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Expression of Cystatin C in the rat endometrium during the peri-implantation period ☆

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Abstract

Endometrial receptivity for embryo implantation in the rat is a transient state occurring on day 5 of pregnancy or pseudopregnancy and is controlled by estrogen and progesterone. To identify genes potentially involved in receptivity, a uterine cDNA library was screened. An interesting pattern for Cystatin C (*Cst3*) expression was discovered with a peak in abundance just prior to embryo implantation (day 4 of pregnancy) followed by a significant drop the following day when implantation is initiated. Histology localized *Cst3* mRNA and CST3 protein to the glandular epithelium on day 4 of pregnancy suggesting that it is secreted into the uterine lumen at this time. In ovariectomized rats endometrial *Cst3* mRNA levels decreased within 3 h of treatment with estradiol; this effect was inhibited by the anti-estrogen, ICI 182, 780. The data suggest that the endometrial expression of the cysteine protease inhibitor, *Cst3*, is modulated by estrogen during the peri-implantation period.

Keywords: Endometrium; Uterus; Cystatin C; Proteinase; Receptivity; Implantation; Rat; Estradiol; Peri-implantation period; Progesterone

In the rat, blastocysts can implant only if the endometrium has been hormonally primed; this requires a minimum of 48 h of progesterone (P_4) followed by estradiol (E_2) and induces a transient state of endometrial receptivity to embryo implantation (reviewed in [1]) that lasts for less than 24 h [2]. The time surrounding the window of receptivity in the rat is referred to as the peri-implantation period and includes days 4, 5, and 6 of pregnancy. The endometrium passes through three distinct phases during this time referred to as the neutral (day 4), receptive (day 5), and refractory (day 6) phases with respect to embryo implantation. Embryo transfer experiments have revealed that blastocysts transferred to the uterine lumen of day 4 pseudopregnant recipients remain in a state of dormancy until the endometrium becomes receptive on day 5 when the process of embryo implantation can be initiated. In contrast blastocysts transferred to a day 6 endometrium encounter a hostile, non-receptive environment and are expelled and/or degraded [1,3]. In response to implanting embryos the underlying endometrial stromal cells undergo decidualization (reviewed in [1]), involving the proliferation and differentiation of endometrial stromal cells into polyploid, glycogen-filled decidual cells which eventually form the maternal portion of the placenta.

Although the hormonal control of receptivity is well documented in the rodent, changes in gene expression and/or molecular cascades that bring about endometrial receptivity are still largely unknown. To identify novel genes, potentially involved in the transition of the endometrium from the neutral (day 4) to receptive (day 5) state, a uterine cDNA library, created using the technique of suppression subtractive hybridization (SSH), was screened [4]. A differentially expressed cDNA clone was isolated from the reverse library and found to encode the sequence for *Rattus norvegicus cystatin C*.

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Cystatin C (CST3) is a cysteine proteinase inhibitor that is secreted into most extracellular fluids including seminal plasma [5], cerebrospinal fluid [6,7], and synovial fluid [8], where it can function as an endogenous protease inhibitor specific to the family C1 cysteine proteases which includes cathepsin B (CTSB), K (CTSK), H (CTSH), L (CTSL), and S (CTSS). CST3 is therefore an endogenous inhibitor of proteolysis of the extracellular matrix (ECM). The ECM of the endometrium contains a network of numerous types of collagen that is continually degraded and turned over as a normal event of the estrous cycle and pregnancy [9]. Part of the degradation of the ECM is achieved by the cysteine proteases CTSK, CTSL, and CTSS which can degrade fibrillar collagen, fibronectin, and laminin (reviewed by [10]), and CTSB which can degrade collagen type IV and X, and fibronectin in vitro [11,12]. ECM remodeling therefore requires the coordinated release of maternal proteinases and their endogenous inhibitors [13].

This study examined the expression of *Cst3* in the rat uterus during the peri-implantation period and the effects of E_2 and P_4 on its expression in ovariectomized animals.

Materials and methods

Animals. Female Sprague–Dawley rats (200–225 g [g] body mass; Charles River, St. Constant, QC) were housed under temperature- and light-controlled conditions (14 h light: 10 h dark; lights on at 05:00 h) with free access to food and water. Animals were ovariectomized under ether anesthesia (EM Science, Gibbstown, NJ) and allowed a minimum of 4 days to recover from surgery. Uteri were differentially sensitized for the decidual cell reaction, using the hormones E_2 and P_4 (Sigma Chemical Co., St. Louis, MO) as described previously [14]. To induce decidualization, animals received a bilateral injection of 0.1 ml of sesame oil into the uterine lumen around noon on the equivalent of Day 5 of pseudopregnancy and were referred to as stimulated (s) as opposed to non-stimulated (ns) [14]. Rats were killed by decapitation between 10:00 am and 11:00 am on the equivalent of days 1 through 8 of pseudopregnancy. Endometrial tissue was separated from myometrial tissue as previously described [4]. For experiments involving pregnant rats, virgin females were placed overnight with males and the presence of sperm in the vaginal smear the following morning was designated as day 1 of pregnancy. To differentiate between implantation and inter-implantation sites on day 6 of pregnancy animals were given an injection of 0.5% Evans blue dye via a tail vein 15 min prior to being killed [1]. Implantation sites were then visualized as areas of dye accumulation and differentiated from inter-implantation sites. All animals were handled in accordance with the guidelines of the Canadian Council on Animal Care and the University of Western Ontario Council on Animal Care.

Suppression subtraction hybridization. Suppression subtractive hybridization (SSH) was performed using the QuickPrep mRNA purification kit (Amersham Pharmacia Biotech, Inc., QC) and the Clontech PCR-Select cDNA Subtraction Kit, (Clontech Laboratories Inc., Palo Alto, CA) as described previously [4]. In brief, poly A+ mRNA from whole uteri was isolated from animals on the equivalent of days 4 or 5 of pseudopregnancy. SSH was performed using double-strand "tester" (day 4) and "driver" (day 5) cDNAs. Nested primers were ligated to the 3′ and 5′ ends of the cDNA sequences to allow for subsequent polymerase chain reaction (PCR) amplification during dot blot analysis. The subtracted cDNA library products were then cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI) for screening. Vectors were transformed into *Escherichia coli* (*E. coli*) and white bacterial colonies were identified as positive for a pGEM-T Easy vector containing a cDNA sequence. Clones identified as differentially expressed, through dot blot screening, were subjected to Northern blot analysis and sequences were compared against the GenBank database.

Northern blot analysis. Total endometrial RNA was collected from two to six animals and pooled for each experimental group. RNA were then extracted by the GTC–phenol–chloroform method [15] and quantified using spectrophotometry (Perkin-Elmer Lambda 3A UV/vis spectrophotometer). In brief, 10 μg samples of total endometrial RNA was resolved by electrophoresis as previously described [16]. RNA was then blotted overnight to a nylon membrane and probed as described previously [17] with modifications . In brief, twenty-five nanograms of the cDNA probe (bases 352–721 of *Rattus norvegicus* cystatin C: GenBank Accession No. XM_215873) was labeled with ^{32}P and hybridization was carried out overnight at 60 °C. The blot was subjected to autoradiography at −70 °C using BioMax MS film (Amersham). To determine the relative amounts of RNA transferred to the membrane, blots were stripped and hybridized with a $\alpha^{32}P$-labeled 18S rRNA probe.

In situ hybridization. The in situ hybridization was performed as described in detail previously [18]. Briefly, uterine horns were immersed in 4% paraformaldehyde for 24 h, rinsed in 1× PBS (2× 10 min), transferred to 70% ethanol, embedded in paraffin, and sectioned at 6 μm. Sections were then hybridized (100 ng of Digoxigenin (DIG)-labeled cRNA probe/slide) overnight, under a coverslip, at 50 °C in a sealed humidified chamber. The sections were then washed in 1× PBS (3× 10 min) and treated with RNase (20 μg/ml) at 37 °C for 20 min. Immunologic detection of the DIG-labeled cRNA probe was achieved using anti-DIG antibodies at a dilution of 1:500 (Roche Molecular Biochemicals, Laval, QC, Canada).

Immunohistochemistry. Paraffin-embedded uteri were sectioned at 6 μm. Sections were blocked in normal rabbit serum (2% NRS in 1× PBS) for 1 h and then incubated overnight at 4 °C with a polyclonal anti-cystatin C antibody produced in rabbits (Cat. # 06-458; Upstate Biotechnology; Lake Placid, NY) diluted to 1:100 in blocking solution (2% NRS in 1× PBS: 400 μl of solution/slide). Slides were then incubated for 2 h with the secondary antibody (Biotinylated goat anti-rabbit IgG antibody raised in goat: Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:800 for 2 h at room temperature. A Vectastain Kit was used (Peroxidase standard: PK-4000, Vector Laboratories Inc.,) as per the manufacturer's instruction, to detect antibody staining which was revealed as the development of a brown color. Sections were then counterstained with hematoxylin (Sigma Chemical Company) for 2 min.

Data analysis. Following Northern blot analysis, relative band intensities, representing hybridization of cDNA and 18S rRNA-specific probes, were quantified by scanning densitometry. The *Cst3* mRNA signal intensity, relative to that seen for 18S rRNA (cDNA signal/18S signal), was then calculated by densitometry (Image Master VDS densitometry software-Pharmacia Biotech). All Northern blot analyses were performed in triplicate with RNA samples obtained from separate groups of animals unless stated otherwise. For experiments performed three times, the means (±SEM) were calculated with the ratios of the signal detected on the equivalent of day 5 of pseudopregnancy, day 5 of pregnancy or the control, as appropriate for the experiment, set to 1. Data were analyzed by within-blocks ANOVA, with experiments being considered blocks. A value of $P < 0.05$ was considered statistically significant.

Results

Tissue distribution of Cst3 mRNA expression

To determine the tissue distribution of *Cst3* expression in the rat, total RNA from various organs and tissues was collected and used for Northern blot analysis. A 0.7 kb transcript was detected at low levels in almost all tissues with a considerably stronger signal detected in brain, lung, oviduct, uterus, vagina, and placental/decidual tissue (Fig. 1).

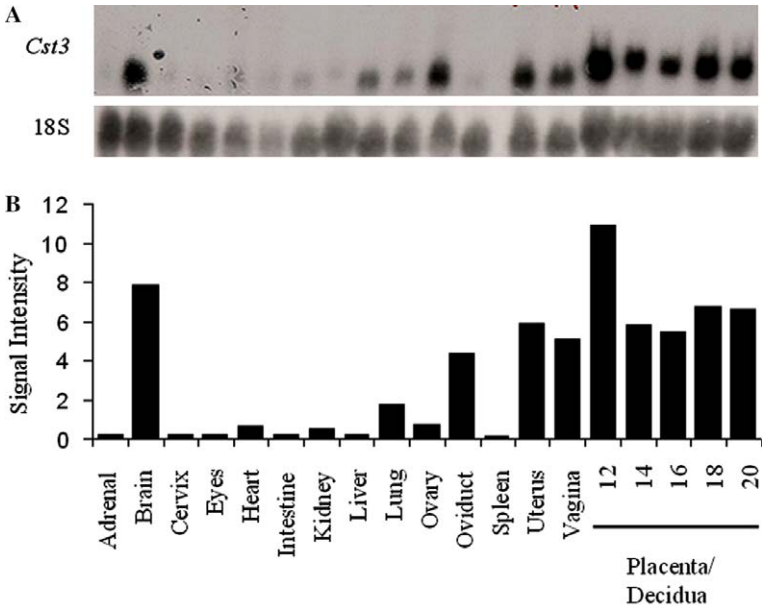

Fig. 1. Tissue distribution of *Cst3* mRNA expression in the rat. Interdigitated placental/decidual tissue was collected on days 12, 14, 16, 18, and 20 of pregnancy as shown. Other tissues were collected from a female rat at a random stage of the estrous cycle. (A) Autoradiography of a membrane probed sequentially with a α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean ratio of *Cst3*/18S rRNA intensities as determined by densitometry ($n = 1$).

Northern blot analysis of Cst3 mRNA expression during pregnancy and pseudopregnancy

To determine *Cst3* mRNA levels during the equivalent of days 1 through 8 of pseudopregnancy, total endometrial RNA was collected and subjected to Northern blot analysis (Fig. 2). *Cst3* mRNA levels were low on days 1 through 3 pseudopregnancy but rose on day 4 ($P < 0.05$). Levels then declined on day 5. Expression on day 4 was 3 times that seen on day 5 ($P < 0.05$). Levels again rose on day 6 in animals not receiving an artificial deciduogenic stimulus, to levels equivalent to those seen on day 4. Levels were therefore high on day 4 when the uterus is referred to as neutral and again on day 6 when the non-stimulated uterus is referred to as refractory. In animals receiving a deciduogenic stimulus on day 5, levels remained low on days 6 and 7, similar to those seen on day 5, and increased on day 8.

To determine if there were similar changes in *Cst3* mRNA levels during pregnancy, total uterine tissue was collected on days 4, 5, and 6 of pregnancy, with implantation sites being separated from inter-implantation sites on day 6. *Cst3* mRNA levels on day 4 were 3 times those detected on day 5 ($P < 0.05$; Fig. 3). Levels rose on day 6 for both implantation and inter-implantation sites relative to those on day 5. *Cst3* transcript abundance at implantation sites was approximately 50% of that detected at inter-implantation sites on day 6 but the difference was not statistically significant ($P > 0.05$).

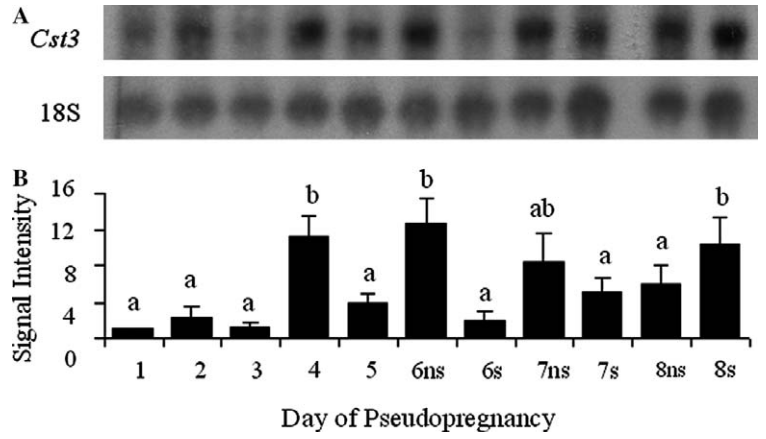

Fig. 2. Northern blot analysis of *Cst3* mRNA levels in endometrial tissue of pseudopregnant rats. (A) Autoradiography of a membrane probed sequentially with a α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean (±SEM; $n = 3$) ratios of *Cst3*/18S rRNA intensities as determined by densitometry with day 1 normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

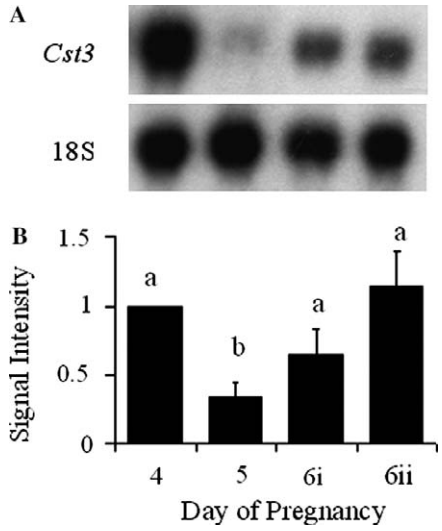

Fig. 3. Northern blot analysis of *Cst3* mRNA levels in uterine tissue of pregnant rats. Implantation [6i] and inter-implantation sites [6ii] were collected from animals on day 6 of pregnancy. (A) Autoradiography of a membrane probed sequentially with a α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean (±SEM; $n = 3$) ratios of *Cst3*/18S rRNA intensities as determined by densitometry, with day 4 normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

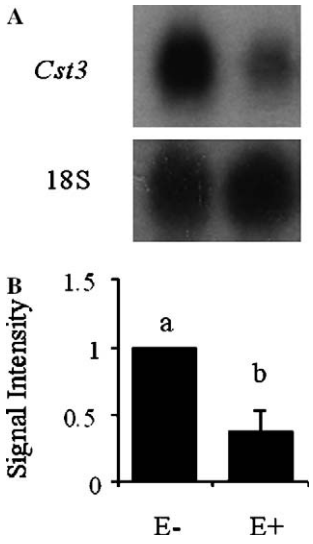

Fig. 4. Northern blot analysis of *Cst3* mRNA levels in a model of delayed implantation. Tissue was collected from animals undergoing the equivalent of implantation delay before E_2 exposure (E−), or after E_2 exposure (E+). (A) Autoradiography of a membrane probed sequentially with a α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean (±SEM; $n = 3$) ratios of *Cst3*/18S rRNA intensities as determined by densitometry, with E− normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

Northern blot analysis of Cst3 mRNA levels during a model of delayed implantation

Delayed implantation occurs in lactating mice and rats. This delay in implantation can be artificially maintained in ovariectomized animals through the continued administration of P_4 and resolved following administration of E_2 which renders the endometrium receptive (reviewed in [1]).To determine *Cst3* mRNA levels during a model of delayed implantation, ovariectomized animals were subjected to an injection protocol [14] with modifications. Animals did not receive an injection of E_2 on the afternoon of the equivalent of day 4 of pseudopregnancy, but instead were maintained on an additional 48 h of P_4 (4 mg/day). Following this, half of the animals received an afternoon injection of both E_2 (0.3 μg) and P_4 (4 mg) while the other half received only P_4 (4 mg). Animals were killed the next morning and designated as E− (animals that had not received an injection of E_2 the evening before) and E+ (animals that had received an injection of E_2 the evening before). Total endometrial RNA was collected from each of the two groups (E− and E+) and used for Northern blot analysis (Fig. 4). *Cst3* mRNA transcript abundance was 2.5-fold higher ($P < 0.05$) in endometrial tissue collected from animals that did not receive E_2 (E−).

Northern blot analysis of the hormonal control of Cst3 mRNA levels

To further determine the hormonal control of *Cst3* mRNA levels, ovariectomized animals were divided into four groups and given a single s.c. injection of either: 1.0 μg of E_2, 4 mg of P_4, 1.0 μg E_2 + 4 mg of P_4, or vehicle (sesame oil). Animals were killed 18 h later and endometrial tissue was collected. The relative abundance of transcripts for *Cst3* in the endometrium from E_2-treated rats was 20% of that seen in controls ($P < 0.05$; Fig. 5). Animals treated with P_4 alone showed no change in *Cst3* mRNA levels when compared to controls, while animals receiving both E_2 and P_4 had levels approximately 50% of those in controls, but this difference was not statistically significant ($P > 0.05$); the levels were, however, higher than those in animals treated with E_2 alone ($P < 0.05$).

Time-course for the effect of estradiol on Cst3 mRNA levels

To determine the time-course of the effect of E_2 on *Cst3* mRNA transcript abundance, animals were ovariectomized and given a s.c injection of E_2 (1 μg). Animals were killed at 0, 3, 6, 12, and 24 h post-injection. Total endometrial RNA was isolated and used for Northern blot analysis (Fig. 6). *Cst3* mRNA levels decreased with time and were approximately 50% of those seen in controls 3 h after E_2 treatment. Levels dropped to 10% of those seen in controls at 12 h and remained low through to 24 h ($P < 0.05$).

Effect of an anti-estrogen, ICI 182, 780, on Cst3 mRNA levels

To further elucidate the effect of E_2 on *Cst3* mRNA levels, a pure anti-estrogen was used. Estradiol can affect target tissues through both "classical" and "non-classical

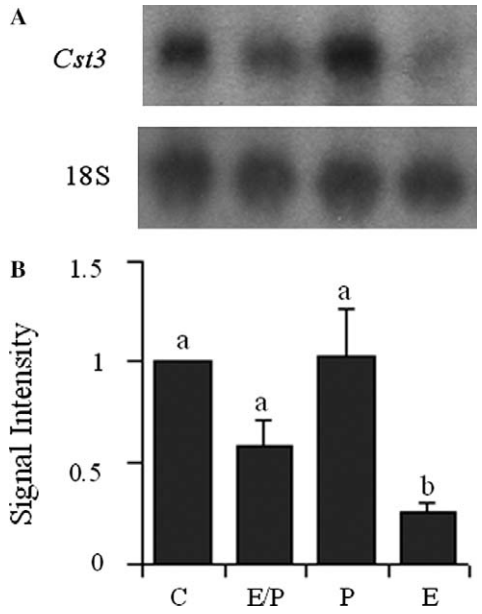

Fig. 5. Northern blot analysis of *Cst3* mRNA levels in endometrial tissue of ovariectomized rats treated with E_2 (E), P_4 (P), both (E/P) or control (C). (A) Autoradiography of a membrane probed sequentially with a α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean ratios of *Cst3*/18S rRNA intensities as determined by densitometry (±SEM; $n = 3$), with control values normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

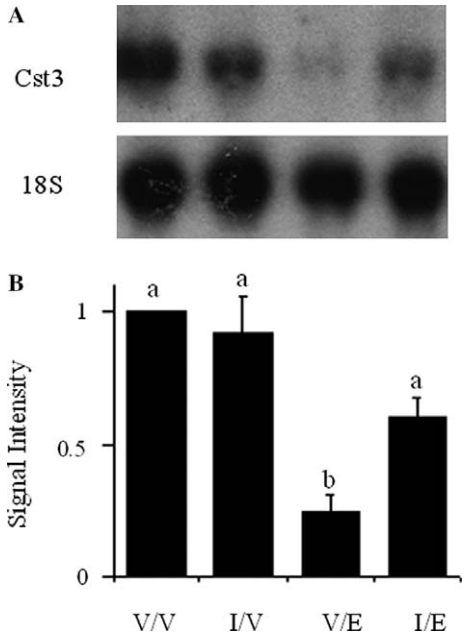

Fig. 7. Effect of an anti-estrogen (ICI 182, 780; I) given 1 h prior to E_2 (E), on *Cst3* mRNA levels in the endometrium of ovariectomized rats. Animals received 0.25 mg ICI in sesame oil and 0.3 μg E_2 (I/E), or vehicle (V/V) or combinations of these two (I/V or V/E) and were killed 12 h later. (A) Autoradiography of a membrane probed sequentially with α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean (±SEM; $n = 3$) ratios of *Cst3*/18S rRNA intensities as determined by densitometry, with vehicle-treated controls normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

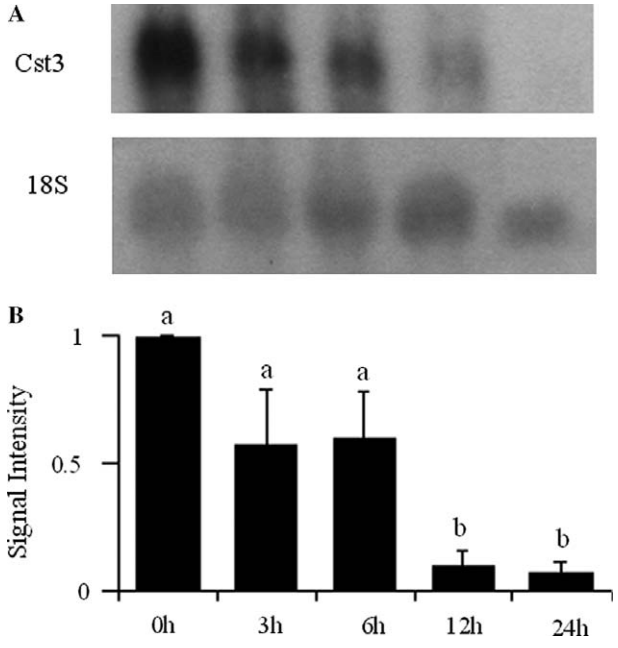

Fig. 6. Effect of E_2 on *Cst3* mRNA levels in endometrium of ovariectomized rats. Animals received a single injection of 1 μg E_2 and endometrium was collected 0, 3, 6, 12, or 24 h later. (A) Autoradiography of a membrane probed sequentially with α^{32}P-labeled cDNA probe for *Cst3* and 18S rRNA. (B) Mean (±SEM; $n = 3$) ratios of *Cst3*/18S rRNA intensities as determined by densitometry, with time 0 data normalized to 1. Bars with the same superscripts are not statistically different from each other ($P > 0.05$).

pathways'' (reviewed in [19]). In the classical pathway, E_2 binds either estrogen receptor (ER) alpha (α) or ERbeta (β) and translocates to the nucleus of target genes to activate or repress transcription rates. Treatment of animals with an ERα and β blocker ICI 182, 780 (Tocris Bioscience, Ellisville, MO; ICI) was used to determine whether *Cst3* mRNA transcription is regulated through this classical pathway. Animals were ovariectomized, randomized to four groups, and treated with or without ICI (0.25 mg/animal) and with or without E_2 (0.3 μg/animal). ICI was administered 1 h prior to E_2 and animals were killed 12 h later (Fig. 4). Exposure of animals to ICI prior to E_2 treatment inhibited the drop in *Cst3* mRNA levels compared to E_2 treatment alone ($P < 0.05$; Fig. 7).

Localization of Cst3 mRNA by in situ hybridization

In situ hybridization was performed to localize *Cst3* mRNA expression within the rat uterus (Fig. 8). On day 4 of pregnancy and on the equivalent of day 4 of pseudopregnancy there were strong signals in the glandular epithelial cells that were reduced but still detectable on day 5. On day 6 of pregnancy the signal was reduced around the implanting embryo with enhanced expression closer to the myometrium. Inter-implantation sites on day 6 of pregnancy showed a slightly stronger diffuse signal throughout the stroma.

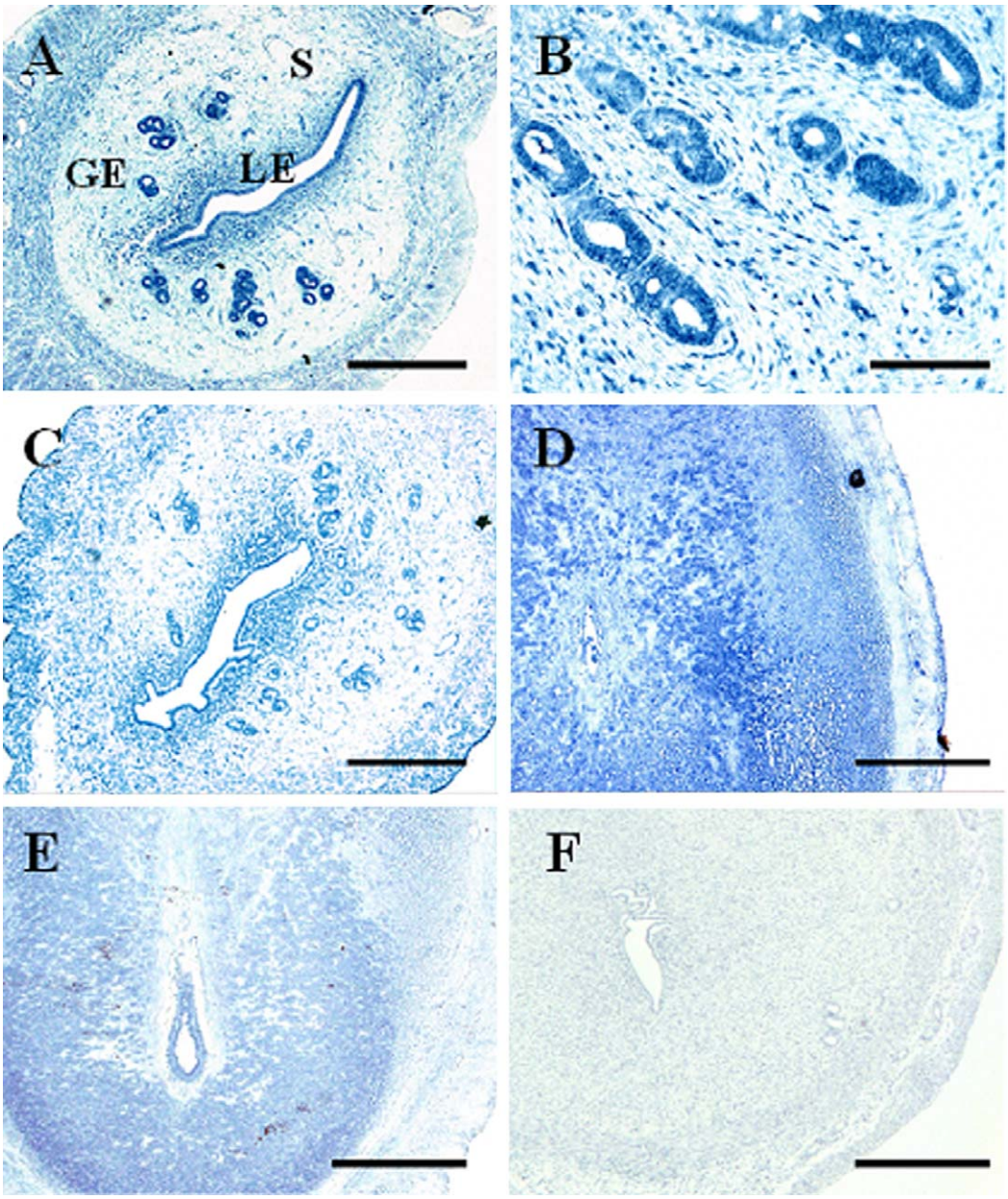

Fig. 8. Localization of *Cst3* mRNA by in situ hybridization. Uterine crosssections from pseudopregnant and pregnant rats were subjected to in situ hybridization using a DIG-labeled *Cst3* cRNA probe. Uteri from Day 4 of pseudopregnancy probed with the antisense probe (A), Day 4 of pregnancy (B), Day 5 of pregnancy (C), Day 6 of pregnancy, inter-implantation (D), Day 6 of pregnancy, implantation site (E), and control (Day 6, inter-implantation, not exposed to cRNA probe) (F). LE, luminal epithelium; GE, glandular epithelium; S, Stroma. Size bars: A,C–F = 500 μm, B = 20 μm.

Localization of CST3 protein by immunohistochemistry

Immunohistochemistry was performed to localize CST3 protein within the rat uterus. On day 4, in both pregnant and the equivalent of pseudopregnant animals, a strong signal was localized to the glandular epithelium (Fig. 9). On day 5 the signal intensity was substantially reduced for both the pseudopregnant and pregnant animals, with primarily glandular epithelial localization. For day 6 animals that received a deciduogenic stimulus, the signal was low in intensity. A stronger diffuse signal was observed in the stroma of animals on day 6 that had not received a deciduogenic stimulus.

Discussion

The current study describes the temporal and hormonal control of *Cst3* mRNA transcript abundance in the rat endometrium during the peri-implantation period. It is the first study to examine the expression of *Cst3* in the rodent endometrium prior to embryo implantation and also the first study to determine the effects of E_2 on subsequent endometrial *Cst3* transcript abundance.

Analysis of tissue distribution of *Cst3* mRNA in the rat revealed low levels of expression in most tissues with a stronger signal being seen in brain, lung, oviduct, uterus, vagina, and interdigitating placental/decidual tissue

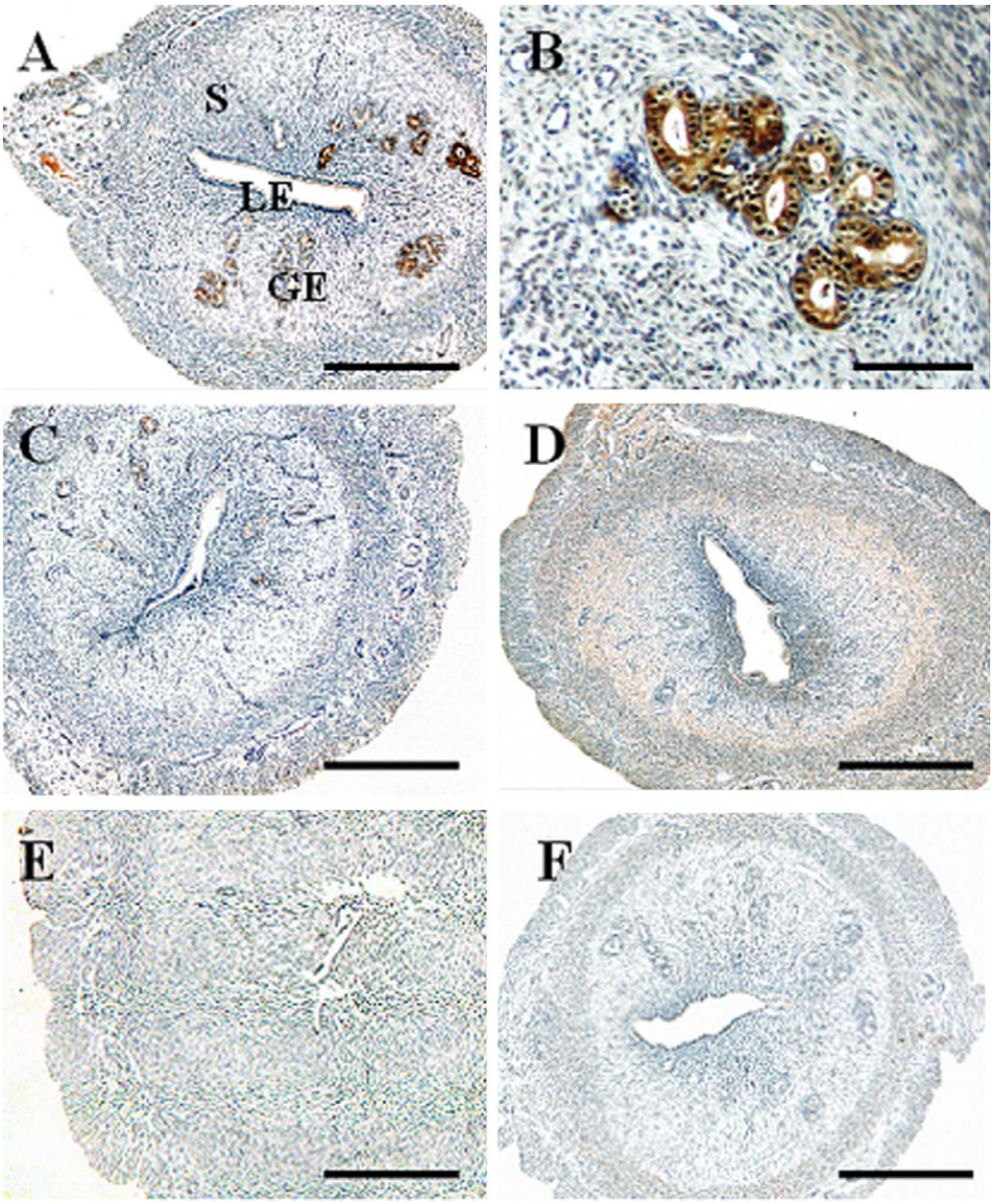

Fig. 9. Localization of CST3 protein by immunohistochemistry in uteri from pseudopregnant and pregnant rats. For day 6 pseudopregnant uteri, some animals received a deciduogenic stimulus on day 5 (day 6s) while others had not received a stimulus (day 6ns). Uterine crosssections were exposed to a polyclonal antibody specific to rat CST3. (A) Day 4 pregnancy, (B) Day 4 pseudopregnancy, (C) Day 5 pregnancy, (D) Day 6ns, (E) Day 6s, (F) Control (day 4 of pregnancy not exposed to the primary antibody). Size bars: A,C–F = 500 μm, B = 20 μm.

(Fig. 1). These results are in agreement with previous studies showing that *Cst3* is strongly expressed in the brain in the rat [7] and human [6], and in decidual tissue in the mouse [20] and sheep [21]. Elevated expression in the oviduct, uterus, and vagina suggests a yet unexplained role for *Cst3* in the female reproductive tract. While the role of CST3 in the female reproductive tract during the estrous cycle and early pregnancy is unknown, previous studies have shown that it is secreted into most extracellular fluids [5–8], including the intrauterine lumen in sheep [21], where it can function as a cysteine protease inhibitor.

The main function of cystatins is to protect cells from the degradative lysosomal enzymes that are released by dying cells, growing cancers, invading microbes, and during ECM turnover [22]. While carcinomas can be highly invasive, trophoblast invasion is tightly regulated by the uterus, with evidence that rapid and organized expression of maternal protease inhibitors at sites of trophoblast cell invasion provides the uterus protection (reviewed in [13]) against uncontrolled trophoblast invasion. For example, it has been shown that invasive trophoblast cells secrete the proteinases matrix metalloproteinase-9 (MMP9) and CTSB while endometrial cells secrete their endogenous

inhibitors, tissue inhibitor of metalloproteinase-3 (TIMP3) and CST3, respectively, at the maternal-fetal interface [20,23] . This co-ordinated interplay of proteinases and their inhibitors limits and directs trophoblast invasion. In the rat, embryonic attachment to the uterine lumen occurs on day 5 of pregnancy followed by initiation of decidualization and trophoblast invasion on day 6. Maternal *Cst3* mRNA levels may therefore decline on day 5 of pregnancy to approximately one-third of that observed on day 4 (Fig. 3) in anticipation of blastocyst invasion which begins on day 6 of pregnancy and requires the release of proteases from the invading trophoblast cells [24].

On the equivalent of day 6 of pseudopregnancy in endometrial tissue undergoing the process of decidualization, *Cst3* mRNA levels are low. This may allow rapid uterine ECM remodeling which consists of the loss of collagen type IV, the laying down of fibronectin, and an increase in cell size as the decidua forms [25,26]. In animals not receiving a deciduogenic stimulus, *Cst3* mRNA levels rose on day 6 to the equivalent of that seen on day 4 (Fig. 4), possibly to minimize remodeling of the ECM.

Following the initialization of decidualization, *Cst3* mRNA levels progressively rose in the endometrium from days 6 through 8, a pattern that has previously been shown to occur in the mouse [20] and sheep [21]. It has also previously been shown that mouse trophoblast cells preferentially express *Ctsb* at the maternal-fetal interface during the process of endometrial invasion. In this model, embryonic expression of the proteinase *Ctsb* is thought to mediate invasion while maternal expression of the proteinase inhibitor *Cst3* limits it.

Other proteinases and their endogenous inhibitors also show changes in expression during early pregnancy. For example, *Timp-1* mRNA levels are elevated in the rat endometrium on the equivalent of day 1 of pseudopregnancy, falling on day 2 and remaining low on days 3 through 5 [27].

Changes in endometrial gene expression in cycling and pregnant animals are usually under hormonal control. The results of this study show that *Cst3* mRNA levels in the ovariectomized rat drop in response to E_2 treatment. This can be seen within 18 h of E_2 treatment in rats undergoing a model of delayed implantation (Fig. 4) and within 3 h (lasting up to 24 h) in animals given a single dose of E_2 (Fig. 6). Interestingly, animals treated with P_4 maintained high mRNA levels, equivalent to those seen for controls, suggesting that P_4 does not inhibit *Cst3* mRNA transcript abundance in the rat endometrium (Fig. 5). In fact, in ovariectomized sheep long-term exposure to P_4 (12 days) results in a rise in *Cst3* mRNA levels. The decline in *Cst3* mRNA levels seen on day 5 of pregnancy and the equivalent of day 5 of pseudopregnancy may therefore be attributed to the nidatory surge of estrogen [1] or injection of E_2 on the evening of day 4 [14]. Hormonal regulation does not however explain the effect of E_2 on day 6 as both stimulated and non-stimulated endometrium are subjected to the same hormone injection regime but show very different *Cst3* mRNA levels. In fact, on day 6 *Cst3* mRNA levels in stimulated endometrium were only 20% of those seen for non-stimulated endometrium, suggesting alternative control of levels at this time (Fig. 2).

In addition to *Cst3*, other mRNA transcripts appear to decrease in abundance in the mouse uterus in response to E_2 including those for soluble guanylyl cyclase (*sGC*) $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ [28]. Ovariectomized rats administered 40 μg of E_2 showed a 90% drop in uterine $sGC_{\alpha 1}$ and a 60% drop in uterine $sGC_{\beta 1}$ mRNA levels within 3 h of treatment. This effect was blocked by the administration of 2 mg/kg of the anti-estrogen ICI 30 min prior to E_2 treatment, suggesting that *sGC* levels are down-regulated through a classical ER-mediated pathway.

In the rat E_2 can bind its receptor in the endometrium, translocate to the nucleus, bind an estrogen response element (ERE) in the promoter region of a target gene, and activate or repress transcription via recruitment of co-activators or co-repressors. *Cst3* does not contain an ERE site in its promoter region [29] but does possess an activator protein-1 (AP-1) site which is able to bind the transcription complex c-Jun/c-Fos and recruit an activated ER complex to modulate transcription. To determine if *Cst3* mRNA levels are regulated through a classical or non-classical pathway, ovariectomized rats were administered ICI 1 h prior to E_2 treatment. ICI is an anti-estrogen that can block both ERα and ERβ [30]. Treatment with ICI prior to E_2 exposure inhibited the decrease in *Cst3* mRNA levels observed following exposure to E_2 alone. These results suggest that *Cst3* mRNA levels change in response to E_2, through a classical estrogen receptor mediated pathway.

Staining patterns for immunohistochemistry and in situ hybridization correlated well, with strong glandular localization of *Cst3* on day 4 of pseudopregnancy and pregnancy followed by weak glandular localization on day 5 (Figs. 8 and 9). Glandular localization of the protein suggests that CST3 may also be secreted into the uterine lumen as previously seen in the sheep [21]. A similar phenomenon is seen by the salivary glands and choroid plexus which both secrete CST3 extracellularly [31,32].

In situ hybridization revealed that *Cst3* mRNA levels are elevated in the non-stimulated compared to the stimulated endometrium on the equivalent of day 6 of pseudopregnancy. It appears that the area of the endometrium surrounding the implanting embryo produces less CST3, presumably to facilitate degradation of the ECM by cysteine proteases.

In conclusion, E_2 inhibits *Cst3* expression in the endometrium of ovariectomized rats. The effect of E_2 on the rat endometrium during pregnancy and the equivalent of pseudopregnancy is more complicated as there is a rise in *Cst3* expression in decidualized tissue being exposed to E_2. It can therefore be stated that E_2 affects *Cst3* expression and that the effects are complicated and change throughout pregnancy. From the overall findings of this study it can be speculated that *Cts3* functions as a protease inhibitor in the rat endometrium during the peri-implantation period with

a decrease in mRNA levels during receptivity and maximal sensitization for the decidual cell reaction.

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