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Matrix Metalloproteinase Expression in the Rat Myometrium During Pregnancy, Term Labor, and Postpartum[1]

Tina Tu-Thu Ngoc Nguyen,[3,4] Oksana Shynlova,[2,3,5] and Stephen J. Lye[3,4,5]

[3]*Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada*
[4]*Department of Physiology, University of Toronto, Canada*
[5]*Department of Obstetrics and Gynecology, University of Toronto, Canada*

ABSTRACT

Pregnancy, spontaneous term labor (TL), and postpartum (PP) involution are associated with changes in the cellular and extracellular matrix composition of the uterus. Both the uterine smooth muscle (myometrium) and the infiltrating peripheral blood leukocytes involved in the activation of labor secrete extracellular matrix-degrading enzymes (matrix metalloproteinases, MMPs) that can modulate cellular behavior and barrier function. MMP expression is induced by mechanical stretch in several tissues. We hypothesized that the expression and activity of myometrial MMPs and their tissue inhibitors (TIMPs) are modulated in preparation for TL and PP involution and are regulated by mechanical stretch of uterine walls imposed by the growing fetus. Myometrial tissues were collected from bilaterally and unilaterally pregnant rats across gestation, TL, and PP. Total RNA and proteins were subjected to real-time PCR and immunoblotting, respectively, and tissue localization and activity was examined by immunohistochemistry and in situ zymography. We found that *Mmp7, Mmp11,* and *Mmp12* mRNA levels were upregulated during TL and PP, while *Mmp2, Mmp3, Mmp8, Mmp9, Mmp10,* and *Mmp13* mRNAs were only upregulated during PP. *Timp1–Timp4* were stably expressed throughout gestation with some fluctuations PP. Active MMP2 was induced in the empty uterine horn during gestation and in the gravid PP uterus, suggesting negative regulation by biological mechanical stretch. We conclude that specific subsets of uterine MMPs are differentially regulated in the rat myometrium in preparation for two major events: TL and PP uterine involution.

extracellular matrix, gene expression, myometrium, parturition, pregnancy

INTRODUCTION

It is well known that uterine smooth muscle cells (SMCs) undergo sequential steps of hyperplasia, hypertrophy, and extracellular matrix (ECM) elaboration in order to adapt to the physiological demands of pregnancy [1–3]. These processes are regulated by both endocrine and mechanical factors [1–3] and are accompanied by extensive remodeling of the uterine ECM [4, 5]. For example, we have previously reported a significant prelabor decrease in the expression of fibrillar collagens (types I and III) and coordinated increases in fibronectin and basement membrane (BM) proteins (laminin and collagen IV) in the rat uterine muscle (myometrium). We found that these in vivo changes in ECM expression are mediated by the ovarian hormone progesterone and by the biological mechanical stretch of the uterus by the growing fetus(es) [2, 5]. We further suggested that phenotypic modulation of myometrial SMCs, focal adhesion proteins, and the biosynthesis of ECM provide cell-matrix stability for forceful labor contractions [6].

ECM biogenesis is accompanied by well-controlled biodegradation by specialized enzymes known as matrix metalloproteinases (MMPs). MMPs are zinc-containing endopeptidases categorized by substrate preference or the organization of their domain constituents, such as collagenases (MMP1, 8 and 13), gelatinases (MMP2 and 9), stromelysins (MMP3, 10, and 11), matrilysin (MMP7), and metalloelastase (MMP12). The expression and activity of MMPs is physiologically controlled by a group of endogenous proteins known as tissue inhibitors of metalloproteinases (TIMPs) that form noncovalent 1:1 complexes with MMPs to either inhibit or promote their enzymatic activity [7]. It is recognized that MMP transcriptional activation can be regulated by several factors, including matrix-integrin receptor signaling, growth factors, and cytokines [8–10]. Typically, MMPs are not expressed by healthy tissues, but they are detected in all diseased, inflamed, or injured tissues [11, 12]. Once activated, MMPs primarily degrade matrix components to modulate the extracellular environment but can also generate chemoattractive peptides and release biological molecules bound to ECM components (i.e., growth factors and cytokines) [13–15].

Several studies investigating the phenotypes of MMP-null mice have concluded that MMPs regulate numerous physiological processes facilitating pregnancy and labor, including ovulation, implantation, cervical ripening, and rupture of fetal membranes [16–18]. MMPs can also promote cellular migration by increasing the permeability between cell-cell junctions [19]. It is well documented that leukocytes utilize both MMPs and TIMPs to infiltrate different compartments of the pregnant uterus near term (myometrium, decidua, and cervix) where they release cytokines and chemokines to produce a sterile inflammatory environment and initiate labor [20–22]. Furthermore, postpartum (PP) uterine involution is also known to be accompanied by extensive ECM remodeling and increased bioavailability of multiple cytokines, chemokines, and growth factors, all modulated by MMP activity [23, 24].

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[2]Correspondence: Oksana Shynlova, Lunenfeld-Tanenbaum Research Institute at Mount Sinai Hospital, 25 Orde Street, Suite 6-1019, Toronto, Ontario, Canada M5G 1X5. E-mail: shynlova@lunenfeld.ca

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The exact role of specific myometrial MMPs during normal gestation and term labor (TL) has not been well characterized. To date, studies of the potential role of MMPs within the pregnant myometrium are limited by both the number of MMPs and the uterine gestational tissues studied [10, 25, 26]. MMPs may play a direct role in labor initiation because increased MMP expression is associated with preterm labor (PTL), and injection of a broad-spectrum MMP inhibitor GM6001 was able to reduce the rate of PTL in mice [27, 28]. Moreover, the role of mechanical stretch in the regulation of MMP was also demonstrated by increased gelatinase expression and activity in stretched myometrium strips isolated from virgin rats [25].

In the current study we aim to fully characterize a gestational profile of MMP expression and activity within the rat myometrium during pregnancy, TL, and PP. Based on the continuous remodeling of the ECM proteins throughout pregnancy, as well as increased inflammation observed toward TL, we hypothesize that MMPs and TIMPs may play a role in the preparation for labor contractions and PP uterine involution. Using two pregnant rat models, we aim to 1) fully characterize the in vivo myometrial expression profile of major secreted MMPs and TIMPs during pregnancy, TL, and PP; 2) investigate whether there is an increase in MMP expression and/or activity associated with TL and PP involution within the myometrium; and 3) investigate if gravidity is one of the putative mechanisms that regulate their expression and/or activity within the myometrium.

MATERIALS AND METHODS

Animal Models

Bilateral pregnancy. We investigated the in vivo myometrial mRNA expression profile of MMPs and TIMPs during pregnancy, at TL, and PP using the rat. Virgin female Wistar rats (12–15 wk, 225–250 g weight) and male Wistar rats (250–300 g weight) were purchased from Charles River Laboratories and housed in the MaRS Toronto Medical Discovery Tower Animal Research Facility. The research ethics board approved these animal studies (AUP no. 2379). Rats were maintained on standard rat chow and water in a 12L:12D cycle. Male (one per cage) and female rats (one per cage) were housed separately and monogamous pairs were only mated overnight. The following morning, male and female rats were separated, and the day when a vaginal plug was detected was considered Day 1 of gestation. Animals were euthanized by carbon dioxide inhalation, and bilaterally pregnant uterine samples were collected on the morning of Gestational Day (GD) 6, 8, 10, 12, 14, 15, 17, 19, 21, 22, 23 (TL), 1 day PP (1PP), and 4PP (n = 6). Labor samples (GD23) were collected during active TL following the delivery of at least one pup.

Unilateral pregnancy. Prior to mating, rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn (gravid horn) according to the method described in detail by Shynlova et al. [5]. Four sets of uterine tissues from the gravid and empty horns were collected separately on GD 6, 12, 15, 19, 21, 23 (TL), and 1PP (n = 4). Additionally, under general anesthesia, unilaterally pregnant rats on GD 19, 20, and 21, underwent insertion of an expandable dried seaweed stem (laminaria, 2-mm width) (MedGyn Products Inc.) into the nongravid uterine horn through midline abdominal incision. The laminaria tube swells as it absorbs moisture (in situ for 12 h to 6–8 mm) and stretches the nongravid uterine horn in both length and diameter by approximately 3-fold to become comparable in size to the gravid horn; the method is described in detail by Shynlova et al. [29]. Rats were euthanized 24 h following tubal insertion, and myometrial tissue was collected (n = 3).

Tissue Collection and Preparation

Animals were sacrificed on specific gestational days; placentae and pups, fat, and connective tissue were carefully removed from the uteri. The myometrium was dissected away from the decidua basalis, scraped on ice to remove the luminal epithelium, and flash frozen in liquid nitrogen. The frozen myometrium was homogenized (TissueLyser II; Qiagen Inc.) to isolate total RNA (RNeasy Universal Mini kit; Qiagen Inc.) and protein using a bicine lysis buffer (25 mM bicine, 150 mM NaCl, pH 7.6). For histological analyses, whole uteri were fixed in 10% neutral-buffered formalin (VWR International), 4% paraformaldehyde (Electron Microscopy Sciences) in phosphate-buffered saline (PBS), or zinc-buffered fixative (100 mM Tris buffer pH 7.4, 0.63 mM calcium acetate, 27.3 mM zinc acetate, 36.7 mM zinc chloride) [30] for 24 h, processed, and then embedded in paraffin wax.

Real-Time Quantitative PCR

Total myometrial RNA was reverse transcribed (iScript supermix; Bio-Rad Laboratories Ltd.) to cDNA and subjected to real-time-quantitative PCR analysis (CFX384 system; Bio-Rad Laboratories Ltd.) of specific primers designed using NCBI Primer Blast software and produced by Eurofins Genomics (Eurofins MWG Operon LLC) (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). Following real-time-quantitative PCR, a dissociation curve was constructed by increasing the temperature from 65°C to 95°C for detection of PCR product specificity. A cycle threshold (Ct) value was recorded for each sample. The Ct value is defined as the number of amplification cycles required to detect a fluorescent signal (SYBR green) that exceeds threshold level. Ct values are inversely proportional to mRNA levels within each sample. PCR reactions were set up in technical triplicates, and the mean of the three Ct values was calculated by the software (CFX Manager, Bio-Rad). A comparative Ct method (ΔΔCt method) was applied to the raw Ct values to find relative gene expression. The mRNA levels of 10 MMPs, that is, collagenases (*Mmp1, Mmp8,* and *Mmp13*), gelatinases (*Mmp2* and *9*), stromelysins (*Mmp3, Mmp10,* and *Mmp11*), matrilysin (*Mmp7*), and metalloelastase (*Mmp12*), as well as *Timp1–Timp4* were detected using SYBR green (Sigma) and normalized to the geometric mean of three housekeeping genes: peptidylprolyl isomerase A (*Ppia*), platelet derived growth factor alpha (*Pdgfa*), and TATA box binding protein (*Tbp*) (Supplemental Table S1). For unilaterally pregnant animals, MMP and TIMP gene expressions were normalized to *Ppia, Pdgfa,* and insulin-like growth factor 1 receptor (*Igf1r*) [3]. The chosen housekeeping genes were adequately expressed and did not display significant changes throughout gestation (Supplemental Figs. S1 and S2). Results were displayed as fold change relative to GD6 (bilaterally pregnant rats) or to GD6 in the empty horn (unilaterally pregnant rats). In addition, a no-template control (RNase-free water instead of cDNA) was analyzed for possible contamination in the master mix.

Protein Isolation, Gelatin Zymography, and Western Blot Analysis

Total protein was extracted from myometrial tissue using a bicine lysis buffer (25 mM bicine, 150 mM NaCl, pH 7.6). The homogenate was centrifuged at 14 000 rpm for 15 min at 4°C. The supernatant was collected and protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). Total protein was denatured in NuPAGE LDS 4× Novex Sample Buffer (Invitrogen, Thermo Fisher Scientific Inc.) with 10% β-merceptoethanol solution (Sigma-Aldrich) at 95°C. Thirty micrograms of protein per well was used for Western blot analysis of MMP8 (1:500) while 70 μg of protein was used to analyze MMP7 (1:200), MMP3 and MMP11 (1:500), and MMP12 (1:1000) (Supplemental Table S2). Protein expression levels were normalized to the housekeeping protein calponin (1:1000). Fifty micrograms of each myometrial sample was used to detect protein activity by gelatin zymography using precast Novex 12% tris-glycine gelatin gels (Invitrogen).

Immunohistochemistry

Formalin-fixed (10%) or paraformaldehyde-fixed (4%) paraffin-embedded rat uteri were sectioned at 5 μm thickness, placed on glass slides (Fisher Scientific), baked at 37°C overnight, deparaffinized in xylene, rehydrated in 100%, 95%, 80%, and 70% ethanol baths for 5 min each, and quenched with 3% H_2O_2 in methanol for 20 min. For MMP7 and MMP11, heat-induced antigen retrieval was performed using a Target Retrieval Solution (pH 9; DAKO) with 0.01% Triton X-100 (Sigma). For MMP3 and MMP12, heat-induced antigen retrieval was performed using a 10 mM sodium citrate solution (pH 6). Slides were then blocked in protein blocking solution (DAKO) and incubated with primary antibody for MMP3, MMP7, MMP11, or MMP12 (all 1:200) at 4°C overnight (Supplemental Table S2). Rabbit immunoglobulin G (IgG) (1:200) was used as the negative control at the same concentration as the primary antibodies. Sections were washed and incubated with a biotinylated secondary anti-rabbit antibody (1:300; Vector Laboratories Inc.) and streptavidin-horseradish peroxidase solution (DAKO), developed with a 3,3′-diaminobenzidine kit (DAB, Vector Laboratories Inc.). Slides were counter-

stained with Gill Accustain hematoxylin (Sigma-Aldrich) and mounted with Surgipath Micromount mounting media (Leica Microsystems Inc.).

In Situ Zymography

Zinc fixation and paraffin embedding was shown to preserve tissue morphology and enzymatic activity better than that of frozen sections [30]. Zinc-fixed and paraffin-embedded sections were sectioned at 5 μm, baked at 37°C for 1 h, deparaffinized in xylene, rehydrated in 100%, 95%, 80%, and 70% ethanol baths for 5 min each. Sections were then incubated with fluorescein-conjugated ECM substrates: DQ gelatin, DQ collagen I, or DQ collagen IV (all from Life Technologies Inc.) diluted 1:50 in a reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide) for 2 h at 37°C. For a negative control, sections were pre-incubated with 0.02M ethylenediaminetetraacetic acid (EDTA) alone, then incubated for 2 h at 37°C with 0.02 M EDTA plus substrate. Following incubation with the fluorescent substrates, sections were postfixed in a 4% neutral buffered formalin (in PBS) solution, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) diluted 1:1000 in PBS, and mounted with a coverslip using Immu-Mount (Thermo Fisher Scientific Inc.), which was adapted from the protocol described in detail by Hadler-Olsen et al. [30].

Statistical Analysis

For gestational profiles, data were natural logarithm (ln)-transformed (unless otherwise stated), and statistical analysis was performed using one-way ANOVA with the Newman-Keuls post hoc test for multiple comparisons. For the unilaterally pregnant rat model, statistical analysis was performed using two-way ANOVA with Bonferroni post hoc test for multiple comparisons using ln-transformed data (unless otherwise stated). Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars that were significantly different from each other after multiple comparisons were indicated by different letters or by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Significant outliers within each data set were determined by the Grubb test (GraphPad Software, Inc.) and removed from analysis. The Kolmogorov-Smirnov normality test was used due to a small sample size (n = 4–6), revealing that ln-transformed data used for parametric analyses were normally distributed. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.).

RESULTS

The level of 10 MMP and four TIMP transcripts was assessed in the pregnant, laboring, and PP rat myometrium (Fig. 1). The most significant findings were in the expression of myometrial *Mmp7, Mmp11,* and *Mmp12* genes. For instance, *Mmp7* myometrial mRNA levels were low throughout rat gestation (GD 6–21) but were dramatically upregulated before labor (GD22, 200-fold increase), during TL (GD23, 350-fold increase), and remained elevated 1PP (150-fold increase) and 4PP (20-fold increase). *Mmp11* levels show a similar expression profile with a significant increase near term and TL (15-fold increase for both) with further increase 1PP (70-fold increase) as compared to early pregnancy (GD6); however, the magnitude of the changes are not as great as for *Mmp7*. The mRNA levels of *Mmp12* were significantly elevated near term (GD22, 155-fold increase) and during the PP period (98-fold increase) compared to early gestation (GD 6–12). Transcript levels of *Mmp2* and *Mmp9* (gelatinases), *Mmp8* and *Mmp13* (collagenases), and *Mmp3* and *Mmp10* (stromelysins) were low throughout gestation and labor with minor fluctuations, but upregulated at 1PP (Fig. 1). Collagenase *Mmp1* transcript levels were barely detectable in the rat myometrium. The mRNA expression of *Timp1–Timp4* was high and relatively stable throughout gestation and TL. *Timp2* mRNA was the most abundant in pregnant myometrium. *Timp1, Timp3,* and *Timp4* expression was relatively unchanged throughout gestation with *Timp1* decreasing and *Timp3* and *Timp4* increasing during the PP period (Fig. 2).

We attempted to assess the role of different MMPs and TIMPs in pregnancy by performing a comparative study (Supplemental Fig. S3). Gene expression was calculated by the ΔΔCt method and normalized to the expression of *Mmp10* on GD6 (the lowest among all detected MMPs and TIMPs, a chosen calibrator). Comparative analysis revealed that relative to all other MMPs, the gelatinase *Mmp2* was most highly expressed during pregnancy (i.e., 200-fold higher than *Mmp10*) but did not change with labor onset. During active TL, only *Mmp7* displayed a dramatic 550-fold relative increase. Other MMPs showed low mRNA abundance throughout gestation, relative increase near term, and induction at 1PP. For instance, immediately after delivery, stromelysins, collagenases, and gelatinases showed at least a 350-fold increase relative to *Mmp10*, whereas *Mmp7* and *Mmp12* showed 150-fold increase. Interestingly, the expression of *Timp*s throughout gestation was similar to that of gelatinases *Mmp2/9*. Further comparison of the *Timp*s family to all *Mmp*s studied (both were standardized to GD6 *Mmp10*) revealed that *Timp* mRNAs were expressed relatively higher within the myometrium throughout gestation. Of the four MMP inhibitors, *Timp2* transcript levels showed the highest abundance throughout gestation, labor, and PP and along with *Timp1* displayed a slight decrease in the PP period. Interestingly, only *Timp3* and *Timp4* were highly upregulated PP (300- to 450-fold), suggesting a role in promoting uterine involution (Fig. 2 and Supplemental Fig. S3).

The effect of gravidity on the expression of MMPs/TIMPs was assessed in unilaterally pregnant rats during gestation and TL. In the gravid uterine horn, MMP transcript levels were similar to that in bilaterally pregnant rats, while expression of all MMP genes in the myometrium of the empty horn was very low. Similar to bilaterally pregnant rats, *Mmp7, Mmp11,* and *Mmp12* exhibited significantly higher transcript levels during active TL in the gravid horn as compared to the empty horn, while other MMPs did not show statistically significant differences in mRNA levels (Fig. 3). Furthermore, *Mmp12* gene expression was already upregulated during late gestation (GD 19 and 21) in the gravid horn as compared to the corresponding empty horn. Surprisingly, in the empty uterine horn, we also detected an increase of *Mmp7* transcript levels during active labor (GD23) as compared to mid- and late gestation (GD 15–21). Interestingly, *Mmp2* gene expression during TL was significantly higher in both uterine horns as compared to GD6 (Fig. 3A). These findings suggest that the transcriptional regulation of MMP genes in the gravid horn may involve mechanisms such as static mechanical stretch, cyclic labor contractions, gestation-related hormonal environment, and/or the presence of the fetal-placental unit.

Therefore, we investigated whether artificial mechanical stretch of the uterine wall, by an expandable material that mimics the effect of gravidity, can regulate MMP gene expression. An intra-uterine expandable tube (IUET) was inserted into the empty horn of unilaterally pregnant rats at late gestation (GD 19–21); myometrial samples were collected 24 h later [29]. As shown in Figure 4, for the majority of MMPs studied, transcript levels in the artificially stretched empty horn were either comparable or higher (i.e., *Mmp7,* $P < 0.05$) than in the gravid horn. Importantly, the expression of *Mmp2, Timp2,* and *Timp4* was significantly downregulated by the IUET, which suggests negative regulation by mechanical stretch. It must be noted that the degree of laminaria-induced uterine stretch was comparable, but not equal, to the stretch seen as a result of gravidity and that the speed of stretching was much faster than during gestation.

Specific antibodies were used to check the expression of MMP7, MMP11, and MMP12 proteins. MMP7 protein was detected at 29 kDa, and its expression was increased at late gestation, during TL, and peaked at 1PP as compared to early gestation (Fig. 5, A and B), exhibiting a pattern similar to that

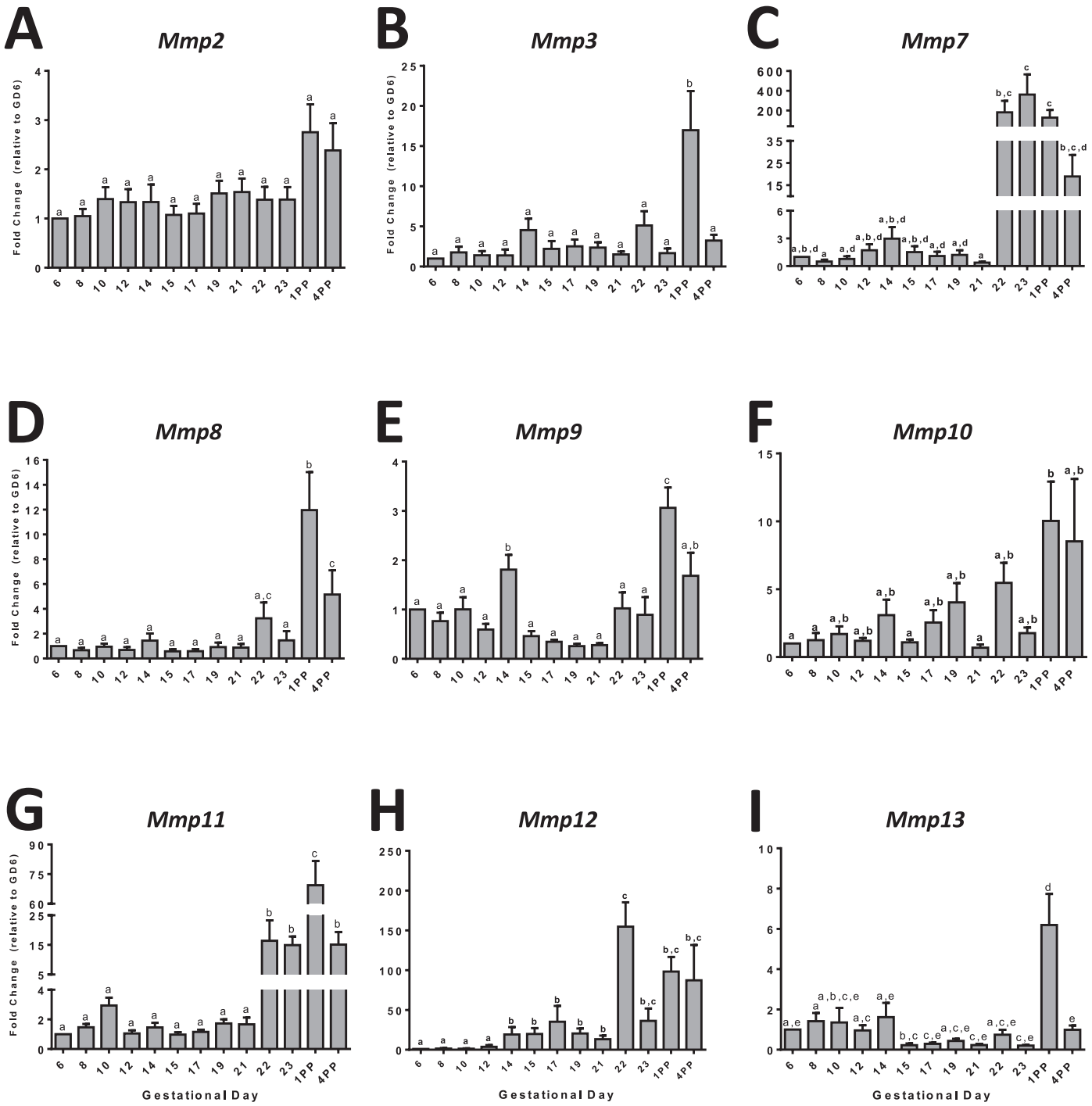

FIG. 1. Gene expression of secreted MMPs in bilaterally pregnant, laboring, and postpartum (PP) rat myometrium. Shown are transcript levels of collagenases *Mmp8* (**D**) and *Mmp13* (**I**); gelatinases *Mmp2* (**A**) and *Mmp9* (**E**); matrilysin *Mmp7* (**C**); stromelysins *Mmp3* (**B**), *Mmp10* (**F**)*, and Mmp11* (**G**); and metalloelastase *Mmp12* (**H**) normalized to three housekeeping genes: peptidylprolyl isomerase A (*Ppia*), platelet derived growth factor alpha (*Pdgfa*), and TATA box binding protein (*Tbp*). The data from each gestational day (GD) is presented as mean ± SEM (n = 4–6/GD). Statistical analysis of each *Mmp*'s gestational profile using one-way ANOVA and the Newman-Keuls multiple comparisons test was performed on the natural log (ln)-transformed normalized expression data and presented as fold changes relative to a corresponding GD6. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters are significantly different from each other.

of *Mmp7* gene expression (Figs. 1 and 4). Active MMP11 protein (54 kDa) also displayed a pattern of increased expression at late gestation beginning from GD17 to 1PP (Fig. 5, A and C). The 45kDa proform of MMP12 was highly and relatively stably expressed throughout gestation, but active MMP12 (25 kDa) was not detected (Fig. 5D). The expression of stromelysin MMP3 and collagenase MMP8 proteins was high and stable in the pregnant, laboring, and PP rat myometrium (Supplemental Fig. S4). Next, we compared the expression of active MMP7 and MMP11 proteins in the empty and gravid horns of unilaterally pregnant rats. Although not statistically significant, as with the bilaterally pregnant myometrium, MMP7 was upregulated in the gravid horn during TL compared to levels during pregnancy. However,

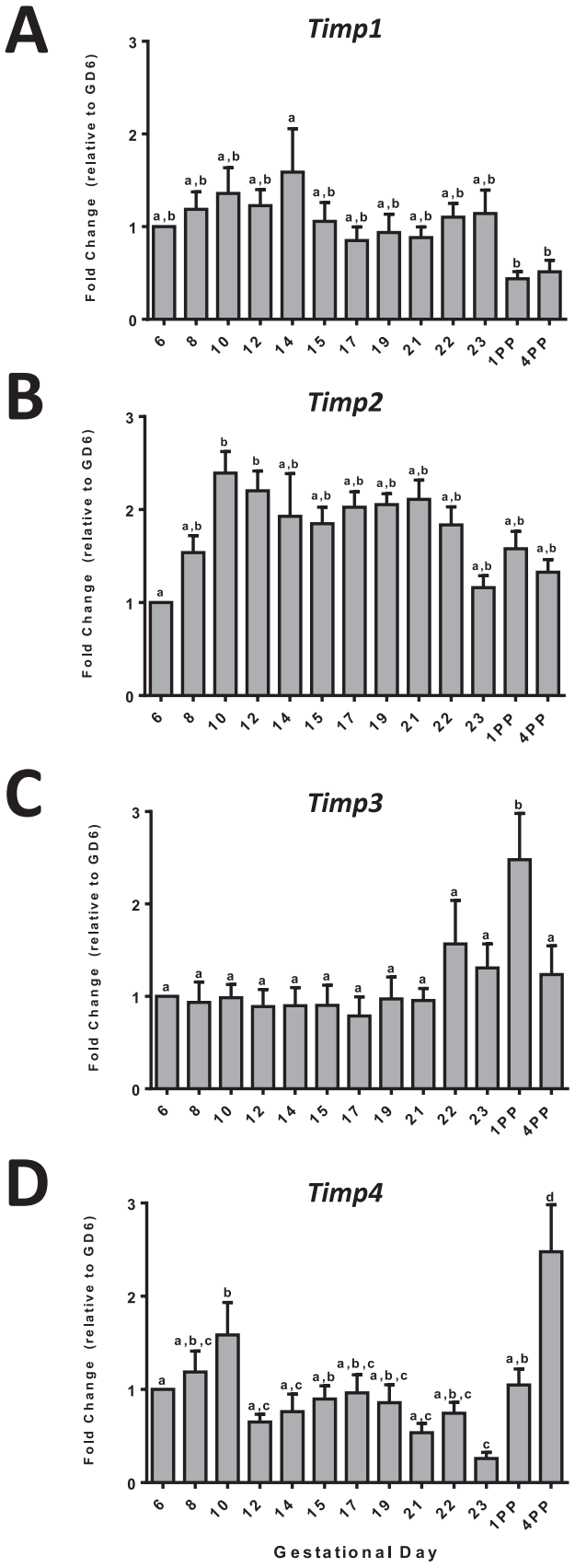

FIG. 2. Expression of TIMP mRNA in bilaterally pregnant, laboring, and postpartum rat myometrium. The mRNA expression of tissue inhibitors of metalloproteinases *Timp1* (**A**), *Timp2* (**B**), *Timp3* (**C**), and *Timp4* (**D**) was normalized to three housekeeping genes (*Ppia, Pdgfa,* and *Tbp)*. The data from each gestational day (GD) is presented as mean ± SEM (n = 5/GD). Statistical analysis of each *Mmp*'s gestational profile using one-way ANOVA and the Newman-Keuls multiple comparisons test was performed on the natural log (ln)-transformed normalized expression data and presented as fold changes relative to a corresponding GD6. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters are significantly different from each other.

MMP11 protein levels in the gravid horn of unilaterally pregnant rats did not display the late increase that was observed in the bilaterally pregnant rat myometrium. We also found no difference in expression of these MMPs between empty and gravid horns (Supplemental Fig. S4, C and D). This inconsistency in protein expression may be due to physiological variability between the two different animal models used.

Spatial localization of MMP7 and MMP11 proteins was assessed in the pregnant rat uterus from midgestation to 1PP by immunohistochemistry (IHC) (Fig. 6). We noticed that MMP7 immunostaining was weak at midgestation (GD15) with an increase within myometrial cells near term (GD21) and was mainly localized to the nuclear and perinuclear regions of SMCs. During TL (GD23) and 1PP, we detected increased expression of MMP7 localized both intracellularly and extracellularly, between SMCs and around the myometrial vasculature. Similarly, MMP11 immunostaining was primarily intracellular but also showed extracellular protein localization. Importantly, both MMP7 and MMP11 immunostaining was also detected in several cells localized around myometrial blood vessels that were morphologically different from myocytes. We speculate these cells might be infiltrating leukocytes that express MMPs (Fig. 6). Similar to protein expression detected by Western Blot analysis, IHC revealed consistent immunostaining of MMP3 and MMP12 throughout gestation and TL primarily in the nuclear and perinuclear region of myocytes, although in the early PP period additional extracellular staining was noticed between SMCs (Supplemental Fig. S5).

The enzymatic activity of MMP2 and MMP9 was also examined by gelatin zymography (Fig. 7A). Pro-MMP2 protein was stably expressed throughout gestation and TL with active-MMP2 protein significantly induced only at 1PP, a profile similar to *MMP2* gene expression (Fig. 1). Active-MMP9 protein activity was very low and could not be accurately analyzed. Gelatin zymography using gravid horn myometrium from unilaterally pregnant rats revealed a pro-MMP2 expression profile similar to that in bilateral pregnancy with no differences between the empty and gravid uterine horns (Fig. 7, A and B). In contrast, active MMP2 protein was higher in the empty uterine horn than in the gravid horn from early gestation until TL (Fig. 7B).

Spatial localization of MMP enzymatic activity in the rat uterus was assessed using in situ zymography (ISZ) with different fluorescein-conjugated ECM substrates (DQ-gelatin, DQ-collagen I, and DQ-collagen IV) as described in [31, 32]. We detected higher levels of gelatin and collagen I degradation in the myometrial parenchyma near term (GD22) and during TL (GD23) as compared to GD15; however the highest MMP activity was detected on 1PP as shown by increased fluorescence, depicting extracellular degradation of gelatin, collagen I, and collagen IV (Fig. 8). Similar to immunostaining results, numerous single cells that were morphologically different from SMCs were observed in myometrial tissue in close proximity to blood vessels.

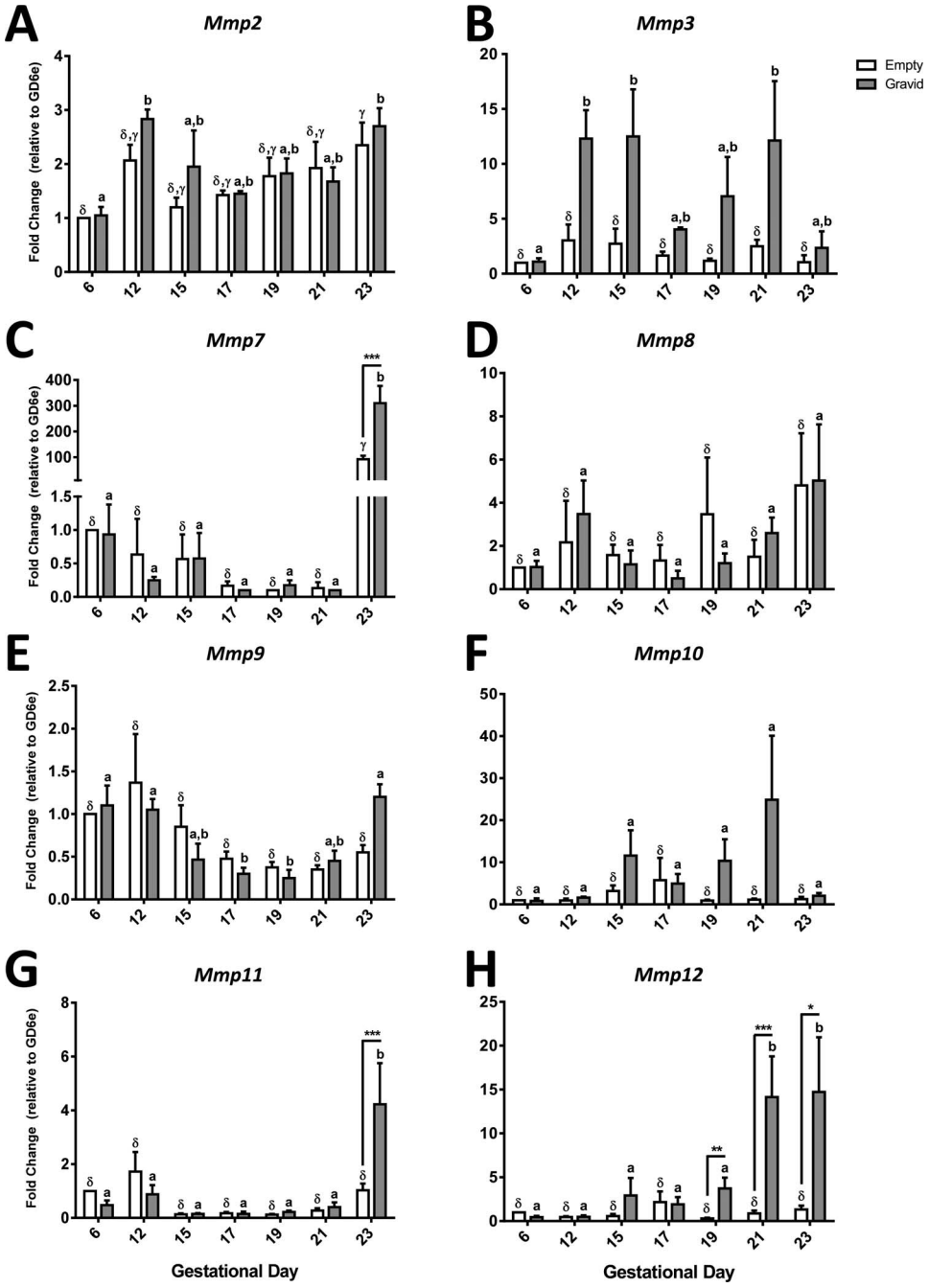

FIG. 3. MMPs gene expression in the unilaterally pregnant and laboring rat myometrium. The mRNA expression of *Mmp2* (**A**), *Mmp3* (**B**), *Mmp7–Mmp12* (**C**–**H**) in the pregnant (gravid) horn (gray bars) compared to the empty (e) horn (white bars) was normalized to three housekeeping genes (*Ppia, Pdgfa,* and *Igf1r)*. Data were presented as fold-change relative to a corresponding GD6e and the bars represent mean ± SEM (n = 4/GD). Statistical analysis was performed on the natural log (ln)-transformed relative fold change data using two-way ANOVA and Bonferroni multiple comparisons test. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters (between gravid horns of each GD) or Greek symbols (between empty horns of each GD) are significantly different from each other. A significant difference between the gravid and empty horn of the same gestational day is indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

DISCUSSION

Until recently, little was known about the expression and regulation of MMPs/TIMPs within the uterine smooth muscle during pregnancy and TL. Using a rat model of gestation, we present here the first comprehensive study of gene and protein analysis of major MMPs and TIMPs, as well as their tissue localization and activity in the pregnant, laboring, and PP myometrium. Our results indicate that the majority of myometrial MMPs that we studied displayed low expression during gestation; only three enzymes, MMP7 (matrilysin, which differs from most MMP family members because it lacks a conserved C-terminal domain), MMP11 (stromelysin-3), and MMP12 (metalloelastase), exhibited increased expression in the myometrium near term, while other MMPs were significantly upregulated PP.

The role of MMPs in several reproductive processes, that is, ovulation, implantation, cervical ripening, and PP involution, is well-established [17, 18, 33]. However, little is known about

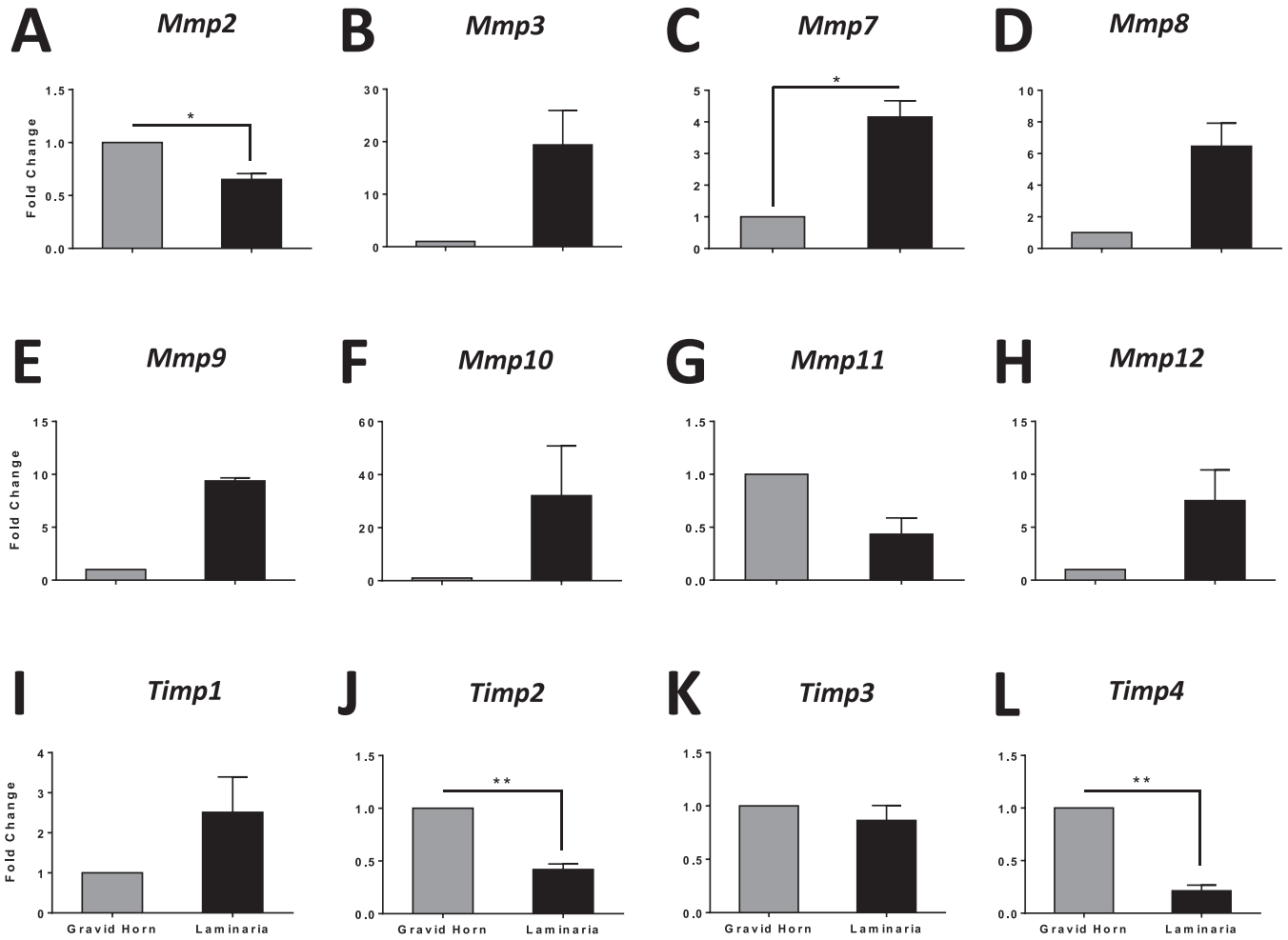

FIG. 4. Artificial mechanical stretching caused by a laminaria regulates MMP gene expression at late gestation (GD 19–21), similar to biological mechanical stretch by growing fetuses. The mRNA expression of collagenase *Mmp8* (**D**); gelatinases *Mmp2* (**A**) and *Mmp9* (**E**); matrilysin *Mmp7* (**C**); stromelysins *Mmp3* (**B**), *Mmp10* (**F**), and *Mmp11* (**G**); metalloelastase *Mmp12* (**H**); and tissue inhibitors of metalloproteinases *Timp1*–*Timp4* (**I**–**L**) in the gravid uterine horn (gray bars) compared to the empty horn stretched by laminaria (black bars) was normalized to three housekeeping genes (*Pdgfa, Ppia,* and *Tbp)*. Laminaria data were expressed as fold-change relative to a corresponding gravid horn and the bars represent mean ± SEM (n = 3). Statistical analysis was performed using the paired *t*-test with two-tailed *P* value. A significant difference between the gravid and luminaria-stretched empty horn is indicated by $*P < 0.05$ and $**P < 0.01$. The majority of *Mmp*s studied (except *Mmp1* that was not detected and *Mmp13* that was very low) exhibited comparable or even higher expression in the artificially stretched empty horn than in the gravid horn. Interestingly, the expression of *Mmp2, Mmp11, Timp2,* and *Mmp4* were downregulated by laminaria-induced stretch, whereas *Timp1* was upregulated and *Timp3* was not changed. It must be noted that the degree of uterine stretch by laminaria was comparable, but not equal, to the stretch seen as a result of gravidity and that the speed of the stretching was much faster than during gestation.

the potential role of MMPs, especially MMP7, MMP11, and MMP12, within the myometrium during gestation and TL. The stromelysins (MMP3, MMP10, and MMP11) and related MMPs (MMP7 and MMP12) are capable of degrading elastin, cell adhesion molecules, proteoglycans, fibronectin, and components of the BM, laminin and collagen type IV [15, 34], suggesting their involvement in modifying the myometrial ECM in preparation for active labor contractions. Our study revealed significant upregulation of *Mmp7, Mmp11,* and *Mmp12* genes before TL with elevated levels sustained during TL and PP. Furthermore, we detected a late increase at the time of TL and PP for MMP7 and MMP11 proteins that resembled the trend observed for *Mmp7* and *Mmp11*. These findings suggest a dual role in promoting both labor and uterine involution.

Throughout gestation, myometrial SMCs transition from a hyperplastic (proliferative) to hypertrophic (or synthetic) phenotype associated with ECM modifications to accommodate the increase in SMC number and size [35]. Williams et al. [36] have previously reported increased α_1, α_3, and β_1 integrin expression localized to myocyte membranes during late gestation and labor, which correlates well with our findings. Because matrix-integrin receptor signaling is known to upregulate MMP transcriptional activation, we speculate this is one of the potential mechanisms in play to regulate myometrial MMP expression [8, 9]. It has been previously reported that MMP-mediated degradation of ECM components can result in the genesis of chemoattractive peptides [37]. Elastin- and fibronectin-derived peptides are known to have chemotactic properties for further monocyte recruitment and can stimulate activated macrophages [37, 38]. Our own and other studies have investigated the role of immune cells and inflammatory mediators contributing to events preceding TL. We identified macrophages as the predominant immune cells in the myometrium and decidua during gestation, and this population was increased in parallel with pro-inflammatory cytokine induction during TL and early PP [39–41]. Monocytes/macrophages have been suggested to support active labor

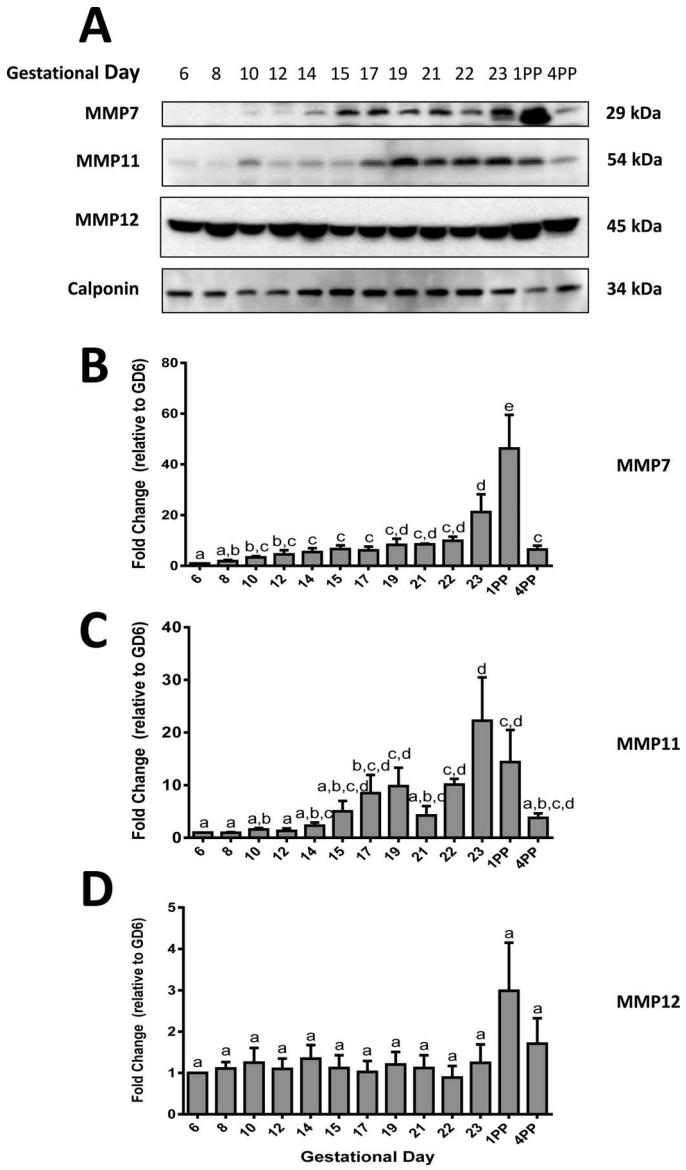

FIG. 5. Protein expression of MMP7, MMP11, and MMP12 in pregnant, laboring, and PP rat myometrium. Representative Western blots (**A**) and densitometric analysis of matrilysin (**B**, MMP7), stromelysin-3 (**C**, MMP11), and metalloelastase (**D**, MMP12) in the rat myometrium. MMP protein expression levels were normalized to Calponin and expressed as fold change relative to GD6. The data from each GD is presented as mean ± SEM (n = 4–6/GD). Statistical analysis of each MMP's gestational profile using one-way ANOVA and the Newman-Keuls multiple comparisons test was performed on the fold change data. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters are significantly different from each other.

contractions by amplifying the inflammatory signal through the secretion of cytokines, including interleukin (IL) 1β, IL6, tumor necrosis factor alpha (TNFα), and MMPs [22]. Not surprisingly, leukocytes, especially macrophages, have been revealed to be able to secrete several soluble MMPs (MMP1–MMP3 and MMP7–MMP13) as well as all four TIMPs [14, 42].

Although uterine MMP7 was first identified in PP tissues [15, 43, 44], we detected a significant increase in MMP7 gene and protein expression near term, which correlates with the increase in myometrial leukocyte recruitment. Apart from promoting chemoattraction, MMP7 has been previously shown to actively cleave vascular endothelial-cadherin, one of the junctional proteins that maintain vascular barrier function [45]. Results from our IHC staining for MMP7 showed particular expression surrounding the myometrial vasculature near term (Fig. 6). We speculate that MMP7 can also promote leukocyte infiltration into the myometrium by upregulating chemotaxis and vascular permeability during late gestation and TL. Interestingly, the increased expression of MMP7 also correlates with our previous reports of increased IGF1 and IGFBP5 expression in the rat myometrium during TL and PP [3]. The family of insulin-like growth factors (i.e., IGF1, IGF2, IGFBPs) are known to stimulate cell proliferation, collagen synthesis, and muscle growth/regeneration, making their bioavailability important to assist PP uterine involution [46–48]. MMP7-mediated cleavage of IGFBP5 has been shown to liberate IGF2 [49], which also suggests its association in upregulating IGF signaling in the PP myometrium.

The role of MMP11 in the myometrium is not well defined; however, previous studies have indicated that it can regulate BM stability [50, 51]. We and others have previously suggested that remodeling of the ECM increases elasticity of the uterus and supports a change in SMC phenotype in preparation for labor contractions by forming an organized, continuous, and regular BM surrounding individual myocytes [6, 52]. We now speculate that the significant increase in MMP11 levels before and during active labor correlates with changes in ECM composition and may contribute to the increased cellular connectivity for coordinated uterine contractions by modulating the BM surrounding individual SMCs [5, 14, 51]. MMP11 has also been reported to cleave a proteinase inhibitor α1-antitrypsin that predominantly inhibits neutrophil elastase-mediated degradation of ECM components and α2-macroglobulin, a general proteinase inhibitor that induces MMP removal by endocytosis [7, 53, 54]. Therefore, the observed elevation of MMP11 protein levels might also contribute to the maintenance of proteolytic activity of other MMPs by preventing their inhibition during TL and PP.

The metalloelastase MMP12 was first believed to be expressed in vivo solely by macrophages [55] and is important for their migration [14] via direct or indirect induction of elastin degradation by targeting the serine proteinase inhibitor α1-antitrypsin [56, 57]. However, it is now known that macrophages are not the only source of MMP12 expression because trophoblasts and vascular SMCs have been shown to express MMP12 to mediate uterine spiral artery remodeling [58]. It is possible that the increase in MMP12 expression is a result of: 1) the activation of resident tissue macrophages by ECM-derived peptides, 2) infiltrating macrophages secreting MMP12, and 3) resident myometrial SMCs producing MMP12 for elastin remodeling before labor.

In many tissues and cell types, expression of MMPs was shown to be upregulated by mechanical stretch [59, 60]. In particular, mechanical stretch was able to increase gelatinase expression and activity in the virgin rat myometrium [25]. Using a unilaterally pregnant rat model, we observed that during labor, gene expression of *Mmp7, Mmp11,* and *Mmp12* (Fig. 3) was significantly increased in the gravid uterine horn as compared to the empty horn. Furthermore, artificially stretching the empty horn in unilaterally pregnant rats using the IUET significantly increased *Mmp7* expression (Fig. 4). However, protein analysis showed a similar increase for MMP7 in both horns (Supplemental Fig. S4) suggesting that these changes are not regulated solely by mechanical stretch but also by other factors such as ovarian hormones or cytokines [14, 61, 62]. Interestingly, our results using the IUET rat model demonstrated a significant inhibition of *Mmp2* expression by

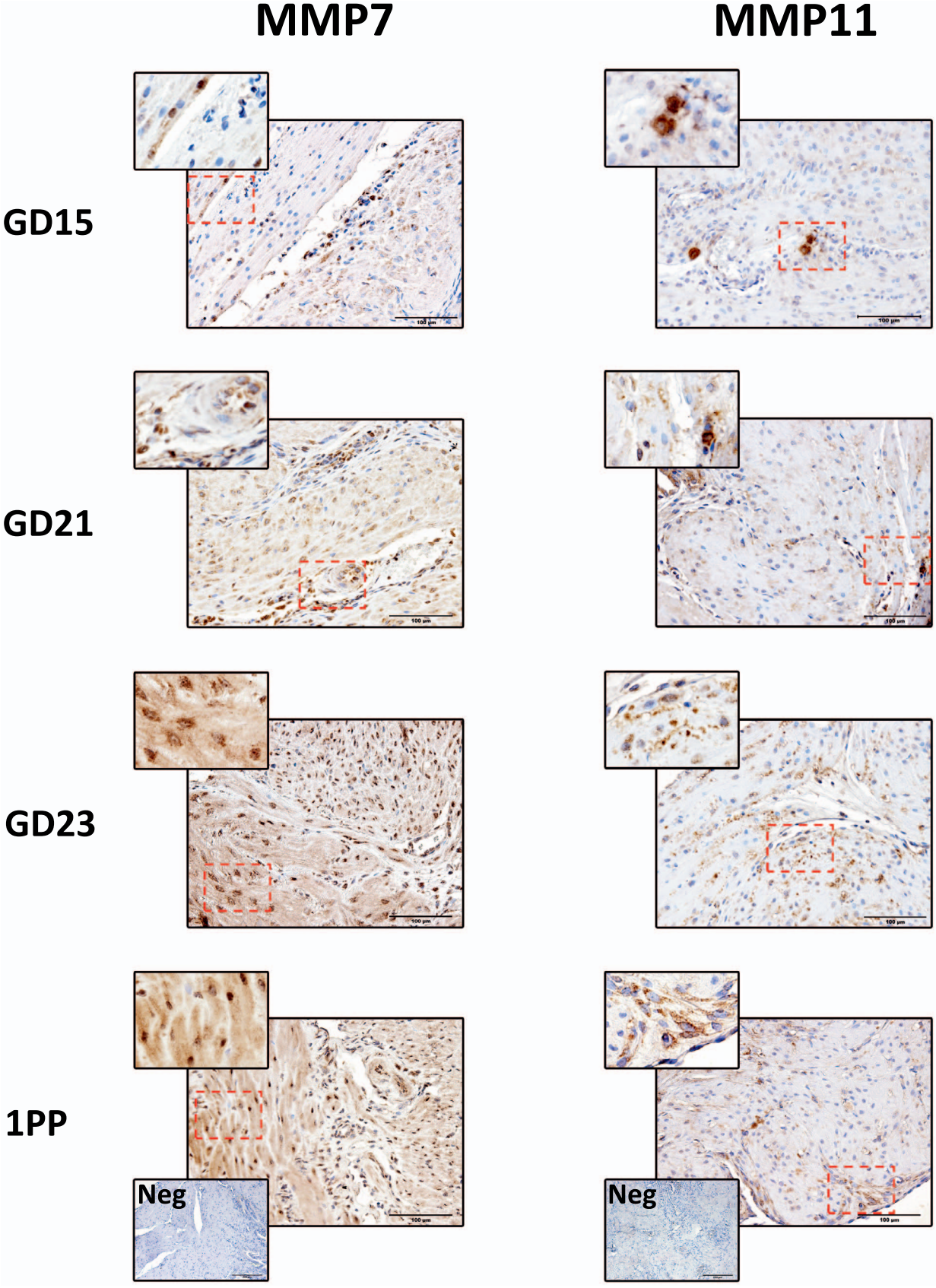

FIG. 6. Spatial and temporal localization of MMP expression in pregnant, laboring, and PP rat myometrium. Tissue sections from GD 15, 21, 23 (laboring), and 1 day PP (1PP) rat uteri were immunostained with anti-MMP7 and anti-MMP11 antibodies. Brown deposition observed inside SMCs in the nuclear and perinuclear area indicates the presence of intracellular proteins in the pregnant myometrium. Both intra- and extracellular immunostaining (between SMCs) was observed for MMP7 and MMP11 proteins during TL at 1PP. It is speculated that positively immunostained single cells around myometrial blood vessels morphologically different from myocytes, are infiltrating leukocytes expressing MMPs. No staining was detected on the negative control slide when primary antibodies were replaced by rabbit IgG at the same concentration (negative control was performed on 1PP). Magnification ×200. Inset magnification ×400.

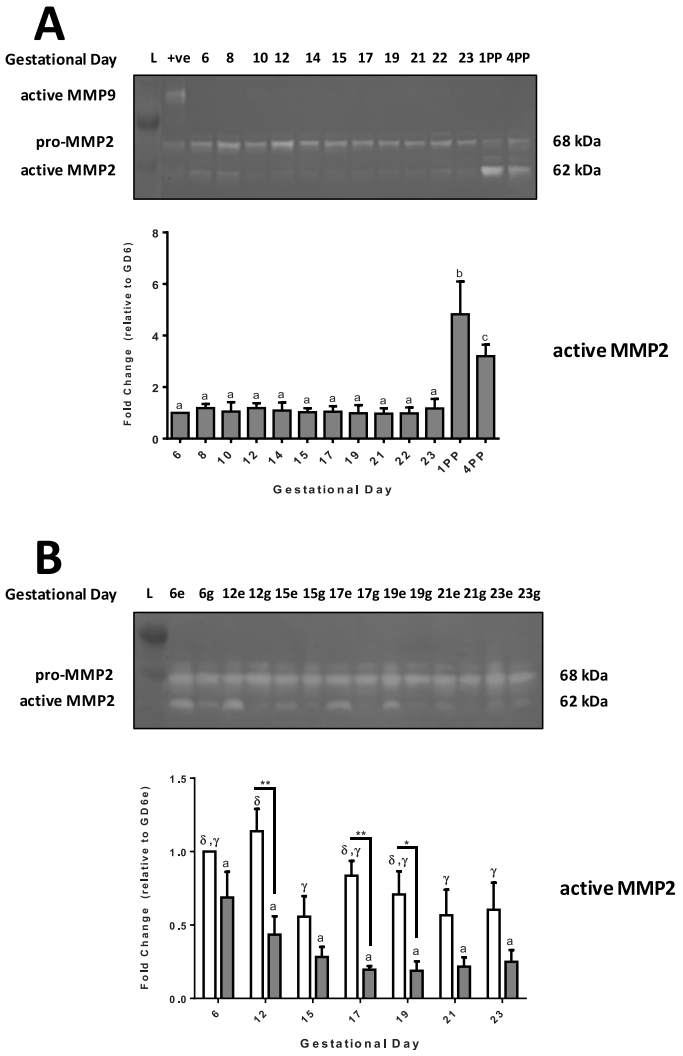

FIG. 7. Representative gelatin zymography and densitometric analysis of active MMP2 in the bilaterally pregnant (**A**) and unilaterally pregnant (**B**) rat models of gestation (L: protein ladder). **A**) Gelatinase activity of total protein in pregnant, laboring, and postpartum (PP) rat myometrium (visualized as light bands). MMP2 activity is observed predominantly at 68 kDa (latent proform) and 62 kDa (active form). MMP9 at 92 kDa (latent proform) and 87 kDa (active form) was very low throughout gestation. MMP9 was observed in the decidua conditioned media used as a positive control (+ve). Densitometric analysis revealed increased MMP2 activity in the PP period compared to pregnancy and labor. The results are presented as fold change (mean ± SEM) relative to a corresponding GD6 (n = 4/GD). Statistical analysis using one-way ANOVA and the Newman-Keuls multiple comparisons test was performed on the fold change data. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters are significantly different from each other. **B**) Gelatinase activity of total protein in unilaterally pregnant rats presented as empty (e, white bars) versus gravid (g, gray bars) and visualized as light bands. Densitometric analysis of active MMP2 revealed higher levels of MMP2 activity in the empty horn compared to the gravid horn during gestation, significantly for GD 12, 17, and 19. Results shown are the mean ± SEM (n = 4/GD) and presented as fold change relative to GD6e. Statistical analysis using two-way ANOVA and Bonferroni multiple comparisons test was performed on the fold change data. Values were considered statistically significant when $P < 0.05$ after multiple comparisons. Bars with different letters (between gravid horns of each GD) or Greek symbols (between empty horns of each GD) are significantly different from each other. A significant difference between the gravid and empty horn of the same GD is indicated by $*P < 0.05$ and $**P < 0.01$.

artificial mechanical stretch (Fig. 4) and an increase in activated MMP2 during PP (after the fetus is expelled). We also observed greater expression of active MMP2 protein in the empty horn compared to the gravid horn from early gestation until TL (Fig. 7B). Based on these findings, we speculate that during gestation MMP2 activity in the gravid horn is repressed by the presence of mechanical stretch by the growing fetus and/or paracrine factors produced by the decidua and fetal-placental unit (e.g., inhibin and TGFβ [63]) to prevent uncontrolled gelatinolysis. Previous studies have demonstrated that members of the transforming growth factor beta (TGFβ) superfamily, activin and inhibin, secreted by the decidua and placenta, have the ability to differentially regulate the expression and secretion of human uterine MMPs. Specifically, Jones et al. [63, 64] revealed that activin A promoted decidualization and trophoblast invasion through upregulated production of MMPs by endometrial, decidual, and cytotrophoblast cells.

The major structural components of the uterine ECM are elastin fibers and collagen fibrils consisting of collagen type I and III [5]. Elastin abundance is important within the uterus to accommodate its expansion in pregnancy, while collagen provides tissue strength [5]. Therefore, the major structural component of the uterine ECM, fibrillar collagen, must be constantly reorganized for pregnancy maintenance [5]. It has been reported that myometrial remodeling during pregnancy involves well-controlled collagenolysis, evident by the disruption and partial degradation of collagen fibrils in late pregnancy [52]. Comparative analysis of overall MMP gene expression indicates that among the MMP genes studied, the collagenases (*Mmp8* and *Mmp13*) and gelatinases (*Mmp2* and *Mmp9*) were highly (yet relatively stably) expressed throughout pregnancy. In addition, the activity of pro-MMP2 was also high and stable throughout gestation. We speculate that the collagenases would cleave fibrillar collagens into fragments that would be denatured into gelatin. Furthermore, the gelatinases would then degrade gelatin into small peptide fragments for rapid removal by infiltrating immune cells (possibly macrophages) [52, 65]. This suggests the existence of very well-controlled and spatially restricted collagenolysis to support phenotypic transitions of SMCs from a hyperplastic to hypertrophic phenotype and to increase elasticity of the myometrium to accommodate the growing fetus(es).

Interestingly, IHC and ISZ analysis detected MMP expression and activity in the nuclear and perinuclear region of myometrial SMCs throughout gestation, TL, and PP involution (Figs. 6 and 8, and Supplemental Fig. S5), which suggests intracellular storage and (possible) activity of these proteins. The intracellular compartmentalization and activity of MMPs has been recently demonstrated in several cell types [66]. Although the mechanism of nuclear translocation is poorly characterized, MMPs have been found inside nuclei and their presence has been associated with the regulation of apoptosis and gene expression. For instance, MMP2 has been shown to process two important factors of the DNA repair machinery capable of protecting cardiac myocytes from apoptosis [67]. Also MMP3 acts as a transcription factor preventing transcriptional repression of the connective tissue growth factor (CTFG/CCN2) gene and promoting chondrocytic proliferation and ECM formation [68]. MMP2 has also been shown to regulate myocyte contractile function by cleavage of myosin light chain-1, a component of the contractile machinery [69]. Furthermore, several cell types (i.e., leukocytes and endothelial cells) can store MMPs intracellularly in exocytic vesicles that are readily available for rapid secretion upon cellular activation [66]. These studies correlate well with our findings suggesting additional roles of myometrial MMPs in 1)

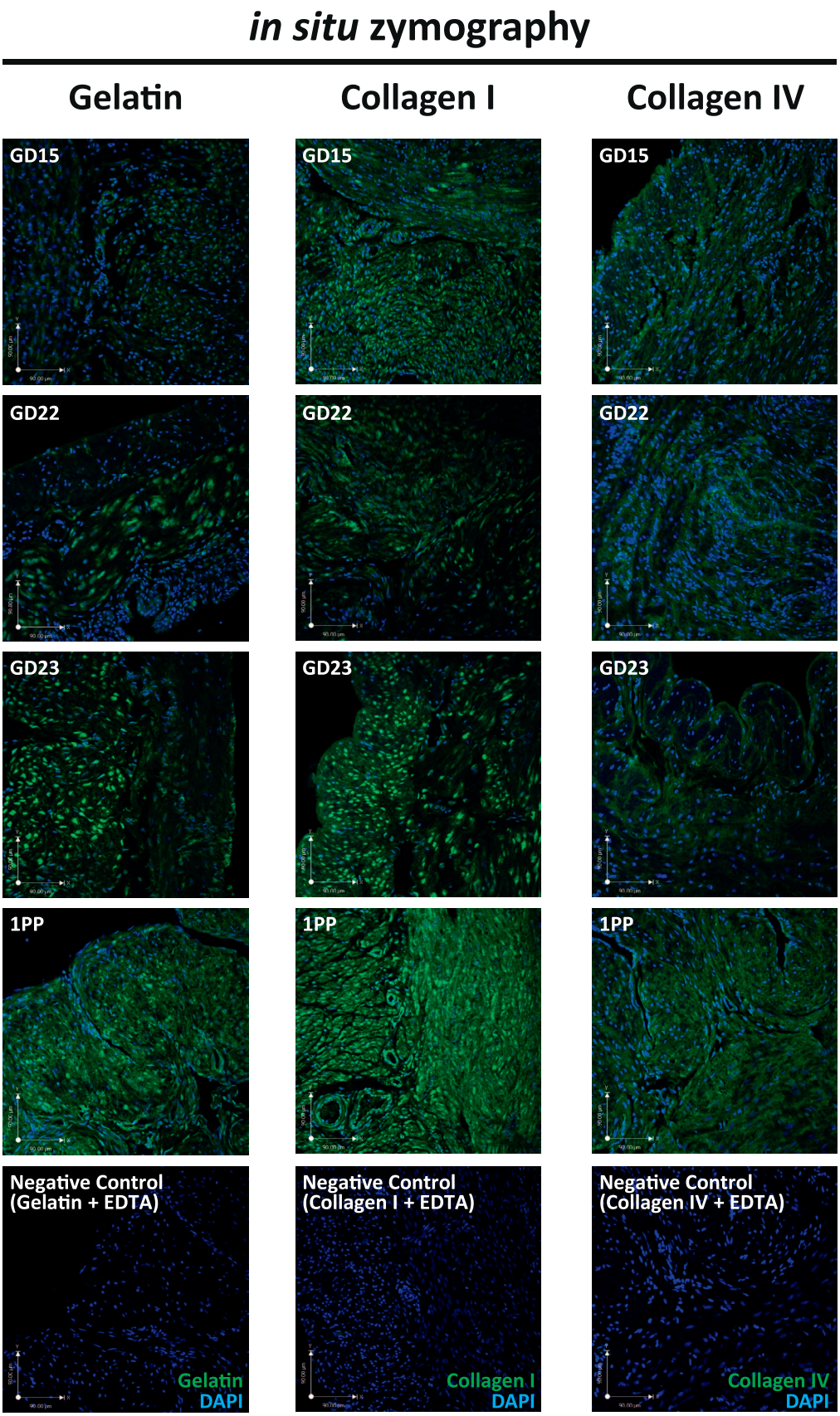

FIG. 8. Spatial and temporal localization of MMP activity in the pregnant, laboring, and PP rat myometrium. Representative images from in situ zymography (ISZ) using gelatin and collagens I and IV as substrates. Increased green fluorescence (depicting substrate degradation) was localized intracellularly to the nuclear and perinuclear region of the myometrial cytoplasm during late gestation (GD22) and laboring (GD23), however during 1PP, fluorescence was detected both intra- and extracellularly in the myometrial parenchyma between SMCs. Negative control sections were pre-incubated with EDTA. Magnification ×100.

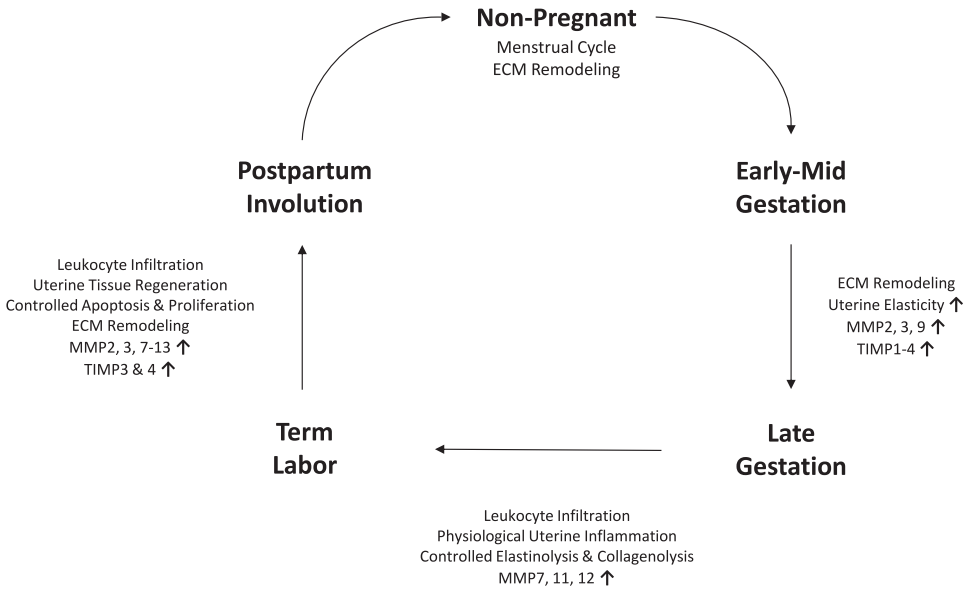

FIG. 9. Schematic presentation of the proposed hypothetical roles of MMPs/TIMPs within the myometrium. 1) Throughout early to midgestation, there is constant remodeling of the ECM by MMPs, counterbalanced by TIMPs, to accommodate the growing fetus by increasing the elasticity of the uterus. 2) Near term and during labor, there is an increase in specific MMPs that promote infiltration of leukocytes, generating sterile physiological inflammation. 3) Following birth, there is a generalized coordinated induction of multiple MMPs/TIMPs to ensure PP uterine tissue regeneration back to a prepregnant state so the female remains fertile.

acting as transcription factors to promote cellular proliferation and ECM formation during gestation and 2) regulating cellular apoptosis and degradation of SMC contractile proteins during PP involution.

Analysis of TIMP expression in the rat myometrium provides a potential mechanism for regulating the expression or activity of MMPs throughout gestation. It is known that TIMP1 and TIMP2 can form a specific complex with MMP9 and MMP2, respectively (via their C-terminus), and further interact with other MMPs via their N-terminus to potentially prevent their activation [70, 71]. Our findings suggest that high, constant expression of major TIMP genes in the rat myometrium throughout gestation ensures an unrestricted formation of inhibitory complexes controlling MMPs activity. Comparative analysis revealed that *Timp2* exhibited the highest expression level of all TIMPs while *Timp1* and *Timp3* have an expression pattern similar to gelatinases. Interestingly, TIMPs are known to have other biological functions that include regulation of cell proliferation, migration, angiogenesis, and apoptosis; specifically, TIMP3 has been shown to have pro-apoptotic function, while TIMP4 is anti-apoptotic [72, 73]. Because we observed induction of both *Timp3* and *Timp4* expression during PP, this may indicate their involvement in the process of uterine involution and tissue regeneration by stimulation of apoptosis of hypertrophied myometrial SMC and proliferation of smaller postpregnancy cells.

The strength of this comprehensive study is the characterization of a gestational profile for expression, activity, and localization of MMPs and TIMPs within the myometrium of pregnant rat, demonstrating the regulated involvement of these enzymes in promoting labor and PP involution. While the processes of uterine proliferation, growth, contraction, and involution are common to human pregnancy, it remains to be determined whether these matrix remodelling enzymes follow a similar regulation in the human myometrium. Furthermore, the putative role of MMPs in promoting ECM remodeling and leukocyte infiltration will need to be confirmed. Future experiments will need to determine and verify the precise role of MMPs in degrading ECM components, liberating chemokines and growth factors, and increasing vascular permeability.

In summary, our results point toward several potentially distinct roles for MMPs within the pregnant, laboring, and PP myometrium. First, we suggest that throughout gestation there is constant remodeling of the ECM by housekeeping MMPs, which are counterbalanced by TIMPs, to accommodate the growing fetus by increasing the elasticity of the uterus through well-controlled restricted collagenolysis and elastolysis [52]. Next, near term and during labor, there is an increase in specific MMPs to promote the infiltration of leukocytes, generating sterile physiological inflammation [41]. Following birth, there is a coordinated induction of multiple MMPs/TIMPs to ensure PP uterine tissue regeneration by promoting cellular renewal through well-controlled apoptosis and proliferation, and remodeling of the uterine ECM back to a prepregnant state for the female to remain fertile (Fig. 9).

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