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Estrogen-induced uterine abnormalities in TIMP-1 deficient mice are associated with elevated plasmin activity and reduced expression of the novel uterine plasmin protease inhibitor serpinb7¹

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Abstract

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a multifunctional protein capable of regulating a variety of biological processes in a wide array of tissue and cell types. We have previously demonstrated that TIMP-1 deficient mice exhibit alterations in normal uterine morphology and physiology. Most notably, absence of TIMP-1 is associated with an altered uterine phenotype characterized by profound branching of the uterine lumen and altered adenogenesis. To begin to assess the mechanism by which TIMP-1 may control these uterine events, we utilized steroid-treated ovariectomized wild-type and TIMP-1 null mice exposed to estrogen for 72 hours. Administration of estrogen to TIMP-1 deficient mice resulted in development of an abnormal uterine histo-architecture characterized by increased endometrial gland density, luminal epithelial cell height, and abnormal lumen structure. To determine the mediators which may contribute to the abnormal uterine morphology in the TIMP-1 deficient mice, cDNA microarray analysis was performed. Analysis revealed that expression of two plasmin inhibitors (serpinb2 and serpinb7) was significantly reduced in the TIMP-1 null mice. Associated with the reduction in expression of these inhibitors was a significant increase in plasmin activity. Localization of the novel uterine serpinb7 revealed that expression was confined to the luminal and glandular epithelial cells. Further, expression of uterine serpinb7 was decreased by estrogen and showed an inverse relationship with plasmin activity. We conclude from these studies that in addition to controlling MMP activity, TIMP-1 may also control activity of serine proteases through modulation of serine protease inhibitors such as serpinb7.

Keywords

Uterus; estrogen; TIMP-1; plasmin; serpinb7; megin

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Introduction

With each reproductive cycle, the uterus continuously undergoes dynamic structural and physiological changes. Structural changes within the uterus involve degradation and remodeling of the extracellular matrix (ECM), which are most prominent during the period of menses while physiological changes ensue in response to numerous growth factors and cytokines. One of the major families of proteases responsible for these changes in tissue turnover is the matrix metalloproteinases (MMPs). MMPs are expressed within the uterus of a variety of species which include human [1-5], monkey [6,7], baboon [8], sheep [9], rat [10,11], and mouse [12]. In menstruating species such as the human, monkey, and baboon, MMPs are implicated to play a role in the active tissue remodeling that occurs during menstruation [1-8]. In non-menstruating species such as the rat and mouse, and during the proliferative stage of the human menstrual cycle, the exact role of MMPs is uncertain but it has been proposed that these proteases may further regulate bioavailability and function of a various growth factor/cytokine networks critical for uterine function [13].

Matrix metalloproteinase activity and, hence, tissue remodeling is controlled at three levels: 1) production and secretion of latent enzymes, 2) activation of latent enzymes, and 3) inhibition of enzyme activity by inhibitors of MMPs [14,15]. The MMP inhibitors can be classified as either serum-borne or tissue-derived. The serum-borne inhibitors include the macroglobulins, such as α_2 -macroglobulin, and are found throughout the body. The exact role of this class of inhibitors in regulating MMP activity at the level of the tissue has been questioned based on their large size (≥ 720 kDa) and ability to traverse endothelial basement membranes. The second class of MMP inhibitors are referred to as the tissue inhibitors of metalloproteinases (TIMPs), which are secreted at the level of the tissue and are postulated to control the site and extent of MMP-induced tissue remodeling/ECM breakdown. Currently, four TIMPs have been identified [16,17], of which TIMP-1 appears to be the most functionally diverse; TIMP-1 has been implicated to play a role in angiogenesis, steroidogenesis, cell proliferation, and mammary gland development. Most recently, it has been demonstrated that disruption of the TIMP-1 gene product is associated with an alteration in the "normal" uterine lumen structure [18]. In fact, TIMP-1 null female mice exhibit increased uterine luminal epithelial cell density, and the luminal epithelial cells appear to invade the surrounding stromal tissue in an uncontrolled fashion. This aberrant phenotype may be a result of uncontrolled MMP activity, which may lead to breakdown of the stromal cell compartment and/or enhanced ability of the luminal epithelial cells to penetrate the surrounding stromal tissue. The breakdown of the supporting ECM of the stroma could further influence cell proliferation by regulation of specific gene expression and cell-matrix interactions [19,20].

The role of TIMP-1 in uterine physiology has been examined through the generation of TIMP-1 deficient mice. Previous findings from our laboratory demonstrated that TIMP-1 may play a role in uterine adenogenesis as well as estrogen-induced uterine edema [18,21,22]. Further, it was demonstrated that during the estrogen-dominated stages of the reproductive cycle, TIMP-1 null mice have significantly larger (greater wet weight) uteri compared to wild-type mice [18], which are associated with elevated MMP activity [23]. In addition, disruption of the TIMP-1 gene product is associated with altered uterine morphology and TIMP-1 deficient females have a reduced reproductive lifespan [24]. To this point, the mechanism by which estrogen coupled with TIMP-1 deficiency imparts these alterations is unknown. As such, the objective of the current study was to examine the mechanisms by which TIMP-1 deficiency results in the aberrant uterine phenotype characteristic of these mice in response to steroid treatment. In doing so, data gleaned from these studies will provide further insight into the multifunctional role of TIMP-1 in uterine physiology under estrogen and progesterone dominant milieus.

Materials and Methods

Animals

Wild-type and TIMP-1 null mice were utilized for all studies and were obtained from our in-house breeding colony. As the TIMP-1 gene is located on the X chromosome, we only evaluated TIMP-1 homozygous null mice (referred here in as TIMP-1 null). Mice deficient in TIMP-1 (SVTER 129 background) were generated by homologous recombination of a neo-containing gene-targeting vector in mouse embryonic stem cells. Transmission of the mutant allele and the genotype of mice were determined by polymerase chain reaction analysis of the neo sequences in genomic tail DNA. TIMP-1 deficiency was confirmed at the transcript and protein level by Northern blot analysis and protease inhibitor assays, respectively [25].

A breeding colony of both genotypes was generated at the University of Kansas Medical Center. Mice were housed within environmentally controlled conditions under the supervision of a licensed veterinarian. All animal procedures for these experiments were approved by the University of Kansas Medical Center Institutional Animal and Use Committee. Mice were maintained on a 14 L : 10 D photo period and provided water and mice chow *ad libitum*. Eight to twelve week-old female mice of both genotypes were ovariectomized and rested for 14 days before they were randomly assigned to respective treatment groups.

Administration of treatments and tissue harvesting

Mice were ovariectomized and treated as previously described [23]. The experiments in this manuscript were divided into two major studies with the mice in all studies on the same background. For all studies wild-type and TIMP-1 null mice were ovariectomized and rested 14 days to allow clearance of endogenous steroid levels. In the first study, mice of both genotypes were injected s.c. with either vehicle (0.1 ml sesame oil) or estradiol-17 β (E₂, 10 μ g/kg BW) for three consecutive days with each injection 24 h apart. Mice were then sacrificed at 72 h after the initial injection.

A second study was conducted to examine whether the E₂ regulation of serine protease activity was mediated via its cognate nuclear receptor. To do so, mice were injected s.c. with estrogen receptor antagonist ICI-182,780 (ICI; Tocris Cookson Inc., Ellisville, MO) 30 min before steroidal treatment.

Assessment of tissue morphology

The middle two thirds of each right uterine horn were used for histological analysis (N = 6 mice/treatment/genotype). Tissues were embedded in paraffin, serial sectioned at 5 μ M, and stained with hematoxylin-eosin. Sections were analyzed for each uterine specimen to assess the structure/ morphology of the uterine lumen, height of the luminal epithelium and the number of endometrium epithelial glands per cross-section. Luminal epithelial height and endometrial gland density were calculated in the same cross-section using the following approach. Luminal epithelium height was measured in 4 random locations/cross section and this was done on every 10th section (approximately 50- μ m intervals) using a minimum of 10 different sections. Using the same cross sections, endometrial gland number was determined by counting the total number of uterine glands in a complete cross-section of the uterine horn portion. The observation of a gland cross-section with a visible open lumen was counted as a single gland. The number of glands was counted on every 10th section using a minimum of 10 different sections, and an overall average number of glands were calculated for each specimen.

Microarray analysis

Uterine tissue was pooled from 2-3 mice/treatment group/genotype and a total of 2 separate analysis were performed (N=2 per treatment group/genotype). Each sample was processed individually for microarray hybridization following the Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, total RNA from uterine tissue was isolated using Trizol (Invitrogen Corp, Carlsbad, CA), digested with deoxyribonuclease (QIAGEN) and then purified by RNeasy Spin Columns (QIAGEN). *In vitro* transcription was subsequently carried out with GeneChip Expression 3'-Amplification IVT Labeling kit (Affymetrix) to generate biotinylated cRNAs. After chemical fragmentation with 5× fragmentation buffer (200 mM Tris-acetate, pH 8.1; 500 mM KOAc; 150 mM MgOAc), biotinylated cRNAs were mixed with controls and were hybridized to Affymetrix GeneChip® 430A 2.0 microarrays on an Affymetrix fluidics station at the University of Kansas Medical Center Microarray Core Facility. Fluorescent labeling and laser confocal scanning were conducted and generated the data for analysis.

The primary data captured using Microarray Suite 5.0 (MAS 5.0) software resulted in a single raw value for each probe set based on the mean of the differences between the hybridization intensity for the perfect match features and the mismatch features for a particular transcript. Three types of normalizations were applied to this data before additional sorting and analysis. The hybridization intensities across the treatments of a particular cell type were first normalized by applying the global normalization algorithm that trims the mean signal intensity of the experiment to the trimmed mean signal of the baseline or control treatment. To facilitate comparison between samples and experiments, the globally normalized data were further subjected to (a) a per chip normalization to account for chip-wide variations in intensity by dividing each intensity value by the 50th percentile of all values on the chip, and (b) a per gene normalization where each gene is divided by the intensity of that gene in the control sample. To perform the global normalizations with high confidence, a regression analysis (Pearson correlation coefficients) was done using the raw signal intensities of various treatments.

These files from expression analyses were then exported via MicroDB and Data Mining Tool (Affymetrix) for additional filtering and analysis. In these analyses, genes designated as significantly changed were those (a) that possessed a reliably detectable signal (absolute call ≠ “Absent” or “Marginal” in the case of repressions, whereas, in the case of inductions, differentially induced genes were taken into analysis), (b) have detection $P \leq 0.05$, and (c) as determined by the statistical algorithm to be changed ≥ 2 -fold (change call ≠ “no change” or “marginal”). To additionally increase the significance of expression changes, we interrogated our data sets for an increase in average difference (intensity) of at least 2-fold at both time points.

Gelatin and Casein zymography

Frozen uteri were homogenized (1:15, w/v) in homogenization buffer (0.5 M Tris-HCl [pH 7.6], 0.2 M NaCl, 0.01 M CaCl₂ and 1.0 % [w/v] Triton X-100). Uterine homogenates were placed on ice for 5 min, followed by centrifugation at 12,000 X g for 30 min at 4 °C. Supernatants were then collected, and an aliquot was subjected to the DC Protein Assay (Bio-Rad Laboratories, Richmond, CA) to determine the total protein concentration. The remaining sample was stored at 4 °C until zymographic analysis.

Protease activity was determined by gelatin and casein zymography as previously described but with minor modification [23]. Briefly, uterine extracts containing equal amount of protein (30 µg) were subjected to substrate gel zymography by electrophoresis in 10 % polyacrylamide gels containing 1 mg/ml of gelatin or in precast, 4-16 % polyacrylamide gels

containing 1 mg/ml of blue casein under non-reducing conditions (Invitrogen, Carlsbad, CA). Following electrophoresis, the gels were washed twice with 2.5 % (v/v) Triton- X 100 for a total of 60 min at room temperature (RT) to remove SDS. The gels were then placed in incubation buffer (50 mM Tris [pH 8.0] and 5 mM CaCl₂) for 30 min at RT. Finally, the gels were incubated in fresh incubation buffer for 18 –24 h at 37 °C to allow proteolysis of the substrates in the gels. Gelatin-impregnated gels were then rinsed with water and stained with Brilliant Blue – R250 dye (2.5 mg/ml) and destained until clear bands became evident. Blue casein-impregnated gels required no staining to allow detection of the caseinolytic activity. Prestained molecular weight marker (Invitrogen, Carlsbad, CA) was co-electrophoresed in all gels to determine the masses of the lysis bands. The relative mass/molecular weight of bands of activity was analyzed using the GDS-8000 System (Ultra Violet Products; Upland, CA).

Inhibition studies were carried out by adding various amounts of inhibitors to the incubation buffer during overnight incubation. 20 mM 1, 10-phenanthroline (Sigma), 20 mM ethylenediaminetetraacetic acid (EDTA, Sigma), 5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 20 µM leupeptin (Sigma) and 50 µM pepstatin A (Sigma) were used for characterizing both the gelatinolytic and caseinolytic activity. Additionally, 1 % (v/v) aprotinin (Sigma), 10 µM E-64 (Sigma) and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, Sigma) were used for characterizing the caseinolytic activity.

Western blot

Expression of plasminogen/plasmin was determined at the protein level by Western blot analysis. Total protein content was extracted from frozen uteri using radioimmuno-precipitation assay buffer (RIPA buffer; 20 mM Tris [pH 7.5], 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing a protease/phosphatase inhibitor cocktail (0.1 mg/ml PMSF, 30 µl/ml aprotinin, 5 µg/ml leupeptin, 1 mM sodium orthovanadate; Sigma). Protein concentration in all samples was determined using the DC Protein Assay (Bio-Rad Laboratories, Richmond, CA). Similar amounts of protein (35 µg) were subjected to 4-12% Bis-Tris (Invitrogen, Carlsbad, CA, USA) gel electrophoresis under non-reducing conditions and electro-blotted onto PVDF membranes (Invitrogen). Immunodetection was carried out using a chromogenic immunodetection kit (Invitrogen). Rabbit antibodies were used for plasminogen/plasmin (0.5 µg/ml; Innovative Research, Southfield, MI, USA) and actin (1 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunodetection of actin was conducted to confirm the equal loading of proteins. Masses of the bands and relative intensity were analyzed using the GDS-8000 System (Ultra Violet Products; Upland, CA).

Assessment of plasmin activity

Plasmin activity was assessed using the plasmin-specific chromogenic substrate D-Val-Leu-Lys-p nitroanilide dihydrochloride (VLKpNA; Sigma; V7127). Briefly, samples (50 µl volume) were incubated with 950 µl of assay buffer (50 mM Tris buffer (pH 7.4) containing 110 mM NaCl) containing 0.6 mM VLKpNA substrate. Samples were incubated at 37 °C for 24 h (time was determined empirically for plasmin activity as was in the linear range). At the end of the incubation period, plasmin activity was determined by reading the samples at 405 nm absorbance. Optical density (OD) was determined and plasmin activity is reported as the change in OD from substrate blanks normalized per mg of protein in each sample.

Quantitative Real-Time RT-PCR for mRNA assessment

To confirm identity and patterns of expression of select genes, quantitative real-time PCR was performed. Total RNA (2 µg) isolated with Trizol reagent was treated with DNase I followed by cDNA synthesis using Maloney murine leukemia virus reverse transcriptase and

random hexamer primer. The resulting cDNAs were diluted 1:10 in sterile water, and 1- μ l aliquots were used in the quantitative real-time PCR reactions. The primers for murine serpinb7/megsin (GenBank NM_027548; forward primer 5' – CTTCACTGCCCTGACCCTAATC – 3' and reverse primer 5' – TGCAGTGCCTTGTCATCTGA – 3') and serpinb2/PAI-2 (GenBank NM_011111; forward primer 5' – TGGAGCCAGACCTGCTAAGC – 3' and reverse primer 5' – AACAGCTCCAAGGCGAAGAG – 3') were designed with Primer Express 2.0 and quantitative RT-PCR was carried out on an Applied Biosystems HT7900 Sequence Detector. To account for differences in starting material, human 18S primers and probe reagents were used. A standard curve was run in each assay, with an arbitrary value assigned to the highest standard and corresponding values to the subsequent dilutions. Each cDNA sample was run in triplicate and the relative abundance of each target divided by the relative abundance of 18S in order to normalize for the starting quantity of cDNA.

In-situ hybridization

To investigate cell-specific distribution of serpinb7 transcript within the uterus, *in-situ* hybridization (ISH) was performed. Briefly, the middle 1/2 of the uterine horns was excised, embedded in O.C.T. tissue freezing medium (Sakura Finetek U.S.A., Inc. Torrance, CA, USA) and snap-frozen on dry ice. 10 μ m cryostat sections were placed on baked, silane-coated (2% 3-aminopropyltriethoxysilane in acetone) slides and stored at –80 °C until use. RNA probes complementary to the mRNA for serpinb7 were synthesized using PCR and digoxigenin labeling. Total RNA isolated with Trizol reagent was treated with DNase I followed by cDNA synthesis using Maloney murine leukemia virus reverse transcriptase and random hexamer primer. Anti-sense and sense primers were designed with Primer Express 2.0 that incorporate T7 or T3 phage transcription promoter sequences at the 5' end. Probes were labeled during *in vitro* transcription with the digoxigenin RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. Tissue sections were post-fixed in 4% paraformaldehyde, denatured and acetylated followed by prehybridization at 68 °C for 45 min. After prehybridization, uterine sections were hybridized to digoxigenin-labeled cRNA probes at 68 °C for 45 min, then cooled to 42 °C for 24 h. Hybridization signal was detected by antidigoxigenin-alkaline phosphatase antibody (Boehringer Mannheim, IN, USA) according to manufacturer's instructions. Negative controls included sense probes which were hybridized in identical fashion to that of the anti-sense probes as well as omission of either probe during hybridization of the tissue sections. To gain a semi-quantitative assessment of serpinb7 expression, HSCORE analysis was performed [Lessey *et al.*, 1994] where $HSCORE = \sum P_i (i + 1)$, where *i* is the intensity of hybridization signal with a value of 0 (absent), 1 (weak), 2 (moderate) or 3 (strong) and *P_i* is the percentage of each cell type (0 -100%) for each intensity. Three cross sections were randomly selected for each treatment for each genotype and the values were averaged to obtain an average HSCORE for each specimen. HSCOREs for each specimen for each treatment/genotype were then statistically analyzed as described below (N=3 treatment/genotype).

Statistical analysis

Unless otherwise indicated, all data was analyzed by one-way ANOVA for comparison across treatment regimes. When an F test indicated statistical significance, post-hoc analysis was made using the Tukey HSD procedure. Unpaired t-tests were used for planned comparisons between genotypes within each specific treatment group. Significance was set at $p < 0.05$ for all comparisons.

Results

Estrogen (E₂) regulation of uterine histo-architecture was examined in mice of both genotypes. Chronic exposure to E₂ (3 injections, 24 h apart) induced a uterine histo-architecture similar to that previously reported in adult intact females [18] which was characterized by precocious luminal folds that exhibited profound branching often extending deep into the stroma to the periphery of the circular smooth muscle (Fig 1). Further, a clearly defined uterine lumen could not be identified in the uteri of the TIMP-1 null mice compared to wild-type counterparts (Fig. 1). In addition, the number of endometrial glands (Fig. 2) and height of the uterine luminal epithelial cells (Fig. 3) were also significantly greater in the TIMP-1 null mice treated with E₂ compared to wild-type counterparts. These data suggest that in the absence of TIMP-1, E₂ induces a profound effect on uterine luminal and glandular epithelial cells which result in an altered uterine histo-architecture similar to that detected in mature, reproductively cycling female mice during the period of estrus.

To begin to assess the mechanisms which may contribute to the altered uterine phenotype, cDNA microarray analysis was performed to compare gene profiles for uterine tissue from wild-type and null mice treated with E₂. For this study, we focused on genes whose expression was significantly increased or decreased at least 3-fold between genotypes. Of the 14,000 genes examined, 22 genes increased at least 3-fold (Table 1) while 115 genes decreased at least 3-fold in the uteri of TIMP-1 null female mice compared to wild-types (Table 2). Most of the genes which displayed the largest decrease in the TIMP-1 null mice are genes which are proposed to play a role in keratinization and cornification (Table 2). The observation that no significant changes (≥ 3 -fold) in MMP inhibitors or MMPs in the null uteri which could be associated with the altered uterine histo-architecture was unanticipated but suggests that under the defined hormonal milieu that compensatory changes in the expression of TIMP-2, -3, and -4 may not occur. Further, assessment of MMP activity by gelatin zymography indicated no changes in MMP-2 or MMP-9 activity between genotypes (data not shown).

In contrast, we did identify two regulators of serine protease activity whose expression was significantly reduced in the uteri of TIMP-1 null mice (Table 2). Serpinb2 (also known as plasminogen activator inhibitor-2 [PAI-2]) was reduced over 8-fold in the uteri of TIMP-1 null mice, while serpinb7 (also known as megin) was reduced over 3-fold (Table 2). To verify the identity and relative changes in the expression levels of these protease regulators, we utilized an aliquot from the pooled uterine tissue used for microarray analysis (N=2/treatment group/genotype) in addition to tissue from 3 additional mice/treatment group/genotype (for a total of 5 separate analysis; N=5). As displayed in Figure 4, serpinb7 levels were reduced in response to E₂ administration, however, the relative level of expression in both 0 and 72 h E₂ treatment groups was approximately 50% lower in the TIMP-1 null mice. In contrast to the E₂-induced reduction in serpinb7 expression, E₂-induced an approximate 7-fold increase in serpinb2 expression over control levels in wild-type mice. However, much like that of serpinb7, serpinb2 expression was approximately 50% lower than that detected in wild-type mice. These data suggest that although regulated by E₂ in a similar manner to that of wild-type mice, both serpinb7 and serpinb2 exhibit markedly reduced levels of expression which may contribute to the elevation in uterine plasmin activity in these mice (see below).

As our microarray assessment indicated that there may be alterations in the plasmin/plasminogen system (due to decrease inhibitor expression) which may lead to the altered uterine histo-architecture in the TIMP-1 null mice, we next wished to assess serine protease activity in these tissues. Assessment of serine protease activity was performed initially by casein zymography. As depicted in Figure 5A, E₂ induced bands of approximately 79 and 75

kDa which were not detected in the uteri of vehicle-treated mice. Surprisingly, no other bands of caseinolytic activity were detected in the uterine samples suggesting that the two detected bands of activity represented the two most abundant proteases (using 30 μ g of protein for analysis). To further confirm identity of the protease responsible for this activity, we conducted the following series of experiments. First, we wished to confirm that the protease activity was due to a serine protease. This would eliminate other classes of proteases which might be able to degrade casein which would display similar molecular weights with the bands of detected activity. The MMP inhibitors (1, 10-phenanthroline and EDTA), the aspartate/acid protease inhibitor (pepstatin A) and the cysteine protease inhibitor (E-64) failed to inhibit the 79/75 kDa band of activity suggesting that this enzyme was not a MMP, aspartate/acid, or cysteine protease (data not shown). In contrast, serine protease inhibitors (PMSF, aprotinin, AEBSF and leupeptin) all inhibited protease activity suggesting that this activity was due to a serine protease (data not shown). To confirm that the serine protease was indeed plasmin, we performed Western analysis. As depicted in Figure 5B, plasmin could be detected by Western analysis in all samples regardless of genotype or treatment. E₂ treatment induced an approximate 2-fold increase in plasmin protein levels when normalized to actin levels in mice of both genotypes. Of interest was the observation that despite rather abundant protein levels for plasmin, plasmin activity was essentially undetectable by zymography in vehicle-treated mice of both genotypes (Fig 5A). Further, although E₂ induced a significant increase in protein levels of plasmin (over vehicle-treated), this increase in protein levels was similar between genotypes. This is in contrast to the difference in plasmin activity between genotypes and may suggest that the difference in plasmin activity may be due to reduction in the activity of plasmin inhibitors. To gain a more quantitative index in the change in plasmin expression between genotypes and verify that the increase was not due to separation of endogenous inhibitors from protease induced by electrophoresis, we performed a quantitative plasmin activity assay. As depicted in Figure 5C, E₂ increased plasmin activity in mice of both genotypes and this increase was significantly greater in the TIMP-1 null mice and is in accord with the qualitative analysis (Fig. 5A) of plasmin activity.

To determine if this effect of E₂ was mediated through its cognate nuclear receptors, a separate group of mice were treated with the estrogen receptor antagonist ICI 182,780 (ICI). As depicted in Figure 6, ICI pretreatment blocked the E₂ increase in plasmin activity verifying the specificity of the E₂ effect. ICI alone had no effect on plasmin activity.

As discussed earlier, serpinb7 has been described as a kidney-specific serpin and expression outside of this organ has yet to be documented. To begin to assess the cellular localization of serpinb7 within the uterus, *in-situ* hybridization was performed. As depicted in Figure 7, serpin7 was localized primarily to the luminal epithelial cells in mice of both genotypes. In vehicle treated wild-type mice, serpinb7 expression was highest in luminal and glandular epithelial cells (Fig. 7) and its expression was significantly reduced in response to E₂ treatment (Figs 7 and 8). Further, wild-type vehicle-treated mice expressed a minimal signal for serpinb7 in stroma underlying the luminal epithelium but expression was not detected in the deeper stromal compartment. This minimal stromal signal was diminished in response to E₂ treatment (Figs 7 and 8). Myometrial expression of serpinb7 was not detected. Overall, serpinb7 was expressed primarily in uterine epithelial cells and this expression was significantly reduced in response to E₂ treatment. In TIMP-1 deficient mice, a very similar pattern of serpinb7 expression was detected with the strongest signal detected in luminal and glandular epithelial cells but the overall level of epithelial cell expression was significantly reduced (Figs. 7 and 8). This level of expression is in agreement with the more quantitative qPCR assessment (Fig. 4) and together these data strongly suggest that uterine serpinb7 expression is primarily localized to epithelial cells, its expression is decreased by E₂, and TIMP-1 null mice exhibit reduced levels of this serpin.

Discussion

TIMP-1 deficient female mice are subfertile [24] and exhibit uterine alterations which include aberrant uterine structure, adenogenesis, and vaginal/uterine epithelial cell cornification [18]. To begin to assess the mechanisms responsible for these abnormalities which occur as a result of disruption of the TIMP-1 gene product, we began by performing microarray gene profile analysis between E₂-treated uterine tissue from wild-type and TIMP-1 null mice. We initially concentrated on identifying alterations in proteases and protease inhibitors whose expression may be altered due to loss of TIMP-1. Surprisingly, we were unable to detect any significant compensatory changes in TIMP-2 or TIMP-3 as well as any of the currently identified murine MMPs in the uteri from these mice. TIMP-1 exhibits the broadest specificity of the four TIMPs and is capable of binding the majority of MMPs, while TIMP-2, TIMP-3 and TIMP-4 exhibit more preferential inhibition of MMPs. Together, these findings may suggest that although there is some redundancy among the TIMPs in their ability to regulate MMP activity [14,15] there is no or very little overlap at the transcriptional level for these MMPs inhibitors in response to deletion of the TIMP-1 gene product.

In contrast to gene expression for the MMP system, we did detect alterations in members of the serine protease system. Most notably, we detected significant reductions in transcript expression for two serine protease inhibitors; *serpinb2* which is also known as plasminogen activator inhibitor type-2 (PAI-2) and *serpinb7* which is also known as megin.

Serpinb2 is expressed in a variety of cell types and linked to a variety of physiological and pathological processes which include differentiation of epidermal keratinocytes [28], monocytes proliferation/differentiation [29], pancreatic adenocarcinoma [30], and endometrial carcinoma [31,32]. Within the context of uterine physiology, *serpinb2* expression has been associated with embryo implantation and pregnancy. PAI-2 (*serpinb2*) was first described as a placental-derived uPA inhibitor by Kawano and colleagues [33] and its expression has been further examined in the human and rhesus monkey by others [34–37]. PAI-2 expression in mouse placenta is uncertain and disruption of the PAI-2 gene product in mice is not associated with an alteration in pregnancy [38]. The E₂ stimulation of uterine PAI-2 to our knowledge is the first description of steroidal regulation of uterine PAI-2 and may suggest that this serine protease inhibitor may play a functional role during the reproductive cycle under estrogen-dominated milieu.

In contrast to the level of expression of *serpinb2*, *serpinb7* expression was abundant in uterine tissue in mice of both genotypes and was down-regulated by E₂. To our knowledge, this is the first description of *serpinb7* in uterine tissue of any species. *Serpinb7* (megsin) was first described in 1998 as a mesangium-predominant gene in human glomeruli [39] and has subsequently been cloned in the rat [40]. Elevated levels of megin have been detected in the glomeruli of patients with IgA nephropathy [41] and in a rodent model of mesangioproliferative glomerulonephritis [40]. Overexpression of megin in transgenic mice [42] is associated with progressive mesangial matrix expansion and increased numbers of mesangial cells. The precise role of megin within the kidney is uncertain. Megsin has been shown to inhibit plasmin activity [42] but the mechanism by which this serpin functions within the context of mesangial disorders remains uncertain. It is postulated that increased levels of megin/*serpinb7* may inhibit protease activity which would normally lead to the degradation of proteins which in turn influence matrix expansion and mesangial cell proliferation.

With respect to our current findings, we propose that *serpinb7* may actually elicit a similar function (regulation of plasmin activity) within the uterus but whose end result might be

opposite to that in the kidney. High levels of uterine serpinb7 (as detected in wild-type vehicle-treated mice) may inhibit plasmin activity as would occur under an environment characterized by low E₂ levels. In response to E₂ administration, serpinb7 expression would be down-regulated and may allow for increases in plasmin activity. Where in the kidney, this increase in protease activity leads to decreased cell proliferation, we postulate that within the uterus, decreased serpinb7 may contribute to the increase in uterine epithelial cell mass associated with E₂ administration. While it is tempting to speculate that decreases in uterine serpinb7 expression may be associated with E₂-associated uterine pathologies which are influenced by unopposed E₂ action, further examination of the role of serpinb7 under such milieus may be warranted.

The observation of altered expression of serine protease inhibitors and plasmin activity in the TIMP-1 null mice was unexpected. Interestingly, no significant difference in plasmin protein levels was observed between the two genotypes, but marked differences were detected in plasmin activity after E₂ treatment. This may be due to presence of inhibitors such as serpinb7 and serpinb2. In wild-type mice, E₂ reduced serpinb7 expression which may allow for increased plasmin activity. In the null mice, the further reduction in serpinb7 coupled with the dampened ability of E₂ to induce serpinb2 may lead to a net result of increased plasmin activity in the null mice compared to wild-type counterparts. As plasmin has been proposed to play a key role in the ECM remodeling in the uterus (34-38), our observations may suggest that elevated activity may contribute to the process of abnormal uterine histo-architecture in the null mice. This is supported by the findings that E₂ induction of elevated plasmin activity was associated with increased luminal epithelial height, increased gland density and abnormal uterine development in the null mice compared to wild-type counterparts.

In summary, the current study demonstrates that in the absence of TIMP-1, E₂ induces a uterine histo-architecture identical to that detected in reproductive age mice during the estrus stage of the estrous cycle. Associated with this phenotype are an increase in uterine plasmin activity and a reduction in mesgin/serpinb7. Further, we describe for the first time the expression of mesgin/serpinb7 within the uterus, demonstrate its modulation by E₂ and its localization to uterine glandular and luminal epithelial cells. The potential role of serpinb7 and its potential modulation through TIMP-1/MMPs within the uterus may provide novel insight into the cross-talk between protease systems within this tissue in both physiological and perhaps pathological processes.

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Fig. 1.

Representative light photomicrographs of uteri from TIMP-1 wild-type and null mice treated with either vehicle or estrogen. Wild-type (+/+) and TIMP-1 null mice (-/-) were ovariectomized, treated with either vehicle (veh) or estrogen (E_2) and uteri were removed and processed for histological assessment as described in *Materials and Methods*. Each photomicrograph is representative of four separate observations per treatment group ($N = 4$). Magnification for Veh +/+ and Veh -/- are X 100 while that of E_2 +/+ and E_2 -/- are X 40.

Fig. 2.

Effect of TIMP-1 disruption on endometrial gland density. The number of endometrial glands per cross-section were calculated as described in *Materials and Methods* and were compiled from 10 different cross-sections per specimen for each treatment group with 4 animals/treatment group ($N = 4$). Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired t -test (* = $P < 0.05$).

Fig. 3. Effect of TIMP-1 disruption on luminal epithelial cell height. Luminal epithelial cell height was calculated as described in *Materials and Methods* and was compiled from 10 different cross-sections per specimen for each treatment group with 4 animals/treatment group ($N = 4$). Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired t -test ($* = P < 0.05$).

Fig. 4.

Quantitative analysis of uterine expression and regulation by estrogen of serpinb7 and serpinb2. RNA was isolated and quantitative real-time PCR was performed as described in *Materials and Methods*. Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired t -test ($* = P < 0.05$).

Fig. 5. Plasmin activity within the uterus of wild-type and TIMP-1 null mice in response to estrogen treatment. Wild-type (+/+) and TIMP-1 null mice (-/-) were treated as described in *Materials and Methods*. (A) Caseinolytic activity was examined by casein zymography and revealed a 81/79 kDa doublet detected only after estrogen treatment (E₂). This activity was more abundant in the null mice. (B) Western analysis revealed that this 81/79 kDa band of activity was plasmin. (C) Plasmin activity was quantitated using a colorimetric plasmin assay and revealed a significant increase in uterine plasmin activity in response to E₂ treatment. This activity was greater in TIMP-1 null mice (-/-) compared to wild-type mice (+/+) treated with E₂. Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired t -test (* = $P < 0.05$).

Fig. 6.

Estrogen induction of plasmin activity occurs via cognate nuclear estrogen receptor pathway. To determine if estrogen (E_2) regulation of plasmin occurs via cognate estrogen receptor pathway, animals were treated with the estrogen receptor antagonist ICI 182,780 (ICI) followed 30 min later by treatment with E_2 . Mice were killed at 72 h after steroid administration, and plasmin activity was analyzed using a colorimetric assay as described in *Materials and Methods*. Data were normalized per mg of protein in the sample and are reported as arbitrary units for four separate observations ($n = 4$ mice/treatment group). Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired t -test ($* = P < 0.05$).

Fig. 7.

In-situ hybridization of uterine serpinb7 in wild-type and TIMP-1 null mice treated with estrogen. Wild-type (+/+) and TIMP-1 null mice (-/-) were ovariectomized, treated with either vehicle (veh) or estrogen (E_2) and uteri were removed, processed and *in-situ* hybridization was performed as described in *Materials and Methods*. Each photomicrograph is representative of results obtained from four separate observations per treatment group per genotype (N = 4). Magnification for Veh +/+ and Veh -/- are X 200 while that of E_2 +/+ and E_2 -/- are X 100.

Fig. 8. Semi-quantitative assessment of uterine serpinb7 expression in uterine tissue. Signal intensity of the serpinb7 *in-situ* hybridization was calculated using the HSCORE system as described in *Materials and Methods* and is displayed for epithelial (A) and stroma cells (B). Data are expressed as the mean $\pm$ SEM for four separate observations for each cell type ($n = 4$ mice/treatment group/genotype). Different letters indicate statistical significance ($P < 0.05$) between treatment groups within genotype by one-way ANOVA (block letters indicate comparisons within wild-type mice while bold letters indicate comparisons within null mice). Asterisks (*) indicate statistically significant differences between genotypes within treatment group by planned comparisons using unpaired *t*-test (* = $P < 0.05$).

Table 1

Genes upregulated in TIMP-1 null mice treated with estrogen/associated with altered uterine phenotype.
Genes with increased expression in TIMP-1 deficient mice after estrogen treatment.

Gene	Function	Accession no.	Change
Stimulated by retinoic acid gene 6	unknown	NM_009291	8.0
Interleukin-1 α	inflammatory response	BC003727	5.8
Transgelin-2	cell structure	AV212626	5.5
Wdr9	transcription	BC024797	4.6
Interleukin-1 β	inflammatory response	BE986062	4.5
Kell blood group	metalloendopeptidase	NM_032540	4.2
TSC22-related inducible leucine zipper 3b	transcription	AF201288	4.1
TRH-degrading ectoenzyme	TRH inactivation	BB245038	4.0
Mast cell protease 6	degranulation	NM_010781	3.9
Viral hemorrhagic septicemia virus induced gene 1	unknown	BB741897	3.7
Thymic stromal lymphopoietin	inflammatory/allergic	NM_021367	3.5
Glucocorticoid-regulated inflammatory prostaglandin synthase	inflammatory response	M88242	3.5
Hemoglobin alpha, adult chain 1	oxygen transport	NM_008218	3.4
Parvin, gamma	cell adhesion	NM_022321	3.4
4-hydroxyphenyl pyruvic acid dioxygenase	catabolism	BC013343	3.3
Small inducible cytokine subfamily, member 2	inflammatory response	NM_009140	3.3
Complexin-1	neurotransmitter release	NM_007756	3.2
Apolipoprotein A-II	HDL metabolism	NM_013474	3.2
Low density lipoprotein receptor related protein 5	cell signaling	BC011374	3.1
Dead ringer homolog 2	DNA binding protein	BM247863	3.0
Zinc finger protein 316	transcription	NM_017467	3.0
Homeobox D11 (Hoxd11)	transcription	NM_008273	2.9

Table 2

Genes down-regulated in TIMP-1 null mice treated with estrogen/associated with altered uterine phenotype.
Genes with decreased expression in TIMP-1 deficient mice after estrogen treatment.

Gene	Function	Accession no.	Change
Keratin complex 1, acidic gene 1	structural	NM_008470	- 236.0
Loricrin	keratinization	NM_008508	- 136.0
Keratinocyte differentiation associated protein	keratinization	AV007306	- 122.1
Calmodulin 4	cell signaling	NM_020036	- 113.3
Repetin	epidermal differentiation	NM_009100	- 96.4
Small proline-rich protein 1B	keratinization	NM_009265	- 92.7
Epidermal keratin subunit II	transcription	K02108	- 85.8
Keratin complex 2, basic gene 1	structural	NM_008473	- 60.5
Small proline-rich like 1	keritization	AV071932	- 58.6
Small proline-rich protein 3	keritization	NM_011478	- 50.8
Keratin complex 1, acidic gene 13	structural	NM_010662	- 50.1
GPI-anchored metastasis-associated protein homolog	unknown	NM_133743	- 44.2
Keratin 14	structural	BC011074	- 42.2
Keratin complex 1, acidic gene 10	structural	AK014360	- 39.9
Prostate stem cell antigen	unknown	AF319173	- 37.4
Small proline-rich protein 9	keritization	NM_026335	- 37.4
Desmocollin 1	cadherin function	NM_013504	- 36.4
Keratin complex 2, basic gene 6b	structural	NM_010669	- 34.0
Keratin complex 2, basic gene 6a	structural	NM_008476	- 33.8
Plakophilin 1	transcription	NM_019645	- 33.7
Small proline-rich like 3	keritization	NM_025984	- 31.6
Small proline-rich like 7	keritization	NM_027137	- 29.5
Involucrin	keritization	AV009441	- 27.1
Small proline-rich protein 2h	keritization	NM_011474	- 25.0
Keratin complex 2, acidic gene 4	structural	NM_008475	- 24.4
Stefin A3	protease inhibitor	NM_025288	- 23.6
Hornerin	cornification	AY027660	- 23.5
Kallikrein 8/Neuropsin	protease	NM_008940	- 21.5
Elovl4	fatty acid biosynthesis	AF277093	- 16.9
Keratin complex 1, acidic gene 15	structural	NM_008469	- 15.1
Small proline-rich like 2	protein binding	NM_028625	- 14.3
Serine protease 20	protease	NM_019974	- 14.1
Kallikrein 7	protease	BB283507	- 13.7
Keratin complex 1, acidic gene 17	structural	AA798563	- 13.7
Filaggrin	keratinization, apoptosis	J03458	- 12.7
Lymphocyte antigen 6 complex	proliferation	NM_010742	- 12.5
Serpib2/Plasminogen activator inhibitor 2	protease inhibitor	NM_011111	- 12.3
Galectin-7	apoptosis	AF038562	- 11.3

Gene	Function	Accession no.	Change
Fatty acid binding protein 5	lipid binding	BC002008	- 10.7
Pit X1	transcription	NM_011097	- 10.6
Defensin b4	barrier/defense response	NM_019728	- 10.6
Haik-1	structural	NM_033373	- 9.5
Trp 63	polarization, differentiation, apoptosis	NM_011641	- 9.1
Small proline-rich like 5	keratinization	NM_026822	- 9.0
Wdt-2	pigmentation	AV298358	- 8.9
Keratin complex 1, acidic gene 14	structural	BE197934	- 8.5
Glutathione-S-transferase alpha 2	metabolism	NM_008182	- 8.5
G-protein coupled receptor 87	signal transduction	NM_032399	- 8.4
Defensin beta-1	barrier, defense response	BC024380	- 8.1
Visinin-like 1	calcium binding	NM_012038	- 8.0
Aquaporin-3	water transport	AF104416	- 7.8
Placental protein 11 – related	proteolysis	AI528824	- 7.4
Ace-related carboxypeptidase	proteolysis	AK008530	- 7.3
TA*p63 alpha	polarization, differentiation, apoptosis	AF075436	- 7.2
Acidic epididymal glycoprotein 1	detoxification	NM_009638	- 7.2
Sine oculis-related homeobox 1 homolog	transcription	BB137929	- 6.3
Calgranulin A	chemotaxis	NM_013650	- 5.9
ACE-2	proteolysis	AB053181	- 5.9
Calponin-3, acidic	smooth muscle contraction	BB490338	- 5.9
Transcription factor AP-2 alpha	transcription	AI507504	- 5.8
3-5 exonuclease TREX 2	DNA repair	AF151108	- 5.7
MDP-77	unknown	AF422244	- 5.7
JNK binding protein-1	signal transduction	BC026444	- 5.6
IL-1, family member 5, alpha	inflammatory response	NM_019451	- 5.5
Fatty acid binding protein-4, adipocyte	inflammatory response	NM_024406	- 5.3
Procollagen Type XVII, alpha I	extracellular matrix	NM_007732	- 5.3
Parathyroid hormone-like peptide	signal transduction	81685341	- 4.9
Epiregulin	differentiation, proliferation	NM_007950	- 4.9
Sciellin	differentiation	NM_022886	- 4.8
Gasdermin	apoptosis	AI506986	- 4.7
Serine protease 18	protease	NM_011177	- 4.7
Cannabinoid receptor (brain)	signal transduction	NM_007726	- 4.6
Cytochrome P450 cyp3a13	steroidogenesis	NM_007819	- 4.3
Nik-related kinase	phosphorylation	NM_013724	- 4.3
Prominin-related protein	membrane-membrane interactions	NM_138750	- 4.2
Gastrotropin	ligand binding	NM_008375	- 4.2
Fibromodulin	collagen fibrinillogenesis	BB504826	- 4.1
Defensin beta 6	barrier/defense response	NM_054074	- 4.1
Guanine nucleotide binding protein alpha 15	signal transduction	NM_010304	- 4.0
Lymphocyte antigen 116	signal transduction	BB199001	- 3.9

Gene	Function	Accession no.	Change
Interleukin-1, family member 9	inflammatory response	AY071843	- 3.9
Discoidin domain receptor family member 1	cell adhesion	BB378700	- 3.8
Chaperonin subunit 4 delta	protein folding	AV216906	- 3.8
Elafin-like protein II	defense response	NM_138684	- 3.8
UCP-3	transport	AB010742	- 3.8
Calsequestrin 2	calcium binding	NM_009814	- 3.7
Annexin A8	calcium binding	NM_013473	- 3.7
Dihydropyrimidinase-like 2	hydrolase activity	NM_009955	- 3.6
Basic domain leucine zipper	transcription	L36435	- 3.6
Cellular retinoic acid binding protein 2	transport/metabolism	BC018397	- 3.5
Phospholipase A2, group IIA	cholesterol transport	NM_011108	- 3.5
Carboxypeptidase N, polypeptide 1	proteolysis	NM_030703	- 3.4
Squamous cell carcinoma antigen 2	cysteine protease inhibitor	NM_009126	- 3.4
Serpinb7 / Megsin	serine protease inhibitor	NM_027548	- 3.4
Mosaic serine protease	serine protease	BF138523	- 3.4
Annexin A9	cell-cell adhesion	AK003395	- 3.3
Apoptosis-associated speck-like protein	apoptosis	BG084230	- 3.3
RAS, guanyl releasing protein 2	signal transduction	BE688720	- 3.3
Septin 2	cell cycle/division	AV304911	- 3.3
Serum amyloid A2	acute phase response	NM_011314	- 3.3
Chloride intracellular channel 3	ion transport	AK009020	- 3.2
Transforming growth factor beta 2	proliferation/differentiation	BF144658	- 3.2
SH3 domain binding protein 1	signal transduction	NM_009164	- 3.2
Fibroblast growth factor 17	proliferation	NM_008004	- 3.2
Fetuin beta	protease inhibitor	NM_021564	- 3.1
Neuronal tyrosine threonine phosphatase 1	dephosphorylation	NM_008748	- 3.1
Carbonic anhydrase 13	metabolism	AK010166	- 3.1
Macrophage galactose N-acetyl-galactosamine specific lectin	calcium binding	NM_010796	- 3.1
Interleukin-1 receptor type II	inflammatory response	NM_010555	- 3.1
Dystonin	structural	AV251091	- 3.1
Lipocalin 5	retinoic acid binding	NM_033145	- 3.0
Pregnancy specific glycoprotein 23	unknown	NM_020261	- 3.0
Calcitonin-related peptide beta	vasodilatation	NM_054084	- 3.0
Flavin-containing monooxygenase 5	electron transport	NM_010232	- 3.0
Endothelin converting enzyme-like 1	proteolysis	NM_021306	- 3.0