Effects of Substrate Stiffness on the Tenoinduction of Human Mesenchymal Stem Cells

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Abstract

Extracellular matrix modulus plays an important role in regulating cell morphology, proliferation and differentiation during regular and diseased states. Although the effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis, there has been relatively little investigation on the effects of this phenomenon on tenogenesis. Furthermore, relative roles of topographical factors (matrix alignment vs. matrix modulus) in inducing tenogenic differentiation is not well understood. In this study we investigated the effects of modulus and topographical alignment of type I collagen substrate on tendon differentiation. Type I collagen sheet substrates with random topographical alignment were fabricated with their moduli tuned in the range of 0.1, 1, 10 and 100 MPa by using electrocomaptation and controlled crosslinking. In one of the groups, topographical alignment was introduced at 10 MPa stiffness, by controlled unidirectional stretching of the sheet. RT-PCR, immunohistochemistry and immunofluorescence results showed that mimicking the tendon topography, i.e. increasing the substrate modulus as well as alignment increased the tenogenic differentiation. Higher substrate modulus increased the expression of COLI, COLIII, COMP and TSP-4 about 2–3 fold and increased the production of COLI, COLIII and TSP-4 about 2–4 fold. Substrate alignment up regulated COLIII and COMP expression by 2 fold. Therefore, the tenoinductive collagen material model developed in this study can be used in the research and development of tissue engineering tendon repair constructs in future.

Graphical abstract
1. Introduction

Modulus of the extracellular matrix plays an important role in regulating cell morphology, proliferation, differentiation during regular and diseased states. For example, 1 kPa stiffness favors differentiation of MSCs into neuronal-like cells, 10 kPa elasticity promotes myogenic differentiation, and a 100 kPa matrix stiffness stimulates osteogenic differentiation. The effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis. However, there has been relatively little investigation of this phenomenon with respect to tenogenesis. Sharma et al. studied the effect of tenogenesis on collagen coated polyacrylamide substrate with modulus gradient in a modulus range less than 0.1 MPa. On the other hand, it is known that the native modulus of the tendon tissue to be as high as 1200 MPa. Therefore, studies to date do not cover a modulus range that is more compatible with the tendon tissue environment.

The literature also reports that unidirectionally aligned fibrous topography as another tenogenic differentiation cue. Tong et al. reported that tendon cell differentiation of hMSCs occurs on collagen coated PDMS substrates which replicate native tendon surface. Yin found that aligned substrate promotes tenogenesis in tendon/stem progenitor cells (TPSC) but did not evaluate MSCs. Furthermore, aligned electrochemically compacted collagen fiber, electrospun polyurethane (PU), silk fibroin, poly (l-lactic acid) and two-dimensional...
(2D) microgrooved surface showed elongated cell morphology and tendon related ECM formation.\textsuperscript{12–19}

To date, the effects of matrix alignment and matrix modulus on tenogenesis have been investigated separately and the synergy between the two variables in terms of inducing tenogenic differentiation is not studied. Our research group created a collagen based material platform to investigate the effect of modulus in a broad range of 0.1 MPa to 100 MPa while controlling the topographical alignment random or unidirectional. In this method, collagen solutions are transformed to highly dense randomly oriented collagen sheets by electrochemical compaction induced by planar electrodes.\textsuperscript{20–22} Matrix alignment is introduced in these sheets by controlled unidirectional stretch of the sheets as molecular alignment lacks when planar electrodes are used to fabricate sheets.\textsuperscript{21} The aim of the current study is to study the effects of matrix modulus and alignment on tenogenic differentiation of human MSCs. Importantly, a broad modulus range of 0.1 MPa to 100 MPa is covered logarithmically for the first time in the literature. Elucidation of the mechanism of topographically induced tenogenesis in human MSCs as such will assist in optimizing materials to produce scaffolds for tendon repair and may provide insight into the differentiation mechanisms of MSCs and other stem cells \textit{in vivo}.

\section*{2. Materials and Methods}

\subsection*{2.1 Collagen Sheet Fabrication by Electrochemical Compaction [Figure 1]}

Electrocompacted collagen sheet was fabricated by the method as described before.\textsuperscript{21} Briefly, two-fold diluted (with RNAase/DNAase free water) acid soluble monomeric Type-I collagen solution (Collagen Solutions, San Jose, CA; 6 mg/ml) was adjusted for pH 8–10 using 1 N NaOH and dialized using ultrapure water for 18 hours. The collagen solution was then used for electrocompaction.

Collagen sheet was fabricated in sheet form by electrocompaction method described before.\textsuperscript{20, 23} Briefly, a 30×10×1.5 mm rectangular window was cut in a plastic piece. The plastic piece was then sandwiched between two planar carbon electrodes by filling the rectangular window with the collagen solution. A 30 VDC electrical potential was applied across the electrodes for 2 min. Collagen molecules are electrophoretically mobilized and compacted due to pH gradients established between the two electrodes under the mechanisms detailed in an earlier publication.\textsuperscript{24} 100–200 microns thick collagen sheets of 30×10 mm dimensions were generated by the electrocompaction. Collagen molecules are randomly oriented within the plane of the sheet. In one of the experimental groups, collagen molecules were unidirectionally aligned by stretching the sheet using a motorized mechanical device as described earlier (Figure 1 & 2). Collagen sheet samples were incubated in phosphate buffered saline (PBS) for six hours at 37 °C to induce fibril formation and treated with 2-propanol solution for 12 hours. Different levels of modulus were attained by crosslinking the sheets as will be described.
2.2 Synthesis of Collagen Gel

Elastically most compliant group was the standard collagen gel which was not subjected to
electrocompaction. Acid soluble monomeric collagen solution was mixed with 10× PBS at
8:1 ratio. pH was adjusted to 7.4 using 0.1 N NaOH. The collagen solution was then placed
in at 37 °C for 1h by pouring in a petri dish to form gel. The gel was then crosslinked with
genipin in 95% ethanol for 3-days as detailed in Table 1.

2.3 Experimental Groups

Four levels of modulus were targeted (0.1, 1, 10 and 100 MPa) for randomly aligned
collagen sheets to investigate the effect of modulus on tenoinduction. These modulus levels
were attained by changing the crosslinking protocols [Table 1]. Crosslinking was carried out
by genipin (Wako Chemical, Japan) in 90% v/v ethanol solution at 37 °C. The fifth group
was a unidirectionally aligned collagen sheet group at 10 MPa along the axis of alignment to
assess the effects of anisotropy. Samples were treated with per acetic acid (Sigma Aldrich,
USA) ethanol solution (2% Acetic acid+96% Ethanol) after the cross linking to bleach out
extra genipin which may keep crosslinking the samples.

2.4 Imaging of 10 MPa Compacted Collagen Sheets for Molecular Alignment

Polarized optical microscope (Olympus BX51, Melville, NY, USA) was used to assess the
alignment of collagen molecules at 10 MPa aligned group as described before. Briefly, as
collagen is a positive birefringent material, the aligned molecules shows blue interference
color along the slower axis of first order wavelength gypsum plate. Therefore, molecular
alignment is indicated by blue in the Compensated Polarized Imaging (CPI) and lack of
alignment is indicated by Magenta color. Alignment was also confirmed by scanning
electron microscopy (SEM-FEI Nova Nanolab 200, Hillsboro, Oregon).

2.5 Assessment of Mechanical Properties

Collagen sheet samples described in Table 1 were tested at a strain rate of 10 mm/min under
monotonic tension (Rheometrics Inc., NJ) (n = 6–8 samples per group) to assess the
attainment of targeted modulus values. Samples were cut into 20 × 2 mm strips and hydrated
in deionized water for 30 minutes before testing. A custom-made micrometer was used to
measure the thickness of the sheet samples where the micrometer closes an electrical circuit
upon contact with the surface of the sample. Stress-strain curves were constructed using the
load-displacement data and sample geometry. Modulus was calculated from the slopes of the
linear regions of stress strain curves.

2.6 Effect of Matrix Modulus and Alignment on Tenogenic Differentiation of Human MSCs

Samples were sterilized in 70% ethanol for 4 hours, washed in 1× PBS and placed into
ultralow attachment 24 well plates (Corning) (n = 3 wells/group). Human mesenchymal
stem cells (MSCs) (Lonza, Walkersville, MD) at passage 5 were seeded at a density of
20,000 cells/cm². The culture medium composed of alpha MEM (Invitrogen) supplemented
with 10% MSC-Qualified FBS (Invitrogen), 1% penicillin/streptomycin and 50 μg/mL
ascorbic acid. Cells were cultured for 21 days with medium changes every 3 days.
Tenogenic differentiation was assessed by RT-PCR at day 3, 14 and 21. At time points 3, 14
and 21 days total RNA was extracted by lysing the cells using TRIZOL reagent (Invitrogen) following manufacturer’s protocol and as described before. cDNA was synthesized from 2 µg of total RNA by reverse transcription using the cDNA Reverse Transcription Kit (Applied Biosystems). Taqman real time PCR mastermix (Applied Biosystems) and Taqman gene expression assays (Applied Biosystems) were used to evaluate the expression of the genes by quantitative real time PCR (Applied Biosystems 7500 Real Time PCR System). For tendon related markers (Collagen I, Collagen III, and COMP) and tendon specific markers (Scleraxis, Thrombospondin) were used. To assess MSC differentiation to chondrogenic, osteogenic adipogenic lineages expressions of COL2, RUNX2 and PPARγ were studied. 2 deltaDeltaCt method was used to quantify relative fold change in the target gene expression by normalizing the target gene expression to RPLP0 as a housekeeping gene and relative to the expression on 0.1 MPa group at day 3.

2.7 Effect of Matrix Modulus and Alignment on Tendon Related Matrix Synthesis

Based on the results of RT-PCR, 1 MPa group was not included in matrix synthesis study. MSCs were seeded on 0.1 MPa, 10 MPa, 10 MPa aligned and 100 MPa groups as described above. MSCs were cultured on the specified groups for 21 days as described in section 2.6. Tendon-related extracellular matrix molecules-i) type-I collagen, ii) type-III collagen and iii) Thrombospondin-4 (TSP-4) were investigated. Immunohistochemistry was performed for type-I and type-III collagen, and for TSP-4 green immunofluorescence was performed.

Samples were fixed and permeabilized in 10% neutral buffered formalin and 0.25% Triton X-100 (in PBS) respectively as described before. Samples were blocked in Phosphate Buffered Saline with Tween (PBST-5% BSA, 22.52 mg/ml glycine and 0.1% Tween 20) for 30 min and then incubated overnight at 4°C with primary antibody. Mouse Anti-Col1A1 (Abcam), rabbit anti-Col III (Abcam), Thrombospondin 4 (Santa-Cruz) were used as primary antibody. Negative control for the secondary antibody was samples incubated with blocking solution without primary antibody. Background negative control was collagen sheet without cells. Alkaline phosphate substrate-chromogen staining was performed using StayRed/AP kit (Abcam) according to manufacturer’s recommendations and as described before. After staining, samples were rinsed in PBS and an Olympus IX83 digital microscope (Olympus Life Science) was used to image the samples using CellSens Dimension software (Olympus Life Science). Anti-Rabbit Alexa-488 conjugated specific secondary antibody (Pierce Protein Biology, Thermo fisher Scientific) was used for immunofluorescence. The samples were then visualized under the same Olympus IX83 digital microscope.

ImageJ (NIH, Maryland, USA) was used to quantify protein staining. ImageJ colour thresholding plug in was employed to calculate the amount of type-I and type-III collagen staining. Corrected total cell fluorescence (CCTF) technique was used to measure the amount of TSP-4 of each cell seeded on different group. Briefly, the CCTF of a cell was measured by deducting the average fluorescence of the background around the cell from the average fluorescence of the cell area. For all the quantifications the areas of interest were picked randomly across the samples.
2.8 Statistical Analysis

One-way analysis of variance (ANOVA) was performed for RT-PCR data and Tuckey’s post hoc analysis was performed for pairwise comparison. Significance was set at p<0.05.

For COL I and COL III quantification Mann-Whitney U test was conducted to compare difference between groups (p<0.05). In case of CCTF quantification of TSP-4, as cell data are involved, a Bonferroni correction was applied and statistical significance was set at p<0.025. Minitab Statistical package (Minitab Inc., State College, PA, USA) was used to perform the statistical analysis.

3. Results

The mechanical test results [Table 1] showed that the targeted modulus levels were attained over 4 order of magnitudes.

Planar stretching introduced molecular as well as fibrillar alignment evident by CPI and SEM image respectively [Figure 2].

SCX expression at day 3 was greater for 100 MPa than for 0.1 MPa (p = 0.02) [Figure 3A]. There was no significant difference between 10 MPa aligned and 10 MPa random groups indicating that alignment doesn’t have any effect at 10 MPa.

COL I expression at day 3 was the lowest for 0.1 MPa level and the highest for 100 MPa [Figure 3B]. Expression at 1–10 MPa range modulus values were positionned intermediately. Between each modulus level, there was about 2-fold upregulation in COL I expression. At day 14, the expression was grouped over two levels, 0.1–1 and 10–100 MPa and there was about 2-fold upregulation between these two modulus levels. By day 21, there was no significant difference between any of the groups. Alignment did not have any effect on COL I expression at 10 MPa groups.

COL III expression was the highest for 100 MPa modulus and it showed more than 2-fold upregulation than 0.1 MPa at different time points [Figure 3C]. Alignment favored COL III expression such that the expression for aligned 10 MPa group at day 14 was two-fold greater than that for random 10 MPa group.

COMP expression for 100 MPa group showed 2 and 3-fold greater upregulation than that of random 0.1–10 MPa groups at day 14 and 21 respectively [Figure 3D]. Alignment increased COMP expression such that 10 MPa aligned group’s expression was greater than 10 MPa random group’s expression. Furthermore, aligned 10 MPa group’s COMP expression did not differ from that of 100 MPa group.

There was a gradual increase in TSP-4 expression with increasing modulus [Figure 3E]. Alignment also significantly favored TSP-4 expression (p = 0.04).

None of the groups showed COL2 expression at any time point. At day 21, RUNX2 expression for 100 MPa group was significantly greater than that of the other groups.
PPARγ expression was detectable at day 3 only and there were no significant differences between the expressions of experimental groups [Figure 4].

After 21 days of culture, Type-I collagen production increased by 2-fold in 10 MPa and 3-fold in 100 MPa group in comparison to the production by the 0.1 MPa group [Figure 5&6]. Alignment increased semi-quantitative COL I staining measurement significantly (p = .031). In case of COL III, there was a gradual increase in semi-quantitative measure of collagen staining with increasing modulus with a 2-fold increase from 0.1 to 100 MPa group [Figure 5&6]. In this case also, alignment increased semi-quantitative COL III staining measurement (p = .034). In case of TSP-4, there was no significant difference between 0.1–10 MPa while 100 MPa showed 4-fold greater TSP-4 production than that of the 0.1 MPa group [Figure 5&6]. There was no significant difference in TSP-4 immunofluorescence level of cells between aligned and random group.

4. Discussion

Natural tendon is composed of highly stiff and aligned type I collagen fibrils. In this study we investigated both the effect of modulus and alignment of type I collagen substrate for tendon differentiation. This study showed that mimicking the tendon topography, i.e. increasing the substrate modulus as well as alignment increased the differentiation of human MSCs to a tenogenic lineage.

Past literature reported the effect of modulus on differentiation (e.g. muscle, neuron) in the kPa range which was then followed by glass which has a modulus around 90 GPa. Therefore, there is a gap in the literature on substrate modulus effects in the range of 100 kPa to 100 MPa range in terms of cellular response. This study not only fills the gap of the modulus ranges that had been studied to date, but also incorporates matrix alignment to investigate tendon differentiation.

Previous studies demonstrated that increasing modulus generally increases cell adhesion, proliferation and multilineage differentiation. Very few of these studies investigated the effect of modulus on tenogenic differentiation on collagen substrate which is the major tendon ECM. Moreover, the studies that investigated tenogenic differentiation used substrate modulus (10–80 kPa) levels which are orders of magnitude softer than tendon and did not consider synergistic effect of alignment. This is the first study to our knowledge, which considers modulus of collagen substrate to MPa level as well as investigates effect of substrate alignment on tendon differentiation.

The current study fabricated type I collagen substrates with modulus values encompassing three orders of magnitudes of change beginning from hundred kPa, to hundred MPa. Moreover, by introducing planar stretch, we were able to generate aligned substrates. Therefore, the collagen material model developed in this study allowed for systematic investigation of the effects of matrix modulus and anisotropy tenogenic differentiation.

Scleraxis is tendon progenitor and important for tendon development. Sharma et al. showed increased SCX expression with increasing collagen coated substrate modulus in their studies related to tendon differentiation. Recently, Chen et al showed arterial
stiffening via SCX upregulation\textsuperscript{48}. This indicates that, substrate modulus is associated with SCX expression. This study showed early increase in the scleraxis expression as at day 3 stiffer groups showed higher expression. Similarly, MSCs seeded on highly anisotropic collagen fibers\textsuperscript{12,22}, knitted silk collagen scaffold\textsuperscript{49} and on culture plates\textsuperscript{50} also induced early increase in scleraxis expression. However, the modulus was not varied systematically in these studies. This study also showed that scleraxis expression levels between groups by day 21 (Figure 3A). Therefore, stiffer substrates seem to benefit tenogenesis by expediting the inception of MSC to tendon differentiation cascade.

Tendon is mostly composed of type-I collagen. Studies showed that matrix stiffening increases collagen I synthesis\textsuperscript{51} and with aging collagen turnover decreases in tendon with decreasing modulus\textsuperscript{52}. The current study showed increased COL I expression with increasing modulus such as 100 > 1–10 > 0.1 MPa at day 3 and 100 > 10 > 0.1–1 MPa at day 14. This indicates that stiffer matrices induce earlier and greater levels of COL I expression than compliant matrices [Figure 3B]. From the protein production data, at day 21, there was a gradual increase in semi-quantitative measure of COL I staining from 0.1 MPa to 100 MPa. At the production level, alignment showed upregulation of semi-quantitative measure of COL I staining and semi-quantitative staining measurement in 10 MPa aligned group was similar to 100 MPa group. This increase in COL I synthesis may be due to the alignment of the matrix as indicated by previous studies which showed that aligned matrix increased tenogenic differentiation\textsuperscript{12,22,53}.

Type-III collagen plays a crucial role for type-I collagen fibrilogenesis\textsuperscript{54,55} and thus another major tendon-associated collagen along with type-I collagen. During tendon injury type-III collagen is produced to quickly repair the damage\textsuperscript{56,57} and after long periods it is remodeled to type-I collagen\textsuperscript{56,58}. In the current study, the trend in COL III expression as well as production was similar to that of COL I. Moreover, in the expression level, 10 MPa aligned group showed similar expression as 100 MPa group both at day 3 and 14. In the production level, 10 MPa aligned group showed higher expression than 10 MPa unaligned at day 21. This indicates that alignment also helps in tenogenesis. Previous studies also showed similar outcome as stiffer matrix\textsuperscript{5} and aligned matrix\textsuperscript{12} increased COL III expression. It can be noticed that the amount of COL III synthesis is higher than COL I. Whether or not COL III is remodeled to COL I as is the case in tendon injury repair sites would require longer duration studies.

TSP-4 is one of the main tendon-related genes\textsuperscript{59} as it shows highest expression in tendon ECM than other tissue types\textsuperscript{59–61}. Cells seeded on decellularized tendon slices, engineered scaffold-free tendon tissue and collagen matrix showed upregulation of TSP-4\textsuperscript{62,63,64}. The current study also showed TSP-4 upregulation both with modulus and alignment after day 21 [Figure 3E]. In matrix synthesis study, after day 21, only 100 MPa group showed higher level of TSP-4 immunofluorescence (Figure 5&6) than the other groups. In tendon, TSP-4 binds to collagen and form complexes with COMP\textsuperscript{61,65}. Smith et. al suggested that COMP has an organizational role in tendon formation as well as COMP is necessary for tendon to resist load\textsuperscript{66,67}. The current study shows, upregulation of COMP expression with stiffer (100 MPa) and aligned (10 MPa aligned) matrix [Figure 3D]. This indicates that, higher
modulus and alignment mimicked tendon topography to an extent and had an influence on tenogenic differentiation.

The level of differentiation induced by alignment was similar to that induced by stiffer but unaligned substrates. Our group and other groups have reported on tenogenic effect of substrate driven cytoskeletal alignment on MSCs previously\textsuperscript{5, 11–16, 21, 68, 69}. Collagen molecules are packed densely when they are registered in parallel (i.e. aligned) in comparison to random. Thus, it is likely that aligned matrix is able to present integrin binding sites more densely than unaligned random configuration. Denser presentation of adhesion sites would result in stronger focal adhesions which in turn is reported to be conducive to actin polymerization\textsuperscript{35, 70, 71}. On stiff substrates, RhoA and Rho-associated kinase (ROCK) activation promotes actomyosin stress fiber assembly and demonstrates long parallel actin stress fiber with elongated cellular shape\textsuperscript{4, 39, 72}. RhoA/ROCK is major molecular pathway that promotes matured focal adhesion, organized stress fibers and elongated cells and this pathway is involved in topography-mediated MSC differentiation\textsuperscript{4, 39, 73–75}. Future studies will test the hypothesis that alignment, independent of stiffness, can activate RhoA/ROCK pathways by presenting integrin adhesion sites more densely due to ordered packing of collagen molecules.

Expressions of chondrogenic, osteogenic and adipogenic markers suggested that the MSCs used in this study did not seem to differentiate towards cartilage, bone or fat formation on the collagen matrices presented to them at various modulus levels. Type II collagen is one of the major markers for chondrogenic differentiation. Differentiating MSCs to chondrogenic lineage requires chondrogenic differentiation media supplemented with growth factors and high density cell seeding \textsuperscript{76–81}. Lack of type-II collagen expression is not surprising given that no chondrogenic growth factors were used and that cell seeding density was low. Only at one time point, RUNX2 was upregulated for 100 MPa group suggesting that such elevated stiffness level my favor osteogenic differentiation. Rigid substrates were reported to provide higher force feedback to cells which in turn increase cell centrality, cell spreading and bone morphogenetic protein (BMP) expression which finally activates RUNX2\textsuperscript{11, 74, 82}. It is interesting to notice that although both 100 MPa and 10 MPa aligned group showed similar level of tenogenesis, only 100 MPa group showed upregulation of osteogenesis. Therefore, it can be speculated that while some level of stiffness is necessary for tenogenesis whereas matrix alignment may be more crucial for tenogenesis than stiffness. Suppression of osteogenesis due to cellular alignment is implied by findings of previous in vitro studies where bone markers were down regulated on aligned collagen threads which have comparable or higher modulus than 100 MPa\textsuperscript{12}. Furthermore, in vivo studies utilizing aligned collagen threads with 100+ MPa stiffness did not result in ectopic mineralization\textsuperscript{83}. PPAR\textgreek{y} is an adipogenic transcription factor\textsuperscript{84, 85} which was expressed only at the earliest time point of day 3. This outcome suggests that MSCs have some adipogenic propensity at the baseline; however, the material model developed in this study does not promote adipogenic differentiation over time.

One limitation of this study was that it did not capture the 3D nature of tendon matrix, which is a common limitation to most biomaterials, except for hydrogel-cell mixtures. On the other hand, hydrogel systems are compliant and it is challenging to attain modulus levels greater
than 1 MPa with most known hydrogel formulations. In future studies, this limitation can be addressed by sandwiching cells between two collagen sheet layers. It may be possible that the stiffness-related outcomes we identified in the current 2D settings may be different from the presentation of stiffness in 3D. Another limitation of the study is that the experimental design of the study included aligned group only for 10 MPa group. The absence of aligned groups at other modulus levels prevents us from reaching any conclusions on synergistic effects alignment and modulus.

Aligned electrocompacted collagen is being developed as a growth-factor free platform to induce tenogenesis topographically. Aligned collagen threads have been implanted in the tendon environment in braided form in vivo. In such orthotopic environment excellent biocompatibility and tissue integration was observed. In environments where endogenous tendon cells or MSCs are absent, the material can be used for priming patient-derived MSCs prior to implantation to treat challenging tendon defects, such as irreparable defects of the rotator cuff. Therefore, aligned and electrocompacted collagen based biomaterial developed in this study has a potential to be use in tendon tissue engineering applications both as a cell-seeded carrier matrix or acellular repair matrix to promote tenogenesis.

5. Conclusions

The current study demonstrates tenogenic differentiation is influenced by both modulus and alignment of the substrate modulus. This material platform developed in this study mimicked both the modulus anisotropy as well as the modulus of the natural tendon and investigated the synergistic effect of these two key factors of tenogenic differentiation. Therefore, the tenoinductive collagen material model developed in this study can be used in the research and development of tissue engineering tendon repair constructs in future.

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References


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Statement of Significance

Although the effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis, there has been relatively little investigation on the effects of this phenomenon on tenogenesis. Furthermore, a relative role of topographical factors (matrix alignment vs. matrix modulus) in inducing tenogenic differentiation is not well understood. We investigated the effects of modulus and topographical alignment of type I collagen substrate on tendon differentiation. This study showed mimicking the tendon topography, i.e. increasing the substrate modulus as well as alignment increased the tenogenic differentiation. Therefore, the tenoinductive collagen material model developed in this study can be used in the research and development of tissue engineering tendon repair constructs in future.
Figure 1.
Overview of fabricating collagen sheets with different modulus levels.
Figure 2.
Manufacturing aligned sheet with mechanical stretching. Molecular alignment is evident by CPI image (Emergence of blue color after stretching indicates molecular alignment). Collagen fibril alignment is evident by SEM images.
Figure 3.
Effect of collagen matrix modulus and anisotropy on tenogenic differentiation of human MSCs. (A) Scleraxis, (B) Collagen-I, (C) Collagen-III, (D) COMP, (E) Thrombospondin-4. Anisotropy and increasing modulus improved tenoinduction. Statistical differences are highlighted by horizontal lines with the corresponding p values. Statistical significance was set at p <0.05.
Figure 4.
Differentiation of human MSCs seeded on collagen matrix towards lineages other than tenocytic differentiation (A) RUNX2, (B) PPARγ. Statistical differences are highlighted by horizontal lines with the corresponding p values. Statistical significance was set at p <0.05.
Figure 5.
Effect of modulus on long term matrix synthesis. After day 21 days of hMSC culture, higher modulus favored matrix synthesis. For collagen I, stiffer groups showed thick collagen fiber formation. For TSP-4, 100 MPA group showed more production. For Collagen III also 100 MPa showed maximum synthesis and alignment (10MPa aligned) increase matrix synthesis. Scale bar 50 μm. Single headed white arrow indicates collagen synthesis; for type-I and type-III collagen. Alignment direction of 10 MPa aligned group is shown by white arrow.
Figure 6.
After 21 days of hMSc culture, 100 MPa and 10 MPa aligned groups showed 3-fold increase in semi-quantitative measurement of staining of Type-I collagen than 0.1 MPa group; cells on 100 MPa group showed 4-fold increase in TSP-4 production than 0.1 MPa group indicated by corrected total cell fluorescence (CCTF); and type-III collagen showed 2-fold upregulation from 0.1 MPa to 100 MPa with a slightly increasing trend in aligned group compared to unaligned group.
Table 1

Experimental groups for different level of modulus range and unidirectionally aligned substrate

<table>
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<th>Groups</th>
<th>Target Modulus (MPa)</th>
<th>Experimental Modulus (MPa)</th>
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<tr>
<td>1</td>
<td>Randomly oriented gel (3day, 0.6% genipin crosslinked)</td>
<td>0.1</td>
<td>0.1 ± 0.04</td>
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<td>2</td>
<td>Randomly oriented compact sheet (1hr, 0.1% genipin crosslinked)</td>
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<td>1.1 ± 0.18</td>
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<tr>
<td>3</td>
<td>Randomly oriented compact sheet (6hr, 0.6% genipin crosslinked)</td>
<td>10</td>
<td>10.4 ± 2.63</td>
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<td>Highly anisotropic and aligned compact sheet (1hr, 0.3% genipin crosslinked)</td>
<td>10</td>
<td>11.8 ± 2.44</td>
</tr>
<tr>
<td>5</td>
<td>Randomly oriented compact sheet (3 day, 2% genipin crosslinked)</td>
<td>100</td>
<td>92.9 ± 15.24</td>
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