Induced Regenerative Elastic Matrix Repair in LOXL1 Knockout Mouse Cell Cultures: Towards Potential Therapy for Pelvic Organ Prolapse

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Abstract

Impaired elastic matrix remodeling occurs in reproductive tissues after vaginal delivery. This has been linked to development of pelvic organ prolapse (POP), for which there currently is no pharmacologic therapy. Hyaluronan oligomers and transforming growth factor beta 1 (termed elastogenic factors, EFs), have been shown to significantly enhance tropoelastin synthesis, elastic fiber assembly and crosslinking by adult vascular smooth muscle cells (SMCs). The goal of this study was to ascertain, if these factors similarly improve the quantity and quality of elastic matrix deposition by vaginal SMCs (VSMCs) isolated from lysyl oxidase like–1 knock out (LOXL1 KO) mouse model of POP. Cells isolated from whole vagina of a LOXL1 KO mouse (multiparous, stage 3 prolapse) were cultured and identified as SMCs by their expression of various SMC markers. Passage 2 vaginal SMCs (VSMCs; 3×10^6 to 10^7/cm^2) were cultured for 21 days with EFs. Cell layers and spent medium aliquots were assessed for elastin content and quality. EF-treated VSMCs proliferated at a similar rate to untreated controls, but synthesized more total elastin, primarily in the form of soluble matrix elastin. Elastin mRNA was also increased compared to controls. The elastic matrix was significantly denser in EF-treated cultures, which was composed of more mature, non-interrupted elastic fibers that were absent in controls. The results are promising towards development of a therapy to enhance regenerative elastic matrix repair in post–partum female pelvic floor tissues.

Keywords: Elastin; Smooth muscle cell; Vagina; Pelvic floor disorders; Prolapse; Growth factors; Elastogenic

Introduction

Chronic enzymatic disruption of, and intrinsic reduction in cellular capacity to repair and/or regenerate highly cross–linked elastic matrix, occurs with aging and contributes significantly to the development and exacerbation of several pathological disorders, characterized by loss of extracellular matrix (ECM) structure [1-4]. One such condition, pelvic organ prolapse (POP), characterized by abnormal herniation of the pelvic organs, correlates with vaginal delivery of children and becomes increasingly more common post–menopause, and with age [5,6]. The lifetime risk of surgery for POP is as high as 11% [78], with a re-operation rate for POP or related conditions, between 6–12% [8-10]. An excess of 1 billion USD is spent annually in surgical treatment of POP [11]. Drug–based therapeutic strategies to reduce the need for re-operation and regress, or prevent the progression of POP are critical to improve the quality of life for patients, and reduce associated costs of long-term treatment; however, no such therapies currently exist.

Clinically, POP and other manifestations of female pelvic floor disorders (FPFD) appear to be associated with heritable genetic alterations in actin, myosin and extracellular matrix (ECM) proteins [12,13]. Expansion and softening of pelvic tissues in preparation for parturition and resorptive involution post–partum occurs via degradation of elastic fibers [14,15]. Defects in homeostasis of the pelvic connective tissues could thus exacerbate tissue damage due to impaired post–partum matrix repair. There is increasing evidence that elastin abnormalities and FPFD may be associated, since POP patients with complete procidentia and women with more than two vaginal deliveries, exhibit reduced elastin content in their uterosacral ligaments [3], which could be due to the reduced elastin mRNA expression and matrix synthesis observed in these patients [16-18]. Neutrophil elastase activity is also increased and α−1 antitrypsin (an inhibitor of neutrophil elastase) expression decreased in vaginal tissues of FPFD patients, suggestive of increased elastic matrix breakdown [19]. Further, genes involved in elastin metabolism and degradation (e.g., elafin) are differentially expressed in the vagina in women with stress urinary incontinence (SUI), suggesting that impaired elastin remodeling is important in the pathophysiology of SUI, as well as POP [20]. Recent insight into the molecular pathogenesis of POP implicates abnormal connective tissue (CT) homeostasis and repair, with aberrations of enzymes responsible for cross–linking tropoelastin, the monomeric precursor molecule, to matrix structures [11]. Such aberrations could lead to the high rate of unsuccessful outcomes with surgical repair, which relies on preserved native tissue structure. Disruption of the CT matrix can potentially lead to increased complications, such as mesh extrusion and protrusion, which is the likely basis for recent warnings, as to use of such meshes by the Food and Drug Administration in the United States [21]. Restoration of disrupted ECM composition, content, and structure in POP tissues may, therefore, prove critical to slow, arrest, or even regress POP development.

In light of evidence that aberrant or frustrated elastic matrix repair occurs in POP tissues, inducing regeneration of elastic matrix may be useful as a therapy to deter the condition. In recent studies, hyaluronan oligomers (HA–o) have been shown to significantly enhance elastin precursor molecule (tropoelastin) synthesis, elastic fiber assembly, and cross–linking by vascular smooth muscle cells [22,23]. Transforming

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growth factor beta−1 (TGF−β1) is also known to enhance elastin production, by suppressing matrix metalloproteinases (MMPs) and elastases [24], and increasing tissue inhibitor of metalloproteinases (TIMPs) [25] and lysyl oxidase (LOX) protein levels [26]. HA−o and TGF−β1, together termed elastogenic factors (EFs), synergistically enhance tropoelastin and matrix elastin synthesis, crosslinking, and fiber formation. This has been demonstrated by EF−treatment, in both healthy [27] and diseased (e.g., aneurysmal) [28,29] adult vascular smooth muscle cell (SMC) culture, known to be inherently poor in regenerating elastic matrix.

To explore future potential for an elastic matrix regeneration−based therapy to treat POP, the present study sought to determine the effects of EFs on vaginal smooth muscle cells (VSMCs) harvested from a lysyl oxidase like−1 (LOXL1)−deficient mouse, a well characterized animal model that simulates clinical POP. VSMCs enable elastic matrix synthesis and cross linking via secretion of precursor molecules and enzymes, such as LOXL1, that are critical to catalyze the formation of mature fibers. This work has the potential to augment current treatments for POP, and complement upcoming therapeutics to improve success rates and reduce the need for re−operation.

Materials and Methods

Isolation and culture of mouse vaginal smooth muscle cells

Whole vagina was harvested just below the cervix to the introitus, from a prolapsed LOXL1 knockout mouse (parity=3; prolapse grade=3), according to animal protocols approved by IACUC. The tissue sample was minced into pieces (2−3 mm long) and incubated in Dubelco’s Modified Eagle Medium (DMEM)−F12 containing 20% v/v fetal bovine serum (PAA Scientific, Ontario, Canada), 1% v/v Penstrep (Thermo Scientific, Rockford, IL), and 175 U/ml collagenase II (Worthington Biochemicals, Lakewood, NJ), for 20 minutes at 37°C. This was followed by a second digestion in the above solution, supplemented with 0.25 mg/ml of elastase III (Worthington Biochemicals, Lakewood, NJ) for 45 minutes. Cells were finally pelleted at 1000 rpm, reconstituted and cultured in DMEM−F12 containing 20% v/v serum and 1% v/v Penstrep. Subsequent passages were cultured in 10% v/v serum and cells at passage 2−3 were used for the study.

To confirm that the isolated cells were VSMCs, they were immunolabeled for the SMC phenotypic markers caldesmon, α−SM actin, and tropomyosin (Abcam, Cambridge, MA). For cell culture studies, confluent VSMCs were trypsinized and seeded in 6−well plates (A=10 cm²/well), at a density of 3×10⁶ cells/well and cultured for 21 days in 10% v/v serum− rich media. Cell cultures supplemented with EFs at a concentration of 10 ng/ml of TGF−β1 (Peprotech Inc., Rocky Hill, NJ) and 2 µg/ml HA–o, served as the ‘Treatment’ group, while those cultured without EFs represented the ‘Control’ group.

The EF dose combination tested was one that has been shown in a series of earlier studies, to elastogenically stimulate both rat and human aortal SMCs, both healthy and diseased (i.e., from abdominal aortic aneurysms) [27,28]. HA−o mixtures used in this study primarily consisted of 4−mers (~75%), with 6−mers and 8−mers, comprising the remaining fraction. They were prepared by digesting high molecular weight HA (1.5 x 10⁶ Da, Genzyme Biosurgery, Cambridge, MA) with testicular hyaluronidase (40 U/mg at 37°C for 18 hours; Worthington Biochemicals, Lakewood, NJ), as previously described [23]. Cell layers were harvested after 21 days of culture for biochemical analysis (n=6 cultures/group). Spent medium from each well was pooled with aliquots, previously removed from the same wells, and frozen at −20°C until biochemically quantified for tropoelastin content at the end of the culture period. For imaging the ECM, additional 2−chamber slides were seeded at 10⁶ cells/well (A=2 cm²) and cultured likewise, with or without the EFs.

DNA assay for cell quantification

The DNA contents within the respective groups of cell cultures were compared to determine the impact of EFs on VSMC growth. For analysis, cell layers were harvested after 21 days of culture in 4 ml aliquots of Pi buffer (50 mM Na₂HPO₄, 2 mM EDTA, 0.02% NaN₃), and then sonicated over ice. Their respective DNA contents were quantified using a Hoechst dye (Gibco−Invitrogen, Carsbad, CA)−based fluorometric assay, as described by Labarca and Paigen [30].

Briefly, sonicated aliquots were incubated with the dye, and dye−bound DNA content was estimated from fluorescence values obtained from excitation at emission peaks at 346 nm and 460 nm, respectively, using a UV−spectrophotometer (SpectraMax M2, Molecular Devices, Inc., Sunnyvale, CA). Cell counts were then calculated assuming 6 pg of DNA/cell [30].

Hydroxy proline assay for collagen content

A hydroxy proline (OH−Pro) assay was used to estimate the collagen content within the EF−treated and control cell layers, as described previously [22]. Briefly, cell layer aliquots harvested in Pi buffer were hydrolyzed in 6 N HCl for 16 hours at 110°C, dried and reconstituted in Milli−Q water. 50 µl aliquots were assayed in duplicates, to measure the OH−Pro content in cell layers. Matrix collagen amounts were then calculated on the basis of the 13.2% w/w OH−Pro content of collagen, and normalized to cell numbers of corresponding cell layers.

Fas tin assay for elastin content

A Fas tin assay (Accurate Scientific and Chemical Corporation, Westbury, NY) was used to quantify the total amount of elastin deposited within cell layers (matrix elastin), and that released into the culture medium as a soluble precursor (tropoelastin). For each treatment group, tropoelastin in the spent medium was collected and pooled over the culture period, and frozen (~20°C ). To isolate matrix elastin following 21 days of culture, the cell layers were harvested in Pi buffer, digested with equal volumes of 0.1 N NaOH (98°C, 1 hour), and centrifuged to yield a less crosslinked, alkali−soluble supernatant fraction (soluble elastin), and a mature, highly crosslinked, insoluble pellet (insoluble elastin). Since the Fas tin assay quantifies only soluble α−elastin, the insoluble elastin was first converted to a soluble form, prior to quantification, by solubilizing it with 0.25 M oxalic acid (95°C, 1 h out), then filter−centrifuging it (3000 RPM, 10 min) in micro centrifuge tubes fitted with low molecular weight (10 kDa) cut−off membranes (Millipore, Bedford, MA). All three elastin fractions (tropoelastin, soluble and insoluble matrix elastin) were quantified using the Fas tin assay kit.

RT−PCR for mRNA expression of matrix proteins

Total RNA was isolated from cell layers at day 21 of culture using an RNeasy kit (Qiagen, Valencia, CA), and reverse transcribed into cDNA using iScript cDNA Synthesis kit (BioRad, Hercules, CA), according to the manufacturers’ instructions. Gene expression for collagen, elastin, LOX, and fibrillin were evaluated using 1 µl of cDNA with Power SYBR®Green Master Mix (Applied Biosystems, Foster, CA) (n = 6/case), in the ABI 7500 Real Time PCR System (Applied Biosystems). Specific primers used for collagen−1 (Col1A1), Elastin (Eln), LOX (LOX), and fibrillin−1 (FBN1) [31] were purchased as optimized primer sets from
Real Time Primers (Elkins Park, PA). A comparative threshold method [32] was used to quantify the mRNA expression of these genes and was reported as $2^{-\Delta\Delta Ct}$, with 18s serving as the internal control.

Transmission Electron Microscopy (TEM) for visualization of elastic matrix

Cells cultured in chamber slides were harvested at 21 days of culture, and fixed in 2.5% v/v gluteraldehyde and 4% v/v paraformaldehyde in 2% w/v sodium cacodylate buffer for 12 hours. Samples were then post-fixed in 1% w/v osmium tetroxide and 1% uranyl acetate (1 hour), and dehydrated in a graded ethanol series (50–100 % v/v). Fixed cell layers were embedded in Epon 812 resin and placed on copper grids embedded in pure Eponate for sectioning. Sections (85 nm thickness) were cut using a diamond knife, and stained with uranyl acetate and lead citrate. Sections were imaged using a Philips CM12 electron microscope, operated at 60 kV.

Statistical analysis

All quantitative results were analyzed from n=6 independent biological replicate cultures, measurements made in triplicate unless otherwise indicated, and reported as mean ± standard deviation. Statistical significance between groups was determined by SPSS software, using a simple t-test assuming unequal variance. Results were deemed significant for $p$ values ≤ 0.05.

Results

Confirmation of SMC phenotype

The cells isolated from the LOXL1 KO mouse vaginal tissue labeled positive for the SMC phenotypic markers caldesmon, α–SM actin, and tropomyosin, confirming their identity as VSMCs (Figure 1).

Cell density and ECM synthesis

There was no statistically significant difference in cell numbers after 21 days of culture between control [(1.6 ± 0.2)×10^6/well] and treatment [(1.7 ± 0.2)×10^6/well] groups ($p=0.289$; Figure 2). Total matrix collagen deposition was also not statistically different between control (346.5 ± 51.4 µg) and treatment (257 ± 78.4 µg) cultures ($p=0.126$) (Figure 3). On a per cell basis, this trend was maintained (216.6 ± 51.4 pg/cell in control vs 151.9 ± 42.4 pg/cell in treated groups, $p=0.092$).

Elastin protein content was quantified in terms of tropoelastin, and alkali–soluble and–insoluble matrix elastin components. Tropoelastin synthesis, though noticeably higher in the treatment group (364.78 ± 54.1 mg/well vs 250.38 ± 56.1 mg/well in control) was not significantly different ($p=0.09$; Figure 4A). Treatment with EFs significantly increased total elastin matrix production ($p=0.004$), primarily in the form of alkali–soluble matrix (6.8±2.1) fold compared to control, $p=0.04$; Figure 4B). No significant differences were observed in alkali–insoluble matrix elastin content.

Expression for matrix protein genes

Elastin mRNA expression in EF–treated cultures were significantly higher (3.6 ± 0.66 fold, $p=0.001$), compared to untreated control cultures (Figure 5). Although, expression of collagen and LOX genes in the EF-treated cultures appeared to be higher than in control cultures, the differences were deemed statistically, not significant ($p=0.132$ and 0.13 vs. control respectively, for collagen and LOX). Likewise, fibrillin

Figure 1: Phenotypic confirmation of primary vaginal cells. Cells isolated by enzymatic digestion of vaginal biopsies were proliferated in DMEM/12 containing 20% v/v FBS. Cells positively labeled for various SMC markers, such as caldesmon (Alexa Fluor 594, red), α–SM actin (Alexa Fluor 555, orange), and tropomyosin (Alexa Fluor 594, red) confirming the SMC phenotype. Nuclei were labeled with DAPI (blue).
Figure 2: Morphology and cell count of cultured VSMCs. (A) Cell layers at day 21. (B) Cell count at day 21 as determined by a fluorometric assay, showing no significant differences between control and treatment groups.

Figure 3: Collagen content quantified in cultures by hydroxyproline assay. No significant difference was observed between the groups.
mRNA expression levels were not significantly different between treated and control groups (p=0.11 vs. controls).

**Visualization of elastic matrix**

TEM revealed sporadic and aberrant elastin fibers in the control cultures, in contrast to the more mature fibers found in abundance in the EF-treated cultures (Figure 6). Elastic fibers were observed in the form of electron-dense regions, surrounded by dark fibrillin microfibrils in the extracellular space, indicating that the cells were in the process of laying down the fibers.

**Discussion**

Connective tissues of the female reproductive tract provide critical structural support to the pelvic organs [33]. Their role in maintaining structural integrity of the pelvic organs can be most appreciated postpartum, when their matrix is known to undergo massive remodeling to repair and restore healthy pelvic tissues, after delivery [34,35]. Specifically, the expression of genes for several matrix proteins essential to elastic matrix assembly (e.g., fibrillin, elastin, fibulin and LOX) and their synthesis, are highly up regulated at this time [36].
This enhanced elastogenic response aids in the reorganization and reassembly of stable pelvic tissue post-partum.

The elastic matrix components of pelvic tissues are known to experience homeostatic disturbances and degradative changes in women with POP [37]. Whether these changes are a causative factor in pelvic floor relaxation and herniation, or are an outcome of the process, is incompletely understood [38]. Animal models of POP suggest that changes to the ECM of the vaginal tissue, occurring during normal pregnancy and parturition, are not restored to a normal, pre–pregnant state post–partum [39]. This implicates a pathologic deterioration of the vaginal tissue ECM that begins decades, before POP clinically manifests [11,40,41].

Among structural ECM proteins, elastin, a major component of elastic fibers, provides resilience and mechanical stability to pelvic tissues [36,42]. Elastic fibers also critically maintain the healthy phenotype and function of SMCs, regulating vital cell signaling pathways. In this regard, it is likely that impaired elastic matrix repair and regeneration post–partum would limit return of the tissue to a pre–pregnancy state, and thus contribute to gradual POP development. Currently, there is no therapy that can prevent, impede or regress this process. The purpose of the present study is to investigate the possibility of an elastogenic therapy that could induce vaginal cells, to regenerate a healthy elastic matrix in pelvic tissues post–partum, towards slowing or even arresting POP development.

The present study utilized VSMCs harvested and isolated from a knock–out mouse lacking the gene expressing LOXL1, a well characterized animal model of POP [43]. LOXL1, a member of the LOX family, is a copper–dependent enzyme that oxidatively deaminates lysine residues on tropoelastin, mediating spontaneous crosslinking between monomers and forming the elastin polymer [44]. LOXL1 colocalizes with both the microfibrillar scaffolding protein fibrin–5 and elastin in the extracellular space [45]. Despite anatomical differences in pelvic floor orientation, POP in LOXL1 knockout mice is etiologically similar to human POP in that (a) prolapse development occurs mainly following parturition, (b) severity of POP increases with increasing parity, (c) cesarean delivery delays and reduces severity of prolapse, (d) heterogeneity of prolapse compartment contents can be observed on MRI, and (e) presence of dysfunctional urinary patterns potentially indicating stress urinary incontinence, a common co–morbid pelvic floor disorder [3,11,43].

While clinical studies support a role for LOXL1 deficiency in POP pathogenesis, the evidence thus far is conflicting and inconsistent. Nonetheless, reports suggest that the disorder is likely to be polygenetic in most women [37,46]. Despite some differences compared to the clinical condition, LOXL1 KO mice exhibit elastic matrix homeostatic disturbances and also develop POP, which provide investigators a pathophysiology–driven model to test novel therapies that address attenuating and restoring these pathological mechanisms.

Data from the present study shows that VSMCs from a LOXL1 KO mouse can synthesize the elastin precursor, tropoelastin, but deposit elastic matrix in the form of sparse, immature and interrupted fiber–like structures. This data also demonstrates that these cells can

**Figure 6:** Example transmission electron microscopy images showing sporadic and aberrant elastin fibers (red arrows in A, C) in control cultures, while more abundant and mature fibers (red arrows in B, D) in EF–treated cultures. Magnification: A and C: 4400x (scale bar = 2µm), B and D: 11,500x (scale bar = 1µm). ECM = extracellular matrix.
be induced to significantly increase both elastin mRNA expressions (Figure 5), as well as deposit higher quantities of matrix elastin (Figure 4). RT–PCR confirmed that increase in elastin synthesis occurred following significant upregulation at the mRNA levels, and was independent of cell proliferation rates. Although, this increase in elastin gene expression was mirrored by increases in tropoelastin protein production, the difference between treated and untreated cultures was deemed statistically insignificant. These results contradict previous studies on aortic SMCs [27,29], wherein EFs stimulated significant increase in tropoelastin production. Despite this, the addition of EFs resulted in a 2.6 ± 1.4-fold increase in total matrix elastin compared to untreated VSMCs. This difference was observed primarily as the less–crosslinked alkali–soluble matrix elastin fraction, similar to that reported in previous studies on EF–treated aortic SMCs [27,45]. Since the quantities of tropoelastin synthesized by cells in both cases were insignificantly different, addition of EFs potentially contributes to matrix assembly processes, downstream from tropoelastin synthesis.

On the other hand, despite the addition of EFs, the lack of any increase in synthesis of the more crosslinked alkali–insoluble matrix–elastin fraction, could be indicative of the importance and indispensability of LOXL1–dependant cross–linking in the formation of mature elastin [47]. Previous studies have shown that the process of formation of mature matrix elastin involves a significant increase in LOX gene expression, and protein synthesis and activity [48]. Although, the tested dose combination of EFs was found to enhance LOX gene expression, this increase was deemed statistically insignificant. Nevertheless, it is certainly possible that any such increase in LOX mRNA might have triggered some increase in LOX protein synthesis (not measured), thereby compensating for absence of LOXL1. Especially, since elastogenetic effects of EFs are highly dose–dependant [27,29,45], further studies with EF dose–variations are required to investigate potential for improving LOX–mediated crosslinking and elastic matrix deposition. One limitation with the present study is that the cultures were analyzed at a single, extended time–point (21 days). It is also possible that compensatory mechanisms to overcome the lack of crosslinking enzymes in a LOXL1–deficient microenvironment may be more pronounced, at culture durations longer than 21 days. The formation and accumulation of elastic fibers may act as nucleation sites, for further tropoelastin deposition and fiber growth at a later time point.

Further, this study investigated results from VSMCs harvested from whole vagina of one multiparous prolapsed LOXL1 KO mouse. Effects of parity, time since previous delivery, age, and prolapse status may all affect the ability of VSMCs, to respond to EFs. Finally, the phenotypic implications of increased matrix elastin on LOXL1 KO VSMCs towards overcoming POP in vivo, are yet unknown and necessitate further studies, using the therapy in vivo.

Despite these limitations, EFs significantly improve overall architecture of elastic matrix. As observed by transmission electron micrographs of control cultures, interrupted/incomplete elastic fibers were distributed between VSMCs. In contrast, EF–treated cultures showed significantly more elastic matrix, which appeared in organized bundles of fibers between cells, with fiber diameters corresponding to that typical for mature elastic fibers (300 nm – 2 µm) [1,42]. Improved elastic fiber assembly may be a likely outcome of organized deposition of fibrillin scaffolds, followed by enhanced tropoelastin recruitment and crosslinking [49]. In addition to new fiber formation, attenuated expression and/or activity of matrix degrading enzymes can greatly help preserve and maintain matrix integrity [50]. Newly formed matrix structures, further serve as nucleation sites for fiber growth and crosslinking [49]. Supporting this, MMPs, specifically elastolytic MMPs –2 and –9, have been shown to be involved in POP development and/or propagation [5,51,52]. There is also evidence that in animal models of POP such as fibrulin–9 KO mice and LOXL1 KO mice, enzyme activities of MMPs –2 and –9 are highly upregulated [5,53,54]. This may account for the presence of interrupted and aberrant elastic fibers in LOXL1 KO mouse VSMCs. In this light, it is quite possible that the EF–induced improvement in overall elastic matrix fiber formation and intactness, are at least in part due to the inhibitory effects of EFs on MMPs [27,29]. Further studies designed to investigate the role of MMPs in the LOXL1 KO mouse, and the regulatory potential of EFs, are warranted.

Conclusion

This study provides evidence that SMCs from prolapsed vaginas of LOXL1 KO mice are responsive to elastogenetic stimulation and improves elastin synthesis, matrix deposition and fiber formation. Developing a pathophysiology–driven preventative–therapy for POP development and progression, and for limiting complications from surger, as well as its recurrence, will significantly improve the quality of life of women suffering from this condition, and at the same time reduce the massive economic burden. The promising results from this study merits further studies to develop therapeutics based on elastogenic induction of cells to overcome development and progression of POP.

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