upper panel, Northern blotting of TIMP-2 mRNA in early (E), mid (M), and late (L) stage bovine CL. The arrows indicate the 3.5 and 1 kb species. The lower panel is the same membrane hybridized with a human cyclophilin probe. **B**) Densitometric ratios of each TIMP-2 mRNA band to cyclophilin in corresponding stages are shown. Dissimilar letters denote significant difference at $p < 0.05$. **C**) A representative Western blot of TIMP-2 in the bovine CL. Molecular masses (kDa) of protein standards are shown on the left. A single 22 kDa TIMP-2 immunoreactive protein band, indicated by the arrow, is present in early (E), mid (M), and late (L) stage CL. **D**) Densitometric analysis of TIMP-2 protein expression among different stages of CL. Dissimilar letters denote significant difference at $p < 0.05$.

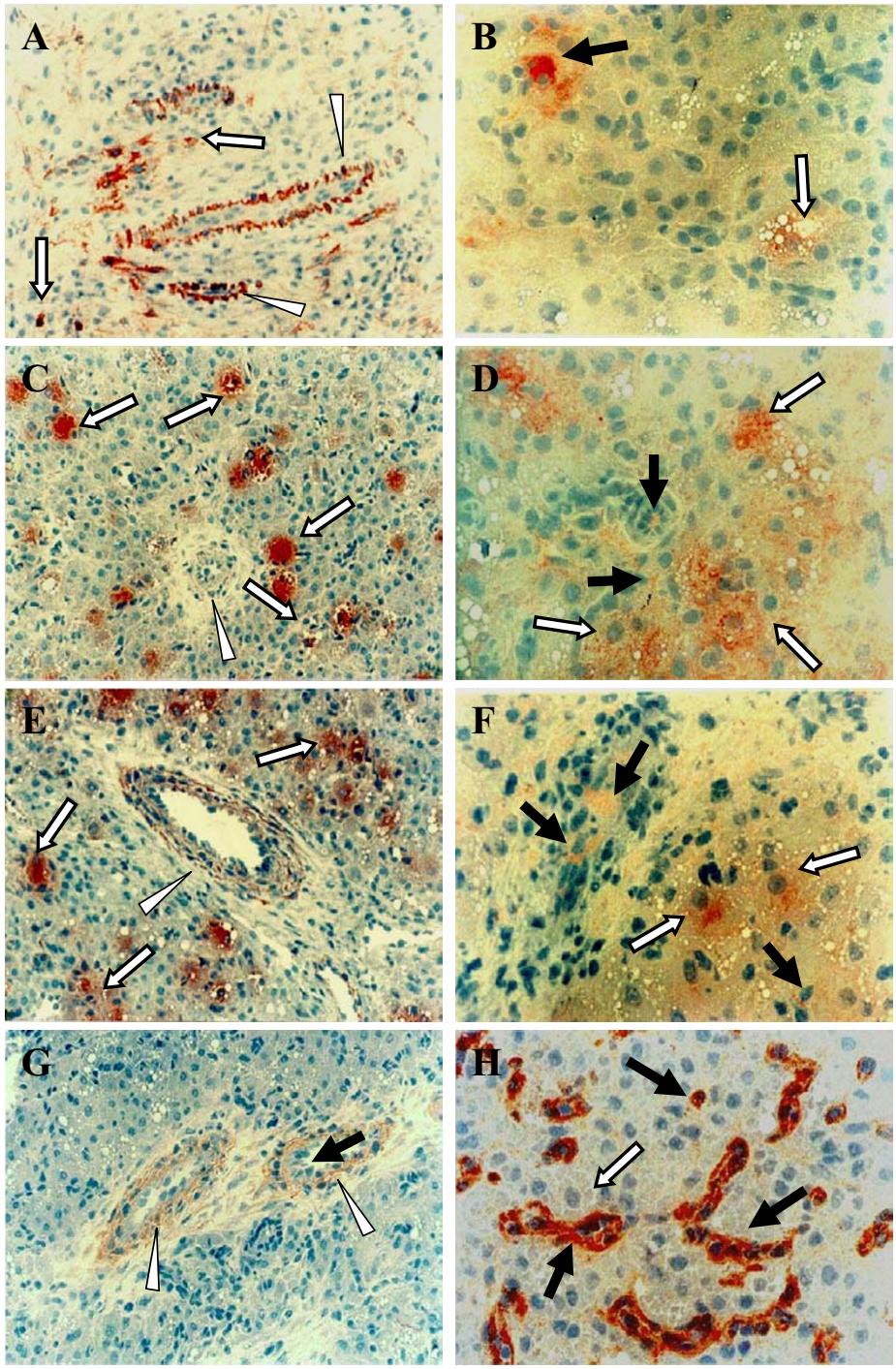

Figure 4
Immunohistochemistry of TIMP-1 (A, C, E; ×200) and TIMP-2 (B, D, F; ×400) in early (A and B), mid (C and D), and late (E and F) stage CL. Vascular smooth muscle cells and endothelial cells are identified by staining with α-actin (G; ×200) and VWF (H; ×400). Positive staining (red color) is observed in large luteal cells (white arrows), endothelial cells (black arrows), and vascular smooth muscle cells (white triangles).

regard to the temporal expression of TIMP-1 transcript in the CL.

The expression of TIMP-1 protein in three ages of bovine CL was also demonstrated in the present study. Reverse zymography demonstrated that TIMP-1 was the predominant TIMP in the bovine CL, similar to the finding in sheep [37]. In the present study, the patterns of TIMP-1 protein and mRNA paralleled each other, being high in the early and mid stages, but decreased in the late cycle CL. The high levels of TIMP-1 in the early and mid cycle CL may participate in regulating the extensive tissue remodeling events that occur during CL formation and development. The reduced TIMP-1 mRNA and protein expression in the late stage CL may portend the decline of this inhibitor observed during luteal regression in bovine [38], ovine [39,40], porcine [35], and primate [19] CL. Collectively, these data implicate TIMP-1 as an important player in the physiology of the CL.

The TIMP-1 protein was localized in large luteal cells of early, mid, and late stage bovine CL. In the ovine CL, TIMP-1 was co-localized with oxytocin in secretory granules of large luteal cells [41]. In addition, TIMP-1 expression was also detected in isolated ovine [16] and porcine [35] large luteal cells, and luteinized human granulosa cells [42]. The presence of TIMP-1 in steroidogenic cells may be associated with its ability to enhance steroid production [13]. This may be related in part to a 124 bp-nucleotide sequence similarity between the protein coding region of the bovine steroidogenic acute regulatory (StAR) gene and the 5' non-coding region of TIMP-1 [43]. Additionally, although female mice lacking the TIMP-1 gene do not show detectable differences in serum estradiol-17β concentrations when compared to the wild type, the TIMP-1 deficient male mice have higher concentrations of total serum testosterone than the wild type [44,45]. Furthermore, cell culture studies demonstrated that TIMP-1 is able to increase estradiol-17β production by granulosa cells from both TIMP-1 deficient and wild-type mice [44], and estradiol-17β and progesterone production from porcine thecal cells [46]. Therefore, the strong expression of TIMP-1 in large luteal cells may be related to its collateral role in steroidogenesis.

Because of the predominant expression of TIMP-1 in large luteal cells, this inhibitor is used as a cellular marker for this cell type [47]. However, other cell types in the CL are positive for TIMP-1 expression as well. For example, the present study showed that TIMP-1 was also localized in the vascular smooth muscle cells (VSMC) of the bovine CL. Although the cellular source was not specified, the capillary compartments in ovine [41] and rat [48] CL stain positively for TIMP-1. The localization of TIMP-1 in the vascular smooth muscle compartment supports its potential role during angiogenesis and vascular maintenance. Indeed, overexpression of TIMP-1 in VSMC by exogenous gene transfer reduced VSMC cell proliferation and migration [49,50]. In addition, stimulation of TIMP-1 expression impaired angiogenesis in a variety of tumor types [51–53]. These data collectively suggest that TIMP-1 may act as a negative regulator of blood vessel formation [32,54].

Extensive angiogenesis occurs during early CL development when theca-derived luteal cells and fibroblasts invade through the breached basement membrane into the cavity of the ruptured follicle. These early events in the angiogenic process require MMP activity. However, in the last step of angiogenesis, recruitment and maintenance of pericytes (VSMC) are critical for vascular maturation and survival [55,56]. Thus, the high level of TIMP-1 expression in VSMC of 4-day old bovine CL may provide an environment where MMP action was curbed as the vasculature matures. As the CL ages, its structure remains relatively stable. Although angiogenesis is ongoing, it is slowed during CL maintenance [57–59]. This may be the reason for the absence of TIMP-1 in the VSMC compartment at this stage of the bovine estrous cycle. In the 16-day old CL, TIMP-1 was detected again in the VSMC compartment. This localized expression of TIMP-1 is consistent with the hypothesis of Redmer et al. [60], who proposed that maintenance of the vasculature may be necessary for the transport of degraded products during luteal regression, which ultimately results in a massive decrease in CL size and weight as regression ensues.

Although TIMP-1 was the most abundant TIMP in luteal tissues, the other three TIMPs were also detected in the bovine CL by reverse zymography. Among them, we chose to focus our attention on TIMP-2. It has been reported that TIMP-2 may be involved in the pro-MMP-2 activation process by binding an MT1-MMP to form a "co-receptor" for pro-MMP-2 on the cell surface [25]. This bound pro-MMP-2 would then be presented to an adjacent MT1-MMP for activation [23,25]. Although a transmembrane-deleted MT1-MMP is capable of activating pro-MMP-2 without the participation of TIMP-2 [61], TIMP-2 deficient mice have a dramatically reduced ability to activate pro-MMP-2 [62]. In the present study, both TIMP-2 mRNA and protein levels were low in the early CL, but significantly increased in the mid and late stages. This expression pattern was similar to that for active MT1-MMP and MMP-2 [31], suggesting that the MT1-MMP/TIMP-2/pro-MMP-2 tri-molecular system may be available for MMP-2 activation in vivo in the bovine CL. The coordinate expression of these three molecules is also observed during embryonic development [63], which supports the presence of this activation system in a variety of tissue types.

In addition to the temporal correlation of TIMP-2 with active MT1-MMP and MMP-2 expression in the CL, TIMP-2 was also co-localized with these two molecules in endothelial and large luteal cells. TIMP-2 has a variety of functions in endothelial cells. On the one hand, the stoichiometrically correlated expression with MT1-MMP and pro-MMP-2 may facilitate the activation process of pro-MMP-2[31], which is critical for the angiogenesis process[64]. On the other hand, inhibition of angiogenesis by TIMP-2 cannot be excluded since TIMP-2 has now been demonstrated to be a potent inhibitor of both physiological and stimulated angiogenesis in vivo[54] and overexpression of TIMP-2 blocks vascular smooth muscle cell invasiveness [65] and reduces angiogenic ability [54,66]. The latter observation is due, in part, to down-regulation of vascular endothelial cell growth factor (VEGF) [67].

The localization of TIMP-2 in large luteal cells may also contribute to the activation of pro-MMP-2 in this cell type by assembling the trimeric complex on the cell membrane. The in situ activated MMP-2 is then able to bind integrin αvβ3 [68], where localized pericellular proteolysis ensues. This resulting degradation of the ECM is needed to accommodate the enlargement of large luteal cells from the early to mid and late stages. Although there is no direct evidence demonstrating involvement of TIMP-2 in steroidogenesis, the dynamic interactions between large luteal cells and their local ECM may induce biochemical changes related to the steroid biosynthetic process in this cell type [69]. For example, disruption of the links between ECM, integrins, and the cytoskeleton [70] may perturb the intracellular transport of substrates, such as cholesterol, for steroidogenesis [71]. Additional in vitro and in vivo studies are needed to elucidate the physiological roles of these TIMPs in this ovarian endocrine gland.

Conclusions

The present study demonstrates that TIMP-1 and TIMP-2 exhibit a coordinated expression pattern in the bovine CL throughout the estrous cycle. The distinct temporal and spatial expression patterns of TIMP-1 and TIMP-2 suggest that these two inhibitors may have multiple and complementary roles in luteal development and angiogenesis during the life span of the CL. Taken together, the expression of these multi-functional TIMPs and MMPs during the bovine estrous cycle suggests that they are key regulators of CL physiology.

Acknowledgements

We thank Gwen Louis for her assistance with the radiometric MMP assay. This research was supported by the Sigma Xi Grant-in-Aid Research Award to BZ, the USDA (98-35208-6654) and the Northeast Regional Project NE-161 to PCWT, and the American Cancer Society (RPG 83821) and the National Institute of Health (R01 CA83106, R01 AT00650, and P01 CA455) to MAM. This is scientific contribution number 2200 from the New Hampshire Agricultural Experiment Station.

References

1. Niswender GD and Nett TM: **Corpus luteum and its control in infraprimate species.** In: *The Physiology of Reproduction Volume 1*. 2nd edition. Edited by: *Knobil E, Neill JD. New York: Raven Press*; 1994:781-816.
2. Matrisian LM: **Metalloproteinases and their inhibitors in matrix remodeling.** *Trends Genet* 1990, **6:**121-125.
3. Massova I, Kotra LP, Fridman R and Mobashery S: **Matrix metalloproteinases: structures, evolution, and diversification.** *FASEB J* 1998, **12:**1075-1095.
4. Woessner JF and Nagase H: **Matrix Metalloproteinases and TIMPs.** *New York: Oxford University Press*; 2000.
5. Docherty AJ, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJ, Murphy G and Reynolds JJ: **Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity.** *Nature* 1985, **318:**66-69.
6. Carmichael DF, Sommer A, Thompson RC, Anderson DC, Smith CG, Welgus HG and Stricklin GP: **Primary structure and cDNA cloning of human fibroblast collagenase inhibitor.** *Proc Natl Acad Sci U S A* 1986, **83:**2407-2411.
7. Stetler-Stevenson WG, Krutzsch HC and Liotta LA: **Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family.** *J Biol Chem* 1989, **264:**17374-17378.
8. De Clerck YA, Yean TD, Ratzkin BJ, Lu HS and Langley KE: **Purification and characterization of two related but distinct metalloproteinase inhibitors secreted by bovine aortic endothelial cells.** *J Biol Chem* 1989, **264:**17445-17453.
9. Pavloff N, Staskus PW, Kishnani NS and Hawkes SP: **A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family.** *J Biol Chem* 1992, **267:**17321-17326.
10. Greene J, Wang M, Liu YE, Raymond LA, Rosen C and Shi YE: **Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4.** *J Biol Chem* 1996, **271:**30375-30380.
11. Itoh Y and Nagase H: **Preferential inactivation of tissue inhibitor of metalloproteinases-1 that is bound to the precursor of matrix metalloproteinase 9 (progelatinase B) by human neutrophil elastase.** *J Biol Chem* 1995, **270:**16518-16521.
12. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E and Iwata K: **Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum.** *FEBS Lett* 1992, **298:**29-32.
13. Boujrad N, Ogwuegbu SO, Garnier M, Lee CH, Martin BM and Papadopoulos V: **Identification of a stimulator of steroid hormone synthesis isolated from testis.** *Science* 1995, **268:**1609-1612.
14. Freudenstein J, Wagner S, Luck RM, Einspanier R and Scheit KH: **mRNA of bovine tissue inhibitor of metalloproteinase: sequence and expression in bovine ovarian tissue.** *Biochem Biophys Res Commun* 1990, **171:**250-256.
15. Goldberg MJ, Moses MA and Tsang PC: **Identification of matrix metalloproteinases and metalloproteinase inhibitors in bovine corpora lutea and their variation during the estrous cycle.** *J Anim Sci* 1996, **74:**849-857.
16. Smith GW, Goetz TL, Anthony RV and Smith MF: **Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of messenger ribonucleic acid expression and in situ localization within preovulatory follicles and luteal tissue.** *Endocrinology* 1994, **134:**344-352.
17. Nothnick WB, Edwards DR, Leco KJ and Curry TE Jr: **Expression and activity of ovarian tissue inhibitors of metalloproteinases during pseudopregnancy in the rat.** *Biol Reprod* 1995, **53:**684-691.
18. Inderdeo DS, Edwards DR, Han VK and Khokha R: **Temporal and spatial expression of tissue inhibitors of metalloproteinases during the natural ovulatory cycle of the mouse.** *Biol Reprod* 1996, **55:**498-508.
19. Duncan WC, Illingworth PJ and Fraser HM: **Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression.** *J Endocrinol* 1996, **151:**203-213.
20. Duncan WC, McNeilly AS and Illingworth PJ: **Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue.** *J Endocrinol* 1996, **148:**59-67.
21. Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S and He CS: **Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2.** *Proc Natl Acad Sci U S A* 1989, **86:**8207-8211.

22. Olson MW, Gervasi DC, Mobashery S and Fridman R: **Kinetic analysis of the binding of human matrix metalloproteinase-2 and-9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2.** *J Biol Chem* 1997, **272:**29975-29983.
23. Deryugina EI, Bourdon MA, Luo GX, Reisfeld RA and Strongin A: **Matrix metalloproteinase-2 activation modulates glioma cell migration.** *J Cell Sci* 1997, **110:**2473-2482.
24. Cao J, Drews M, Lee HM, Conner C, Bahou WF and Zucker S: **The propeptide domain of membrane type 1 matrix metalloproteinase is required for binding of tissue inhibitor of metalloproteinases and for activation of pro-gelatinase A.** *J Biol Chem* 1998, **273:**34745-34752.
25. Butler GS, Butler MJ, Atkinson SJ, Will H, Tamura T, van Westrum SS, Crabbe T, Clements J, d'Ortho MP and Murphy G: **The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. A kinetic study.** *J Biol Chem* 1998, **273:**871-880.
26. Hayakawa T, Yamashita K, Ohuchi E and Shinagawa A: **Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2).** *J Cell Sci* 1994, **107:**2373-2379.
27. Smith GW, McCrone S, Petersen SL and Smith MF: **Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea.** *Endocrinology* 1995, **136:**570-576.
28. Smith GW, Juengel JL, McLntush EW, Youngquist RS, Garverick HA and Smith MF: **Ontogenies of messenger RNA encoding tissue inhibitor of metalloproteinases 1 and 2 within bovine periovulatory follicles and luteal tissue.** *Domest Anim Endocrinol* 1996, **13:**151-160.
29. Duncan WC, McNeilly AS and Illingworth PJ: **The effect of luteal "rescue" on the expression and localization of matrix metalloproteinases and their tissue inhibitors in the human corpus luteum.** *J Clin Endocrinol Metab* 1998, **83:**2470-2478.
30. Simpson KS, Byers MJ and Curry TE Jr: **Spatiotemporal messenger ribonucleic acid expression of ovarian tissue inhibitors of metalloproteinases throughout the rat estrous cycle.** *Endocrinology* 2001, **142:**2058-2069.
31. Zhang B, Yan L, Moses MA and Tsang PC: **Bovine membrane-type 1 matrix metalloproteinase: molecular cloning and expression in the corpus luteum.** *Biol Reprod* 2002, **67:**99-106.
32. Moses MA, Sudhalter J and Langer R: **Identification of an inhibitor of neovascularization from cartilage.** *Science* 1990, **248:**1408-1410.
33. Waterhouse P, Khokha R and Denhardt DT: **Modulation of translation by the 5' leader sequence of the mRNA encoding murine tissue inhibitor of metalloproteinases.** *J Biol Chem* 1990, **265:**5585-5589.
34. Morrell NW, Grieshaber SS, Danilov SM, Majack RA and Stenmark KR: **Developmental regulation of angiotensin converting enzyme and angiotensin type 1 receptor in the rat pulmonary circulation.** *Am J Respir Cell Mol Biol* 1996, **14:**526-537.
35. Pitzel L, Ludemann S and Wuttke W: **Secretion and gene expression of metalloproteinases and gene expression of their inhibitors in porcine corpora lutea at different stages of the luteal phase.** *Biol Reprod* 2000, **62:**1121-1127.
36. Liu K, Olofsson JI, Wahlberg P and Ny T: **Distinct expression of gelatinase A [matrix metalloproteinase (MMP)-2], collagenase-3 (MMP-13), membrane type MMP 1 (MMP-14), and tissue inhibitor of MMPs type 1 mediated by physiological signals during formation and regression of the rat corpus luteum.** *Endocrinology* 1999, **140:**5330-5338.
37. Smith GW, Moor RM and Smith MF: **Identification of a 30,000 M(r) polypeptide secreted by cultured ovine granulosa cells and luteal tissue as a tissue inhibitor of metalloproteinases.** *Biol Reprod* 1993, **48:**125-132.
38. Juengel JL, Smith GW, Smith MF, Youngquist RS and Garverick HA: **Pattern of protein production by bovine corpora lutea during luteolysis and characterization of expression of two major secretory products of regressing corpora lutea.** *J Reprod Fertil* 1994, **100:**515-520.
39. Towle TA, Tsang PC, Milvae RA, Newbury MK and McCracken JA: **Dynamic in vivo changes in tissue inhibitors of metalloproteinases 1 and 2, and matrix metalloproteinases 2 and 9, during prostaglandin F2α-induced luteolysis in sheep.** *Biol Reprod* 2002, **66:**1515-1521.
40. Ricke WA, Smith GW and Smith MF: **Matrix metalloproteinase expression and activity following prostaglandin F2α-induced luteolysis.** *Biol Reprod* 2002, **66:**685-691.
41. McIntush EW, Pletz JD, Smith GD, Long DK, Sawyer HR and Smith MF: **Immunolocalization of tissue inhibitor of metalloproteinases-1 within ovine periovulatory follicular and luteal tissues.** *Biol Reprod* 1996, **54:**871-878.
42. Aston KE, Stamouli A, Thomas EJ, Vyas S, Iredale JP, Arthur MJ and Richardson MC: **Effect of gonadotrophin on cell and matrix retention and expression of metalloproteinases and their inhibitor in cultured human granulosa cells modelling corpus luteum function.** *Mol Hum Reprod* 1996, **2:**26-30.
43. Hartung S, Rust W, Balvers M and Ivell R: **Molecular cloning and in vivo expression of the bovine steroidogenic acute regulatory protein.** *Biochem Biophys Res Commun* 1995, **215:**646-653.
44. Nothnick WB, Soloway P and Curry TE Jr: **Assessment of the role of tissue inhibitor of metalloproteinase-1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene.** *Biol Reprod* 1997, **56:**1181-1188.
45. Nothnick WB, Soloway PD and Curry TE Jr: **Pattern of messenger ribonucleic acid expression of tissue inhibitors of metalloproteinases (TIMPs) during testicular maturation in male mice lacking a functional TIMP-1 gene.** *Biol Reprod* 1998, **59:**364-370.
46. Shores EM and Hunter MG: **Production of tissue inhibitors of metalloproteinases (TIMPs) by pig ovarian cells in vivo and the effect of TIMP-1 on steroidogenesis in vitro.** *J Reprod Fertil* 2000, **120:**73-81.
47. Haworth JD, Rollyson MK, Silva P, McIntush EW and Niswender GD: **Messenger ribonucleic acid encoding monocyte chemoattractant protein-1 is expressed by the ovine corpus luteum in response to prostaglandin F2α.** *Biol Reprod* 1998, **58:**169-174.
48. Bagavandoss P: **Differential distribution of gelatinases and tissue inhibitor of metalloproteinase-1 in the rat ovary.** *J Endocrinol* 1998, **158:**221-228.
49. Forough R, Koyama N, Hasenstab D, Lea H, Clowes M, Nikkari ST and Clowes AW: **Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions in vitro and in vivo.** *Circ Res* 1996, **79:**812-820.
50. George SJ: **Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis.** *Curr Opin Lipidol* 1998, **9:**413-423.
51. Martin DC, Sanchez-Sweatman OH, Ho AT, Inderdeo DS, Tsao MS and Khokha R: **Transgenic TIMP-1 inhibits simian virus 40 T antigen-induced hepatocarcinogenesis by impairment of hepatocellular proliferation and tumor angiogenesis.** *Lab Invest* 1999, **79:**225-234.
52. Guedez L, McMarlin AJ, Kingma DW, Bennett TA, Stetler-Stevenson M and Stetler-Stevenson WG: **Tissue inhibitor of metalloproteinase-1 alters the tumorigenicity of Burkitt's lymphoma via divergent effects on tumor growth and angiogenesis.** *Am J Pathol* 2001, **158:**1207-1215.
53. Bloomston M, Shafii A, Zervos EE and Rosemurgy AS: **TIMP-1 overexpression in pancreatic cancer attenuates tumor growth, decreases implantation and metastasis, and inhibits angiogenesis.** *J Surg Res* 2002, **102:**39-44.
54. Fernandez CA, Butterfield C, Jackson G and Moses MA: **Structural and functional uncoupling of the enzymatic and angiogenic inhibitory activities of TIMP-2: Loop 6 is a novel angiogenesis inhibitor.** *J Biol Chem* 2003 in press.
55. Nehls V, Denzer K and Drenckhahn D: **Pericyte involvement in capillary sprouting during angiogenesis in situ.** *Cell Tissue Res* 1992, **270:**469-474.
56. Hirschi KK and D'Amore PA: **Pericytes in the microvasculature.** *Cardiovasc Res* 1996, **32:**687-698.
57. Tsukada K, Matsushima T and Yamanaka N: **Neovascularization of the corpus luteum of rats during the estrus cycle.** *Pathol Int* 1996, **46:**408-416.
58. Amselgruber WM, Schafer M and Sinowatz F: **Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study.** *Anat Histol Embryol* 1999, **28:**157-166.
59. Dickson SE, Bicknell R and Fraser HM: **Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor.** *J Endocrinol* 2001, **168:**409-416.

60. Redmer DA, Grazul AT, Kirsch JD and Reynolds LP: **Angiogenic activity of bovine corpora lutea at several stages of luteal development.** *J Reprod Fertil* 1988, **82:**627-634.
61. Pei D and Weiss SJ: **Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity.** *J Biol Chem* 1996, **271:**9135-9140.
62. Wang Z, Juttermann R and Soloway PD: **TIMP-2 is required for efficient activation of proMMP-2 in vivo.** *J Biol Chem* 2000, **275:**26411-26415.
63. Apte SS, Fukai N, Beier DR and Olsen BR: **The matrix metalloproteinase-14 (MMP-14) gene is structurally distinct from other MMP genes and is co-expressed with the TIMP-2 gene during mouse embryogenesis.** *J Biol Chem* 1997, **272:**25511-25517.
64. Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, Harper J, Tamvakopoulos G and Moses MA: **Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model.** *Proc Natl Acad Sci U S A* 2000, **97:**3884-3889.
65. Cheng L, Mantile G, Pauly R, Nater C, Felici A, Monticone R, Bilato C, Gluzband YA, Crow MT, Stetler-Stevenson W and Capogrossi MC: **Adenovirus-mediated gene transfer of the human tissue inhibitor of metalloproteinase-2 blocks vascular smooth muscle cell invasiveness in vitro and modulates neointimal development in vivo.** *Circulation* 1998, **98:**2195-2201.
66. Li H, Lindenmeyer F, Grenet C, Opolon P, Menashi S, Soria C, Yeh P, Perricaudet M and Lu H: **AdTIMP-2 inhibits tumor growth, angiogenesis, and metastasis, and prolongs survival in mice.** *Hum Gene Ther* 2001, **12:**515-526.
67. Hajitou A, Sounni NE, Devy L, Grignet-Debrus C, Lewalle JM, Li H, Deroanne CF, Lu H, Colige A, Nusgens BV, Frankenne F, Maron A, Yeh P, Perricaudet M, Chang Y, Soria C, Calberg-Bacq CM, Foidart JM and Noel A: **Down-regulation of vascular endothelial growth factor by tissue inhibitor of metalloproteinase-2: effect on in vivo mammary tumor growth and angiogenesis.** *Cancer Res* 2001, **61:**3450-3457.
68. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP and Cheresh DA: **Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha v\beta 3$.** *Cell* 1996, **85:**683-693.
69. Smith MF, McIntush EW, Ricke WA, Kojima FN and Smith GW: **Regulation of ovarian extracellular matrix remodeling by metalloproteinases and their tissue inhibitors: effects on follicular development, ovulation and luteal function.** *J Reprod Fertil Suppl* 1999, **54:**367-381.
70. Murdoch WJ: **Microtubular dynamics in granulosa cells of periovulatory follicles and granulosa-derived (large) lutein cells of sheep: relationships to the steroidogenic folliculo-luteal shift and functional luteolysis.** *Biol Reprod* 1996, **54:**1135-1140.
71. Niswender GD, Juengel JL, Silva PJ, Rollyson MK and McIntush EW: **Mechanisms controlling the function and life span of the corpus luteum.** *Physiol Rev* 2000, **80:**1-29.