CHAPTER 3

Functional characteristics of vaginal fibroblastic cells from premenopausal women with pelvic organ prolapse

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ABSTRACT

Pelvic organ prolapse (POP) remains a great therapeutic challenge with no optimal treatment available. Tissue maintenance and remodelling are performed by fibroblasts, therefore altered cellular functionality may influence tissue quality. In this study, we evaluated functional characteristics of fibroblastic cells from tissues involved in POP. To rule out normal ageing tissue degeneration, biopsies from 18 premenopausal women were collected from the precervical region (non-POP site) after hysterectomy of 8 healthy and 10 POP cystocele cases (POP-Q stage ≥ II). Extra tissues from the prolapsed sites were taken in the POP cases to distinguish between intrinsic and acquired cellular defects. Twenty-eight primary fibroblastic cultures were studied *in vitro*. A contractility assay was used to test fibroblast-mediated collagen contraction. Cellular mechanoresponses on collagen-coated or uncoated substrates were evaluated by measuring matrix remodelling factors at protein or gene expression levels. No differences were found between fibroblasts from the controls and the non-POP site of the case group. Fibroblastic cells from the prolapsed site showed delayed fibroblast-mediated collagen contraction and lower production of matrix metalloproteinase-2 (MMP-2) on collagen coated plates. On uncoated surfaces the gene MMP-2 and its tissue inhibitor of metalloproteinases-2 were up-regulated in POP site fibroblastic cells. In conclusion, fibroblastic cells derived from prolapsed tissues of patients with cystocele, display altered *in vitro* functional characteristics depending on the surface substrate and compared with non-prolapsed site. This implies an acquired rather than an intrinsic defect for most patients with cystocele, and should be taken into account when trying to improve treatments for POP.

INTRODUCTION

Pelvic organ prolapse (POP) affects the quality of life of women world-wide, but remains a great therapeutic challenge[1-4]. Patients with POP may suffer from urinary and faecal incontinence, sexual dysfunction, chronic pelvic pain, vaginal relaxation, and social isolation[5-7]. A recent systematic review showed that there is a strong genetic component in some women affected with POP[8], nevertheless environmental factors (parity, physiological ageing and obesity) seem to play a major role in the development of prolapse in most patients. However, the pathogenesis remains unclear[2,3,9].

Among types of POP, cystocele has the greatest incidence and is characterized by the protrusion of the bladder into the anterior vaginal wall and outside the body[10,11]. In the vaginal wall, passive tissue strength is given by the connective tissue layer which consists of a small number of cells embedded in a ground component, *i.e.* the extracellular matrix (ECM). The ECM obtains its strength from the fibrillar proteins collagen I, collagen III and elastin[12], and is remodelled and maintained by fibroblasts. These mechanosensitive cells produce anabolic proteins such as collagens, and activate catabolic enzymes, such as matrix metalloproteinases (MMPs). They remodel their surrounding matrix in response to mechanical and biochemical stimuli. If fibroblasts are affected, extracellular matrix balance could be distorted leading to weak tissues that could fail and eventually prolapse. The biomechanical microenvironment may be further compromised if, in surgical treatments for prolapse, non-resorbable polymeric meshes are used to replace tissue function. In spite of this fact, little is known about the role of fibroblasts in the pathogenesis of, and treatments for POP.

It is clear that current treatments are far from optimal and that new therapies are needed[11,13]. New approaches that promote tissue regeneration are promising alternatives[14], including autologous cell-based therapies which would be feasible to treat only acquired (and not genetic) diseases. Therefore the aims of the present study are: (1) to evaluate the contractile capacity of vaginal wall fibroblasts'; (2) to evaluate vaginal wall fibroblast mechanoresponses to two different substrates; and (3) to investigate the probability that alterations of fibroblast functions in premenopausal women with cystocele, are acquired or intrinsic. The study design followed a novel approach that we recently used to identify changes in tissue composition at histological and biochemical levels[15]. In this previous study, each POP patient was used as her own control as biopsies were taken from the anterior vaginal wall from prolapsed and non-prolapsed sites within the same woman and it was shown that changes in the connective tissue of the anterior vaginal wall from women with cystocele are an acquired, rather than an intrinsic defect in POP[15]. We hypothesize that the altered characteristics of the tissues may be governed primarily by acquired changes in the fibroblast phenotype. This hypothesis was tested by assessing cellular functional properties, *i.e.* contractile capacities and biomechanical responses, of cells derived from adjacent tissues of the same patient cohort.

MATERIALS AND METHODS

Patient selection, tissue processing, and cell culture

The retrieval of biopsies from patients was approved by the medical ethics committee of Kennemer Gasthuis Hospital (Haarlem, The Netherlands) and informed consent was acquired. Participants were recruited between March 2009 and 2011 following the strict criteria described by Kerkhof *et al.*[15]. Exclusion criteria included a history of pelvic surgery or endometriosis, the use of steroids or progestin-only hormone regimen, pelvic malignancy or connective tissue disease affecting tissue remodelling, adhesions or scarring at the biopsy site, surgeons' judgment that a biopsy may harm the patient, morbid obesity (body mass index [BMI]> 35 kg/m^2), diabetes, chronic inflammatory disease or infections, and inability to provide informed consent. Two groups of Caucasian premenopausal women were included: (1) a control group of women undergoing abdominal or laparoscopic hysterectomy (n=8) for benign gynaecological diseases with no sign of POP during gynaecological examination; and (2) a case group of patients undergoing vaginal hysterectomy and reconstructive pelvic surgery of the anterior vaginal compartment because of a cystocele (n=10). Patients and controls were matched for age and parity (Table I). Standardized demographic and pertinent clinical information was recorded prospectively and stored in a dedicated database. Full thickness (1 cm^2) anterior vaginal wall tissue biopsies were taken from the vaginal cuff at the anterior midline portion of the vaginal wall (non-POP site). From POP cases, extra biopsies were collected from the prolapsed anterior vaginal wall (POP site). Of these 28 samples, primary cell cultures were set-up within 24 hours after tissue extraction and cultured as described previously[16]. Cells were grown with cultured medium (Dulbecco's modified Eagle's medium-DMEM; Gibco-Life technologies, Paisley, UK) supplemented with 10% v/v fetal bovine serum (FBS, HyClone, South Logan, UT, USA), 100 µg/ml streptomycin, 100 U/ml penicillin, and 250 µg/ml amphotericin-B (Sigma, St. Louis, MO, USA). All the experiments were performed with cells from the early passages 2 or 3.

Immunohistochemistry

The primary cells were characterized by immunohistochemistry with the markers for mesenchymal (vimentin), smooth muscle (desmin), and endothelial cells (Ulex Europaeus Agglutinin-I). A list of antibodies and titrations can be found in Table II. Cells were cultured in 96-well plates with a density of 10,000 cells/cm^2 for 24 hours and fixed with 4% v/v formaldehyde, then incubated with blocking buffer (BB: 0.5% v/w BSA, 0.1% v/v Triton-x-100, PBS) for 30 minutes followed by 1 hour incubation with the primary antibody at 4°C. After three washings with BB, secondary antibody was added for 1 hour, subsequently washed with PBS and a drop of Vectashield mounting medium with DAPI was added to visualize the nuclei (Vectro Laboratories, Burlingame, CA, USA). Analysis was performed by counting cells in three independent wells using an inverted fluorescent microscope (Leica DMIL Microsystems, Wetzlar, Germany).

Table I. Patient characteristics

Characteristic	Control (n = 8)	Case (n = 10)	P-value
Age [a]	45.13 ± 5.4	42.50 ±6.2	0.360 †
Parity [b]	2.5 (1-3)	2 (1-4)	0.336 †
BMI [a]	24.31 ± 5.43	27.86 ± 6.68	0.163 †
Smoking [c]			
Current (%)	3 (37.5%)	2 (20%)	0.608 ‡
Previous (%)	4 (50%)	5 (50%)	0.588 ‡

Data is presented as: mean ± SD [a], median (range) [b], or number of patients (%) [c]. Non-parametric statistical tests: † Mann-Whitney; ‡ Fisher's exact.

Table II. List of antibodies for immunohistochemistry

Antibody		Dilution	Source
Monoclonal mouse anti-vimentin, clone V9	Primary	1:150	DakoCytomation, Copenhagen, Denmark
Desmin mouse anti-human clone D33	Primary	1:250	DakoCytomation, Copenhagen, Denmark
Fluorescein labelled Ulex Europaeus Agglutinin – I (UEA-1)	Primary	1:200	Vector Laboratories, Burlingame, CA, USA
Goat anti-mouse Alexa fluor 555	Secondary	1:800	Life technologies, Paisley, UK
Goat anti-mouse Alexa fluor 488	Secondary	1:800	Life technologies, Paisley, UK

Proliferation assay

To find differences in the proliferation rate of the studied cells, we seeded fibroblastic cells in 48-well plates with a starting population of 10,000 cells/cm^2, and tested in quadruple at different time points: 1, 2, 3, 4 and 7 days. CyQuant cell proliferation assay kit (Molecular Probes Inc., Life Technologies) was used to evaluate proliferation rate by following manufacturers' instructions. Fluorescence was measured using a micro-plate reader (Synergy^{TM}HT, Biotek Instruments Inc., Vermont, USA).

Contractility assay

A contractility assay was used to evaluate fibroblast-mediated collagen I contraction using a protocol adopted from Lu *et al.*[17]. Hydrogels were prepared by mixing: 2.5 mg/ml rat tail collagen type I (BD Biosciences, MA, USA), one part 10x DMEM (Sigma), one part reaction buffer (262 mM $NaHCO_3$, 0.05 N NaOH, 200 mM HEPES), and one part cell suspension (1,500,000 cells/ml) in culture media. A mixture volume of 100 µl/well was added to 96-well plates, under sterile conditions and on ice. Polymerization was achieved by incubating for 30 minutes at pH 7.4, 37°C and 5% CO_2. Thereafter, gels were covered with culture media and refreshed every 4 days up to 8 days. Pictures were acquired using a BiospectrumAC Imaging System (UVP, Cambridge, UK) at different time points: 0, 2, 3, 4 and 8 days. Image J 1.44p software (National Institutes of Health, USA), was used to calculate the percentage of initial surface area which was inversely related to the cells-mediated contraction.

Rheology of collagen hydrogels

The viscoelastic properties of the collagen hydrogels were assessed with a stress-controlled cone-plate rheometer (Paar Physica MCR501; Anton Paar, Graz, Austria) and steel plates. The top cone-plate had 40 mm diameter, an angle of 1° and 49 µm of truncation[18]. For the measurements, 300 µl of hydrogel without cells was placed on the bottom plate of the rheometer at constant 37°C and 5% humidity. Polymerization of the samples was followed under small amplitude oscillating shear measurement with 0.5% strain and 0.5 Hz frequency until reaching plateau. Frequency sweep measurements were performed decreasing from 100 to 0.01 Hz, at constant 0.5% strain amplitude. The elastic (G') and the viscous modulus (G") were obtained from values with an angular frequency of 1 Hz (2π rad/sec). The shear modulus (G*) was calculated by the formula: $|G^*| = \sqrt{(G'^2 + G''^2)}$.

Cyclic mechanical loading

A dynamic *in vitro* model was used to assess the effects of two elastic surface substrates and cellular mechanoresponses to loading mimicking continuous respiration: sinusoidal wave, 0.2 Hz, 10% elongation[19]. We recently reported that vaginal fibroblasts increased secretion of MMP-2 from 24 to 48 hours, particularly when mechanically loaded[16]. Based on these results, in the present study we applied the loading regimen for 48 hours. Fibroblastic cells were seeded at a density of 15600 cells/cm^2 on collagen I-coated or uncoated Bioflex® plates (BioFlex, Flexcell International Corp., McKeesport, PA, USA), and left to attach for 48 hours in culture media supplemented with 10% v/v FBS (HyClone). Then, 10%-culture media was replaced by culture media containing 1% v/v FBS (HyClone), in order to be able to detect released matrix metalloproteinases (MMPs)-2 and -9, thereafter cyclic mechanical loading was applied using a Flexercell FX4000 device (Flexcell International Corp). Unloaded cells cultured under the same conditions were used to evaluate substrate effect, and as static controls. After the loading period, conditioned media was collected and analysed for *secreted MMP-2, MMP-9 and TIMP*-2. Thereafter, for *total DNA* and *gene expression analysis*, cells were lysed in a solution (1:100) of β-mercaptoethanol (Sigma-Aldrich) and RA1 buffer (Macherey-Nagel, Bioke, Leiden, The Netherlands). According to the suppliers' specifications, DNA and RNA were isolated using NucleoSpin TriPrep kit (Bioke).

Total DNA quantification

Total DNA was measured using a CyQuant kit and following manufacturers' instructions (Molecular Probes Inc., Life Technologies). Fluorescence was measured with a Synergy^{TM}HT (Biotek Instruments Inc.).

Gene expression analysis

Total RNA had a final concentration of 250 ng/ml, and was reverse-transcribed using SuperScript VILO cDNA synthesis kit (Life technologies). Gene expression of KI67, alpha-1(I) procollagen (Col 1α1), alpha-1(III)procollagen (Col 3α1), matrix metalloproteinases

(MMP)-2, -9, -14 and tissue inhibitor of metalloproteinases (TIMP)-1, -2 and -3 were normalized to the housekeeping genes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta popypeptide (*Ywhaz)* and hypoxanthine-guanine phosphoribosyltransferase (*HPRT)*. Genes were evaluated using the primers listed in Table III (Life technologies), with the SYBR Green Reaction Kit following suppliers' specifications (Roche, Diagnostics, Mannheim, Germany) and measured by RT-PCR in a Light Cycler 480 device (Roche). Gene expression levels were normalized using a factor derived from the equation $\sqrt{(}$*Ywhaz x HPRT*). Crossing points were assessed using the Light Cycler software (version 4) and plotted versus serial dilutions of cDNA derived from a human universal reference total RNA (Clontech Laboratories Palo Alto, CA, USA).

Table III. Primer sequences used for RT-PCR

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product Size (bp)
KI67	Forward	5' CCCTCAGCAAGCCTGAGAA 3'	57	202
	Reverse	5' AGAGGCGTATTAGGAGGCAAG 3'		
Col 1α1	Forward	5' TCCAACGAGATCGAGATCC 3'	57	191
	Reverse	5' AAGCCGAATTCCTGGTCT 3'		
Col 3α1	Forward	5' GATCCGTTCTCTGCGATGAC 3'	56	279
	Reverse	5' AGTTCTGAGGACCAGTAGGG 3'		
MMP-2	Forward	5' GGCAGTGCAATACCTGAACA 3'	56	253
	Reverse	5' AGGTGTGTAGCCAATGATCCT 3'		
MMP-9	Forward	5' TGACAGCGACAAGAAGTG 3'	57	219
	Reverse	5' CGTGGCTCAGGTTCAGG 3'		
MMP-14	Forward	5' CTGAGATCAAGGCCAATGTTC 3'	56	206
	Reverse	5' CTCACGGATGTAGGCATAGG 3'		
TIMP-1	Forward	5' CACAGACGGCCTTCTGCAA 3'	63	211
	Reverse	5' TTGTGGGACCTGTGGAAGT 3'		
TIMP-2	Forward	5' CTGAACCACAGGTACCAGAT 3'	63	237
	Reverse	5' TGCTTATGGGTCCTCGATG 3'		
TIMP-3	Forward	5' AGGACGCCTTCTGCAACTC 3'	63	163
	Reverse	5' GCTTCCGTATGGATGTACTG3'		
Ywhaz	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	56	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'		
HPRT	Forward	5' GCTGACCTGCTGGATTACAT 3'	56	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'		

Col1α1, α1(I)procollagen; Col3α1, α1(III)procollagen; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

Secreted MMP-2 and MMP-9

The enzymatic activities of MMPs -2 and -9 present in the conditioned media were analysed using Novex zymogram gels (10% zymogram gelatin gel, Life Technologies) following manufacturers' protocol. Visualization of dark bands of gelatinolytic activity was facilitated by eStain protein device (GeneScript, Piscataway, NJ, USA). Images were acquired with BiospectrumAC (UVP) and zymogram quantification of the density of the bands was performed using Image J (NIH). Values were calculated as follows: Total MMP-2 = inactive MMP-2 + active MMP-2; and percentage of active MMP-2 = (active MMP-2 x 100)/Total MMP-2.

Secreted TIMP-2

Since we found MMP-2 and not MMP-9 in our samples, we quantified released tissue inhibitor of metalloproteinase (TIMP)-2 because it inhibits MMP-2 (or collagenase IV) with a 1:1 stoichiometry[20]. Secreted TIMP-2 was quantified using the TIMP-2 Human ELISA kit ab100653 (Abcam plc., Cambridge, UK) following the supplier's instructions. Color intensity was measured at 450 nm using Synergy^{TM}HT (Biotek).

Power analysis

The primary outcome of the study was to detect a difference in fibroblast-mediated collagen contraction from cells derived from prolapsed and non-prolapsed tissues from premenopausal women with cystocele. In a previous study, the average of the contraction factor after 48 hours calculated as the ratio of the diameter of a contracted gel to the initial diameter of the well from pelvic floor myofibroblasts of women with and without severe prolapse was 1.8 ± 0.3 and 4.4 ± 1.9 respectively[21]. Based on these data, 8 women were required in each group to detect a difference of at least 25% for a power of 80% at the 0.05 significance level using an independent sample t-test.

Statistical analyses

Statistical analyses were performed using the software Prism version 5.02 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as the mean with either the standard deviation (SD) for individual measurements, or the standard error of the mean (SEM) for grouped values. Comparisons between the control and case non-POP site were done with unpaired t-test. Paired t-test was used in POP cases to evaluate differences between POP and non-POP sites. One-sample t-test compared to 1 was used to compare: (1) the contractility of POP and non-POP cells within the same patient at the different time points by using the ratio between percentage of collagen contraction by fibroblastic cells derived from POP and non-POP site from each case evaluated; and (2) the effects of 48 hours of cyclic mechanical loading (CML) and two surface substrates on secreted total MMP-2 and TIMP-2 by using the ratio between released protein with and without CML (+CML/-CML). One-way analysis of variance (ANOVA) followed by Tukey-Kramer's post-hoc test was used to test differences between all the groups. Patient characteristics

were evaluated by Mann-Whitney or Fisher's exact non-parametric tests. Differences were considered significant at 5% level ($p<0.05$).

RESULTS

Immunohistochemistry analysis showed that the cells studied were from the mesenchymal lineage (vimentin positive), and that they were all UEA-1 negative demonstrating that no endothelial cells were present. Cultures were at least 99% desmin negative, *i.e.* smooth muscle free (Supplementary figure 1). Proliferation rates were similar in all cells studied, independent of the biopsy site (Fig.1).

A contractility assay was performed to evaluate fibroblastic cells contractile capacities over time. We used collagen I hydrogels with shear modulus (G*) of 12.7 ± 6.00 Pa, elastic modulus (G') of 13.7 ± 4.94 Pa, and viscous modulus (G") of 2.3 ± 0.79 Pa. Fibroblasts-mediated collagen contraction of the POP site within the same patient was lower than the non-POP site (Fig.2B) in 80% of the cases studied (Supplementary figure 2). No differences were found between contraction of fibroblastic cells derived from healthy controls and non-POP site from POP cases (Fig.2A).

The effects of two surface substrates were evaluated using elastic silicone membranes, coated with collagen I or uncoated, and cellular mechanoresponses were tested by stretching fibroblastic cells for 48 hours. On this occasion, we used total DNA as an indicator of cell attachment and the gene KI67 as an indicator of cell proliferation (Fig.3). Cell attachment was facilitated by collagen-coated surfaces (Fig.3A), and proliferation was up-regulated on uncoated plates (Fig.3D). Cyclic mechanical loading inhibited cell proliferation (Fig.3E and F) and decreased cell attachment on uncoated (Fig.3C) but not on collagen-coated plates (Fig.3B). No apparent differences in cell attachment and proliferation were seen between cells from prolapsed (POP site) and non-prolapsed tissues (control and non-POP site).

To evaluate the effects of the different experimental conditions on the capacity of the fibroblastic cells to remodel the extracellular matrix (ECM), matrix metalloproteinase (MMP)-2, -9 and tissue inhibitor of metalloproteinase-2 (TIMP-2) protein secretion was evaluated. No differences were found in total levels of released MMP-2 between controls and non-POP site fibroblastic cells from POP cases. Both cell populations secreted significantly more MMP-2 in uncoated than in collagen-coated plates. POP site fibroblastic cells secreted less MMP-2 than control and non-POP site fibroblastic cells on collagen-coated plates (Fig.4B). Stretching fibroblastic cells on uncoated plates induced production (Fig.4C) and activation of MMP-2 which was more pronounced in non-POP than in POP fibroblastic cells (Fig.4E). Secreted TIMP-2 was not affected by loading (Fig.4D). Released MMP-9 (Fig.4A) and active MMP-2 on collagen-coated plates (Fig.4A, left panel), were below detection levels or completely absent.

A panel of ECM remodelling related genes were evaluated and results can be found in Table IV. Gene expressions of Col 3α1, MMP-9 and TIMP-2 were up-regulated in fibroblastic cells cultured on collagen-coated plates. In uncoated plates, cyclic mechanical loading increased gene expression of MMP-14 in all the cells studied, but MMP-2 and TIMP-2 only in fibroblastic cells from the prolapsed site.

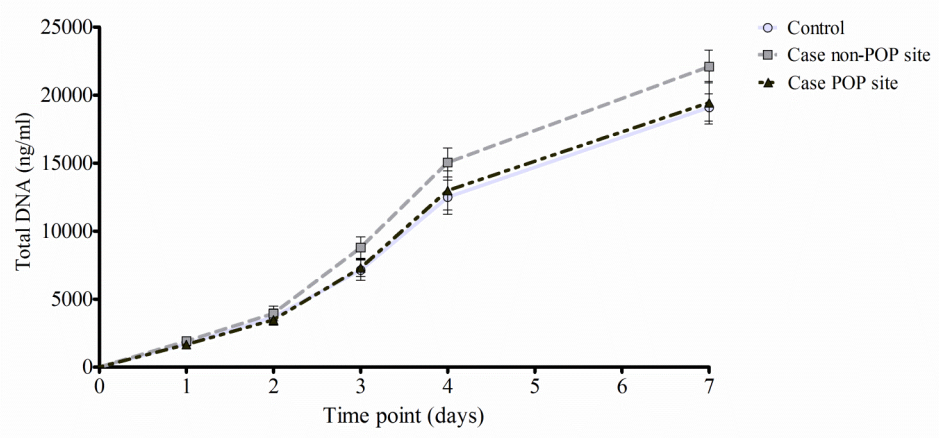

Figure 1. Proliferation rate of fibroblastic cells derived from non-prolapsed (control and case non-POP site) and prolapsed (case POP site) tissues. The cells were cultured on 48-well plates at different time points and total DNA was measured by CyQuant. Data represent the average of the means of replicates within each subject ± SEM. No differences were found by ANOVA followed by Tukey-Kramer's post-hoc test.

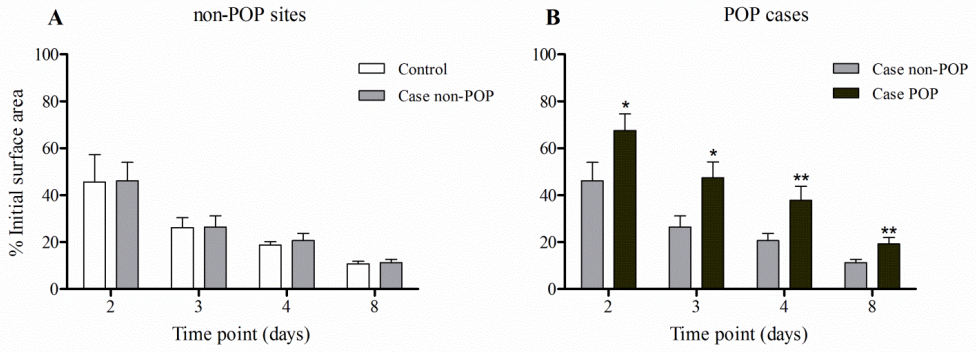

Figure 2. Fibroblastic cell-mediated collagen contraction. The figure shows the percentage (%) of the initial surface area – which is inversely proportional to the cell-mediated collagen contraction – at different time points for **(A)** non-POP sites (Control vs. Case non-POP), and for **(B)** POP cases (Case non-POP vs. Case POP). Each bar represents the average of the means of replicates within each subject for control (n=8) or POP cases (n=10) ± SEM. *p<0.05, **p<0.01; comparing non-POP vs. POP site by paired t-test.

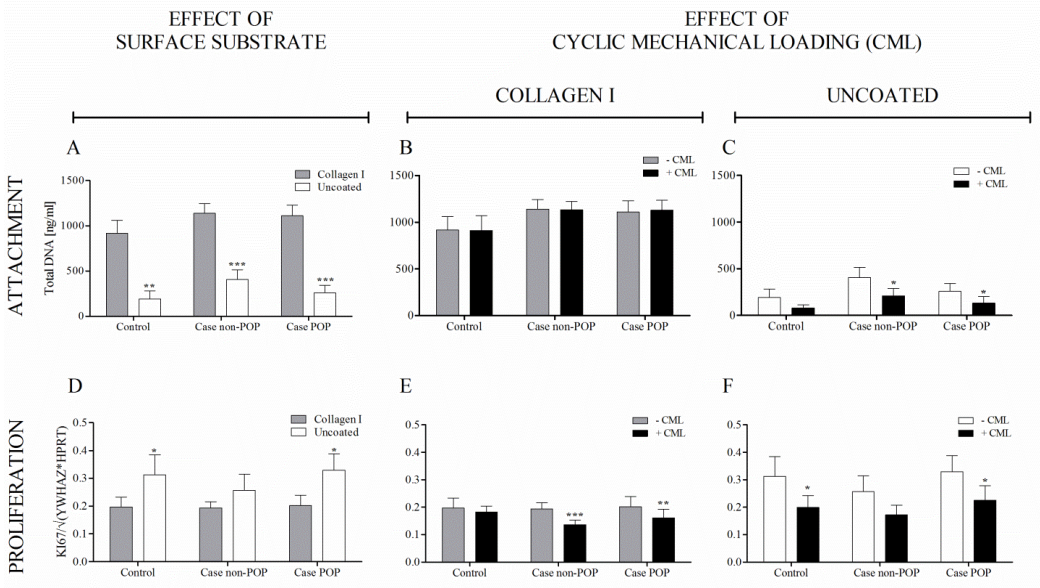

Figure 3. Effects of surface substrate and loading on fibroblastic cells attachment and proliferation. Cells were cultured on collagen (**A, B, D** and **E**) or uncoated silicone plates (**A, C, D** and **F**) and subjected or not to 48 hours of cyclic mechanical loading (+/- CML). We used total DNA measured by CyQuant as an indicator of cell attachment, and the gene KI67 as an indicator of cell proliferation. The effect of surface substrate is shown in figures **(A)** and **(D)**; while the effect of mechanical loading is shown for collagen I in figures **(B)** and **(E)**, and for uncoated plates in figures **(C)** and **(F)**. Each bar represents the average of the means of replicates within each subject ± SEM. *p<0.05, **p<0.01 or ***p<0.001 by paired t-test.

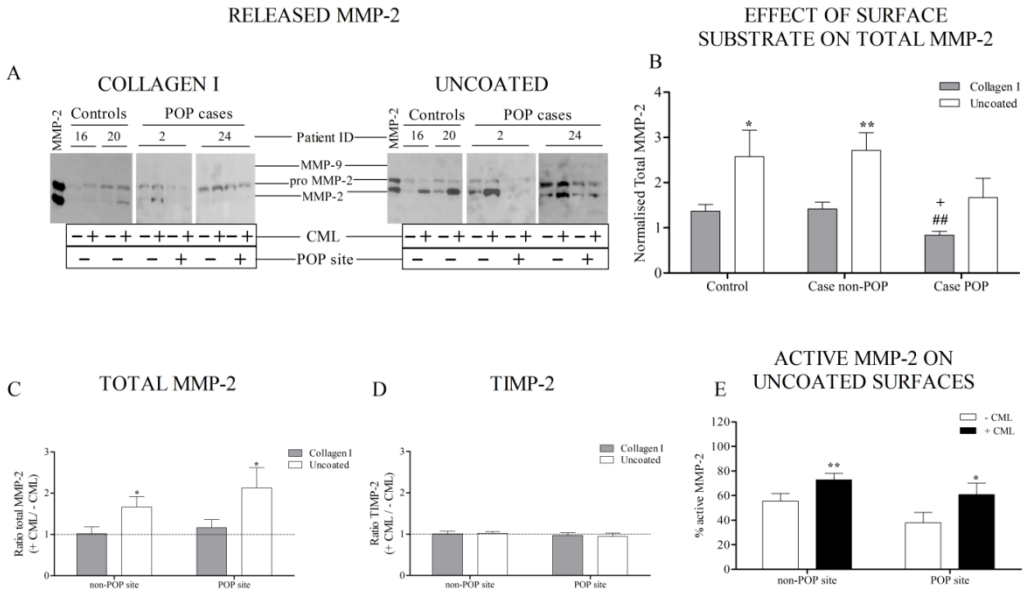

Figure 4. Effects of surface substrate and loading on secreted MMP-2 and TIMP-2. Conditioned media released by fibroblastic cells from controls (- POP site) or POP cases (-/+ POP site) cultured on collagen or uncoated silicone plates after 48 hours of cyclic mechanical loading (-/+ CML) were analysed for: MMP-2, MMP-9 and TIMP-2. **(A)** Representative zymograms showing MMP-2 and -9. Figure **(B)** depict quantitative data from zymograms comparing released MMP-2 on collagen vs. uncoated plates without loading (- CML). Total MMP-2 = (pro MMP-2 + active MMP-2)/(static pro MMP-2). The effects of mechanical loading on: **(C)** total MMP-2 and **(D)** TIMP-2 were evaluated by one sample t-test compared to 1 with *p<0.05. CML on uncoated plates promoted secretion of total MMP-2 and not TIMP-2 by fibroblastic cells. TIMP-2 was measured by ELISA. Figure **(E)** shows the percentage (%) of activation of MMP-2 relative to total MMP-2 on uncoated plates. Each bar represents the average of the means of replicates within each subject ± SEM. Differences between control vs. POP site (+p<0.05) and between case non-POP vs. POP site (##p<0.01) were identified by ANOVA followed by Tukey-Kramer's post-hoc test. Paired t-test was used to compare collagen I vs. uncoated plates: *p<0.05 or **p<0.01.

Table IV. Extracellular matrix remodelling related genes on two different substrates after 48 hours of cyclic mechanical loading.

	Control (n=8)				Case non-POP site (n=10)				Case POP site (n=10)			
GENE	Collagen I		Uncoated		Collagen I		Uncoated		Collagen I		Uncoated	
(normalised)	- CML	+ CML	- CML	+ CML	- CML	+ CML	- CML	+ CML	- CML	+ CML	- CML	+ CML
COL 1A1	1.04 ± 0.22	1.03 ± 0.20	0.75 ± 0.09	0.90 ± 0.11	0.90 ± 0.15	0.94 ± 0.16	0.83 ± 0.21	0.86 ± 0.14	0.95 ± 0.12	0.96 ± 0.14	0.73 ± 0.14	0.75 ± 0.12
COL 3A1	0.74 ± 0.08	0.75 ± 0.11	0.45 ± 0.04**	0.47 ± 0.07	0.74 ± 0.06	0.78 ± 0.06	0.46 ± 0.09**	0.54 ± 0.06	0.78 ± 0.06	0.76 ± 0.11	0.46 ± 0.05***	0.48 ± 0.06
MMP-2	1.29 ± 0.13	1.22 ± 0.06	1.10 ± 0.12	1.14 ± 0.08	1.41 ± 0.10	1.19 ± 0.11	1.28 ± 0.15	1.41 ± 0.11	1.19 ± 0.07	1.06 ± 0.11	1.13 ± 0.06	1.35 ± 0.09^{b}
MMP-9	0.68 ± 0.11	0.72 ± 0.18	0.39 ± 0.05*	0.31 ± 0.04	0.71 ± 0.07	0.66 ± 0.08	0.35 ± 0.06**	0.32 ± 0.04	0.84 ± 0.17	0.57 ± 0.13	0.33 ± 0.04*	0.44 ± 0.12
MMP-14	0.95 ± 0.06	0.94 ± 0.10	0.66 ± 0.07*	0.96 ± 0.10^{b}	1.01 ± 0.08	0.92 ± 0.10	0.74 ± 0.09	1.04 ± 0.12^{bbb}	0.81 ± 0.05	0.82 ± 0.07	0.60 ± 0.04**	0.96 ± 0.10^{bb}
TIMP-1	0.76 ± 0.13	0.74 ± 0.13	0.94 ± 0.23	1.06 ± 0.27^{b}	0.71 ± 0.09	0.63 ± 0.08	0.89 ± 0.17	0.99 ± 0.16	0.57 ± 0.05	0.50 ± 0.05	0.67 ± 0.07	0.77 ± 0.07^{b}
TIMP-2	1.09 ± 0.06	0.95 ± 0.08	0.71 ± 0.03***	0.81 ± 0.07	1.24 ± 0.08	0.92 ± 0.09^{a}	0.84 ± 0.09**	0.88 ± 0.03	1.06 ± 0.06	0.87 ± 0.09	0.77 ± 0.04*	0.92 ± 0.06^{b}
TIMP-3	0.53 ± 0.06	0.49 ± 0.05	0.75 ± 0.11	0.69 ± 0.09	0.50 ± 0.03	0.44 ± 0.04	0.66 ± 0.05	0.64 ± 0.09	0.58 ± 0.07	0.54 ± 0.08	0.81 ± 0.10	0.89 ± 0.11

CML: cyclic mechanical loading.
Each gene was normalised using the equation: Gene/√(Ywhaz x HPRT).
Data are the mean ± SEM. Paired t-test was used to compare: the effect of coating collagen I vs. uncoated (* p<0.05, ** p<0.01, *** p < 0.001);the effect of loading on collagen I coated plates - CML vs. + CML (^{a} p<0.05); and the effect of loading on uncoated plates - CML vs. + CML (^{b} p<0.05, ^{bb} p<0.01, ^{bbb} p < 0.001.

DISCUSSION

In pelvic organ prolapse (POP) tissue strength is lost and quality of the extracellular matrix (ECM) is compromised[3,15]. Alterations in cells derived from prolapsed tissues have also been observed[16,21,22]. Whether these changes in fibroblastic cells are induced by the modified matrix in the vaginal tissue of patients with POP, or whether there is an intrinsic defect of the cells itself, still remains unclear. Basic understanding about cell-matrix interactions in women with POP is necessary to develop new therapeutic strategies. It is important to discriminate between acquired and intrinsic defects because for genetic diseases treatments with autologous cells would not be an appropriate therapy. In the present study, we evaluated the functional characteristics of vaginal wall fibroblastic cells of healthy controls and POP patients by assessing their contractile properties and their response to cyclic mechanical loading. Furthermore we analysed whether this response is affected by the presence of artificial polymeric substrates, which is of importance in the development of new scaffolds.

We recently published an *in vitro* dynamic model using a physiological stretching regime to compare fibroblast mechanoresponses to artificial collagen-I-coated and uncoated substrates made of silicone[16]. In this pilot study, we found lower mechanoresponses in POP fibroblasts compared to a healthy control and especially on uncoated plates, *i.e.* when cells were being stretched and stressed. We found that surface substrate affects cellular behaviour, and that cell-matrix interactions seem to be impaired in POP fibroblasts. In the present study we included cells derived from a very strict patient cohort[15], and we evaluated the same parameters *in vitro* to corroborate our results and further included other experimental parameters to evaluate cellular functionalities and not just mechanoresponses.

We used a novel approach in which study samples were paired in POP patients, where each patient was her own control, and which was conducted under strict patient inclusion criteria, ruling out any deterioration due to ageing processes and menopause. Tissue samples were taken and processed in a very standardized manner, and cell cultures were at least 99% free of smooth muscle cells. No differences were found in proliferation rates of the cells studied, suggesting that the quality and not the quantity of the fibroblastic cells was responsible for the results.

Our data shows that fibroblast mechanoresponses from the non-POP site of the anterior vaginal wall from patients with prolapse do not differ within any of the parameters evaluated with cells derived from the same site in healthy controls. However, there were clear differences between fibroblastic cells derived from prolapsed and non-prolapsed tissues within the same women. Fibroblastic cells from the POP site showed delayed fibroblast-mediated collagen contraction and lower production of MMP-2 on collagen-coated silicone plates. Mechanoresponses to cyclic mechanical loading on uncoated silicone plates were also different: activation of MMP-2 was more pronounced in cells from non-prolapsed tissues, whereas up-regulation of MMP-2 and TIMP-2 gene expressions were only seen in POP-site fibroblastic cells.

Aberrant contractile capacities of fibroblastic cells have been associated with impaired wound-healing in soft tissues[23-25]. The hydrogels used in the present study had a shear modulus of 12.73 Pa, which is comparable to the elastic modulus of the ECM in early wounds[26]. A decreased fibroblast-mediated contraction of collagen gels by cells from the vaginal wall of severe POP patients compared to healthy controls has been reported previously[21,22]. By observing the intra-patient comparisons we could: (1) confirm these findings; and (2) demonstrate that in 80% of cases the lower cellular contractile capacities are an acquired feature in the POP prolapsed tissues.

In concordance with the trend of lower functional characteristics, we found that fibroblastic cells from the POP site secreted lower total MMP-2 than the non-POP site cells. Previous studies showed no differences on enzymatic activity on collagen coated plates between vaginal fibroblasts from women with prolapse; we suggest that this may be due to the low sample size[27]. Recently, our group reported delayed cell alignment and lower activation of MMP-2 by mechanical loading in uncoated silicone plates by severe POP fibroblasts[16]. Results were confirmed here as enzymatic activation on uncoated surfaces seemed lower in fibroblastic cells from the prolapsed site. Interestingly, after 48 hours of cyclic mechanical loading, MMP-2 and TIMP-2 gene expressions were found up-regulated only in POP-site fibroblastic cells. We speculate that this apparent discrepancy between gene expression and enzymatic activity may be due to the generally delayed mechanoresponses in POP fibroblastic cells. If so, mechanoresponsive genes in the non-POP fibroblastic cells might have reached plateau levels faster, *e.g.* at 24 hours, allowing synthesis and secretion of active enzyme to occur within 48 hours, with subsequent normalization of mRNA levels in the 24-48 hour time frame. In contrast, the reduced mechanosensitivity of POP fibroblastic cells might result in a delayed mRNA up-regulation and consequently the peak level of these genes might be reached after 48 hours with concomitant delayed secretion of MMP-2. Unfortunately, due to limited number of early passage primary cells, we were unable to verify this speculation.

The surface substrate also appeared to influence fibroblastic cells responses and cell-matrix interactions. Collagen-coating promotes cell attachment and alignment[16]. Coating also increased gene expression of the extracellular matrix remodelling factors: collagen 3α1, TIMP-2 and MMP-9, showing that vaginal fibroblastic cells are mechanoresponsive and can sense and remodel their surrounding matrix. The current study also indicates that collagen coating improves cell-substrate interactions *in vitro*.

What does this mean for our understanding of POP and the design of optimized treatment modalities? Clinically, development and progression of prolapse has been associated with conditions that overstretch the vaginal wall by excessive mechanical loading such as giving birth, chronic coughing or obesity[5,6,7]. Proper tissue remodelling, wound healing and repair are fundamental to maintain mechanical stability of the supportive tissues in the pelvic floor. If healing is frustrated, the vaginal tissue would not regain its strength and prolapse could eventually occur after continued excessive mechanical loading. In current clinical practice, increasing numbers and types of surgical

implants have been launched over the last decade to restore the biomechanical balance[28]. However, evidence of efficacy for these products is lacking and rates of complications as erosions, pain, infection and vaginal shrinkage are unacceptably high at 10% [29]. Our findings of delayed cellular mechanoresponses in POP fibroblastic cells suggest altered functional characteristics of fibroblasts from prolapsed tissues in women with POP. Thus, prolapsed fibroblastic cells may be unable to respond and restore ECM homeostasis, in the same manner as cells from non-prolapsed tissues to changes in the microenvironment. This may in part explain the high failure rate in native tissue repair. In the case of artificial polymeric meshes/substrates being used in vaginal reconstructive surgery, our data indicate that collagen-coating of meshes may improve treatment outcome. This is supported by recent findings that highly purified collagen coating enhances tissue integration of polypropylene meshes in rats[30].

Cell based tissue engineering strategies potentially provide attractive alternatives to current surgical reconstruction of POP[14]. Combining biomaterials with unaffected autologous cells, such as fibroblasts or stem cells, or even induced pluripotent stem cell (iPSC) lines from vaginal tissue in which the effects of age can be potentially erased[31], could stimulate vaginal tissue repair. These new approaches can only be implemented in patients with an acquired defect and not with a genetic condition. Since our data shows that fibroblastic cells from prolapsed tissues have altered functional characteristics compared to cells from non-prolapsed tissues within the same patient and that the cells derived from non-prolapsed tissues show similar functional characteristics to healthy controls, we conclude that for most patients the prolapse condition is an acquired rather than an intrinsic defect. This conclusion is in line with our recent findings on tissue samples from the same premenopausal women where we showed that the changes in the ECM composition of those tissues are an acquired defect in POP[15]. Therefore autologous cell-based therapies could be considered as alternative treatments for most cases.

Since prolapse affects both tissue components of the anterior vaginal wall – the extracellular matrix and the cells that are embedded within – development of new treatments for POP should be supported by research on disease-treatment-specific models, both *in vitro* and *in vivo*.

It is important to note that results from this study were obtained *in vitro* under controlled experimental set-ups, allowing sound conclusions to be drawn about specific parameters, but they may not completely reflect the *in vivo* situation. Nevertheless, our models provide valuable information about the influence of the prolapsed tissue in fibroblastic cells behaviour and the possible implications for current and future treatments for pelvic organ prolapse.

In conclusion, fibroblastic cells from the prolapsed anterior vaginal wall from POP patients show altered functional characteristics compared to non-POP sites and healthy cells. Such cellular alterations are acquired rather than intrinsic, and seem to be the effect rather than the cause of the disease. This information should be taken into account when improving treatments for pelvic organ prolapse.

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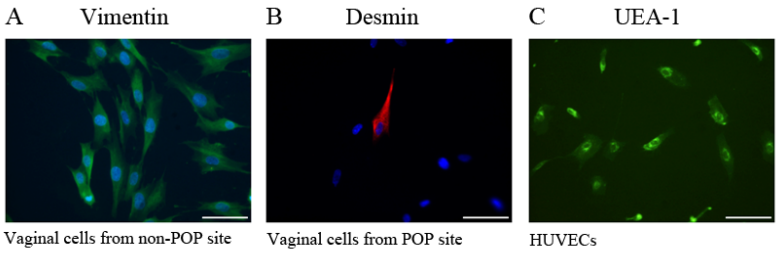
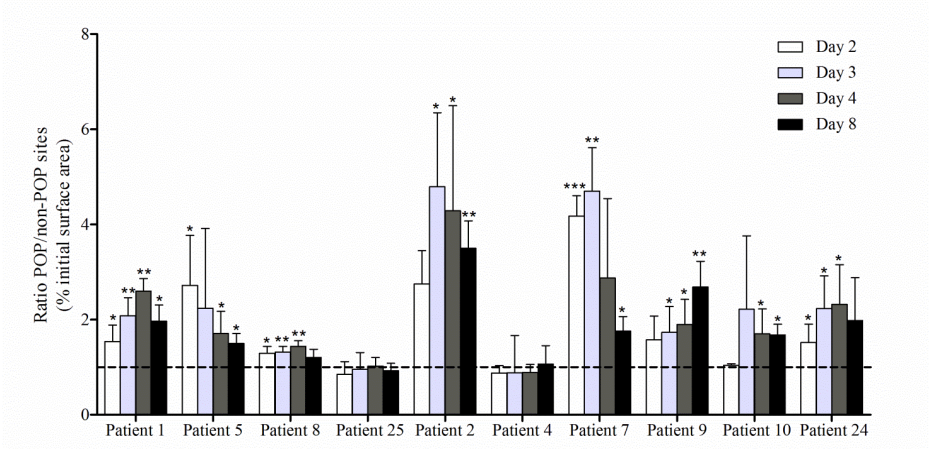
SUPPLEMENTARY DATA



Supplementary figure 1. Representative immunohistochemistry micrographs. The figure shows human primary cells stained for **(A)** vimentin which is a mesenchymal marker (green), **(B)** desmin which is a smooth muscle marker (red), and **(C)** Ulex Europaeus Agglutinin-I (UEA-I) which is an endothelial cell marker (green). Nuclei were stained with DAPI (blue). In our population of cells no positive UEA-1 cells were found, therefore figure **(C)** is showing the positive control: primary human umbilical vein endothelial cells (HUVECs). Images were acquired with the 10x objective of a Leica microscope, bar is 100µm.



Supplementary figure 2. The ratio between collagen I contraction by fibroblastic cells derived from prolapsed (POP) and non-prolapsed (non-POP) site from each case evaluated. Each bar represents the mean of five measurements ± SD. No differences = 1; POP site less contractile than non-POP site > 1; POP site more contractile than non-POP site < 1. *p<0.05, **p<0.01, ***p<0.001; by one-sample t-test compared to 1.

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