Supporting Information

An anisotropic nanocomposite hydrogel guides aligned orientation and enhances tenogenesis of human tendon stem/progenitor cells

Yichi Xu[‡a,b], Heyong Yin[‡c,d], Jin Chu[c], David Eglin[a], Tiziano Serra[*a], Denitsa Docheva[*c]

[a]AO Research Institute Davos, Clavadelerstrasse 8, Davos Platz, Switzerland
[b]Department of Plastic and Reconstructive Surgery, The First Medical Center, Chinese P.L.A. General Hospital, Beijing, China
[c]Experimental Trauma Surgery, Department of Trauma Surgery, University Regensburg Medical Centre, Regensburg, Germany
[d]Department of Orthopedics, Beijing Friendship Hospital, Capital Medical University, Beijing, China

Corresponding authors:
Tiziano Serra, E-mail address: tiziano.serra@aofoundation.org
Denitsa Docheva, E-mail address: denitsa.docheva@ukr.de

‡These authors contributed equally to this work.

S1.Gene name abbreviations

Table S1. List of target genes, full names and abbreviation

Target gene	Abbreviation	Category
Actin alpha 2	ACTA2	Tenogenic-related genes
Cartilage oligomeric matrix protein	COMP	
Ephrin type-A receptor 4	EPHA4	
Proteoglycan 4	PRG4	
Thrombospondin 2	THBS2	
Thrombospondin 4	THBS4	
Tenascin C	TNC	
Transforming growth factor beta 1	TGFB1	
Tenomodulin	TNMD	
Collagen type I alpha 1	COL1A1	Collagen genes
Collagen type III alpha 1	COL3A1	
Collagen type V alpha 1	COL5A1	
Collagen type VI alpha 1	COL6A1	
Collagen type XII alpha 1	COL12A1	
Collagen type XIV alpha	COL14A1	
Collagen type XV alpha 1	COL15A1	
Early growth response 1	EGR1	Transcription factor genes
Early growth response 2	EGR2	
Eyes absent homolog 1	EYA1	
Eyes absent homolog 2	EYA2	

Mohawk homeobox	MKX	
Scleraxis homolog A	SCX	
SIX homeobox 1	SIX1	
SIX homeobox 2	SIX2	
Aggrecan	AGCAN	Chondrogenic-related genes
Collagen, type II, alpha 1	COL2A1	
Sex determining region Y box 9	SOX9	
Lipoprotein lipase	LPL	Adipogenic-related genes
Peroxisome proliferator-activated receptor gamma	PPARG	
Transcription factor AP-2 alpha	TFAP2A	
Integrin-binding sialoprotein	IBSP	Osteogenic-related genes
Runt-related transcription factor 2	RUNX2	
Sp7 transcription factor	SP7	
Desmin	DES	Myogenic-related genes
Myogenic differentiation 1	MYOD1	
Myogenin	MYOG	
Fucosyltransferase 4	FUT4	Embryonic-related genes
Nanog homeobox pseudogene 8	NANOG	
POUdomain, class 5, transcription factor 1	POU5F1	
Asporin	ASPN	Collagen cross-linking genes
Biglycan	BGN	
Decorin	DCN	
Fibromodulin	FMOD	
Fibronectin	FN	
Lumican	LUM	
Lysyl oxidase	LOX	
Procollagen-lysine 5-dioxygenases	PLOD1	
Transglutaminase 2	TGM2	

S2. Cell isolation and expansion

Human TSPCs isolated from non-ruptured Achilles tendon biopsies of three young and healthy human patients were previously described and profoundly characterized by Kohler *et al.* [1] with approval by the Ethical Commission of the LMU Medical Faculty (Ethical Grant No. 166-08). TSPCs were isolated according to established protocols described previously by Kohler et al [1]. Briefly, Achilles tendon tissue was minced into small pieces, digested with 0.15% collagenase II (Worthington, Lakewood, NJ, USA) over night in a 37°C incubator, filtered with 100µm pore size nylon mesh, and centrifuged for 10 min at 500g. The cell pellet was then resuspended in DMEM/Ham's F-12 (1:1 mixture) supplemented with stabile glutamine, 1% MEM amino acids (Biochrom, Berlin, Germany), 10% FBS, and 1% L-ascorbic acid-2- phosphate (Sigma-Aldrich, Munich, Germany), and TSPCs were maintained at a constant temperature of 37°C under 5% CO2 humidified atmosphere. Cells at passage 8–10 were used in the experiments.

S3. Preparation of Iron Oxide nanoparticles (IOPs)

PEG capped IOPs were synthesized using a co-precipitation and solvothermic method as described previously [2]. Briefly, 10^{-1} M ferric and 5×10^{-3} M ferrous chloride were dissolved in a mixture of diethylene glycol (DEG) and ethylene glycol (EG) with a ratio of 30:10 (w/w). Next, one molar sodium acetate was added and the solution was stirred thoroughly at room temperature for 3 hours. Afterwards, 2.5% PEG (MW = 4000 g.mol-1) was added in the case of PEG-capped IOPs and the solution placed in a ventilated oven at 190 °C for 6 hours. Finally, a dark black precipitate was collected by a magnet and washed 3 times in isopropyl alcohol and 3 times in ultrapure Milli-Q water.

S4. Cultivation of hTSPCs in the anisotropic COL I-IOPs nanocomposite hydrogels

For the cultivation of hTSPCs in the COL I-IOPs nanocomposite hydrogels, 1 ml mixture of IOPs (0.3 mg/ml), hTSPC (1×10^6/ml) and COL I (1.25 mg/ml) were mixed thoroughly. The pH was adjusted to 7.4 using 1 N NaOH (Sigma). The induction of biological anisotropy of the nanocomposite hydrogel was achieved under remote magnetic field: two pieces of neodymium magnet (NdFeB, a permanent magnet made from an alloy of neodymium, iron, and boron forming the Nd2Fe14B tetragonal crystalline structure) were set on a Teflon plate in an ice box with a distance of 5 cm, which provided 0.02 Tesla linear and homogeneous magnetic field environment. Briefly, a mixture of COL I-IOPs-hTSPCs in a petri dish (35 mm diameter) was placed in the middle of the two magnets. After 20 minutes of exposure to the magnetic field in the ice box, the mixture was then transferred into 37℃ incubator and cross-linking and gelation occurred in approx. 15 minutes.

HTSPCs embedded in COL I with random IOPs (COL I-R/IOPs-hTSPCs) or pure COL I (COL I-hTSPCs) served as control groups.

S5. Cell viability and proliferation assays

Cell viability and proliferation were assessed by Live/Dead staining (Sigma, St. Louis, USA) and Resazurin cell metabolic assay. For Live/Dead staining, the whole hydrogels were washed with PBS 3 times and then stained for 5 min at room temperature in 10μl/ml Calcein AM and 1mm Ethidium homodimer (EthHD). To ensure the uniform and smooth diffusion of the staining dye during the whole process, the samples incubated under 4℃ in dark for 3 hours first, and then incubated in a 5% CO2 incubator at 37℃ in dark for another 1 hour. Fluorescence images were taken with laser confocal microscope (LSM 800, Laser Scanning Confocal, Carl Zeiss Microscopy, Thornwood, USA). For quantification of cell viability for each study group and time point, three images per donor and three visual fields per image were analyzed by counting the number of green-labelled cells (live) and red-labelled cell (dead) and their sum provided the total cell number. The data was expressed in % of live cells from total cell number. Resazurin, a cell metabolic assay (Sigma-Aldrich), was performed according to the manufacturer's protocol. Live/Dead staining and Resazurin assays were repeated independently twice with 3 donors in triplicates.

S6. F-actin staining

F-actin staining was used to visualize cell morphology in the 3D hydrogels. After fixing with 4% paraformaldehyde (PFA) buffer solution, the sample was then washed twice with 1XPBS solution.Next, 0.25% TritonX-100 reagent was added at room temperature for 15 minutes to facilitate penetration. Afterwards, 1:500 diluted Phalloidin TRITC (red) dye (Life technologies) was

incubated with the samples at room temperature in dark for 45 minutes. Lastly, PBS solution was discarded and 1:500 diluted DAPI dye (working concentration: 1ug/ml) was used for nuclear counter-staining for 10 minutes in dark at room temperature. Fluorescence images were observed and photographed with Laser Confocal microscope (LSM 800, Laser Scanning Confocal, Carl Zeiss Microscopy, Thornwood, USA). F-actin staining was independently reproduced twice with the representative 3 donors in triplicates.

S7. Cell aspect ratio analysis

Cell aspect ratio (width/length) (Figure S1) was analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to demonstrate cell elongation during cultivation in the hydrogels over a period of 7 days. Three donors per group, each represented by three images, were analyzed manually with an Image J measure tool at three different time points (day 0, 3 and 7). The data was expressed as aspect ratio and represents the average of 90 cells/group.

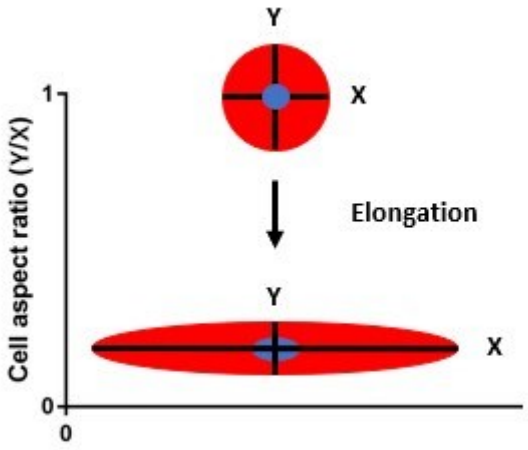

Figure S1. Schematic diagram of measurement and calculation of cell aspect ratio.

S8. Formation of cell rows or chains

The formation of cell rows or chains (indicative of cell fusion index) was analyzed with F-actin images. The number of individual cells (n1), cell chain of 2 or more cells (n2) and total nuclei (n3) from each view were counted, then the value of (n1 + n2)/ n3 was calculated to evaluate cell row formation, as per formula the lower the result value is, the higher is the cell chain formation, which is indicative of increased cell fusion via cell-cell contact. Three different horizons were randomly selected from each cell donor, then calculated the number of rows and number of cells in each row, as the X axis and Y axis respectively, to draw dot distribution, further reflected the degree of cell linear alignment.

S9. Bright-field optical microscopy for analysis of IOPs alignment

The alignment of IOPs was evaluated with a bright-field optical image and analyzed using two-dimensional fast Fourier transform (2D-FFT) performed with ImageJ software [2]. The pictures were acquired with an optical microscope (AxioVert. A1, 3846001621, Carl Zeiss, Germany). All the images were initially converted to grayscale and an unsharped mask with a radius of 20-30 pixels

was applied. Then, a 2D-FFT was performed on the original images, the resulting 'frequency' domain images were tilted of 90°, to invert the intrinsic rotation due to the 2D-FFT. The pixel intensities were summed along the radius for each angle of the circular projection and plotted as a function of the corresponding angle of acquisition using Oval Profile plugin [3]. In order to avoid differences in sample thickness, camera performance, operator preference for contrast/brightness, and other variables, all the alignment plots were normalized and shifted to a straight baseline of zero [4]. The fibers diameter distribution was obtained measuring manually the length of the fibers along the minor axis of the elongated IOPs pathways.

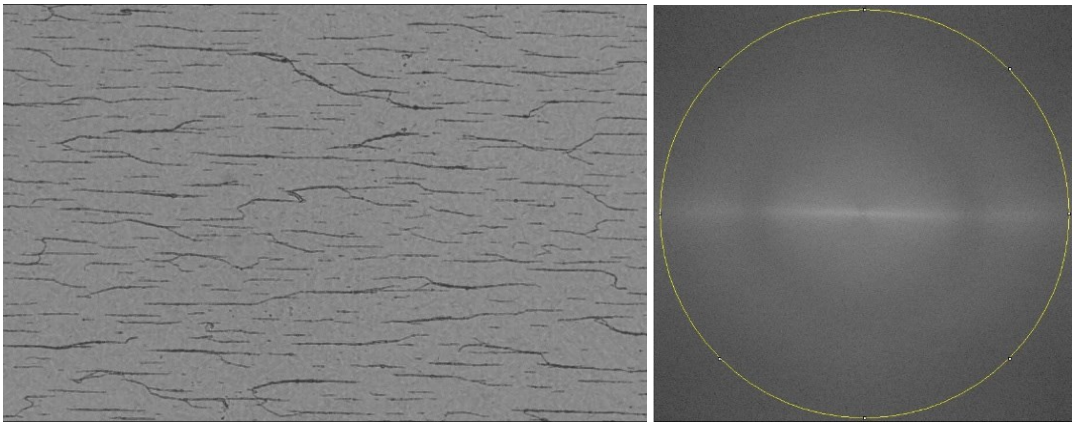

Figure S2. Aligned IOPs processed by 8-bit gray scale image and the schematic diagram of gray scale superposition after fast Fourier transform with ImageJ software. The yellow circle indicated that the region of interest was selected for the next step of gray value quantization.

S10. RNAisolation and qPCR

The total RNA from the hydrogels with three hTSPC donors was extracted using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and used for qRT-PCR. For cDNA synthesis, 1 µg total RNA and a Transcriptor First-Strand cDNA Synthesis Kit (Roche) were used. Quantitative PCR of tenogenic, lineage, and cross-linking gene markers, as listed in table 1, was performed using RealTime PCR Ready Custom design plates with format 96-well/32+ [4] according to the manufacturer's instructions (Roche, Penzberg, Germany). Briefly, PCR reactions were pipetted on ice and each well contained 10 µl LightCycler 480 probes master mix, 0.2 µl undiluted cDNA and 9.8 µl PCR grade water. Plates were subsequently sealed and centrifuged down for 15 s at 2100 rpm. The ΔΔCt method ($2^{-\Delta\Delta Ct}$) was used to analyze the qPCR results. Gene expression was calculated as fold change compared to COL I gel. The PCR results were attained with 3 donors.

S11. Collagen type 1, collagen type 6 and Scleraxis staining

For immunofluorescence staining COL I-A/IOPs, COL I-R/IOPs, COL I-A/IOPs-hTSPCs and COL I-R/IOPs-hTSPCs hydrogels (two gels per group) were fixed at day 7 with 4% PFA/PBS, cryoprotected by sucrose gradient and embedded in cryoprotective media. Cryosections (10 µm thickness) were collected (cryotome Leica, Wetzlar, Germany), and stored at -20°C until use. Prior to staining, sections were equilibrated to room temperature and rehydrated with PBS for 5 minutes. For COL 1 and 6 staining, sections were blocked by 10% goat serum (Sigma-Aldrich, Germany, Lot. Nr. SLCD5403) at room temperature for 1 hour, and then incubated with corresponding primary antibodies (ab34710 for COL 1 and ab6588 for COL 6, Abcam, Germany) at 4℃ overnight (1:100

dilution). For SCX staining, permeabilization with 0.2% TritonX-100 reagent was performed at room temperature for 15 minutes. Afterwards, 10% goat serum and 1:100 diluted primary antibody (ab58655, Abcam) were applied as described above. Next, secondary antibody (1:200 diluted goat anti-rabbit secondary antibody conjugated with cy3, 111-165-144, Jackson ImmunoResearch, Germany, Lot. Nr. 111-165-144) was given for 1 hour at room temperature. Sections receiving only secondary antibody served as negative control. Lastly, DAPI (working concentration: 1ug/ml) was used for nuclear counter-staining for 10 minutes at room temperature. Representative fluorescence images were taken on inverted microscope equipped with CCD camera (Carl Zeiss Microscopy, Germany).

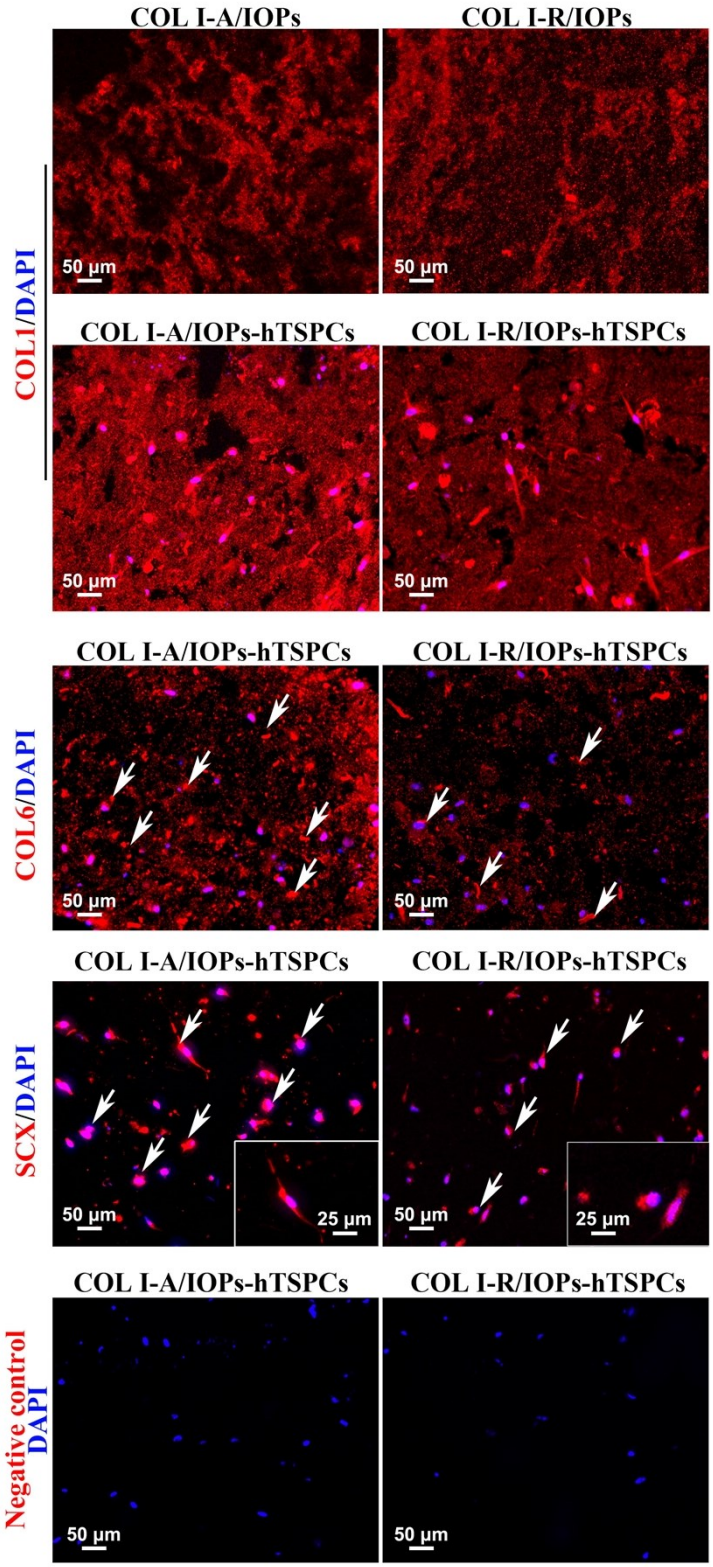

Figure S3. Representative fluorescent images. COL I was ubiquitously stained since the carrier used is 0.1% COL I hydrogel. COL 6 was deposited mainly in the pericellular matrix (white arrows). SCX expression was detected mainly in the nucleus (white arrows). Nuclear counterstaining: DAPI. Negative control: only secondary cy3 antibody.

S12. Statistical analysis

Data was presented as Mean ± Standard Deviation (SD). Statistical differences between two groups were determined using unpaired two-tailed non-parametric Student's t-test with GraphPad Prism (Version 7.00, GraphPad Software lnc., USA). Analysis of variance (ANOVA) was used to determine statistical significant between multiple groups. *$p<0.05$ indicates statistically significant difference between the groups, while #$p<0.05$ and ##$p<0.01$ indicates statistically significant difference between different time points in one group.

References

[1] Kohler J et al. Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration[J]. Aging Cell, 2013, 12(6:988-999.

[2] Tognato R et al. A Stimuli-Responsive Nanocomposite for 3D Anisotropic Cell-Guidance and Magnetic Soft Robotics. Advanced Functional Materials, 2019, 29(9:1804647.1-1804647.10.

[3] Ayres C E et al. Measuring fiber alignment in electrospun scaffolds: a users guide to the 2D fast Fourier transform approach. Journal of Biomaterials Science, Polymer Edition, 2008, 19(5):603-621.

[4] Hsieh C F et al. In Vitro Comparison of 2D-Cell Culture and 3D-Cell Sheets of Scleraxis-Programmed Bone Marrow Derived Mesenchymal Stem Cells to Primary Tendon Stem/Progenitor Cells for Tendon Repair. International Journal of Molecular Science. 2018, 19(8):2272.