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 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.

Key words: pelvic organ prolapse / vaginal fibroblasts / contractility assay / cyclic mechanical loading / matrix remodelling factors

Introduction

Pelvic organ prolapse (POP) affects the quality of life of women worldwide, but remains a great therapeutic challenge (Bartoletti, 2006; Kerkhof et al., 2009; Word et al., 2009; Smith et al., 2010). Patients with POP may suffer from urinary and faecal incontinence, sexual dysfunction, chronic pelvic pain, vaginal relaxation and social isolation (DeLancey, 1993; Weber and Ritcher, 2005; Jelovsek et al., 2007). A recent systematic review showed that there is a strong genetic component in some women affected with POP (Lince et al., 2012), 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 (Kerkhof et al., 2009; Word et al., 2009; Levin et al., 2012).

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 (Hendrix et al., 2002; Lensen et al., 2013). 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 (Abramowitch et al., 2009), 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, ECM 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 (Deprest and Feola, 2013; Lensen et al., 2013).
New approaches that promote tissue regeneration are promising alter-
natives (Boenneylck et al., 2013), including autologous cell-based ther-
apies which would be feasible to treat only acquired (and not genetic)
diseases. Therefore, the aims of the present study are: (i) to evaluate
the contractile capacity of vaginal wall fibroblasts’; (ii) to evaluate
vaginal wall fibroblast mecanoresponses to two different substrates;
and (iii) to investigate the probability that alterations of fibroblast func-
tions 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 (Kerkhof et al., 2013). 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 an-
terior vaginal wall from women with cystocele are an acquired, rather
than an intrinsic defect in POP (Kerkhof et al., 2013). 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 capac-
ties 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. (2013). Exclusion criteria included a history of pelvic surgery or endo-
metriosis, the use of steroids or progestin-only hormone regimen, pelvic ma-
lignancy or connective tissue disease affecting tissue remodelling, adhesions
or scarring at the biopsy site, surgeons’ judgment that a biopsy may harm the
woman and it was shown that changes in the connective tissue of the an-
terior vaginal wall from women with cystocele are an acquired, rather
than an intrinsic defect in POP (Kerkhof et al., 2013). In this previous study, each POP patient
was used as her own control as biopsies were taken from the anterior
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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 capac-
ties and biomechanical responses, of cells derived from adjacent tissues
of the same patient cohort.

Table I Patient characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 8)</th>
<th>Case (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.13 ± 5.4</td>
<td>42.50 ± 6.3</td>
<td>0.360†</td>
</tr>
<tr>
<td>Parity</td>
<td>2.5 (1–3)</td>
<td>2 (1–4)</td>
<td>0.336‡</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.31 ± 5.43</td>
<td>27.86 ± 6.68</td>
<td>0.163‡</td>
</tr>
<tr>
<td>Smoking</td>
<td>Current (%)</td>
<td>3 (37.5%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td></td>
<td>Previous (%)</td>
<td>4 (50%)</td>
<td>5 (50%)</td>
</tr>
</tbody>
</table>

*Data are presented as: mean ± SD, median (range) or number of patients (%).
Non-parametric statistical tests: †Mann–Whitney; ‡Fisher’s exact.

Table II List of antibodies for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Kind</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-vimentin, clone V9</td>
<td>Primary</td>
<td>1:150</td>
<td>DakoCytomation, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Mouse anti-human clone D33</td>
<td>Primary</td>
<td>1:250</td>
<td>DakoCytomation, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Ulex Europaeus Agglutinin-I (UEA-1)</td>
<td>Primary</td>
<td>1:200</td>
<td>Vectro Laboratories, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Goat anti-mouse Alexa fluor 555</td>
<td>Secondary</td>
<td>1:800</td>
<td>Life technologies, Paisley, UK</td>
</tr>
<tr>
<td>Goat anti-mouse Alexa fluor 488</td>
<td>Secondary</td>
<td>1:800</td>
<td>Life technologies, Paisley, UK</td>
</tr>
</tbody>
</table>

Table II List of antibodies for immunohistochemistry.

<table>
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<td>Secondary</td>
<td>1:800</td>
<td>Life technologies, Paisley, UK</td>
</tr>
</tbody>
</table>

bovine serum (FBS, HyClone, South Logan, UT, USA), 100 μg/ml strepto-
mycin, 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 passage 2 or 3.

Immunohistochemistry

The primary cells were characterized by immunohistochemistry with the
markers for mesenchymal (vimentin), smooth muscle (desmin) and endothe-
llial cells (Ulex Europaeus Agglutinin-I, UEA-1). A list of antibodies and titra-
tions can be found in Table II. Cells were cultured in 96-well plates with a
density of 10 000 cells/cm² for 24 h and fixed with 4% v/v formaldehyde,
then incubated with blocking buffer [BB: 0.5% v/v BSA, 0.1% v/v
Triton-x-100, phosphate-buffered saline (PBS)] for 30 min followed by 1 h
incubation with the primary antibody at 4°C. After three washings with BB,
secondary antibody was added for 1 h, 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 per-
formed by counting cells in three independent wells using an inverted fluores-
cent microscope (Leica DMIL Microsystems, Wetzlar, Germany).

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/
cm², and tested in quadruple at different time points: 1, 2, 3, 4 and 7 days.
CyQuant cell proliferation assay kit (Molecular Probes Inc., Life Technolo-
gies) was used to evaluate proliferation rate by following manufacturers’
instructions. Fluorescence was measured using a micro-plate reader (Synergy™-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. (2010). 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 NaHCO3, 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 min at pH 7.4, 37°C and 5% CO2. 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. ImageJ 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 (Piechocka et al., 2011). For the measurement, 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/s). The shear modulus (G”) was calculated by the formula: |G”| = √(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 (Blaauboer et al., 2011). We recently reported that vaginal fibroblasts increased secretion of MMP-2 from 24 to 48 h, particularly when mechanically loaded (Ruz Zapata et al., 2013). Based on these results, in the present study we applied the loading regimen for 48 h. Fibroblastic cells were seeded at a density of 15 600 cells/cm2 on collagen I-coated or uncoated BioFlex® plates (BioFlex, Flexcell International Corp., McKeensport, PA, USA), and left to attach for 48 h 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 MMPs-2 and -9, thereafter cyclic mechanical loading (CML) 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™-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), MMPs-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 \( \sqrt{\text{Ywhaz} \times \text{HPRT}} \). 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).

**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 ImageJ (NIH). Values were calculated as follows: Total MMP-2 = inactive MMP-2 + active MMP-2; and percentage of active MMP-2 = (active MMP-2 × 100)/Total MMP-2.

**Secreted TIMP-2**

Since we found MMP-2 and not MMP-9 in our samples, we quantified released TIMP-2 because it inhibits MMP-2 (or collagenase IV) with a 1:1 stoichiometry (Goldberg et al., 1989). Secreted TIMP-2 was quantified using the TIMP-2 Human ELISA kit ab100653 (Abcam plc., Cambridge, UK) following the supplier’s instructions. Colour intensity was measured at 450 nm using Synergy™-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 h 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 (Meyer et al., 2008). Based on these data, eight 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 with 1 was used to compare: (i) 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...
Table III  Primer sequences used for RT–PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI67</td>
<td>Forward</td>
<td>5′ CCCTCAGCAAGCCTGAGAA 3′</td>
<td>57</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ AGAGGCGTATTAGGAGGCAAG 3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col 1α1</td>
<td>Forward</td>
<td>5′ TCCAACGAGATCGAGATCC 3′</td>
<td>57</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ AAGCCGAATTCTCTGTGCT 3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col 3α1</td>
<td>Forward</td>
<td>5′ GATCCGTCTCTGGATGACG 3′</td>
<td>56</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ AGTTCTGAGGACATGAGGG 3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Forward</td>
<td>5′ GGCAGTGCATCATCCTGACA 3′</td>
<td>56</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ AGGTGTGTAGCCAATGACTCT 3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward</td>
<td>5′ TGCACAGCGACAAGATG 3′</td>
<td>57</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ CGTGCTCAGGTTGAGG 3′</td>
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<td></td>
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<td>MMP-14</td>
<td>Forward</td>
<td>5′ CTGAGATCAAGGCAATGTTC 3′</td>
<td>56</td>
<td>206</td>
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<td></td>
<td>Reverse</td>
<td>5′ CTCAGGAGATGAGGAGGG 3′</td>
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<tr>
<td>TIMP-1</td>
<td>Forward</td>
<td>5′ CACAGCCGGCCTTCTGCAA 3′</td>
<td>63</td>
<td>211</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′ TGTGAGATCGGCTGAGG 3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Forward</td>
<td>5′ CTGACCACAGGTACGAGAT 3′</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′ TGGATGCTGCTGAGG 3′</td>
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<td>TIMP-3</td>
<td>Forward</td>
<td>5′ AGGACGCCCTCTGCATTCTG 3′</td>
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<td>163</td>
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<td></td>
<td>Reverse</td>
<td>5′ GCTTCCCTAGGTGATGTATC 3′</td>
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<td></td>
</tr>
<tr>
<td>Ywhaz</td>
<td>Forward</td>
<td>5′ GCTAGACCAGGCTGATTGCT 3′</td>
<td>56</td>
<td>229</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′ CTATTGTGGGACAGGTGATGG 3′</td>
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</tr>
<tr>
<td>HPRT</td>
<td>Forward</td>
<td>5′ GCTGACCTGCTTGATTATCAT 3′</td>
<td>56</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ CCTGGCGACCTTGACCACATCT 3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 data, Fig. S1). 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 was within the same range of the patient was lower than the non-POP site (Fig. 2B) in 80% of the cases studied (Supplementary data, Fig. S2). 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 h. 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). CML inhibited non-POP site from each case evaluated; and (ii) the effects of 48 h of 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).
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 ECM, MMP-2, -9 and 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 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, CML 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.

**Discussion**

In POP tissue strength is lost and quality of the ECM is compromised (Word et al., 2009; Kerkhof et al., 2013). Alterations in cells derived from prolapsed tissues have also been observed (Poncet et al., 2005; Meyer et al., 2008; Ruiz-Zapata et al., 2013). 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 CML. 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 (Ruiz-Zapata et al., 2013). In this pilot study, we found lower mechanoresponses in POP fibroblasts compared with 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 (Kerkhof et al., 2013), 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.
Table IV  ECM remodelling related genes on two different substrates after 48 h of CML.

<table>
<thead>
<tr>
<th>GEN</th>
<th>Control (n=8)</th>
<th>Case, POP site (n=10)</th>
<th>Case, non-POP site (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncoated</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>Collagen I + CML</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>MMP-1</td>
<td>0.95 ± 0.12</td>
<td>0.96 ± 0.14</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.78 ± 0.11</td>
<td>0.76 ± 0.11</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.13 ± 0.11</td>
<td>0.13 ± 0.11</td>
<td>0.13 ± 0.11</td>
</tr>
<tr>
<td>MMP-14</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.09</td>
</tr>
</tbody>
</table>

Each gene was normalized using the equation: Gene/×HPRT). The effect of CML versus CML (a, b). The effect of loading on collagen-coated silicone plates. Data are the mean ± SEM. Paired t-test; *P < 0.05; **P < 0.01; ***P < 0.001; the effect of loading on uncoated silicone plates seemed lower in fibroblastic cells from the prolapse 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. Mechanosponses to CML 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 (Tomasek et al., 2002; Hinz and Gabbiani, 2003; Li and Wang, 2011). 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 (Wipff and Hinz, 2009). A decreased fibroblast-mediated contraction of collagen gels by cells from the vaginal wall of severe POP patients compared with healthy controls has been reported previously (Poncet et al., 2005; Meyer et al., 2008). By observing the intra-patient comparisons we could: (i) confirm these findings; and (ii) 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 (Zong et al., 2010). Recently, our group reported delayed cell alignment and lower activation of MMP-2 by mechanical loading in uncoated silicone plates by severe POP fibroblasts (Ruiz-Zapata et al., 2013). Results were confirmed here as enzymatic activation on uncoated surfaces seemed lower in fibroblastic cells from the prolapsed site. Interestingly, after 48 h of CML, 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 h, allowing synthesis and secretion of active enzyme to occur within 48 h, with subsequent normalization of mRNA levels in the 24–48 h 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 h 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 cell responses and cell–matrix interactions. Collagen-coating promotes...
cell attachment and alignment (Ruiz-Zapata et al., 2013). Coating also increased gene expression of the ECM 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 (DeLancey, 1993; Weber and Ritcher, 2005; Jelovsek et al., 2007). 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 (Gupta-Rogo et al., 2012). 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% (Abed et al., 2011). 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 (Siniscalchi et al., 2013).

Cell-based tissue engineering strategies potentially provide attractive alternatives to current surgical reconstruction of POP (Boenneylcycke et al., 2013). Combining biomaterials with unaffected autologous cells, such as fibroblasts or stem cells, or even induced pluripotent stem cell lines from vaginal tissue in which the effects of age can be potentially erased (Wen et al., 2013), 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 with 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 (Kerkhof et al., 2013). 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 ECM 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 setups, 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 POP.

In conclusion, fibroblastic cells from the prolapsed anterior vaginal wall from POP patients show altered functional characteristics compared with 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 POP.

## Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

## Acknowledgements

Authors would like to thank the AMOLF Institute Amsterdam and Suzanne Detiger from the VU medical centre for assistance with the rheological measurements; Robert Szulcek from the VU medical centre for kindly donating primary human umbilical vein endothelial cells (HUVECs); and Alan Brind for proof reading the manuscript.

## Authors’ roles

A.M.R.-Z and M.H.K.: 1) Substantial contributions to conception and design; acquisition of data, analysis and interpretation of data. 2) Drafting the manuscript and revising it critically for important intellectual content. 3) Final approval. B.Z.-D. and M.N.H: 1) Substantial contributions to conception and design; acquisition of data, analysis and interpretation of data. 2) Revising the manuscript critically for important intellectual content. 3) Final approval. H.A.M.B. and T.H.S.: 1) Analysis and interpretation of data. 2) Revising the manuscript critically for important intellectual content. 3) Final approval.

## Funding

This work was supported by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (FES0908 to A.M.R.-Z.).

## Conflict of interest

None declared.

## References


