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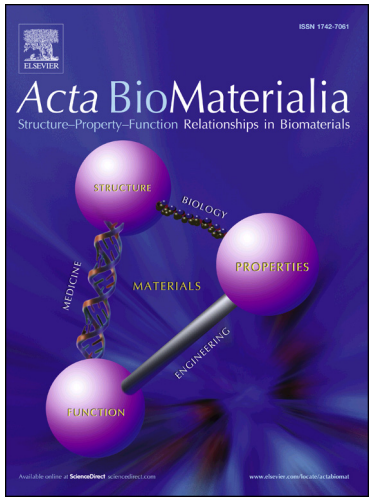

Full length article

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Gentle cyclic straining of human fibroblasts on electrospun scaffolds enhances their regenerative potential

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Abstract

The extracellular matrix of fascia-like tissues is a resilient network of collagenous fibers that withstand the forces of daily life. When overstretched, the matrix may tear, with serious consequences like pelvic organ prolapse (POP). Synthetic implants can provide mechanical support and evoke a host response that induces new matrix production, thus reinforcing the fascia. However, there is considerable risk of scar formation and tissue contraction which result in severe complications. Matrix producing fibroblasts are both mechanosensitive and contractile; their behavior depends on the implant's surface texture and mechanical straining. Here we investigate the effect of both in a newly-designed experimental setting.

Electrospun scaffolds of Nylon and PLGA/PCL and a non-porous PLGA/PCL film were clamped like a drumhead and seeded with fibroblasts of POP patients. Upon confluency, scaffolds were cyclically strained for 24 or 72h at 10% and 0.2Hz, mimicking gentle breathing. Non-loading condition was control. Strained fibroblasts loosened their actin-fibers, thereby preventing myofibroblastic differentiation. Mechanical loading upregulated genes involved in matrix synthesis (collagen I, III, V and elastin), matrix remodeling (α-SMA, TGF-β1, MMP-2) and inflammation (COX-2, TNF-α, IL8, IL1-β). Collagen genes were expressed earlier under mechanical loading and the ratio of I/III collagen increased. Matrix synthesis and remodeling were stronger on the electrospun scaffolds, while inflammation was more prominent on the non-porous film. Our findings indicate that mechanical straining enhances the regenerative potential of fibroblasts for the regeneration of fascia-type tissues and limit the risk of scar tissue formation. These effects are stronger on an electrospun texture.

Key words: fascia, fibroblasts, dynamic loading, electrospinning, pelvic organ prolapse

1. Introduction

Soft tissue injuries in the abdomino-pelvic disorders, such as pelvic organ prolapse (POP), are frequently associated with symptoms of pelvic floor dysfunction that reduce the quality of life. There is much evidence that mechanical loading plays a significant role in pathophysiology of such disorders, like obesity or excessive straining experienced during child delivery [1]. Injuries like these can rupture the fascia, a resilient collagenous network that mechanically supports and protects skin, muscles and organs. During the last two decades, physicians have attempted to improve the outcome of POP surgery by using synthetic knitted meshes that provide mechanical support for the organs in the pelvic cavity, while the damaged tissues regenerate and regain their strength. However, these meshes bear a considerable risk for serious, sometimes irreversible adverse events, like dyspareunia, pelvic pain, and exposure to the vagina [2][3][4].

Electrospun scaffolds have recently been suggested as alternative implants for fascia regeneration in general and reconstructive pelvic surgery in particular [5–7]. The reason is that the surface texture of the electrospun scaffolds provides a structural resemblance to natural extracellular matrix (ECM) to which cells better attach, proliferate and function [8]. Furthermore, electrospun scaffolds can provide good strength and biocompatibility [6][9]. Our group has recently shown that human vaginal fibroblasts from both prolapsed and non-prolapsed vaginal tissue are able to proliferate and deposit new collagenous matrix on electrospun scaffolds under static *in-vitro* conditions [10]. There is also an important role for mechanical stimuli, which may critically affect tissue remodeling during the wound healing process [11]. On the other hand, lack of mechanical straining, *e.g.* as a result of implanting a stiff mesh, disturbs the remodeling processes at the tissue-implant interface. Soft tissue erosion is an example of a complication that may arise due to the rigidities in the interfacial region [12,13]. It is suggested that mechanical stimulation improves cell behavior when

seeded on an electrospun scaffold [14]. Fibroblasts maintain ECM-remodeling of soft tissues in response to naturally-occurring forces in the pelvic floor like breathing [15]. Cells do this by producing ECM proteins such as collagen and elastin and by releasing matrix-degrading enzymes like matrix metalloproteinases (MMPs). However, the capacity of fibroblasts for modulating this process through their metabolic activities alters in pelvic floor disorders [16], and also in the presence of an implant [17]. Thus, both mechanical loading and surface structure of the implant influences the responses of host tissue cells once cells encounter the implant.

The aim of the study was to evaluate the effect of breath-mimicking strain on the behavior of POP-fibroblasts. Furthermore, we hypothesize that an electrospun biomimetic may enhance the mechano-biological response of fibroblasts because cells better adhere to such a surface. To investigate this, we designed a novel *in-vitro* cell culture system to stimulate POP-fibroblasts by a hammock-like straining simulating abdominal pressure. Cells are seeded on electrospun scaffolds of Nylon (non-degradable) and PLGA/PCL (degradable), or on non-porous casted films of PLGA/PCL. The materials were chosen for their clinical relevance, with Nylon as a permanent, non-degradable polymer suitable for older, post-menopausal women with limited regenerative capacity, and PGLA/PLC as a resorbable scaffold for the younger, pre-menopausal patient who may have sufficient capacity for fascia regeneration. We subjected the cell-seeded scaffolds to static or cyclic loading for 24 or 72 hours (see experimental design in figure 1). We characterized cells for their morphology and regenerative capacity by analyzing genes involved in i) cell proliferation, ii) ECM-synthesis, iii) ECM-remodeling, and iv) inflammation.

2. Materials and Methods

2.1. Preparation of the scaffolds

75:25 PLGA/PCL (Purac, Netherlands) was dissolved in chloroform (15% w/v) and medical grade Nylon polyamide 12 ($[HN(CH_2)_{11}CO]_n$, Sigma, Netherlands) was prepared in formic acid. Scaffolds were produced in an electrospinning device (IME Technologies, The Netherlands) at room temperature using a grounded static collector and a syringe pump (Harvard apparatus, PHD 2000, USA). Non-porous films were casted from the PLGA/PCL solution overnight. The grounded collector was placed horizontally at a distance of 15cm from the needle. With a syringe pump (Harvard apparatus, PHD 2000, USA) 0.8 ml of each polymer solution was extruded at flow rates between 0.5 and 1 ml/h, under an applied voltage of 20 kV. Fibers were collected on aluminum foil at room temperature and humidity. Circular samples with 2.5 cm in diameter were vacuumed overnight to remove the residual solvents. At this point, all of the scaffolds were clamped in between two sample clamps rings using pre-designed screws (fig.2, C-E schematically shows how the samples were clamped like a drumhead using the screw system). The sample clamps were made of PEEK and designed with screws to increase the tightening of the scaffolds (fig.2, C-E). After being clamped, scaffolds were disinfected by complete immersion in two rinsing steps of 70% ethanol, and overnight incubation in 1% antimicrobial culture medium at 37°C.

2.2. Cell isolation and pre-culturing on scaffolds

Tissue collection was approved by the medical ethical committees of VU University Medical Centre (Amsterdam) and Kennemer Gasthuis Hospital (Haarlem). Full-thickness (1 cm^2) biopsies of the anterior vaginal wall were taken from a patient undergoing reconstructive surgery of the anterior vaginal compartment. The patient was in her menopause and had a

cystocele stage 2 (POP_Q classification). Within 24 h, cells were isolated and cultured as described previously [15]. Prior to the experiments, cells were grown until passage 3 in an incubator at 37 ºC, 95% humidity and 5% CO2, with culture medium: DMEM supplemented with 10% FBS, 100mg/ml streptomycin, 100 U/ml penicillin, and 250mg/ml amphotericin-B. Cells were counted and their viability was determined using a Count and Viability assay and a Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany). Cells were cultured with 10%-culture medium on all clamped scaffolds (n=24 for each scaffold type) at a density of 150,000 cells/cm^2 for 3 days until confluency.

2.3. Dynamic multiaxial mechanical loading

The mechanical loading experiment was performed with a custom-made instrument (fig.2, A) capable of generating sinusoidal displacements with a resolution of 0.01 mm at rates between 0.01-30 Hz as described previously [18]. The instrument consists of six culture chambers with stainless-steel indenters with a diameter of 6 mm. Culture chambers and indenters were autoclaved prior to the experiment. The cell-seeded scaffolds (total n=72) were clamped upside down (cells below) like a drumhead (fig.2, C-E), put inside the chambers and filled with culture medium. The indenters were brought into contact with the scaffold's surface (fig.2, B) and fixed. Indenter displacement was controlled by a custom-made software (implemented in LabVIEW 8.2, National Instruments, Austin TX). Figure 1 shows a scheme of our study design. After three days of pre-culture with POP-cells, samples of each scaffold group (n=24) were subjected to a cyclic strain dynamic of 10% at a frequency of 0.2 Hz (n=12), or left unloaded (static control, n=12). Samples were harvested after 24h to observe the immediate effects of dynamic strain (n=6) or after 72h to evaluate the effect of continuous straining (n=6). A strain magnitude of 10% and a frequency of 0.2 Hz were chosen to mimic continuous respiration as reported previously [19].

2.4. Cell morphology

Cell morphology and adhesion were qualitatively evaluated by scanning electron microscopy (SEM) after 72h. Cell-seeded samples were fixed in 4% formaldehyde (pH 7.2) and underwent serial dehydration with ethanol and then sputter-coated with gold. Several magnification pictures were obtained from random spots on each sample.

2.5. F-actin staining

Cell alignment and attachment was qualitatively evaluated by F-actin staining after 72h. Cells were fixed with 4%formaldehyde and stained with Alexa Fluor 488 phalloidin (Molecular Probes, Leiden, The Netherlands). We used an inverted Leica DMIL microscope (Leica Microsystems, Germany) for evaluation.

2.6. Gene expression analysis

For gene expression analysis, cell-seeded samples were washed with PBS, and the total RNA was extracted using TRIzol (Gibco) according to the manufacturer's protocol to a final concentration of 250 ng/ml. Extracted RNA was reverse transcribed to complementary DNA (cDNA) using Fermentas synthesis kit (Life Technologies, USA), following the manufacturer's protocol. Gene expression of KI-67, COL1A1, Col3A, COL5A1, elastin, α-SMA, TGF-β1, COX2, IL1-β, IL-8, TNF-α and MMP-2 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 1 (Invitrogen, Thermos Fisher), with the SYBR Green Reaction Kit following suppliers' specifications (Invitrogen, Thermo Fisher) and measured by reverse transcriptase polymerase chain reaction (RT–PCR) in a Light Cycler 480 device (Roche, Germany). Gene expression levels were normalized

using a factor derived from the equation √(Ywhaz × 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).

2.7. Statistical analysis

Three independent experiments were performed in duplicate (n=6) for each of the scaffold types and culture times. Data are expressed as mean ± standard error of the mean (SEM) or shown in graphs as mean ±standard deviation (SD). Statistical analysis was performed using Graphpad (Prism version 5.02, GraphPad Software Inc., La Jolla, CA, USA). Unpaired *t-test* was used to identify the differences between static versus dynamic values, and between 24h versus 72h values with a level of significance at $p < 0.05$. One-way analysis of variance (ANOVA) followed by Tukey–Kramer's *post hoc* test was used to identify statistical differences between three scaffolds, with a level of significance at $p < 0.001$.

3. Results

3.1. Characteristics of scaffolds

The scaffolds were previously characterized for structural (fiber size and porosity) and mechanical properties including [9][6]; thickness, fiber size, pore size and uniaxial tensile stiffness and strength. Table 2 provides an overview of these properties. Nylon had thinner fibers (0.1μm) and smaller pores (1.3 μm^2) than those of PLGA/PCL (with values of about 1 μm and 8.8 μm^2, respectively). The mechanical properties of the electrospun scaffolds were similar and were about 2.5 times stronger and 20 times stiffer than the non-porous film (Table 2).

3.2. Cell morphology

Under gentle cyclic loading the cells remained intact and well attached to the scaffolds (fig.3). However, cyclic loading disturbed the actin-mediated cell alignment. Therefore, protein bundles and myofibroblastic-like (elongated instead of spindle-shaped) cells were observed on Nylon and film under static conditions, but not under dynamic loading (figure 3).

3.3. F-actin staining

F-actin staining (fig.4) confirms the outcome results of the SEM experiment. Actin fibers are more vividly active and similarly aligned under static condition, while cyclic loading disturbed the configuration of fibers in all samples. Actin fibers are irregular and unaligned under dynamic conditions.

3.4. Gene expression analysis

The expression of mRNA was affected by mechanical loading (fig.5) in most of the genes monitored and the effect was more significant in the first 24h. On Nylon, the cells were slightly more mechano-biologically responsive than on PLGA/PCL and the non-porous film. Expression of inflammatory mediator genes had a decreasing trend on electrospun PLGA/PCL from 24h to 72h, while it increased on non-porous film.

KI-67, a gene associated with cell proliferation, increased over time in all samples, with no significant difference between the static or dynamic condition. Matrix synthesis genes (Col I, III and V, elastin) generally upregulated over time, and faster in the first 24h. Collagen I/III ratio increased under mechanical loading conditions, particularly at 24h on the electrospun scaffolds. Elastin showed a low gene expression on all conditions. Mechanical loading also increased the relative expression of genes associated with matrix remodeling (MMP-2, TGF-β1 and α-SMA), particularly after the first 24h. Furthermore, mechanical loading generally

increased the expression level of inflammatory mediator genes (TNF-α, IL1- β, IL-8 and COX-2) over time. The expression increased on the non-porous film and electrospun Nylon, but the expression was comparatively lower on electrospun PLGA/PCL. Statistical differences between the time point values (24h versus 72h) and the scaffolds are represented in supplementary data in suppl.fig.1-3.

4. Discussion

Mechano-biological events that occur at the tissue-implant interface affect tissue integration and long-term clinical outcomes of an implant in reconstructive surgery [2,20]. The implant surface should be gentle to cells and have elastic moduli similar to the adjacent soft tissues, to avoid high interfacial shear stresses and the formation of fibrous tissue and thus prevention of proper integration [13,19,21]. Electrospun scaffolds allow adhesion of host cells and their ECM synthesis; therefore, they can be potential alternatives for vaginal polypropylene meshes [7][8][6]. In the current study, we designed an *in-vitro* model to evaluate the effect of gentle cyclic straining on the behavior of human vaginal fibroblasts on electrospun scaffolds. Gentle loading reduces the myofibroblastic differentiation (the cells responsible for scar tissue formation and contraction) and at the same time enhances the expression level of matrix synthesis and remodeling genes. We further observe that an electrospun surface texture may contribute to a beneficial mechano-responsive behavior.

POP-cells exhibited an elongated shape (myofibroblastic morphology) on both electrospun scaffolds and non-porous film under static conditions. Mechanical loading disturbed actin-mediated stretching, which indicates that mechanical loading interferes with myofibroblastic differentiation. This result is in agreement with the observations by Blaauboer *et al.*[22] who showed that dynamic mechanical loading prevents myofibroblastic differentiation of lung

fibroblasts. Actin fibers are cellular mechanical tools to respond to mechanical tensions [23], and we qualitatively show here that they are disturbed under mechanical loading. One reason might be a lack of collagen; mature collagen (which has proper alignment), can dictate cellular alignments [24], while in the dynamic condition of this study, collagen is not yet present on the scaffolds (or is very immature). It may also be that fibroblasts attachment is frustrated under cyclic loading: cells adhere to their surface prior to loading in the pre-culture period but they become disoriented after loading. As the actin cytoskeleton requires cellular tension, cells cannot find the proper counterpart to create tension and thus disorient. Prolonged experiments are required to address actin-mediate adhesion of cells under such loading conditions.

The mechano-responsive genes are mainly categorized into three types: ECM-synthesis, ECM-remodeling and inflammation [25]. Our results showed that mechanical loading upregulates all of these genes with some major findings. First, consistent with previous studies, our results showed that the enhancing effect of loading is more in the first 24h and the effect reduces after 72h [26]. When cells presumably enter matrix remodeling pathways, they release enzymes like matrix metalloproteinases that can lower their collagen production rate [27]; thus the expression is higher in the first 24h. Second, matrix synthesis genes (particularly collagen I and III which are relevant for fascia remodeling;[28] are significantly upregulated under dynamic loading, while cell proliferation was not affected as much. This implies that mechanical loading enhances the capacity of cells for regulation of their collagen genes, and the results are not a mere cause of increase in cell number. Elastin, another fascia-relevant gene [28], expressed low without significant increase over time. The reason may be that elastin development starts at later stages of remodeling and it also depends on strain magnitude, frequency and duration [29], so our study was too short-time for a proper response. The collagen I/III ratio increased by mechanical stimulation. This ratio is important

for the load-bearing characteristics of connective tissues, as it is shown to decrease in e.g. hernia patients [30]. Together, the results suggest that the mechanical loading enhances the ability of cells in regulating their matrix synthesis genes.

Our third finding highlights the simultaneous upregulation of the genes during experiment, and depicts an ongoing remodeling, as well as the mutual effects of genes on one another. For instance, three genes upregulated at the first 24h of loading which influence each other subsequently; α-SMA, collagen and TGF-β1. α-SMA is a precursor for collagen synthesis and cells produce collagen when differentiating to myofibroblasts (characterized by α-SMA) [29]. On the other hand, TGF-β1 is a growth factor required for both myofibroblast differentiation and collagen production [31]. Under mechanical loading, fibroblasts secrete TGF-β1 directly into the matrix which induces collagen synthesis and maturation [32]. Together, this may explain that all these genes upregulate at first 24h to induce/initiate matrix deposition, and their expression slows down after 72h. After 72h, the matrix enters a new pathway to sustain its turn-over and preventing from accumulation and scar formation; cells activate their genes for matrix-degrading enzymes (like MMPs) and release them into the matrix, while downregulating their α-SMA gene [32]. This sustaining pathway is particularly important in wound healing, because an excessive production of collagen can lead to accumulation and stiffening of the matrix [31], which creates problems such as implant contraction and pain. Thus, a gentle straining is required for the cells on a scaffold, that induces cells to regulate their new matrix, but at the same time prevents the abovementioned adverse events. We showed here that gentle cyclic mechanical loading of cells on the electrospun scaffolds, is beneficial because it reduces myofibroblastic differentiation, while it enhances the matrix-regulating of cells.

Our fourth finding regards the expression of inflammatory markers. Inflammation is a key aspect in wound healing after implantation of a biomaterial [33], because cells produce

catabolic markers and inflammatory mediators to identify a foreign material and to attract cells to break down damaged tissues and stimulate vascularization for repair [34]. In our study, catabolic genes generally upregulated under loading, except for TNF-α gene which was expressed very low and thus was not conclusive. Simultaneous upregulation of IL1-β and MMP-2 under cyclic loading are commensurate with previous findings that these two genes have mutual effects on one another, because inflammatory cytokines such as IL1-β upregulate MMPs in the presence of mechanical loading [32,35]. The simultaneous upregulation of COX-2 also suggests that the release of IL1-β under mechanical stimulation may be a synergetic trigger on matrix remodeling/destruction via COX-2 expression [32]. Supp.fig3 is an overview of gene expression over time for the three scaffolds. It is well appreciated that inflammatory markers play an important role in regulating the foreign body reactions and the healing process in-vivo, as long as their expression does not exceed the necessary level [36]. In scar tissue formation, the proper balance between inflammatory markers and synthesis of ECM is disturbed and excessive amount of matrix is produced which leads to scar tissue formation, and this also stimulates further inflammation. The exact moment and level when the inflammatory responses should slow down to let a proper remodeling occur is not known. But it is known that such response is required to some extent (specially at the beginning) for the mesh not to be encapsulated [37][38]. Thus, upregulation of these markers is not necessarily considered as negative, but a more quantitative studu of the inflammatory reactions is required to determine what level of response is healthy and what level leads to scar formation.

Finally, we observed that cells expressed relatively higher levels of mechano-responsive genes on electrospun scaffolds than on non-porous film, except for the inflammatory markers which expressed more strongly on the non-porous film. Cellular mechano-responsiveness is based on the ability of their focal adhesion proteins to maintain attachment to a surface [36].

An electrospun texture improves cell adhesion (because it modulates the focal adhesions) and thereby enhances cells mechano-responsive behavior. Differences in surface characteristics of the scaffolds also plays a role in activating different genes. The electrospun PLGA/PCL fibers are larger than Nylon fibers, allowing the ingrowth of cells and matrix in the former, but less so in the latter [6]. On the other hand, electrospun Nylon has stiffer surface than PLGA/PCL [10]. This way, surface texture may induce different levels of inflammatory pathways. Our observations suggest an interesting effect of surface topography, which should be studied in more systematic long-term experiments where cell infiltration occurs and becomes a factor. Also, other topographies of the scaffold, like alignment of the fibers, remain to be addressed in future studies.

This study shows that POP-cells respond to cyclic mechanical loading and that the behavior depends on the scaffold's surface texture as well as on the particular loading condition, as shown by others before [7,13,39,40]. To the best of our knowledge, this is the first study to use electrospun scaffolds in combination with mechanical loading in a hammock-like, bi-directional stretch model instead of uniaxial tension; our model thereby better mimics the normal breathing strains occurring in the pelvic floor [15,22]. There is one recent study that showed upregulation of elastin by cells cultured on an eletrospun balloon-model [41] and revealed rather similar outcomes compared to ours. The set-up can also be used to study cell-implant interactions in other load-bearing tissues, like pulse-like abdominal pressure peaks mimicking coughing or laughing. Such experiments give a more quantitative understanding of the effect of mechanical loading on cell-matrix behavior and can also be performed to pre-clinically evaluate new implants. We need to find an optimum regime of mechanical loading that is essential for cells function without creating fibrotic tissue.

A limitation of the present study is the rather low number of samples tested per condition and culture time (n=6); this resulted in quite some non-significant differences in the qPCR

expression studies. Nevertheless, we did find significant differences, especially between static and dynamic loading and also between 24 and 72 hours of loading time. Further, we found differences in morphology by SEM and F-actin staining (Figures 3 and 4), which were assessed qualitatively rather than quantitatively. With the results of the current study, we can calculate the appropriate statistical power for future studies.

The cells used for this study were expanded from one single patient. Naturally, there is large variation in cell behavior within and between individuals. However, we were interested in the effect of mechanical straining and surface texture, and therefore kept the other parameters of the study, in particular cell source, constant. We do not expect, however, that cells from another location or individual would behave essentially different, because similar effects of gentle mechanical loading on cell behavior in general and the cytoskeleton in particular, have been observed before [39].

A further limitation is the relatively short follow-up time, which only allows for gene profiling but much less for protein expression, which only occurs in substantial amounts after several weeks of culturing. Still, morphological outcomes and differences in gene expression are indicative of the strong effect of dynamic strain and scaffold properties on cell behavior. This remains a limitation of current study, which needs to be addressed in long-term experiments in future.

We disinfected the samples in this study by two rinsing steps of 70% ethanol and overnight incubation in 1% antimicrobial culture medium at 37°C. This is a routine way of disinfecting scaffolds in laboratories, because it is effective and relatively easy and cheap. However, it is different from the sterilization of polymer implants for clinical usage, which includes gamma-radiation, e-beam, and gas-sterilization. One may speculate about the effect of treatment on the mechanical and surface properties and on the behavior of the cells.

Radiation, for example is known to affect molecular weight of the polymers and thereby mechanical properties and degradation kinetics [42]. Furthermore, radiation and gas-sterilization affect surface properties and thereby the attachment and behavior of cells [43]. We do not exclude that sterilization effects are also relevant for electrospun polymers, but in the current study we were just interested in the effect of mechanical loading and the structure of the scaffolds.

The experiments described are, of course, a simple model for what might be relevant for the *in vivo* situation. The advantage of our experimental set-up is that we are able to control the mechanical and biochemical environment and thus systematically study cell behavior under well-controlled conditions. This way, we can conclude that gentle cyclic loading is beneficial for tissue regeneration and that POP fibroblasts have less chance of differentiation into myofibroblasts. This may help in the definition of the proper stiffness of a supportive scaffold for pelvic floor repair: it should be stiff enough to provide mechanical support, but not so stiff that cells do not sense any straining. Obviously, the conditions investigated here do not entirely reflect the mechanical complexities of the pelvic floor during the activities of daily life (in particular the peak pressures that occur during laughing, coughing, obstipation, etc). One also may consider the complexities of different types of tissues and cells and the heterogeneities in anatomy and chemistry. These may be investigated using other specific experimental conditions, which will be subject of future studies.

Conclusion:

Cells change their morphological characteristics such as actin-fibers expression in response to mechanical stimuli. Measured at gene level, matrix synthesis and remodeling (in particular collagen) started earlier and significantly enhanced by mechanical loading. Inflammatory

mediator markers upregulated by loading, and expressed at a higher level on non-porous films. Our findings suggest that dynamic conditions significantly enhance the behavior of the POP-cells on scaffolds in terms of adhesion, morphology and expression of their measured mechano-responsive genes. Both parameters should be considered in mesh-based prolapse surgery. Electrospun scaffolds thereby appear to provide attachment for the cells and thus higher mechano-sensitivity.

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[1] I. Milsom, D. Altman, M.C. Lapitan, R. Nelson, U. Sillén, D. Thom, Epidemiology of Urinary (UI) and Faecal (FI) Incontinence and Pelvic Organ Prolapse (POP), Ics. (2009) 35–112.

[2] a L. Olsen, V.J. Smith, J.O. Bergstrom, J.C. Colling, a L. Clark, Epidemiology of surgically managed pelvic organ prolapsed and urinary incontinence, Obs. Gynecol. 89 (1997) 501–506.

[3] D. Lee, J. Chang, P.E. Zimmern, Iatrogenic Pelvic Pain: Surgical and Mesh Complications, Phys. Med. Rehabil. Clin. N. Am. (2017). doi:10.1016/j.pmr.2017.03.010.

[4] S. Todros, P.G. Pavan, A.N. Natali, Biomechanical properties of synthetic surgical meshes for pelvic prolapse repair, J. Mech. Behav. Biomed. Mater. (2016). doi:10.1016/j.jmbbm.2015.10.024.

[5] S. Roman, N. Mangir, J. Bissoli, C.R. Chapple, S. Macneil, Biodegradable scaffolds designed to mimic fascia-like properties for the treatment of pelvic organ prolapse and stress urinary incontinence, 30 (2016) 1578–1588. doi:10.1177/0885328216633373.

[6] M. Vashaghian, B. Zandieh, J.P. Roovers, T.H. Smit, Electrospun matrices for pelvic floor repair: effect of fiber diameter on mechanical properties and cell behavior, Tissue Eng. Part A. (2016) ten.TEA.2016.0194. doi:10.1089/ten.TEA.2016.0194.

[7] S. Roman Regueros, M. Albersen, S. Manodoro, S. Zia, N.I. Osman, A.J. Bullock, C.R. Chapple, J. Deprest, S. MacNeil, Acute in vivo response to an alternative implant for urogynecology., Biomed Res. Int. 2014 (2014) 853610. doi:10.1155/2014/853610.

[8] M. Vashaghian, S.J. Zaat, T.H. Smit, J.P. Roovers, Biomimetic implants for pelvic floor repair, Neurourol. Urodyn. 37 (2018). doi:10.1002/nau.23367.

[9] M. Vashaghian, A.M. Ruiz-Zapata, M.H. Kerkhof, B. Zandieh-Doulabi, A. Werner, J.P. Roovers, T.H. Smit, Toward a new generation of pelvic floor implants with electrospun nanofibrous matrices: A feasibility study, Neurourol. Urodyn. 36 (2017). doi:10.1002/nau.22969.

[10] M. Vashaghian, A.M. Ruiz-Zapata, M.H. Kerkhof, B. Zandieh-Doulabi, A. Werner, J.P. Roovers, T.H. Smit, Toward a new generation of pelvic floor implants with electrospun nanofibrous matrices: A feasibility study, Neurourol. Urodyn. (2017). doi:10.1002/nau.22969.

[11] M. Xie, L. Song, J. Wang, S. Fan, Y. Zhang, Y. Xu, Evaluation of stretched electrospun silk fibroin matrices seeded with urothelial cells for urethra reconstruction., J. Surg. Res. 184 (2013) 774–81. doi:10.1016/j.jss.2013.04.016.

[12] a Feola, S. Abramowitch, Z. Jallah, S. Stein, W. Barone, S. Palcsey, P. Moalli, Deterioration in biomechanical properties of the vagina following implantation of a high-stiffness prolapse mesh., BJOG. 120 (2013) 224–32. doi:10.1111/1471-0528.12077.

[13] R. Liang, S. Abramowitch, K. Knight, S. Palcsey, a Nolfi, a Feola, S. Stein, P. a Moalli, Vaginal degeneration following implantation of synthetic mesh with increased stiffness., BJOG. 120 (2013) 233–43. doi:10.1111/1471-0528.12085.

[14] R.D. Cardwell, J. a. Kluge, P.S. Thayer, S. a. Guelcher, L. a. Dahlgren, D.L. Kaplan, A.S. Goldstein, Static and Cyclic Mechanical Loading of Mesenchymal Stem Cells on Elastomeric, Electrospun Polyurethane Meshes, J. Biomech. Eng. 137 (2015) 071010. doi:10.1115/1.4030404.

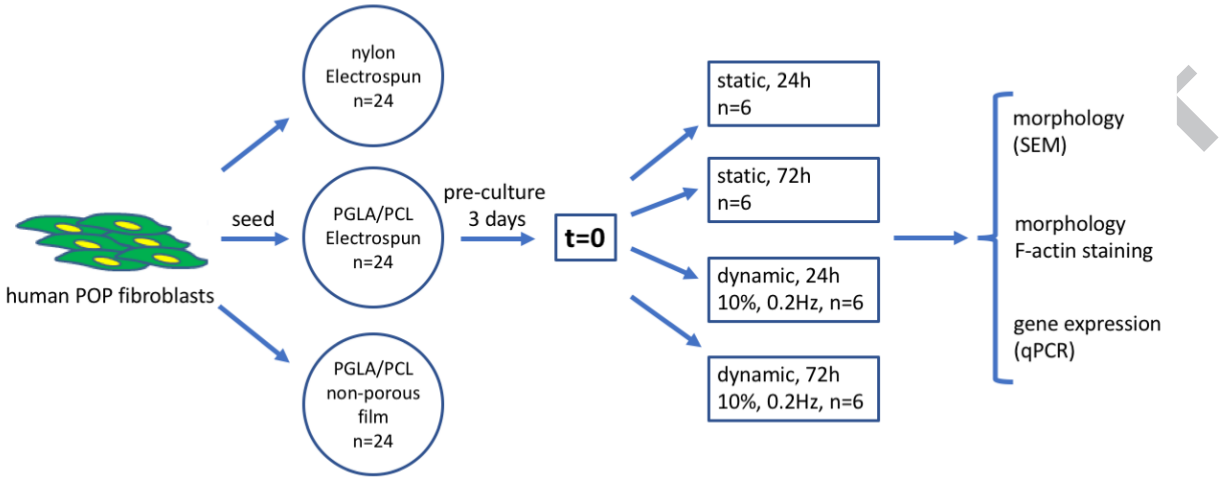
[15] A.M. Ruiz-Zapata, M.H. Kerkhof, B. Zandieh-Doulabi, H. a M. Brölmann, T.H. Smit, M.N. Helder, Fibroblasts from women with pelvic organ prolapse show differential mechanoresponses depending on surface substrates., Int. Urogynecol. J. 24 (2013) 1567–75. doi:10.1007/s00192-013-2069-z.

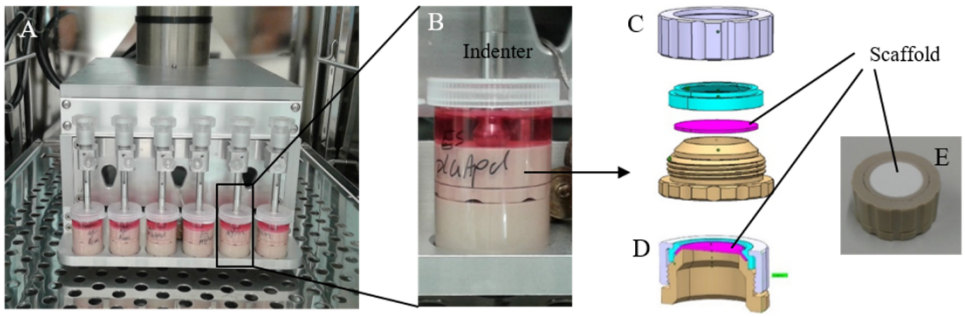
[16] D.J. Tschumperlin, S.S. Margulies, J. Daniel, Alveolar epithelial surface area-volum

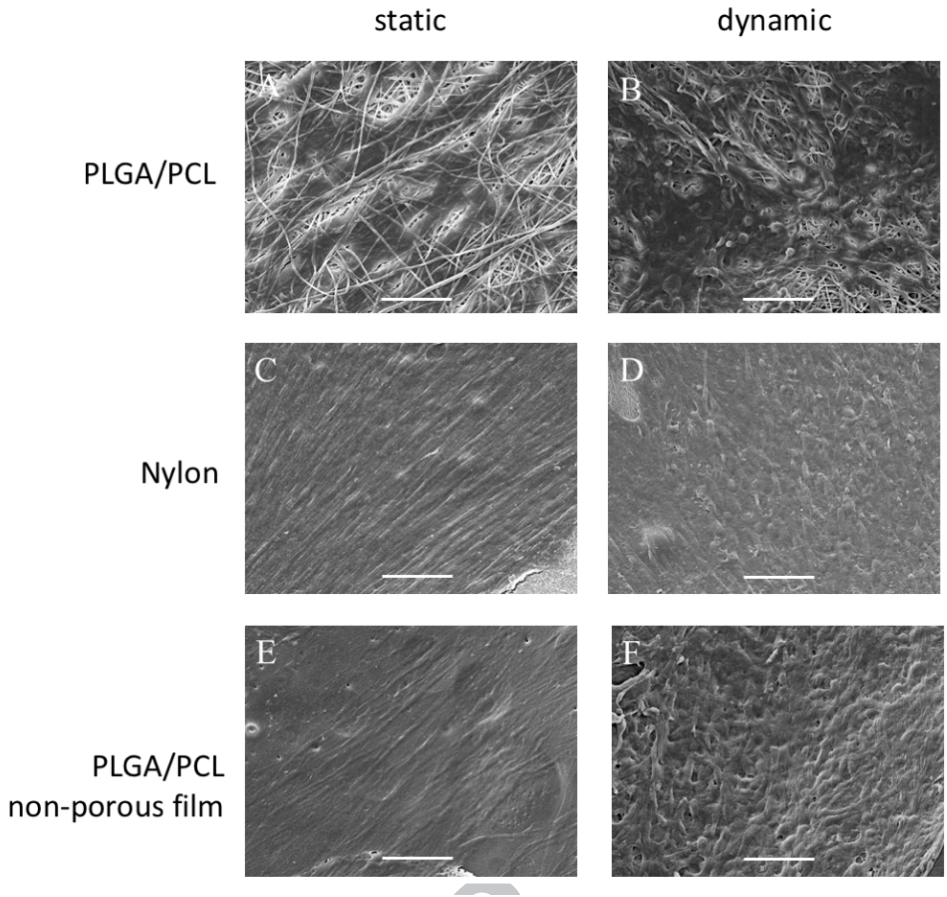
relationship in Isolated Rat Lungs, (1999).
[17] R.D. Cardwell, J.A. Kluge, P.A. Thayer, S.A. Guelcher, L.A. Dahlgren, D.L. Kaplan, A.S. Goldstein, Static and Cyclic Mechanical Loading of Mesenchymal Stem Cells on Elastomeric, Electrospun Polyurethane Meshes, J. Biomech. Eng. 137 (2015) 071010. doi:10.1115/1.4030404.
[18] A.D. Berendsen, T. Smit, X.F. Walboomers, V. Everts, J. Jansen, T. Bronckers, Three-Dimensional Loading Model for Periodontal Ligament Regeneration in vitro., Tissue Eng. Part C. Methods. 15 (2009) 1–10. doi:10.1089/ten.TEC.2008.0336.
[19] A. Mangera, A.J. Bullock, S. Roman, C.R. Chapple, S. MacNeil, Comparison of candidate scaffolds for tissue engineering for stress urinary incontinence and pelvic organ prolapse repair., BJU Int. 112 (2013) 674–85. doi:10.1111/bju.12186.
[20] H. Kufaishi, M. Alarab, H. Drutz, S. Lye, O. Shynlova, Static Mechanical Loading Influences the Expression of Extracellular Matrix and Cell Adhesion Proteins in Vaginal Cells Derived From Premenopausal Women With Severe Pelvic Organ Prolapse, Reprod. Sci. (2016). doi:10.1177/1933719115625844.
[21] R.M. Wazen, J.A. Currey, H. Guo, J.B. Brunski, J.A. Helms, A. Nanci, Micromotion-induced strain fields influence early stages of repair at bone-implant interfaces, Acta Biomater. 9 (2013) 6663–6674. doi:10.1016/j.actbio.2013.01.014.
[22] M.E. Blaauboer, F.R. Boeijen, C.L. Emson, S.M. Turner, B. Zandieh-Doulabi, R. Hanemaaijer, T.H. Smit, R. Stoop, V. Everts, Extracellular matrix proteins: A positive feedback loop in lung fibrosis?, Matrix Biol. 34 (2013) 2–10. doi:10.1016/j.matbio.2013.11.002.
[23] L. Lu, S.J. Oswald, H. Ngu, F.C.-P. Yin, Mechanical properties of actin stress fibers in living cells., Biophys. J. 95 (2008) 6060–71. doi:10.1529/biophysj.108.133462.
[24] J. Foolen, V.S. Deshpande, F.M.W. Kanters, F.P.T. Baaijens, The influence of matrix integrity on stress-fiber remodeling in 3D, Biomaterials. 33 (2012) 7508–7518. doi:10.1016/j.biomaterials.2012.06.103.
[25] J.H.C. Wang, B.P. Thampatty, J.S. Lin, H.J. Im, Mechanoregulation of gene expression in fibroblasts, Gene. 391 (2007) 1–15. doi:10.1016/j.gene.2007.01.014.
[26] D. van Geemen, A. Driessen-Mol, F.P.T. Baaijens, C.V.C. Bouten, Understanding strain-induced collagen matrix development in engineered cardiovascular tissues from gene expression profiles., Cell Tissue Res. 352 (2013) 727–37. doi:10.1007/s00441-013-1573-2.
[27] A.C. Newby, Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates, Cardiovasc. Res. 69 (2006) 614–624. doi:10.1016/j.cardiores.2005.08.002.
[28] M. Kumka, J. Bonar, Fascia: a morphological description and classification system based on a literature review., J. Can. Chiropr. Assoc. (2012). doi:10.1002/ase.1584.
[29] J.J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, R. a Brown, Myofibroblasts and mechano-regulation of connective tissue remodelling., Nat. Rev. Mol. Cell Biol. 3 (2002) 349–63. doi:10.1038/nrm809.
[30] K. Junge, U. Klinge, R. Rosch, P.R. Mertens, J. Kirch, B. Klosterhalfen, P. Lynen, V. Schumpelick, Decreased collagen type I/III ratio in patients with recurring hernia after implantation of alloplastic prostheses, Langenbeck's Arch. Surg. 389 (2004) 17–22. doi:10.1007/s00423-003-0429-8.
[31] B. Hinz, Tissue stiffness, latent TGF-β1 Activation, and mechanical signal transduction: Implications for the pathogenesis and treatment of fibrosis, Curr. Rheumatol. Rep. 11 (2009) 120–126. doi:10.1007/s11926-009-0017-1.
[32] G. Yang, H.J. Im, J.H.C. Wang, Repetitive mechanical stretching modulates IL-1beta induced COX-2, MMP-1 expression, and PGE2 production in human patellar tendon

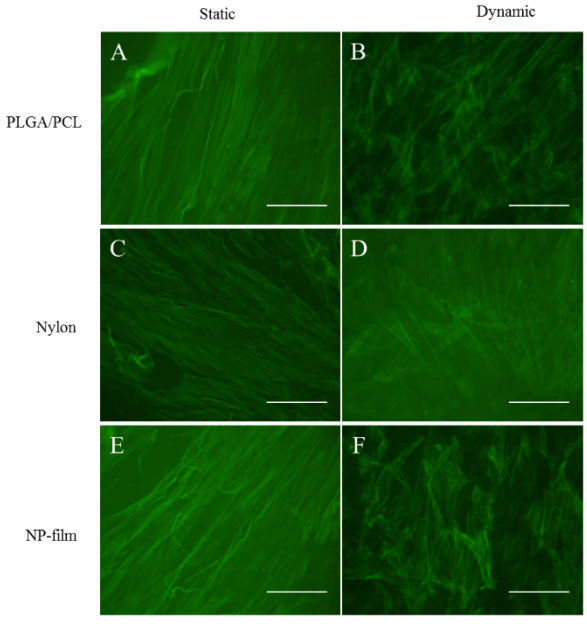
fibroblasts, Gene. 363 (2005) 166–172. doi:10.1016/j.gene.2005.08.006.
[33] Z. Sheikh, P.J. Brooks, O. Barzilay, N. Fine, M. Glogauer, Macrophages, foreign body giant cells and their response to implantable biomaterials, Materials (Basel). 8 (2015) 5671–5701. doi:10.3390/ma8095269.
[34] M. Colaco, J. Mettu, G. Badlani, The scientific basis for the use of biomaterials in SUI and POP., BJU Int. (2014). doi:10.1111/bju.12819.
[35] T.T. Chowdhury, S. Arghandawi, J. Brand, O.O. Akanji, D.L. Bader, D.M. Salter, D. a Lee, Dynamic compression counteracts IL-1beta induced inducible nitric oxide synthase and cyclo-oxygenase-2 expression in chondrocyte/agarose constructs., Arthritis Res. Ther. 10 (2008) R35. doi:10.1186/ar2389.
[36] K.C. Rustad, V.W. Wong, G.C. Gurtner, The role of focal adhesion complexes in fibroblast mechanotransduction during scar formation, Differentiation. 86 (2013) 87–91. doi:10.1016/j.diff.2013.02.003.
[37] K. Junge, M. Binnebösel, K.T. Von Trotha, R. Rosch, U. Klinge, U.P. Neumann, P.L. Jansen, Mesh biocompatibility: Effects of cellular inflammation and tissue remodelling, Langenbeck's Arch. Surg. (2012). doi:10.1007/s00423-011-0780-0.
[38] M. Xue, C.J. Jackson, Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring, Adv. Wound Care. (2015). doi:10.1089/wound.2013.0485.
[39] M.E. Blaauboer, T.H. Smit, R. Hanemaaijer, R. Stoop, V. Everts, Cyclic mechanical stretch reduces myofibroblast differentiation of primary lung fibroblasts., Biochem. Biophys. Res. Commun. 404 (2011) 23–7. doi:10.1016/j.bbrc.2010.11.033.
[40] S. Wang, Z. Zhang, D. Lü, Q. Xu, Effects of Mechanical Stretching on the Morphology and Cytoskeleton of Vaginal Fibroblasts from Women with Pelvic Organ Prolapse, Int. J. Mol. Sci. (2014) 9406–9419. doi:10.3390/ijms16059406.
[41] F.J. Bye, L. Wang, A.J. Bullock, K.A. Blackwood, A.J. Ryan, S. MacNeil, Postproduction Processing of Electrospun Fibres for Tissue Engineering, J. Vis. Exp. (2012). doi:10.3791/4172.
[42] T.H. Smit, K.A. Thomas, R.J.W. Hoogendoorn, G.J. Strijkers, M.N. Helder, P.I.J.M. Wuisman, Sterilization and strength of 70/30 polylactide cages: e-beam versus ethylene oxide, Spine (Phila. Pa. 1976). (2007). doi:10.1097/01.brs.0000259057.94986.3b.
[43] R.J. Kroeze, M.N. Helder, W.H. Roos, G.J.L. Wuite, R.A. Bank, T.H. Smit, The effect of ethylene oxide, glow discharge and electron beam on the surface characteristics of poly(L-lactide-co-caprolactone) and the corresponding cellular response of adipose stem cells, Acta Biomater. (2010). doi:10.1016/j.actbio.2009.11.022.

Pelvic organ prolapsed is a dysfunctional disease in female pelvic floor that can reduce the quality of life women. Currently, trans-vaginal knitted meshes are used to anatomically correct the dysfunctional tissues. However, the meshes can create sever adverse complications in some patients (e.g. chronic pain) in longer-term. As an alternative, we developed nanofibrous matrices by electrospinning based on different materials. We designed an *in-vitro* culture system and subjected cell-seeded matrices to cyclic mechanical loading. Results revealed that gentle straining of POP-cells on electrospun matrices, advances their regenerative potential at morphological and gene expression levels. Our findings, provide a proof-of-concept for using electrospun matrices as an alternative implant for pelvic floor repair, given that the parameters are designed efficiently and safely.









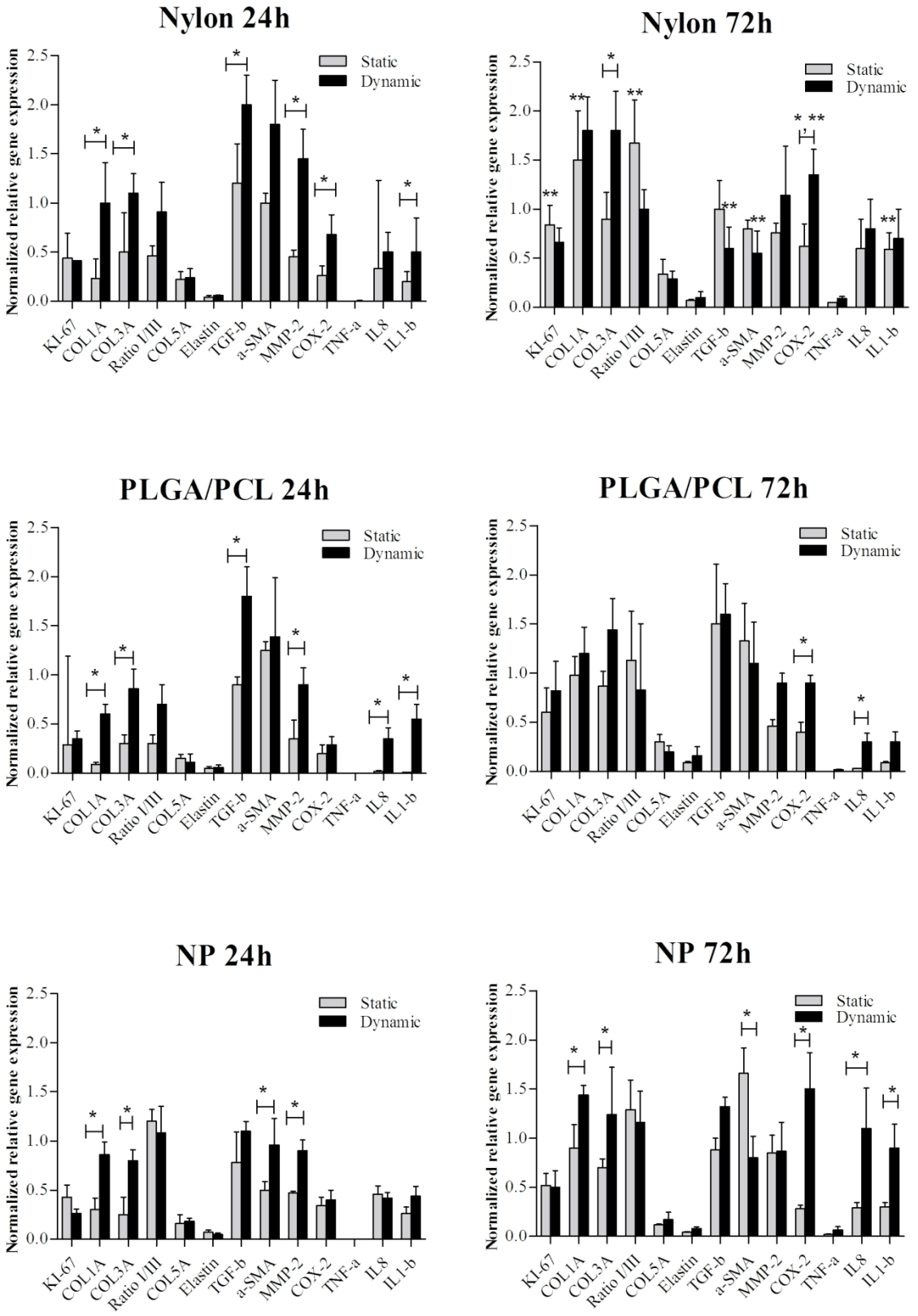


Table 1. Primer sequences used for reverse transcriptase polymerase chain reaction (RT-PCR)

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)	Gene role
KI67	Forward Reverse	5'CCCTCAGCAAGCCTGAGAA 3' 5'AGAGGCGTATTAGGAGGCAAG 3'	57	202	Cell proliferation
COL1A1	Forward Reverse	5' TCCGGCTCCTGCTCCTCTTA 3' 5' GGCCAGTGTCTCCCTTG 3'	57	336	Matrix synthesis
COL3A	Forward Reverse	5' GATCCGTTCTCTGCGATGAC 3' 5' AGTTCTGAGGACCAGTAGGG 3'	56	279	Matrix synthesis
COL5A1	Forward Reverse	5' CAGGCCGATCCTGTGGATG 3' 5' GTGGCCTTCTGGAAAGAGT 3'	56	174	Matrix synthesis
Elastin	Forward Reverse	5' TTCCTGGAATTGGAGGCATCG 3' 5' AGCTCCTGGGACACCAACTA 3'	56	152	Matrix synthesis
α-SMA	Forward Reverse	5' TGGACCAACATAGTGGTGTTCT 3' 5' GAGAGGCTTTAATGTACCAGTT 3'	56	234	Matrix remodeling
MMP-2	Forward Reverse	5' GGCAGTGCAATACCTGAACA 3' 5' AGGTGTGTAGCCAATGATCCT 3'	56	232	Matrix remodeling
TGF-β1	Forward Reverse	5' CTACTACGCCAAGGAGGTCA 3' 5' CACGTGCTGCTCCACTTT 3'	56	199	Matrix remodeling
COX-2	Forward Reverse	5' GCATTCTTTGCCCAGCACTT 3' 5' AGACCAGGCACCAGACCAAAGA 3'	57	299	Inflammation mediator
IL1-β	Forward Reverse	5' TGGAGCAACAAGTGGTGTTCT 3' 5' GAGAGGTGCTGATGTACCAGTT 3'	56	270	Inflammation mediator
IL-8	Forward Reverse	5' TCTGCAGCTCTGTGTGAAG 3' 5' TGTGTTGGCGCAGTGTGG 3'	56	147	Inflammation mediator
TNF-α	Forward Reverse	5' AGAGGGCCTGTACCTCATCT 3' 5' AGGGCAATGATCCCAAAGTAG 3'	56	315	Inflammation mediator
Ywhaz	Forward Reverse	5'GATGAAGCCATTGCTGAACTTG 3' 5'CTATTTGTGGGACAGCATGGA 3'	56	229	House-keeping
HPRT	Forward Reverse	5' GCTGACCTGCTGGATTACAT 3' 5' CTTGCGACCTTGACCATCT 3'	56	260	House-keeping

Table 2. Characteristics of different scaffolds used in this study.

Scaffold type	Thickness (μm)	Fiber size (nm)	Pore size (μm^2)	Porosity (%)	Tensile strength (MPa)	Stiffness (MPa)	Weight (g/m^2)
Nylon	50 ± 4.5	117 ±7.8*	1.3 ± 0.1*	69 ± 4	15.4 ±3.3	13.74 ± 0.8	35 ± 6
PLGA/PCL	136 ± 27	994 ± 115	8.8 ± 0.6	81 ± 6	13.8 ±0.9	13.8 ± 2	55 ± 3.2
Non-porous film	55 ± 12	n.a.	n.a.	n.a.	6.3 ±0.06	0.62 ± 0.03*	117 ± 7

*Data are presented as mean ±standard deviation (*P<0.05, analyzed with t-test for Nylon and PLGA/PCL, and one-way ANOVA for all three scaffolds, n=3)*