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Localization and temporal regulation of tissue inhibitor of metalloproteinases-4 in mouse ovary

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Abstract

Tissue inhibitors of metalloproteinases (TIMPs) are potential regulators of tissue remodeling in the ovary. The aim of the present study was to examine the localization and temporal regulation of TIMP-4 protein in the mouse ovary. An induced superovulation model (eCG/hCG) was employed in immature mice to evaluate TIMP-4 protein expression profiles in ovaries collected during the follicular phase, the pre ovulatory period, and the luteal lifespan. Immunofluorescence results indicated that TIMP-4 protein was localized to theca of both antral and preovulatory follicles and adjacent ovarian stroma. After the initiation of luteinization with hCG, TIMP-4 was observed within the luteinizing granulosa cells and persisted throughout the lifespan of the corpus luteum. In the cycling ovary, TIMP-4 signaling localized to corpus luteum from previous estrous cycles, the theca of preovulatory follicles, and appeared to be lower in newly forming corpus luteum. Western analysis further showed that the levels of TIMP-4 increased significantly during the luteinization process of granulosa cells, but no significant change was found among all corpus luteum stages. A putative regulatory mechanism of TIMP-4 expression was identified utilizing an in vitro model. Treatment of cultured granulosa cells with hCG significantly augmented TIMP-4 protein expression levels. Together our data indicate that the luteinization process of granulosa cells is associated with up-regulation of TIMP-4 and that TIMP-4 might play an essential role in maintenance of the luteal function during the whole lifespan of corpus luteum.

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Introduction

Mammalian ovaries consist of follicles as basic functional units (Hirshfield 1991). The ovarian follicle undergoes extensive remodeling during its growth and development, as well as after ovulation and corpus luteum (CL) formation (Murphy 2000). The CL develops by extensive cellular reorganization and neovascularization of the remnants of the evacuated follicle following ovulation (Murphy 2000). In the mouse, the lifespan of the CL is either several days (estrous cycle) or about 3 weeks (pregnant). Both the cycling CL and pregnant CL are with a temporal change including the formation, maintain and regression (Greenwald and Rothchild, 1968). Associated with the repetitive cycles of luteal development and regression is extensive connective tissue remodeling. These remodeling events require the participation of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Liu *et al.* 1999, Smith *et al.* 1999, Curry & Osteen 2001, 2003, Bakke *et al.* 2002, Ricke *et al.* 2002, Young *et al.* 2002, Liu *et al.* 2003, Zhang *et al.* 2003, Young & Stouffer 2004).

Currently, four distinct TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified and characterized based on their molecular weight, biological activity, or cDNA cloning (Gomez *et al.* 1997, Brew *et al.* 2000). The primary recognized function of TIMP is the ability to inhibit the active forms of MMPs (Brew *et al.* 2000). In addition to their classical role as MMPs inhibitors, TIMPs have other non-classical actions including stimulation of cell growth (Hayakawa *et al.* 1992, Murphy *et al.* 1993), impact of angiogenesis (Johnson *et al.* 1994), inducement of apoptosis (Bond *et al.* 2002, Guo *et al.* 2004), and regulation of ovarian steroidogenesis (Boujrad *et al.* 1995, Nothnick 2000). All of these physiological actions are important for overall luteal function (Machell and Farookhi 2003).

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TIMP-4, the newest family member, was identified in 1997 by Leco *et al.* and has been demonstrated to be present in ovaries of mice (Rahkonen *et al.* 2002), rats (Simpson *et al.* 2003), horse (Riley *et al.* 2001), bovine (Li *et al.* 2004) and humans (Robinson *et al.* 2001). Despite the characterization of the gene in mammalian ovary, the regulatory mechanisms of TIMP-4 protein expression as well as its function remain ambiguous. The initial objective of this research was to characterize the spatial and temporal expression pattern of TIMP-4 in the mouse ovary during induced ovulation and CL, and in the naturally cycling adult mouse. Subsequent studies addressed the regulation of TIMP-4 protein expression in cultured granulosa cells.

Materials and Methods

Major chemicals

Rabbit Anti-TIMP-4 was obtained from Santa Cruz (SC-9375, USA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Animal treatment

The Guidelines for the Care and Use of Animals in Research were followed. Synchronized folliculogenesis was initiated in prepubertal (21-day-old) Kunming white strain mice (purchased from the Experimental Animal Center, Institute of Zoology, Chinese Academy of Sciences, PR China) by administration of 5 IU eCG followed 48 h later by 5 IU hCG to induce ovulation and pseudopregnancy. In this model, ovulation occurred approximately 12–16 h post-hCG administration. Ovaries were collected during follicular development (0, 24, and 48 h after eCG), and over the peri-ovulatory period (12, 24, and 48 h after hCG; $n=3$–6 animals/time point). To evaluate TIMP-4 expression in the CL of pseudopregnancy, we collected ovaries ($n=3$ animals/time point) representative of luteal function (peak progesterone production; 4 and 8 d after hCG) and after functional luteal regression (12 d after hCG) as previously established (Chang *et al.* 2004). Expression of ovarian TIMP-4 across the estrous cycle was evaluated in mature animals exhibiting normal cycle (>3 consecutive cycles as determined by vaginal lavage); ovaries were collected and snap frozen at 1 000 h on the day of estrus, metestrus, diestrus, and proestrus ($n=3$–6 animals/time point). One ovary from each animal was mounted in optimal cutting temperature (OCT) compound for localization studies; the second ovary was used to isolate nonluteinized granulosa cells, luteinizing cells, or CL under the microscope.

Indirect immunofluorescence

Four ovaries from four mice per time point were evaluated and three sections per ovary were used for indirect immunofluoresence. Frozen ovarian sections (10 μm) were fixed in 4% paraformaldehyde solution and blocked with 5% bovine serum albumin (BSA) before incubation (4 °C overnight) with goat Anti-TIMP-4 (1:200). Then the sections were incubated in FITC-conjugated secondary antibody at a dilution of 1:100 in PBS for 1 h at 37 °C. Nuclei were stained with 0.01 mg/ml propidium iodide (PI) for 10 min and viewed under a laser scanning confocal microscope (Leica, Heidelberg, Germany). For negative control, parallel experiments were performed with sections using preimmune goat serum.

Isolation of nonluteinized granulosa and luteinizing granulosa cells

Granulosa cells were collected by needle puncture from the ovaries of immature mice treated 48 h prior with 5 IU equine chorionic gonadotropin (eCG) to initiate follicular growth. Oocytes were excluded from collection by mesh filtration (70 μm). The pooled, PMSG-primed granulosa cells were plated into an Eppendorf tube in serum-free DMEM/F12 media containing 3% BSA, 2 mM L-GLN, 5 μg/ml insulin-transferrin-sodium selenite (ITS). After isolation, cells were washed three times with PBS.

Generally, mouse granulosa cells become luteal cells between 48 and 96 h after hCG injection. So the luteinizing granulosa cells were harvested at 72 h post-hCG injection as the references (Hampl *et al.* 2000). Undamaged, easily recognizable corpora lutea at 72 h post-hCG injection were microdissected according to their morphology using 27-gauge needles under the microscope and then transferred into an Eppendorf tube. Immediately after their isolation, tissues were washed three times with PBS, and mechanically disintegrated and lysed in ice-cold lysis buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM sodium chloride, 1% triton X-100, 1 mM EDTA. The following protease inhibitors were included: PMSF (100 μM), leupeptin (1 μg/ml), aprotinin (1 μg/ml), Soybean trypsin inhibitor (10 μg/ml), and tosylphenylalanine chloromethane (10 μg/ml). After 30 min of extraction on ice, lysates were cleared by centrifugation at 15 000×g for 20 min at 4 °C, and the concentrations of total protein in supernatants were determined using the Bradford assay. Extracts were equalized for total protein and then used for Western blot analysis.

Total RNA extraction and RT-PCR

Total RNA was extracted using Trizol Reagents (Invitrogen, Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA was dissolved in 20 μl nuclease-free water. RT-PCR was

performed according to a coupled one-step procedure using Access RT-PCR System (Promega). Briefly, 2 μg of total RNA was reverse transcribed at 42 °C for 1 h, denatured at 94 °C for 2 min, and amplified for 33 (beta-actin) or 33 (TIMP-4) cycles of denaturation at 94 °C of 30 s, primer annealing at 56 °C for 30 s, and extension at 72 °C for 45 s, with a final extension step of 10 min at 72 °C. The amplified products were analyzed by electrophoresis on 1.5% agarose gels and cloned, sequenced and utilized for the expression purpose. The primers used for RT-PCR of the TIMP-4 and β-actin genes with the accession number and their amplified segments were listed in Table 1.

Western blot analysis

Proteins obtained from nonluteinized granulosa cells, luteinizing granulosa cells or CL were quantified using the Bradford assay. About 20 μg protein from each sample were loaded onto 12.5% polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were first incubated for 2 h in PBS–Tween (PBST) containing 5% low-fat milk. The blocked membranes were then incubated overnight at 4 °C with antibody diluted 1:100 in PBST containing 5% low-fat milk. After incubation, the membranes were incubated with Alkaline phosphatase–conjugated Rabbit Anti-Goat IgG at 37 °C for 1 h. The blot was detected using the NBT-BCIP detection system according to the instructions of the manufacturer. After incubation with TIMP-4 antibody, membranes were stripped using Genotech Re-Probe buffer and then reprobed with the actin antibody. The intensities of TIMP-4 bands were corrected by comparison of corresponding β-actin levels.

Granulosa cell culture

The pooled, eCG-primed granulosa cells were plated into 6 cm culture dishes (1×10^6 cells/ml) in Myos5A media (with 2 mM L-GLN) with treatment or control vehicle and incubated for 12, 24, or 48 h at 37 °C, 5% CO_2. After treatment with 1 IU/ml hCG or control media ($n=4$ dishes/treatment), cells were collected by scraping and centrifugation. Total protein was collected for analysis by Western blot as described above.

Progesterone assay

Samples (conditioned granulosa cell medium) were diluted as necessary and assayed by sequential competitive immunoassay according to the manufacturer's instructions.

Statistical analysis

Values were presented as mean ± S.E.M. The data were analyzed using one-way ANOVA as appropriate. P values <0.05 were considered statistically significant.

Results

Localization of TIMP-4 protein to periovulatory follicles and to corpora lutea of mouse ovaries

During follicular growth (0 h post-eCG–0 h post-hCG), extensive immunolabeling was apparent in thecal and stromal compartments, but was absent from the granulosa cells (Fig. 1A). After hCG injection, a notable increase in labeling intensity was observed in newly formed CL (24–48 h post-hCG; Fig. 1A). Specificity of immunoreactivity was verified by the use of preimmune serum instead of primary antibody, which resulted in the absence of reaction product (Fig. 1A).

Expression of TIMP-4 protein persisted in luteal tissue through the functional lifespan of the CL (4 and 8 d post-hCG; Fig. 1B), and was readily detectable in CL after functional regression (12 d post-hCG; Fig. 1B). The intensity of TIMP-4 protein was also present in stroma, thecal tissue, and luteinized follicles. Immunostaining of tissue sections with the preimmune serum resulted in little or no background immunofluoresence in luteal cells or any other cells including stroma (Fig. 1B), indicating that the staining observed was specific for TIMP-4 protein.

To confirm that the expression profile we characterized in the induced-ovulation model was representative of TIMP-4 expression during normal (i.e. unstimulated) ovarian function, we evaluated TIMP-4 expression in ovaries collected daily over the 4-day estrous cycle from mature, naturally cycling mice. Results of our analysis demonstrated corresponding patterns of TIMP-4 expression between the induced and naturally ovulating models. TIMP-4 protein was localized to the thecal cells

Table 1 Primers used for determinations of the mouse TIMP-4 and β-actin mRNA by RT-PCR.

Gene product	Primer sequence	Genbank Acc. No.	Region amplified
TIMP-4	F: 5′-TACACGCCATTTGACTCTT-3′ R: 5′-TGGTTCCTGGTCCCTACT-3′	gi31981527	Nt. 350–761
β-actin	F: 5′-GGCCCAGAGCAAGAGAGGTATCC-3; R: 5′-ACGCACGATTTCCCTCTCAGC-3′	gi6671508	Nt. 251–710

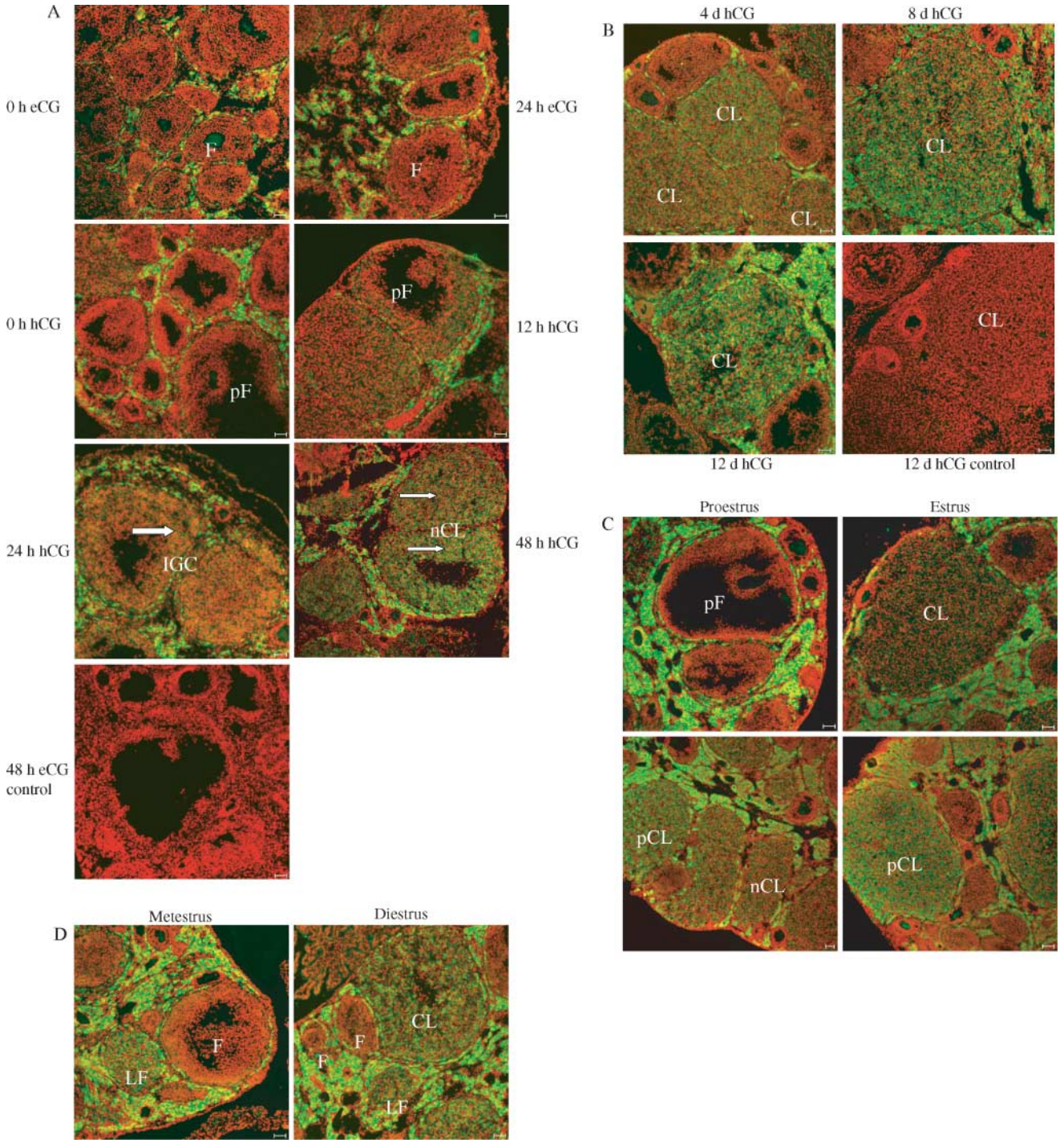

Figure 1 Indirect immunofluoresence analysis of TIMP-4 protein expression in the mouse ovary using rabbit Anti-TIMP-4 antibody. Green represents TIMP-4 staining and red indicates nuclear staining. Yellow coloration represents an overlap of green and red. A: TIMP-4 expression during induced follicular growth and ovulation. During follicular growth (0 h post-eCG–0 h post-hCG), extensive immunolabeling was apparent in thecal and stromal compartments, but was absent from the granulosa cells. After hCG injection, a notable increase in labeling intensity was observed in newly formed CL (24–48 h post-hCG). B: Luteal expression of TIMP-4 during induced pseudopregnancy. During the whole pseudopregnancy, the green immunofluoresence for TIMP-4 protein was not only present in the CL and sroma, but also localized to the theca cells in the adjacent follicles. C: Expression of TIMP-4 protein in ovaries of naturally cycling mature mice. A strong level of TIMP-4 protein was detected in the thecal layers and the adjacent ovarian stroma at proestrus. At estrus, faint staining was detected in the newly formed CL. Whereas at diestrus and metestrus, strong signal was detected in the previous CL and faint signal was present in the newly formed CL. D: TIMP-4 protein expression in normal vs. luteinized follicles. The normal follicles expressed high levels of TIMP-4 protein in the theca, whereas the luteinized follicles expressed high levels of TIMP-4 protein throughout. GC, granulosa cells; TC, theca cells; nCL: new corpus luteum; pF: preovulatory follicles; S: stroma. Bar = 50 μm.

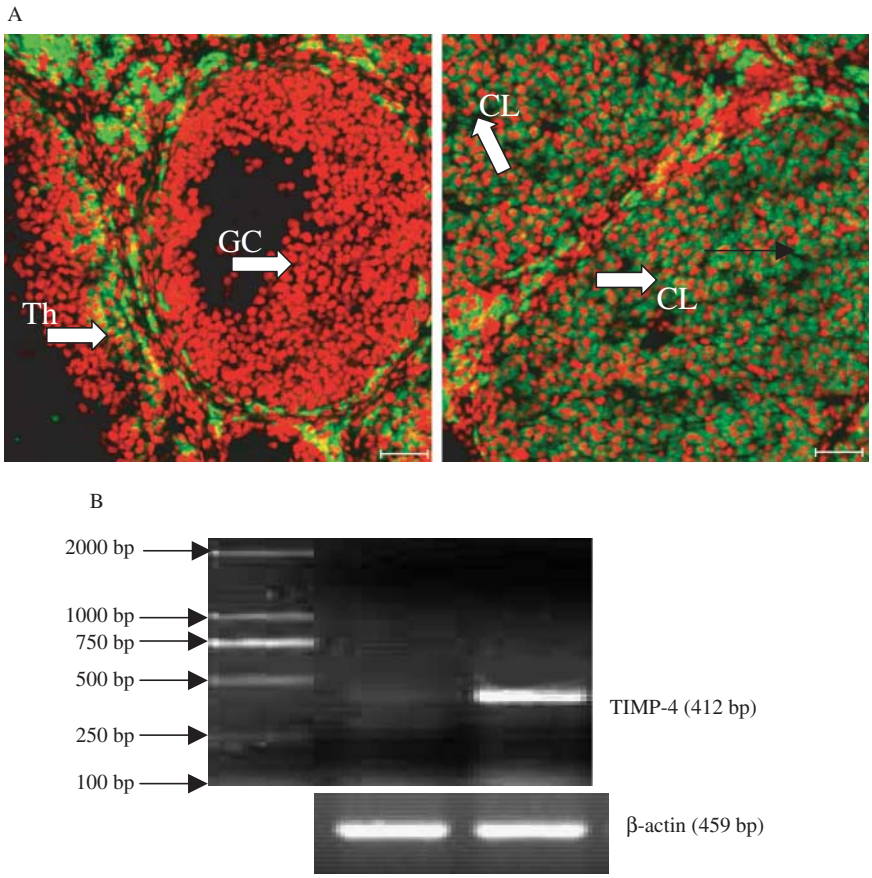

Figure 2 Comparison of TIMP-4 expression in granulosa cells and luteinizing granulosa cells. Green represents TIMP-4 staining and red indicates nuclear staining. Yellow coloration represents an overlap of green and red. A: Indirect immunofluoresence analysis of TIMP-4 expression in ovaries collected at 46–48 h after eCG injection and 72 h after hCG injection. A strong level of green immunofluoresence was present in the luteinizing cells including the granulosa cell derived (black arrowheads), but was not present in the nonluteinized granulosa cells. GC, granulosa cells; CL, corpus luteum; Th, theca cells. Bar= 50 μm. B: RT-PCR analysis of TIMP-4 mRNA expression in nonluteinized granulosa cells (collected at 46–48 h after eCG injection) and luteinizing cells (collected at 72 h after hCG injection). A PCR product of the expected size was not produced from the nonluteinized granulosa cells, but produced from the luteinizing cells.

of preovulatory follicles, CL, and stroma on the whole estrous cycle (Fig. 1C).

Luteinized follicles are a later stage in the regression of follicles. The small follicles identified as luteinized follicles are based upon their lack of apoptotic cells, their lack of a granulosa cell basement membrane, and their cellular morphology. In luteinized follicles, the granulosa cells and oocyte have become atretic, regressed, and been removed, whereas the cells of the thecal layer have hypertrophied and become more luteal-like (Guraya & Greenwald 1964). The difference between a normal follicle and a luteinized follicle and their expression of TIMP-4 protein can be seen in Fig. 1D. The normal follicles expressed high levels of TIMP-4 protein in the theca, whereas the luteinized follicles expressed high levels of TIMP-4 protein throughout.

Comparative expression of TIMP-4 in the nonluteinized granulosa cells and luteinizing granulosa cells

When the ovarian tissue sections were examined by indirect immunofluorescence it was found that the nonluteinized granulosa cells (48h post-eCG) contained a low to undetectable levels of TIMP-4 protein (Fig. 2A); Whereas the luteinizing granulosa cells (72h post-hCG) had high levels of TIMP-4 protein (Fig. 2A). To elucidate whether TIMP-4 mRNA was being expressed in the nonluteinized granulosa cells, granulosa cells were isolated from preovulatory follicles and the RNA from the cells amplified by RT-PCR. A PCR product of the expected size was not produced from the nonluteinized granulosa cells, but produced from the luteinizing cells (Fig. 2B).

The change of TIMP-4 protein in the corpus luteum stage

In order to examine the change of TIMP-4 protein in all stages of CL, we collected nonluteinized granulosa cells (day 0), luteinizing granulosa cells (day 3 post-hCG) and CL (days 8, 12, and 16 post-hCG) from the whole ovaries. Western analysis demonstrated that the levels of TIMP-4 protein increased significantly during the luteinization process of granulosa cells, but no significant change was found among all CL stages (Fig. 3).

TIMP-4 in granulosa cells luteinized in vitro

The elevated levels of TIMP-4 protein in luteinizing granulosa cells prompted us to test its regulation by

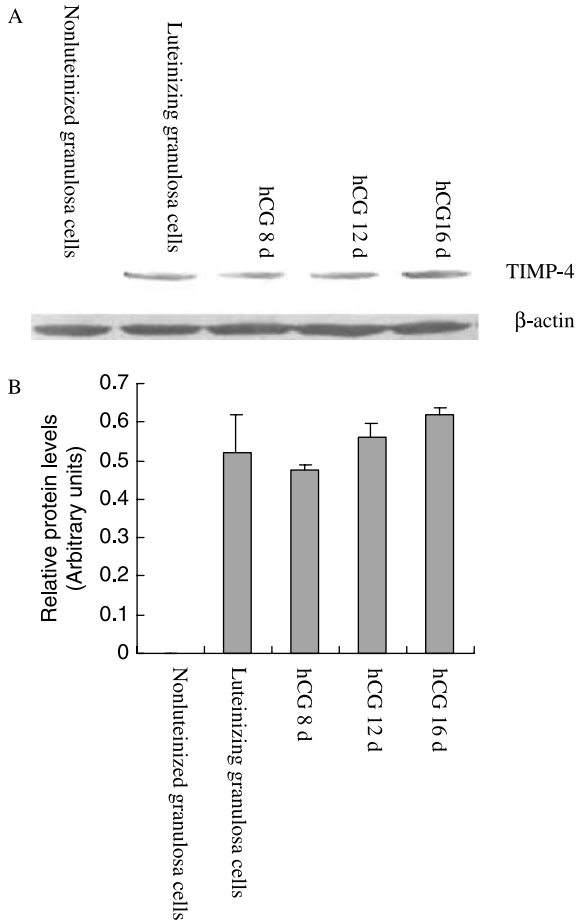

Figure 3 Western blot analysis of TIMP-4 protein expression in nonluteinized granulosa cells (collected at 46–48 h after eCG injection), luteinizing cells (collected at 72 h after hCG injection), fully luteinized cells of corpora lutea of pseudopregnancy (collected at days 8, 12, and 16 after hCG injection). An antibody against β-actin was used in the same Western blot as a loading control. A: Representative immunoblots from analysis of TIMP-4 and β-actin expression. B: Densitometric analysis of the shown blots about TIMP-4. Results are shown as mean±S.E.M. of three replicates. ADU (arbitrary densitometric unit) was defined as percentage of target protein densitometric value compared with β-actin. Statistical analysis was performed using the Student t-test (*$P<0.05$). Values on luteinizing granulosa cells to days 16 were significantly ($P<0.05$) increased compared with nonluteinized granulosa cells. Values on day 8 to day 16, no significant differences compared with luteinizing granulosa cells ($P>0.05$).

a luteotropic hormone, hCG. Progesterone production by cultured granulosa cells was induced by hCG as measured in conditioned media at 6, 12, 24, and 48 h. Cells cultured with hCG showed a significant increase in progesterone production compared with cells cultured without hCG. This difference was seen after 6 h in culture and persisted over the entire 2-day culture period (Fig. 4).

Addition of 1 IU/ml hCG to the culture media for 24 h stimulated the expression of TIMP-4 protein compared to control cells (Fig. 5). TIMP-4 protein expression was significantly elevated in hCG-stimulated cells by 24 h of

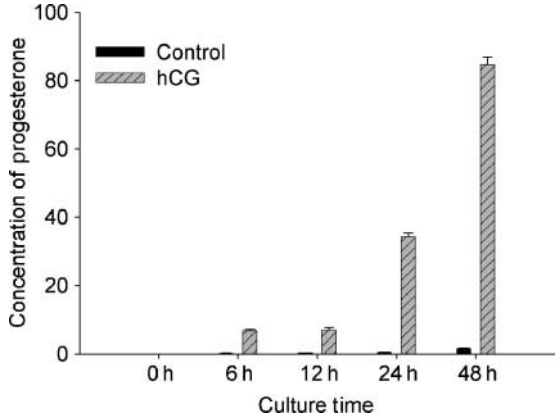

Figure 4 Progesterone production *in vitro* by luteinizing granulosa cells. Granulosa cells, isolated from equine chorionic gonadotropin (eCG)-treated prepubertal mice, were cultured in either the absence or presence of 1 IU hCG for 48 h. Progesterone production was assessed each 24 h by measurement of the progesterone concentration in the media. Dissimilar superscripts denote significant differences between treatments (control vs hCG) within each time point as well as differences over time within a treatment ($P<0.05$; $n=4$).

culture, but not in control cells until 48 h ($P<0.05$). By 48 h of culture, control and treated cells expressed approximately equivalent levels of TIMP-4 protein (Fig. 5).

Discussion

The current study clearly demonstrates that the luteinization process of granulosa cells is associated with

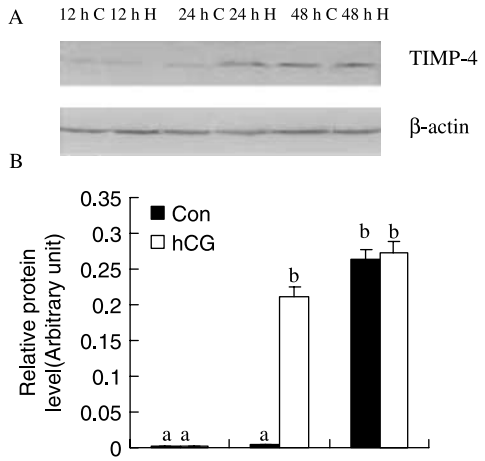

Figure 5 TIMP-4 is expressed in vitro by luteinizing mouse granulosa cells. A: Representative immunoblots from analysis of TIMP-4 and β-actin expression. Control: C; hCG: H; B: Densitometric analysis of the shown blots about TIMP-4. Results are shown as mean±S.E.M. of three replicates. TIMP-4 protein expression of granulosa cells cultured in vitro without (black bars) and with (white bars) hCG treatment. ADU (arbitrary densitometric unit) was defined as percentage of target protein densitometric value compared with β-actin. Statistical analysis was performed using the Student t-test (*$P<0.05$). Dissimilar superscripts denote significant differences between treatments (control vs hCG) within each time point as well as differences over time within a treatment ($P<0.05$; $n=4$).

up-regulation of TIMP-4 and TIMP-4 protein is present throughout the lifespan of the CL in both the induced model of ovulation and naturally cycling model. These findings support the postulate that TIMP-4 might play an important role in ovarian physiology during the establishment and/or maintenance of the differentiated CL phenotype.

Because follicular development, ovulation, and CL formation and regression are associated with extensive tissue remodeling, the mammalian ovary provides an excellent model for the study of developmental and regressive events in the adult organism (Greenward & Rothchild 1968). Many studies have indicated that MMPs and their inhibitors are important regulators in the tissue remodeling of the above-mentioned process (Hagglund *et al.* 1999, Nothnick 2000, Simpson *et al.* 2003, Goldman & Shalev 2004, Li *et al.* 2004). Unlike its counterparts TIMP-1, -2, -3, the localization and temporal regulation of TIMP-4 in rodent ovary have so far not been investigated in detail (Curry & Wheeler 2002). In the present study, TIMP-4 protein was expressed in thecal and stromal compartments of the ovary, with the background levels of expression in granulosa cells of antral or preovulatory follicles during follicular growth (0 h post-eCG to 0 h post-hCG), a period of significant structural reorganization. After ovulation, TIMP-4 protein expression was localized to newly forming CL (48 h post-hCG), possibly implicating a role for this inhibitor in the dynamics of luteinization (e.g. cell differentiation, extracellular matrix remodeling, and angiogenesis).

Simpson and colleagues were unable to block the antibody with TIMP-4 peptide and, thus, could not ascertain whether the localization of TIMP-4 protein was similar to that of its mRNA in rat ovary (Simpson *et al.* 2003). Our present study extended their results and indicated that TIMP-4 protein expression in mouse ovary exhibited a similar localization pattern of its mRNA in the cycling ovary of rats. For example, both TIMP-4 mRNA and protein were detected in the theca of follicles in the proestrus and in the CL of previous cycles. The similarity between the TIMP-4 protein expression in mouse in the current study and previous reports in the rat suggests a conserved function for TIMP-4 in rodent ovary.

The current findings of the localization of TIMP-4 protein in the mouse follicles were in contrast to the localization of TIMP-4 protein in the horse and bovine (Riley *et al.* 2001, Li *et al.* 2004). For example, TIMP-4 protein was found in the granulosa cells of bovine and horse preovulatory follicles, but it did not present in the granulosa cells of mouse preovulatory follicles (present study). The disparate results highlight the species variation.

Investigation of TIMP-4 protein localization in the present study showed that TIMP-4 protein expression in the cycling ovary exhibited a similar pattern of follicular and stromal expression as reported in PMSG/hCG-treated prepubertal mice. In the cycling mice, TIMP-4 protein was detected in the theca of follicles and in CL, but also present in stroma and luteinized follicles. In the PMSG/hCG primed mice, as the CL is forming, there are fewer luteal cells present. Thus, the quantitative levels of TIMP-4 expression are lower in this period (day 2) in the present study. By day 3, the CL is fully formed, has increased in size, and TIMP-4 is expressed throughout the CL, resulting in an overall increase in the levels of TIMP-4. Both the appearance of TIMP-4 in the CL throughout pseudopregnancy and the finding that the CL is the predominant cellular source of this inhibitor support the concept that TIMP-4 is turned on the healthy preovulatory follicles that become CL. The role of TIMP-4 in this process of transition, however, is unknown. An attractive hypothesis is that the expression of this matrix metalloproteinase inhibitor throughout the CL may act to protect the CL from proteolytic degradation. Moreover, a role of TIMP-4 in luteal cells is supported by the finding that its expression in these cells is up-regulated by the luteotropic hormone, hCG.

Although TIMP-4 has generally been described as the inhibitor of MMPs, recent studies provide evidence for some other novel function. Tummaapalli and colleagues reported that TIMP-4 controlled normal cardiac fibroblast transformation and induced apoptosis in transformed cells (Tummalapalli *et al.* 2001). Further, TIMP-4 is believed to play an important role in regulating angiogenesis; expression of TIMP-4 increases following vascular injury, and TIMP-4 has the ability to reduce the migration of vascular smooth muscle cells (Dollery *et al.* 1999). All of these reported actions of TIMP-4 in other tissues may have a physiological foundation for controlling ECM remodeling during the formation, maintenance and regression of CL, the exact role for TIMP-4 in mouse ovary remains further study.

One of the intriguing findings in this study was the high level of TIMP-4 protein expression in small luteinized follicles. Luteinized follicles, which are the thecal remnants of follicles that have undergone atresia, make up a large part of the interstitial tissue (Guraya & Greenwald 1964). Thus these structures may influence the functions or characteristics of other ovarian structures. Although no TIMP-4 protein was detected in the granulosa cells of atretic follicles, it was highly expressed in luteinized follicles. The high level of TIMP-4 protein points to a role for this gene in the differentiation of luteinized follicles. As luteinized follicles are steroidogenic (Bukovsky *et al.* 1993), establishment of a role of TIMP-4 in steroidogenesis that is independent of regulation of ECM remodeling will require further investigation.

In conclusion, TIMP-4 protein expression was characterized within the mouse ovary for the first time. TIMP-4 protein increased during the luteinization process of granulosa cells. The up-regulated levels of TIMP-4 might be necessary for maintaining the fully differentiated phenotype of luteal cells in vivo. Further studies are needed to determine the exact role of TIMP-4 in CL function.

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