

# Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice

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## Abstract

Cervical remodeling during pregnancy and parturition is a single progressive process that can be loosely divided into four overlapping phases termed softening, ripening, dilation/labor, and *post partum* repair. Elucidating the molecular mechanisms that facilitate all phases of cervical remodeling is critical for an understanding of parturition and for identifying processes that are misregulated in preterm labor, a significant cause of perinatal morbidity. In the present study, biomechanical measurements indicate that softening was initiated between gestation days 10 and 12 of mouse pregnancy, and in contrast to cervical ripening on day 18, the softened cervix maintains tissue strength. Although preceded by increased collagen solubility, cervical softening is not characterized by significant increases in cell proliferation, tissue hydration or changes in the distribution of inflammatory cells. Gene expression studies reveal a potentially important role of cervical epithelia during softening and ripening in maintenance of an immunomucosal barrier that protects the stromal compartment during matrix remodeling. Expression of two genes involved in repair and protection of the epithelial permeability barrier in the gut (trefoil factor 1) and skin (serine protease inhibitor Kazal type 5) were increased during softening and/or ripening. Another gene whose function remains to be elucidated, purkinje cell protein 4, declines in expression as remodeling progressed. Collectively, these results indicate that cervical softening during pregnancy is a unique phase of the tissue remodeling process characterized by increased collagen solubility, maintenance of tissue strength, and upregulation of genes involved in mucosal protection.

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## Introduction

Premature birth is one of the most significant causes of perinatal morbidity in developed countries, and its incidence over the last decade has been increasing (Branum & Schoendorf 2002, Ananth *et al.* 2005). Investigations to define the causes of preterm birth remain a challenge in the obstetrical field as phenotypes of preterm labor and delivery vary extensively. As an example, precocious remodeling of the cervix in the absence of uterine contractions, clinically known as cervical incompetence or insufficiency in humans, is associated with preterm birth (Papiernik *et al.* 1986).

The cervix is a metabolically active organ in pregnancy composed of extracellular matrix (ECM) components such as collagen, elastin, proteoglycans, and hyaluronan in addition to stromal, epithelial, and smooth muscle cells (Leppert 1995). The cervix is extensively remodeled throughout gestation in order to open sufficiently for safe passage of the fetus during childbirth. Cervical remodeling during pregnancy and

parturition is a single continuous process that can be loosely divided into four overlapping phases termed softening (phase 1), ripening (phase 2), dilation/labor (phase 3), and *post partum* repair (phase 4; Liggins 1978, Word *et al.* 2007).

In 1895, Hegar first described ‘softening’ of the lower uterine segment in association with human pregnancy at 4–6 weeks. The Hegar sign refers to the characteristic softening of the uterus in cervix that is evident on physical examination during early pregnancy, and it was customarily used to clinically diagnose pregnancy until the discovery of human chorionic gonadotropin many years later (Hegar 1895). Cervical softening can be defined as a change in the biomechanical properties of the cervix when compared with the nonpregnant cervix and is characterized by a progressive decrease in tissue stiffness without loss of tensile strength (Leppert & Yu 1994). The ability of the cervix to soften yet remain resistant to forces exerted upon it requires dual mechanisms in which there is an increase in the geometry (size) of the cervix along with maintained

stiffness of the cervical wall (Drzewiecki *et al.* 2005). Physiologic softening of the rat cervix was first recognized by Harkness & Harkness (1959) who noted that distensibility of cervical tissue increased dramatically starting midgestation between days 11 and 12 (Harkness & Harkness 1959). An increase in the diameter of the cervical canal between gestation days 10 and 12 was used initially as a crude measurement to define cervical softening in the mouse (Leppi 1964). The change in diameter, however, was <1 mm and statistical analysis was not performed. Changes in tissue compliance during softening are proposed to be facilitated by changes in the composition or structure of the ECM in humans and rats (Danforth *et al.* 1974, Liggins 1978, Leppert 1995). This is supported by the fact that cervical incompetence is increased in women with inherited defects in collagen and elastin synthesis or assembly (e.g. Ehlers-Danlos and Marfan syndromes; Paternoster *et al.* 1998, Rahman *et al.* 2003). In addition, mice deficient in the ECM protein, thrombospondin 2, have altered collagen fibril morphology and premature cervical softening (Kokenyesi *et al.* 2004).

Cervical softening begins early in mammalian pregnancies and overlaps at the end of gestation in a second phase of matrix remodeling commonly referred to as cervical ripening. While the endocrine events that herald this stage differ from species to species, the series of events that bring about ripening are very similar. Cervical ripening has been studied much more extensively than the process of cervical softening. Ripening is characterized by an increase in hyaluronan content, loosening of the collagen matrix, increased collagen solubility (Hillier & Wallis 1982, Granstrom *et al.* 1989), changes in the distribution of inflammatory cells, increased tissue growth and hydration, and loss of tensile strength (Uldbjerg *et al.* 1983, Buhimschi *et al.* 2004, Straach *et al.* 2005, Timmons & Mahendroo 2006). In contrast to cervical ripening, the biochemical and molecular changes that occur during cervical softening are not well-defined. We propose that processes regulating softening events may differ from cervical ripening and are crucial to our understanding of cervical remodeling for successful delivery of young. The objective of the current series of experiments is to understand the midgestation cervical softening process in mice and to identify genes that may be integral to one or all stages of the cervical remodeling process.

## Materials and Methods

### Mice

Animals were housed under a 12L:12D photoperiod (lights-on, 0600–1800 h) at 22 °C. Mice used in the present studies were of mixed strain (C57BL/6 × 129SvEv). The C57BL/6 × 129SvEv mice were generated and maintained as a breeder colony at the University of

Texas Southwestern Medical Center (Dallas, TX, USA). In general, mice in these studies were 3–6 months old and nulliparous. Female mice were housed overnight with males and checked at midday for vaginal plugs in order to obtain accurately timed pregnant mice. The day of plug formation was counted as day 0, and birth occurred in the early morning hours of day 19. Cervices collected before noon on the day of birth were estimated to be 10 h *post partum* and those collected before noon on the day after birth were estimated to be 24 h *post partum*. All studies were conducted in accordance with the standards of humane animal care as described in the NIH Guide for the Care and Use of Laboratory Animals. The research protocols were approved by the institutional animal care and research advisory committee.

### Cervical tissue collection

Animals were given anesthesia with 1.25% tribromoethanol (25 µl/g of body weight injected intraperitoneally). After euthanasia by cervical dislocation, the uterine horns, cervix, and vagina were removed. Using a dissecting microscope, the cervix was isolated by transection at the utero-cervical junction (caudal to the uterine bifurcation) and removal of all vaginal tissue from cervical tissue specimens. All specimens for RNA extraction were immediately snap frozen in liquid nitrogen.

### Tissue biomechanics

Tensile distensibility and maximum stretch of isolated cervical tissues was conducted according to a method developed by Harkness & Harkness (1959) and adapted in our laboratory. The excised cervix was mounted by means of two pins inserted through the cervical canal. One pin was attached to a calibrated mechanical drive and the other pin to a force transducer. Tissues were incubated in a water-jacketed bath containing physiologic saline solution (NaCl (120.5 mM), KCl (4.8 mM), MgCl<sub>2</sub> (1.2 mM), CaCl<sub>2</sub> (1.6 mM), NaH<sub>2</sub>PO<sub>4</sub> (1.2 mM), NaHCO<sub>3</sub> (20.4 mM), dextrose (10 mM), and pyruvate (1.0 mM), pH 7.4 at 37 °C) bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Baseline cervical dilatation (i.e. resting diameter of cervical os) was quantified by determining the difference in cervical diameter at 0 (pins juxtaposed with no tension) and the inner cervical diameter at the initiation of tension as the pins were separated. Thereafter, the inner diameter of the cervix was increased isometrically in 0.5 mm increments at 2-min intervals. The amount of force required to distend the cervix and the tension exerted by the stretched tissue were recorded. The diameter was increased until either forces exerted by the tissue reached a plateau or the tissue tore. Force was plotted as a function of cervical diameter. Cross-sectional area and stress (force/cross-sectional area)

were calculated. The slope of the linear portion of the force–strain curve was computed as an index of tissue stiffness and elasticity (Young's modulus).

### Water content

Cervix tissue was weighed immediately after tissue collection (wet weight) and again after freeze–drying overnight (dry weight). Water content was determined by the ratio of the difference between the wet and dry cervical weights to the dry cervical weight (wet weight–dry weight)/wet weight (Anderson *et al.* 2006).

### Collagen solubility

Dry cervical samples were homogenized and incubated in 0.5 ml of 0.5 M acetic acid, 1 mg/ml pepsin (Sigma, catalog no. P7012-1G) at 4 °C for 24 h with gentle agitation. Samples were spun at 12 000 r.p.m. (13 400 g) and supernatant containing soluble collagen was separated from the insoluble pellet. Soluble collagen was hydrolyzed by the addition of an equal volume of 12 M HCl for a final concentration of 6 M HCl. The insoluble collagen was hydrolyzed by the addition of 6 M HCl. Both fractions were incubated at 100 °C for 20 h. Samples were allowed to cool uncapped and incubated at 100 °C until HCl was evaporated. The dried hydrolysates were dissolved in 250 µl of water and the hydroxyproline concentration in each fraction measured by a colorimetric method according to methods as described (Stegemann & Stalder 1967). Briefly, 20 µl sample or standard was diluted in 500 µl water. Two hundred and fifty microliters of chloramine-T solution (1.41 g chloramine-T (Sigma), 10 ml H<sub>2</sub>O, 10 ml *n*-propanol, and 80 ml OH-pro buffer) were added to each tube and incubated at RT for 20 min. (OH-pro buffer: 50 g citric acid 1 H<sub>2</sub>O, 12 ml acetic acid, 120 g sodium acetate 3H<sub>2</sub>O, 24 g NaOH. H<sub>2</sub>O is added to bring volume up to 1200 ml with water. pH to 6.0 with 6 N NaOH and then add 300 ml *n*-propanol). Two hundred and fifty microliters of aldehyde/perchloric acid solution (1 g *p*-dimethyl-amino-benzaldehyde (JT Baker, Phillipsburg, NJ, USA), 4 ml *n*-propanol, and 1.73 ml 70% perchloric acid) was added to all samples and standards, vortexed, and incubated at 60 °C for 15 min. Two hundred and fifty microliters of each sample were aliquoted into a 96-well microtiter plate and absorbance was measured at 558 nm on a Tecan Saffire2 microplate reader (Tecan, San Jose, CA, USA). A *trans*-4-hydroxy-L-proline (Sigma catalog no. 56250) standard series (6, 3, 2, 1, 0.5, and 0.2 µg) and blank (water) were included in the experiment. All measurements were carried out in duplicate, and samples that had an absorbance higher than that of the most concentrated standard were diluted further and measured again. The hydroxyproline concentration in the soluble and insoluble fraction was

interpolated from the standard curve and expressed as a percentage of the total OH-Pro in the sample. Total OH-Pro in the sample was determined by adding OH-Pro in soluble and insoluble fractions. The amount of total collagen was calculated by assuming the hydroxyproline content of collagen was 14% (Horgan *et al.* 1990). The concentration of collagen was determined by dividing the total collagen (µg) by the wet weight (mg) of the tissue. Four to six cervixes were measured individually for each time point and data represent an average ± S.E.M.

### Immunohistochemistry

Freshly excised cervixes were embedded in OCT compound (Tissue Tek; Bayer Corp., Elkhart, IN, USA) and frozen immediately in liquid nitrogen. Air-dried tissue sections (thickness, 5 µm) were fixed for 10 min in acetone. Nonspecific binding was blocked using 1.5% normal donkey serum for 20 min. Sections were incubated for 30 min at 25 °C with the monoclonal antibody, neutrophil 7/4, a rat anti-mouse neutrophil, and monocyte-specific antibody at a working dilution of 0.01 mg/ml (Serotec, Raleigh, NC, USA) or rat anti-mouse BM8, a macrophage-specific antibody (1:800 of a 0.5 µg/ml stock; BACEM Biosciences Inc., King of Prussia, PA, USA). Neutrophil 7/4 recognizes myeloid lineage leukocytes, including neutrophils and monocytes, but not macrophages (Henderson *et al.* 2003, Taylor *et al.* 2003). BM8 is a pan-macrophage marker that recognizes the F4/80 antigen found on cell membranes and the cytosol of mononuclear phagocytes (Mackler *et al.* 1999). Biotinylated donkey anti-rat (1:200; Jackson Laboratories, Westgrove, PA, USA) and alkaline phosphatase-conjugated avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) were applied in sequence followed with Vector Red substrate (Vector Laboratories). Tissues were counterstained in hematoxylin for 10 s. The primary antibody was replaced with rat IgG2a (Caltag Laboratories, Burlingame, CA, USA) as a negative control. Three animals were tested for each time point. Nonpregnant cervixes were collected during metestrus.

### RNA isolation and quantitative real-time PCR

Total RNA was extracted from frozen tissue using RNA Stat 60 (Tel-test B, Friendswood, TX, USA). Total RNA was treated with DNase I (DNA free; Ambion, Austin, TX, USA) to remove any genomic DNA contamination. cDNA was synthesized using a TaqMan cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed using SYBR Green and a PRISM 7900HT Sequence Detection System (Applied Biosystems). Aliquots (20 ng) of total cDNA were used for each PCR and were performed in triplicate. Expression of each gene was expressed relative to that of the housekeeping

gene cyclophilin (*Ppib*). Relative levels of gene expression were determined by the ddCt method (Applied Biosystems User Bulletin #2).

### Microarray

RNA from three separate cervical samples were obtained on days 10 and 12 of gestation and subjected to microarray experiments. Gene Spring 5.0 (Silicon Genetics, Redwood City, CA, USA) software was used in data analysis and statistical calculations. Affymetrix mouse expression chip 430 2.0 data were exported to the gene analysis software. Per chip normalization to the 50th percentile expression level and per gene normalization to the median expression of all samples was performed. Only probes with two out of three samples scored as either present or marginal were used in the data analysis. Probe sets with average expression of 40 or less in both experimental and control groups were excluded. Fold change expression was calculated as the median expression of day 12 over the expression of day 10 in mouse cervix tissue. Annotations for the probe sets were derived from the Affymetrix web site (<http://www.affymetrix.com/>) and were updated with publicly accessible databases: National Center for Biotechnology Information (<http://www.ncbi.nih.gov/>), Information Hyperlinked over Proteins (<http://www.iHop-net.org/UniPub/iHOP/>), and Swiss Prot (<http://www.ebi.ac.uk/swissprot/>) to verify gene identity and update annotations from current literature references. After generating a comprehensive gene list, the function and ontology of each gene were examined and then scored to determine possible relevance to cervical remodeling. From this list, a number of genes were chosen for further validation and analysis.

### Statistical tests

Means of data were compared with Student's *t*-test where appropriate. Two-tailed *P*-value  $\leq 0.05$  was considered statistically significant. For multiple comparisons, a Kruskal–Wallis one way ANOVA on ranks was performed followed by the Dunnett or Tukey method. For the microarray analysis, a gene was considered significant if one of two conditions were satisfied: first, if there was a two-fold change in mean expression of the gene from days 10 to 12 of gestation as calculated by Gene Spring, or second, if there was a statistically significant change in expression as determined by parametric testing adjusted for multiple comparisons. These two methods were combined to create a comprehensive and inclusive gene list. Statistical significance was defined in this context using a Benjamini and Hochberg false discovery rate (FDR) of  $< 40\%$ . An FDR  $< 40\%$  has been considered appropriate for exploratory research and gene discovery

(Welle *et al.* 2002, Reiner *et al.* 2003). Real-time PCR was used to confirm the differential expression of genes found to be significant by the microarray analysis to reduce the possibility of finding false positives from the less stringent microarray analysis.

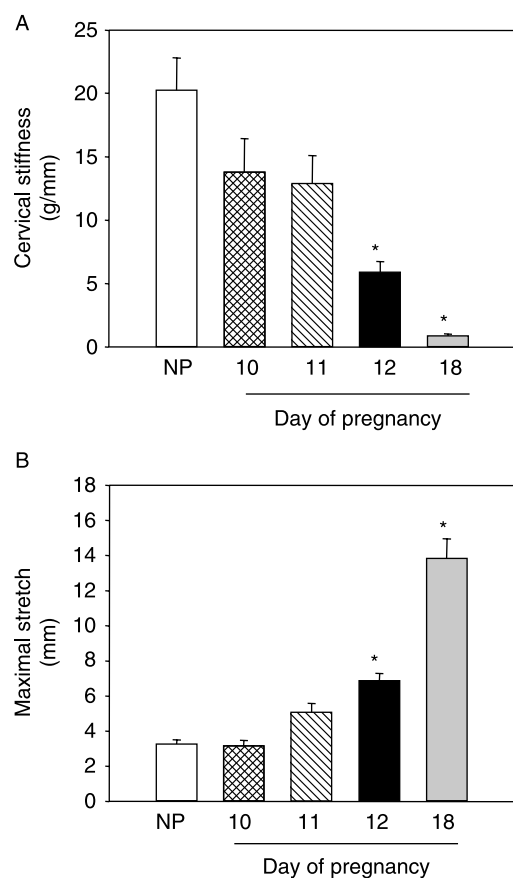
## Results

### Biomechanical characterization of cervical softening

To better define the time point at which cervical softening begins in the mouse when compared with earlier studies (Leppi 1964), biomechanical studies were carried out in cervices from nonpregnant and time-mated pregnant mice on gestation days 10, 11, 12, and 18 using methods that have been described previously (Word *et al.* 2005). Data shown for gestation day 18 is previously published and is shown in Fig. 1 for comparison to days 10–12 (Word *et al.* 2005). Cervical stiffness on day 10 (day 10) was not statistically significant from that of nonpregnant animals. Cervical tissues from nonpregnant animals and pregnant mice on day 10 were not distensible as the cervix could not be stretched to more than 3.5 mm before failure (Fig. 1A and B). By day 12, cervical stiffness (g/mm) decreased from  $20.2 \pm 2.6$  g/mm in nonpregnant animals to  $6.2 \pm 0.9$  g/mm ( $P \leq 0.001$ , Fig. 1A), and cervical distensibility increased from  $3.2 \pm 0.2$  mm in nonpregnant animals to  $6.9 \pm 0.5$  mm on day 12 ( $P \leq 0.001$ ). Cervical stiffness declined further from days 12 to 18 ( $P < 0.001$ ), and distensibility also increased dramatically from days 12 to 18 ( $P < 0.001$ ; Fig. 1A and B). Despite decreased tissue stiffness and increased cervical distensibility on day 12, the maximal load before failure was similar in nonpregnant and pregnant animals on days 10 and 12 ( $99 \pm 19$  mN/mm<sup>2</sup> when compared with  $129 \pm 17$  mN/mm<sup>2</sup>,  $p = \text{NS}$ , Tukey) suggesting that collagen integrity is maintained (Table 1). In contrast, the maximal load before failure decreased on late day 18 to  $41 \pm 8$  mN/mm<sup>2</sup> (when compared with day 12,  $P < 0.05$ , Tukey), suggesting that, in contrast to the softened cervix, the ripened cervix lacks cervical strength (Table 1). Collectively, these data indicate that, in the mouse, a significant change in biomechanical properties of the cervix (i.e. cervical softening) occurs by gestation day 12, and that certain biomechanical changes during the ripening phase are distinct from those during cervical softening.

### Physiological properties of the cervix during pregnancy: hydration and collagen solubility

Having verified the time point at which softening begins, studies were undertaken to identify physiological changes that may facilitate this process. To determine if tissue growth or changes in tissue hydration occur during softening, cervical wet weight, dry weight, and water



**Figure 1** Biomechanical properties of the mouse cervix during early pregnancy. (A) Tissue stiffness was determined from the slope of the stress-strain relationship in cervixes from nonpregnant (NP, open bar,  $n=8$ ) and pregnant mice on gestation days 10 (hatched bar,  $n=6$ ), 11 (diagonal lines,  $n=7$ ), 12 (solid bar,  $n=8$ ), and 18 and 19 (gray bar,  $n=11$ ). \* $P \leq 0.001$  when compared with NP. (B) Cervices from nonpregnant (NP) and pregnant mice on gestation days 10, 11, 12, and 18 and 19 were distended in 0.5 mm increments every 2 min until failure. Data are expressed as maximal distension in mm and represent mean  $\pm$  S.E.M. of 6–11 cervixes per time point. \* $P \leq 0.001$  when compared with nonpregnant.

content were measured in multiple cervixes from nonpregnant and pregnant animals at selected intervals throughout gestation (Fig. 2). The wet and dry cervical weights were similar in nonpregnant and pregnant animals in early gestation (i.e. days 10–12). This suggests that increases in cervical tissue mass either through increased synthesis of ECM, cell proliferation, or tissue hydration were not significant during early gestation. Even though cervical dry weight increased 1.9-fold from early pregnancy to shortly before birth on day 18.75 ( $P < 0.001$ ), the increase in cervical wet weight was 2.5-fold (from  $9.1 \pm 0.4$  to  $22.2 \pm 1.3$  mg,  $P < 0.001$ ) suggesting that increases in cervical weight during late gestation are due to both increased protein synthesis as well as a modest increase in water content. The finding that tissue hydration increased from  $74.7 \pm 5.4\%$  (days 10–12) to  $78.6 \pm 1.8\%$  on day 18.75 is in agreement with

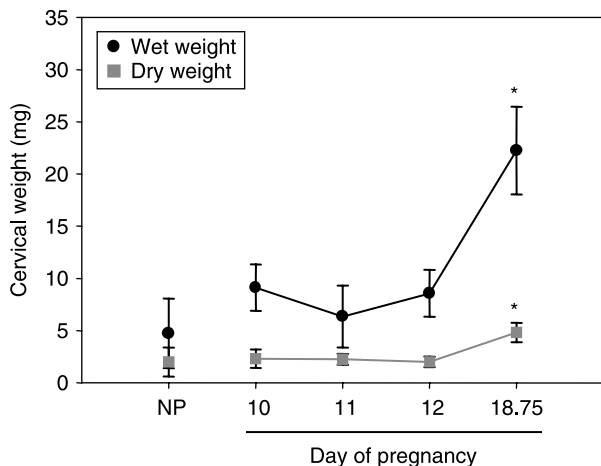
**Table 1** Maximal load before tissue failure for non-pregnant (NP) and gestational age days 10 (day 10), 12 (day 12), and 18 (day 18) mouse cervix ( $n=6-8$  per time point).

Stage	Average	S.E.M.
Non-pregnancy	168.0*	25.1
Pregnancy		
Day 10	99.0	19.0
Day 12	128.7 <sup>†</sup>	17.2
Day 18	41.0*, <sup>†</sup>	7.7

\*Indicates  $P < 0.001$  and <sup>†</sup>refers to  $P < 0.05$  for comparison.

this interpretation and previously reported studies (Rimmer 1973, Anderson *et al.* 2006).

Changes in the solubility characteristics of collagen depend on the number of covalent cross-links between collagen triple helices which are required for the formation of stable collagen fibrils (Robins *et al.* 2003). Previous studies in humans confirm increased solubility of collagen in the presence of acetic acid and pepsin during cervical ripening (Granstrom *et al.* 1989). We sought to determine if the progressive increase in tissue compliance observed during softening and ripening correlates with progressive changes in collagen solubility. Cervices from nonpregnant (early metestrus), gestation days 7, 10, 12, 18, and 10 or 24 h *post partum* were homogenized and extracted in 0.5 M acetic acid containing 1 mg/ml pepsin for 24 h. The percentage of collagen in the soluble and insoluble fraction was determined by quantitative measurement of hydroxyproline (Stegemann & Stalder 1967). Forty-three percent of collagen were soluble in pepsin in nonpregnant mice (Fig. 3A). As early as day 7 the solubility increased to 61% (nonpregnant versus day 7,  $P = 0.03$ ) and reached a peak of 74% at gestation day 12 (day 7 vs day 12,  $p = \text{NS}$ ) with no further increases in solubility during cervical ripening on gestation day 18 (73%) (day 12 vs day 18,  $p = \text{NS}$ ). The percentage of collagen in the soluble fraction rapidly declined in the *post partum* (PP) period and within 24 h after birth is similar to nonpregnant mice (day 18 vs 1 dPP,  $P < 0.01$ ). This data suggest that an increase in soluble collagen accompanies the changes in biomechanical properties of the cervix during softening but the progressive increase in tissue compliance during cervical ripening is not correlated with further increases in collagen solubility. Compared to nonpregnant cervix (metestrus), there were no significant changes in collagen content during pregnancy in contrast to the *post partum* period when there is a significant increase (Fig. 3B). While there appeared to be a decline in collagen concentration right before parturition when compared with nonpregnant (Fig. 3C), this trend did not reach statistical significance. Collagen concentration was at its maximal on day 7 and significantly declined on days 12, 18, and 10 h *post partum* when compared with day 7 ( $P < 0.05$ , Tukey test).



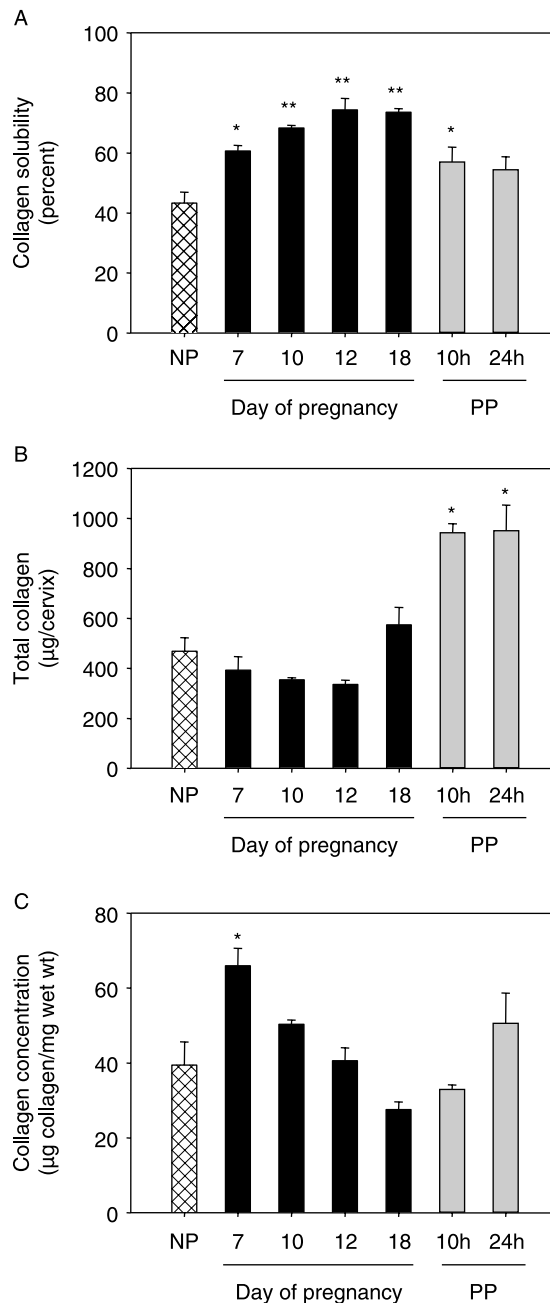
**Figure 2** Regulation of cervical weight in pregnant mice during pregnancy. Cervical dry (gray square) and wet (black circle) weights were determined in nonpregnant (NP) and pregnant mice on gestation days 10, 11, 12, and 18.75. Both the mean cervical wet and dry weights were greater just prior to delivery at day 18.75 when compared with cervical tissues from nonpregnant or pregnant mice earlier in gestation ( $*P < 0.001$ , Dunnett). ( $n = 5-11$  cervices at each time point).

### Role of inflammatory cells during cervical softening

To determine if the softening process is accompanied by changes in inflammatory cell distribution, immunohistochemical detection of macrophages, neutrophils, and monocytes was carried out using frozen cervical sections from nonpregnant and pregnant mice at gestation days 10 and 12 with antibodies that detect monocytes and neutrophils (7/4) and macrophages (BM8) as described previously (Mackler *et al.* 1999, Henderson *et al.* 2003, Timmons & Mahendroo 2006). 7/4- (Fig. 4, upper panels) and BM8-positive cells (Fig. 4, lower panels) were observed in both pregnant and nonpregnant sections with 7/4-positive cells predominantly lining the cervical epithelium and BM8-positive cells residing within the stroma near the subepithelial regions. This distribution pattern of immune cells at days 10 and 12 is similar to nonpregnant cervical tissues in metestrus (Fig. 4) and differs significantly to changes in distribution of immune cells observed during ripening (Timmons & Mahendroo 2006). This suggests that although a significant number of inflammatory cells are present in the cervix at this time in pregnancy, there appears to be little change in the distribution of neutrophils, monocytes or macrophages within the cervix from days 10 to 12 during cervical softening.

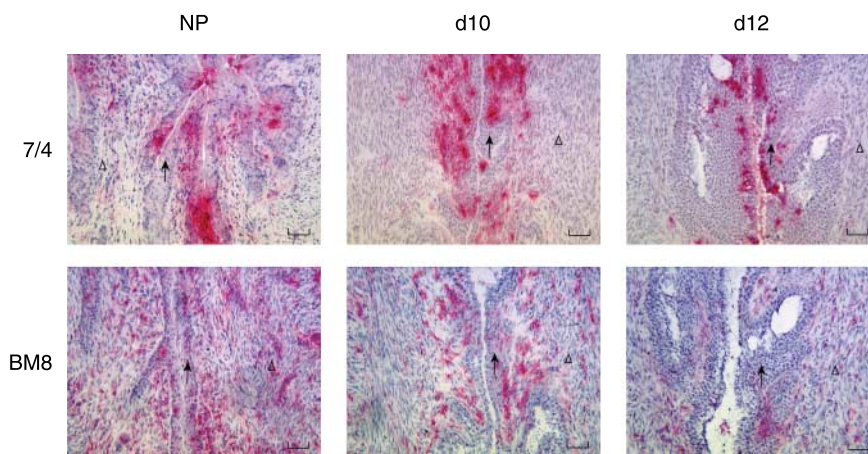
### Differential expression of genes in the cervix during cervical softening: results of microarray analysis

Further studies were carried out to identify genes that may be transcriptionally regulated during cervical softening. Total RNA from three cervixes each at gestation days 10 and 12 was hybridized to six Affymetrix microarray chips.



**Figure 3** Collagen solubility, total collagen, and collagen concentration in cervical tissues from nonpregnant (NP), pregnant (days 7, 10, 12, and 18) and *post partum* (PP) mice. Percentage of solubilized collagen was determined in nonpregnant metestrus (hatched bar), pregnant (black bars), and *post partum* (grey bars) (A). Total amount of collagen in the cervix and concentration of collagen normalized to cervical wet weight are shown in (B and C) respectively.  $*P < 0.05$ ;  $**P < 0.001$ , ANOVA Dunn test comparison with NP ( $n = 4-6$  cervices at each time point).

Of 45 037 sequence tags on the array, expression of 21 544 genes was detectable in cervical tissue. One hundred and forty-six genes were significantly upregulated and 96 were downregulated in cervical tissues on day 12 when compared with day 10 ( $\chi^2$ ,  $P = 0.001$ ).



**Figure 4** Immunolocalization of leukocytes (neutrophils and monocytes) and macrophages in cervical tissues from nonpregnant (NP) and pregnant mice on gestation days 10 and 12. Tissue sections were oriented in the sagittal plane through the cervical os. The cervical lumen lined by epithelial cells is indicated by arrows and cervical stroma by open arrowheads. Immunohistochemistry was conducted using MAB 7/4 (upper panels) and BM8 (lower panels). Positive immunoreactivity is indicated by red staining. 7/4-positive cells (leukocytes and monocytes) were predominantly localized in the epithelium, whereas macrophages were primarily localized in the subepithelial and stromal compartments of the cervix. Sections are representative of three cervixes from each gestation day. Bar represents 20  $\mu\text{m}$ .

The first criterion for differential expression of genes during cervical softening (mean difference in expression from days 10 to 12 by  $\geq$  two-fold) yielded a gene list with 64 entries and is represented in Table 2 (upregulated genes) and Table 3 (downregulated genes). Using parametric statistical tests adjusting for multiple comparisons generated an additional gene list of 247 entries. This list is available as supplemental material (Supplemental Table 1, which can be viewed online at [www.reproduction-online.org/supplemental/](http://www.reproduction-online.org/supplemental/)). Candidate genes identified in microarray analysis based on a two-fold change (*Cxcl5*, *Mcp7*, *Pcp4*, *Spink5*, and *Tff1*; Tables 2 and 3) were more often confirmed by RT-PCR than genes selected based on parametric statistical analysis (*Aspn* and *Sdc2*; Supplement).

The function and ontology of genes identified by mean difference in expression (Tables 2 and 3) or statistical tests (Supplemental table) were reviewed to identify those genes with a potential role in cervical softening. Contrary to our hypothesis, relatively few genes encoding ECM proteins were changed in expression between the two time points. Cervical softening was associated with increased expression of genes encoding protease inhibitors (serine protease inhibitor Kazal type 5 (*Spink5*); serine proteinase inhibitor, clade B, member 5; and serine protease inhibitor, Kunitz type 2), mucosal epithelial cell repair/anti-inflammatory proteins (trefoil factor 1, chitinase 3 like-1, decay accelerating factor, and platelet-activating factor acetylhydrolase, isoform 1b, beta 1 subunit), and neutrophil chemoattractants (chemokine C-X-C ligand 5). Of the total gene list, eight genes were selected for further analysis based on their likelihood of contributing to cervical softening.

Two genes, asporin (*Aspn*) and syndecan 2 (*Sdc2*), were selected as they encode ECM proteins that could

influence changes in the cervical ECM during softening. Other candidate genes included chemokine ligand 5 (*Cxcl5*, a neutrophil chemoattractant expressed by epithelial cells), mast cell protease 7 (*Mcp7*, a serine protease expressed in mast cells), trefoil factor 1 (*Tff1*, a secreted protein involved in repair and protection of gastrointestinal mucosal epithelia), purkinje cell protein 4 (*Pcp4*, a neuronal specific calmodulin regulatory protein that inhibits apoptosis), serine proteinase inhibitor lympho-epithelial Kazal-type inhibitor (*Spink5*, a protease inhibitor that prevents degradation of proteins required for maintenance of an intact epithelial barrier), and decay accelerating factor (*Daf1*, a protein that suppresses inflammatory responses via inhibition of activation of the complement cascade; Lin *et al.* 2001, Kanamori *et al.* 2003, Singh *et al.* 2004, Yang *et al.* 2004, Funaba *et al.* 2006, Masui *et al.* 2006).

### Regulation of gene expression in the cervix during pregnancy and parturition

Relative mRNA levels of selected genes were determined in cervical tissues from mice on gestation days 10 and 12. Asporin (*Aspn*), syndecan 2 (*Sdc2*), mast cell protease 7 (*Mcp7*), and decay accelerating factor 1 (*Daf1*) were found to be differentially expressed on the microarray, but further analysis with RT-PCR did not substantiate these differences (Fig. 5). Four genes with confirmed changes are indicated (Fig. 5). The analysis of relative mRNA levels of these genes was subsequently expanded to include cervical samples that were obtained throughout gestation (from day 8 through day 1 *post partum*) using RT-PCR (Fig. 6). Chemokine C-X-C ligand 5 (*Cxcl5*) encodes a neutrophil chemoattractant and was found to be upregulated on day 12 (Fig. 5).

**Table 2** Upregulated genes on day 12 when compared with day 10 of gestation in mouse cervical tissue.

Entrez gene ID	Gene title	Fold change	s.d.*	P value
Transport functions				
12870	Ceruloplasmin	2.38	1.73	0.092
56774	Solute carrier family 6 (neurotransmitter transporter), member 14	2.21	2.95	0.184
23844	Chloride channel calcium activated 3	2.19	0.89	0.027
Cytoskeletal components				
19240	Thymosin, beta 10	4.59	5.13	0.132
16660	Keratin complex 1, acidic, gene 1	2.68		0.296
16687	Keratin complex 2, basic, gene 6a	2.33	1.67	0.211
18472	Platelet-activating factor acetylhydrolase, isoform 1b, $\beta$ 1 subunit	2.16	1.41	0.471
Enzymes				
241230	ST8 alpha-N-acetyl-neuraminide $\alpha$ -2,8-sialyltransferase 6	3.02	1.89	0.126
14933	Glycerol kinase	2.46	1.30	0.070
68631	Crystallin, $\lambda$ 1	2.21		0.422
14538	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	2.08	0.89	0.316
Cellular growth and differentiation				
21784	Trefoil factor 1	2.19	0.68	0.040
21946	Peptidoglycan recognition protein 1	2.03	0.37	0.061
Hormones				
57262	Resistin-like $\alpha$	2.24	1.19	0.612
Immune function				
20311	Chemokine (C-X-C motif) ligand 5	3.92	3.11	0.147
12654	Chitinase 3-like 1	3.34	8.43	0.359
Matrix molecules/cellular adhesion				
21922	C-type lectin domain family 3, member b	2.38	0.27	0.141
Undefined function				
328660	Brain expressed, X-linked 6	2.48	1.54	0.218
17141	Melanoma antigen, family A, 5	2.47		0.225
68718	Ring finger protein 166	2.25	0.64	0.390
170654	Keratin-associated protein 16-4	2.04	1.08	0.389
Protease and protease inhibitors				
72432	Serine protease inhibitor, Kazal type 5 ( <i>Spink5</i> )	4.36	3.46	0.140
70574	Carboxypeptidase M	3.01	1.37	0.153
Cell surface receptors				
12638	Cystic fibrosis transmembrane conductance regulator homolog	2.61	0.84	0.151
21942	Tumor necrosis factor receptor superfamily, member 9	2.50	2.09	0.161
Signaling cascade				
76757	Triadin	2.63	0.16	0.081
243659	Serine/threonine/tyrosine kinase 1	2.51		0.252
14609	Gap junction membrane channel protein $\alpha$ 1	2.43	2.12	0.121
100986	A kinase (PRKA) anchor protein (yotiao) 9	2.27	3.12	0.215
252837	Chemokine (C-C motif) receptor-like 1	2.02	0.88	0.138
Transcription factors				
20861	Stefin A1	3.00	1.62	0.242
268885	Stefin A2-like 1	2.83		0.561
268903	Nuclear receptor interacting protein 1	2.44		0.335
19655	RNA-binding motif protein, X chromosome	2.13	0.75	0.065
80913	Pumilio 2 ( <i>Drosophila</i> )	2.06	1.45	0.506
15220	Forkhead box Q1	2.02	1.15	0.043

\*s.d. values are not shown for probe sets with only two expression values.

Though *Cxcl5* expression varied throughout gestation, *Cxcl5* was consistently expressed at higher levels during pregnancy than nonpregnancy (Fig. 5), but there was no specific pattern of expression with respect to duration of pregnancy (data not shown).

Purkinje cell protein 4 (*Pcp4*) was expressed in cervix, and similar to the microarray data, its expression declined on day 12 (Fig. 5). When the expression of this gene was evaluated throughout gestation and in the nonpregnant cervix, it was found to be highly expressed early in pregnancy and to gradually decline towards term (Fig. 6, upper panel). Given the potential role of this protein as an apoptosis inhibitor (Erhardt *et al.* 2000),

*Pcp4* may play a role in suppression of apoptosis in the cervix during pregnancy. In the rat, apoptosis has been shown to be suppressed in the cervix during pregnancy and increases during parturition and *post partum* day 1 (Leppert & Yu 1994, Ramos *et al.* 2002). Further experiments will determine if the gradual decline in expression of *Pcp4* facilitates increased apoptosis of cervical cells during parturition and *post partum* repair.

Both *Tff1* and Kazal-type serine peptidase inhibitor 5 (*Spink5*) were found to be expressed in cervix and upregulated on day 12 of pregnancy (Fig. 5). In addition, both genes were found to be highly upregulated with advancing gestation (Fig. 6, middle and lower panel).



**Table 3** Downregulated genes on day 12 when compared with day 10 of gestation in mouse cervical tissue.

Entrez gene ID	Gene title	Fold change	s.d.*	P value
Transport functions				
11576	$\alpha$ fetoprotein	-7.63	5.98	0.348
108052	Solute carrier family 14 (urea transporter), member 1	-2.07	0.69	0.013
Cytoskeletal components				
16561	Kinesin family member 1B	-2.62		0.518
11459	Actin, alpha 1, skeletal muscle	-2.62	2.03	0.429
245049	Myosin VIIA and Rab-interacting protein	-2.41	1.54	0.229
58187	Claudin 10	-2.36	1.59	0.295
DNA repair/RNA associated				
109113	Ubiquitin-like, containing PHD and RING finger domains 2	-2.69	0.85	0.050
Enzymes				
17523	Myeloperoxidase	-2.47		0.354
11687	Arachidonate 15-lipoxygenase	-2.26	1.21	0.031
11676	Aldolase 3, C isoform	-2.18	0.35	0.088
77219	Zinc-binding alcohol dehydrogenase, domain containing 1	-2.16	1.16	0.204
16891	Lipase, endothelial	-2.09	0.71	0.008
20249	Stearoyl-coenzyme A desaturase 1	-2.08	0.59	0.036
52815	Lactate dehydrogenase D	-2.04	1.25	0.163
12411	Cystathionine beta-synthase	-2.01	0.79	0.056
Hormones				
18776	Chorionic somatomammotropin hormone 2	-3.51	2.43	0.338
Immune function				
76933	Interferon, $\alpha$ -inducible protein 27	-2.13	1.03	0.213
Matrix molecules/cellular adhesion				
66996	CEA-related cell adhesion molecule 11	-8.72	9.18	0.355
Undefined function				
21984	Trophoblast-specific protein $\alpha$	-6.70	4.57	0.329
327959	F-box protein 39	-2.28	1.42	0.444
545422	Similar to low density lipoprotein receptor-related protein 2	-2.22	1.39	0.142
Protease and protease inhibitors				
17230	Mast cell protease 7	-3.46	5.87	0.297
Cell surface receptors				
26563	Receptor tyrosine kinase-like orphan receptor 1	-2.15	1.43	0.113
239849	Cd200 receptor 4	-2.00	0.72	0.407
Signaling cascade				
18546	Purkinje cell protein 4	-3.80	2.64	0.269
211577	MAS-related GPR, member F	-2.01		0.287
Transcription factors				
69188	Myeloid/lymphoid or mixed-lineage leukemia 5	-2.18		0.916
353187	Nuclear receptor subfamily 1, group D, member 2	-2.08	1.28	0.202
17877	Myogenic factor 5	-2.06		0.923

\*s.d. values are not shown for probe sets with only two expression values.

*Tff1* mRNA levels were low in cervical tissues from nonpregnant and pregnant animals on days 8 and 9. During cervical softening, expression of *Tff1* increased 2.8-fold from days 10 to 12 (from  $2.6 \pm 0.6$  to  $7.3 \pm 1.4$  relative units,  $P=0.007$ ). Elevations in *Tff1* during cervical softening was followed by a more dramatic increase in *Tff1* mRNA prior to cervical ripening at term, such that *Tff1* mRNA levels on late-gestation day 18 and after birth were over 1000-fold that in cervical tissues from nonpregnant or early pregnant animals (Fig. 6, middle panel).

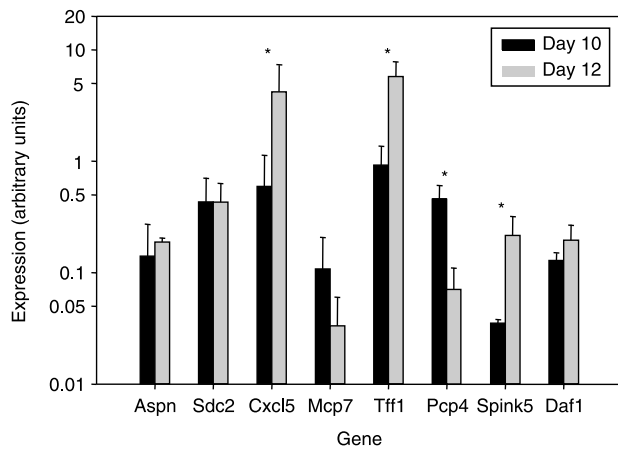
Expression of *Spink5* was also regulated in the cervix during softening and ripening with a gestational age-dependent pattern of expression similar to *Tff1*. Cervical mRNA levels of *Spink5* increased exponentially during cervical softening, throughout parturition and the

*post partum* period. Expression of *Spink5* during cervical softening was increased 2.1-fold on day 12 versus day 10 ( $0.7 \pm 0.02$  vs  $0.15 \pm 0.04$  relative units,  $P=0.05$ ) (Fig. 4). Compared with early gestation (day 8), expression of *Spink5* increased to greater than 650-fold after birth (PP; Fig. 6, lower panel).

## Discussion

### **Molecular events regulating cervical softening are distinct from cervical ripening or dilation at term**

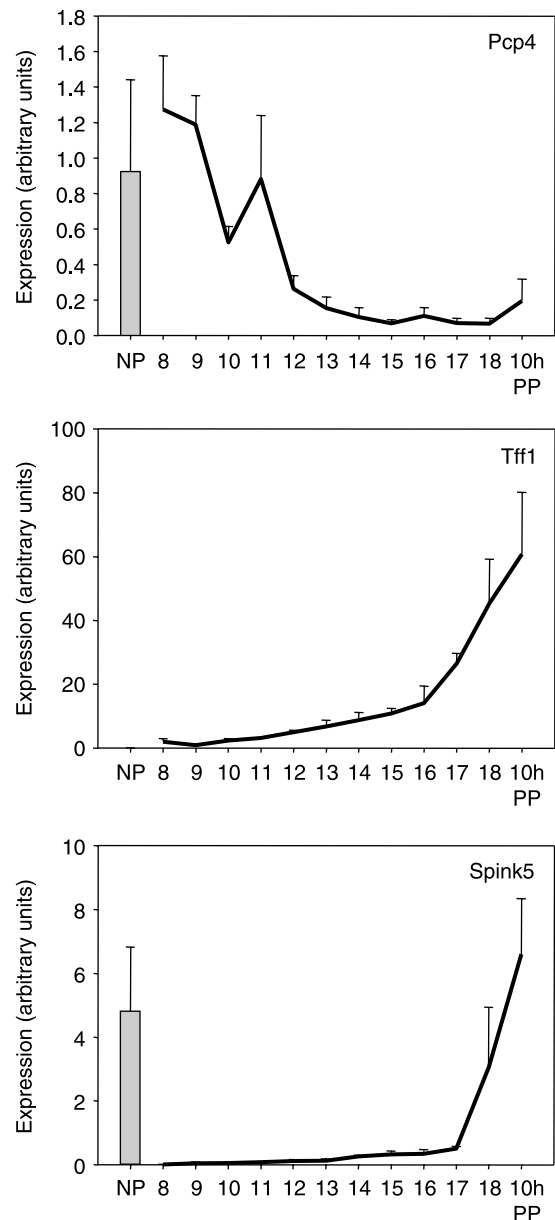
Studies outlined in this work suggest that the molecular processes of cervical softening differ significantly from ripening. Figure 7 illustrates some of these differences. Increases in tissue proliferation, hydration or distribution



**Figure 5** RT-PCR confirmation of differentially regulated genes identified in microarray comparisons between days 10 and 12 of gestation. Chemokine ligand 5 (*Cxcl5*), Kazal-type serine protease inhibitor 5 (*Spink5*), and trefoil factor 1 (*Tff1*) were upregulated on day 12, while purkinje cell protein 4 (*Pcp4*) was downregulated on day 12. Significant changes in Asporin1 (*Aspnl*), Syndecan 2 (*Sdc2*), mast cell protease (*Mcp7*), and decay-accelerating factor (*Daf1*) mRNA were not detected by QRT-PCR. Expression values are displayed in logarithmic scale and represent mean  $\pm$  S.E.M. of six cervixes at each time point. \* $P < 0.05$  when compared with day 10.

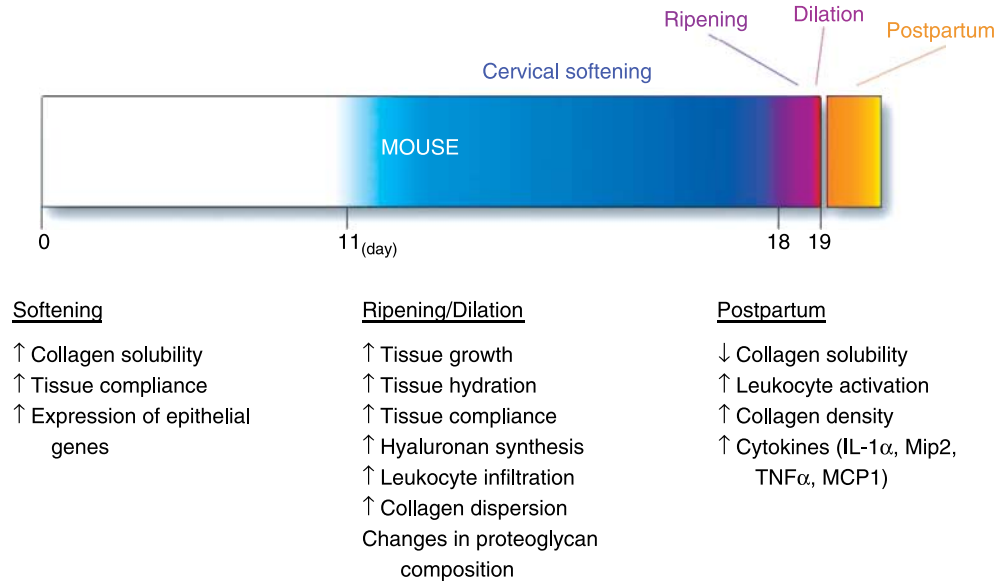
of inflammatory cells in cervical stromal matrix were not evident during cervical softening. Increases in collagen solubility appear to precede biomechanical evidence of softening, indicating that this is one of the earlier events in the remodeling process. Further studies are required to determine the steps in collagen synthesis, cross-linking and fibril assembly that may be altered in early pregnancy, thus explaining the observed changes in our collagen solubility data. Changes in solubility are not predicted to result from increased collagen degradation as the overall cervical collagen content did not decline significantly during softening or ripening and was actually increased after parturition (Fig. 3). Previous studies are inconsistent with our findings as they report small increases in collagen content in mouse cervixes prior to parturition; however, no statistical analysis was carried out (Rimmer 1973). Similar to previous studies, collagen concentration was greatest early in pregnancy (day 7) and lowest during cervical ripening (day 18) and 10 h *post partum* (Rimmer 1973). This suggests that the processes leading to increased collagen solubility precede the molecular events that control cervical tissue compliance during cervical softening and ripening (Fig. 1).

An important question in the understanding of cervical remodeling is how the cervix can change its characteristics to promote softening throughout pregnancy yet remain closed to retain the developing fetus within the uterus. Biomechanical studies in the rat provide some insight into a dual mechanism that affects both cervical geometry and tissue stiffness. Cervical softening is influenced by the increase in cervical diameter with no change in cervical wall thickness and biomechanical



**Figure 6** Expression of purkinje cell protein 4 (*Pcp4*, upper panel), Trefoil factor 1 (*Tff1*, middle panel), and Kazal-type serine protease inhibitor 5 (*Spink5*, lower panel) in cervical tissues from nonpregnant (NP) and pregnant mice. Cervixes were obtained from nonpregnant (NP) and pregnant mice throughout gestation and the morning after parturition (10hPP). Expression of *Pcp4*, *Tff1*, and *Spink5* was quantified using RT-PCR and normalized to that of cyclophilin B (*Ppib*) as described in Materials and Methods. Data represent mean  $\pm$  S.E.M. of an average of three cervixes per time point with the exception of days 10 and 12 in which data are estimated from nine cervixes per time point.  $P < 0.001$  by ANOVA Kruskal–Wallis test day 8 through 10hPP for all three genes analyzed.

integrity of the cervix is maintained until late in gestation (Drzewiecki *et al.* 2005). Studies outlined in this study may provide insights into the biochemical changes that result in the dual biomechanical changes that promote softening.



**Figure 7** Representative schematic of the phases of mouse pregnancy. The changes in cervical physiology during mouse gestation can be divided into distinct yet overlapping phases: softening, ripening/dilation, and *post partum* repair. The ripening and dilation phases are described together as it is still not clear what processes may be distinct to these two phases. Processes described under *post partum* repair are those that occur in the immediate *post partum* period (within 24 h *post partum*).

In contrast to our observations with cervical softening, cervical ripening at the end of pregnancy is characterized by increased cell proliferation as well as reduced apoptosis of both the epithelia and the stroma which result in an increased circumference of the cervical lumen (Lee & Sherwood 2005, Lee *et al.* 2005). Tissue water content is also increased presumably due to the increased expression of aquaporin water channel proteins (Anderson *et al.* 2006) and hydrophilic glycosaminoglycans (ElMaradny *et al.* 1997, Straach *et al.* 2005). Increases in the glycosaminoglycan, hyaluronan, result in disruption of the collagen matrix which, along with tissue growth, facilitates cervical ripening (ElMaradny *et al.* 1997, Straach *et al.* 2005). These differences between softening and ripening which are illustrated in Fig. 7, may explain why in the ripened cervix there is loss of structural integrity, tensile strength, as well as an increase in dilation in response to uterine contractions of labor, yet during softening, the cervix retains its structural integrity, tensile strength, and is resistant to forces exerted upon it.

### **Distribution of inflammatory cells during cervical softening**

Inflammatory cells and cytokines are increased during cervical dilation of labor in all mammalian species studied (Bokstrom *et al.* 1997, Thomson *et al.* 1999, Sennstrom *et al.* 2000, Kelly 2002). The role of inflammatory cells in ripening before dilation; however, is not clear in women or mice (Sakamoto *et al.* 2004, Sakamoto *et al.* 2005, Timmons & Mahendroo 2006).

In the mouse, cervical ripening (Phase 2) is characterized by a change in distribution of inflammatory cells with increased staining of monocytes and/or neutrophils within the cervical stroma (Timmons & Mahendroo 2006). Studies conducted in cervical tissues from women and mice before and after cervical ripening (but before labor) failed to find significant increases in the number of granulocytes in the ripened cervix before labor (Sakamoto *et al.* 2004, 2005, Timmons & Mahendroo 2006). Macrophage numbers are reported to increase in women and one study in mice during ripening but the antibodies used may also recognize tissue monocytes thus a functional role of these cells in ripening remains unclear (Mackler *et al.* 1999, Sakamoto *et al.* 2004).

In contrast to the later phases of cervical remodeling, midtrimester cervical softening phase was associated with little change in the distribution of macrophages or neutrophils. In addition, relatively few differentially expressed genes were identified that are involved with activation of the immune system by gene microarray comparisons. One exception was the expression of *Cxcl5*, a neutrophil chemoattractant made by epithelial cells (Walz *et al.* 1991, Imaizumi *et al.* 2003). *Cxcl5* was expressed throughout gestation (data not shown) and is consistent with the fact that neutrophils are confined to the epithelial region at both gestation days 10 and 12 as well as later in pregnancy (Timmons & Mahendroo 2006). We hypothesize that the localization of neutrophils in the epithelial region (Fig. 4, upper panel) helps to ensure that numerous surveillance mechanisms are in place during pregnancy to prevent ascending infections

and protect the vulnerable, remodeling stromal matrix. Future studies to determine activation of inflammatory cells in a functional assay or measure cytokine levels directly will be required to verify that major inflammatory events do not mediate the softening process.

### **Expression of epithelial-specific genes during cervical softening**

We initially hypothesized that changes in expression of genes affecting structure of collagen or elastin fibers would be identified in cervical tissues before and after cervical softening. This hypothesis is supported by i) decreased activity of the cross-linking enzyme, lysyl oxidase, in the mouse cervix during pregnancy, ii) changes in proteoglycan composition in the human and rat cervix during softening, and iii) the finding that mutations in the ECM proteins fibrillin-1 and thrombospondin 2 lead to premature cervical softening in women and mice respectively (Ozasa *et al.* 1981, Osmers *et al.* 1993, Kyriakides *et al.* 1998, Rahman *et al.* 2003, Kokenyesi *et al.* 2004). Contrary to our expectations, however, we were unable to detect significant changes in expression of ECM genes in the cervix from days 10 to 12. There are several possible explanations for these results. First, regulation of ECM proteins may occur at the post-transcriptional level that would not be reflected by changes in transcript expression using microarrays or RT-PCR. This possibility is suggested by studies that report no decrease in transcripts for small proteoglycans such as decorin, biglycan, and fibromodulin in the cervix from women at term despite a 50% reduction of total proteoglycan concentration (Westergren-Thorsson *et al.* 1998). Second, cervical epithelial cells undergo hyperplasia with advancing gestation such that the ratio of epithelial to stromal cells may change and therefore bias the microarray results towards epithelial-specific genes (Burger & Sherwood 1998, Lee *et al.* 2005). Since, in these studies, whole cervix was used instead of fractionating the epithelial from the stromal compartment, the contribution of potentially important stromal genes may have been diluted. Finally, significant changes in mRNA levels of proteins affecting the ECM may have occurred prior to day 10. Although changes in biophysical properties of the cervix were not detected until gestation day 12, significant changes in mRNA transcripts may have preceded cervical softening by several days. The fact that increases in collagen solubility (day 7) preceded changes in tissue compliance (day 12) further supports this idea. Studies are in progress to determine the biochemical and molecular changes that bring about increases in collagen solubility during early pregnancy.

Nevertheless, although genes encoding ECM molecules were not found to be differentially regulated during cervical softening, increased expression of several epithelial-specific genes were identified in the

softened cervix on gestation day 12. The genes we identified are important in maintaining the integrity and barrier function of epithelia in the gut or skin. *Tff1* and *Spink5* are two genes that were significantly upregulated during cervical softening, and their expression progressively increased to peak at term and *post partum*. Based on their functions in gut and skin respectively, we propose that these transcripts encode proteins that may protect the cervix from infection and maintain the integrity and barrier function of the epithelia. Both of these genes are reported to be expressed in the female reproductive tract but have not been carefully explored with respect to female reproductive tract physiology (Wiede *et al.* 2001, Masui *et al.* 2006). The *Tff1* gene encodes the estrogen-responsive protein, pS2, which is expressed in the gastric mucosa of humans and mice and involved in protection and restitution of mucosal epithelia (Shaoul *et al.* 2004, Hoffmann 2005). *Tff1*-deficient mice (*Tff1*<sup>-/-</sup>) develop multiple antral gastric tumors from the loss of mucosal protection afforded by the pS2 protein (Lefebvre *et al.* 1996).

*Spink5* is expressed in the differentiated layers of stratified epithelial tissue, and mutations in this gene have been shown to be the cause of autosomal-recessive Netherton syndrome (Netherton, 1958). Patients with this condition suffer from frequent bacterial infections. *Spink5*<sup>-/-</sup> mice die a few hours after birth from dehydration due to the lack of an intact permeability barrier of skin (Hewett *et al.* 2005). The gene product, lymphoepithelial Kazal-type related inhibitor, is a serine protease inhibitor that attenuates the functions of stratum corneum tryptic enzymes, which degrade desmoglein 1 as well as other proteins involved in barrier formation and maintenance (Descargues *et al.* 2005).

The transcriptional upregulation of *Spink5* and *Tff1* during cervical softening and continuing through parturition suggests that the cervical epithelia may play a greater role in the process of parturition than was previously recognized. Changes in stromal matrix must orchestrate, and are integral to, the change in biomechanical properties of the cervix. In addition, epithelial cells together with neutrophils lining the cervical os, may serve an immunomucosal protective role to prevent inappropriate access of invading microorganisms to the stromal matrix, which may be in a vulnerable, 'softened' state as well as prevent ascending infections into the upper reproductive tract. As the cervix loses structural integrity during ripening, parturition and *post partum* recovery of the cervix, the need for epithelial barrier protection potentially becomes even greater. Further studies on the role of *Spink5* and *Tff1* in cervical remodeling may enhance our understanding of molecular events that control parturition and determine if alterations in expression or function of these epithelial-specific genes could result in accelerated remodeling of the cervical ECM, thereby leading to the complications of cervical insufficiency and preterm birth.

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