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Metabolic, Endocrine and Genitourinary Pathobiology

Failure of Elastic Fiber Homeostasis Leads to Pelvic Floor Disorders

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Pelvic floor disorders, a group of conditions affecting adult women, include pelvic organ prolapse and urinary incontinence. Vaginal childbirth and aging are risk factors, and weakening of the pelvic support structures is a major aspect of the pathology. However, the underlying molecular mechanism remains unknown. Female reproductive organs are rich in elastic fibers that turn over slowly in most adult tissues but undergo massive remodeling in the reproductive organs through pregnancy and birth. Here we show that a failure to maintain elastic fiber homeostasis in mice causes pelvic floor disorders. Lysyl oxidase-like-1 (LOXL1), a protein essential for the postnatal deposition of elastic fibers, was highly expressed and regulated in the reproductive tract of the mouse, and its expression was diminished during aging. LOXL1 deficiency caused an inability of reproductive tissues to replenish elastic fibers after parturition, leading to pelvic organ prolapse, weakening of the vaginal wall, paraurethral pathology, and lower urinary tract dysfunction. These data demonstrate the importance of elastic fibers for maintaining structural and functional integrity of the female pelvic floor. Our findings raise the possibility that a failure of elastic fiber homeostasis, either due to genetic predisposition or advancing age, could underlie the etiology of pelvic floor dysfunction in women. ***(Am J Pathol 2006, 168:519–528; DOI: 10.2353/ajpath.2006.050399)***

Pelvic floor disorders refer to a group of conditions that include pelvic organ prolapse, urinary incontinence, and other sensory and emptying abnormalities of the lower urinary tract. These conditions constitute a major health and quality-of-life problem affecting adult women in their reproductive and menopausal years.[1–6] Urinary incontinence can be classified into different types based on symptoms and clinical observations, including stress, urge, or mixed types.[7] Stress urinary incontinence is defined as involuntary leakage of urine on effort or exertion, such as when laughing or coughing, which raises abdominal pressure. Urge urinary incontinence is an involuntary leakage accompanied by urgency and is related to bladder overactivity. Stress urinary incontinence, in contrast, stems primarily not from bladder dysfunction but rather from ineffective urethra closure.[5,8] Urinary incontinence among elderly women is one of the major geriatric concerns.[2,9,10] Although not life threatening, its negative impact on quality of life and its economic cost to society are enormous.[11] Long recognized as an important disease entity, this condition has been aptly described as "an affection beginning in the middle life, most common in multiparae. It begins as a rule with slight leakage, which gradually grows worse, leading to complete incontinence with all its unfortunate and repellent sequelae."[12]

Effective closure of the urethra requires the concerted action of various pelvic floor structures in addition to proper function of urethral musculature. It is widely accepted that the suburethral vaginal wall and the paraurethral connective tissues are key factors in maintaining continence.[4,5,8] Two epidemiological factors most strongly associated with stress urinary incontinence are vaginal childbirth[10,13] and advancing age.[10] Vaginal delivery can injure the nerve, muscle, and connective tissues responsible for maintaining continence.[14,15] Physiological changes with age also contribute to the development of urinary incontinence,[14,16] although the detailed mechanism is unclear. Other risk factors may

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include genetic predisposition, lifestyle, and certain medical conditions.[17–20]

Pelvic organ prolapse, another major clinical manifestation of pelvic floor disorders, has a prevalence and associated risk factors similar to those of urinary incontinence.[3,21–23] Pelvic organ prolapse and urinary incontinence frequently occur together or at different times in the same patients. Urinary incontinence may be masked in a patient with pelvic organ prolapse due to urinary retention from obstruction of the urethra by the prolapsed organ. Such complications have been well described in the literature.[24,25]

It has been suggested that the etiology of urinary incontinence and pelvic organ prolapse is multifactorial, with different factors acting or interacting to produce clinical conditions in different women.[17] Yet, a clear understanding of the pathophysiology of pelvic floor disorders is lacking. Animal models could be particularly useful in studying female pelvic floor disorders because these conditions are described by a wide variety of symptoms and have multiple causative factors whose interrelationships are not fully understood. A number of injury-induced rat models of pelvic floor disorders have been studied and were recently reviewed by Weber and colleagues.[17] No genetic animal models, however, have thus far been described.[17] Connective tissues in the pelvic floor are critical for its tensile strength and provide support to the pelvic organs that are subjected to intra-abdominal pressures. Research into the role of connective tissues in pelvic floor disorder has traditionally focused on changes in fibrillar collagens.[8] Published reports indicate decreased collagen content in vesicovaginal fascia,[26] abdominal skin, and round ligament[8] in women with urinary incontinence compared with controls. There have also been reports suggesting increased prevalence of pelvic floor disorders in genetic conditions characterized by collagen and connective tissue defects, such as Ehlers-Danlos and Marfan syndromes.[27,28] It remains unclear if a primary defect in collagen metabolism constitutes a contributing factor to the development of clinical pelvic floor disorders.

In addition to collagens, female pelvic tissues are extremely rich in elastic fibers. Elastic fibers are components of the extracellular matrix and confer resilience.[29] The latter property is presumably important for reproductive tissues to accommodate the enormous expansion in pregnancy and involution after parturition. Elastic fibers are turned over slowly in most adult tissues[30] except for the female reproductive organs, where they undergo massive remodeling.[31,32] The major component of elastic fibers is an amorphous polymer composed of the protein elastin, known as tropoelastin in its monomeric form. Polymerization requires an initial step of oxidative deamination of lysine residues catalyzed lysyl oxidases (LOXs).[33] Mammalian genomes have five related genes coding for the prototypic LOX and four LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4).[33] Recently, we have shown that LOXL1 is essential for elastic fiber homeostasis in multiple tissues including the female pelvic organs.[34] In all tissues examined, LOXL1 always colocalizes with elastic fibers. Mice lacking LOXL1 are unable to synthesize elastin polymers in adult tissues, whereas collagen synthesis appears to proceed normally. These observations suggest that the function of LOXL1 is dedicated to elastic fiber homeostasis. Because elastic fibers undergo active remodeling through pregnancy and parturition in the female genito-urinary organs, these tissues are expected to be sensitive to gene defects affecting elastogenesis. In this study, we examined the impact of failed elastic fiber homeostasis on female pelvic organs and development of voiding abnormalities.

Materials and Methods

Animals

Generation of LOXL1-deficient mice was described previously.[34] Both the mutant and wild-type (WT) control mice were of a mixed C57BL/6 and 129Sv backgrounds. *Loxl1*$^{-/-}$ mice manifest a host of pathologies that can be attributed to elastic fiber defects.[34] Approximately one-third of female *Loxl1*$^{-/-}$ mice develop severe pelvic organ prolapse after the first litter, and all of the remaining two-thirds develop prolapse after the second litter. No female *Loxl1*$^{-/-}$ mice were ever found to give birth to a third litter. The acute stage of prolapse, in which a long stretch of vaginal/uterine tissues is exposed outside of the body cavity, typically lasts 1 to 2 weeks. This is followed by a permanent and moderate prolapse, as indicated by the descent of pelvic organs forming a bulge at the urogenital region, which remains little changed for the remainder of the animal's lifespan (stable stage).

Female *Loxl1*$^{-/-}$ mice that had given birth to one or two litters, and were between 4 and 7 months of age, were selected at random for the examination of pelvic organ pathology ($n = 32$), and for urinary behavior measurement ($n = 8$; from within the group of 32). This group did not include any mouse with apparent signs of urinary retention (see below). At the time of study, mice were in the stable stage of pelvic organ prolapse and were between 3 and 10 weeks after their most recent parturition. Age-matched WT females that had given birth to two or three litters were randomly chosen and included as controls in the study of pelvic organ pathology ($n = 30$) and urinary behavior measurement ($n = 7$). The WT control females matched or exceeded the parity of the mutant mice. On rare occasions, female *Loxl1*$^{-/-}$ mice showed signs of urinary retention as indicated by an enormous pelvic bulge in the pelvic region that far exceeded those typically seen in mice with prolapse. They also had difficulty walking. These mice were visually identified and tested for voiding behavior followed by gross pathology examinations ($n = 3$; with results shown in Figure 4).

The mutant and WT mice were processed in parallel and under identical conditions, and the results from the two groups of animals were compared. For studies examining expression of LOXL1 at different gestational time points, nulliparous WT females were used. Mice were mated and gestational days were calculated after the

detection of a vaginal plug. For studies examining expression of LOXL1 at different ages, WT mice, at 2 and 18 months of age, were used. Blood urea nitrogen was analyzed at Anilytics, Inc. (Gaithersburg, MD). All experiments involving animals were performed following protocols approved by the institutional Animal Care and Use Committee.

Examinations of Gross Pelvic Pathology and Histopathology

Mice were euthanized by CO_2 inhalation, and pelvic organs were examined and photographed under a dissecting microscope. Afterward, tissues were rinsed in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde/PBS overnight. Tissues were embedded in paraffin. Transverse sections through the middle portion of the vagina/urethra were cut at 4-μm thickness. Sections were stained with hematoxylin and eosin (H&E). A total of 32 *Loxl1*$^{-/-}$ mice and 30 WT mice were examined.

Measurements of Urinary Behavior

A mouse micturition chamber, designed to measure urinary output in real time, was custom built by Columbus Instruments (Columbus, OH). This chamber was adapted from the standard mouse metabolic cage offered by Columbus Instruments. Key features of the micturition chamber included a wire mesh bottom (mesh 4), which was connected to a funnel. The bottom of the chamber was designed for unobstructed collection of urine droplets, but it would not sequester solid droppings. The inside surface of the funnel was coated with molten paraffin and was recoated after several uses to minimize trapping of liquid droplets inside the funnel. Directly below the bottom opening of the funnel was a collection tube, which was placed on a balance (Mettler Toledo electronic balance, model PL83 with 0.001 g weight resolution). The data port of the balance was connected to a computer. The bottom of the funnel and the collection tube were encased in a Plexiglas outer casing, which served to cut down evaporation and reduce the effect of air draft. Changes in the weight of the collection tube were recorded at a sampling speed of six times/minute. Initial tests of this system had confirmed that urine droplets as small as 50 μl in volume could be reliably collected and recorded. Before placement inside the chamber, mice were given a residue-free diet (Lactaid brand whole milk, lactose-free) for 24 hours. The liquid diet was given to prevent feces droppings from interfering with measurement of urine output. During the entire test period, mice continued to have free access to this liquid diet. Pilot tests had confirmed that this diet was well accepted by mice and that it produced no apparent adverse effects (in contrast, regular milk that contained lactose produced diarrhea).

Loxl1$^{-/-}$ mutant and WT control mice were tested in this chamber one at a time, each lasting a duration of 24 hours. Mutant and WT mice were tested in an alternating sequence. The climate control included 12 hours of light and 12 hours of darkness that were synchronized with the light/dark cycle in the animal facility. Data were analyzed and plotted using the Multi-Device Interface software provided by Columbus Instruments.

Antibodies, Immunoblotting, and Immunofluorescence

LOXL1N and LOXL1C antibodies, which recognize the N- and C-termini of LOXL1, respectively, were described previously.[34] Elastin antibodies were obtained from Elastin Products Company (Owensville, MO): PR385 (rabbit anti-mouse elastin, exons 6 to 17) and RT675 (goat anti-rat elastin). Immunoblotting and immunofluorescence staining of unfixed cryosections were performed as described.[34] For immunoblotting analysis, 5 μg of total proteins were loaded per lane, separated on sodium dodecyl sulfate-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. For immunostaining on frozen sections, transverse sections were cut at 10 μm through the middle portion of the vaginas/urethras. Unfixed frozen sections were used in immunofluorescence procedures. Cell nuclei were stained blue with Hoechst dye 33342.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY). First-strand cDNA synthesis was primed with oligo(dT)$_{20}$ using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). PCR primers for amplifying LOXL1 were P1 (5′-CGCGTTACGAGGACTACGGAG-3′) and P2 (5′-GACCATTCTGGTTGGGTCGGT-3′). PCR primers for LOX were P7 (5′-GCAGGAACCGACCTGGATACGGCAC-3′) and P8 (5′-CAGCCTGAGGCATAGGCATGATGTC-3′). PCR primers for elastin were P3 (5′-CTGGATCGCTGGCTGCATCCA-3′) and P4 (5′-GTCCAAAGCCAGGTCTTGCTG-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified together with LOXL1 and elastin targets in the same tube as an internal standard for quantification. PCR primers for GAPDH were P5 (5′TGAAGGTCGGTGTGAACGGATTTGGC-3′) and P6 (5′-CATGTAGGCCATGAGGTCCACCAC-3′). Pilot experiments were done to determine the optimal primer concentrations in these mixed PCR reactions. Finally, P1, P2, P3, P4, P7, and P8 primers were used at 0.15 μmol/L. P5 and P6 primers were used at 0.1 μmol/L. PCR products were separated on 1.5% agarose gels and the images were captured by Fluor-S MultiImager. PCR reactions were terminated at different cycle numbers (20, 25, 30, and 35) to ensure that amplifications did not reach a plateau. Quantification was performed using the Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA).

Data Analyses

Mean volume per urinary event was calculated for each animal. Data analyses included univariate statistics to

calculate group means, standard deviations, and plots of frequency distributions. Mean group differences were evaluated by *t*-test. $P < 0.05$ indicated a significant difference between groups.

Results

Pelvic Organ Prolapse and Gross Pathology in LOXL1-Deficient Females

The pelvic organ defects in the LOXL1 mutant animals were striking. *Loxl1* mutant mice developed pelvic organ prolapse after giving birth to either their first or second litter of pups. Severe prolapse was seen 1 to 3 days postpartum. Exposed vaginal/uterine tissues varied from a quarter to one inch in length (Figure 1A, left). The prolapsed tissues typically retracted throughout a period of 1 to 2 weeks, but a large bulge remained apparent in the urogenital region indicating internal pelvic organ descent (Figure 1A, middle). Mice would remain in this stable state of moderate prolapse indefinitely. *Loxl1* mutant females were also prone to develop mild rectal prolapse. Rectal prolapse generally appeared later than vaginal prolapse and was not seen in all of the mutant mice that had developed vaginal prolapse (~50%). Female *Loxl1*$^{-/-}$ mice appeared to lose fecundity afterward, as none had been found to give birth for a third time. Parturition appeared to be the single most important trigger for pelvic organ prolapse in female *Loxl1*$^{-/-}$ mice because virgin females did not develop prolapse in this age range (3 to 7 months). Spontaneous pelvic floor problems did develop slowly in nulliparous *Loxl1*$^{-/-}$ females, so that by 1 year of age ~50% of them showed sign of pelvic organ descent that appeared similar to the one shown in Figure 1A (middle). Pelvic floor defects in nulliparous *Loxl1*$^{-/-}$ females were not examined further. WT females, up to 18 months of age and regardless of parity, showed no sign of pelvic organ prolapse or descent.

A cohort of female *Loxl1* mutant mice, which had recovered from acute prolapse after parturition, was examined for gross pelvic organ pathology and histopathology in comparison to the WT control group. On dissection of the pelvic cavity, WT controls were found to maintain well-defined uterine, cervical, and vaginal structures. The urethra was tightly adhered to the suburethral vaginal wall along its entire length, and the urinary bladder was firmly attached at a position near uterine cervix (Figure 1B, left). In contrast to the WT mice, all mutant mice showed descent of the uterine, bladder, and upper vaginal tissues into the lower vaginal cavity, creating a ring-like fold in the upper middle position of the vaginal wall (Figure 1B, middle and right; Figure 1C). The severity of the pelvic organ descent was such that the urinary bladder was seen trapped into this vaginal fold (Figure 1B, right). The ring-shaped fold in the vaginal wall appeared to mark the position where uterine and vaginal tissues folded inside out during the acute phase of prolapse.

Without exception, mutant mice had enormously distended lower vaginal walls (Figure 1B). The upper portion

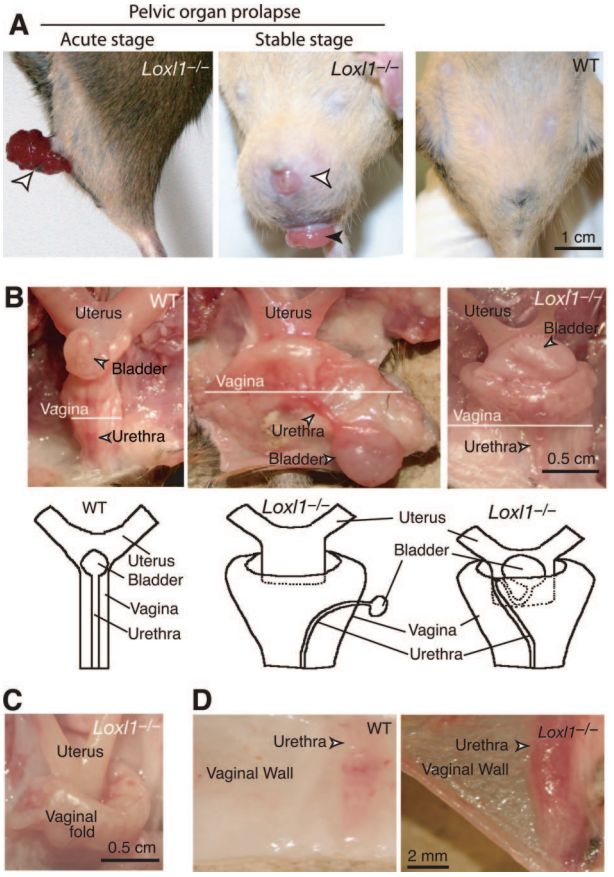

Figure 1. Representative images of gross pathological findings in the pelvic organs of parous *Loxl1*$^{-/-}$ females. **A:** Acute (**left**) and stable (**middle**) stages of pelvic organ prolapse in the mutant (**white arrowheads**). An age-matched WT control is shown at the **right**. Note the large bulge at the pelvic region of the mutant animal (**middle**). There is also mild rectal prolapse (**black arrowhead**) in this animal, which often but not always co-exists with vaginal prolapse. **B:** Examination of dissected pelvic organs. **Top**: Pelvic organs in WT (**left**) and mutant (**middle** and **right**) parous females are shown. **White horizontal lines** mark the mid-section of the vaginas, showing enormous distension in the mutant. Unlike the WT, the mutant urethra was typically detached from the vaginal wall and the bladder had a much greater degree of free movement (compare **left** and **middle panels**). In the **middle panel**, the uterus was lifted up from a descended position. In the **right panel**, prolapsed tissues were unperturbed, which shows the urinary bladder partly trapped into the ring-shaped fold in the vaginal wall. These findings are summarized in the schematic diagrams shown in the **bottom panels**. **C:** The same pathology as that shown in the **right panel** of **A**, with the bladder pulled away to fully expose the ring-shaped fold. **D:** Compared to the WT, mutant vaginal walls became thinned and appeared transparent.

of the urethra in the mutant was typically detached from the vaginal wall, potentially allowing for a much greater degree of movement of the urethra and bladder. In addition, the lower portion of the vaginal walls in the mutant had a totally different texture and appearance compared to the WT (Figure 1D). Whereas the WT tissues appeared thick and exhibited considerable tensile strength during dissection, the mutant tissues were membrane-thin and tore at the slightest application of force.

Elastic Fiber Defects and Histopathology of the Pelvic Floor and Paraurethral Tissues

After parturition, there were signs of increased elastin polymer deposition in the WT vaginal wall tissues such as

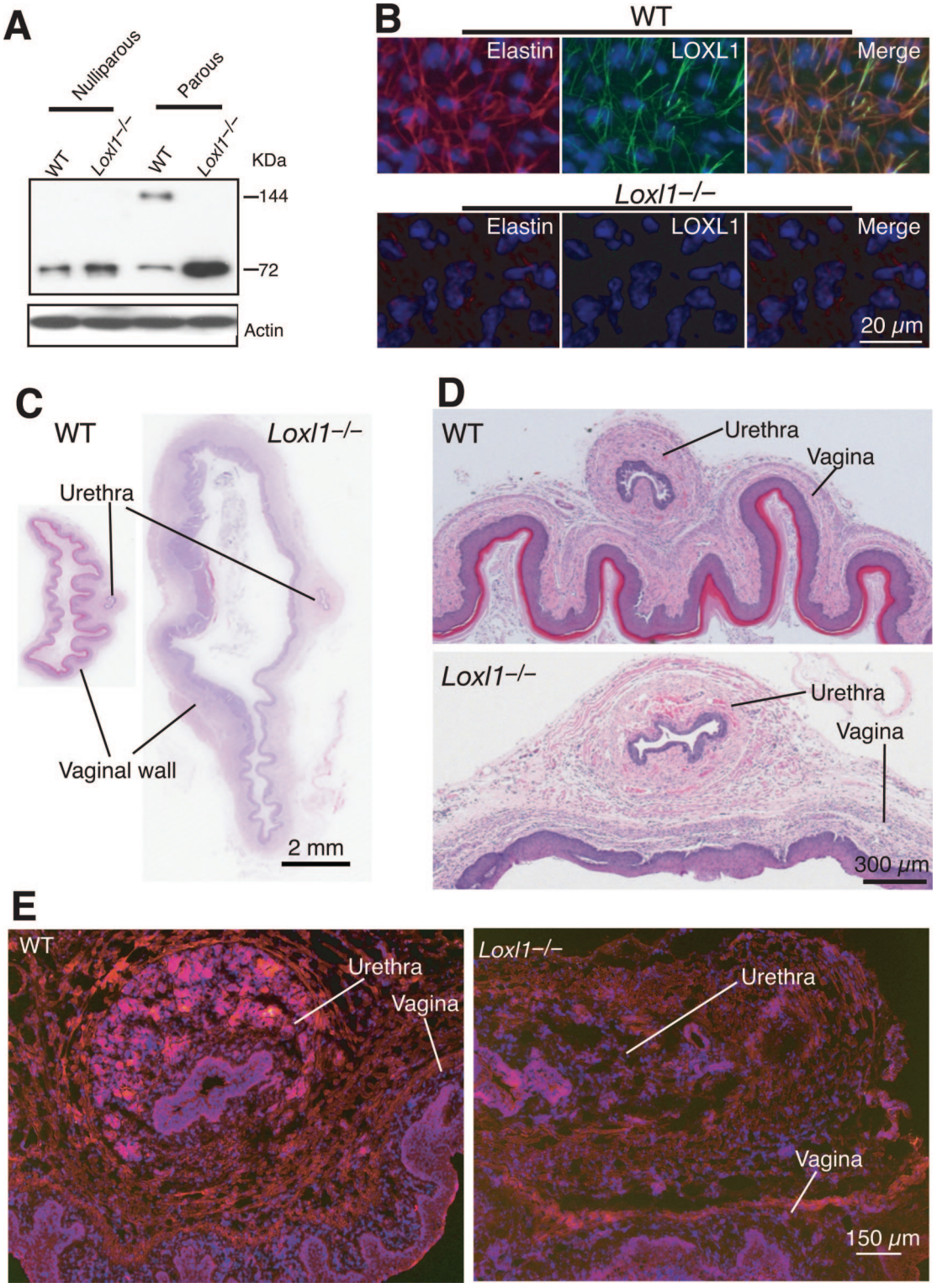

Figure 2. Failure of elastin polymer deposition and histopathology in the vaginal wall, urethra, and paraurethral connective tissues. **A:** Immunoblots of vaginal wall/urethral homogenates detected by an elastin antibody. After parturition, the mutant showed a substantial accumulation of elastin monomer (72 kd) and an absence of cross-linked products (eg, elastin dimer, 144 kd). In contrast, there was little difference between nulliparous WT and mutant animals. **B:** Elastic fibers in the urethral wall were reduced and disorganized as revealed by immunostaining for elastin (red) and LOXL1 (green), which normally co-localizes with elastic fibers. **C:** Vaginal wall circumference was greatly enlarged in the mutant (H&E staining). The mutant vagina also lacked infoldings. **D:** An enlarged view of **C** focusing on the urethra and suburethral vaginal wall. The urethral walls and the paraurethral connective tissues were loosely packed and disorganized in the mutant. Prominent infoldings in the WT mice were absent in the mutant. In addition, the vaginal wall in WT mice was lined with a uniform keratinized layer (red), which was completely lacking in the mutant. **E:** Immunostaining for smooth muscle actin (red) showed reduced and disorganized smooth muscle cell layers in the urethral wall. Cell nuclei were counterstained blue with Hoechst dye 33342.

the appearance of cross-linked intermediates (Figure 2A). In contrast, there was an accumulation of tropoelastin monomers but no appearance of dimers in the mutant vaginal and paraurethral tissues, suggesting a block in elastin polymer formation (Figure 2A). As a result, elastic fibers in the paraurethral connective tissues were reduced in the mutant in comparison to the WT (Figure 2B).

The vaginal wall circumference in the mutant was several-fold larger than that in the WT (Figure 2C). The vaginal wall in the mutant lost most of its infoldings, which were abundant and evenly distributed in the WT (Figure 2, C and D). This would appear to have resulted from the mutant vaginal wall being in an overstretched state. Both the urethral walls and the paraurethral connective tissues appeared loosely packed and disorganized in the mutant (Figure 2D). The smooth muscle cell layer in the mutant urethra was reduced and severely disorganized as shown by immunostaining for smooth muscle actin (Figure 2E).

Abnormal Voiding Behavior in the LOXL1-Deficient Females

We hypothesized that a history of pelvic prolapse and tissue damage would negatively impact the urethral function and could lead to a functional deficit of the lower urinary tract. Several earlier observations suggested that parous *Loxl1*$^{-/-}$ females appeared unable to maintain normal urine storage. There was no voiding of the bladder on euthanasia by CO_2 inhalation, as would invariably occur in the WT and *Loxl1*$^{-/-}$ males. Dissection of pelvic organs of the parous *Loxl1*$^{-/-}$ females found the urinary bladders relaxed and empty in most cases, thus ruling out urinary retention as the cause for lack of voiding. Absence of urine could also be caused by kidney failure, which would shut down urine production. We therefore performed tests for the filtration function (blood urea nitrogen) and histological examinations of the kidney. Both were found normal, thus excluding any overt kidney disease.

To demonstrate a urinary dysfunction directly, we evaluated the urinary behaviors of the parous LOXL1 mutant and WT mice in a custom-built metabolic chamber. We found that the mutant mice had a 10-fold higher frequency of urinary events throughout a 24-hour period while urinary output per event in the mutant mice was only one-tenth that of the WT mice (Figure 3). The total urinary output and total amount of fluid intake throughout the same period were comparable between the mutant and WT mice. These data show that the *Loxl1*$^{-/-}$ mice produced urine normally but had an abnormal voiding pattern.

Paradoxically, occasional mutant animals exhibited evidence of bladder outlet obstruction. These animals were identified by an enormous bulge in the pelvic region and reduced mobility. Voiding behavior measurements showed that they had very infrequent but exceedingly high-output urinary events (Figure 4A). Output per urinary event reached 3.5 ml, which was more than 10% of the total body weight of the animals. An output of 3.5 ml is an

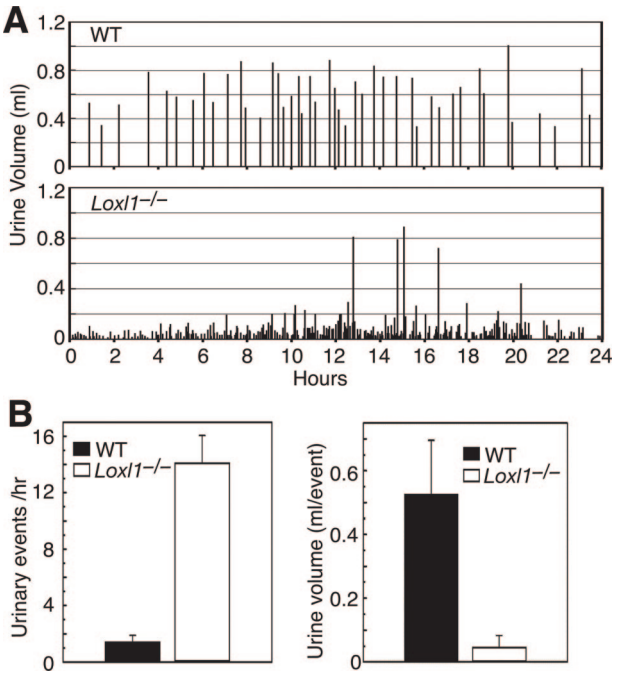

Figure 3. Urination frequency and output of parous *Loxl1*$^{-/-}$ and WT control females measured in a micturition chamber. **A:** Representative mutant and control urinary profiles recorded throughout a 24-hour period. Note the higher urination frequency but lower output per urinary event in the mutant. **B:** The urinary profiles of *Loxl1*$^{-/-}$ (n = 8) and WT females (n = 7) were analyzed and compared. The mean urinary frequency of the mutant was 14.144 ± 1.895 events/hour, almost 10-fold higher than that of the WT control (1.450 ± 0.430 events/hour). Accordingly, urinary volume/event in the mutant (0.049 ± 0.034 ml/event) was 10-fold less than that of the WT control (0.528 ± 0.167 ml/event). The differences in both frequency and volume were statistically highly significant ($P < 0.001$). The total urine output and the total liquid drank throughout a 24-hour period were in the range of 16 to 18 ml for both groups, showing no significant differences.

exceptionally large volume that was never observed in the WT control animals. The highest output we recorded among the WT animals was ~1 ml. Bladder outlet obstruction was further confirmed by pelvic organ dissection, which revealed an extraordinarily large, overfilled urinary bladder (Figure 4B). The overfilled bladder could be dissected out without leakage, with only a small amount of urethra and supportive tissues attached, suggesting that the point of obstruction was close to the bladder. We estimate that ~5 to 10% of the parous mutant animals may exhibit bladder outlet obstruction at some point in time. Bladder outlet obstruction appeared transient and was found to resolve itself without intervention. This issue, however, was not analyzed further in detail.

Changes in LOXL1 Expression through the Reproductive Cycle and with Age

The uterine tract undergoes enormous expansion during pregnancy and rapid resorptive involution postpartum. Being a key component for tissue remodeling through this period, LOXL1 expression might be expected to fluctuate in response to changing physiological needs. Indeed we found that LOXL1 mRNA in the uterine cervix of WT mice fell to 20% of baseline perinatally and re-

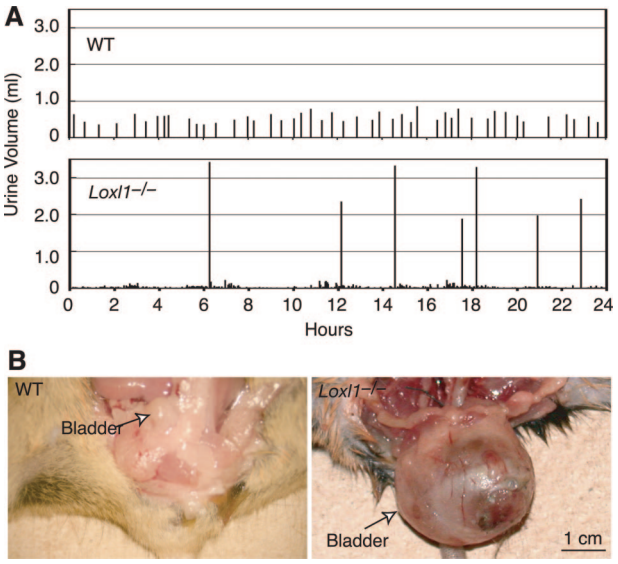

Figure 4. Bladder outlet obstruction is an infrequent complication in the LOXL1-deficient mutant mice. **A:** Urinary profile from a *Loxl1*$^{-/-}$ female showing clear signs of urinary retention (**bottom**). Urinary profile from a WT control is shown in comparison (**top**). The mutant registered infrequent but exceptionally high-output urinary events, often exceeding 3 ml/event. In contrast, output/event in the WT averages 0.5 ml (see Figure 2) and never exceeds 1 ml. In a 24-hour period, the mutants with urinary retention gave 7 ± 2 major events and the mean urine volume for major events is 2.661 ± 0.431 ml ($n = 3$). This is significantly higher than that of the WT ($P < 0.05$). The total urinary output and total amount of fluid drank throughout a 24-hour period were not significantly different between the mutant and WT mice. **B:** Urinary retention in the mutant as indicated by a greatly distended urinary bladder filled with urine (**right**). This particular mouse had not registered a single voiding event in the micturition chamber throughout an 8-hour period, before euthanasia and pelvic dissection. **Left**: Control WT mouse with a normal urinary bladder.

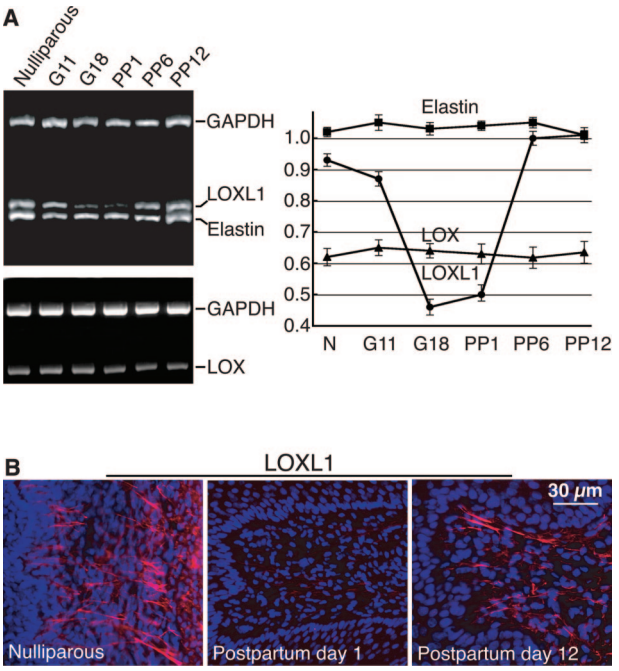

Figure 5. Suppression of LOXL1 expression perinatally in the uterine cervix of WT mice. **A:** Analysis of LOXL1 mRNA through the reproductive cycle by RT-PCR. **Left**: Representative agarose gel analysis of the PCR products. **Right**: Graphic representation of the changes in LOXL1 expression. Vertical axis shows arbitrary units with the level in a nonpregnant female (N) set as 1.0. Values are the averages of independent samples from three mice. GAPDH (glutaraldehyde phosphate dehydrogenase) levels were used as internal normalization standards. Elastin and LOX mRNAs were unchanged through this period. G, gestational days; PP, postpartum days. $n = 3$ at each time point. **B:** Immunofluorescence for LOXL1 shows absence of LOXL1 fibers at 1 day postpartum in the uterine cervix. Red, LOXL1 (anti-LOXL1C antibody); blue, nuclear stain with Hoechst dye 33342.

turned to baseline 6 days postpartum in WT mice (Figure 5A). LOXL1 protein also became undetectable in the uterine cervix at day 1 postpartum and subsequently returned to normal (Figure 5B). In contrast, expression of the tropoelastin and LOX mRNAs (Figure 5A) remained unchanged through this period.

LOXL1 expression became deficient in the reproductive tract as the WT mice aged, along with cessation of reproductive activity. At 18 months, an age when estropause (analogous to menopause) has taken place in WT mice,[35,36] LOXL1 mRNA (Figure 6A) and protein (Figure 6, B–D) were diminished. In contrast, elastin (Figure 6A) and LOX (not shown) mRNAs remained little changed. As a result, tropoelastin accumulated to a very high level (Figure 6B). By immunofluorescence, elastic fibers were reduced and replaced by random elastin aggregates, concomitant with the decline of LOXL1 expression (Figure 6, C and D). Thus, LOXL1 expression is reduced just before parturition and again in old age.

Discussion

In this study, we show that mutant mice lacking LOXL1 develop complex and severe pelvic floor disorders. The pelvic floor disorders initially present as an acute vaginal and uterine prolapse within a few days after giving birth. The acute phase of prolapse is succeeded by a stable state of pelvic disorders characterized by marked pelvic organ descent and widespread damage to the pelvic floor tissues. It is not entirely clear what biological process drives the initial pelvic organ prolapse and what leads to the partial recovery. Because there is a lag of 1 to 3 days between parturition and the appearance of prolapse, it is unlikely that muscle contraction during delivery played a direct role. It is clear, however, that pregnancy and parturition are a key risk factor for developing pelvic floor disorders in the mutant mice. Changes in the pelvic floor organs, fascia, and ligament structures that hold the organs in place presumably all play a role. Because LOXL1 is dedicated to elastic fiber synthesis,[34] we conclude that elastic fiber defects underlie the primary pathophysiology of the pelvic floor disorders in the mutant mice.

During pregnancy and parturition, tissues in the reproductive tract undergo profound changes that include breakdown and resynthesis of elastic fibers. An inability to rebuild the elastic fiber network might be anticipated to produce some structural and functional deficits in these tissues, but the severity of disease was surprising and unexpected. The data suggest that the resilience conferred by elastic fibers is important for involution of the pelvic tissues after parturition. It remains to be determined, however, whether such massive pelvic floor disorders arise merely as the result of altered mechanical

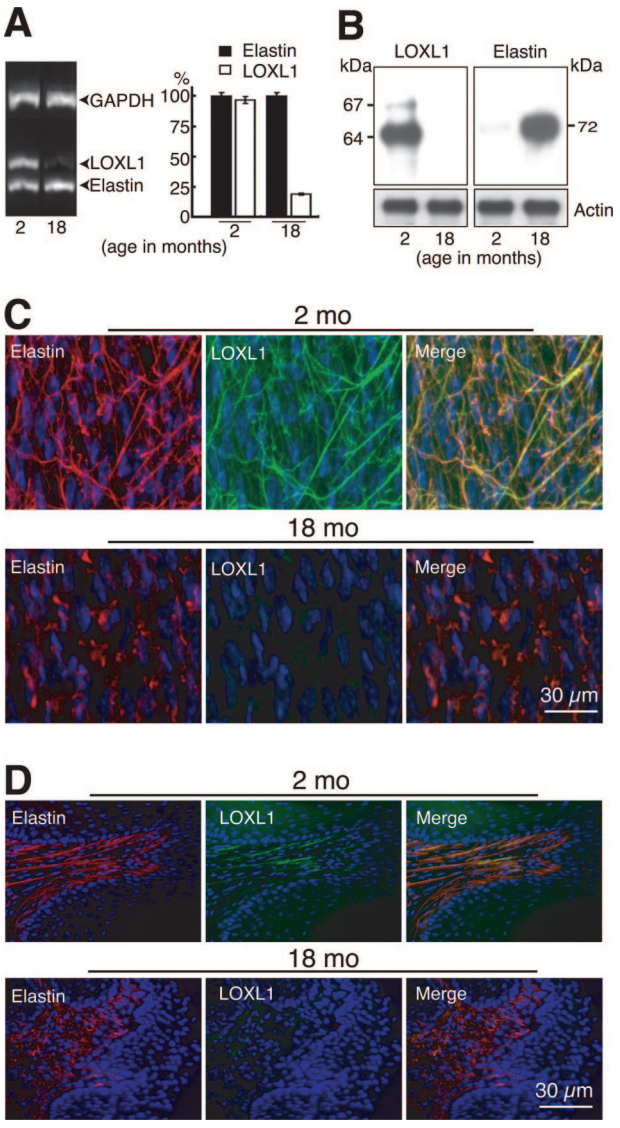

Figure 6. Age-dependent decline of LOXL1 expression in the uterus and cervix. WT mice at 2 months (young, nulliparous) and 18 months (old, parous) of age were examined. **A:** Analysis of LOXL1 mRNA in the uteri of young and old mice by RT-PCR. **Left**: A representative agarose gel analysis of PCR product. **Right**: Bar graph representation of the percentage changes in LOXL1 expression. Values are the averages of three independent samples. GAPDH levels were used as internal normalization standards. $n = 3$ at each age. **B:** Comparison of LOXL1 protein levels in the uteri of young and old mice by immunoblotting (**left**). LOXL1 is undetectable in the older animals. Loss of LOXL1 expression is correlated with an accumulation of tropoelastin in the older animals (**right**). **C:** Examination of LOXL1 and elastic fibers by immunofluorescence in the uteri (the middle layer of myometrium) of young and old mice. **D:** Examination of LOXL1 and elastic fibers by immunofluorescence in the uterine cervix of 2-month-old and 18-month-old mice. Green, LOXL1 (anti-LOXL1N antibody); red, elastin (RT675 antibody). Cell nuclei were counterstained blue with Hoechst dye 33342.

properties of the pelvic tissues. Elastin polymers and soluble elastin-derived peptides have been reported to play important signaling roles in cell migration and proliferation, among others.[37,38] It is possible that changes in the content of elastin polymer and accumulation of soluble elastin, hence their cellular signaling roles, may also contribute to the development of pelvic floor defects.

The pelvic floor disorders in the mutant mice include a profound lower urinary tract dysfunction, manifesting as a large increase in voiding frequency. Compared to the WT controls, the mutant mice have an ~10-fold higher voiding frequency with a corresponding decrease in volume. The total urinary output throughout a 24-hour period is comparable to that of the WT controls, and we have ruled out a kidney disease. Hence, the problem lies with the lower urinary tract and could be associated with the bladder, the urethra, or both. Occasionally the lower urinary tract dysfunction presents as severe bladder outlet obstruction. Although a seemingly opposite manifestation to increased frequency, the underlying etiology is probably the same. This is because pelvic prolapse and connective tissue damage could lead to hypermobility of pelvic organs including the bladder and the urethra. When the latter becomes twisted or otherwise develops hard kinks along its lengths, urine flow could be blocked off. Urine flow could resume only after the bladder and urethral pressures elevate to a sufficient level to force open the blockage or when positions of the pelvic organs shift and relieve the kink. Bladder outlet obstruction and the resultant severe bladder distention could further damage the bladder and sphincter and exacerbate incontinence once the urethra reopens. Such clinical complications are well documented in human patients.[25] These findings suggest common aspects in the pathophysiology of lower urinary dysfunction between this mouse model and human patients.

The increased voiding frequency accompanied by a corresponding decrease in volume in this mouse model is reminiscent of human urinary incontinence. However, incontinence is distinguished from normal voiding by one's intent or lack thereof, which cannot be determined in rodents. Given the massive damage to the pelvic floor and paraurethral tissues, it would be reasonable to assume that ineffectual urethral closure exists. Therefore an element of stress urinary incontinence may account for at least a part of the voiding abnormality in this model. Considering that the urinary bladder is trapped inside the vaginal cavity during prolapse (cystocele; Figure 1B) and that the bladder wall itself is normally rich in elastic fibers, damage to the bladder muscle and its innervation are likely. This in turn could lead to overactivity of the bladder (detruser overactivity). Thus, contribution from an urge type of urinary incontinence cannot be ruled out. A better understanding of the urinary dysfunction in this model will await more specialized measurements such as cystometry and leak point pressures, which are standard in rats[15,39–44] and could be adapted for use in mice.

We found LOXL1 expression to be regulated through the reproductive cycle, with the mRNA and protein levels decreasing near parturition and returning to baseline postpartum. The temporal pattern of LOXL1 expression in the uterine cervix coincides with a physiological process in late pregnancy known as cervical ripening.[45] In preparation for parturition, cervical ripening leads to the breakdown of collagen and elastic fibers and softening of the uterine wall.[45,46] This process appears to be regulated by relaxin and is also suggested to be mediated through a down-regulation of estrogen receptors.[47] LOXL1 expression in the reproductive tract of female mice is diminished at an advanced age and is accom-

panied by diminished and fragmented elastic fibers. Interestingly, LOXL1 expression levels remain little changed in the lungs and aortas of the same aged animals. These data suggest that the expression of LOXL1 in the pelvic organs may be under hormonal regulation. Further studies will be necessary to delineate how LOXL1 expression is regulated in the reproductive organs and shed light on pathophysiology of pelvic floor disorders among adult women.

To our knowledge, the *Loxl1*$^{-/-}$ female mice represent the first genetic animal model that simulates major aspects of human pelvic floor disorders. The important question is whether a failure of elastic fiber homeostasis also underlies the etiology of common pelvic floor disorders among older women. There is no strong evidence in support of this theory at present.[48,49] A gradual loss of elastic fibers is considered a normal part of connective tissue aging. Thus, the line between a normal and pathological loss of elastic fibers may not always be clear-cut. Nevertheless, concerted and focused investigations on elastin metabolism in relation to pelvic floor disorders are warranted in light of our findings. Elastic fiber homeostasis depends on a balance between degradation and resynthesis of elastin polymers. Reduced elastin polymer synthesis and excessive elastolytic activity could both lead to loss of elastic fibers.[50,51] Elastic fiber homeostasis would therefore require the proper synthesis of tropoelastin,[52] the scaffolding function of fibulin-5,[53,54] cross-linking by LOXs,[33] and regulation of elastolytic activities by endogenous inhibitors. In theory, allelic differences in genes involved in elastic fiber homeostasis and a host of environment factors could tip the balance from a state of homeostasis into one of gradual diminution of elastic fibers. Finally, regardless of the primary etiology therapeutic interventions aimed at boosting endogenous LOXL1 or providing exogenous replacement LOXL1, in the hope of promoting elastin synthesis, may provide a potentially effective treatment for this clinically important condition.

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