CHAPTER 7

Influences of extracellular matrices on myofibroblast differentiation in pelvic organ prolapse – a pilot study

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ABSTRACT

Pelvic organ prolapse (POP) is the weakening of the pelvic floor supportive tissues with subsequent prolapse of the pelvic organs outside the body. This common, multifactorial soft-tissue condition has no optimal treatment. POP affects tissue composition, tissue properties and cell behaviour, but little is known about the cell-matrix interactions in the pathophysiology of prolapse. Decellularized (DT) tissues have been successfully used as cell culture systems to study cell-matrix interactions in lung fibrosis. The aim of this pilot study was to evaluate the feasibility of using DT-matrices derived from vaginal tissues as cell culture systems to study cell-matrix interactions in pelvic organ prolapse. In particular, we investigated the effects of prolapse and non-prolapsed DT-matrices on vaginal fibroblast to myofibroblast differentiation in cells derived from prolapsed and non-prolapsed tissues. Fibroblasts survived and migrated into the exogenous DT-matrices. Control cells showed better attachment to the DT-matrices than POP cells. The stiffer muscularis layer of the DT-matrices induced myofibroblast differentiation of POP cells, but not of control fibroblasts. The fibroblast to myofibroblast differentiation of POP cells was higher in the prolapsed matrices. These preliminary results suggest that fibroblast to myofibroblast differentiation is different in cells from prolapsed tissues and dependent on the extracellular matrix encountered. This pilot study shows that decellularized vaginal wall matrices can be useful *in vitro* culture systems to study cell-matrix interactions in pelvic organ prolapse.

INTRODUCTION

Pelvic organ prolapse (POP) is the weakening of the pelvic floor supportive connective tissues with subsequent prolapse of the uterus, rectum, and/or most commonly, the bladder outside the body[1-3]. To date, his multifactorial soft-tissue disease has no optimal treatment and affects almost 50% of postmenopausal women world-wide[1,2]. Under normal conditions, the bladder is kept in place by the connective tissue layer of the anterior vaginal wall. Soft connective tissues are made of dense extracellular matrices rich in collagens, elastin and glycosaminoglycans (GAGs), and are typically maintained and remodelled by fibroblastic cells, *i.e.* fibroblasts and myofibroblasts. Fibroblastic cells respond to mechanical and biomechanical cues and keep tissue strength by maintaining homeostasis between tissue secretion and degradation. However, fibroblastic cells derived from prolapsed tissues are altered as they have been shown to have lower contractile capacities[4-6], altered mechanoresponses[6,7], lower responses to specific growth factors[8], and altered matrix production[9]. At tissue level, women suffering from prolapse have connective tissues with altered extracellular matrix composition[10-19] and altered mechanical properties with higher stiffness than their control counterparts[18,20-22]. Nevertheless, the effect of prolapsed tissues, matrix composition and mechanical properties on cell behaviour is still unclear.

To date, there is no adequate animal model to study the development of pelvic organ prolapse. The animal models that have been used to study prolapse are difficult to interpret since they are far from resembling human female anatomy, tissue biomechanics and soft tissue remodelling. *In vitro* studies using human primary cells have been useful to answer specific questions, but standard *in vitro* culture approaches are of unclear physiological relevance because they do not mimic the composition, mechanical properties or architecture of living tissues. Decellularized tissues would be a better physiological substrate than tissue culture plates because they retain native tissue proteins, architecture, and mechanical properties, and can be used as *in vitro* culture systems[23]. For this purpose, we adopted a cell culture system from Booth *et al.*[23], that uses decellularized soft tissue matrices to study cell-matrix interactions in lung fibrosis. The aim of this pilot study was to evaluate the feasibility of using DT-matrices derived from vaginal tissues as cell culture systems in order to study cell-matrix interactions in pelvic organ prolapse. In particular, we investigated the effects of prolapse and non-prolapsed DT-matrices on vaginal fibroblast to myofibroblast differentiation in cells derived from prolapsed and non-prolapsed tissues. To that end we measured the surface stiffness of the matrices with a micro-indentor and used alpha smooth muscle actin (α-SMA) as a myofibroblast marker, detected by western immunoblotting.

MATERIALS AND METHODS

Biopsy collection

Full thickness biopsies (> 1cm^2) were retrieved from four women: two patients with severe pelvic organ prolapse (with POP-Q stage IV), and two healthy controls who were operated for benign gynaecological reasons. For ethical reasons, the biopsy site from the POP patients was the prolapsed anterior vaginal wall whereas for the healthy controls this was the pericervical anterior vaginal wall. Tissues were collected in PBS at 4°C and decellularized within 24 hours. The tissues used in this study were collected from waste material after surgery and therefore not subject to Ethical Committee approval. Patient informed consent was obtained.

Decellularization of vaginal wall tissue biopsies

In this pilot study we decellularized matrices derived from the anterior vaginal wall of four donors: two without prolapse named 1.DT-Control and 2.DT-Control, and two with severe prolapse named 3.DT-POP and 4.DT-POP. We used a Triton x-100/sodium-deoxycholate decellularization treatment that was reported to maintain the mechanical properties of porcine descending aorta[24]. Briefly, tissues were incubated at 37ºC and constant agitation for 24 hours in 0.25% w/w Triton X-100 and 0.25% w/w sodium-deoxycholate (Sigma-Aldrich, St.Louis, MO, USA) in PBS, washed with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life technologies, Auckland, NZ) for 72 hours at 4ºC and treated with DNase I (150 IU/ml) with 50 mmol $MgCl_2$ (Sigma-Aldrich) in PBS for 24 hours at 37ºC. The samples were then incubated in DMEM (Gibco) for 24 hours at 4ºC, extensively washed with PBS and further incubated for 24 hours at 37ºC in 1% penicillin/streptomycin PBS (100 U/ml penicillin and 100µg/ml streptomycin; Sigma-Aldrich). Decellularized biopsies were kept in PBS at 4ºC until further use.

Hematoxylin and eosin staining

Hematoxylin and eosin staining (H&E) was performed in vaginal tissues and compared to decellularized biopsies to verify the decellularization process. Samples were snap frozen in Tissue-Tek (Sakura Finetek, Zoeterwoude, NL) in N_2 (l), cryosectioned in series of 10 µm on the sagittal plane and mounted on poly-lysine coated slides. Slides were then fixed for 10 minutes in 4% formaldehyde prior H&E staining. Images were acquired with a Leica DMRA microscope equipped with a DFC300FX camera and using QWinPro software (Leica Microsystems, Heidelberg, Germany).

Biochemical analysis

The amount of three extracellular matrix proteins – collagen, glycosaminoglycan (GAG) and elastin – were measured using biochemical assays in three samples of each decellularized vaginal tissue. To measure the amount of collagen and glycosaminoglycans (GAGs), samples were lyophilized overnight and then digested in papain solution at 60°C

for 16 hours[25]. Collagen amount was assessed by measuring the hydroxyproline amount (HYP) per dry weight using a modification of the assay protocol described by Huszar *et al.*[26], and with standard curves prepared from trans-4-hydroxyproline (Sigma, USA). The amount of chondroitin sulphate, the most abundant GAG in the body, was determined according to a modified protocol described earlier by Farndale *et al.*[27], and a standard curve prepared from chondroitin sulphate from shark cartilage (Sigma, USA)[25].

The elastin content was measured with Fastin Elastin Assay kit and following manufacturer's specifications (kit F2000; Biocolor, Carrickfergus, United Kingdom). Briefly, oxalic acid (0.25 M) was added to the wet samples and heated for 1 hour at 98°C. The supernatant was collected after centrifugation and the procedure with oxalic acid was repeated two more times to make sure all elastin was extracted from the sediment. The total elastin amount per wet weight was calculated based on the standard curve obtained from five concentrations of α-elastin.

Cell culture

Primary human anterior vaginal wall fibroblasts used by our group in previous studies from one woman with severe POP and one healthy control were isolated as previously described[7]. All the experiments were performed with cells from passage 5 cultured in DMEM (Gibco) supplemented with 1g/L glucose, 10% foetal calf serum (FBS; HyClone (South Logan, UT, USA), 1% penicillin/streptomycin (100 U/ml penicillin and 100µg/ml streptomycin; Sigma-Aldrich) and 1% fungizone (Gibco).

Preparation of decellularized (DT)-matrices for tissue culture

To keep the culture system as homogeneous as possible, we made cylinders of the decellularized vaginal tissues with a sterile 6-mm biopsy punch. Biopsies were snap frozen in Tissue-Tek (Sakura Finetek), and cut in series of 50 µm thick on the transversal plane starting from the epithelial layer. Matrices were mounted on poly-lysine coated slides (two samples per slide). Sections were dried for 1 hour at room temperature and washed with PBS, then placed in a petri dish, sterilised by a quick wash with 70% ethanol solution and kept at 4ºC in 1% penicillin/streptomycin PBS until further use. The anterior vaginal wall has different layers that we identified by histology in a previous study[19]. We use that data here as a reference to locate the different layers within the tissue from top to bottom: the epithelium is approximately the first 30 µm, the mucosal layer is approximately between 50 µm to 400 µm deep, and the muscularis and adventitia layers are deeper than 450 µm. All the experiments on the mucosal layers were performed on DT-matrices corresponding to depths between 100 µm to 250 µm. The experiments on the muscularis layers were performed on DT-matrices corresponding to depths between 500 µm to 1700 µm.

Reseeding DT-matrices

Fibroblasts were harvested and counted with the Muse® Count and Viability kit and the Muse® Cell Analyzer device (Merk Millipore, Darmstadt, Germany). Cells were re-

suspended in supplemented DMEM medium at a concentration of 1 x 10^7 cells/ml, seeded on top of the decellularized matrices (5 µl/matrix) and incubated for 30 minutes at 37ºC. 12 ml of extra media were then carefully added to the petri dish. Cells were cultured for 48 hours. Conditioned media were used to measure the toxicity of the DT-matrices on the cells with ToxiLight bioassay kit and following manufacturer's instructions (Lonza, Rockland, ME, USE). Samples were collected for live and dead staining, total DNA or western immunoblotting (see sections below).

Live and dead staining

The viability of the fibroblasts seeded on the DT-matrices was stained with the LIVE/DEAD® Viability/Cytotoxicity kit and following manufacturer's instructions (Molecular probes, Life technologies) and imaged with a Zeiss Apotome.2 microscope (Carl Zeiss, Oberkochen, Germany).

Total DNA

Samples from two DT-matrices were homogenized in 350 µl of Lysis-M buffer including proteinase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). After three freeze and thaw cycles, total DNA of duplicate samples was measured with the commercial CyQuant cell proliferation assay kit according to the manufacturer's specifications (Molecular Probes Inc., Life Technologies). Fluorescence was measured using the Synergy™HT multi-mode microplate reader (Biotek Instruments Inc.). Unseeded decellularized matrices were used as negative controls.

Surface micro-stiffness

The mechanical properties of the DT-matrices were measured using a newly-developed micro-indentor designed to test the surface stiffness of biological tissues (PIUMA, Optics11, Amsterdam, the Netherlands). Samples were indented in PBS with the PIUMA device, using a soft cantilever on the top of a ferruled optical fibre[28,29] with a radius of 95 µm and a stiffness of 0.5 N/m. The indentations were depth controlled (15 µm) and the loading and unloading periods were set to 3 s. Based on the load and displacement curves, the effective Young's modulus was automatically calculated by the software from the PIUMA (Optics11). To calibrate the probe and the measurement technique, a stiff surface was used before each series of tests.

Western immunobloting analysis

Fibroblasts cultured on two DT-matrices were homogenized in 350 µl of Lysis-M buffer including proteinase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). Equal volumes of samples were reduced with dithiothreitol (Roche), denatured for 5 min, separated by electrophoresis on a Bolt® 4-12% Bis-Tris Plus gel, and transferred to iBlot® PVDF membrane (Life Technologies). Blots were blocked for 1 hour at room temperature with a blocking buffer (PBS with 0.5% Tween-20 and 1% bovine serum albumin; Sigma-

Aldrich), then incubated with anti-α-SMA monoclonal antibody (clone 1A4, DakoCytomation, Copenhagen, Denmark) diluted 1:500 in blocking buffer for 1 hour at room temperature and overnight at 4°C, along with anti-tubulin polyclonal antibody rabbit anti-human (ab59680, Abcam plc, Cambridge, UK) diluted 1:4,000. After washing with PBS, blots were incubated for 45 minutes at room temperature with the secondary antibodies (1:10,000): IRDye- 680CW-conjugated goat anti-mouse IgG (H+L) highly cross-adsorbed (LI-COR; #926-32220) and IRDye- 800CW-conjugated goat anti-rabbit IgG (H+L) highly cross-adsorbed (LI-COR; #926-32211). Bound antibodies were visualized at 680-nm for α-SMA and 800-nm for tubulin using the LICOR OdysseyH scanner (LI-COR Biosciences, Nebraska, USA). Quantification of band densities was performed using Image J 1.44p software (NIH). Unseeded DT-matrices were used as negative controls and cells seeded on 24 well plates were used as positive controls.

Statistical analysis

Three independent experiments were performed in duplicate and data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using unpaired t-test test or one-sample t-test (Prism version 5.02, GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at 5% level ($p<0.05$).

RESULTS AND DISCUSSION

Biopsies from four women with and without pelvic organ prolapse (POP) were decellularized with a Triton x-100/sodium-deoxycholate treatment that has been shown to be most effective for tissue decellularization of the porcine aorta without decreasing the mechanical properties of the tissue[24]. Efficacy of the decellularization process was macroscopically observed by the white aspect of the tissues (Fig.1C) compared to fresh biopsies (Fig.1A), and confirmed microscopically by the absence of nuclei within the decellularized matrices as visualized by hematoxylin-eosin staining (Fig.1B and D).

Biochemical analysis showed that all the decellularized tissues had the three most characteristic extracellular matrix proteins: collagen (by hydroxyproline content), chondroitin sulphate – the most abundant glycosaminoglycan (GAG) in the body[30] – and elastin. In general, the decellularized tissues (DT) contained more collagen than GAG or elastin. The matrix composition was very different in the four tissues. Results showed that specimen 4.DT-POP had the highest content of collagen and GAG, whereas 1.DT-Control had the lowest content of collagen, GAG and elastin. The amount of GAG and elastin was similar in the two DT-controls, while the DT-POP samples showed a slightly higher content of GAG than elastin (Table 1).

Table 1. Biochemical analysis of extracellular matrix proteins from the decellularized tissues

Protein	1.DT-Control	2.DT-Control	3.DT-POP	4.DT-POP
Collagen (HYP)[a]	52.3 (± 7.67)	97.4 (± 3.16)	62.4 (± 9.38)	204.9 (± 31.99)
GAG[a]	1.38 (± 0.09)	3.40 (± 0.62)	3.10 (± 0.77)	4.33 (± 0.57)
Elastin[b]	1.78 (± 0.29)	3.047 (± 0.001)	2.66 (± 0.11)	1.82 (± 0.10)

HYP: hydroxyproline, and GAG: glycosaminoglycan. Data are in µg/mg of dry weight (a) or in µg/mg of wet weight (b), and are presented as: mean ± SD.

Fibroblasts seeded on exogenous DT-matrices survived 48 hours of culture (Fig.2A and D) and were able to migrate from the top to the bottom, and also from the centre to the periphery, of the DT-matrices (Fig.3 and supplementary movie). None of the DT-matrices was toxic to the cells as the levels of adenylate kinase released by cells seeded on exogenous DT-matrices were comparable to those released by the same cells seeded on plastic tissue culture plates (data not shown). Only a few dead cells were seen inside and towards the centre of the matrices (Fig.3 and supplementary movie). The amount of DNA from the POP fibroblasts was lower than that of the control cells, suggesting lower cell attachment to the DT-matrices (Fig.2B, C, E and F). Notably the amount of DNA of control cells found on DT-POP matrices was higher than the amount of DNA corresponding to the seeded cell density (600 ng; Fig.2E and F). These results indicate that proliferation of control cells on exogenous matrices is tissue-dependent, with DT-POP matrices inducing more proliferation than DT-Controls. POP fibroblasts did not seem to proliferate on the DT-matrices as the DNA detected was not higher than the initial amount of DNA (Fig.2C, E and F), and in 1.DT-Control it was significantly lower (Fig.2B). In our previous studies we have seen that the *in vitro* proliferation of cells derived from women with prolapse was comparable to control cells when grown on tissue culture plates[6,7]. Here we show that matrices derived from prolapsed and non-prolapsed tissues influence cell attachment and proliferation in a different way. These results emphasize the influence of the matrix on cell behaviour, and underline the added value of using natural DT-matrices instead of 2D plastic culture plates to study cell-matrix interactions.

Myofibroblast differentiation was evaluated by western immunoblotting of alpha smooth muscle actin (α-SMA). After 48 hours of culture we were able to detect α-SMA in all the cells that were studied (Fig.4 and Fig.5), except for the POP fibroblasts seeded on the mucosal layer of 1.DT-Control (Fig.4C). The protein expression of α-SMA in control fibroblasts is dependent on the substrate encountered by the cells, which seems to be donor- and tissue layer-dependent (Fig.4A, 4B and Fig.5). Fibroblast to myofibroblast differentiation of POP cells seems to be different from control cells. The levels of α-SMA protein detected in POP cells on the muscularis layer of DT-Controls were slightly lower compared to control cells (Fig.5A and B); and the opposite was found in DT-POP matrices (Fig.5C and D). The DT-POP matrices seem to promote myofibroblast differentiation in POP cells but not in control cells (Fig.5C and D).

The surface micro-stiffness of the DT-matrices was measured by micro-indentation. Results showed that the mean effective Young modulus, in three of the four tissues tested from the DT-matrices derived from the muscularis layers, was increased compared to the mucosal layers by: 55% in 1.DT-Control (Fig.6A), 32% in 2.DT-Control (Fig.6B), and 25% in 3.DT-POP (Fig.6C). The surface micro-stiffness of 4.DT-POP was similar in both tissue layers (Fig.6D). POP cells showed more α-SMA proteins on the stiffer muscularis layers than on the mucosal layers of the DT-Controls (Fig.4C and D). The control cells did not show this pattern as the stiffest DT-matrix (2.DT-Control muscularis; Fig.6C) induced the lowest myofibroblast differentiation after 48 hours of culture (Fig.4B).

Together these results showed that fibroblasts from women with prolapse had a phenotypical change towards myofibroblasts when exposed to stiffer matrices, particularly in DT-POP. Interestingly, this did not seem to be the case in control fibroblasts. Results should be confirmed in further studies, nevertheless from this pilot study we can deduce that fibroblast to myofibroblast differentiation seems to be altered in cells from prolapsed tissues and that this process depends on the extracellular matrix encountered by the cells.

Results from this study should be interpreted within their limitations. This is a pilot study with a very limited number of samples using an *in vitro* set-up to study cell-matrix interactions in pelvic organ prolapse. Since tissue samples from individuals inevitably vary in their composition, and cannot be controlled quantitatively, the composition of each sample should be determined and a larger number of samples will be required to statistically determine the effect of certain parameters on cell behaviour. We are currently in the process of expanding our sample size to be able to investigate in more detail the effect of specific parameters, such as matrix composition and mechanical properties, on cell behaviour. It is also important to realise that although DT-matrices resemble the native microenvironment of the cells more than tissue culture plates, the effect of the decellularization treatments on the tissues are unknown, so our system reflects some, but likely not all, *in vivo* parameters.

In spite of these limitations, the data presented above suggests that matrix composition of non-prolapsed tissues plays a more dominant role than matrix stiffness in controlling vaginal fibroblast to myofibroblast differentiation at 48 hours. That is in line with recent data from Sazonova *et al.*[31], who showed that matrix composition has a direct effect on vascular smooth muscle cells' mechano-transductions. The study indicated that specific matrix components nullified the effect of substrate mechanics, *e.g.* laminin, which suggests that different pathways could be involved in cellular responses to surface stiffness and could have a direct effect on cardiac pathologies[31]. Another possibility could be that prolapsed and non-prolapsed vaginal tissues differ in their composition or in their growth factor content. Moreover, cells derived from prolapsed tissues could have a lowered response to the myofibroblast differentiation-promoting cytokine transforming growth factor (TGF)-β, as has recently been suggested[8]. Last but not least, the apparent lack of cell attachment of the POP cells seen on DT-matrices suggests a possible role of integrins, which serve as mechanosensors that facilitate cell attachment to the proteins of the

extracellular matrix and also transmit the external forces to the cytoskeleton and the nucleus[32]. Alterations to these processes might partly explain the differences in mechanoresponses seen in POP cells.

Taken as a whole, the decellularized tissue culture system as presented here provides a more physiological microenvironment for the cells than tissue culture plates, enabling us to study cell-matrix interactions in a disease-specific manner. In follow-up studies it would be interesting to identify in more detail which pelvic floor matrix components, integrins, and/or growth factors within the ECM dictate the phenotype of the fibroblasts, and whether dynamic biomechanical conditions may influence the phenotypic transitions as well. Since fibroblast to myofibroblast differentiation seems to be altered in cells from prolapsed tissues, identification of different pathways that lead this differentiation could provide interesting insights into the pathogenesis of prolapse.

In summary, this pilot study shows that DT-matrices from vaginal tissues can be useful *in vitro* culture systems to study cell-matrix interactions in pelvic organ prolapse. In particular, we observed differences in fibroblast to myofibroblast differentiation between cells from control and prolapsed tissues on the different DT-matrices evaluated. Results should be confirmed in a larger study.

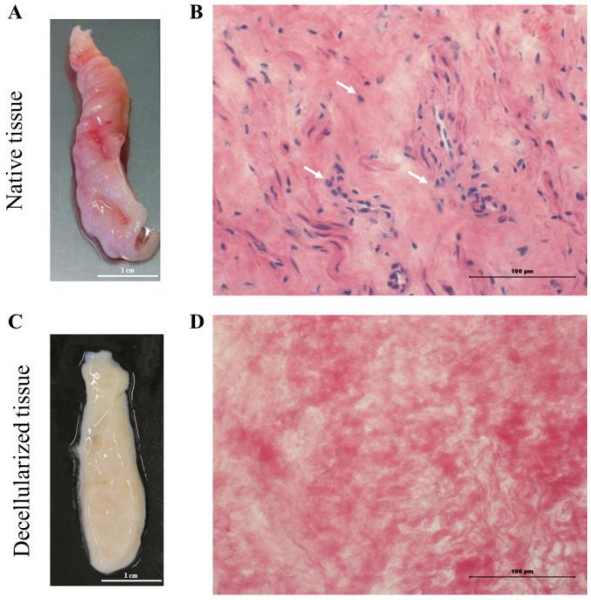

Figure 1. Anterior vaginal wall tissues were successfully decellularized. Biopsies from women with and without prolapse were decellularized with a Triton x-100/sodium-deoxycholate treatment. The figure shows representative photographs of biopsy samples before (A) and after decellularization process (C). Decellularization process was confirmed by hematoxylin-eosin staining of native (B) and decellularized tissues (D) where matrix is red and the nuclei are blue (white arrows). The white scale bars are 1 cm (A and C) and the black scale bars are 100 µm (B and C).

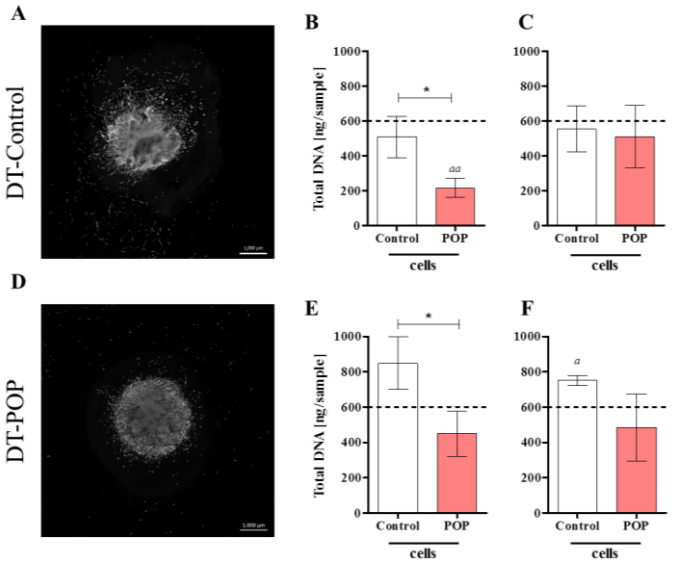

Figure 2. Vaginal fibroblasts were successfully reseeded on DT-matrices. Fibroblasts derived from control or from POP patients were seeded for 48 hours on exogenous decellularized (DT)-matrices from control (DT-control; A, B and C) or from patients with prolapse (DT-POP; D, E and F). Representative images of living cells seeded on DT-control (A) and DT-POP (D) that were stained with fluorescent calcein (white). The scale bar is 1000 µm. The amount of cells was quantified by measuring the total DNA of control or POP cells seeded on two DT-controls (B and C) and on two DT-POP (E and F). Data represents the mean ± SD (n=3). Dot lines correspond to the initial concentration of total DNA at the time of seeding (600 ng). Differences between control and POP cells seeded on each DT-matrix were detected by unpaired t-test (* $p < 0.05$). One-sample t-test compared to 600 ng (^{a} $p < 0.05$; ^{aa} $p < 0.01$).

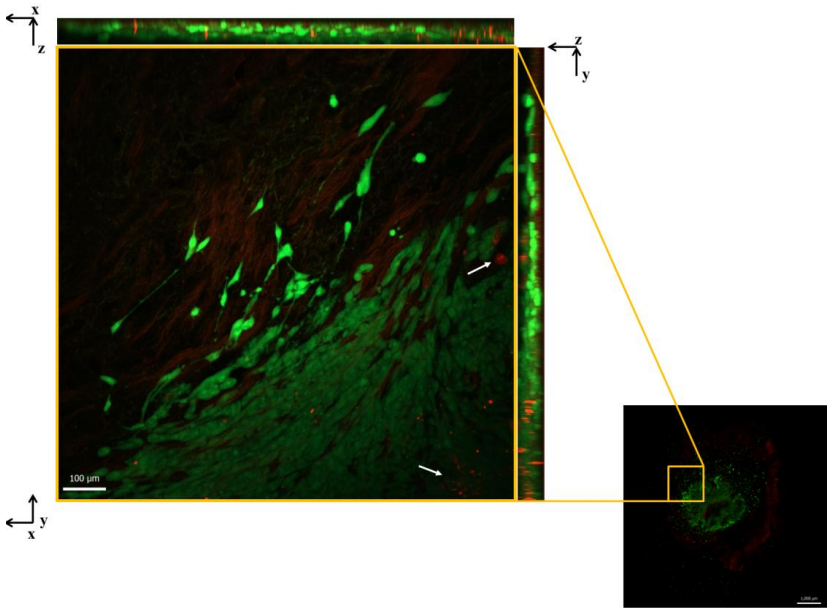

Figure 3. Vaginal fibroblasts migrated into the exogenous DT-matrices. A close-up of the living fibroblasts (green) show that they could migrate into the DT-matrix in the z-plane (as shown in the orthogonal views) and also from the centre to the outside of the matrix. Dead cells (red, white arrows) were mainly found inside and towards the centre of the DT-matrix. Images were acquired with Zeiss Apotome.2 microscope with an apotome optical sectioner. The left image is an insert of the image on the right and is a maximum intensity projection with the orthogonal sections on the sides. The scale bar of the insert is 100 µm and of the image on the right is 1 mm.

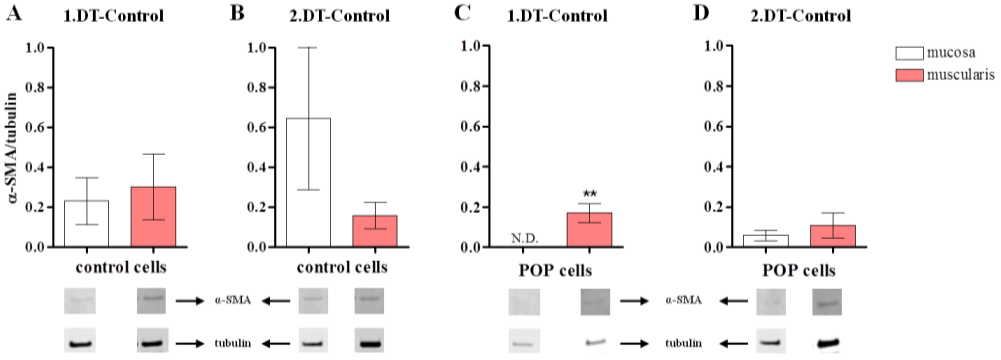

Figure 4. Fibroblast to myofibroblast differentiation of cells derived from prolapsed tissues was higher in muscularis than in the mucosal layer of DT-Controls. Cells were seeded for 48 hours on two DT-control matrices derived from the mucosal layer (depth: 100 µm to 250 µm) or the muscularis layer (depth: 800 µm to 1700 µm). Myofibroblast differentiation of control cells (A and B) and POP cells (C and D) was detected by western immunoblotting of α-smooth muscle actin (α-SMA). Blots were quantified by densitometry analysis of the bands and normalised to tubulin. Data represents the mean ± SD (n=3). In one of the samples α-SMA was not detectable (N.D.), so **$p < 0.01$ corresponds to a one-sample t-test compared to 0.

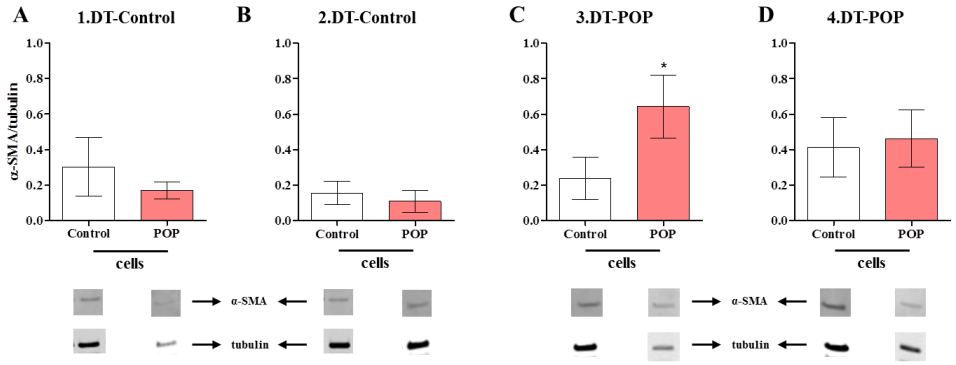

Figure 5. Muscularis layers of DT-POP matrices promoted fibroblast to myofibroblast differentiation of cells derived from prolapsed tissues. Cells derived from prolapsed (POP) and control tissues were seeded for 48 hours on DT-matrices derived from the muscularis layer (depth: 500 µm to 1700 µm) of two controls (DT-Control; A and B) and two prolapsed tissues (DT-POP; C and D). Myofibroblast differentiation was detected by western immunoblotting of α-smooth muscle actin (α-SMA). Blots were quantified by densitometry analysis of the bands and normalised to tubulin. Data represents the mean ± SD (n=3). * $p < 0.05$ by unpaired t-test.

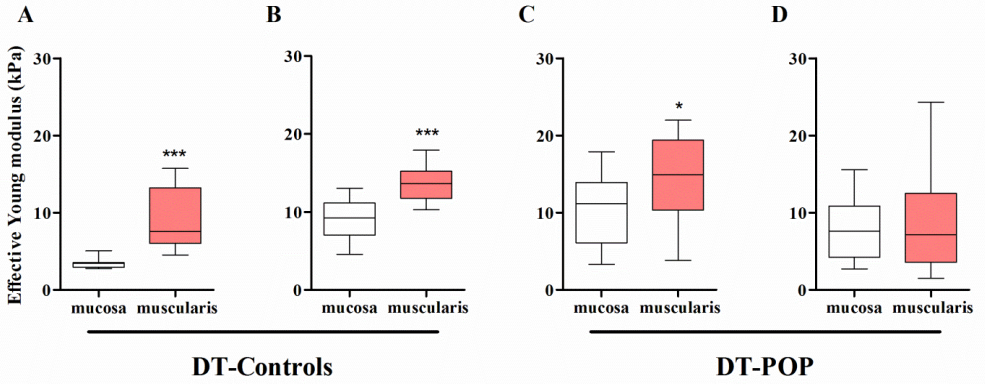

Figure 6. Surface stiffness of the DT-matrices prior tissue culture. The surface micro-stiffness of decellularized (DT) matrices from two controls (A: 1.DT-control and B: 2.DT-Control) or from two prolapsed patients (C: 3.DT-POP and D: 4.DT-POP) was measured with a PIUMA device under wet conditions. The matrices derived from the muscularis layer (depth: 1000 µm to 1500 µm) were stiffer than the mucosal layer (depth: 100 µm to 250 µm) in the two DT-controls (A and B) and in one DT-POP (C). The surface stiffness of the other DT-POP (D) was similar between the layers. The box plots correspond to the effective Young modulus of at least 16 measurements per DT. * $p < 0.05$ and *** $p < 0.0001$ by unpaired t-test.

ACKNOWLEDGEMENTS
The authors would like to thank Prof. Jan-Paul Roovers and Prof. Hans A.M. Brölmann for kindly providing tissue biopsies, Marion van Duin and Jolanda Hogervorst for technical assistance, Dr. Dimitra Micha for kindly donating the secondary antibodies, and Alan Brind for proof reading the manuscript. A.M. Ruiz-Zapata acknowledges financial support from a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908), and S. Ghazanfari acknowledges financial support from a ZonMW-VICI grant 918.11.635 (The Netherlands).

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