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Total number of authors:  
14

Published in:  
Biomedical Materials (Bristol)

Link to article, DOI:  
[10.1088/1748-605x/ab6709](https://doi.org/10.1088/1748-605x/ab6709)

Publication date:  
2020

Document Version  
Peer reviewed version

[Link back to DTU Orbit](#)

### Citation (APA):

Haramshahi, S. M. A., Bonakdar, S., Moghtadaei, M., Kamguyan, K., Thormann, E., Tanbakooei, S., Simorgh, S., Milan, P. B., Amini, N., Latifi, N. A., Joghataei, M. T., Samadikuchaksaraei, A., Katebi, M., & Soleimani, M. (2020). Tenocyte-imprinted substrate: a topography-based inducer for tenogenic differentiation in adipose tissue-derived mesenchymal stem cells. *Biomedical Materials (Bristol)*, 15, Article 035014. <https://doi.org/10.1088/1748-605x/ab6709>

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To cite this article before publication: Seyed Mohammad Amin Haramshahi *et al* 2020 *Biomed. Mater.* in press <https://doi.org/10.1088/1748-605X/ab6709>

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# Tenocyte-imprinted substrate; a topography-based inducer for tenogenic differentiation in adipose tissue-derived mesenchymal stem cells

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## Abstract

Tendon tissue engineering based on stem cell differentiation has attracted a great deal of attention in recent years. Previous studies have examined the effect of cell-imprinted polydimethylsiloxane (PDMS) substrate on induction differentiation in stem cells. In this study, we used tenocyte morphology as a positive mold to create a tenocyte-imprinted substrate on PDMS. The morphology and topography of this tenocyte replica on PDMS was evaluated with scanning electron microscopy and atomic force microscopy. Then the tenogenic differentiation induction capacity of tenocytes replica in ADSCs was investigated and compared with other groups including tissue replica (Which was produced similar to the tenocyte replica and was evaluated by SEM), decellularized tendon, and BMP-12 as other potentially inducers. This comparison gives us an estimate of the ability of tenocyte-imprinted PDMS (which called cell replica in the present study) to induce differentiation compared to other inducers. For this reason ADSCs were divided into 5 groups including control, cell replica, tissue replica, decellularized tendon and BMP-12. ADSCs were seeded on each group separately and investigated by real-time RT-PCR after 7 and 14 days. Our results showed that in spite of the higher effect of growth factor on tenogenic differentiation, cell replica can also induce tenocyte marker expression (Scleraxis and Tenomodulin) in ADSCs. Moreover, tenogenic differentiation induction capacity of cell replica was greater than tissue replica. Immunocytochemistry analysis revealed that ADSCs seeding on cell replica for 14 days led to Scleraxis and Tenomodulin expression at the protein level. Also, immunohistochemistry indicated that contrary to the promising results *in vitro*, there was little difference between ADSCs cultured on tenocyte-imprinted PDMS and untreated ADSCs. The results of such studies could lead to the production of inexpensive cell culture plates or biomaterials that can induce differentiation in stem cells without growth factors or other supplements.

Keywords:

Cell and tissue Imprinting, Tenogenic differentiation, ADSCs

## 1. Introduction

The emerging aspect of musculoskeletal tissue engineering has been attempting to address serious tendon problems-which usually occur during exercise and other intense physical activities- through a variety of new techniques (1-3). Due to low cellularity and vascularity and limited innervation, the regeneration of tendon injuries is usually not performed spontaneously (4-7). Clinical interventions including autograft and allograft transplantation as well as suturing, are associated with some problems and obstacles such as donor site morbidity, infection risk, and injury relapse (8, 9). Hope for the production of engineered or three-dimensional personalized tendon tissues that are designed to promote the healing of damaged tissues rise due to a recent innovation in the field of regenerative medicine and material science (10, 11). To treat defects, various tendon substitutes were developed using synthetic and natural polymers such as poly-lactic glycolic acid (PLGA), Polycaprolactone (PCL)(12, 13), chitosan and collagen subclasses and deploying advanced technologies (14-17).

To develop functional tendon, cell-based tissue engineering can be the most promising solution. Since the tendon matrix is made by fully differentiated cells called tenocytes, these cells are the first choice to repair the tendon injuries(18). However, the isolation of tenocytes requires an invasive surgery to harvest the tendon tissue and results in donor site morbidity(19). Also the proliferation of tenocytes in the culture medium can be time-consuming and result in dedifferentiation of these cells (3, 18, 20). As a result, in recent years many studies have been focused on tendon tissue engineering using stem cells (21, 22). Adipose tissue-derived mesenchymal stem cells are considered as an available candidate for the stem cell-based strategies for tendon regeneration due to their large number in the adult body, high proliferation, and differentiation ability, and ease of isolation with little donor site morbidity (3, 23).

It is well understood that biochemical factors can change the behavior and fate of stem cells(24). Recent researches are promising in presenting that the tenogenesis could be improved through differentiation induction using BMP-12, BMP-13, BMP-14, TGF-beta III, and CTGF signaling molecules(25-28). Despite promising results, the use of growth factors has some limitations. Studies have shown that different growth factors are active in different phases of tendon healing process. Different growth factors play different roles in tendon healing(29). Therefore growth factors must be customized for each condition. In other hands, growth factors can trigger unwanted

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6 molecular pathways and lead to stem cell differentiation to other lineage(18, 30). Further studies  
7 are needed to improve the efficacy of growth factor-based approaches and to better understand the  
8 molecular pathways involved, as well as the effect of growth factors on the stem cell commitment  
9 process(31).

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12 In addition, microenvironment-mediated biophysical signals can also affect stem cell proliferation,  
13 migration, and differentiation (32-34). Currently, biomaterials are designed to transmit specific  
14 messages to the cells and control their behavior(2). Many studies have been performed to  
15 investigate the effect of biophysical factors on induction differentiation in stem cells. Islam and  
16 colleagues have shown the importance of surface stiffness on induction tenogenic differentiation  
17 in bone marrow mesenchymal stem cells (35). Other studies demonstrated that changing geometry,  
18 cell shape, and matrix elasticity in 2D substrates and 3D microenvironment can change cell fate  
19 and lineage commitment (36-40). So far, many studies have been conducted to examine  
20 topographical cues as a biophysical factor on stem cell differentiation into many types of cells  
21 including tenocytes (41-45). It is demonstrated that nanotopography with alteration in cellular  
22 morphology and F-actin alignment can differentiate neural stem cells toward astrocytes and  
23 neurons (46). It has also been reported that the topography can alter the human embryonic stem  
24 cell response to bFGF (47). Topographical cues also have been used to induce myogenic  
25 differentiation in human bone marrow mesenchymal stem cells and it was found that biomimetic  
26 nanotopography in combination with electro-conductivity can increase myogenic marker  
27 expression(48). Due to the specific structure of the tendon tissue and the arrangement of collagen  
28 fibers, the role of the substrate and especially the topography in inducing the tenogenic  
29 differentiation in the cells have received much attention. Many studies have focused on the  
30 induction of tenogenic differentiation in stem cells using topography(49, 50). In a study, it was  
31 demonstrated that aligned fibers with or without BMP-12 can increase tendon makers *in vitro* (51).  
32 In addition, Zhang et al. utilized ultrafine fibers to induce tenogenic differentiation in human  
33 induced pluripotent stem cells based on mimicking tendon microstructure and topographical  
34 features (2).

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37 Various methods including replica modeling, Nanoimprint lithography, Photolithography,  
38 Microcontact printing, etc. have been used to create surface topography so far(52). In some studies,  
39 cell or tissue morphology has been used as a template for producing negative molding substrates  
40 on polydimethylsiloxane (PDMS) (30, 53). The basis of this method is to imprinting the  
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6 morphology of cells and tissues at the micrometer and nanometer scales in PDMS. Mahmoudi et  
7 al., induced chondrogenic differentiation in rabbit adipose tissue-derived mesenchymal stem cell  
8 using chondrocyte-imprinted substrate (53). Mashinchian and colleagues indicated that stem cell  
9 culturing on a substrate patterned by keratinocyte cell shape influences the nucleus shape of stem  
10 cells and gene expression (54). In other studies, cell imprinting method was used to induce  
11 differentiation in mesenchymal stem cells and modulate trans-differentiation (30, 55). Despite the  
12 testing of various methods based on biochemical and biophysical cues, the best way to induce  
13 tenogenic differentiation is still unclear. On the other hand, the efficacy of inducing differentiation  
14 using biophysical factors such as topography is still controversial (3, 56). In this study, it has been  
15 attempted to investigate whether imprinting tenocyte morphology on PDMS can produce a sheet  
16 capable of enhancing the expression of tenocyte markers in mesenchymal stem cells. Various  
17 studies have used BMP-12 as an inducer of tenogenic differentiation. Therefore, the potential of  
18 induction tenogenic differentiation of tenocyte-imprinted PDMS substrate was compared with the  
19 BMP-12 (as a positive control group) and other potential inducers including tendon tissue  
20 imprinted PDMS and decellularized tendon. In the next part of this study, the expression of specific  
21 tenocyte protein markers in mesenchymal stem cells cultured on tenocyte-imprinted PDMS was  
22 investigated, and finally, the behavior of cells cultured on this sheet was studied *in vivo*. Studying  
23 this method can help to provide inexpensive, reliable and effective PDMS sheets that can be used  
24 for induction tenogenic differentiation in mesenchymal stem cells.  
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## 41 **2. Material and methods**

### 42 **2.1. Ethical approval**

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46 This study was confirmed by the ethical committee of Iran University of Medical Sciences  
47 (IUMS ethical code: IR.IUMS.REC1395.9221565204) to use human-derived tissues and animal  
48 experiments and conducted based on the national guidelines.  
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## 2.2. Study design

This study is divided into *in vitro* and *in vivo* sections. A) *In vitro* investigations: in order to evaluate and compare the tenogenic marker expression in mesenchymal stem cells cultured on human tenocyte imprinted PDMS with other groups including tendon tissue imprinted PDMS, decellularized tendon and BMP-12 on induction tenogenic marker expression in human ADSCs, we designed 5 study groups which were individually affected by different conditions. These five groups are 1) ADSCs with no treatment as a control group. 2) ADSCs that were treated with 100 ng/ml BMP-12 (GF; Growth factor group). 3) ADSCs which was seeded on human tenocyte replica on PDMS without additional treatment (CR; cell replica group). 4) ADSCs which were seeded on human tendon tissue replica on PDMS without additional treatment (TR; tendon replica group) and 5) ADSCs cultured on the human decellularized tendon (DT group). The capacity of experimental groups to induction tenogenic differentiation was evaluated and compared using real-time RT-PCR analysis. The potency of replicas to the induction of tenogenic markers expression at the protein level was evaluated using immunocytochemistry.

B) *In vivo* investigations. Rat cells were used for *in vivo* investigations. After tenocyte isolation and characterization from rat, rat tenocyte replica was fabricated. Then rat adipose tissue-derived mesenchymal stem cells were seeded on rat tenocyte replica for 14 days and then were transplanted into rat subcutaneously using an electrospun PCL mat. Also, differentiation of rat stem cells to tenocytes after 14 days of culture on cell replica was confirmed by immunocytochemistry (results not shown).

## 2.2. Isolation, culture, and characterization of ADSCs

Human adipose tissue-derived stem cells have been isolated based on published protocols (57). Briefly, the adipose tissue samples were obtained from 5 healthy 30-35 years old men, during abdominal liposuction and transferred to the laboratory in a sterile dish containing Phosphate Buffer Saline (PBS) and antibiotic. Next, the samples were chopped into the 1 mm<sup>3</sup> pieces, incubated with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK) containing 1 mg/ml collagenase type I at 37°C. The mixture was gently shaken every 15 minutes during digestion. After 1 hour, the prepared suspension centrifuged at 1200 rpm for 5 min at 25°C. Then supernatant was removed and cell pellets were cultured in DMEM/Ham's F-12 (Gibco, UK) supplemented



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6 with 10 % Fetal Bovine Serum (FBS, Gibco, UK) and 1 % penicillin (100 IU/ml, Sigma Aldrich,  
7 US)/streptomycin (100 µg/mL, Sigma Aldrich, US) and incubated (5% CO<sub>2</sub>, 95% humidity). After  
8 48 h, the supernatant containing debris, dead cells, and erythrocytes was replaced by a fresh  
9 medium. The isolated cells were characterized using flow cytometry and multi-lineage  
10 differentiation assay after passage 3. ADSCs from 5 adult rats (250-300 g, 6-8 weeks old, male)  
11 were similarly isolated.  
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16 The isolated mesenchymal stem cells from adipose tissue with fibroblast-like morphology  
17 expressed mesenchymal lineage-specific markers including CD73, CD90, and CD105 (98.75%,  
18 99.4%, and 99.3%, respectively) and did not express CD34 and CD45 according to flow cytometry  
19 results. In addition, Tri-lineage differentiation assay was performed and ADSCs successfully  
20 differentiated into osteocyte, chondrocyte, and adipocyte (see supplementary data 1).  
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### 29 **2.3. Isolation and culture of tenocytes**

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31 Human tendons were harvested from surgical waste obtained from allograft rotator cuff surgery in  
32 5 healthy men (25-30 years old) under ethical consent. Samples were transferred to the lab using  
33 a sterile dish containing 3% Penicillin (100 IU/ml)/Streptomycin (100 µg/ml) dissolved in PBS.  
34 For tenocyte isolation, surrounding tissues were removed by a forceps and the tendon was cut into  
35 pieces under 1 mm<sup>3</sup>. After washing with sterile PBS, slices were digested with DMEM containing  
36 5 mg/ml collagenase type I at 37°C overnight. Afterward, the suspension was centrifuged at 1200  
37 RPM for 5 minutes at 25°C. The cell pellet was cultured after resuspension with DMEM/Ham's  
38 F12, supplemented with 10% FBS and 1% Penicillin/Streptomycin, and kept in a humidified  
39 incubator (37°C and 5% CO<sub>2</sub>). The medium was refreshed every 3 days. After collection, Cells  
40 were characterized individually and then, the cells were pooled and used for subsequent studies.  
41 Rat tenocytes (r-tenocytes) also isolated from Achilles tendon of 5 adult male Wistar rats.  
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### 50 **2.4. Tenocyte characterization using immunocytochemistry**

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52 For characterization of isolated tenocytes, immunocytochemistry was performed. The tenocytes  
53 were rinsed twice with PBS followed by fixation using paraformaldehyde 4% in PBS (pH=7.2) for  
54 20 minutes at 25°C. After removing the fixative solution, the cells were washed with PBS for 3  
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6 times and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 40 minutes. Following that, cells  
7 were washed using PBS (3 times) and incubated with BSA 1% in PBS at 37°C for 1 h to block  
8 non-specific antigen-antibody reactions. Next, they were incubated with primary antibodies  
9 against scleraxis (1:250, ab58655, Abcam, UK) and Tenomodulin (1:200, orb101154, Biorbyt,  
10 UK) at 4°C overnight. Finally, anti SCX treated samples were incubated with Alexa Fluor® 647-  
11 conjugated secondary antibody (1:300, ab150079, Abcam, UK) and Anti tenomodulin treated  
12 samples were incubated with Alexa Fluor® 488-conjugated secondary antibody (1:300, ab150077,  
13 Abcam, UK) for 1 h in the darkness at 25°C. DAPI staining (1µg/ML, Sigma) was used for nuclei  
14 detection. After immunocytochemistry, the samples were imaged using fluorescent microscopy  
15 (Olympus, Japan). Then by dividing the cells expressing TNMD (as a specific marker of adult  
16 tenocytes) by the total number of cells, it was shown that in different samples, on average, 93 %  
17 of cells expressed TNMD.  
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#### 27 **2.4. Fabrication of Cell and Tissue replica**

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29 Cell and tissue replicas were fabricated based on previously published protocols (53). Briefly  
30 Human and rat tenocytes (h-tenocytes and r-tenocytes) at passage 1 were cultured (Separately) at  
31 a density of  $3 \times 10^3/\text{cm}^2$  in 6 well plates. After 48 hours, the medium was removed and cells were  
32 rinsed using PBS and fixed by glutaraldehyde 2.5% (V/V) at 4°C overnight. Polydimethylsiloxane  
33 (PDMS, SYLGARD 184, RTV, Dow Corning, USA) was mixed with the curing agent (1:10) and the  
34 mixture was cast onto the cells and they were kept at 37°C. After 48 hours, the PDMS that was  
35 perfectly molded was gently removed from the cells(53, 54).  
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41 For human tendon tissue imprinting, human rotator cuff tendon was sliced into 60 µm  
42 diameter slices with longitudinal cuts using cryostat. Then, the slices were placed at the bottom of  
43 the 6 well cell culture plate and after drying, the tendon replica was prepared via casting PDMS  
44 on the slices. Prior to using the PDMS samples, chemical and biological materials that could have  
45 remained on them were eliminated using 1M NaOH solution at 90°C.  
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#### 52 **2.5. Cell seeding on Cell Replica and Tendon replica**

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54 Human ADSCs ( $10 \times 10^3$  cells/cm<sup>2</sup> in 50 µL of culture medium) were seeded on the cell  
55 and tissue replica and then the samples were incubated at 37°C. After 3 h of incubation,  
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6 DMEM/Ham's F12 medium supplemented with FBS 10% (v/v) was added to each sample,  
7 covering the sheet surface completely. The medium was refreshed every 3 days. Cells were  
8 collected after 7 and 14 days for gene expression analysis. Also after 14 days of seeding ADSCs  
9 on cell replica and tissue replica, cells were fixed for immunocytochemistry. For evaluation cell  
10 alignment on PDMS after immunocytochemistry the average aspect ratio of cell nuclei (major  
11 axis/minor axis) was measured using ImageJ software. 100 cell nuclei were used in each groups.  
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## 19 **2.6. Tendon decellularization and recellularization**

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21 Human rotator cuff tendon was cut into 1 cm<sup>3</sup> segments and then kept in sterile deionized  
22 water for 2h. Afterward, tissue segments were washed using 1% EDTA in deionized water at 25°C  
23 for 4 hours. Samples were rinsed 3 times using PBS containing 3% Penicillin/Streptomycin and  
24 then were treated with 0.1% SDS solution in PBS for 24 h. After 3 times washing with PBS, tendon  
25 segments were incubated for 24 h with a hypertonic solution containing potassium chloride (KCl)  
26 5 Molar and 1% (w/v) Triton X-100. Samples were preserved at -80 C before use. The tendon  
27 decellularization was evaluated by Hematoxylin and Eosin (H&E) staining and DNA content  
28 assay. Also, the decellularized tendon was imaged by SEM to investigate the structure and integrity  
29 of the tendon tissue after decellularization. To recellularize, h-ADSCs were seeded at a density of  
30  $1 \times 10^6$  /cm<sup>2</sup> in 20  $\mu$ L culture medium and left for 2 hours. Ultimately culture medium was added  
31 to all samples to cover tissues.  
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## 44 **2.7. Scanning Electron Microscopy (SEM) and Atomic force microscopy (AFM)**

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46 SEM microscopy was performed to investigate the microstructure of tendon tissue, pattern,  
47 and topography of cell replica and tissue replica and cell morphology on sheets. For cell fixation,  
48 2.5% glutaraldehyde was added to all samples so as to cover all the sheets. All samples were  
49 dehydrated using ethanol with increasing concentration (serial dilution 50-100%). After drying,  
50 the specimens were coated with a thin layer of gold and then were viewed under a TESCAN  
51 VEGA3 SEM at an accelerating voltage of 30 kV. In order to investigate the topography of cell  
52 replica, AFM (NanoWizard 4a NanoScience, JPK Instruments AG, Berlin, Germany) contact  
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mode imaging was performed. A rectangular cantilever (HQ:NSC18/Al BS, MikroMasch, Bulgaria) was used with spring constant of 2.8 N/m and a conic tip of 8 nm radius. Using a scan rate of 0.05 Hz and setpoint force of 0.5 nN, surface areas up to 100<sup>2</sup> μm were scanned. Image analysis was done using standard software of the instrument (JPKSPM Data Processing).

## 2.8. Real-time polymerase chain reaction (PCR)

To extract RNA, all samples were directly lysed using Trizol according to manufacturer protocol. Genomic DNA was eliminated using DNase I. RNA concentration and purification were evaluated by 260/280 absorbance ratio using nanodrop (Thermo scientific, USA). A mix solution containing 1 μg of RNA, 0.5 μl of oligo dTs, and 0.5 μl of random hexamer were combined. Finally, the cDNA construct was synthesized using the cDNA Synthesis Kit (PrimeScript RT Master Mix, TAKARA, Kyoto, Japan) according to the manufacturer's protocol.

For quantitative PCR, 1 μl of synthesized cDNA, 13 μl Maxima SYBR Green/ROX qPCR Master Mix kit (Thermofisher, USA), 0.5 μl of each primer, and 10 μl deionized water were mixed at 25 μl total volume and PCR was performed using Rotor-Gene 6000 instrument (Corbett Life Science, Australia). Scleraxis (SCX), Tenomodulin (TNMD), and Tenascin C (TNC) were used as tenogenic differentiation markers and GAPDH was used as a housekeeping gene. ADSCs without any treatment has been considered as a control group for normalizing target gene expression. Primer pairs are demonstrated in table1. Relative expressions were determined using the  $\Delta\Delta C_t$  method relative to gene expression values for control samples. Each PCR reaction was carried out in triplicate.

[Table 1]

## 2.9. *In vivo* investigations

To study *in vivo* behavior of topography-induced tenogenic differentiation of ADSCs, the cultured rat ADSCs (r-ADSCs) on a cell replica of rat tenocytes, after 14 days of incubation, were

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6 detached by trypsinization, and then were labeled with DiI stain (Sigma), seeded on a Poly ( $\epsilon$ -  
7 caprolactone)-based electrospun mat and kept for 24 h. Wistar rats (6 male, 250-300 g) were  
8 anesthetized via injecting the mixture of ketamine and Xylazine (70 mg/kg and 10 mg/kg,  
9 respectively). A pocket was made through an incision in the median backside and electrospun mat  
10 containing cells was transplanted into the pocket. Cyclosporine A as an immunosuppressive drug  
11 (10 mg/Kg) was injected intravenously every day. At the end of week 4 rats (in each group) were  
12 sacrificed using CO<sub>2</sub> inhalation and the tissue samples were harvested for histological evaluations.  
13 Also, PCL electrospun mats without cells were used as control negative group and ADSCs group  
14 including electrospun mats carrying untreated ADSCs were transplanted into rats. Collagen type  
15 I expression were imaged in each group by fluorescence microscopy with 100x magnifications  
16 (olympus ix71, Japan). In each group, different fields were randomly imaged and 5 images were  
17 analyzed in each group. The amount of Collagen I expression (green) was analyzed by ImageJ  
18 software and revealed as color intensity percent. GraphPad Prism software was used to analyze the  
19 results.  
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#### 41 **2.10. Histological evaluations using Hematoxylin and Eosin (H&E) staining and** 42 **immunohistochemistry** 43 44

45 For H&E staining, samples were fixed in 10% formalin and embedded in paraffin after dehydration  
46 and clearing. Then paraffin blocks were sectioned in 5  $\mu$ m slices and stained with hematoxylin and  
47 eosin. To investigate the difference of the collagen type I (as the main component of tendon ECM)  
48 production capacity in tenogenic differentiation induced ADSCs with the control group, the  
49 Immunohistochemistry technique was performed.. Briefly, after deparaffinization and hydration  
50 of tissue samples, antigen retrieval was performed via incubation in sodium citrate (0.01 M) at  
51 120°C for 11 minutes using an autoclave (Memert, Germany). Tissue sections were treated with a  
52 blocking solution consisting of 1% bovine serum albumin (BSA; Sigma Aldrich) and 1% Triton  
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6 X-100 (Sigma Aldrich) in PBS for 60 minutes at 25°C. The specific primary antibody against  
7 collagen type I (1:200, ab34710, Abcam, UK) was added to each sample and incubated at 4°C  
8 overnight. Next, the Alexa Fluor® 488-conjugated secondary antibody (1:300, ab150077, Abcam,  
9 UK) was added to each sample and samples were kept at 25°C and in darkness for 45 minutes. For  
10 nuclei staining, samples were exposed to DAPI (1 µg/mL, Sigma Aldrich) for 10 minutes at 25°C.  
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## 18 **2.11. Statistical analysis**

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20 All data were expressed as means  $\pm$  SD of three separate experiments. Statistical significance was  
21 evaluated by two-way ANOVA and Fisher's LSD (GraphPad Prism ver. 7.04). A *P* value less than  
22 0.05 was regarded as statistically significant.  
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## 29 **3. Results and discussion**

### 30 **3.1. Microscopic observations**

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32 The isolated tenocytes from human rotator cuff illustrate bipolar and spindle-shaped morphology  
33 like fibroblasts (Fig. 1, a1-a4). Immunocytochemistry assay demonstrated that most of cells  
34 expressed Scleraxis and Tenomodulin proteins as specialized tendon markers (Fig. 1, b1-b6).  
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39 As can be seen in figure 2 (a-d and g), cell morphology is well imprinted on PDMS substrate and  
40 the appearance of the cell replica on PDMS was similar to the cell morphology in 2D culture.  
41 According to the SEM images, the morphology of h-tenocytes and tendon tissue sections were  
42 successfully imprinted on PDMS (Fig. 2e-2g). Data showed that alignment and microstructure of  
43 the tendon tissue were similar to its replica (Fig. 2e and 2f). Furthermore, AFM analysis revealed  
44 that the h/r-ADSCs and the h/r-tenocyte-imprinted PDMS (as called cell replica; CR) showed  
45 differences in height and morphology (see Fig. 3e). According to these results, the footprint of  
46 cells in each group (stem cells and tenocytes) seems to be different from others and specific (Fig.  
47 3e). The differences in surface topography between h-tenocyte and h-ADSCs can be considered as  
48 a result of the existing differences in cell cytoskeleton and cell membrane. As mentioned in the  
49 literature, cytoskeleton composition and structure plays an essential role in cellular  
50 morphology(58, 59). Furthermore, the similarity of surface topography between h/r-tenocytes  
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6 seems be higher than the similarity between the tenocytes and ADSCs in the same species based  
7 on AFM analysis (see Fig. 3e). Recently, researchers found that the cell surface pattern can be  
8 imprinted in nano and micro-scale using PDMS(53). On the other hand, it has been shown that the  
9 cytoskeleton structure influences nucleoskeleton organization resulting in chromosome  
10 remodeling which may have regulatory effects on gene expression profile (54, 60, 61). There is  
11 increasing evidence that the nuclear structure can control heterochromatinization(62). Also,  
12 studies have been shown the nucleus structure can affect gene expression via controlling  
13 transportation and sequestration of transcription factors(62). In this study, the molecular pathways  
14 of differentiation induction have not been investigated. However, by reviewing similar studies, we  
15 can find that integrin can be one of the important candidates for mediating differentiation induction  
16 via biophysical signals. The integrins transmit biophysical signals to the nucleus through focal  
17 adhesions and actin, which contributes to the structure of the cytoskeleton. The transmission of  
18 these signals, resulting from the molecular interactions between focal adhesion, the cytoskeleton  
19 and the structure of the cell and nucleus, ultimately leads to the regulation of gene expression(63).  
20 So far, cell shape and especially nucleus structure have been neglected in designing biomaterials.

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32 [Figure 1]

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### 37 38 39 40 41 **3.2. Decellularized tendon investigation**

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43 Natural extracellular matrix of the tendon was prepared by decellularization method using  
44 chemical materials. H&E staining confirmed that all cells were removed from the fresh tendon  
45 tissue while its structure was preserved. Additionally, the collagenous structure of the  
46 decellularized tendon was similar to the native one (Fig. 4). The results of the DNA content assay  
47 also showed that the cells were well removed from the tissue. As shown in the Fig 4c, the DNA  
48 content decreased significantly after tendon decellularization as compared to native tissue  
49 ( $p < 0.0001$ ). The DNA content assay results were in good agreement with the H&E images of the  
50 decellularized tendon. Also, SEM image showed that the tendon tissue integrity was maintained  
51 after decellularization.  
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6 In the studies undertaken, both SDS and Triton X-100 caused human tendon  
7 decellularization successfully (64). It also demonstrated that tendon collagen and its mechanical  
8 properties were successfully preserved after decellularization using chemical detergents (64, 65).  
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11 [Figure 4]  
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### 16 **3.3. Cell attachment on Cell Replica and Tendon Replica**

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18 As shown in Figure 5, the cells were well attached to the surface of PDMS. Also ADSCs were  
19 expanded on PDMS during 7 and 14 days of incubation. After stem cell seeding on cell replica,  
20 the cells began to aggregate and created colony-like structures. At the end of the first week  
21 following the cell seeding, in both CR and TR group, the cells began to organize and by the end of the  
22 second week, the seeded cells in CR group, developed well-organized and well-aligned structures (Fig. 5,  
23 a, b, e and h). As shown in figure 5a, 5c, 5d, after seven days of seeding h-ADSCs on cell and tissue  
24 replica, they started to aggregate and formed round structures. These aggregations are  
25 interconnected through cellular processes and then, cell aggregations joined together and formed  
26 aligned structures in the CR group (Fig. 5, a-c, e and h). Consistent with the previous studies  
27 results, cell replica successfully effects on cell fates (30, 53, 54). More specifically, after culturing,  
28 cell alignment could be seen as a sign of altering cell behavior Fig.5 (e and h) clearly shows the  
29 aligned structures constructed by the cells seeded on cell replica. As can be seen in the Fig. 5 (b, e  
30 and h), these structures are present in the center of the substrate. These structures are found in all  
31 CR substrates 14 days after seeding ADSCs. The results also showed that aspect ratio in CR group  
32 was significantly higher than the TR and the control group (see supplementary data 2) ( $p < 0.0001$ ).  
33 The results of the aspect ratio measurement are quite consistent with what is observe in the Fig. 5  
34 (b, e and h). This cellular behavior, which is associated with the expression of tenocytic markers  
35 on the 14th day, can be a sign of cell differentiation and their tendency to form tissue-like  
36 structures. Additionally, the effect of substrate on alignment and orientation of cells has been  
37 studied (66, 67). The effect of cell alignment on tenogenic differentiation has been evaluated in  
38 some studies. Wu and his colleagues showed that aligned fibers can promote cell proliferation and  
39 cell alignment as well as tenogenic marker expression(68). It has also been shown that the  
40 differentiation of stem cells into tenocytes are associated with increased cell alignment(69). The  
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6 results of the present study also confirm that the increased expression of tenocyte markers is  
7 directly related to cell alignment.  
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10 [Figure 5]  
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### 12 **3.4. Real-time PCR analysis**

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14 Tenogenic markers expression including SCX, TNMD, and TNC were found following 7  
15 days of culturing. In fact, a high level of SCX and TNMD as early and late tenocyte markers were  
16 detected only in the GF group compared to other groups (3.1 Fold and 4.3 Fold respectively,  
17  $p < 0.01$  for SCX and  $p < 0.05$  for TNMD), while, the level of TNC expression (as a marker of tendon  
18 extracellular matrix) only in TR (tendon replica) group was higher significantly than the control  
19 group ( $p < 0.05$ ). Moreover, at this time, the level of SCX expression in the GF group was  
20 significantly higher than the CR group ( $p < 0.05$ ) while there was no significant difference between  
21 GF and other groups. In addition, no significant difference was detected in TNMD expression  
22 between the GF and other groups after 7 days of culturing. These findings mean that it was not  
23 enough time for physical cues or even the natural tissue (decellularized tendon) to induce tenogenic  
24 differentiation in the cultured h-ADSCs. Also, this study was undertaken to examine the expression  
25 of tenogenic markers at days 14. The GF and CR groups showed significant high expression of  
26 SCX in comparison with the control group ( $p < 0.001$  for GF and  $p < 0.05$  for CR). In addition the  
27 expression of TNMD in GF and CR was significantly higher than the control group ( $p < 0.0001$  for  
28 GF and  $p < 0.01$  for CR). The SCX was increased 2.6 fold and also the TNMD increased 2.5 fold in  
29 the CR group when compared to the control. Also, TNMD expression in the GF group was  
30 significantly higher than the CR ( $p < 0.05$ ), TR ( $p < 0.001$ ), and DT ( $p < 0.01$ ). Meanwhile, there was  
31 no significant increase in TNC expression in comparison with the control group at this time. Also,  
32 the expression level of SCX and TNMD in the TR group increased about 1.7 and 2 fold compared  
33 to the control group, however this increase was not significant. The results showed that the TR can  
34 induce a non-significant increasing expression of tenocytic markers as well. However, the effect  
35 of this substrate on the expression of the Tenascin C marker, which is a marker of the extracellular  
36 matrix, is more than its effect on the expression of SCX and TNMD as tenocyte markers.  
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54 Consequently, data showed that CR in comparison with TR is significantly more effective  
55 in increasing the expression of SCX and TNMD markers than the expression of TNC. In contrast,  
56 the TR group increased TNC gene expression. However, this increase does not reach to a  
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6 significant level. In addition, the level of TNC expression of DT group was significantly high  
7 compared to the control group after 14 days ( $p < 0.05$ ).  
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10 Results also revealed that the level of SCX expression increased in the DT group up to 2  
11 folds after 7 days and 1.8 after 14 days. Moreover, TNMD expression increased in the DT group  
12 rather than the control group after 7 and 14 days, although the increase was not significant.  
13 Contrary to these results, in some studies, significant increases in the tenocyte markers in stem  
14 cells that have been treated with biophysical and/or biochemical cues have been observed after 7  
15 days (70) While, other studies have shown that the most expression of tenogenic markers are  
16 observed after 14 days (18, 30). Shen et al showed that the treatment of mesenchymal stem cells  
17 with BMP-12 could induce the highest expression of SCX at day 14 post-treatment (18). Also,  
18 Bonakdar and colleagues observed that culturing mesenchymal stem cells on patterned sheets for  
19 14 days can increase TNMD expression significantly(30). The results of these studies are in  
20 agreement with the results of the present study.  
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29 According to results, BMP-12 and decellularized tendon increased the expression of tendon  
30 markers in ADSCs. These results are not surprising due to some other studies. Previously, many  
31 articles have indicated the effect of BMP-12 in the induction of tenogenic differentiation in stem  
32 cells. Zarychta-Wisniewska et al. found that BMP-12 can activate tenogenic differentiation in  
33 human ASCs and can be a suitable candidate for acute tendon injuries but not for chronic  
34 injuries(71). In other studies, it has been suggested that BMP-12 can induce tenogenic  
35 differentiation in 2D and 3D conditions *in vitro* alone and in combination with other growth factors  
36 such as TGF- $\beta$  and bFGF (51, 72, 73). The effect of this growth factor in tendon healing *in vivo* is  
37 also demonstrated in some studies (74, 75). Also, as shown in some other studies, stem cells  
38 cultured on the decellularized tendon tissue can induce tenogenic differentiation and increase  
39 tendon specific marker expression (76, 77). In this study we did not investigate the molecular  
40 pathway of tenogenic differentiation. However, various studies have shown that treatment of cells  
41 with BMP-12 increases the expression of Smad family molecules, which ultimately results in  
42 increasing the expression of the SCX transcription factor(78). Increased levels of SCX in the cell  
43 nucleus result in the expression of various genes, including TNMD(78, 79). Biophysical factors  
44 (such as surface topography) can also enhance SCX expression through different molecular  
45 pathways(78, 80). Our observations in this study also confirm the results of these articles. In this  
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6 study, we observed that treatment of cells with BMP-12 growth factor, as well as cell culture on  
7 decellularized tendon, could increase expression of tenocytic markers. Our studies also confirm the  
8 studies that have used surface topography to induce tenogenic differentiation in mesenchymal stem cells.  
9 Using cell culture on aligned multilayered electrospun, Orr and his colleagues were able to induce  
10 the expression of tenocyte markers in adipose tissue mesenchymal stem cells. However, the results  
11 of this study showed that the highest expression was observed on day 7 after seeding, which is in  
12 contrast to our results(81). The aim of this study was to evaluate the expression of tenocyte markers  
13 in stem cells cultured on human tenocyte-imprinted PDMS (human tenocyte replica on PDMS).  
14 Comparison of tenocyte markers expression by growth factor-treated stem cells with topography  
15 induced cells shows that expression level of tenocyte markers in cells cultured on cell replica is  
16 lower than the expression of these markers in growth factor-treated cells. A comparison of gene  
17 expression results in the CR group and the GF group shows that the biophysical signal induced by  
18 the CR group, although weaker than the biochemical signal of the GF group, is an important and  
19 significant inducer. In fact, in this study, the GF group acts as a positive control, allowing us to  
20 evaluate the true biophysical signal strength of CR.  
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[Figure 6]

### 3.5. Immunocytochemistry

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39 Immunocytochemistry was performed to determine tenogenic differentiation in ADSCs,  
40 14 days after seeding on cell replica and tissue replica. According to figure 7, single cells as well  
41 as aligned structures that were mentioned above expressed SCX and TNMD. Data showed that  
42 tendon marker expression in ADSCs cultured on tenocyte-imprinted PDMS (CR group) was  
43 greater than ones cultured on tissue-imprinted PDMS. In single cells, there was no significant  
44 difference detected in the cell morphology and nucleus, however, in cells present in aligned  
45 structures, the nucleus was more aligned. Also, cell aggregations were lower and smaller in ADSCs  
46 cultured on tissue replica as well as tenocyte marker expressions (Fig. 7). Additionally,  
47 immunocytochemistry images demonstrated that the topography-induced ADSCs, successfully  
48 express tenogenic markers at protein level after 14 days of culturing on the patterned sheet  
49 (including CR and TR), indicating the successful differentiation of these cells. However, the cell  
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6 morphology and SCX and TNMD expression in CR and TR group after 14 days were similar to  
7 adult tenocytes (that illustrated in Fig. 1). Cultured cells on cell replica formed larger and more  
8 regular structures than cells on tissue replica. These results are consistent with the results of the  
9 gene expression which indicate that CR has a stronger effect on induction of differentiation than  
10 TR. It has been well known that the Scleraxis is a member of class II basic helix-loop-helix  
11 transcription factors that is important for tendon formation and development during embryogenesis  
12 and tendon regeneration. It has also been shown that scleraxis plays an important role in tenogenic  
13 differentiation in mesenchymal stem cells (84, 85). Tenomodulin (TNMD) is a type II glycoprotein  
14 transmembrane glycoprotein which is highly expressed in mature tendon and ligaments and it is  
15 known as a marker for tenocytes detection(79, 86). Furthermore, studies have shown that SCX acts  
16 as one of the upstream regulators of TNMD(79, 87).  
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25 [Figure 7]

### 26 27 28 **3.6 Immunohistochemistry and H&E staining**

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30 IHC staining showed that the DiI-labeled ADSCs that induced by cell replica topography  
31 on the electrospun mat can express collagen type I, which is the main component of the tendon  
32 ECM, . Despite the results of *in vitro* tests, cells cultured on cellular replica did not show significant  
33 extracellular tissue production *in vivo* compared to untreated stem cells. Collagen type I formation  
34 is expected in all three groups due to the migration of host cells to the electrospun mat. The  
35 production of type I collagen in CR group and ADSC group was higher than acellular electrospun  
36 mat group, however, there is little difference between ADSCs and cells cultured in CR, which  
37 could be the result of higher differentiation of cells of the CR group (Fig 8, a1-c4). The absence  
38 of significant differences between CR and ADSCs may be due to subcutaneous inappropriate  
39 conditions for tendon-like tissue production.  
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48 As can be seen in Figure 8d, H&E staining also showed that the tendon ECM like structures were  
49 formed in the CR group (Fig. 8, d2) and it seems at a high level in comparison with the control  
50 negative group (Fig. 8, d1). Although the ADSC group also contains large numbers of cells as well  
51 as significant ECM-like structures (Fig. 8, d3), the shape of these structures differ from that of the  
52 CR group and do not form tendon-like structures. The nuclei morphologically were oval-shaped  
53 and aligned with the direction of the tissue-like structure while in the control group and in ADSC  
54 group the nuclei were round with no specific orientation (Fig. 8, d1-3).  
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6 Despite the many studies about the differentiation of stem cells into tenocytes using  
7 physical cues such as topography, the results of these studies are still controversial. English et al.,  
8 in their study, concluded that although topography is effective in maintaining tenogenic phenotype  
9 *in vitro*, it cannot produce tissue in the *in vivo* and ectopic conditions(56). In the present study, the  
10 results are almost similar to those of English et al., and despite the promising *in vitro* results, the  
11 *in vivo* results are not very satisfactory. However, there are other studies showing that cells treated  
12 with biophysical factors can also repair damaged tendon tissue *in vivo*. Xu et al. using heterotopic  
13 transplantation, evaluated neo-tendon maturation potency of their tendon derived stem cell-seeded  
14 constructs (88). In another study, Ni and colleagues were able to produce tendon-like tissue  
15 subcutaneously without using scaffolds and only using tendon-derived mesenchymal stem  
16 cells(89). The results of the Ni study, in contrast to the results of the present study, show that *in*  
17 *vitro* treated mesenchymal stem cells are capable of producing tendon-like tissue *in vivo*.  
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27 [Figure 8]  
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#### 29 **4. Conclusion**

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31 The focus of this study lies in evaluating the impact of tenocyte-imprinted PDMS (as called cell  
32 replica or tenocyte replica in this study) as a topography based approach on induction tenogenic  
33 differentiation in mesenchymal stem cells.  
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37 What is revealed through this study is that the cell-based topography has a significant effect on  
38 tenogenic differentiation, although the effect of topography is lower than the growth factor. On the  
39 other hand, the results showed that the topography of tendon fibers also has a significant effect on  
40 tenogenic marker expression in tendon replica and decellularized tendon. Our results showed that  
41 cell replica is more effective on induction tenogenic marker expression than tissue replica.  
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46 Our findings may be used to design biomaterials since they have been more focused on the shape  
47 and structure of the extracellular matrix. Although the effect of growth factor on tenogenic  
48 differentiation is more than cell and tissue imprinted substrate, the remarkable impact and the  
49 advantage of physical cues in inducing tenogenic differentiation can lead us to further study on the  
50 effects of topography.  
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6 It seems that the biophysical cues (e.g. topography) are safe and more cost-effective than chemical  
7 cues (e.g. growth factors) for clinical applications, using these factors should be given more  
8 attention.  
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11 On the other hand, despite the advantages of the cell imprinting method, it has some limitations.  
12 One of these limitations is the low efficiency of this method compared to biochemical factors such  
13 as growth factors. Also, cell culture on these substrates is stochastic, and cell placement in cell  
14 replica cannot be controlled. After all, there are still many questions about the effectiveness of  
15 biophysical based methods. Overall, it seems that studies need to continue in this regard to address  
16 all the ambiguities.  
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## 27 **Acknowledgment**

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29 This research was supported by grant No 94-05-87-27505 from Iran university of medical sciences.  
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## 31 **Figure captions:**

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33 Supplementary data 1. h-ADSCs characterization. (a) 5 days after isolation. (b) 15 days after  
34 isolation. Oil red O, Alizarin red S and toluidine blue staining was used to confirm adipogenic (c),  
35 osteogenic (d) and chondrogenic (e) differentiation capacity of adipose tissue-derived cells (scale  
36 bar = 100  $\mu\text{m}$ ). Also, flow cytometry results confirmed that isolated cells express mesenchymal  
37 stem cell markers and do not express hematopoietic markers (f).  
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43 Supplementary data 2. The effect of substrate topography on aspect ratio of cell nuclei. Aspect  
44 ratio of ADSCs nuclei cultured on cell replica was significantly higher than that cultured on TR  
45 and control group ( $p < 0.0001$ ).  
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49 Supplementary data 3. Immunocytochemistry staining to confirm tenogenic differentiation in h-  
50 ADSCs, 14 days after seeding on cell replica. (a1 and b1) Cell nuclei stained by DAPI (blue), SCX  
51 and TNMD protein expression stained by Alexa Fluor® 488-conjugated goat anti-rabbit antibody  
52 (green). (a2 and a3) SCX expression in aligned structure of ADSCs cultured on the h-tenocyte  
53 replica (scale bar = 200  $\mu\text{m}$ ), (b2-b3) TNMD expression in single h-ADSC cultured on the h-  
54 tenocyte replica (scale bar = 100  $\mu\text{m}$ ).  
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Fig 1. Human tenocyte characterization 10 days after isolation. (a-d) Morphology and size of isolated cells from human tendon under invert microscope was identical with h-tenocyte (days 5, 10, 15 and 20)(scale bar = 100  $\mu$ m), (b1) Scleraxis expression and (b4) Tenomodulin expression, (b2) and (b5) show nuclei with DAPI staining, (b3) and (b6) show overlay images of DAPI and protein expression (scale bar = 100  $\mu$ m).

Fig 2. Cell and tissue imprinting on PDMS. Invert microscope images of (a) Fixed h-tenocytes, (b) tenocytes replica on PDMS, (c) Fixed h-ADSCs, (d) h-ADSCs replica on PDMS (a-d, scale bar = 100  $\mu$ m), SEM images of (e) human tendon section, (f) tendon tissue replica on PDMS, (g) a single h-tenocyte replica on PDMS.

Fig 3. AFM image analysis of cell replica topography. (a) h-ADSC, (b) r-ADSC, (c) h-tenocyte, (d) r-tenocyte. (e) Image analysis by the JPKSPM Data Processing software shows some differences in the height and shape of the cell surface of the cell replicas on PDMS, reflecting the topographic difference at the cell surface.

Fig 4. Human tendon decellularization. H&E images of Native human tendon (a) and decellularized tendon (DT) (b). The results of DNA content assay (c) and SEM image of decellularized tendon (d).

Fig 5. Cell attachment on PDMS (CR and TR). The left column shows the h-ADSCs attachment on cell replica 7 days after seeding. The middle column shows the h-ADSCs attachment on cell replica 14 days after seeding. The right column shows the h-ADSCs attachment on tissue replica 7 days after seeding. Cells did not show much change after 14 days of culture on TR, and therefore the image of day 14 was deleted. (a-c) invert microscope image of ADSCs aggregations on h-

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6 tenocytes and tendon tissue replica (scale bar = 200  $\mu\text{m}$ ), (a) invert microscope images of cell  
7 aggregates 7 days after seeding ADSCs on CR, (b) invert microscope images of cell alignment, 14  
8 days after seeding ADSCs on CR, (c) h-ADSCs attachment on TR 7 days after seeding, (d) SEM  
9 image of h-ADSCs aggregates on cell replica, 7 days after seeding. Black arrows indicate cell  
10 aggregations. (e) and (h) SEM images of h-ADSCs alignment on cell replica, 14 days after seeding  
11 (f) and (i) SEM images of h-ADSCs aggregates on TR, 7 days after seeding, (g) a single ADSC  
12 on cell replica 7 days after seeding  
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21 Fig 6. Gene expression of hADSC, 7 and 14 days after treatment with BMP-12 and seeding on  
22 CR, TR, and DT. (a) expression of SCX as a marker of early tenocytes, (b) expression of TNMD  
23 as a marker of adult tenocytes, (c) expression of TNC as a marker of the extracellular matrix of  
24 tendons.  
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30 Fig 7. Immunocytochemistry staining to confirm tenogenic differentiation in h-ADSCs, 14 days  
31 after seeding on cell replica and tissue replica. Cell nuclei stained by DAPI (blue), SCX and TNMD  
32 protein expression stained by Alexa Fluor® 488-conjugated goat anti-rabbit antibody (green). (a1-  
33 a4) SCX expression in h-ADSCs cultured on the h-tenocyte replica (scale bar = 200  $\mu\text{m}$ ), (b1-b4)  
34 TNMD expression in h-ADSCs cultured on the h-tenocyte replica. In b4, the arrow is pointed to  
35 tenocyte replica on PDMS. (scale bar = 100  $\mu\text{m}$ ), (c1-c4) SCX expression in h-ADSCs cultured  
36 on human tendon replica, (d1-d4) TNMD expression in h-ADSCs cultured on h-tenocyte replica  
37 (scale bar = 200  $\mu\text{m}$ ).  
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47 Fig 8. Evaluation of Collagen I expression and tendon-like tissue formation in CR-induced  
48 tenogenic differentiation in r-ADSCs after implantation in ectopic condition. Cells labeled by DiI  
49 before implantation (red), Nuclei stained by DAPI (blue) and protein expression stained by Alexa  
50 Fluor® 488-conjugated (green). (a1-a4) control group including PCL electrospun mat without  
51 cells, (b1-b4) collagen I expression in r-ADSC group cultured on PCL mat, (c1-c4) collagen I  
52 expression in cell replica group cultured on PCL mat (scale bar = 200  $\mu\text{m}$ ). (d1-d3) H&E staining  
53 in cell-containing and cell-free mats. The d1 demonstrate PCL electrospun mat without cells, d2  
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6 demonstrate PCL electrospun mat containing cells of CR group and d3 demonstrate PCL  
7 electrospun mat containing r-ADSCs without any treatment 4 weeks after implantation. In d2,  
8 arrows are pointing to tendon-ECM like structures that formed in PCL mat containing CR cells 4  
9 weeks after implantation (scale bar = 100  $\mu\text{m}$ ). (e) Percent of color density related to collagen I  
10 expression in (left) PCL electrospun mat (middle) r-ADSC group cultured on PCL electrospun mat  
11 (right) cell replica group cultured on PCL mat.  
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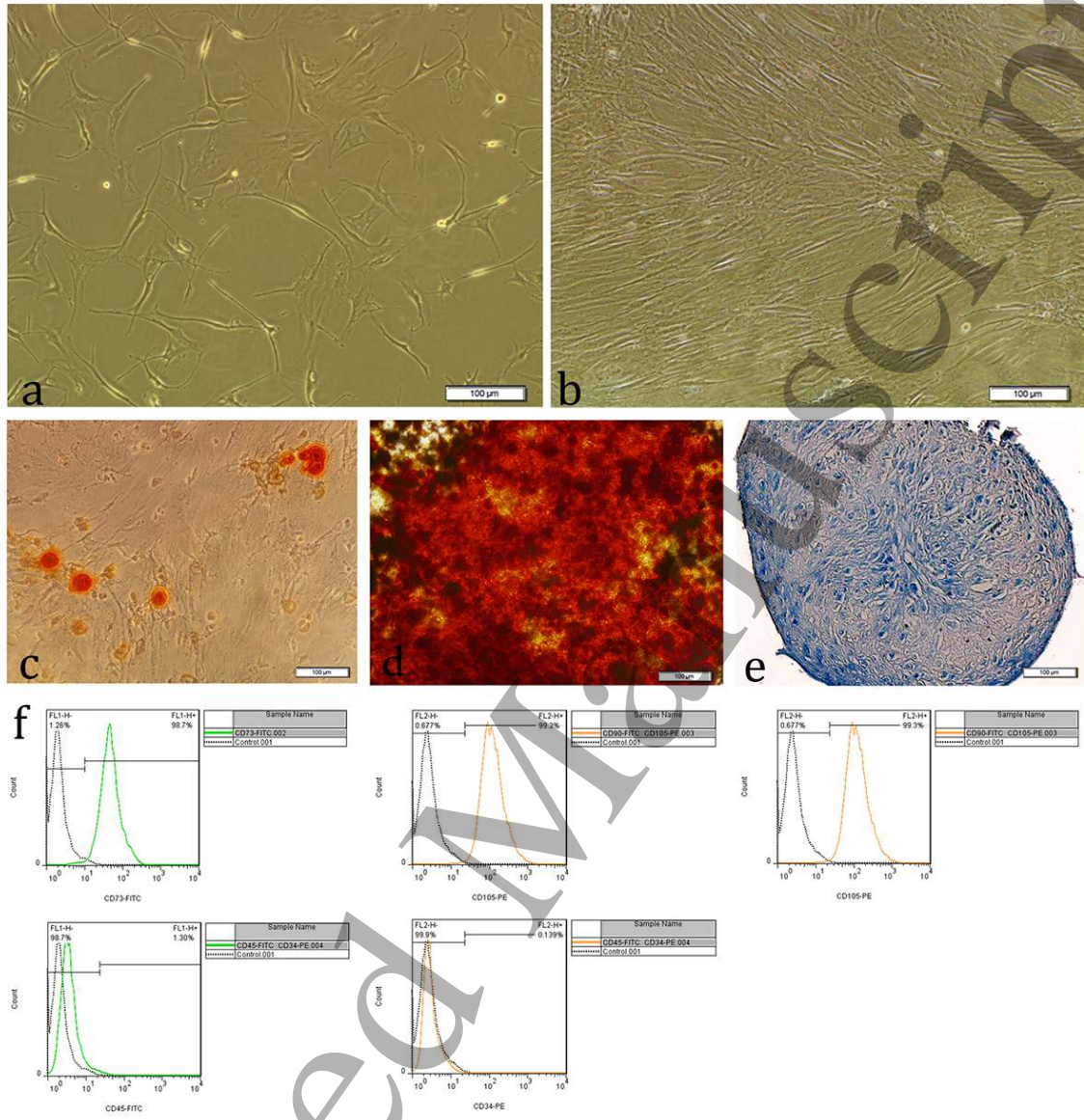
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Table 1. Sequences of Primers Used in Real-Time PCR

Primer	Sequence (5'-3')
TNMD-F	TGTGGACTGGTGTGGTATCC
TNMD-R	CCATTGCTGTAGAAAGTGTGCTC
SCX-F	ACACCCAGCCCAAACAGATC
SCX-R	GCCACCTCCTAACTGCGAATC
TNC-F	CAGCCAAAGAGACCTTCACAAC
TNC-R	CTTGCCATTCTCCATTCCAG
Gapdh-F	TCAAGATCATCAGCAATGCCTC
Gapdh-R	GTCATGAGTCCTTCCACGATAC

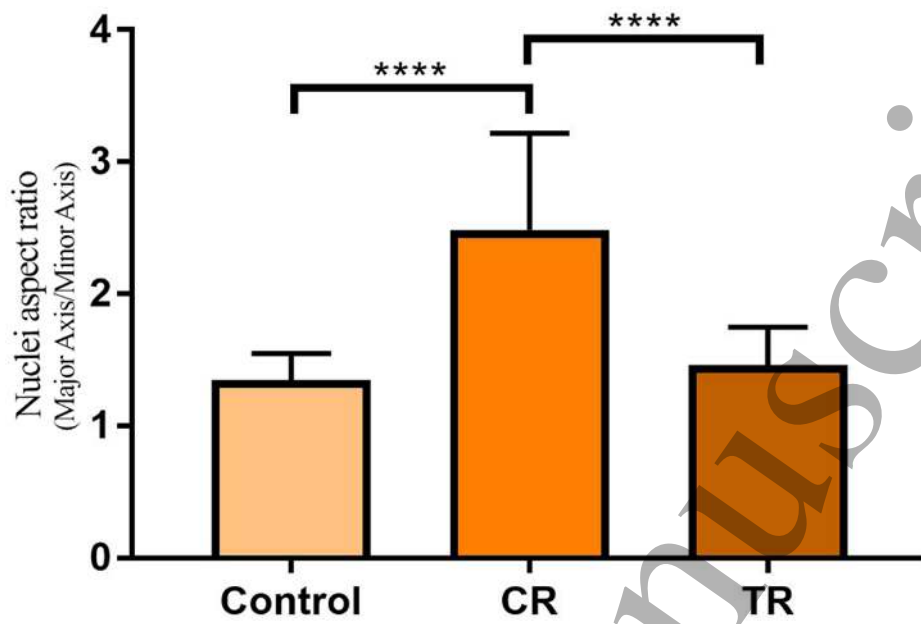
F: forward and R: reverse



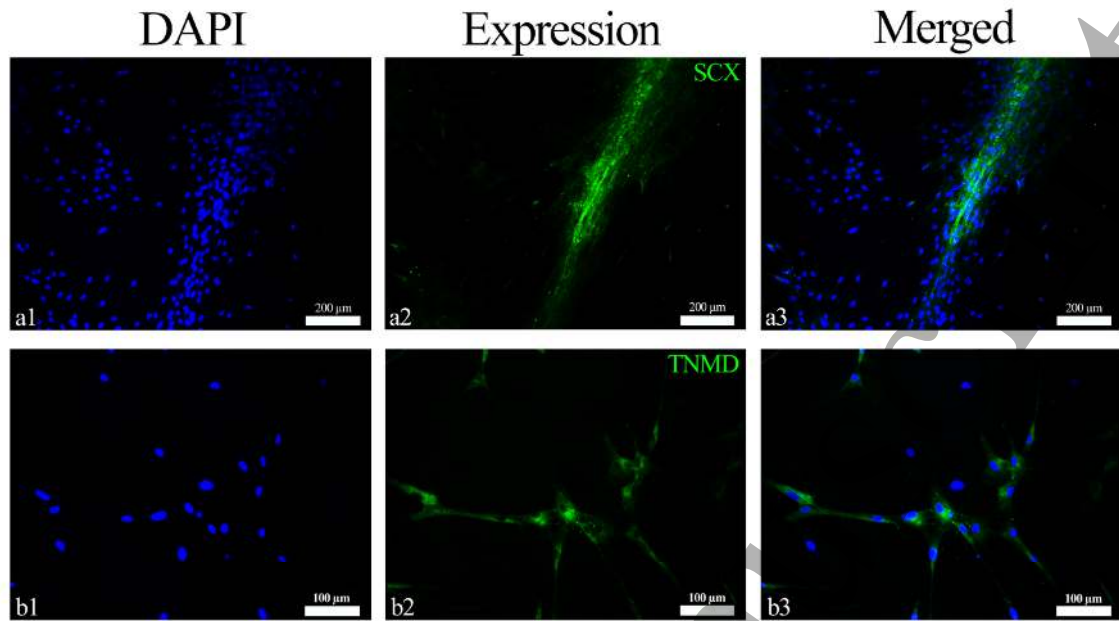
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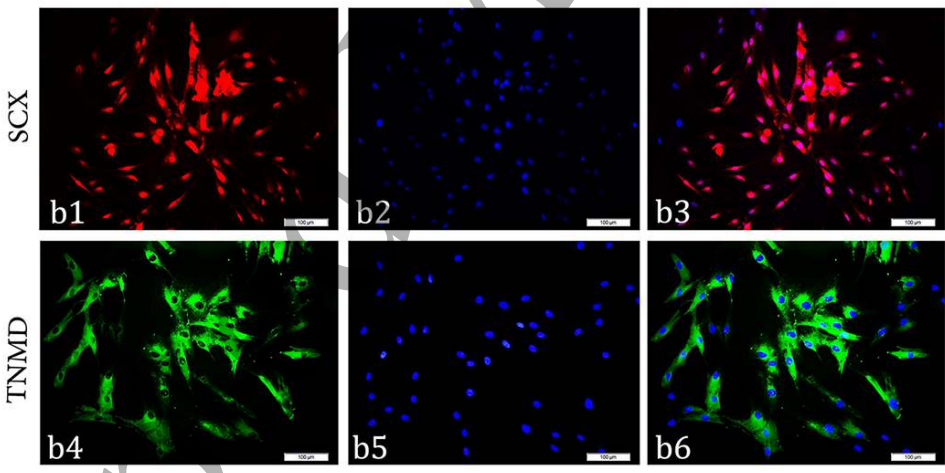
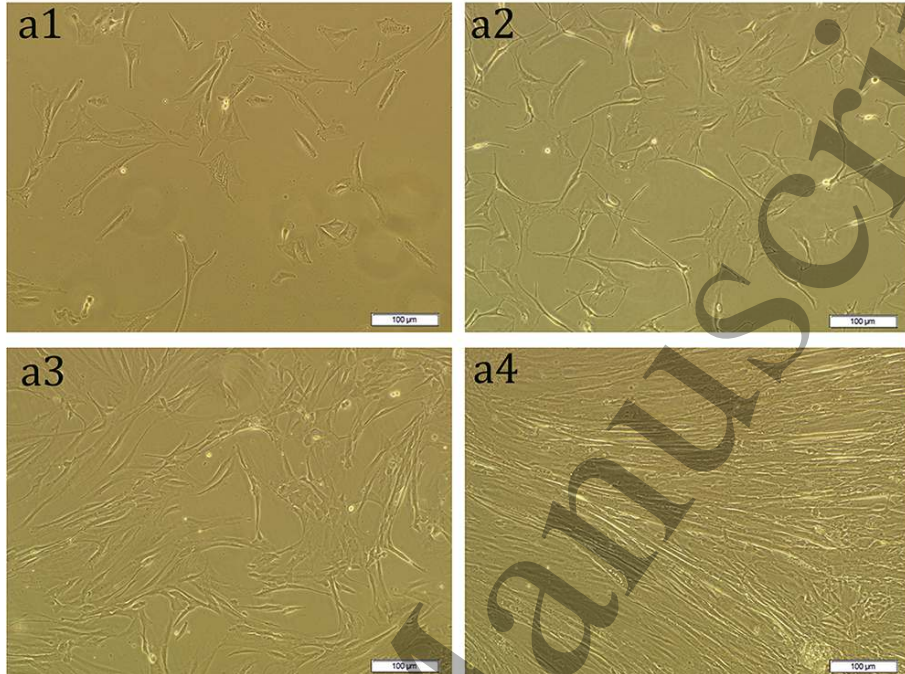




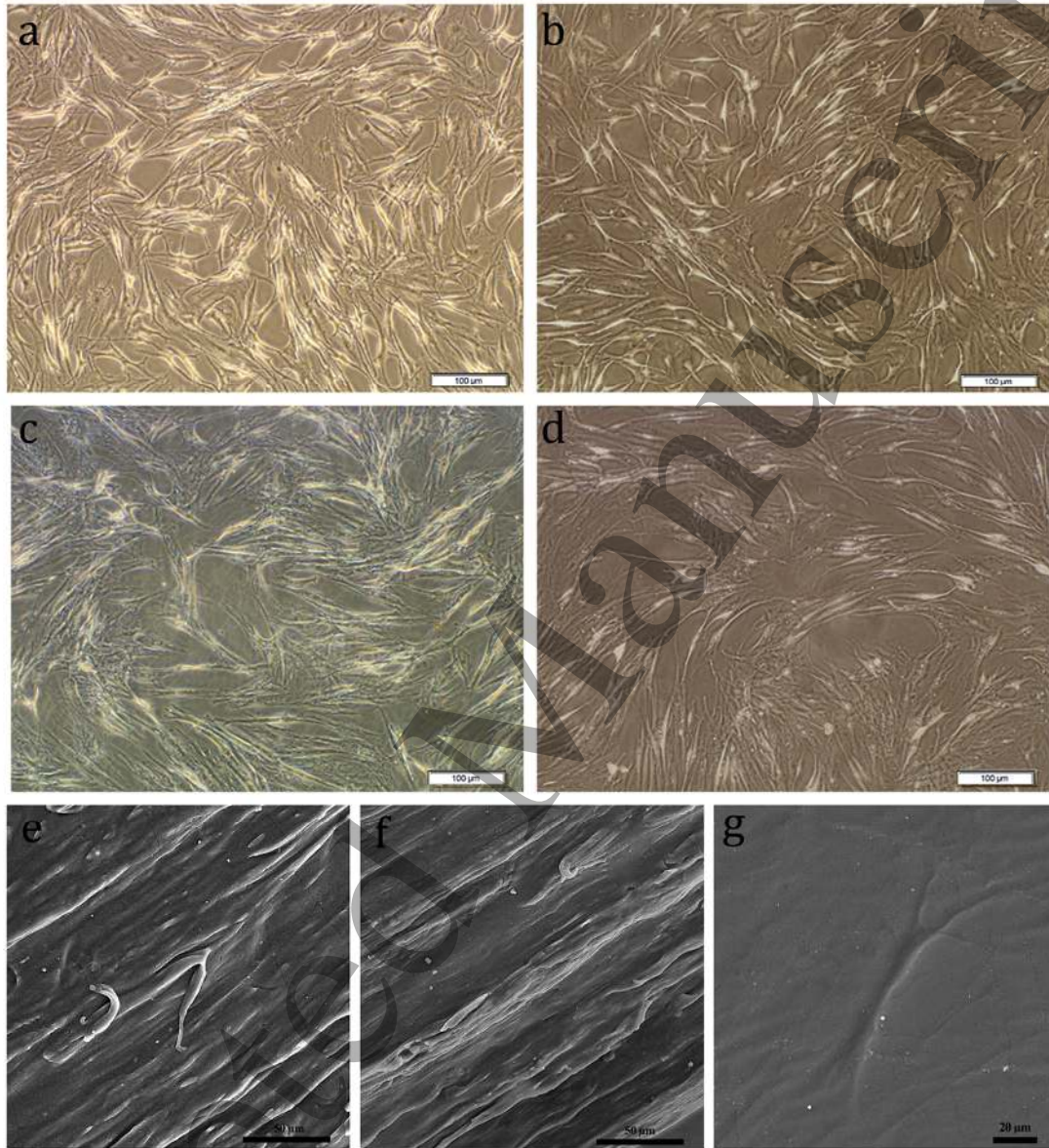
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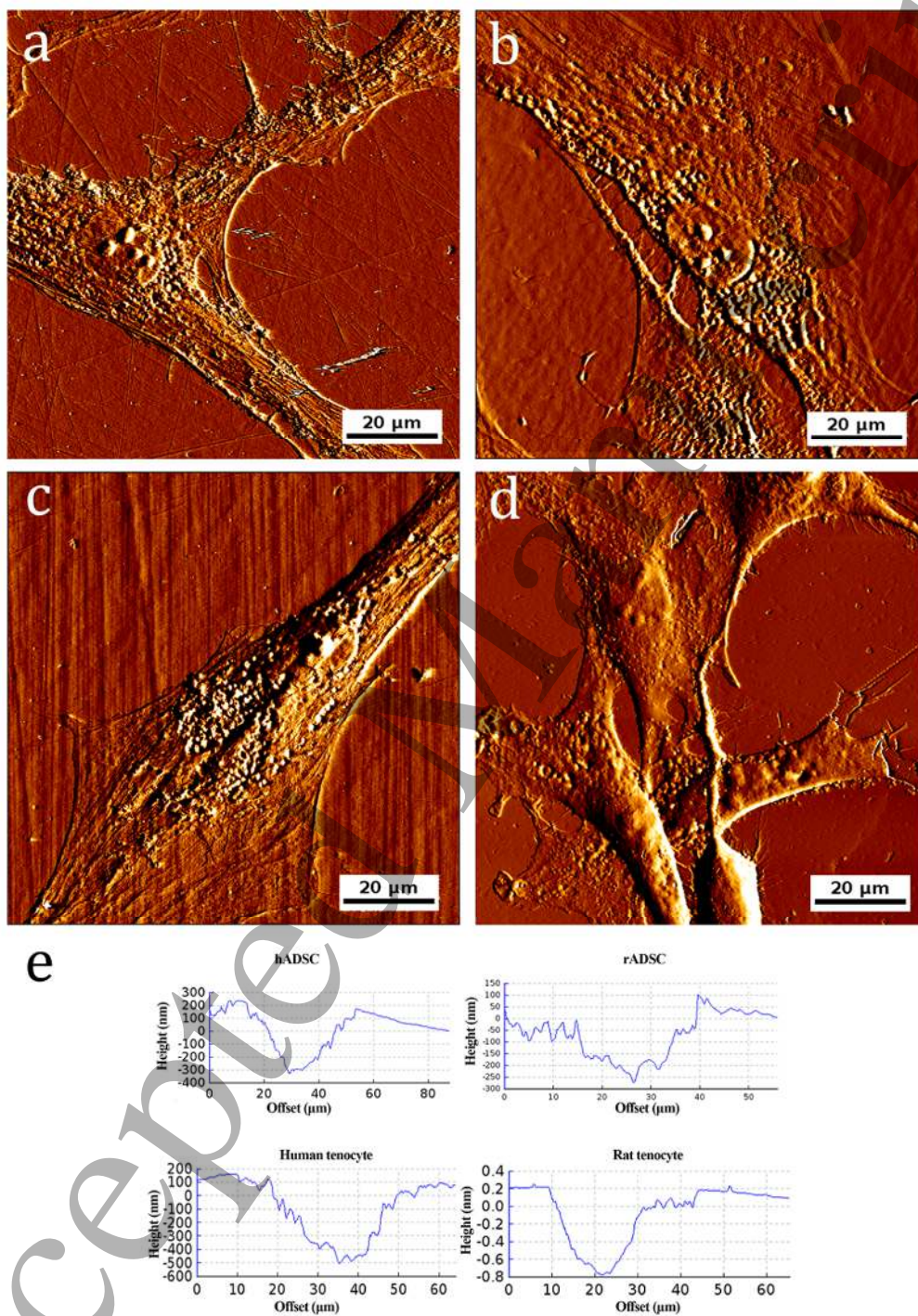


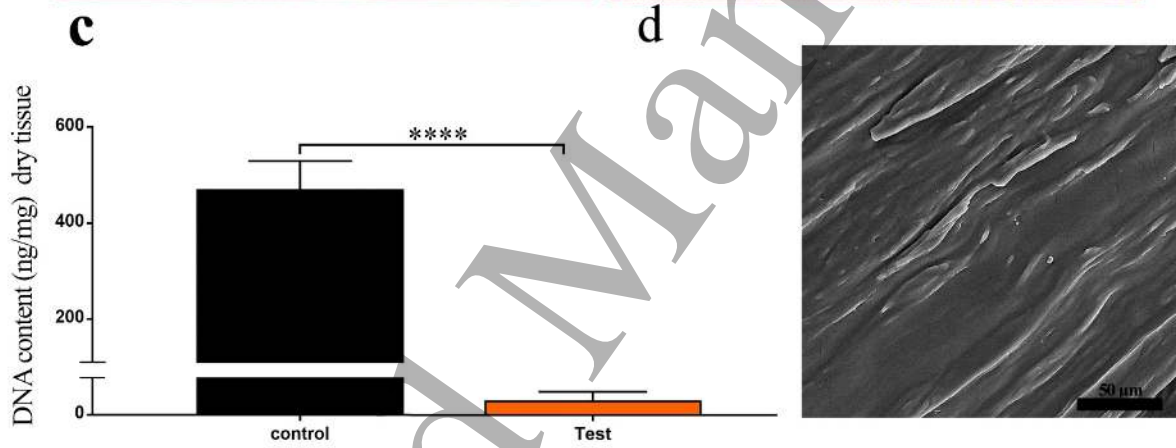
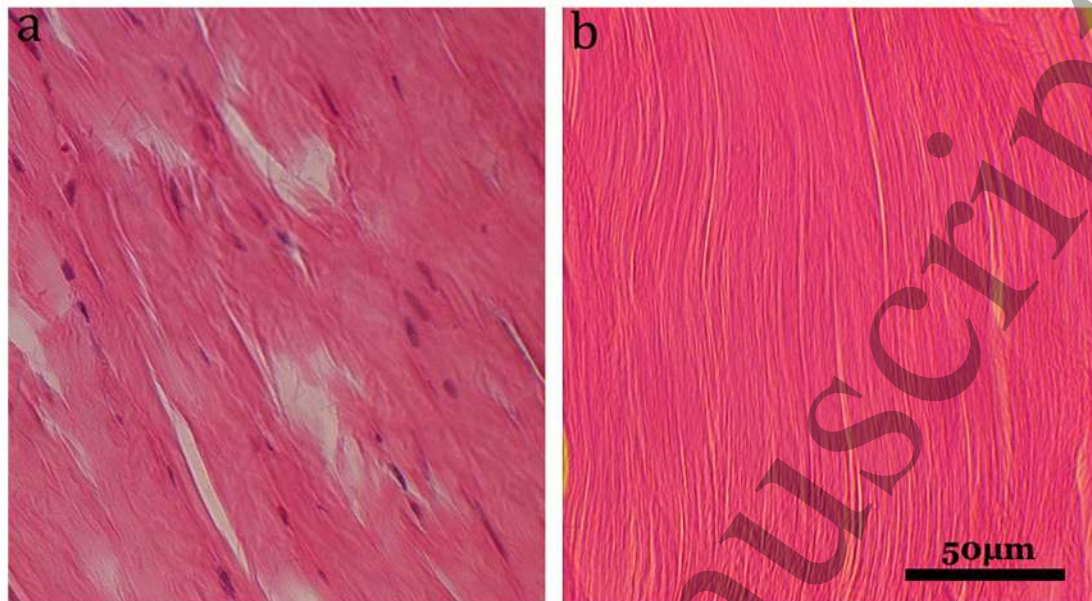
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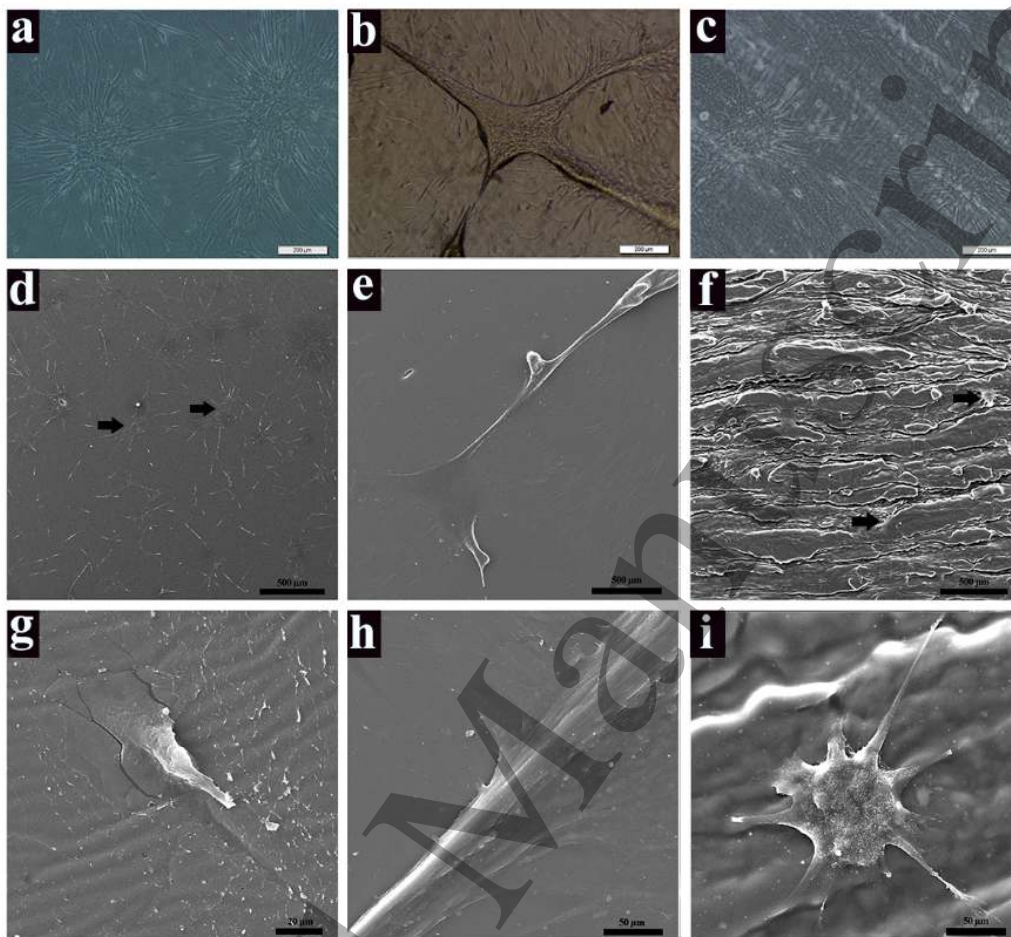


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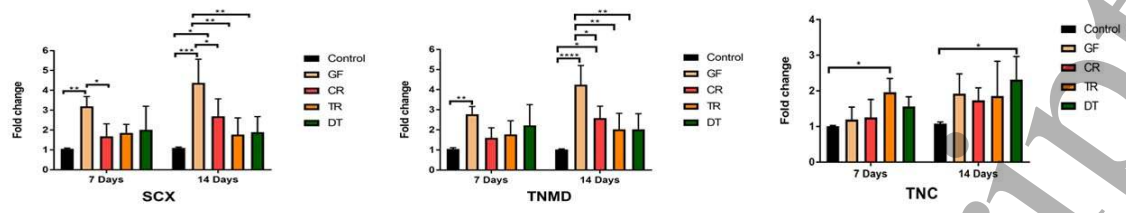






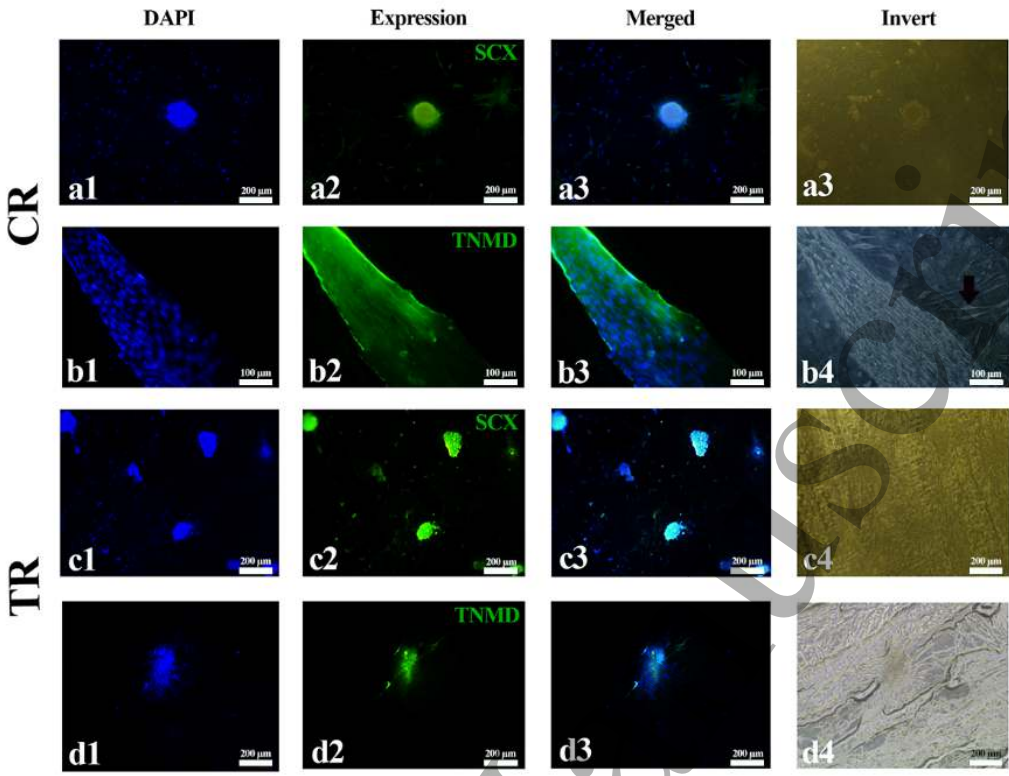
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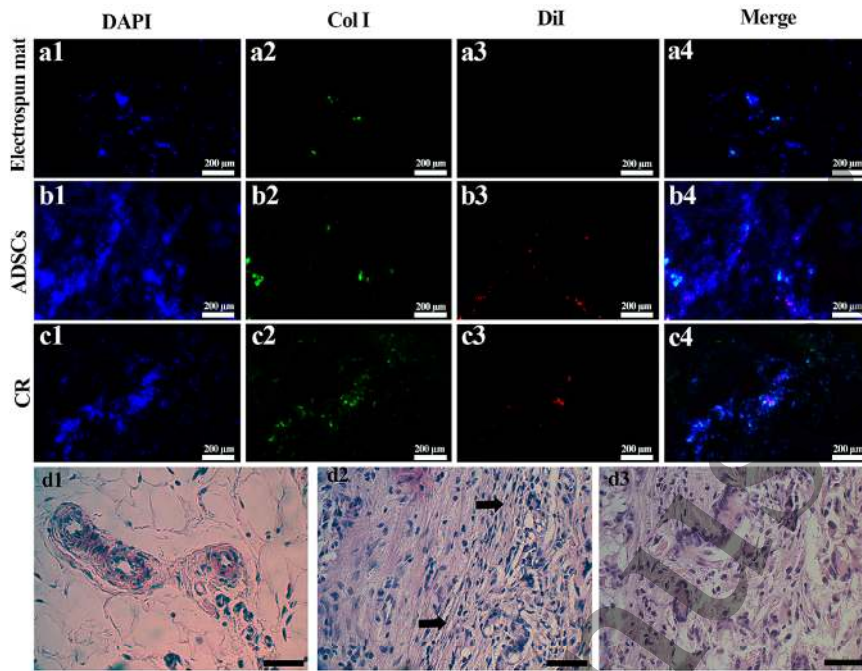
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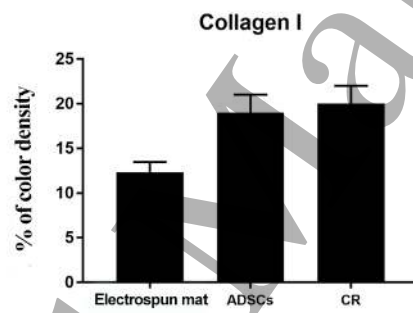
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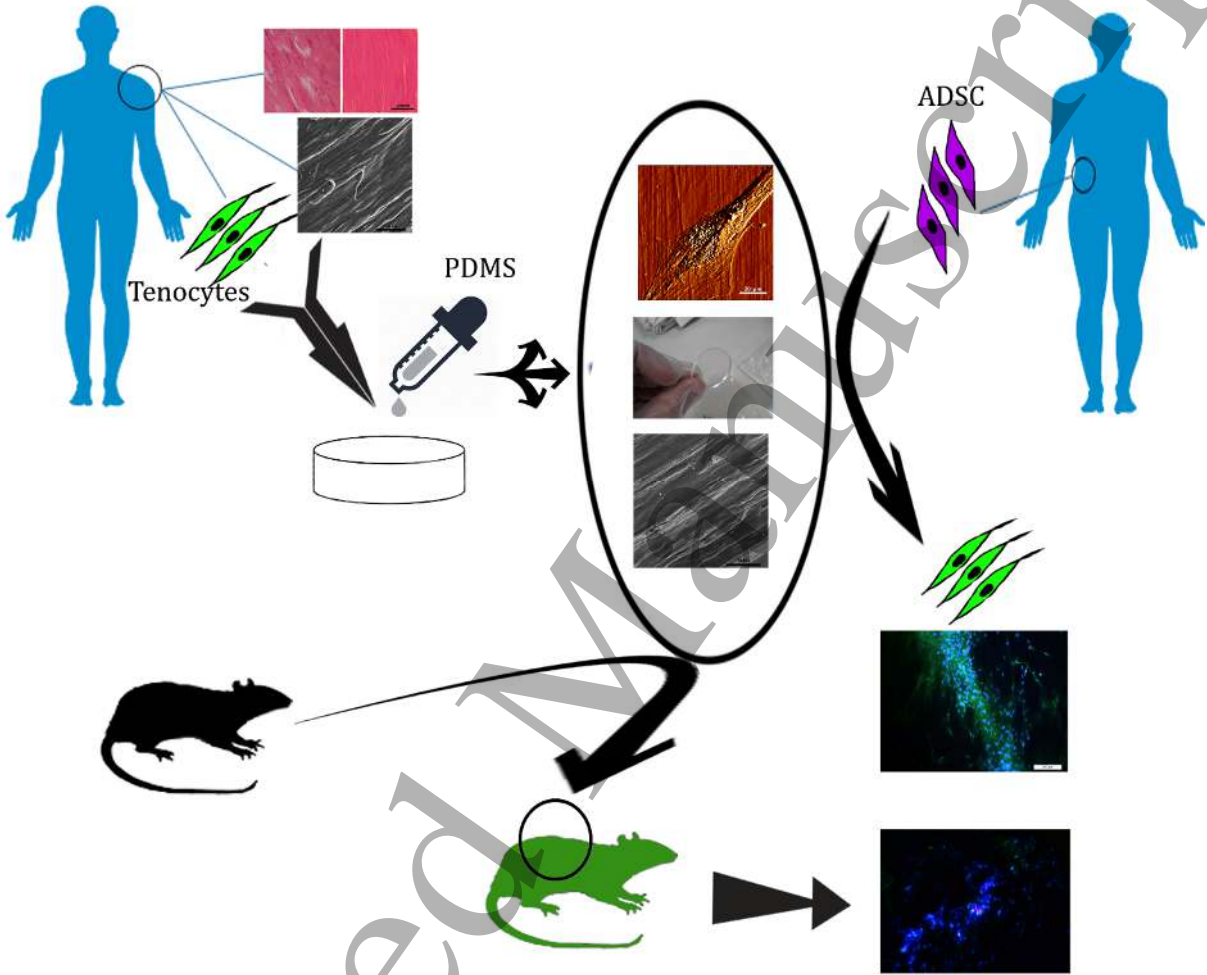
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Graphical abstract



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