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Abstract

Tenocytes, the main cell type of the tendon, require mechanical stimuli for their proper function. When the tenocyte environment changes due to tissue damage or by transferring tenocytes from their native environment into cell culture, the signals from the tenocyte niche are lost, leading towards a decline of phenotypic markers. It is known that micro-topographies can influence cell fate by the physical cues they provide. To identify the optimal topography-induced biomechanical niche *in vitro*, we seeded tenocytes on the TopoChip, a micro-topographical screening platform, and measured expression of the tendon transcription factor Scleraxis. Through machine learning algorithms, we associated elevated Scleraxis levels with topological design parameters. Fabricating micro-topographies with optimal surface characteristics on larger surfaces allowed finding an improved expression of multiple tenogenic markers. However, long-term confluent culture conditions coincided with osteogenic marker expression and the loss of morphological characteristics. In contrast, passaging tenocytes which migrated from the tendon directly on the topography resulted in prolonged elongated morphology and elevated Scleraxis levels. This research provides new insights into how micro-topographies influence tenocyte cell fate, and supports the notion that micro-topographical design can be implemented in a new generation of tissue culture platforms for supporting the phenotype of tenocytes.

Keywords

Micro-topography; tenocytes; phenotypic maintenance; machine learning; cell morphology

1. Introduction

The tendon is a connective tissue enabling bone movement through the transmission of mechanical forces originating from the muscle. Injuries in this tissue are common and represent 30% of musculoskeletal disorders [1]. Low cellular content and hypovascularity in tendons go hand in hand with limited regenerative capability, often leading towards scarred and inferior tissue [2]. Clinical interventions usually do not recover the original tendon architecture, emphasizing the need for new tissue engineering approaches. Recent scientific advances such as the regeneration of a complete epidermis [3] illustrate the potential of the tissue engineering field for clinical applications. Tissue engineering a functional tendon, however, is hindered by the rapid loss of cellular phenotype *in vitro,* evidenced by loss of spindle-shaped morphology and tenogenic phenotypical markers [4][5][6].

The occurrence of phenotypic drift in cell culture is a generic problem in the tissue engineering field and not only limited to cells of the tenogenic lineage. For example, chondrocytes grown in a monolayer cell culture likewise lose their morphologic characteristics and chondrogenic gene expression [7][8]. Nevertheless, adjusting culture conditions by reducing oxygen tension [9], substrate composition [10] or the addition of growth factors [11] can positively influence chondrocyte phenotype. Expanding cells in an improved culture environment is not only beneficial for phenotypic characteristics; it allows priming cells for *in vivo* applications. Examples include the use of cAMP by our lab to improve osteogenic differentiation of mesenchymal stem cells for bone repair [12] or the self-renewal of muscle stem cells for muscle regeneration through changing the stiffness of the substrate material [13]. These examples illustrate that growing cells in an optimized culture dish can direct cell fate for tissue engineering applications.

Also for tenocytes, external stimuli such as growth factors [14][15] and small molecules [16] can positively influence tenocyte characteristics. *In vivo*, mechanical stimulation enables maintenance of

the collagen-rich tendon matrix [17] and stimulates the expression of tenogenic genes [18][19]. Since tenocytes are mechanosensitive cells, we reasoned that micro-topographical architectures might improve tenogenic characteristics by eliciting topography-induced biomechanical cues. In light of this, other types of surface structures like micro- and nanogrooves improved the expression of tendon-related genes [20][21][22], with microgrooves having a beneficial effect on the alignment of collagen-I [20]. In general, geometric sensing can influence cell fate, ranging from inducing proliferation or apoptosis [23], lineage differentiation [24][25] and maintaining phenotypic characteristics [26]. It is further known that altered geometric environments can activate the MAPK/ERK pathway [27] or RhoA signaling [28], both known to effect Early-Growth-Response-Factor 1 (Egr-1) [29][30], a tendon related transcription factor [31]. These findings provide an interesting theoretical framework for studying the effects of micro-topographies on tenocyte phenotype.

Currently, there is no marker uniquely expressed in tenocytes. Nevertheless, since the tendon tissue is composed of an organized assembly of collagen fibers, an optimal expression of matrix proteins is essential for proper tendon maintenance. The most prominent tendon related matrix protein is Collagen-I (Col-I), which compromises the majority of the tendon dry mass [32]. Collagen-III (Col-III) is the next most abundant collagen protein and its expression is associated with wound healing after injury [33]. Other tendon matrix-associated proteins include Tenascin-C (Tn-c), which plays a role in collagen fiber orientation [34] and is known to be upregulated upon mechanical loading [35]. The proteoglycans Decorin (Dec) and Biglycan (Bgn) are essential for proper fiber orientation, the mechanical properties of the tendon [36][37], and play important roles in repair mechanisms [38]. Also, Tenomodulin (Tnmd), a transmembrane glycoprotein and predominantly expressed in tendons, is essential for proper tenocyte proliferation and collagen fibril maturation [39]. Knock-out models of the transcription factors Scleraxis (Scx), Mohawk Homeobox Protein (Mkx) and Egr-1 coincide with reduced expression of the before mentioned proteins and lead to disturbed tendon development [31][40][41]. Both Egr-1 and Mkx can upregulate the expression of Tnmd and Col-I upon mechanical stimuli [19], with Egr-1 also influencing the expression of Scx [42]. In tendons, Scx is upregulated upon physical stimulation [43] and acts upon the expression of Col-I [44] and Tnmd [45]. Due to the strong relationship between mechanical

stimulation and tenogenic gene expression, it is not surprising that a loss of mechanical stimuli is detrimental to healing after injury, while mechanical stimulation improving clinical outcome [46]. Of interest, overloading can cause the expression of chondrogenic and osteogenic markers [18], potentially leading towards tendinopathy. These research findings illustrate the importance of mechanical stimulation on proper tenocyte function for maintaining a healthy tendon.

To find an optimal topography-induced biomechanical niche, we utilized the TopoChip, a high-throughput screening platform with 2176 uniquely designed micro-topographies [47]. Previous screens performed with this platform successfully identified topographies enhancing osteogenic differentiation of mesenchymal stem cells [48] and chondrogenic marker expression of the ATDC5 cell line [49]. Due to its importance for proper tenocyte function, and known activation upon mechanical stimuli, we chose Scx as screening target. We further utilized Scx promoting micro-topographical surfaces for studying the behavior of tenocytes on these culture platforms and their exerted influence on tenogenic and non-tenogenic marker expression. In this study, we illustrate that micro-topographical designs can be implemented in tissue culture platforms for improving the phenotype of *in vitro* cultured tenocytes.

2. Materials and methods

2.1 TopoChip and large area surface fabrication

A detailed description of the surface fabrication procedures can be found elsewhere [50]. In short, the in silico design of the TopoChip is generated using an algorithm that combines triangles, circles, and rectangles to generate complex shapes. The combination of these shapes in varying orientations, numbers and sizes leads towards the generation of 154.320.600 unique topographies. For the TopoChip design, 2176 topographies were randomly selected from this pool and placed in duplicate in areas of 290x290 µm, separated by walls of 30 µm. All topographies have a height profile of 10 µm. 4 flat control surfaces were also included on the TopoChip. The inverse pattern of the topographies was etched from a silicon wafer, by directional reactive ion etching (DRIE), generating a silicon master mould for hot embossing. To facilitate the demoulding procedure, the master was coated with a layer of perfluorodecyltrichlorosilane (FDTS, Sigma-Aldrich). Polydimethylsiloxane (PDMS; Down Corning) was used to generate a positive mould. The PDMS mould was subsequently used to create a second negative mould in OrmoStamp® hybrid polymer (micro resist technology Gmbh), which serves as the mould for hot embossing the PS films (Goodfellow). The conditions for the hot embossing were 140°C for 5 min at a pressure of 10 Bar, with a demoulding temperature of 90°C. For micro-topographies imprinted in larger surface areas (⌀ 1-5 cm) the same procedures were performed. For these surfaces, micro-topographies with the same feature parameters as the TopoChip are etched across larger areas and without 30 µm walls. Before cell culture, the PS topographies were treated with oxygen plasma to improve cell adhesion. The plasma treatment was performed for 30 s at 75 mTor, 50 sccm O_2, and 50W. Quality of the fabricated imprints was assessed using a Keyence VK-H1XM-131 profilometer.

The topographical numbering utilized in this manuscript is based on our TopoUnit database, a repository containing the design parameters of each individual micro-topography. For example, T2-PS-0304 refers to the second generation of TopoChip design (T2), of which the micro-topographies are imprinted on polystyrene (PS). The first two digits represent the row number starting from the top of a

TopoChip, while the second two digits represent the column number. Throughout the manuscript, we will apply the abbreviation PS-0304.

2.2 Tenocyte isolation and cell culture

A protocol describing the isolation of rat tenocytes is described elsewhere [21]. Rat tendon tissues were harvested post-mortem from rats enrolled in studies approved by the institutional Ethics Committee of NUI Galway. The isolated tenocytes were placed in culture flasks in DMEM low glucose (Merck) supplemented with 10% (v/v) FCS (Merck) and Penicillin/Streptomycin (100 U/ml; ThermoFisher). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2. Media was changed every 2-3 days. Cells were seeded at 5000 cell/cm^2 at passage 3 unless stated otherwise.

2.3 Immunocytochemistry

After cell culture, the cells were washed with phosphate buffered saline (PBS; Merck) and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 5 min at 37 °C. After washing three times, cells were permeabilized with 0.01% (v/v) Triton X-100 (Acros Organics) and blocked with goat serum (1:100; Sigma-Aldrich) in PBT (PBS + 0.02% Triton-X-100, 0.5% BSA) for 1h. Afterwards, cells were incubated with the primary antibody dissolved in PBT for 1h. Cells were washed three times and incubated with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 647 (1:500; ThermoFisher), together with Phalloidin conjugated to Alexa Fluor 568 (1:500; ThermoFisher) in PBT for 1h. After washing, the nucleus was counterstained with Hoechst 33258 (1:1000; Sigma-Aldrich) for 20 min. After washing three times, surfaces were mounted on glass cover slides with mounting media (Dako). All washing steps were performed with PBT. Primary antibodies used in this study were: anti-Scx antibody (1:200; Abcam; ab58655) and anti-Egr1 antibody (1:200; Thermo Fisher Scientific; T.126.1). For live cell imaging, Celltracker Green CMDFA (Thermo Fisher Scientific) was used according to manufacturer's instructions.

2.4 Image Analysis

Fixed samples were inverted and fluorescent images were acquired through the glass coverslip using a fully automated Nikon Eclipse Ti-U microscope in combination with an Andor Zyla 5.5 4MP camera. For live cell imaging, the same microscopy setup was applied, yet whereby cells were held inside an incubation chamber at 37 °C and a 5% CO_2 atmosphere. Images were analyzed through CellProfiler [51] with custom-made pipelines. After illumination corrections, nuclei morphology was captured using the Otsu adaptive thresholding method applied on the Hoechst 33258 channel. Subsequently, cell morphology was determined by applying Otsu adaptive thresholding and appropriate propagation algorithms on the Phalloidin channel. To prevent the influence of the walls on the cell morphology measurements, cells touching the walls were filtered out of the dataset. After background correction, the SCX intensity value of each pixel inside the segmented nuclear area is summarized to calculate the integrated SCX value of each individual nuclei.

2.5 Random forest classification to associate SCX levels with micro-topography design features and cell morphology

To identify surface design parameters that influence SCX expression, the top 200 micro-topographies inducing the highest and statistically significant median integrated SCX values were selected and defined as positive hits. The top 200 micro-topographies inducing the lowest and non-significant median integrated SCX values were defined as negative hits. This division in two classes allowed the use of a random forest binary classification for creating a predictive model associating feature design parameters with SCX levels of the micro-topographies belonging to these top and negative hits. For associating morphological parameters with SCX levels, we measured the median value of each morphological parameter (e.g. cell and nuclear area) from each cell grown on the micro-topography belonging to the selected hit topographies. The Random Forest algorithm was run in R ver. 3.3.3 [52]. The accuracy of the model was depicted by the receiver operating curve (ROC), which illustrates the performance of the binary classifier by plotting the true positive rate, against the false positive rate, at various threshold settings. In order to have a training set for testing the accuracy of the model, the data set was split into 2 parts. The first part contained 75% of the data and was used for model training and the remaining 25% was used for model testing. The models were trained with 10 fold cross validation

in “caret” package version 6.0 [53]. ROC curves and the scatter plots separating high and low SCX hits were visualized through package “ggplot2” [54].

2.6 Quantitative PCR (RT-qPCR)

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. Reverse transcription was carried out using an iScript™ cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad) in a CFX96™ Real-Time PCR Detection Kit (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and relative expression was determined using the ΔΔCt method. Primer sequences are listed in **Supplementary Data File 3**.

2.7 Statistical analysis

Statistical significance of integrated SCX intensity values between flat and surface micro-topographies was determined by Dunns post-hoc test. A p-value smaller than 0.05 after Benjamini and Hochberg correction for multiple testing is considered significant. Statistical significance of RT-qPCR experiments is determined by one sample t-test after log transformation of expression fold changes.

3. Results

3.1 Tenocytes lose morphological characteristics *in vitro* and SCX levels in confluent culture conditions

In order to assess the effect of topographical cues on tenocyte markers, we first investigated the behavior of tenocytes on standard, flat polystyrene. When comparing the morphology after passaging, it became clear that their original spindle shape characteristics were lost towards a spread morphology with a denser F-actin fiber architecture, coinciding with a flattened nucleus (**Fig. 1a**). In order to have sufficient cell numbers for downstream experiments, we expanded these tenocytes and investigated how *in vitro* culture conditions affect Scleraxis (SCX) levels, a tenogenic related transcription factor. All cells showed a positive SCX signal, which was, as expected, localized in the nucleus. We noticed that SCX levels remained constant when cells were passaged every 3-4 days and fixed 24 hours later (**Fig. 1b**). We further validated this observation by quantifying SCX nuclear intensity levels and found no differences at these different passages (**Fig. 1c**). Next, we were interested in evaluating SCX levels of cells grown in prolonged confluent culture conditions. In contrast to cells that were passaged, we observed a lower SCX nuclear signal after 7 culture days. This decline continued and after 21 days SCX nuclear localization was absent (**Fig. 1d**). Quantification of SCX intensity levels verified this phenomenon (**Fig. 1e**; * $P<0.05$, ** $P<0.01$). These observations confirm that tenocytes rapidly lose their morphological characteristics *in vitro* and that the culture method (passaging or a confluent monolayer) can have a profound effect on tenogenic marker expression.

3.2 TopoChip screen identifies micro-topographies elevating Scleraxis levels in tenocytes

Since *in vitro* culture conditions induce changes in tenocyte phenotype, we investigated if micro-topographical architectures can positively influence SCX levels and morphological characteristics of these dedifferentiated tenocytes. For this, we produced TopoChips of polystyrene (PS), the standard material used in cell culture. The quality of the spatial dimensions of the topographical features (10 μm height) and the walls (30 μm height) separating the individual units (290x290 μm) each containing a micro-topographical design was assessed by profilometric imaging (**Fig. 2a**). The height profile of the TopoChip can be found in **Supplementary Figure 1**. Next, tenocytes were seeded on 6 TopoChips and

cultured for 48 hours after which F-actin, DNA, and SCX were fluorescently labeled. This enabled high-content imaging whereby 24 flat surfaces and 12 replicas of each unique micro-topography were analyzed. We identified 29,534 cellular objects with an average of 13.5 cells for each unique micro-topography and a total of 25 cells located on the flat control surfaces. Nuclear SCX intensity values of each cell was measured and used to calculate the SCX median intensity per TopoUnit, thus allowing evaluation of the performance of each individual micro-topographical design on SCX expression. The flat surface belonged to the 100 surfaces inducing lowest SCX expression, which was approximately 2-fold lower compared to the 100 surfaces inducing highest SCX expression (**Fig. 2b;** * $P<0.001$). No significant difference was measured between the flat surfaces and the lowest top 100 scoring micro-topographies. However, a significant difference was found on 1023/2176 surfaces which induced higher SCX levels compared to flat ($P<0.05$). **Fig. 2c** further illustrates that the majority of micro-topographies induce higher SCX levels compared to the flat surface. Visual inspection of the morphological characteristics of cells cultured on the topographies revealed cell morphology heterogeneity between topographies and their replicas (**Fig. 2d**). Tenocytes either spread out on top of the micro-topographies, similar to when grown on a flat surface, while others obtained elongated characteristics. These observations demonstrate that micro-topographies can induce higher SCX levels in tenocytes, yet accompanied by a heterogeneous response in cell morphology.

3.3 Scleraxis expression is associated with topographical and morphological shape descriptors.

After observing that the majority of micro-topographies induce higher SCX levels, we were interested in identifying the relationship between surface design, cell morphological parameters, and SCX expression. The 2176 surface architectures can each be described through multiple parameters, including feature size, pattern area, the number of primitives used and wavenumbers (WN). A complete description of these parameters can be found in **Supplementary Data File 1**. In order to characterize the relationship between these surface parameters and SCX expression, we employed a Random Forest algorithm with binary classification. For creating the model we used 75% of the 200 lowest and highest SCX scoring micro-topographies. The accuracy of the model was assessed on the remaining 25%.

Models were trained with 10-fold cross-validation. Here, we found that SCX expression can be predicted most prominently based on pattern area and wavenumber 0.1 (**Fig. 3a**). Applying this model can predict with high accuracy (77% accuracy; 79% area under the curve (AUC)) SCX intensity levels based on surface parameters (**Fig. 3b**). An AUC > 0.6 is typically regarded as signifying a positive signal.

The same models were applied to correlate cell morphological features to SCX expression. Through CellProfiler [51], we extracted multiple quantitative parameters describing morphologic characteristics. These include cell and nuclear area, yet also compactness and solidity, which can describe cell elongation or branching. An overview of morphological feature parameters and their explanation can be found in **Supplementary Data File 2**. Applying the same random forest algorithm identified cell and nuclear area as the most prominent morphological features for distinguishing cells grown on the lowest and highest SCX inducing TopoUnits (**Fig. 3c**). Here, the model can predict with very high accuracy (92% accuracy; 83% AUC) SCX intensity levels based on morphological parameters (**Fig. 3d**). Of interest, parameters that define elongation or branching were unimportant in determining SCX levels, which is in sharp contrast with previous screens performed with mesenchymal stem cells, which exhibited a more diverse morphologic response when grown on the micro-topographies [48]. This indicates that the morphologic response that micro-topographies elicit on cells can vary strongly depending on the cell type used.

Fig. 4a illustrates how the Random Forest model can successfully separate the lowest 200 and highest 200 SCX inducing micro-topographical designs from the TopoChip platform based on WN0.1 and pattern area. A low WN0.1 and an intermediate to high pattern area favors elevated SCX expression. Low values of this particular wavenumber are generally associated with higher pattern areas in our micro-topographical design space (**Supplementary Figure 2**). Despite the achieved separation, negative SCX hit topographies can still be found with a low WN0.1 and high pattern area, and positive SCX hit topographies with a high WN0.1 and low pattern area. This reflects the inability of the Random Forest algorithm to achieve a full separation based on the feature parameters we utilize to describe the

micro-topographical designs. For these micro-topographies, technical variability, or other unknown design parameters, might attribute for this lack of separation. **Fig. 4b** illustrates the differences in low, intermediate and high micro-topographical pattern areas. **Fig. 4c** highlights the separation of low and high SCX inducing units based on cell and nuclear area. In general, we found that cells grown on micro-topographies with a lower pattern area exhibited a less spread out morphology. Cells belonging to the highest SCX hits are characterized by a larger cell spreading compared to flat due to a more profound micro-topographical pattern area (**Fig. 4d)**.

3.4 Selection and large area fabrication of SCX promoting micro-topographies allow in-depth investigations into the dynamic behavior of tenocytes.

To further investigate the influence topographies exert on tenocytes, we fabricated equivalent micro-topographies from the TopoChip in larger surface areas (ø 1 cm), while maintaining the original feature dimensions. We randomly selected surfaces PS-0264, PS-0304, and PS-1538 from the top 200 SCX inducing micro-topographies. These surfaces induced significant higher SCX levels on tenocytes compared to flat when cultured on the TopoChip platform (**Fig. 5a; *** $P<0.001$). Surface PS-0264 contains a low WN0.1 of 0.071 and an intermediate pattern area of 8.54, while PS-0304 consist a low WN0.1 of 0.042 and an intermediate pattern area of 6.83, which are both in the range as what the random forest algorithm determined important for inducing elevated SCX levels. Of interest, for a SCX inducing surface, PS-1538 contains a slightly atypical surface configuration with a low WN0.1 of 0.11 and a low pattern area of 4.25 (**Fig. 5b**). These enlarged surface areas allow the use of other experimental techniques besides immunocytochemistry (ICC). **Fig. 5c** depicts the *in silico* design of surface architecture PS-0304. After fabrication of these surfaces, we applied a quality control with the profilometer to verify structural integrity. In contrast to the TopoChip platform, these constructs do not contain walls of 30 μm (**Fig. 5d**). A detailed height profile of micro-topography PS-0304 is found in **Supplementary Figure 3**. Representative ICC images of tenocytes cultured on surfaces PS-0264, PS-0304 and PS-1538 on the TopoChip platform with nuclear, F-Actin and SCX staining can be found in **Supplementary Figure 4**.

During the analysis of the TopoChip screen, we noticed cell morphology heterogeneity of cells grown on the replicas of the individual micro-topographical designs. Despite the strong association between increased cell spreading and SCX levels, we did observe that SCX positive cells with an elongated morphology contributed to the observed cell morphology heterogeneity. Therefore we wanted to investigate if cell shape is dynamic in nature on the micro-topographies by monitoring tenocyte behavior for 16 hours. Cells were allowed to adapt and grow for 24 hours on the PS-0304 surface prior to the start of the live cell imaging. At the start of the monitoring, cells had attained heterogeneous morphologic characteristics on the surface, similar as seen on the TopoChip screen. Cells were either spindle-shaped and localized between the micro-topographies, or were spreading out and engulfing the structures (**Fig. 5e**), which were identified by the Random Forest algorithm as associated with the highest elevated SCX levels. We observed that the spindle shape cells were able to migrate in between the structures, while the spread cells seemed to be immobilized. Cells with both shapes were able to proliferate. Of interest, we noticed that the elongated cells during proliferation were able to either keep their cell shape (**Fig. 5f**) or become spread and immobilized upon the topography (**Fig. 5g**). However, these immobilized and flattened cells were able to reobtain an elongated morphology during the 16 hour culture period (**Fig. 5h**). These observations demonstrate that the offspring of dividing tenocytes contribute to the observed cell heterogeneity. Previous TopoChip screens utilizing mesenchymal stem cells (MSCs) show less morphological heterogeneity when cultured on the same surface [48]. We reason that this might be related to the increased stress fiber content in tenocytes, which is associated with increased cell stiffness [55], making them less flexible to adapt to the micro-topographical environment compared with MSCs. The dynamic behavior of tenocytes on the PS-0304 surface can be studied in further detail in **Movie file 1**. These observations demonstrate that micro-topographies elicit dynamic cell morphological remodeling on tenocytes.

3.5 Micro-topographies increase tenogenic marker expression yet coincides with the expression of chondrogenic and osteogenic markers at later culture times.

To further assess if micro-topographies elicit a beneficial effect on tenocytes or induce dedifferentiation, we performed gene expression analysis on tenogenic and chondrogenic/osteogenic transcription factors, downstream target genes, and tendon matrix-related proteins, both after 48 hours (**Fig. 6a**) and 7 days (**Fig. 6b**). Through immunocytochemistry, we observed an increase of SCX on these large are surfaces after 48 hours, excluding the possibility that the walls of the TopoChip, or growth factors secreted by neighboring TopoUnits, influenced SCX levels (data not shown). Through qPCR, we further validated the observation that micro-topographies can upregulate *Scx* gene expression after 48 hours. Of interest, this effect persisted after 7 days with even a 2-fold increase in *Scx* transcription levels present compared to the 48 hour culture time. Likewise, we found mild but significantly higher *Mkx* levels for surfaces PS-0264 and PS-1538 compared to flat (* $P<0.05$) after 48 hours. However, also here the observed *Mkx* levels showed a higher elevation for all surfaces after 7 days. *Col-I*, a downstream target of the before mentioned transcription factors, followed a similar trend as the *Mkx* gene expression pattern on these surfaces. For the downstream target *Tnmd*, increased levels were observed both after 48 hours and 7 days. Of interest, no upregulation was found for the transcription factor *Egr-1* at the time points investigated.

Sox9, a chondrogenic transcription factor also expressed by tenocytes at the junction between tendon and bone, likewise followed a similar gene expression pattern as *Mkx* and *Col-I* for which elevated levels were found on PS-0264 and PS-1538 after 48 hours. Also here, *Sox9* transcription levels were markedly higher after 7 days on all surfaces. For the osteogenic associated transcription factor *Runx2*, elevated levels were observed after 7 days, highlighting that also osteogenic transcription is activated at this time point.

Of the matrix related proteins, *Tn-c* showed increased levels both after 48 hours and 7 days with elevated levels of *Col-III* observable after 7 days. Of interest, *Bgn* and *Dec* were not elevated, with even a decrease of *Bgn* detected at 48 hours for surfaces PS-0264 and PS-0304, which returned to similar levels against flat after 7 days. The expression levels of Aggrecan (*Acan*), a protein present in both tendons and cartilage and regulated by *Sox9*, was markedly higher after 7 days. Furthermore, also *Col-II*,

regulated by *Sox9*, and *Alp* regulated by *Runx2*, displayed increased expression levels after 7 days. An overview of the expression profiles of these genes can be found in **Supplementary Figure 5**.

Since we did not observe differences in the expression levels of the transcription factor *Egr-1* at 48 hours and 7 days, we wondered if we could detect differences in expression levels at earlier time points. For this, we applied immunocytochemistry and detected an increased presence of EGR-1 2 hours after cell seeding on the surfaces (**Fig. 6c**). Of interest, we observed that at this early time point, all cells exhibit spreading across the micro-topographies. This demonstrates that the morphologic remodeling of tenocytes on micro-topographies is already active at the initial cell-substrate contacts.

The qPCR data confirms that topographies have a positive effect on the gene expression levels of tendon-related markers. However, after 7 days, the upregulation of tenogenic markers coincides with increased levels of chondrogenic and osteogenic markers, indicating that the positive effect of micro-topographies needs further guidance by other environmental stimuli.

3.6 Evaluation of serial passaging and confluent culture conditions on tenocytes cultured directly on the PS-0304 surface.

Previous experiments indicate that micro-topographies can have a beneficial effect on tenogenic marker expression, yet with heterogeneous morphologic characteristics and the expression of chondrogenic/osteogenic markers at later time-points. However, until now, we utilized tenocytes which exhibited dedifferentiated morphologic characteristics before seeding on the micro-topographies. For tissue engineering applications, it would be most interesting to evaluate the performance of micro-topographies on tenocytes isolated directly from a tendon tissue. Therefore, we selected the PS-0304 surface, which we manufactured in a 50 mm diameter dimension. Achilles tendon tissue was isolated from rats and placed on flat and the PS-0304 surface which allowed the migration of tenocytes directly from a tendon tissue. After 5 days, we observed that cells started migrating on the surface (**Fig. 7a-b**). Two days later, cells were either fixed for ICC (Passage 0; P0) or passaged. These passaged cells (P1) were either grown to confluency or passaged further every 3 days either on the PS-0304 surface or flat,

after which SCX levels were evaluated by ICC after 48 hours. We found only a slight and non-significant increase in SCX expression of the P0 tenocytes grown on the PS-0304 surface compared to flat. However, once cells were reseeded on the topography, we noticed improved SCX levels, and this effect was maintained upon multiple passaging steps (**Fig. 7c**; * P<0.001). This highlights the necessity of cell reseeding, with direct growth on the micro-topographies being insufficient for inducing higher SCX levels. Furthermore, we observed that cells homogenously retained a small and elongated nuclear and cell morphology (**Fig. 7d-e**), which is in contrast with the dedifferentiated cells used previously which showed a heterogeneous cell morphological response with a strong spreading across the micro-topographies. Quantification of cell spreading revealed that cells grown on topographies had a similar spreading as P0 tenocytes across multiple passages, with confluent cells on PS-0304 losing their spindle-shaped morphology and re-obtaining characteristics similar as cells cultured on flat (**Supplementary Figure 5a**; * P < 0.05; ** P <0.001). Of interest, P0 tenocytes grown on PS-0304 had even smaller spreading compared to cells grown on flat. Nuclear dimensions of tenocytes cultured on PS-0304 remained similar as P0 tenocytes and was markedly different compared to tenocytes grown on the flat surface (**Supplementary Figure 5b**; ** P < 0.001). For tenocytes that were allowed to grow confluent, SCX levels remained elevated compared to flat after 7 days, a similar observation as seen with the qPCR experiments. However, after 14 days SCX levels dropped and micro-topographies were unable to counteract SCX loss as seen with confluent cells grown on the flat surface (**Fig. 7f**). The loss of spindle-shaped characteristics of tenocytes allowed to grow confluent on PS-0304 demonstrates that micro-topographies are unable to counteract dedifferentiation and the loss of SCX levels in long-term culture conditions. Nevertheless, it seems that passaging cells on the topographies repeatedly leads to increased SCX expression and maintenance of elongated morphological characteristics, which is best applied with tenocytes isolated directly from the tendon tissue and which are not preconditioned in classic culture conditions.

4. Discussion

This study is the first to assess the performance of substrate structures in micro-topographical dimensions on tenocyte phenotype. Previously, other research groups have investigated the influence of alternative surface structures on tenocyte phenotype by utilizing groove structures in nano- and micrometer dimensions [20][21][22]. When comparing these studies with our research results, some interesting similarities are observed. In the study of English *et al.*, grooves in both nano- and micrometer dimensions led to improved elongated morphological characteristics in tenocytes and also gave rise to an upregulation of Scx and other tenogenic markers [21]. However, similar as in our study, an upregulation of chondrogenic and osteogenic genes was observed, which depended on the culture time point and width of the grooves. In another study, micro-grooves in larger dimensions (50-250 μm) increased the elongation of tenocytes with an improvement in Col-I and Col-III levels [20]. Of interest, in the study of Zhu *et al.*, culturing tenocytes on grooves in the μm range increased the expression of both Tnmd and Col-I across multiple passages [22], an approach similar as in this study where we found a repeated upregulation of SCX, which is upstream of Tnmd and Col-I [44][45]. These research findings support the notion that substrate structures can positively modulate tenogenic gene expression, favoring a culture system where cells are passaged upon these substrates.

The previous statement is further highlighted by the observation that tenocytes reaching confluency on the micro-topographies adapt a spread shape similar as when cultured on flat. This confluent condition is further associated with elevated osteogenic and chondrogenic markers and eventually a decline in SCX levels. Of interest are the elevated levels of EGR-1 at the initial moments of cell-substrate contact, a mechanosensitive gene known to be upregulated shortly upon mechanical stimulation [56], which might be linked with the upregulation of SCX. Although it is beyond the scope of this study to investigate the pathways involved in this phenomenon, a plausible explanation might be RhoA dynamics, previously shown to play a role in increasing the expression of Tnmd and Col-I on microgrooves [22], and tenogenic differentiation of MSCs [57][58]. The influence of RhoA on Egr-1 might be due to the pathways' involvement in ERK signaling [59]. In light of this, we observed active cell shape remodeling, known to play a role in RhoA signaling [60], both upon initial cell-substrate

contacts and after proliferation events, where spread cells on the topographies obtained a smaller and elongated morphology. This might explain why machine learning algorithms indicate an association between the strongest SCX expression, cell spreading and an intermediate to high pattern area, since this will require more cytoskeletal reorganization to adapt to the topographical environment. Further research unto the pathways underlying this topography-induced biomechanical stimulation might shed light into the mechanisms responsible for SCX upregulation and if this is related with *in vivo* mechanical stimulation.

In light of this, we found only a slight and non-significant increase in SCX levels of P0 tenocytes migrating on micro-topographies, even with elongated morphological characteristics. However, these tenocytes did not receive any mechanical loading during the first week they were in culture, further emphasizing the observation that cell seeding is required to initiate a topography-induced biomechanical stimulus. The beneficial effect on SCX expression was observed for dedifferentiated tenocytes used for the TopoChip screen, yet was more pronounced with the serial passaging of P0 tenocytes, which also maintained a more homologous cell population with elongated morphologic characteristics upon multiple passages. This discrepancy might be due to the higher presence of F-Actin fibers on the dedifferentiated tenocytes, which is associated with increased cell stiffness [55] and might result in a decreased ability to adapt to the micro-topographical environment. Utilizing SCX reporter cell lines might provide deeper insights how SCX expression is coincided with cell morphology dynamics, while increasing the pillar height might elucidate if preventing spreading during proliferation and cell seeding inhibits SCX upregulation.

When considering the use of micro-topographies in cell culture platforms, the multifactorial nature of the culture system should be taken into account. Although *Egr-1* regulates tenogenic signaling upon mechanical stimulation [42], it is not expressed solely in cells of the tenogenic lineage, yet is involved in chondro- and osteogenesis as well [61][62]. This might explain the observed early elevation of Sox9, a chondrogenic transcription factor [63]. Since tenocyte gene expression is dependent on the location of the tenocytes in the tendon [64], with even a SCX and SOX9 positive subpopulation existing [65], it is difficult to assess if the observed gene signature is similar as when tenocytes are *in vivo* subjected

with mechanical stimulation. Nevertheless, since other tenocyte subtypes do not readily express chondrogenic or osteogenic markers, we believe that the physical cues presented by the micro-topographies needs additional guidance from the culture environment. Examples for improving *in vitro* culture conditions include changing the polystyrene material towards a collagen substrate or altering the growth factor composition of the medium.

For tissue engineering applications, it is imperative that cultivated tenocytes maintain their phenotypic identity. After obtaining sufficient cell numbers, cells cultured on micro-topographies might improve the clinical outcome when injected in a damaged tendon tissue of which pilot studies already hinted towards beneficial effects [66]. Alternatively, these cells can be harnessed for integrating into a scaffold, which can replace damaged tissue, and provide both mechanical support and a cell source for aiding tendon repair [67]. Furthermore, tenocytes with improved characteristics can be applied for the complex 3D construction of a tendon tissue in a bioreactor [68]. We conclude by stating that micro-topographical architectures can provide tenocytes with phenotypic supportive cues, otherwise absent in classical culture platforms, which might allow improved clinical outcomes in tissue engineering applications.

5. Conclusion

This study illustrates that micro-topographies can positively modulate the expression of tenogenic associated markers. Furthermore, we demonstrated that this is largely dependent on the cell culture context, whereby culturing cells during multiple passages is more advantageous compared to confluent culture conditions. This work supports the concept that micro-topographies can provide xenofree physical cues for supporting the phenotype of tenocytes.

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Disclosure Statement

JdB is a co-founder of Materiomics b.v. The other authors do not have any financial interests to declare.

Figure Legends

Fig. 1: Tenocytes lose phenotypic characteristics in vitro. a) Representative ICC images of passage 0 and 1 rat tenocytes cultured on a PS flat surface. Tenocytes obtained a spread, wing-like cell morphology and flattened nucleus after passaging. Tenocytes were stained with Phalloidin-568 to visualize F-actin filaments (green) and counterstained with Hoechst to visualize the nucleus (blue). **b)** Tenocytes stained positive for SCX, and serial passaging resulted in constant SCX levels. **c)** Quantitative analysis of SCX intensities confirmed constant SCX levels when cells are passaged. Bar chart represents the median of integrated SCX intensities measured in the nucleus. Error bars represent 95% confidence interval. **d)** In a confluent culture, a steady decline in SCX levels was observed. At 21 days, SCX nuclear localization was undetectable. For visibility purposes, the contrast was enhanced for the 21-day time point. **e)** Quantitative analysis confirmed a steady decline in SCX intensity levels for confluent culture conditions. Bar chart represents the median of integrated SCX intensities measured in the nucleus. Error bars represent 95% confidence intervals (* $P<0.05$; ** $P<0.01$). For **a**, **b** and **d**, the scale bars represents 100 µm.

Fig. 2: TopoChip screen identified micro-topographies inducing elevated SCX levels in tenocytes after 48 hours of culturing. a) Profilometric quality control before cell seeding confirms structural integrity of TopoChip features. **b)** Dot chart represents median SCX intensity levels of individual TopoUnits of either the top 100 lowest or highest SCX expressing units (* $P<0.001$). Median is represented by a horizontal line. Errorbars represent interquartile range. **c)** Dot chart representing median SCX integrated expression levels of tenocytes cultured on the TopoChip of each individual TopoUnit, ranked according to statistical significance. 1023/2176 surfaces were considered to be statistical significant ($P<0.05$). The blue dot represents Scleraxis median integrated intensity of the flat surfaces on the TopoChip. **d)** Representative ICC images of tenocytes cultured on flat, low and high hit topographies. Two replicas per topography are shown. Cells were stained for F-Actin through

Phalloidin-568 and counterstained with Hoechst for nuclear visualization. Alexafluor-647 was used to visualize SCX after primary antibody binding. Scale bar represents 100 μm.

Fig. 3: Identification of topographical and morphological feature parameters associated with increased Scleraxis expression. a) Bar diagram illustrating the importance of topographical feature design parameters as predictive factors for SCX expression through random forest algorithms. Pattern area and wavenumber 0.1 are the most prominent features to distinguish low and high SCX inducing TopoUnits. **b)** ROC curve showing the prediction performance of the random forest classification algorithm. The AUC is the area under the curve which indicates the predictive power of the model**. c)** Bar diagram representing the importance of morphological parameters as predictive factors for SCX expression through random forest algorithms. The cell area is the most prominent feature next to the nuclear area to distinguish low from high SCX expressing TopoUnits. **d)** ROC curve showing the prediction performance of the random forest classification algorithm. The AUC is the area under the curve which indicates the predictive power of the model**.**

Fig. 4: High pattern area and low WN0.1 promote Scleraxis expression. a) Scatter plot representing the distribution of the pattern area and wavenumber 0.1 belonging to the highest (blue; top 200 hits) and lowest (orange; bottom 200 hits) SCX inducing micro-topographies on the TopoChip. **b)** Scatter plot representing the distribution of the median cell and nuclear area for cells growing on the lowest (orange; bottom 200 hits) or highest (blue; top 200 hits) SCX inducing micro-topographies on the TopoChip. **c)** Illustration of topographical designs with low, intermediate and high pattern area. **d)** Representative images of tenocytes cultured on a low and high pattern area. Tenocytes cultured on high pattern areas are associated with more cell spreading and elevated SCX levels. Cells were stained for F-actin through Phalloidin-568 and counterstained with Hoechst for nuclear visualization. Alexafluor-647 was used to visualize SCX after primary antibody binding. For **c** and **d**, the scale bars represent 100 μm.

Fig. 5: Micro-topographies fabricated in large surface areas allow in-depth investigation of micro-topographical influence on tenocyte behavior. a) Performance of the selected surfaces on the

TopoChip platform. Each of the selected surfaces induced significant higher SCX levels compared to flat control ($P<0.001$). The black dot represents the median, error bars represent interquartile range. **b)** WN0.1 and pattern area design parameters of the selected surfaces inducing significant SCX levels. **c)** *In silico* pattern design of micro-topography PS-0304 used for downstream experiments. **d)** Profilometric quality control of PS-0304 confirms structural integrity before cell seeding. **e)** After 24h of culture on the PS-0304 surface, tenocytes exhibited either a flattened or elongated morphology. Cells were stained with CellTracker Green to track cell morphology dynamics. **f)** During proliferation, tenocytes either kept their elongated morphology or **g)** lost this characteristic and spread across the micro-topographies immobilizing their migration. **h)** The spread and immobilized tenocytes were able to reobtain elongated characteristics during the 16 hours of culture time. For **c**, **d**, **e, g** and **h** the scale bars represents 100 μm.

Fig. 6: Gene expression profile of tenocytes grown on the micro-topographical surfaces PS-0264, PS-0304, and PS-1538. a) Bar chart representation of tenogenic, chondrogenic and osteogenic expression levels of tenocytes grown on micro-topographies after 48 hours. Increased levels for the majority of tenogenic markers are observed (* $P< 0.05$). **b)** Tenocytes cultured on micro-topographies after 7 days exhibit increased tenogenic marker expression compared to flat, yet coinciding with increased chondrogenic and osteogenic marker expression (* $P<0.05$). **c)** Immunocytochemistry reveals that the mechanosensitive transcription factor EGR-1 is activated early upon seeding on micro-topographies. Cells were stained for F-actin through phalloidin-568. Alexafluor-647 was used to visualize EGR-1 after primary antibody incubation. Scale bar represents 100 μm.

Fig. 7: Evaluation of serial passaging and confluent culture conditions on tenocytes cultured directly on the PS-0304 platform. a) Bright field image of tenocytes migrating out of the tendon tissue on PS-0304. The white arrow directs towards the tendon tissue. The black arrow guides towards tenocytes migrating on PS-0304. **b)** Close-up of P0 tenocytes migrating from the tendon tissue on PS-0304. **c)** ICC quantification of nuclear SCX in tenocytes after serial passaging reveals elevated protein levels. P0 tenocytes grown on the topography show only a slight non-significant elevation. Bar chart represents the median of integrated Scx intensities measured in the nucleus. Error bars represent 95%

confidence interval (* P<0.01). **d)** Representative ICC image of P0 tenocytes grown on flat or PS-0304. **e)** Representative ICC images of P3 tenocytes reveals that cells grown on PS-0304 retain an elongated morphology. **f)** A drop in SCX intensity levels is observed for tenocytes cultured in confluent conditions on PS-0304 after 14 days. Tenocytes furthermore lose their morphological characteristics. Cells were stained with phalloidin-568 to visualize F-actin filaments. Alexafluor-647 was used to visualize SCX. For **a**, **b**, **d** and **f**, the scale bar represents 100 μm.

Supplementary Figure 1: Optical profilometric imaging of the TopoChip platform. **a)** 3D reconstruction reveals the wall and feature structures of the TopoChip. The black line represents cross-section taken for measuring the height profile. **b)** The height profile confirms a 10 μm height of the feature structures and a 30 μm height of the walls dividing the individual TopoUnits in a 290x290 μm area.

Supplementary Figure 2: Scatter plot representation of WN0.1 and pattern area of all 2176 micro-topographical architectures on the TopoChip. The flat surface is represented by a WN0.1 and pattern area of 0. Higher micro-topographical pattern areas are associated with lower WN0.1 values.

Supplementary Figure 3: Optical profilometric imaging of the PS-0304 surface. **a)** 3D reconstruction reveals the feature architecture of PS-0304. The black line represents cross-section taken for measuring the height profile. **b)** The height profile confirms a 10 μm height of the PS-0304 feature structures with a side length of 20 μm.

Supplementary Figure 4: Representative ICC images of tenocytes cultured on surfaces PS-Flat, PS-0264, PS-0304, and PS-1538 located on the TopoChip platform. Cells were stained for F-actin through phalloidin-568 (green) and counterstained with Hoechst for nuclear visualization (blue). Alexafluor-647 was used to visualize SCX after primary antibody incubation. Scale bars represents 100 μm.

Supplementary Figure 5: Bar chart representation of gene expression levels of tendon-related matrix genes and the osteogenic marker *Alp* and the chondrogenic marker *Col-II* of tenocytes cultured on the PS-Flat, PS-0264, PS-0304 and PS-1538 surface (* P <0.05).

Supplementary Figure 6: a) Quantification of cell area reveals that cells grown on PS-0304 had smaller spreading compared to cells cultured on flat and similar levels as P0 tenocytes on flat (* $P < 0.05$; ** $P < 0.001$). **b**) Quantification of the nuclear area reveals that cells grown on PS-0304 obtained smaller nuclear dimensions compared to cells grown on the flat surface (* $P < 0.001$) and similar levels as P0 tenocytes. Median is represented by a black dot. Error bars represent interquartile range.

Supplementary Data File 1: Description of the micro-topographical feature design parameters.

Supplementary Data File 2: Description of cell morphological feature measurements (derived from the CellProfiler version 2.1.1 measure-object-size-shape module).

Supplementary Data File 3: Primer sequences used for RT-qPCR.

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