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Expression of Gelatinases and Their Tissue Inhibitors in Rat Corpus Luteum During Pregnancy and Postpartum

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ABSTRACT Extensive tissue remodeling occurs in the corpus luteum (CL) during both formation and luteolysis. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are believed to play pivotal roles in these processes. In the present study, to evaluate the potential roles of matrix degrading proteases in luteal development and regression, we examined gelatinases and TIMP-1, -2, -3 mRNA expressions, as well as gelatinase activity in rat CL during pregnancy and postpartum using Northern blot, in situ hybridization, and gelatin zymography, respectively. The results showed that MMP-2 mRNA was only expressed at the early stages of pregnancy; TIMP-2 mRNA was highly expressed at the early and late pregnancy and day 1 postpartum, but could not be detected during the mid-phase of pregnancy; TIMP-3 mRNA expression was abundant during early pregnancy and peaked at day 7, but was absent from other time points examined. MMP-9 and TIMP-1 mRNAs in rat CL were below detectable level in the current study. Furthermore, the active MMP-2 was only present during the early stages of pregnancy, and no MMP-9 activity was observed in the zymogram. Taken together, our results suggest that MMP-2 and TIMP-3 may have functional roles in rat luteal formation, while TIMP-2 may be implicated in both formation and regression of the pregnant CL. *Mol. Reprod. Dev. 63: 273–281, 2002.* © 2002 Wiley-Liss, Inc.

Key Words: matrix metalloproteinases; Northern blot; in situ hybridization; zymography; luteolysis

INTRODUCTION

Normal ovarian function depends on cyclical remodeling of the extracellular matrix (ECM) which is implicated in the processes of follicular development and atresia, ovulation, and the development, maintenance and regression of corpora lutea (Hulboy et al., 1997; Smith et al., 1999). The corpus luteum (CL) is a dynamic organ within the ovary and undergoes dramatic structural changes and tissue remodeling during its life span (Liu et al., 1999). At present, the molecular mechanisms whereby CL is rescued and regressed during pregnancy remain to be addressed. Recently, there are experimental data indicating that ECM components enhance luteinization, whereas loss of ECM results in luteal cell death (Smith et al., 1999). Matrix metalloproteinases (MMPs) or matrixins, and their tissue inhibitors known as TIMPs, are postulated to be responsible for the remarkable remodeling of the ECM, and therefore they are postulated to play key roles in both formation and regression of the CL (Fata et al., 2000). The MMPs are a multigene family of zinc-dependent proteinases that degrade compounds of the ECM. Over 20 of structurally related members of MMPs have been identified so far based on their substrate specificity, amino acid sequence, and cellular localization (Brenner et al., 1996; Rudolph-Owen et al., 1998; Park et al., 2000). Among the identified MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have attracted much more attention for their prominent roles in ovarian processes (Curry et al., 2001).

MMPs are secreted as latent zymogens and require activation to exhibit catalytical activity to cleave their substrates (Nagase, 1997; Murphy et al., 1999). The activity of MMPs is tightly controlled by TIMPs, of which four have been described to date (Stetler-Stevenson et al., 1989; Apte et al., 1994; Greene et al., 1996; Bigg et al., 1997; Gomez et al., 1997; Brew et al., 2000). The generally accepted roles of TIMPs are confined to their inhibition of MMPs (Brew et al., 2000). Recent studies have suggested additional roles for TIMPs including pro-MMP activation, steroidogenesis, and cell growth activity (Goldberg et al., 1989; Stetler-Stevenson et al., 1992; Boujrad et al., 1995).

Although investigators have proposed important roles for matrix degrading proteases and their tissue inhibitors in luteal function, most research on the role of MMPs/TIMPs in luteal formation and regression has

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been conducted by utilizing gonadotropin primed animals so far (Nothnick et al., 1995; Duncan et al., 1996b; Liu et al., 1999; Curry et al., 2001). Since the pseudopregnant animal model does not always parallel and reflect the situation of natural pregnancy, the changes of MMPs/TIMPs in the CL during pseudopregnancy may not correlate well with those during natural gestation. Moreover, detailed information about the expression and localization of gelatinases and TIMPs in rat CL during pregnancy and postpartum has not been available to date. Therefore, in the current study, to evaluate the potential roles of matrix degrading proteases in luteal development and regression, we focused on the expression and localization of gelatinases and TIMP-1, -2, -3 mRNAs, as well as the variation of gelatinases activities in rat CL during pregnancy and postpartum.

MATERIALS AND METHODS

Animals and Tissue Recovery

Adult male and female Sprague–Dawley rats were obtained from the Experimental Animal Center of Institute of Zoology, Chinese Academy of Sciences. The rats were housed under controlled conditions, with free access to water and food. Illumination was between 0700 and 1900 hr. Experimental protocols were approved by the ethical committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Female rats were mated overnight. Day 1 (D1) of pregnancy was designated the morning (between 0800 and 0900 hr) on which spermatozoa were found in a vaginal smear under microscopic examination. To confirm early pregnancy, rats were killed on day 1–5 and blastocysts were recovered from the reproductive tract. Rats were killed at various stages (D1, D3, D7, D9, D18, and D22) of pregnancy and day 1 postpartum (P1) ($n = 9$ for each time point). At each time point, six ovaries from three rats ($n = 3$) were imbedded in OCT (Triangle Biochemical Sciences, Durham, NC) and stored at -80°C for in situ hybridization of MMP-2, -9 and TIMP-1, -2, -3 mRNA expressions. Corpora lutea (CLs) were dissected from the remaining ovaries ($n = 6$) under stereoscopic microscope as previously described (Telleria et al., 1998), and the CLs from every two rats were pooled and frozen at -80°C until processed for total RNA extraction and protein preparation, followed by Northern blot and gelatin zymography analysis, respectively.

cRNA Probe Labeling

Plasmids (pBluescript) containing the cDNA fragments encoding rat MMP-2, -9 and TIMP-1, -2, -3 were kindly provided by Dr. Akiko Okada (Institute de Genetique et de Biologie Moleculaire et Cellulaire, Université Louis Pasteur, France). The riboprobes were generated from the linearized plasmids using the following restriction enzymes and RNA polymerases (Promega Corp., Madison, WI): MMP-2, *Hin*d III with T3 RNA polymerase (antisense), and *Xba* I with T7 RNA polymerase (sense); MMP-9, *Hin*d III with T3 polymerase (antisense), and *Not* I with T7 RNA polymerase (sense); TIMP-1 and TIMP-2, *Hin*d III with T3 RNA polymerase (antisense), and *Bam*H I with T7 RNA polymerase (sense); and TIMP-3, *Xho* I with T7 RNA polymerase (antisense), and *Bam*H I with T3 RNA polymerase (sense). The probes used for Northern blot and in situ hybridization were labeled with a digoxigenin (Dig) RNA labeling kit (Roche Molecular Biochemicals, Mennheim, Germany) according to the manufacturer's instructions. The probe labeling efficiency was confirmed by dot assay, and the purified probes were stored in DEPC-treated water at -80°C.

Northern Blot

Total RNA from rat CLs was isolated using Trizol reagent (Gibco BRL Life Technologies, Inc., Rockville, MD) and the concentration was determined by absorption at 260 nm. Equivalent RNA (20 μg) was denatured, electrophoresed in a 1.0% formaldehyde-agarose gel and transferred to a positively charged nylon membrane (Roche Molecular Biochemicals) by capillary action in $20 \times$ SSC. Then the transferred RNA was fixed onto the membranes by baking at 80°C for 2 hr. Membranes were prehybridized for 2 hr in 10 ml of prehybridization buffer [50% formamide, $5 \times$ SSC, 2% Blocking reagent (Roche Molecular Biochemicals), 0.02% SDS] at 66°C. Dig-labeled riboprobes of gelatinases or TIMPs were then added to the prehybridization buffer. After hybridization at 66°C for 20 h, the membranes were washed at room temperature (RT) in $2 \times$ SSC/0.1% SDS for 30 min, and twice in $0.1 \times$ SSC/0.1% SDS (15 min/wash). The blots were visualized in NBT/BCIP reagents (Roche Molecular Biochemicals). To ensure the loading accuracy of RNA, Dig-labeled probes of actin beta (a gift from Dr. Jian-Guo Qi, Institute of Virology, Chinese Academy of Medical Sciences) was included in the hybridization system as control since it does not change in rat CL during pregnancy (Townson et al., 1996).

In Situ Hybridization

In situ hybridization was performed according to the method recently developed by Braissant and Wahli (1998) with slight modifications. Frozen sections (10 μm) on poly-L-lysine-coated slides were quickly thawed and fixed in 4% PFA (Sigma Chemical Co., St. Louis, MO) in DEPC-treated PBS for 15 min at RT. The slides were washed 2×15 min in PBS containing 0.1% active DEPC before equilibration in $5 \times$ SSC (DEPC-treated) for 15 min. Prehybridization was conducted at 52°C for 3 hr in a buffer containing 50% formamide, $5 \times$ SSC, and 120 μg/ml sDNA. Slides were then hybridized with 400 ng/ml of Dig-labeled probe in prehybridization buffer overnight at 52°C in a moist chamber. The sections were serially washed in $2 \times$ SSC at RT for 30 min, $2 \times$ SSC at 65°C for 1 hr, $0.1 \times$ SSC at 65°C for 1 hr. Slides were rinsed in buffer A (100 mM Tris, 150 mM NaCl, pH 7.5) and then incubated for 2 hr with anti-digoxigenin-alkaline phosphatase antibody (1:3000, Roche Molecular Biochemicals) in buffer B (buffer A with 0.5% Roche Blocking reagent). The slides were

further washed in buffer A for 2×15 min before equilibration in Buffer C (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. The hybridization signals were visualized with NBT/BCIP reagents. In each experiment, the sense probes were also included to ensure the specificity of hybridization. The results were recorded with SPOT digital camera system (Diagnostic Instruments, Inc., Michigan).

Gelatin Zymography

Protein extraction procedure was provided with the Trizol reagent (Wang et al., 2001). The protein concentration was measured according to the method of Bradford (1976). CLs extracts (40 μg of protein in sample buffer) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel containing 0.5 mg/ml of gelatin under nonreducing conditions. After two washes in Tris buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, pH 7.5), the gel was incubated in the same buffer in the absence of Triton X-100 for 18 hr at 37°C. After stained with Coomassie Brilliant Blue R-250, the gel was destained with 10% (v/v) acetic acid, and the nonstaining bands resulting from digestion of the substrate by gelatinase enzymes were then visualized.

Statistical Analysis

The intensities of the Northern blots were normalized to those of actin beta in corresponding samples (intensity of MMP-2, TIMP-2 and -3 mRNAs divided by that of respective actin beta mRNA $\times$ 100). All values are presented as mean $\pm$ SEM. The mRNA signals and gelatinase activity in the respective Northern blot and zymography experiments were semi-quantified by Meta View image analyzing system (version 4.50, Universal Imaging Corp., Pennsylvania). Statistical comparisons among groups were analyzed by one-way ANOVA followed by Student's *t*-test using SPSS software package (version 10.0.1, SPSS, Inc., Chicago, IL). A value of $P < 0.05$ was considered significant.

RESULTS

Detection of MMP-2, -9 and TIMP-1, -2, -3 mRNAs in Rat CL by Northern Blot Analysis

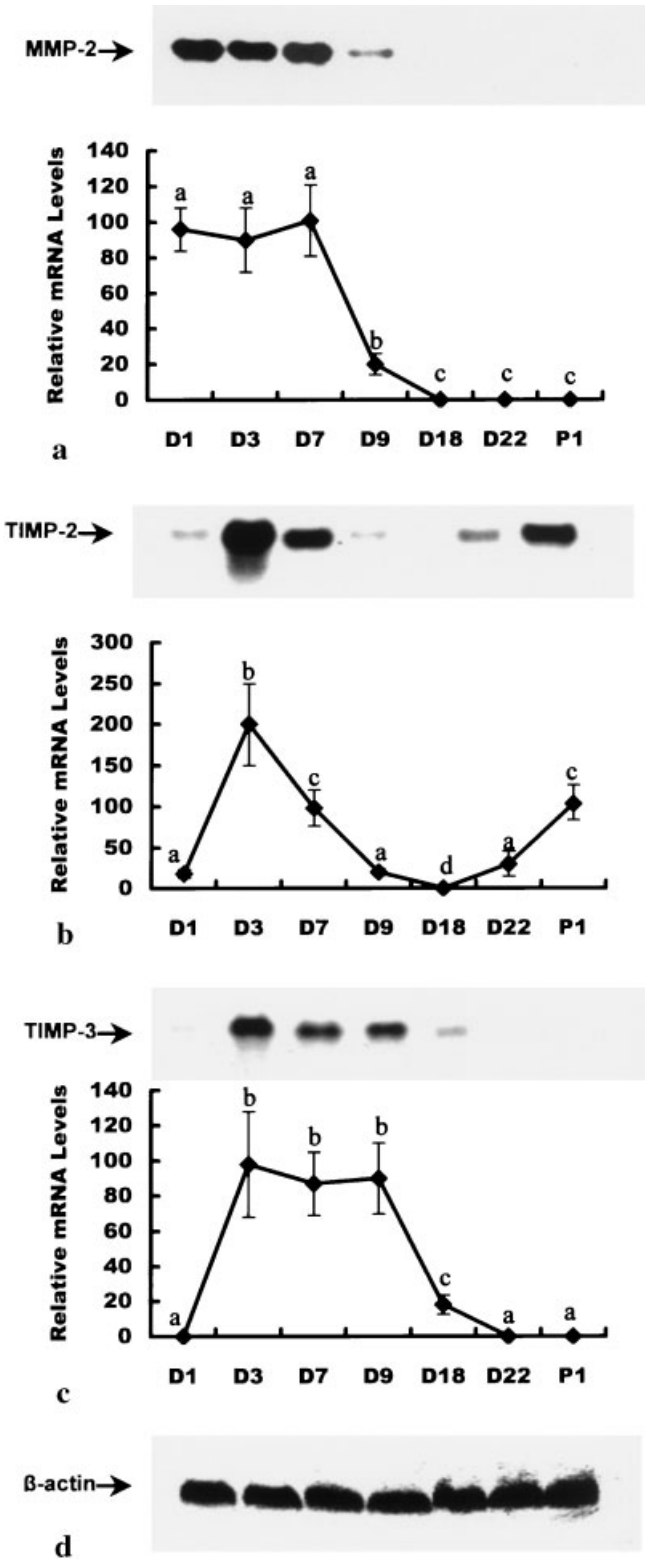
Northern blot was used to explore the expression patterns of MMP-2, -9, and TIMP-1, -2, -3 mRNAs in rat CL at different time points of pregnancy and day 1 postpartum when obvious structural luteal regression takes place. MMP-2 and TIMP-2, -3 mRNAs with respective molecular sizes of 3.1, 1.0, and 4.5 kb were detected in rat CL (Fig. 1a–c), whereas MMP-9 and TIMP-1 mRNAs were undetectable in rat CL (data not shown) at all the examined time points. Furthermore, the intensities of the hybridization bands of MMP-2, TIMP-2, -3 mRNAs were normalized to those of actin beta (Fig. 1d), and then subjected to semi-quantitative analysis for the variation. The results revealed that MMP-2 mRNA was abundantly expressed at day 1, 3, and 7, but was remarkably decreased at day 9 ($P < 0.05$), and was undetectable at day 18, 22, and 1 postpartum (Fig. 1a). TIMP-2 mRNA was low but detectable at day 1 of pregnancy, increased substantially at day 3 and 7 ($P < 0.05$). The staining dramatically declined at day 9 and disappeared at day 18, but occurred at day 22 again and was elevated significantly at day 1 postpartum ($P < 0.05$, Fig. 1b), coinciding with structural luteolysis. Expression of TIMP-3 mRNA was faint at day 1 of pregnancy, but significantly increased ($P < 0.05$) and remained high at day 3, 7, and 9. The staining signal markedly declined at day 18 ($P < 0.05$) and was absent from the CL thereafter (Fig. 1c). MMP-2 and TIMP-3 mRNA expression during early stages of pregnancy may hint their possible roles in luteal development, while TIMP-2 mRNA expression at the early and late gestation, as well as day 1 postpartum implies that it may be implicated in both formation and regression of the pregnant CL.

In Situ Localization of MMP-2, -9 and TIMP-1, -2, -3 mRNAs in the CL of Pregnant and Postpartum Rats

In order to get a more detailed picture of the expression of MMP-2, -9, and TIMP-1, -2, -3 mRNAs at cellular level, in situ hybridization was performed using the whole rat ovary at different time points of pregnancy and day 1 postpartum. The results were summarized in Figures 2 and 3. MMP-2 mRNA was localized in rat CL at early pregnancy as illustrated in Figure 2a,b. CLs from day 18 and day 1 postpartum did not exhibit positive hybridization signals (Fig. 2c,d). In accordance with the results of Northern blot, MMP-9 mRNA was not expressed in rat CL at the various time points examined, although positive signals were visualized in the granulosa cells of some ovarian follicles (Fig. 2f–i). Figure 2e,j were representative control sections hybridized with the respective MMP-2 and MMP-9 sense probes. It is interesting to note that TIMP-1 mRNA was specifically and constitutively localized to the encircling stroma of rat CL, as well as the granulosa cells and/or theca cells of some ovarian follicles, but not in CL per se (Fig. 3a–d). In contrast to TIMP-1 mRNA expression, TIMP-2 mRNA was evenly distributed in the CLs on day 3, 7, and 1 postpartum (Fig. 3f,g,i), with no discernible expression on day 18 (Fig. 3h). Expression of TIMP-3 mRNA displayed a punctate pattern in rat CLs at day 3, 7, and 18 (Fig. 3k–m), and hybridization signal was almost undetectable the day following delivery (Fig. 3n). Sense RNA probes for TIMP-1, -2, -3 did not produce specific hybridization signals (Fig. 3e, j,o).

Gelatinolytic Activity in Rat CL During Pregnancy and Postpartum

To further characterize the gelatinolytic activity in rat CL throughout the pregnancy and day 1 postpartum, total proteins from the CLs were extracted and subjected to gelatin zymography analysis. A representative zymogram is depicted in Figure 4. Two clear bands of proteins migrating at a relative molecular mass of 72 kDa and 66 kDa of gelatin activity were identified in rat CL.



The 72- and 66-kDa bands are consistent with the previously reported pro-MMP-2 and active MMP-2, respectively (Woessner, 1991). The pro-MMP-2 was defined at day 1, 3, 7, 9, 18, and 22, whereas the active form of gelatinase A was only present at day 1, 3, 7, and 9. No bands of pro-MMP-2 or active MMP-2 were observed at day 1 postpartum. To better understand the variation of MMP-2 activity at the examined time points, the intensity of each band on the zymogram was analyzed using Metaview image analyzing system. The data demonstrated no significant differences among day 1, 3, and 7 of pregnancy, however, the gelatinolytic activity markedly declined at day 9 of pregnancy ($P < 0.01$). The active gelatinase A activity observed in the zymogram at day 1, 3 and 7 suggests that MMP-2 may participate in rat luteal formation during early pregnancy. No MMP-9 activity was observed in zymogram, implying the functional gelatinase in pregnant rat CL is MMP-2, rather than MMP-9.

DISCUSSION

Recently, MMPs and TIMPs which are key enzymes in the degradation of ECM components are shown to be implicated in luteal formation and regression in mouse (Waterhouse et al., 1993), rat (Bagavandoss, 1998; Liu et al., 1999; Simpson et al., 2001), sow (Pitzel et al., 2000), sheep (McIntush et al., 1996), cattle (Goldberg et al., 1996), and human (Bogusiewicz et al., 2000; Duncan, 2000). This study was undertaken to further investigate the expression of MMP-2, -9, and TIMP-1, -2, -3 mRNAs, as well as gelatinase activity in the CL of pregnant and postpartum rats.

The present observation that MMP-2 mRNA was only expressed during early pregnancy in rat CL instead of late pregnancy and day 1 postpartum corresponds, in part, with the previous finding in pseudopregnant rat model (Liu et al., 1999). Moreover, the relatively high intensity of MMP-2 mRNA declined after day 7 of pregnancy. Hence, it is suggested that MMP-2 may be involved in luteal formation during early gestation. MMP-2 and -9 are synthesized as latent proenzymes and must be activated in order to exhibit their proteolytic activities (Murphy et al., 1999). To further evaluate gelatinolytic activity in pregnant rat CL, we performed gelatin zymography analysis, which mainly detects the gelatinase activity. The gelatinolytic activity of pro-MMP-2 was found throughout pregnancy, whereas active MMP-2 was only observed at early pregnancy, and its activity decreased abruptly at day 9, which

Fig. 1. Representative Northern blot analysis of MMP-2 and TIMP-2, -3 mRNA expressions in rat CL at different time points of pregnancy and day 1 postpartum. **a**–**c**: They show the representative Northern blot and relative abundance of MMP-2, TIMP-2 and TIMP-3 mRNAs, respectively, as calculated by correcting for the relative transcription levels of actin beta mRNA (**d**) in each individual sample. Values are mean ± SEM from three experiments (two animals for each time point/experiment). D1-P1, represent the time point of pregnancy and postpartum in the corresponding lanes of Northern blot. Bars with different letters on the top are significantly different ($P < 0.05$).

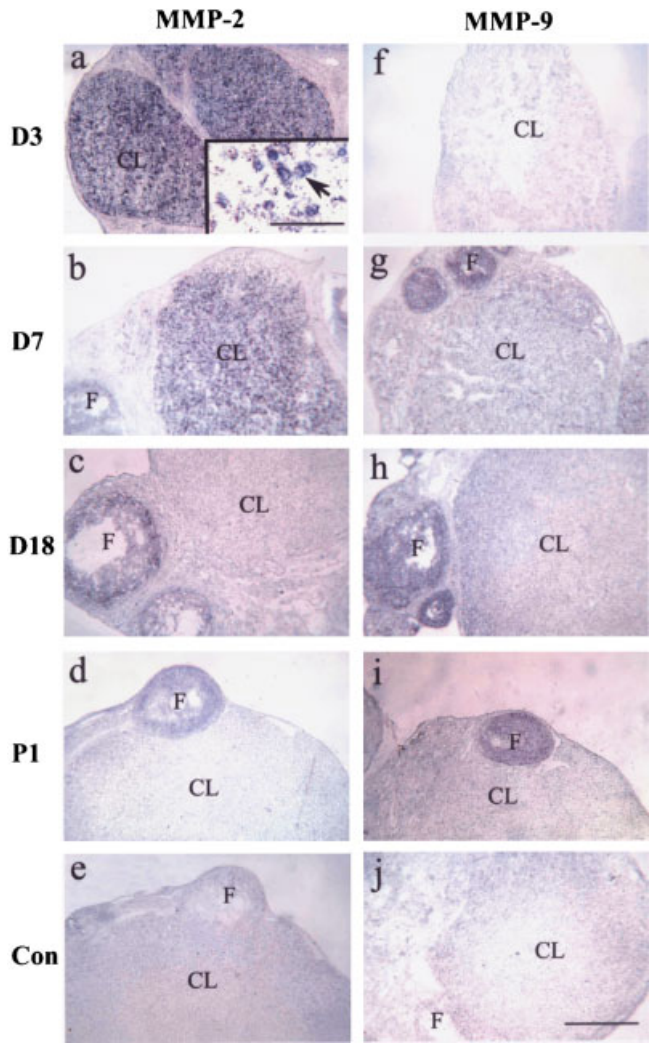


Fig. 2. In situ hybridization of MMP-2, -9 mRNAs in rat CL at various stages of pregnancy and day 1 postpartum. Representative sections in the first and second vertical rows were hybridized with MMP-2 and MMP-9 antisense cRNA probes, respectively. Representative sections in the first, second, third, and fourth horizontal rows were from day 3, 7, 18 of pregnancy and day 1 postpartum, respectively. The fifth horizontal row represents control sections hybridized with sense probes. MMP-2 mRNA was localized in rat corpus luteum (CL) at day 3 and 7 of pregnancy, whereas CLs from day 18 and day 1 postpartum did not produce positive hybridization signals. MMP-9 mRNA was not expressed in rat CL at all the stages examined, although positive hybridization signals were visualized in the granulosa cells of some ovarian follicles (F). Inset in (**a**) shows a higher magnification of the positively stained cells in rat CL from D3. The arrow indicates positive hybridization signals. D3, 7, 18, and P1 indicate day 3, 7, 18 of pregnancy and day 1 postpartum, respectively; Con, control. The experiments were repeated at least three times with similar results. All pictures except the inset are of identical magnification as indicated in (**j**), scale bar = 200 μm; inset, scare bar = 50 μm.

indicates a role of MMP-2 in luteal formation. Recent studies by Duncan et al. (1998) demonstrated that exposure to hCG during luteal rescue in vivo might cause a reduction in the expression and activity of MMP-2 in human CL. In consideration of the role of MMPs in extracellular matrix degradation, we therefore hypothesize that the down-regulation of gelatinase A activity in rat CL at mid pregnancy may contribute to the ECM stabilization of CL, thus maintaining the luteal structure and function. The absence of MMP-9 mRNA and enzyme activity in the pregnant CL implies that gelatinase B is not functional in pregnant rat CL.

The activities of MMPs are partially regulated by TIMPs. The balance between active MMPs and TIMPs is important in maintaining the ECM microenvironment conducive to the differentiation of follicular-derived cells into luteal cells (Smith et al., 1999), and perturbation in these gene families may impact the biology of ovary (Fata et al., 2000). TIMPs differ in their enzyme specificities and patterns of action (Leco et al., 1994, 1997; Gomez et al., 1997). They were shown to be produced by the CL in a variety of species and the expression patterns vary from species (Waterhouse et al., 1993; Nothnick et al., 1995; Duncan et al., 1996a,b; Goldberg et al., 1996; McIntush et al., 1996; Liu et al., 1999; Pitzel et al., 2000). The normally high level of TIMP-1 mRNA seen in the ovaries of virgin mice was low during gestation, and the level was not elevated until day 18 and postpartum (Waterhouse et al., 1993). In contrast, TIMP-1 mRNA expression in pseudopregnant rat CL was highest at day 1, decreased abruptly from day 2 to 12, and up-regulated again at day 14–16 (Nothnick et al., 1995). In primate CL, TIMP-1 was reported to be constitutively synthesized throughout the luteal phases (Duncan et al., 1996a,b). However, unique to the current study was the observation that TIMP-1 mRNA was specifically localized to the stroma encircling CL and some ovarian follicles, rather than the CL. The significance of the constitutive expression of TIMP-1 mRNA encircling the CL shown by in situ hybridization remains unclear. It should be noted that at late pregnancy and day 1 postpartum, no gelatinases mRNAs or active enzymes could be detected, hence, it is reasonable to propose other MMP(s) are responsible for the ECM remodeling implicated in luteal regression. This is supported by the observation that both collagenase-3 (MMP-13) and the membrane type MMP-1 (MMP-14) are localized in rat CL during luteal regression (Liu et al., 1999). Thus, it is tempting to speculate that TIMP-1 may serve as an in vivo "house-keeper" inhibitor of MMPs encircling the CL during pregnancy and postpartum. Unlike TIMP-1, TIMP-2 mRNA mainly existed in rat CL at early pregnancy, and was down-regulated during mid-pregnancy, but increased again at late pregnancy and day 1 postpartum. During rat gestation, progesterone production declines by day 22 of pregnancy (functional regression of the CL), but the weight of the pregnant CL does not fall until after parturition (structural regression of the CL), and continues to decline over numerous estrous cycles following parturition (Roughton et al., 1999). Therefore, the data suggest a likely role of TIMP-2 in the tissue remodeling during luteal formation and regression. Our finding is incontrast with that of Nothnick et al. (1995) who showed in pseudopregnant rat model that TIMP-2 was

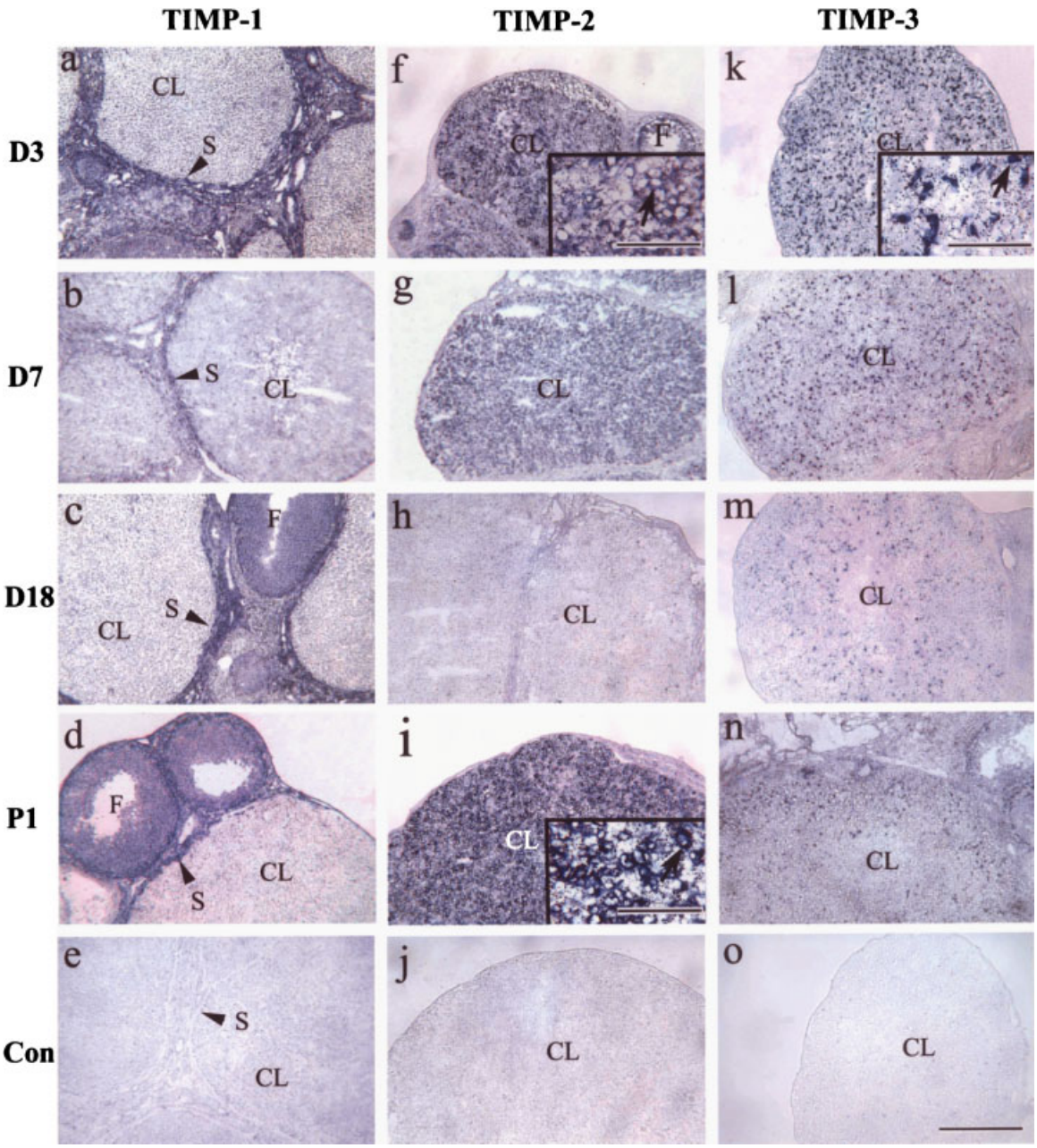


Fig. 3. In situ hybridization of TIMP-1, -2, -3 mRNAs in rat CL at various stages of pregnancy and day 1 postpartum. Representative sections in the first, second, and third vertical rows were hybridized with TIMP-1, -2, and -3 antisense cRNA probes, respectively. Representative sections in the first, second, third, and fourth horizontal rows were from day 3, 7, 18 of pregnancy and day 1 postpartum, respectively. The fifth horizontal row represents control sections hybridized with sense probes. Expression of TIMP-1 mRNA was detected in the encircling stroma (S) of rat corpus luteum (CL), as well as the grannulosa cells and/or theca cells of some ovarian follicles (F). TIMP-2 hybridization signals were evenly distributed in rat CL on day 3, 7, and day 1 postpartum, with no expression at day 18. TIMP-3 mRNA expression demonstrated a punctate pattern in rat CL at day 3, 7, and 18, and was almost undetectable at day 1 postpartum. Insets in (**f**), (**i**), and (**k**) show higher magnifications of the positively stained cells in the corresponding figures. Arrows indicate the positive hybridization signals. D3, 7, 18, and P1 indicate days 3, 7, 18 of pregnancy and day 1 postpartum, respectively; Con, control. The experiments were repeated at least three times with similar results. All pictures except the insets are of identical magnification as indicated in (**o**), scale bar = 200 μm; inset, scare bar = 50 μm.

constitutively expressed and might not have functional roles in CL formation and regression. Interestingly, the transcript size for TIMP-2 was 1.0 kb and 3.5 kb in rat ovary during estrous cycle, however, Northern blot for TIMP-2 resulted in a single band of 1.0 kb in rat CL during pregnancy and postpartum under the current investigation. Other researchers (Duncan et al., 1998) also demonstrated that TIMP-2 mRNA transcript was detected as a single band in human CL after luteal "rescue" compared to the normal human tissue

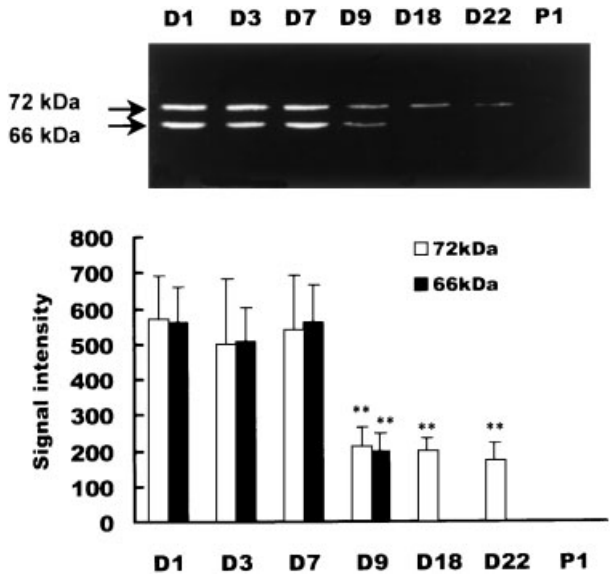


Fig. 4. Representative gelatin zymogram of rat CL extracts from different stages of pregnancy and day 1 postpartum. The bands are bright against a dark background, and the molecular size of each band in kiloDaltons is indicated on the left. D1-P1, represent the time point of pregnancy and postpartum. The 72-kDa band was visualized at day 1, 3, 7, 9, 18, and 22, while the 66-kDa band was only detected at day 1, 3, 7, and 9. No gelatinase activity was detected at day 1 postpartum. The variations of gelatinase activity in the zymogram were analyzed by Meta View image analyzing system. Data are mean ± SEM from three experiments (two animals for each time point/experiment). Compared with day 1, $^{**}P < 0.01$.

(Stetler-Stevenson et al., 1990). Thus, it seems that TIMP-2 mRNA may be selectively transcribed at different physiological status.

The discrepancy of MMP-2 and TIMP-1, -2 mRNA expressions between pseudopregnant and pregnant rat is not clear. It is likely due to the adoption of different animal models. Many differences exist between pseudopregnancy and pregnancy. For example, although the serum progesterone concentration in rat during early pregnancy and pseudopregnancy is similar, it declines markedly at day 11 of pseudopregnancy, while remains relative high at day 20 of pregnancy (Pepe and Rothchild, 1974). Recently, a link between hormones and MMPs/TIMPs was demonstrated (Iwamasa et al., 1992; Salamonsen and Woolley, 1996; Chaffin and Stouffer, 1999; Luo and Liao, 2001), and Chaffin and Stouffer (1999) further showed that the action of progesterone, and possibly other steroids, was to regulate the expression of ovarian proteases and their inhibitors including MMPs/TIMs during ovulation and luteinization of the primate follicle. In the human CL, MMP-2 was down-regulated after administration of exogenous hCG to mimic the hormonal changes of early pregnancy (Duncan et al., 1998). Taken together, the different expression patterns of gelatinases and TIMPs between pseudopregnant and pregnant rat may be, in part, associated with the differences of hormone level, time span and status of luteal development, maintenance and regression. High level of TIMP-3 mRNA transcripts were detected during early pregnancy, but the hybridization signal became weak during mid-pregnancy, and diminished to an undetectable level at late pregnancy and day 1 postpartum. The finding is consistent with a recent research in the rat by Curry et al. (2001) who demonstrated that luteal cells of the newly forming CL induced by hCG administration expressed TIMP-3 mRNA, but in contrast to that of Duncan et al. (1998) in human, and our previous investigation in rhesus monkey (Li et al., 2001), which showed TIMP-3 mRNA was not expressed in human and macaque CL. Collectively, the co-expression of MMP-2 and TIMP-2, -3 mRNAs at day 1, 3, 7, and 9 points to their possible roles in tissue remolding during rat luteal development. In addition, several additional functions including activation of pro-MMP, and regulation of cell proliferation and differentiation, angiogenesis, steroidogenesis, and apoptosis have been attributed to certain members of TIMP family (Goldberg et al., 1989; Stetler-Stevenson et al., 1992; Yang and Hawkes, 1992; Boujrad et al., 1995; Chesler et al., 1995; Gomez et al., 1997; Bond et al., 2000). Ovarian TIMPs were also proposed to have some of the above roles during follicular growth, ovulation, and luteal formation (Curry et al., 2001). Since development of the CL is associated with extensive tissue remodeling as well as neovascularization, and cell proliferation and differentiation (Liu et al., 1999), other roles (e.g., regulation of cell proliferation and differentiation) besides ECM degradation may be assumed for TIMP-2 and -3 in pregnant rat CL during luteal formation.

In summary, we have shown for the first time, to our knowledge, the distinct expression patterns of MMP-2 and TIMP-2, -3 mRNAs, as well as gelatinolytic activity in rat CL during pregnancy and postpartum. Our findings suggest that MMP-2, and TIMP-2, -3 under study may have regulatory roles in the pregnant rat CL, and provide support to the hypothesis that MMPs/TIMPs may be involved in the formation or destruction of ovarian CL, and the expression patterns may vary among species. Further investigations involving the construction and study of specific gene-deficient animal model perhaps will illuminate the functional roles of MMPs and TIMPs in CL.

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