Metabolic, Endocrine and Genitourinary Pathobiology

Pelvic Organ Prolapse in Fibulin-5 Knockout Mice

Pregnancy-Induced Changes in Elastic Fiber Homeostasis in Mouse Vagina

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Pelvic organ prolapse is strongly associated with a history of vaginal delivery. The mechanisms by which pregnancy and parturition lead to failure of pelvic organ support, however, are not known. Recently, it was reported that mice with null mutations in lysyl oxidase-like 1 (LOXL1) develop pelvic organ prolapse. Elastin is a substrate for lysyl oxidase (LOX) and LOXL1, and LOXL1 interacts with fibulin-5 (FBLN5). Therefore, to clarify the potential role of elastic fiber assembly in the pathogenesis of pelvic organ prolapse, pelvic organ support was characterized in *Fbln5*^{-/-} mice, and changes in elastic fiber homeostasis in the mouse vagina during pregnancy and parturition were determined. Pelvic organ prolapse in *Fbln5*^{-/-} mice was remarkably similar to that in primates. The temporal relationship between LOX mRNA and protein, processing of LOXL1 protein, FBLN5 and tropoelastin protein, and desmosine content in the vagina suggest that a burst of elastic fiber assembly and cross linking occurs in the vaginal wall postpartum. Together with the phenotype of *Fbln5*^{-/-} mice, the results suggest that synthesis and assembly of elastic fibers are crucial for recovery of pelvic organ support after vaginal delivery and that disordered elastic fiber homeostasis is a primary event in the pathogenesis of pelvic organ prolapse in mice. (*Am J Pathol* 2007, 170:578–589; DOI: 10.2353/ajpath.2007.060662)

Pelvic organ prolapse is a common condition that negatively impacts the quality of life of millions of women.¹ In one study, 11% of women had surgery for urinary incontinence or pelvic organ prolapse during their lifetime.¹ For hundreds of years, it has been recognized that the process of pregnancy, labor, and delivery is associated with the development of prolapse.^{2,3} In addition to vaginal birth, epidemiological studies suggest that aging is also a significant risk factor for developing pelvic organ prolapse. The mechanisms by which pregnancy, parturition, and aging lead to failure of pelvic organ support, however, are not known. Furthermore, the mechanisms that mediate the delayed manifestations of childbirth-associated injuries of the pelvic floor during childbirth are not understood.

Support of the pelvic viscera is maintained by fibromuscular connective tissues of the female pelvic floor and a group of skeletal muscles known as the levator ani. It is believed that the levator ani can sustain direct or neurological damage during childbirth and other neuropathic processes. Even so, defects in the levator ani (or even the reduced ability of the levator to contract) do not correlate with pelvic organ prolapse in many women.^{4–7} Thus, a potential role of fibromuscular connective tissue in the pathophysiology of pelvic organ prolapse has been proposed by us^{8,9} and others.¹⁰ The supportive connective tissues of the vagina, although essentially a continuous sheet, are generally subdivided into three levels of support: the uterosacral and cardinal ligaments (level I), paravaginal connective tissues suspending the lateral vaginal walls to the arcus tendineus and fascia of the levator ani (level II), and the perineal membrane and perineal body (level III).¹¹ Studies conducted in the vaginal wall of women with pelvic organ prolapse reveal marked abnormalities in histomorphology,^{8,12} biochemistry,⁹ gene expression,¹³ and ultrastructural morpholo-

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gy.¹⁴ Studies conducted with vaginal tissues from women with or without pelvic organ prolapse reveal information only about the tissue differences at the time of surgery but little regarding the pathogenesis of prolapse. Prolonged stretch, mechanical stress, and hypoxia within the vaginal wall may produce secondary effects that may contribute to progressive deterioration of pelvic organ support, but may be unrelated to its primary pathogenesis.

Recent findings in mice with null mutations in the gene encoding lysyl oxidase-like 1 (LOXL1) suggest that LOXL1 is crucial for pelvic organ support.¹⁵ LOXL1 knockout mice are viable and appear grossly normal except for elastic fiber defects in the skin, lung, and postpartum uterus.¹⁵ Interestingly, mice lacking LOXL1 develop pelvic organ prolapse 1 to 2 days after giving birth. Because elastin cross-links were markedly decreased in the postpartum uterus, but not in the virgin uterus, of LOXL1 knockout mice,^{15,16} failure to form elastic fibers in the postpartum uterine wall was suggested to account for the phenotype of pelvic organ prolapse in LOXL1-null mice. The specific role of elastic fiber remodeling and homeostasis in the phenotype of pelvic organ prolapse in mice, however, is not clear. Although hydroxyproline content was reported as normal in the postpartum uterus of LOXL1^{-/-} mice,¹⁵ it is unlikely that remodeling of the uterus per se contributes to pelvic organ support. Furthermore, we find that regulation of lysyl oxidases is tissue-specific and hydroxyproline content is reduced 29% in the aorta and 23% in lung tissue from LOXL1-null mice (I.H., personal communication), suggesting that decreased formation of mature, crosslinked collagen fibrils may contribute to the phenotype of LOXL1-deficient animals.

Elastic fiber synthesis and assembly is a complex process that is incompletely understood. Precursor elastin monomers (tropoelastin) are secreted primarily from fibroblasts and smooth muscle cells. Microfibrils consist of several proteins (including fibrillin and microfibril-associated glycoprotein) that form a scaffold on which elastin is deposited before it is displaced to the periphery of the growing fiber where it is cross-linked by one or more copper-requiring extracellular enzyme(s), the lysyl oxidases. There are five members of the LOX family [lysyl oxidase (LOX) and lysyl oxidase-like 1 to 4 (LOXL 1 to 4)]. Fibulin-5 (FBLN5) is an elastin-binding protein crucial for elastogenesis.^{17,18} Mice with null mutations in fibulin-5 (*Fbln5*^{-/-}) also demonstrate numerous signs of elastinopathy including lax skin, emphysematous lungs, and dilated and tortuous great vessels. LOXL1 binds to FBLN5, and it has been suggested that FBLN5 targets LOXL1 to elastic fiber assembly sites in the extracellular matrix.¹⁵ To clarify the role of elastic fiber assembly in the pathogenesis of pelvic organ prolapse, herein we characterized pelvic organ support in *Fbln5*^{-/-} mice and determined the regulation of elastic fiber synthesis and assembly in normal mouse vagina during pregnancy and parturition. In this investigation, we focused on the vaginal wall, together with its attachments to the adjacent pubocaudalis (level II support) to represent the supportive connective tissues of the female pelvic organs. The

results suggest that synthesis and assembly of elastic fibers are crucial for recovery of pelvic organ support after vaginal delivery and that disordered elastic fiber homeostasis plays a primary role in development of pelvic organ prolapse in mice.

Materials and Methods

Mice

Animals were housed under a 12-hour light cycle (lights on, 6:00 AM to 6:00 PM) at 22°C. All mice used in these studies were of C3BL/6J or mixed strain (C57BL/6 × 129SvEv). To obtain timed-pregnant animals, females less than 7 months of age were housed with males for 4 to 6 hours and checked at midday for vaginal plugs. Plug day was considered day 0. Birth occurred in the early morning hours (2 to 6 AM) of day 19. Mice were sacrificed at nonpregnant ($n = 40$), pregnant (day 18, $n = 58$), parturient (in labor after delivery of the first pup, $n = 8$), or postpartum time points (2 hours, $n = 9$; 4 hours, $n = 5$; 12, $n = 4$; 24 hours, $n = 25$; 48 hours, $n = 12$; 72 hours, $n = 9$; 1 week, $n = 11$; 2 weeks, $n = 11$; and 4 weeks, $n = 10$). Maternal body weight increased during pregnancy (from 27.5 ± 0.5 g to 42.2 ± 0.8 g). By 48 hours, maternal weight was 30.8 ± 0.6 g. All animals delivered at least three pups with an average litter size of 7.8 ± 0.3 . The average number of pups delivered did not vary among animals at each time point. After disarticulation of the pubic symphysis, uterine horns together with the bladder, cervix, and vagina were dissected down to the perineal skin. The vaginal dissection extended to the connective tissue suspending the vaginal wall to the pubocaudalis. Using microinstruments and a dissection microscope, the uterine horns were removed at the level of the cervicovaginal junction. Perineal skin was removed and the bladder and urethra dissected from the anterior vaginal wall. Wet weight of vagina and cervix was determined. Thereafter, the cervix was removed from the vaginal tube and weighed. Tissues were stored at -20°C in RNA_{Later} (Ambion, Austin, TX). All studies were conducted in accordance with the standards of humane animal care described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, using protocols approved by an institutional animal care and research advisory committee.

Homogenization of Tissue and Protein Extraction

On thawing in RNA_{Later}, tissues were blotted, weighed, and pulverized with a liquid nitrogen-chilled mortar and pestle. Tissue powder was then homogenized in basic buffer containing protease inhibitors (16 mmol/L potassium phosphate, pH 7.8, 0.12 mol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 10 $\mu\text{g/ml}$ pepstatin A, and 10 $\mu\text{g/ml}$ leupeptin), and then centrifuged at $10,000 \times g$. The supernatant was then removed and the previous homogenization step repeated after resuspending the

remaining tissue pellet in basic buffer. After removal of the second supernatant, the remaining tissue pellet was suspended in urea buffer (6.0 mol/L urea in above basic buffer), homogenized, and placed on a rotating rack for overnight extraction at 4°C. Thereafter, the samples were centrifuged ($10,000 \times g$ for 30 minutes) and the supernatant removed. Protein concentrations were determined using a bicinchoninic acid protein assay and standard curves of bovine serum albumin in appropriate buffers.

Immunoblot Analysis

Total protein (10 μ g/lane) was applied to 4 to 20% Criterion gradient polyacrylamide gels (Bio-Rad, Hercules, CA), separated by electrophoresis, and transferred to nitrocellulose membranes overnight at 4°C. To ensure equal protein loading, identical gels were run side-by-side for Coomassie staining. Nitrocellulose membranes were placed in blocking buffer (10 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, 0.1% Tween 20, 2% nonfat powdered milk, and 0.01% thimerosol) for 1 hour at 37°C and incubated with primary antibody for 1 hour at 30°C. Membranes were then washed with TBST (10 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.1% Tween 20) for 5 minutes $\times$ 3, an enhanced detergent wash (TBST, Nonidet P-40 0.05%, 3 mmol/L sodium deoxycholate, and 0.1% sodium dodecyl sulfate) for 7 minutes $\times$ 3, and again with TBST for 5 minutes $\times$ 3. Thereafter, the blot was incubated with a second antibody (goat IgG-horse-radish peroxidase conjugate, 1:8000) at room temperature for 1 hour. The membrane wash protocol was repeated, followed by incubation with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA) for 2 minutes. Chemiluminescence images were obtained on a Chemimager 4400 (Alpha Innotech Corp., San Leandro, CA). Signal strength was quantified using α Ease v5.5 software (Alpha Innotech). The relative signal strength per μ g of urea-extracted protein was calculated and normalized to external standards of nulliparous non-pregnant vaginal protein extract present on each blot. The amount of protein loaded and exposure time was determined to be in the linear range.

Rabbit anti-rat FBLN5 (BSYN1923) was used at 1:250 dilution. This antibody is weakly immunoreactive with cytokeratin. To ensure specificity of immunoreactivity for FBLN5, two experiments were conducted. First, a second FBLN5 antibody (UT65), which recognizes the sixth Ca^{2+} -binding domain of FBLN5, yielded results virtually identical to those obtained with BSYN1923. Second, membranes were incubated with a pancytokeratin antibody (PAN-CK 5/6/8/18; Novocastra Laboratories, Newcastle on Tyne, UK), and, unlike the results with FBLN5 antibodies, the signal intensity of immunoreactivity at 65 kd was similar in all samples throughout gestation. Rabbit anti-mouse tropoelastin was obtained from Elastin Products (Owensville, MO). LOX antibodies were used as described previously (University of Hawaii, Honolulu, HI).¹⁹ Polyclonal LOXL1 antibody (anti-LOXL1, 204-393) was generated by immunizing rabbits with C-terminal two-thirds (45 kd, starting with the sequence VYYRG) of

recombinant mouse LOXL1 as described previously.²⁰ A smaller 20-kd fragment consisting of the N-terminal region of the 45-kd protein ending with VGSVY and not containing the catalytic domain was expressed in bacteria and purified. Antiserum to the 45-kd fragment was affinity-purified by absorption against the smaller 20-kd recombinant fragment that was covalently bound to ultra-link resin. This purification removed antibodies immunoreactive to the common catalytic domain, yielding antibodies specific for LOXL1 as described previously.²⁰

Quantification of Desmosine

Elastic fiber content was assessed by radioimmunoassay for desmosine, an amino acid crosslink found only in elastin. After urea extraction, pelleted tissue extracts were hydrolyzed in 6 N HCl at 100°C for 24 hours. An aliquot was evaporated again to dryness and redissolved in 100 μ l of H_2O , vortexed, microfuged, and assayed for desmosine as previously described.²¹

Real-Time Polymerase Chain Reaction (PCR)

Tissues were minced and homogenized in 4 mol/L guanidinium isothiocyanate buffer and layered over 5.7 mol/L cesium chloride and centrifuged overnight at $237,000 \times g$ to extract RNA. Concentration of RNA was measured and purity confirmed by spectroscopy. Reverse transcription reactions were conducted with 2 μ g of total RNA in a reaction volume of 20 μ l. Each reaction contained 10 mmol/L dithiothreitol, 0.5 mmol/L dNTPs, 0.015 μ g/ μ l random primers, 40 U of RNase inhibitor (no. 10777-019; Invitrogen, Carlsbad, CA), and 200 U of reverse transcriptase (no. 18064-014; Invitrogen). Primer sequences for amplifications were chosen using published cDNA sequences and the Primer Express program (Applied Biosystems, Foster City, CA). Primers were chosen to differentiate the LOX isoforms when possible, such that the resulting amplicons would cross an exon junction, thereby eliminating false-positive signals from genomic DNA contamination (Table 1). SYBR Green was used for amplicon detection. Gene expression was normalized to expression of the housekeeping gene β 2-microglobulin (β 2M). Positive (heart, lung, aorta) and negative (liver) controls were run on each plate including no template controls. All primer sets were tested to ensure that efficiency of amplification throughout a wide range of template concentrations was equivalent to that of β 2M. Positive and negative tissue controls for each primer set were included in each reaction (aorta, lung, heart, and liver). PCR reactions were performed in the ABI Prism 7000 sequence detection system (Applied Biosystems). The reverse transcription product from 50 ng of RNA was used as template, and reaction volumes (30 μ l) contained $1 \times$ Master Mix (no. 4309155; Applied Biosystems). Primer concentrations were 900 nmol/L. Cycling conditions were 2 minutes at 50°C, followed by 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A preprogrammed dissociation protocol was used after amplification to ensure that all

Table 1. Sequences of Primers Used for Quantification of Gene Expression by Real-Time PCR

Gene	Genbank accession no.	Forward primer (location)	Reverse primer (location)
mLOX	M65142.1	5'-TGCCAGTGGATTGATATTACAGATGT-3' (1325 to 1350)	5'-AGCGAATGTCACAGCGTACAA-3' (1448 to 1428)
mLOXL1	BC037999.1	5'-AAGGCACAGCGACTTTCTC-3' (1695 to 1714)	5'-GAACTCGTCCATGCTGTGGTAA-3' (1783 to 1762)
mLOXL2	BC086801.1	5'-CAACCCCAAAGCCTATAAACCT-3' (1005 to 1027)	5'-GCCCGTGCAGTTTCATAGAAAA-3' (1080 to 1060)
mLOXL3	BC011298.1	5'-GGGTGGACTCATAGTGCCAAATA-3' (367 to 389)	5'-TCCCGTGCAGCTCAGATT-3' (439 to 421)
mLOXL4	BC068307.1	5'-TGGTGACCTGTCGGCAACT-3' (1429 to 1447)	5'-GAACTCCACTCATCACCATTCTTT-3' (1536 to 1512)

samples exhibited a single amplicon. Levels of mRNA were determined using the ddCt method (Applied Biosystems) and expressed relative to an external calibrator present on each plate. To estimate the relative abundance of various lysyl oxidase mRNA transcripts in tissues from nonpregnant and pregnant mice, expression of lysyl oxidase (LOX) was used as the calibrator and levels of other enzymes expressed relative to that of LOX in the same tissues.

Elastase Treatment

Purified porcine elastase (Elastin Products Inc.) was solubilized in phosphate-buffered saline. Five U of elastase in 200 μ l of phosphate-buffered saline was administered through a 30-gauge needle into the posterior vaginal wall of eight postpartum mice on the day of delivery. Controls comprised 14 postpartum animals injected with saline ($n = 8$) or heat-inactivated elastase (5 U, $n = 6$). Heat inactivation was accomplished by incubating the solubilized elastase in a water bath at 100°C for 2 minutes. The presence or absence of a perineal bulge was recorded after 2 to 4 hours, 24, 48, and 72 hours by investigators blinded to treatment status. For statistical purposes, the 72-hour time point was used, although all animals with prolapse at 72 hours exhibited a perineal bulge at earlier time points.

Statistical Analysis

Quantified signal strengths of chemiluminescence and mRNA levels were compared statistically using SigmaStat software (Jandel Scientific, San Rafael, CA). Analysis of variance, Kruskal-Wallis analysis of variance on ranks, and Bonferroni t -test were used to compare the groups, using nulliparous nonpregnant mouse vagina as controls. Pilot studies for quantification of Fbln-5 indicated that three to five animals at each time point would be required to achieve a difference of twofold at the $P \leq 0.05$ level. To determine whether elastase-treated animals were different from saline- or heat-inactivated elastase controls, the presence or absence of a perineal bulge at 72 hours was considered a nominal value and a Fischer's exact test performed. A P value <0.05 was considered statistically significant.

Results

Urogenital Phenotype of *Fbln5*^{-/-} Mice

As early as 3 months of age, virginal *Fbln5*^{-/-} mice exhibited a genitourinary bulge (Figure 1). The degree of prolapse varied among mice but increased progressively with age. By 6 months, 92% of *Fbln5*^{-/-} females (33 of 36) exhibited pelvic organ prolapse. Although the size of the bulge did not increase appreciably during pregnancy, increased severity of prolapse was seen within 1 week postpartum, and mice ≥ 6 months of age (both parous and nulliparous) developed severe prolapse.

In wild-type mice, the vaginal tube, urethra, bladder, and bifurcation of the uterine horns were suspended 1- to 2-cm cephalad to the pubic symphysis (Figure 2A). Dissection of the female reproductive tract in *Fbln5*^{-/-} mice revealed that the urogenital bulge was comprised of the urethra and a patulous vaginal wall (Figure 2B). Bifurcation of the uterine horns was just beneath the pubic symphysis. In some animals, the bladder was caudal to the pubic symphysis and was thereby a component of the bulge. The dissections indicate true urogenital prolapse in *Fbln5*^{-/-} mice, not vulvar edema or laxity of vulvar skin. The anatomical features of complete pelvic organ prolapse in *Fbln5*^{-/-} mice are remarkably similar to that of prolapse in nonhuman primates and women in that the vagina and cervix are descended, stretched, and herniating through the pelvic floor musculature. Like women with pelvic organ prolapse, the vaginal wall was patulous and the genital hiatus appeared to be increased in *Fbln5*^{-/-} mice.

Figure 1. Pelvic organ prolapse in *Fbln5*^{-/-} mice. Anogenital region of 6-month-old nonpregnant virginal *Fbln5*^{-/-} mice (**B**) is compared with age-matched wild-type females (**A**). Both mice were of mixed strain (C57BL/6;129SvEv). Note urogenital bulge (arrow) and visible cervix at the vaginal opening in the knockout.

Figure 2. Surgical anatomy of urogenital bulge in *Fbln5*^{-/-} mice. In wild-type mice (**A**), the bifurcation of the uterine horns (Ut), urethra (U), and bladder (B) are superior to the pubic ligament (PL). (The pubic ligament is disarticulated.) In *Fbln5*^{-/-} mice with pelvic organ prolapse (**B**), the bladder and underlying vagina are caudal to the pubic ligament. Bifurcation of uterine horns and vagina are also caudal to the pubic ligament (not visible in photograph). V, vagina; PL, pubic ligament; Ut, uterine horn; U, urethra.

Pelvic Organ Support in Wild-Type and *Fbln5*^{-/-} Mice

Anatomical dissections of the female reproductive tract in wild-type mice revealed that the upper vagina was suspended by uterosacral ligaments extending from the posterior cervix and upper vagina to the sacrum. In both upper and mid-vagina, the vaginal tube and rectum were enveloped by connective tissue suspending the vagina and rectum to the pubocaudalis musculature. The pubocaudalis extended from the pelvic sidewalls to the paravaginal and pararectal suspensory connective tissue (level II support in women). In *Fbln5*^{-/-} mice, this suspensory connective tissue was either absent or poorly developed. Furthermore, uterosacral ligaments (level I support in women) were either absent or attenuated in older (≥ 6 months) *Fbln5*^{-/-} mice (Figure 3). Although bladder weights were modestly increased in *Fbln5*^{-/-} mice, this was primarily attributable to marked increases in bladder size in 3 of 26 knockout females, all of which were older animals (8 to 11 months) with longstanding urogenital prolapse. In contrast, vaginal size and wet weight were increased significantly in the majority of *Fbln5*^{-/-} mice regardless of age (127 ± 13.3 mg compared with 49 ± 3.5 mg, $P < 0.01$; Figure 4).

Figure 3. Absence of pelvic support structures in *Fbln5*^{-/-} mice. Uterine horns (Ut) from wild-type (left) and *Fbln5*^{-/-} (right) mice are dissected from the ovarian suspensory ligaments and brought forward through the disarticulated pubic ligament (PL) to visualize the rectum (R) and uterosacral ligaments that suspend the upper vagina (V) and cervix (CX) to the pelvis posteriorly (arrows). Note absence of uterosacral ligaments and the flattened patulous vagina (V) in the *Fbln5* knockout animal. Both mice were of mixed strain (C57BL/6;129SvEv).

Figure 4. Vaginal mass is increased in *Fbln5*^{-/-} mice. Vaginal size is increased in vaginal specimens from *Fbln5*^{-/-} mice (**B**) compared with wild-type controls (**A**). ut, uterus; vag, vagina. **C:** Bladder and vaginal wet weight was determined in wild-type (WT) and *Fbln5*^{-/-} (*Fbln5* KO) mice. Each bar represents mean $\pm$ SEM of 34 virginal wild-type (C3BL/6) and 26 (bladder) or 33 (vaginal) virginal *Fbln5*^{-/-} (C57BL/6;129SvEv) females. ** $P < 0.001$ compared with WT.

Expression of FBLN5 and Tropoelastin in the Vaginal Wall during Pregnancy

To determine the regulation of FBLN5 and tropoelastin in normal pregnancy and parturition, full-length vagina was harvested from nonpregnant wild-type (C3BL/6) mice and pregnant mice at various time points during gestation, labor, and the puerperium. Levels of urea-extracted and soluble proteins were quantified by immunoblot analysis (Figures 5 and 6). Although FBLN5 and tropoelastin were not expressed in the soluble protein fractions at any time point, these proteins were highly expressed in urea extracts of the vaginal wall of nonpregnant and postpar-

Figure 5. Immunoblot analysis of FBLN5 and tropoelastin protein in mouse vagina during pregnancy, parturition, and the puerperium. Total protein (10 μ g/lane) from soluble and urea extracts of vaginal tissues were applied to 5 to 20% gradient polyacrylamide gels, separated by electrophoresis, transferred to nitrocellulose, and probed for FBLN5 (top blot) and tropoelastin (middle blot) with specific antibodies. Results were compared with total protein in side-by-side Coomassie-stained gels (bottom). Tropoelastin and FBLN5 were enriched in urea extracts (Urea Ext) but absent in the soluble fraction (Sup). Protein extract from a *Fbln5* KO uterus was used as a negative control. The immunoreactive band detected in the *Fbln5*^{-/-} lane using the elastin antibody is nonspecific. NP, nonpregnant; D18, pregnant D18; 2h, 24h, 48h, and 72h indicate hours postpartum.

Figure 6. Quantification of FBLN5 and tropoelastin protein and desmosine in mouse vagina during pregnancy, parturition, and postpartum time period. Staining intensity of immunoreactive FBLN5 (**A**) and tropoelastin (**B**) was quantified with chemiluminescence and compared with a nonpregnant standard on each blot. Each bar represents mean $\pm$ SEM of 3 to 11 samples at each time point. $^{**}P \leq 0.001$ compared with nonpregnant; $^{*}P \leq 0.05$ compared with pregnant D18. **C:** Desmosine content was determined in nonextractable pellets from vaginal homogenates and expressed as pmol per mg protein. NP, nonpregnant; Preg D18, pregnant D18; In Labor, tissues collected after delivery of first pup; 2, 4, 12, 24, 48, and 72 hours indicate hours postpartum; 7 days PP, 7 days postpartum.

tum animals, but not in pregnant mice (Figure 5). Levels of FBLN5 and tropoelastin protein were quantified and expressed relative to nulliparous nonpregnant control tissues, which served as a standard on each gel (Figure 6). In wild-type mice, FBLN5 content decreased eightfold during pregnancy. A burst in FBLN5 protein was observed in vaginal extracts 12 to 24 hours postpartum. Thereafter, levels decreased until 7 days after delivery. Full recovery of nonpregnant levels of FBLN5 was apparent in parous mice 1 week after weaning (ie, 4 weeks postpartum; not shown).

Tropoelastin decreased 2.5-fold during pregnancy compared with vaginal tissues from nonpregnant animals. Like FBLN5, tropoelastin increased 12 to 24 hours postpartum and returned to nonpregnant levels 4 weeks after delivery (Figure 6). Desmosine content (indicative of mature cross-linked elastic fibers) was similar in nonpregnant and pregnant animals. However, desmosine content in the vagina increased significantly 48 hours to 7 days postpartum (after the burst of FBLN-5 and tropoelastin in the early postpartum period).

Elastin is a remarkably stable protein with slow turnover rates in adult tissues under physiological conditions.^{22,23} To ascertain overall changes in elastic fibers and elastic fiber assembly in the vagina during pregnancy and the postpartum time period, the total content of FBLN5, tropoelastin, and desmosine in the nonpregnant, pregnant, and postpartum vagina was computed, thereby adjusting for increased vaginal weight and protein synthesis in the vaginal wall during pregnancy (Table

2). The content of FBLN5 in the vagina was decreased significantly during pregnancy, although tropoelastin per vagina did not change appreciably ($P = 0.08$). Desmosine content was increased significantly in the vaginal wall postpartum. Moreover, desmosine content, tropoelastin, and FBLN5 protein in the vaginal wall was increased significantly in the vaginal wall of parous mice (1 week after weaning) compared with that of nulliparous animals (Table 2). This was not attributable to increased vaginal weight because vaginal weight in nonpregnant virginal and parous mice was similar (59.4 ± 2.4 mg compared with 67.7 ± 4.8 mg). Collectively, these data indicate that, in mice under normal physiological conditions, elastic fiber biosynthesis is temporally regulated in the vaginal wall during pregnancy and parturition and that synthesis of new elastic fibers occurs in the postpartum vagina.

Elastic Fiber Morphology in the Vaginal Wall Postpartum

To examine the morphological changes in elastic fibers during pregnancy and parturition, tissues were fixed in formalin and sections were stained for elastic fibers using Hart's stain (Figure 7). An extensive network of long, straight elastic fibers bridged the interface between the vaginal adventitia and the levator ani in late pregnancy. However, elastic fibers were fragmented and disoriented 2 hours after delivery (Figure 7). Disrupted elastic fibers were noted at

Table 2. FBLN5, Tropoelastin, and Desmosine Content of the Vagina from Nonpregnant, Pregnant, and Postpartum Mice

Time point	FBLN5 (relative U/vagina)	Tropoelastin (relative U/vagina)	Desmosine (pmol/vagina)
Nonpregnant nulliparous	31.0 $\pm$ 5.9	35.9 $\pm$ 6.4	157.0 $\pm$ 11.5
Pregnant late gestation	3.7 $\pm$ 0.9 [†]	25.4 $\pm$ 4.5	193.5 $\pm$ 30.2
Postpartum			
24 hours	22.0 $\pm$ 7.8 [*]	50.4 $\pm$ 14.7	268.7 $\pm$ 29.6
48 hours	11.0 $\pm$ 3.5 [†]	30.6 $\pm$ 8.9	307.1 $\pm$ 59.2 [†]
72 hours	6.6 $\pm$ 2.3 [†]	16.2 $\pm$ 5.2 [†]	326.8 $\pm$ 97.6 [†]
7 days	3.9 $\pm$ 2.0 [†]	5.7 $\pm$ 1.2 [†]	207.5 $\pm$ 22.9
4 weeks	40.1 $\pm$ 5.7	71.6 $\pm$ 10.9	285.0 $\pm$ 51.4 [†]

Data represent mean $\pm$ SEM of 4 to 13 samples at each time point. For FBLN5 and tropoelastin, data were obtained by immunoblot analysis as relative U per mg protein, which was then normalized to wet weight of the vagina. Desmosine is expressed as pmol per vagina.

^{*} $P \leq 0.05$ compared with pregnant late gestation.

[†] $P \leq 0.05$ compared with nonpregnant nulliparous.

Figure 7. Elastic fibers in upper and mid vaginal wall from pregnant (D16) and postpartum mice (2 hours after delivery). Hart's stain was used to visualize elastic fibers (black) in sagittal sections of vaginal tissues from pregnant (D16, **A** and **C**) and postpartum (2 hours, **B** and **D**) C3/BL6 wild-type mice. Note elastic fiber morphology in vaginal muscularis suspending the vaginal wall to the levator ani muscle (l ani). Sections were obtained from the upper and middle thirds of the vagina. epi, epithelium; m, muscularis. **E** and **F**: Masson's trichrome stain of vaginal wall from pregnant (D16, **E**) and postpartum (2 hours, **F**) mice. **Arrows** in **F** indicate many of the infiltrating inflammatory cells that are associated with dissolution of the collagenous extracellular matrix (stained blue). Original magnifications: $\times 250$ (**A–D**); $\times 200$ (**E**, **F**).

this time period in sections taken in both sagittal and transverse planes. Numerous infiltrating inflammatory cells (predominantly monocytes) were also noted (Figure 7).

Effect of Pregnancy and Parturition on LOX Isoforms in the Vagina

To determine regulation of lysyl oxidase and its family members during pregnancy and parturition, mRNA levels of LOX and LOXL-1, -2, -3, and -4 were quantified using real-time PCR and RNA isolated from urogenital tissues of nonpregnant, pregnant, and parturient mice. The efficiency of amplification was similar among primer pairs for each gene of interest. Although all isoforms were expressed, based on the cycle thresholds of amplification, LOX, LOXL1, and LOXL2 were the predominant isoforms expressed in all tissues examined (Figure 8). Regulation of various lysyl oxidase mRNAs during pregnancy was both gene- and tissue-specific. For example, LOX transcripts were down-regulated in the vagina and cervix during pregnancy but not in the uterus (Figure 8). On the other hand, in all tissues examined, LOXL1 mRNA was not regulated during pregnancy except in the cervix in

Figure 8. Regulation of LOX isoform mRNA expression in urogenital tissues during pregnancy and the postpartum time period. The relative abundance of LOX and LOXL1–4 mRNA was determined by real-time PCR in vagina, cervix, uterus, and bladder from nonpregnant (open bar), pregnant D18 (hatched bar), and 24 hours postpartum (closed bar) wild-type (C3BL/6) mice. Transcript levels are expressed relative to that of LOX in nonpregnant vagina. Each bar represents mean $\pm$ SEM of 3 to 16 tissues. * $P \leq 0.05$ compared with corresponding nonpregnant tissue; ** $P \leq 0.05$ compared with corresponding postpartum tissue.

which LOXL1 mRNA levels were increased on gestation D18 compared with 24 hours postpartum. In general, transcripts for lysyl oxidases in the bladder were not regulated in pregnancy and were less than that expressed in the female reproductive tract (Figure 8).

Further studies were conducted to address the regulation of LOX and LOXL1 in the vaginal wall during pregnancy and the postpartum time period (Figure 9). In early pregnancy, LOX expression in the vagina decreased 10-fold compared with vaginal tissue from nonpregnant animals (Figure 9A). LOX mRNA remained suppressed until the day before parturition (D18) in which mRNA levels increased. After the onset of labor and in the early postpartum time period, LOX mRNA levels increased further (Figure 9A). In contrast, LOXL1 transcripts were not regulated in the vaginal wall during pregnancy (Figure 9D). LOX and LOXL1 are secreted as proenzymes that are cleaved to active enzymes by peptidases in the extracellular matrix.²⁰ To determine the regulation of LOX and LOXL1 in the vaginal wall during pregnancy, urea extracts of vaginal protein from nonpregnant, pregnant, and postpartum mice were subjected to immunoblot analysis using antibodies that recognize both immature and mature forms of the enzymes (Figure 9, B–F). In agreement with the mRNA results, LOX protein levels (both pro- and mature forms) were decreased significantly in the vagina during pregnancy compared with that of nonpregnant animals. Both mature and immature enzymes increased in the postpartum time period (Figure 9, B and C). The pattern of LOXL1 protein levels in the vagina during pregnancy was distinct from that of LOX. Consistent with mRNA results, the total amount of immunoreactive LOXL1 protein did not change during pregnancy. Processing of immature LOXL1 to the mature active enzyme, however, increased in the vagina during late pregnancy and the postpartum time period (Figure 9, E and F). In contrast to the vaginal wall, both LOX and LOXL1 increased considerably in the uterus on D18 returning to nonpregnant levels 24 hours postpartum (data

Figure 9. Regulation of LOX and LOXL1 in vaginal tissues during pregnancy, parturition, and the puerperium. **A** and **D**: Real-time PCR was used to determine the relative abundance of *LOX* (**A**) and *LOXL1* (**D**) mRNA in vaginal tissues from nonpregnant (np) and pregnant wild-type (C3/BL6) mice at various time points in gestation or postpartum. Data are expressed relative to β 2-microglobulin and normalized to a nonpregnant vagina standard. Each data point represents mean $\pm$ SEM of 5 to 13 tissues at each time point except 72 hours ($n = 3$). Labor, in labor after delivery of the first pup; 24h, 24 hours postpartum; 48h, 48 hours postpartum; 72h, 72 hours postpartum; 1 wk, 1 week postpartum; 2 wk, 2 weeks postpartum. * $P \leq 0.05$ compared with early pregnancy time points. **B** and **E**: Immunoblot analysis of LOX (**B**) and LOXL1 (**E**) with antibodies that recognize pro- and mature enzymes in vaginal tissues from nonpregnant, pregnant D18, and postpartum (12 to 24 hours) mice. The blot in **E** is overexposed to detect mature LOXL1 protein because epitopes for antibody recognition are fewer in the mature form compared with the proenzymes. **C** and **F**: Relative intensity of chemiluminescence of mature LOX (**C**) and LOXL1 (**F**) isoforms in vaginal tissues from nonpregnant (NP, open bars), pregnant D18 (hatched bars), and postpartum (PP, solid bars). LOX and LOXL1 indicate proforms of the enzyme, whereas mature LOX and LOXL1 indicate the final processed forms. Ten μ g of urea-extracted protein was applied in each lane. Data represent mean $\pm$ SEM of six to eight tissues in each group. * $P \leq 0.05$ compared with nonpregnant.

not shown). Some of the differences in expression of LOX and LOXL1 in uterine and vaginal tissues may be attributable to differences in tissue expansion during pregnancy. For example, whereas vaginal weight increases 2.5-fold during pregnancy (from 36 ± 5.6 mg to 93 ± 7.1 mg), uterine weight increases >8 -fold (from 69 ± 5.2 mg to 521 ± 59 mg) and the uterus expands >10 -fold in diameter.

Elastase Induces Pelvic Organ Prolapse in Mice

Experimental results indicated that elastic fiber synthesis and assembly were suppressed in the vagina during pregnancy and immediately postpartum, suggesting that the vagina may be especially susceptible to elastolytic enzymes during this period. During postpartum remodeling of the vagina, we noted marked increases in monocytes and macrophages in the vaginal wall 2 to 24 hours postpartum. To test the hypothesis that elastic fiber integrity is important in pelvic organ support during the postpartum time period (ie, when elastic fiber assembly is compromised), we injected 5 U of purified porcine elas-

tase into the posterior vaginal wall of postpartum mice. Saline-injected or heat-inactivated elastase-injected postpartum mice served as controls. Significant protrusion of the posterior vaginal wall was observed 2 to 4 hours after injection in six of eight elastase-treated animals. By 72 hours, prolapse of the posterior vagina was observed in six of eight elastase-treated mice but not in vehicle controls (none of eight) or in animals treated with heat-inactivated elastase (none of six) ($P \leq 0.05$, compared with elastase-treated mice). Except for the relative sparing of the anterior vagina (which was not injected), the phenotype was remarkably similar to prolapse observed in *Fbln5*^{-/-} mice (Figure 10A). At the time of sacrifice, a distinct separation of the rectum and vagina was observed with dissolution of the posterior vaginal muscularis and vaginal wall attachments (Figure 10B).

Discussion

Pelvic floor disorders affect up to one-third of adult women.²⁴ One of the most prevalent pelvic floor disorders is

Figure 10. Effect of elastase injection on pelvic organ prolapse in postpartum mice. **A:** Parturient (C3BL/6) mice were injected with saline (left) or 5 U of purified pancreatic elastase (right) on the day of delivery and examined for pelvic organ prolapse. **Arrow** denotes urogenital bulge in elastase-treated animals. Results are representative of six of eight elastase-treated mice and eight animals in the control group. **B:** Vaginal wall tissues of postpartum mice injected with saline (control) or elastase (elastase) were obtained 72 hours after injection, mounted in the transverse section, and stained for elastic fibers. Marked dissolution of the extracellular matrix and shortened, frayed elastic fibers were noted in the posterior vaginal wall of elastase-injected animals. epi, epithelium; m, muscularis.

pelvic organ prolapse, a condition in which the pelvic organs (bladder, vagina, cervix, and uterus) herniate through the vaginal opening. Although multiple mechanisms have been hypothesized to contribute to the development of pelvic organ prolapse, none fully explain the origin and natural history of this process. Whereas vaginal birth and aging are two major risk factors for developing pelvic organ prolapse, the specific effects of pregnancy, parturition, and aging on pelvic floor support mechanisms have not been identified.

Recently, we reported that pelvic organ prolapse is associated with increased expression of human macrophage metalloelastase in women who smoke,¹⁴ and that smoking is an independent risk factor for pelvic organ prolapse.^{14,25} Another study indicated that other matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 that also degrade elastic fibers are increased in pelvic floor connective tissues from women with pelvic organ prolapse.²⁶ Proteases such as plasminogen activators, serine elastases, and cathepsins may also contribute to degeneration of vaginal wall connective tissue during aging. Studies in LOXL1-deficient mice, together with those of the current investigation, lend insight into potential mechanisms by which failure of pelvic organ support may occur during parturition and aging. The results suggest that although mechanisms by which aging, vaginal delivery, and smoking lead to pelvic organ prolapse may be different, each may share a final common pathway involving a net decrease in the number of functional elastic fibers in the vaginal wall and paravaginal connective tissue.

In many respects, pelvic organ prolapse may be considered the end result of a multifactorial process leading to destruction of vaginal wall connective tissue. Mechanisms regulating connective tissue metabolism have received much emphasis in other degenerative conditions such as abdominal aortic aneurysms,²⁷ inguinal hernias,^{15,28} rheumatoid arthritis,²⁹ and chronic obstructive lung disease.³⁰ However, the importance of these mechanisms in mediating pelvic organ prolapse is not well defined. Like other connective tissues, the most important structural elements in the vagina are elastin and

interstitial collagens. Production of elastin is unique among connective tissue proteins in that, in most organs, elastin biosynthesis is limited to a brief period of development. Elastic fiber assembly is complete by maturity when tropoelastin synthesis ceases. In undisturbed tissues, elastic fibers produced in the third trimester of fetal life³¹ may last throughout the entire human lifespan.³² In adults and elderly patients, elastic fibers gradually become tortuous, frayed, and porous.³³ Unlike other adult organs, however, elastic fiber turnover appears to be continuous in the female reproductive tract and accelerated after childbirth.^{22,34} Results from the current investigation suggest that, in the vagina, this unique adaptation in elastic fiber degradation and renewal allows the vagina to expand during parturition and recover from childbirth with increased deposition of mature elastic fibers. The effect of aging on this process is not known, as we did not closely control for age in this study.

Elastase activity has been identified as a key underlying cause of elastic fiber degradation during the onset and progression of certain types of degenerative diseases.^{15,35,36} Elastic fiber degradation in the postpartum vagina has not been shown. However, the vaginal wall is infiltrated with monocytes during labor and during the first 2 to 12 hours postpartum,¹⁴ and these cells represent a rich source for elastolytic enzymes. Biologically active elastin degradation peptides are also chemotactic for numerous cell types and induce expression and release of proteolytic enzymes by stromal cells.³⁷ Thus, elastin degradation may be involved in leukocyte recruitment and vaginal wall infiltration during labor. Activation of MMP-9 and MMP-2 in monocytes may lead to elastic fiber degradation as well as breakdown of interstitial collagens in the vaginal matrix. Because FBLN5 is crucial for elastic fiber assembly^{17,18} and is believed to act as a bridge between cells and tropoelastin for effective cross-linking and assembly of tropoelastin into mature elastic fibers, increased synthesis of tropoelastin and FBLN5 may be necessary to counteract disrupted elastic fibers and to regenerate vaginal wall elastic fibers postpartum. In addition to its role in elastic fiber assembly, FBLN5 may have additional biological properties. For example, stud-

ies using *Fbln5*^{-/-} mice indicate that FBLN5 is required for binding of extracellular superoxide dismutase (ecSOD) to vascular tissue.³⁸ Decreases in tissue-bound ecSOD levels in aortas from *Fbln5*^{-/-} mice were associated with an increase in vascular oxygen-free radicals.³⁸ FBLN5 may therefore regulate oxidative stress of the vaginal wall after delivery or other physiological processes involved in recovery of the vagina from childbirth.

Although we focused on the specific regulation of Fbln5, tropoelastin, LOX, and LOXL1 in elastic fiber regeneration in the vaginal wall postpartum, other proteins may be involved. For example, null mutations in fibulin-1 result in perinatal death from extensive hemorrhaging because of abnormal endothelial cells.³⁹ Fibulin-4 deficiency is also associated with death shortly after birth because of profound defects in elastic fiber formation in the lung and the vasculature.⁴⁰ Thus, the role of fibulin-1 or fibulin-4 in the process of elastic fiber regeneration in the vagina after pregnancy is not known. The biological effect of each fibulin may depend on the relative expression level of these proteins in each organ or cell type. The current investigation suggests that Fbln5 is likely to represent a major fibulin in the vaginal wall, but other fibulins may also play an important role. Surprisingly, inactivation of genes encoding fibrillin-1 or fibrillin-2 has little effect on elastic fiber formation.⁴¹⁻⁴³ Fibrillin-1, however, is important in maintaining tissue homeostasis,⁴¹ and deletion of both fibrillin-1 and -2 results in mice with severe defects in elastic fiber formation suggesting a redundant role of fibrillin proteins in elastic fiber assembly.⁴⁴ The role of these proteins in pelvic organ support has not been examined. Interestingly, relaxin inhibits fibrillin-2 expression.⁴⁵ The peptide hormone relaxin is increased during pregnancy and is important for growth of the vagina during gestation.⁴⁶⁻⁴⁸ Relaxin-induced decreases in fibrillin-2 expression are consistent with our results demonstrating down-regulation of other proteins involved in elastic fiber assembly during pregnancy.

In contrast to the pregnant vagina, tropoelastin monomeric protein was readily extracted from extracellular matrix protein extracts from nonpregnant and postpartum animals, suggesting that either tropoelastin is synthesized in amounts excess of that needed for incorporation into elastic fibers or that the rate of elastic fiber turnover is increased. The postpartum burst of FBLN5 and tropoelastin and increased vaginal desmosine content after parturition is consistent with the idea that elastic fibers are regenerated during the postpartum time period. The significance of increased expression of FBLN5, LOX, LOXL1, and tropoelastin in the vagina of nonpregnant animals, however, is not readily apparent. The rate of elastic fiber turnover may be increased in the nonpregnant vagina in response to fluctuating levels of steroid and peptide hormones during the estrus cycle. Estrogen increases expression of LOX and LOXL1 mRNA in the vaginal wall, whereas progesterone inhibits estrogen-induced increases in LOX and LOXL1 gene expression (unpublished results). These findings support the conclusion that extracellular matrix turnover is increased in nonpregnant mice under various hormonal conditions.

Whereas the postpartum burst of elastic fiber synthesis may be crucial for recovery of the vaginal wall from parturition, down-regulation of Fbln5 and, to a lesser extent, tropoelastin, may be an important adaptation of the vagina in pregnancy. Remodeling of the vaginal wall during pregnancy involves growth, smooth muscle cell hypertrophy, epithelial cell proliferation, and a net increase in extracellular matrix proteins.^{46,49,50} We speculate that down-regulation of Fbln5 and tropoelastin in the vaginal wall facilitates growth of the vagina because 1) elastin inhibits proliferation of smooth muscle cells,⁵¹ 2) *Fbln5*^{-/-} mice are more susceptible to severe neointima formation during remodeling of the vasculature,⁵² and 3) Fbln5-deficient vascular smooth muscle cells exhibit increased proliferation and migratory responses to mitogenic stimulation which is suppressed by overexpression of Fbln5.⁵² Interestingly, thrombus formation is also common in *Fbln5*^{-/-} mice during vascular remodeling.⁵² It has been suggested that Fbln5, either alone or through elastic fibers, may be important in regulating fibrinolysis. Thus, down-regulation of Fbln5 during pregnancy may also facilitate vascular hemostasis during parturition. Further experiments are necessary to test these hypotheses.

In summary, the pelvic floor is a complex dynamic system that supports the vagina and pelvic viscera. Studies reported herein indicate that elastic fiber assembly is crucial for normal pelvic organ support and that elastic fiber assembly is a dynamic process in the vaginal wall after parturition. Results suggest that parturition results in a distinct remodeling process of the vaginal wall that involves specific proteins in elastic fiber homeostatic pathways. These findings in mice lead us to propose that the inability to repair or synthesize new elastic fibers because of genetic defects in elastic fiber synthesis or assembly may lead to pelvic organ prolapse in some women. We suggest that disturbances in the balance between synthesis/assembly and degradation of elastic fibers in the vaginal wall during aging, parturition, and smoking result in pelvic organ prolapse, and that therapies designed to prevent or abrogate elastic fiber degradation in the vagina may be successful in preventing or ameliorating the clinical manifestations of this disease.

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