



Engineered substrates incapable of induction of chondrogenic differentiation compared to the chondrocyte imprinted substrates

Taheri, Shiva; Ghazali, Zahra Sadat; Montazeri, Leila; Ebrahim, Fatemeh Ale; Javadpour, Jafar; Kamguyan, Khorshid; Thormann, Esben; Renaud, Philippe; Bonakdar, Shahin

Published in:
Biomedical Materials (Bristol)

Link to article, DOI:
[10.1088/1748-605X/acb5d7](https://doi.org/10.1088/1748-605X/acb5d7)

Publication date:
2023

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Taheri, S., Ghazali, Z. S., Montazeri, L., Ebrahim, F. A., Javadpour, J., Kamguyan, K., Thormann, E., Renaud, P., & Bonakdar, S. (2023). Engineered substrates incapable of induction of chondrogenic differentiation compared to the chondrocyte imprinted substrates. *Biomedical Materials (Bristol)*, 18(2), Article 025006. <https://doi.org/10.1088/1748-605X/acb5d7>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

ACCEPTED MANUSCRIPT

Engineered substrates incapable of induction of chondrogenic differentiation compared to the chondrocyte imprinted substrates

To cite this article before publication: Shiva Taheri *et al* 2023 *Biomed. Mater.* in press <https://doi.org/10.1088/1748-605X/acb5d7>

Manuscript version: Accepted Manuscript

Accepted Manuscript is “the version of the article accepted for publication including all changes made as a result of the peer review process, and which may also include the addition to the article by IOP Publishing of a header, an article ID, a cover sheet and/or an ‘Accepted Manuscript’ watermark, but excluding any other editing, typesetting or other changes made by IOP Publishing and/or its licensors”

This Accepted Manuscript is © 2023 IOP Publishing Ltd.

During the embargo period (the 12 month period from the publication of the Version of Record of this article), the Accepted Manuscript is fully protected by copyright and cannot be reused or reposted elsewhere.

As the Version of Record of this article is going to be / has been published on a subscription basis, this Accepted Manuscript is available for reuse under a CC BY-NC-ND 3.0 licence after the 12 month embargo period.

After the embargo period, everyone is permitted to use copy and redistribute this article for non-commercial purposes only, provided that they adhere to all the terms of the licence <https://creativecommons.org/licences/by-nc-nd/3.0>

Although reasonable endeavours have been taken to obtain all necessary permissions from third parties to include their copyrighted content within this article, their full citation and copyright line may not be present in this Accepted Manuscript version. Before using any content from this article, please refer to the Version of Record on IOPscience once published for full citation and copyright details, as permissions will likely be required. All third party content is fully copyright protected, unless specifically stated otherwise in the figure caption in the Version of Record.

View the [article online](#) for updates and enhancements.

Engineered substrates incapable of induction of chondrogenic differentiation compared to the chondrocyte imprinted substrates

Shiva Taheri^{1,2}, Zahra Sadat Ghazali^{3,*}, Leila Montazeri⁴, Fatemeh Ale Ebrahim¹, Jafar Javadpour², Khorshid Kamguyan⁵, Esben Thormann⁶, Philippe Renaud⁷, Shahin Bonakdar^{1,*}

¹ National Cell Bank Department, Iran Pasteur Institute, Tehran, Iran

² Department of Materials Engineering, Iran University of Science and Technology, Tehran, Iran

³ Department of Biomedical Engineering, Amirkabir University of Technology, Tehran, Iran

⁴ Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

⁵ Department of Health Technology, Technical University of Denmark, 2800, Kongens Lyngby, Denmark

⁶ Department of Chemistry, Technical University of Denmark, 2800, Kgs. Lyngby, Denmark.

⁷ STI-IMT-LMIS4, Station 17, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

*. Corresponding authors: shahinbonakdar@yahoo.com

mahya.ghazali@yahoo.com

Abstract

It is well established that surface topography can affect cell functions. However, finding a reproducible and reliable method for regulating stem cell behavior is still under investigation. It has been shown that cell imprinted substrates contain micro- and nanoscale structures of the cell membrane that serve as hierarchical substrates, can successfully alter stem cell fate. This study investigated the effect of the overall cell shape by fabricating silicon wafers containing pit structure in the average size of spherical-like chondrocytes using photolithography technique. We also used chondrocyte cell line (C28/I2) with spindle-like shape to produce cell imprinted substrates. The effect of all substrates on the differentiation of adipose-derived mesenchymal stem cells (ADSCs) has been studied. The AFM and SEM images of the prepared substrates demonstrated that the desired shapes were successfully transferred to the substrates. Differentiation of ADSCs was investigated by immunostaining for mature chondrocyte marker, collagen II, and gene expression of collagen II, Sox9, and aggrecan markers. C28/I2 imprinted substrate could effectively enhanced chondrogenic differentiation compared to regular pit patterns on the wafer. It can be concluded that cell imprinted substrates can induce differentiation signals better than engineered lithographic substrates. The nanostructures on the cell-imprinted patterns play a crucial role in harnessing cell fate. Therefore, the patterns must include the nano-topographies to have reliable and reproducible engineered substrates.

Keywords: Chondrogenic differentiation, Cell imprinting, lithography, nano-topography

1-Introduction

In the past few years, due to the increased need for replacement or treatment of the damaged or diseased tissues in the human body, tissue engineering has played an essential role in designing artificial constructs to keep or improve the function of different organs^{1,2}. One of the fundamental challenges in tissue development is the lack of an adequate amount of cells capable of self-renewal and differentiating toward specific lineage³. Among different tissues, cartilage tissue was believed to be efficiently engineered; however, due to its complex architectural construct and biomechanical properties, it has been challenging to reach the clinical stage^{4,5}. It is also reported that the original phenotype of spherical chondrocytes changes to spindle fibroblast-like after isolation from the body and cultivation in a monolayer condition. The expression of specific marker collagen type II is changed to collagen type I within 2 or 3 passages^{6,7}. Therefore, while maintaining the chondrocyte phenotype in vitro is hardly achievable, induction of chondrogenic differentiation in stem cells becomes more challenging^{8,9}. Even though it has been tried to treat cartilage defects with expanding chondrocytes in culture, providing an ample amount of cells considering the low proliferative capacity of the derived chondrocytes and limited cartilage tissue accessibility, is remained challenging^{10,11}. Also, keeping cells for a long time in culture leads to dedifferentiation of chondrocytes and compromising their functionality¹². Tissue engineering and stem cell differentiation toward chondrocytes provide a promising approach to overcoming present obstacles^{11,13,14}. Cells in their microenvironment are directed by biochemical factors, biological cues, and biophysical features such as topography^{15,16}. Understanding cell-substrate interactions with microfabrication technologies helps to design a smart micro/nano environment more similar to the origin extracellular matrix to control cell shapes, behavior, and fate¹⁷⁻¹⁹. In the last two decades, micropatterning techniques have enabled scientists to resemble the complex structure of tissues by designing substrates on various scales and positioning the cells on the prepared substrates to precisely control cell morphology and function in vitro^{20,21}. However, lately, nanofabrication methods such as e-beam lithography have attracted much attention to develop highly ordered nanopatterns on the substrates^{22,23}. Nano-pillar and nano-hole topographies are among the first studies that have changed the morphology and proliferation of mesenchymal stem cells to induce chondrogenic differentiation¹². In the same

study, nano-grill topography significantly delayed chondrogenesis. The former topographies showed high expression of type II collagen and hyaline chondrogenic marker; however, the latter increased the expression of type I collagen, fibrocartilage marker, and proliferation continued until 14 days even in chondrogenic induction condition¹². Although controlling cell functions is enabled by nano-topography, it is challenging to mimic natural bio-interfaces and tissues since most contain structures that range in different sizes from nano- to micrometer scales²⁴. In this regard, nano/microtopography, inspired by cell shapes, which has been used for inducing differentiation to several lineages^{25–27}, was suggested to induce chondrogenic differentiation of MSCs²⁸. Interestingly, MSCs which are cultured on the cell imprinted substrate expressed specified chondrocytes markers, collagen type II, and aggrecan, while MSCs on the unpatterned substrate did not express mature markers²⁸. Further, cell imprinted substrates based on spherical chondrocyte shapes are used to induce differentiation and transdifferentiation²⁹. It was shown that chondrocyte templates could successfully direct the spindle morphology of MSCs and semifibroblasts toward spherical morphology. Moreover, the immunostaining results and gene expression analysis indicated a high collagen type II expression level for both MSCs and semifibroblasts cultured on cell imprinted substrate²⁹. Osteoblasts imprinted substrate was also investigated and compared with the stem cells template. Although both groups approximately pose spindle shape, AFM height-profile analysis revealed that osteoblasts imprinted substrate imposed pyramidal geometry while stem cells contained a cubical shape. MSCs differentiation to osteogenic lineage was also concluded from gene expression analysis, immunostaining, and osteocalcin measurements³⁰. It was also reported that each cell type has specific nano-topographies on imprinted substrates³⁰.

Similar studies raise the question of which ranges of topography could more effectively regulate the stem cell behavior on these substrates. Our objective was to evaluate the impact of spherical micro-pits with the patterns imprinted by spherical and spindle like topographies. For this purpose, we utilized the chondrocyte template on poly-dimethyl siloxane (PDMS) together with silicon wafers containing average chondrocyte dimensions developed by the photolithography method. In fact, the features on these wafers only simulate the spherical-like shape of chondrocytes, not the nano topography of the membrane. Chondrogenic differentiation on chondrocyte imprinted substrates was compared with the silicon

wafer substrates. Additionally, to investigate the role of nanoscale topography of these substrates on the ADSC differentiation, we used the C28/I2 cells (human chondrocyte cell line), which have spindle-like morphology and express collagen type II. Cell imprinting was performed, and the behavior of chondrogenic differentiation of stem cells was evaluated on substrates containing the pattern of these cells (Figure 1).

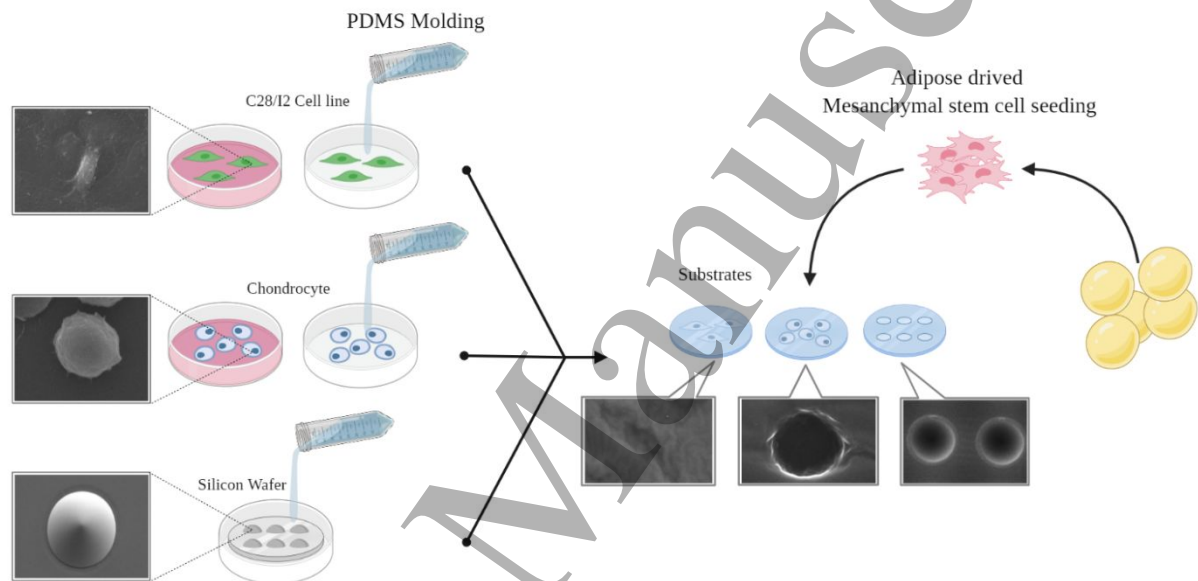


Figure 1: Schematic of the imprinted surfaces: Human C28/I2 cell lines and chondrocytes are used to prepare the imprinted substrates. In the next step, ADSCs are cultured on the prepared substrate. Further, we examine the potential of chondrogenic differentiation of ADSCs on these substrates.

2. Materials and Methods

Cell isolation procedures were performed according to the protocols by the National Cell Bank- Pasteur Institute of Iran and the ethical issues were approved by the Pasteur Institute of Iran's ethical committee (IR.PII.REC.1399.048).

2.1. Human chondrocyte cell line culture

The human chondrocyte cell line (C28/I2) was obtained from Iran's national cell bank and cultured according to their protocols. C28/I2 cells cultured at 37°C in 5% CO₂ containing DMEM/ Ham's F12 (Gibco, Switzerland) supplemented with 10% FBS (Gibco) and 100 U/ml penicillin plus 100 mg/mL of streptomycin (Sigma,

USA). In five days, cells filled up to 80 percent of the culture plates and prepared for fixation.

2.2 Rabbit chondrocyte isolation and culture

Rabbit chondrocytes were isolated according to the previously published methods^{33,34} and approved protocols of Pasteur Institute of Iran. Hyaline cartilage slices were put in Petri dishes, washed for three times with PBS, and then chopped into small pieces utilizing a scalpel blade. The slices were treated with trypsin-EDTA for 30 minutes and digested in 0.08 mg/mL collagenase type II (Sigma, USA) for 16 hours in an incubator at 37°C and 5% CO₂. Cells pellets were obtained by centrifuging the prepared suspension at 1300 rpm for 5 min and were transferred to a culture medium, DMEM/ Ham's F12 with 10% FBS and 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator (37°C, 5% CO₂). The cells were freshly fixed with 4% glutaraldehyde solution in PBS after 24 h.

2.3. Fabrication of silicon wafers by photolithography

The chip was fabricated by general photolithography steps, including photoresist coating, exposure (MLA150, Heidelberg Instruments), developing and deep reactive-ion etching (Alcatel AMS 200 SE) of the silicon wafer. Afterward, the microlense method was applied by heating the silicon wafer at 110°C for 1 minute.

2.4. Cell-Imprinted Substrates fabrication procedure

After cells fixation in 4% glutaraldehyde solution in PBS (GLA, Sigma) at 4 °C for 24 h, two-part of the silicon elastomer kit (PDMS, SYLGARD 184, RTV, Dow Corning, USA) were mixed with a 10:1 ratio. The imprinting substrates was made by pouring PDMS on the cells for 24 h at 37 °C. Further, the PDMS was separated and heated at 100 °C for 10 minutes to cure completely. The prepared substrates were characterized by scanning electron microscopy (SEM, Vega2 Tescan, Czech), atomic force microscopy (AFM, NanoWizard 4a NanoScience, JPK Instruments AG, Germany), and optical profilometry (Veeco WykoNT 1100).

2.5. Isolation and Culturing of Adipose Derived Stem Cells (ADSCs)

As mentioned in the previously published methods, ADSCs were freshly isolated from healthy human bodies according to the instructions at the National Cell Bank

of Iran under their ethical committee approval²⁹. The same protocol was performed to isolate ADSCs from the interscapular region of the rabbits³⁴. Adipose derived stem cells were cultured in (DMEM)/Ham's F12 supplemented with 10% FBS (Gibco) and 100 U/ml penicillin plus 100 µg/mL streptomycin (Sigma, USA) in a humidified incubator (37°C, 5% CO₂). 3×10^3 cells/cm² in 100 µL of culture medium was seeded on each substrate. After 24 h, 500 µL/cm² fresh medium was added to the well plates and incubated for two weeks. During this time the medium was changed every two days.

2.6. Scanning Electron Microscopy (SEM) and Atomic Force Microscopy Imaging (AFM)

The topography of the fabricated substrates and the cells morphology cultured on the imprinted substrates after 14 days were visualized by SEM (Vega2 Tescan, Czech). Before imaging, cells were fixed in 4% glutaraldehyde solution for 45 minutes followed by dehydration with graded dilutions of ethanol (10–100% v/v). Additionally, samples were coated in gold, and SEM was carried out at various magnifications.

The morphology and topography of the substrates and cultured cells on samples were also analyzed using AFM contact mode imaging (NanoWizard 4a NanoScience, JPK Instruments AG, Germany). The instrument software (JPKSPM Data Processing) was used to analyze and obtain the cross-section height profiles of the images.

2.7. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde solution for 10 minutes and washed with PBS (pH 7.4). Further, Triton X-100 in PBS (0.2%) solution for 10 minutes at 25°C is used to permeabilize cells, followed by blocking with BSA for 30 minutes. The primary antibodies used on the samples for 1 hour at 25°C are as follows: mouse monoclonal collagen II (Novus antibody), rabbit polyclonal collagen I. The secondary antibodies are goat polyclonal Anti-Mouse and Anti-Rabbit and cell nuclei stained with Hoechst dye. Imaging was performed with Olympus Microscope. Alcian blue staining was done to observe glycosaminoglycan

formation in ADSCs cultured on different substrates. For this purpose, we prepared alcian blue solution by dissolving alcian blue dye in 100 mL of 3% (w/v) acetic acid. Further, the cells were fixed with 2% paraformaldehyde and incubated in the prepared solution for 30 minutes. In the next step, cells were washed three times with PBS and the samples were observed under a light microscope.

2.8. Gene expression analysis

The real-time PCR was done to analyze the expression of chondrocytes markers. Total RNA was extracted after 14 days of cultured ADSCs on either the patterned substrates or the TCP as our control using the YektaTajhiz Kit (Iran). RNA quality was checked with a NanoDrop spectrophotometer (Eppendorf, Germany). Further, genomic DNA was removed with DNase I (TaKaRa, Japan). Afterwards, the complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit Kit (Takara, Japan). Finally, the real-time PCR assay was conducted utilizing SYBR Premix Ex Taq II (TaKaRa, Japan). Gene expression amounts were acquired from Ct values and normalized to GAPDH. For final comparisons, the expression levels of each group were normalized to its TCP value.

2.9. Statistical analysis

Student's t-test was used to compare two experimental conditions. Multiple comparisons were done in Graphpad Prism 8.0 (two-way ANOVA with Tukey's method). Significance was denoted when P values were less than 0.05. To normalize gene expression analysis, ADSCs cultured on TCP, as our control, are considered equal to 1.0 in every condition. For quantifying protein expression (Col II), at least three random, non-overlapping fields in independent experiments were taken at the same magnification. Cells that showed positive reactions for Col II antibody were counted using ImageJ software. For better understanding the difference of cell morphologies of the three different cell types (Adipose derived stem cells, Chondrocytes and C28/I2 cell line), automated ellipse fitted of ImageJ software was used to measure major and minor axes lengths from the SEM images (at least three various images for each cell types), followed by calculating the aspect ratio (major axis /minor axis) of the cell shapes.

3. Results

3.1. Optical profilometry

Optical profilometry was used to scan the cell surface topography to depict the overall cell shape of a single chondrocyte (figure 2(a)). Based on the chondrocytes spherical shape and average diameter (between 7 to 30 μm ³⁵), we have produced the silicon wafer containing pits structure with a diameter of 18 μm to enable us to recapitulate the micro-scale topography of the overall cell shape. SEM image of the wafer is shown in figure 2(b). Further, the pattern was transferred to PDMS. Figure 2((c)-(d)) show SEM and AFM images of the patterned PDMS.

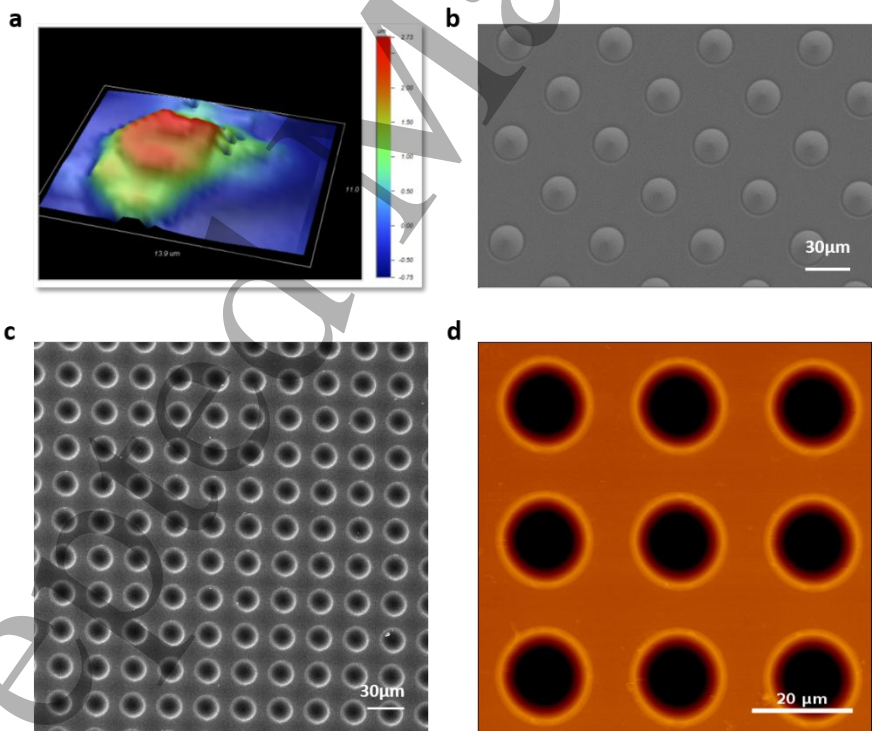


Figure 2: a) optical profilometry of a single chondrocyte. b) SEM image of the wafer. c) SEM image of the patterned PDMS. d) AFM image of the patterned PDMS.

Calculating the aspect ratios of C28/I2 cell line and chondrocytes clearly shows that the morphology of the cells are completely different and they are also distinct from ADSCs which are further cultured on the imprinted substrates (figure 3). Further, the AFM images of the individual cells prove that besides the micro-scale topography, the cell surface includes nanometric topographies which we cannot simply simulate on the wafers by the lithography method (figure 4).

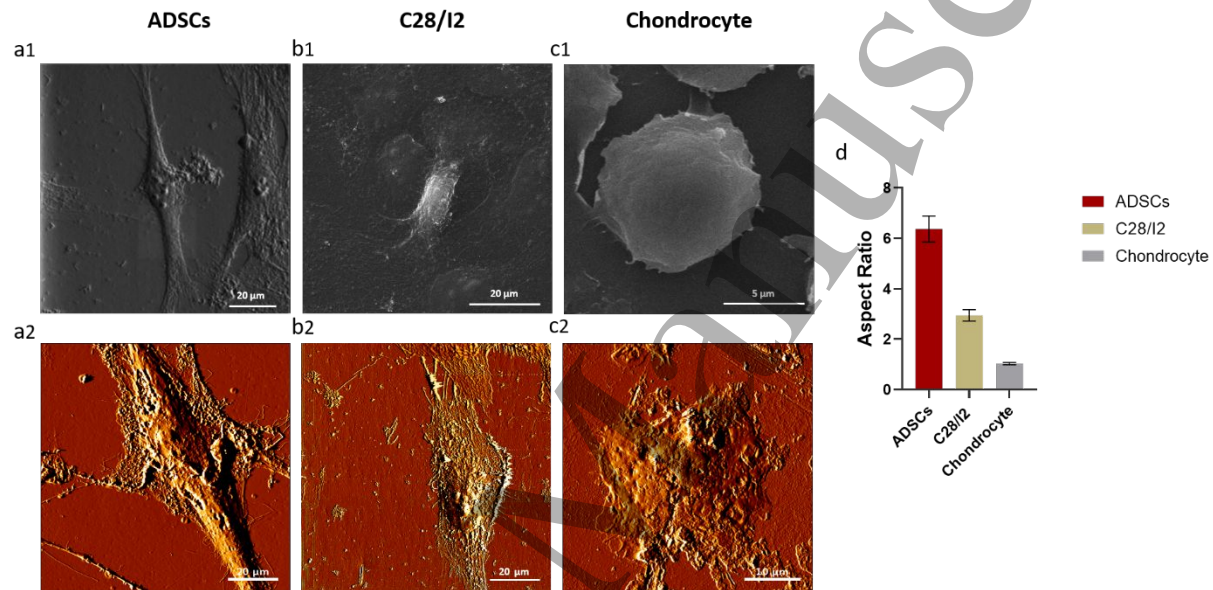


Figure 3: SEM and AFM vertical deflection images of the cells at first day of culture. a1-a2) SEM and AFM vertical deflection images of ADSCs. b1-b2) SEM and AFM vertical deflection images of C28/I2 cell line. c1-c2) SEM and AFM vertical deflection images of chondrocytes. d) The aspect ratio (major axis/ minor axis) of the cell shapes.

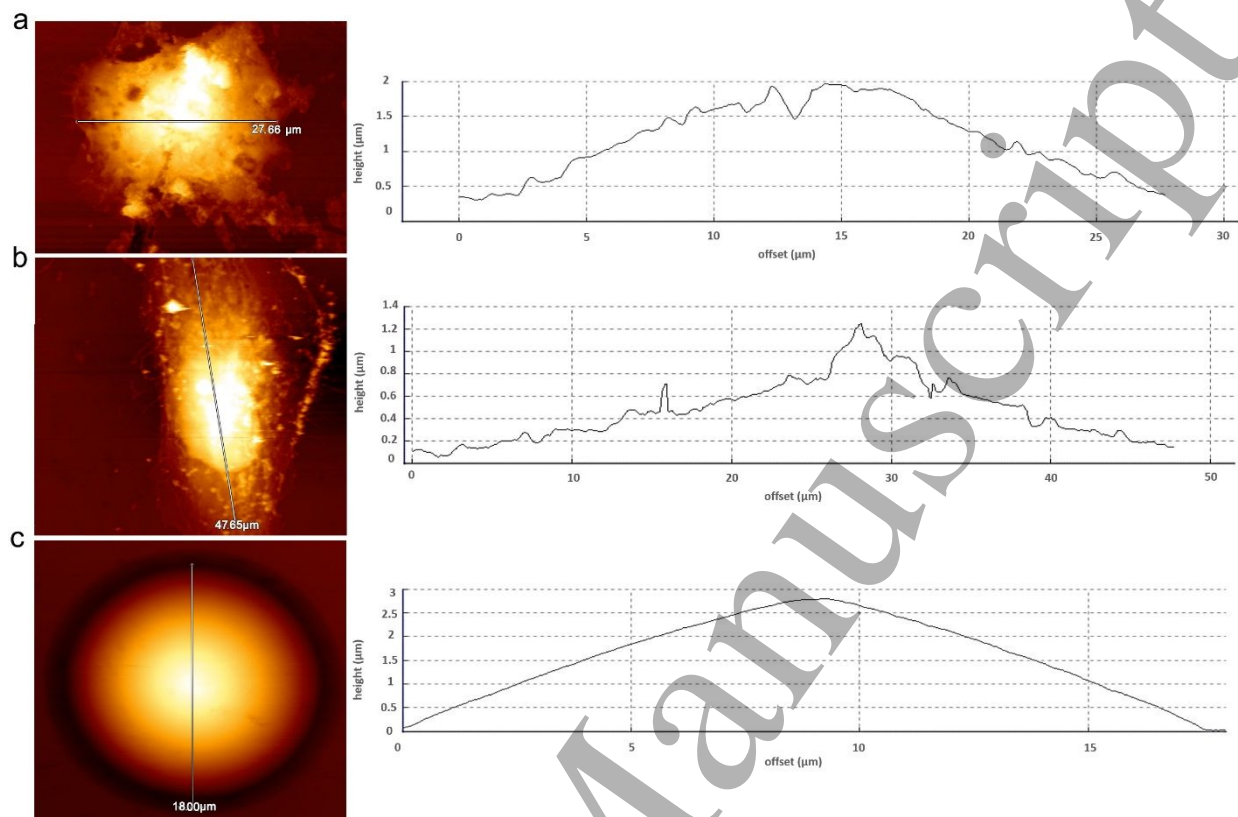


Figure 4: height profile and AFM images of cells: a) chondrocytes, b) C28/I2 cell line, c) silicon wafer

3.2. Microscopy observations

The SEM and AFM images indicate an approximate hemispherical morphology of rabbit-derived chondrocytes and a spindle-like morphology of the C28/I2 cell line. Figure 5 confirmed the morphology of the fixed cells used for preparing imprinted substrates. Figure 4(c) and 5(c) show the AFM and SEM images of the prepared wafer and the imprinted substrate. We further cultured ADSCs on the imprinted PDMS substrates. Figure 3(a) shows the morphology of ADSCs at first day of culture and figure 5 ((d)-(e)-(f)) indicate the morphology of the cultured cells after 14 days. It can be concluded that the substrate topography changed the morphology of ADSCs.

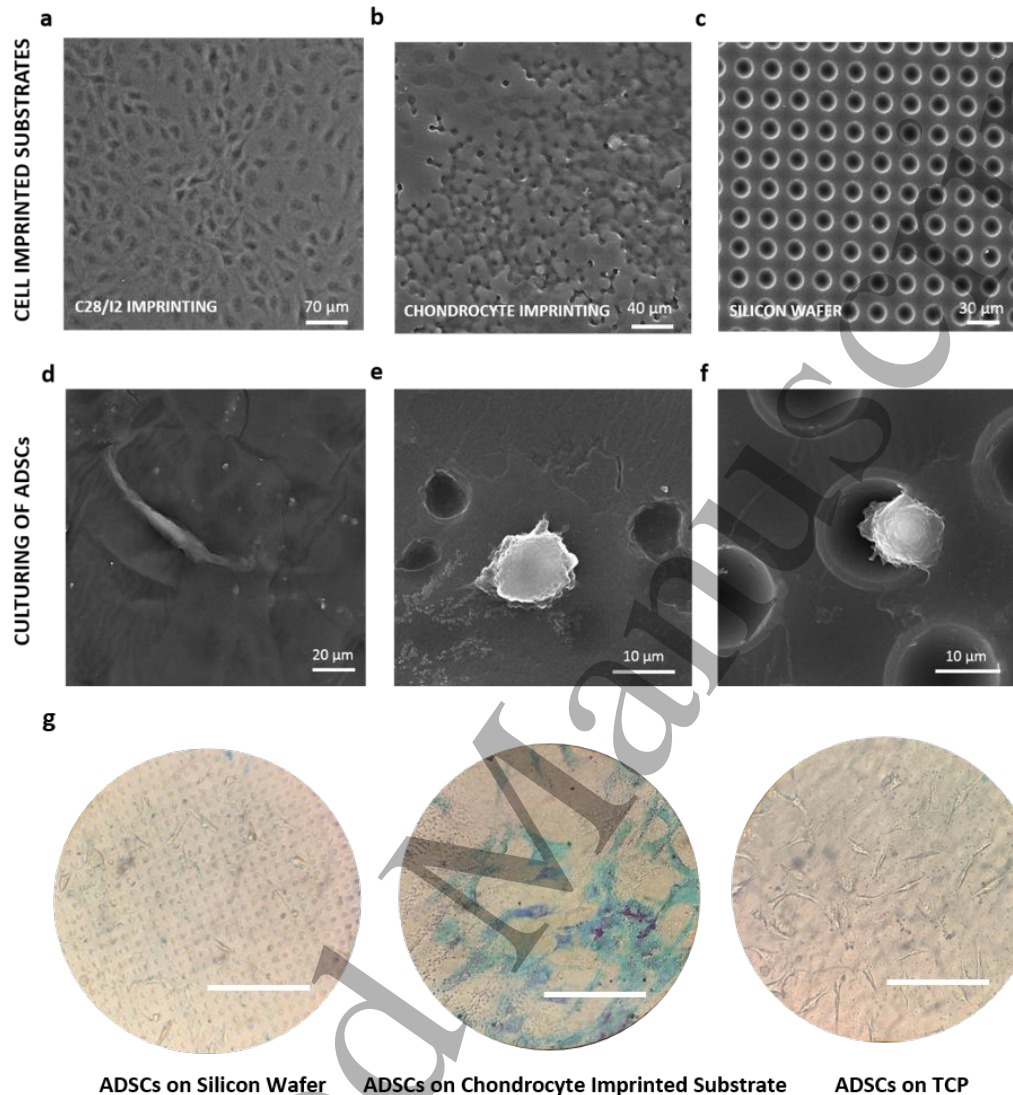


Figure 5: SEM images of the patterned substrates and ADSCs cultured on the prepared substrate. (a): SEM image of C28/I2 imprinted substrate (b) SEM image of chondrocyte imprinted substrate (c) SEM image of silicon wafer imprinted substrate (d) ADSCs on C28/I2 imprinted substrate (e) ADSCs on Chondrocyte imprinted substrate (f) ADSCs on silicon wafer imprinted substrate. (g) Alcian blue staining of ADSCs cultured on silicon wafer, chondrocyte imprinted substrate and TCP (control). Scale bar: 250 μm.

3.3. Differentiation of ADSCs on the prepared substrates

In our next step, we used qPCR analysis to investigate the effect of various patterns on gene expression profiles. Also, for comparing the results, we chose ADSCs cultured on tissue culture polystyrene (TCP) as our negative control and C28/I2 cultured on TCP as our positive control. As shown in figure 6, the expression levels of chondrocyte markers, collagen II, Sox9, and aggrecan, were increased in ADSCs

cultured on chondrocyte ($P < 0.0001$, $P = 0.0003$ and $P < 0.0001$ respectively against ADSCs cultured on the silicon wafer) and for C28/I2 imprinted substrate ($P < 0.0001$, $P = 0.0097$ and $P = 0.0006$ respectively). It can be concluded that the ordered pits pattern on the wafer cannot significantly increase the expression of specific chondrocyte markers in compare with the imprinted substrates, although they imitated the spherical shapes of the chondrocytes on a micrometer scale.

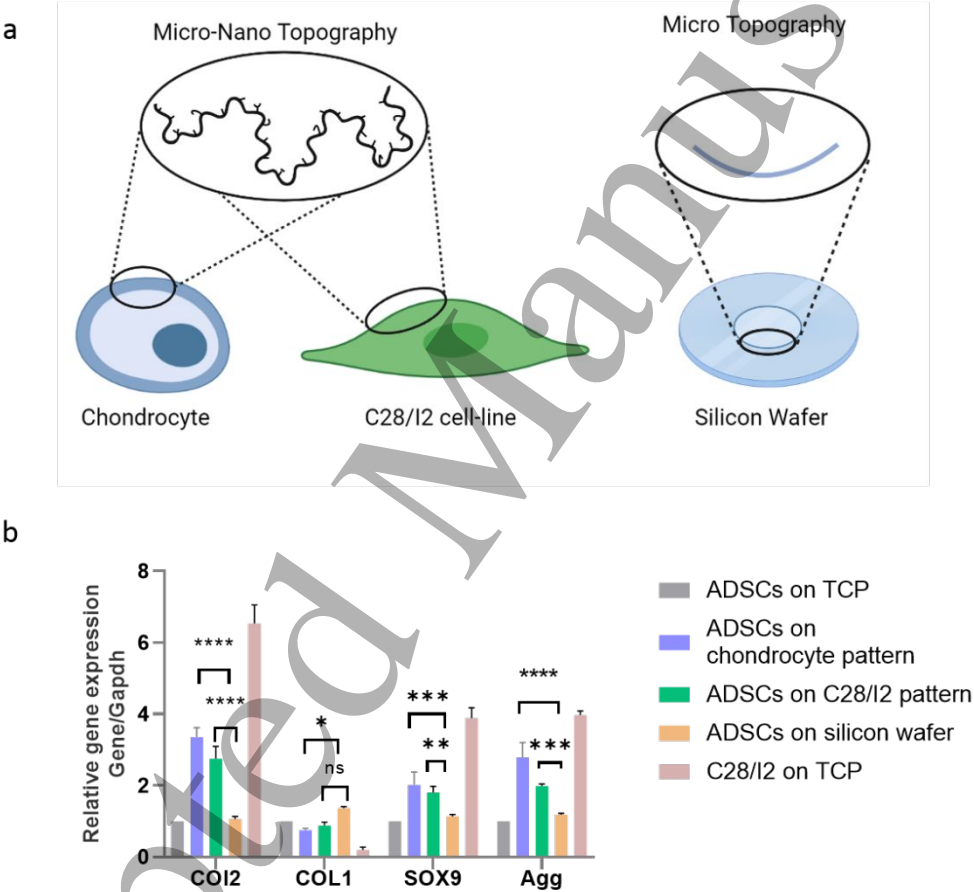


Figure 6: a) Schematic of the three different samples chosen for gene expression analysis. b) Gene expression analysis of ADSCs cultured on the prepared substrates.

Alcian blue staining was done for confirmation of GAG deposition in ADSCs cultured on the cell imprinted substrate (figure 5 (g)). Also, to evaluate the differentiation of stem cells toward chondrogenic lineage, collagen II; the specific marker for hyaline cartilage chondrocytes; and collagen I were examined with immunofluorescent staining. Figure 7 represents immunostaining results for collagen I and collagen II markers for ADSC cells cultured on TCP on the first day

((a1) – (a2)), on the silicon wafer on day 14 ((c1) – (c2)), on C28/I2 imprinted substrate on day 14 ((d1) – (d2)) and also C28/I2 cells on TCP at the first day as our positive control ((b1) – (b2)).

The results suggested that ADSCs cultured on the C28/I2 imprinted substrate expressed collagen II marker significantly in comparison with ADSCs on the wafer ($P= 0.0039$).

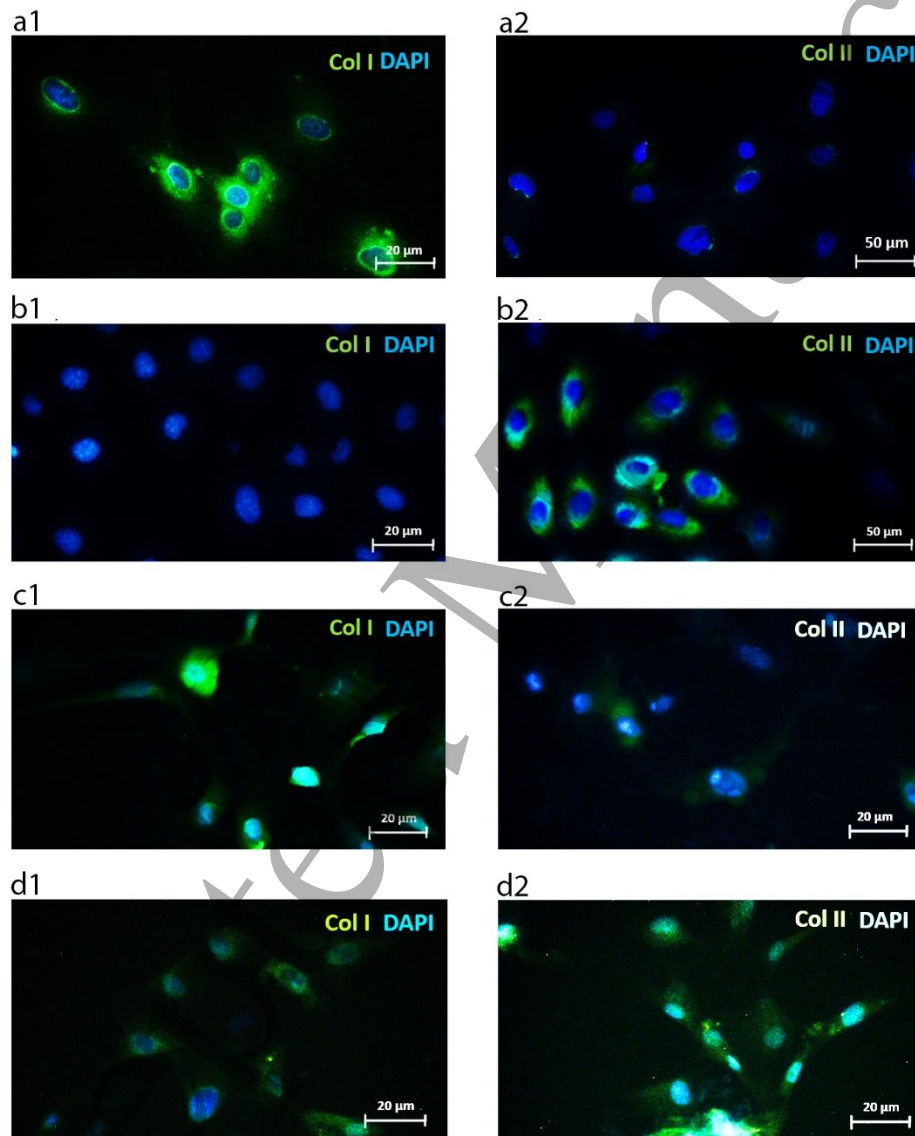


Figure 7: Immunostaining of ADSCs and C28/I2 cells cultured on different substrates: (a1) Immunostaining of ADSCs at one day of culture on TCP for Col I (green) and DNA (with DAPI, blue). (a2) Immunostaining of ADSCs at one day of culture on TCP for Col II (green) and DNA (with DAPI, blue). (b1) Immunostaining of C28/I2 cells at one day of culture for Col I (green) and DNA (with DAPI, blue). (b2) Immunostaining of C28/I2 cells at one day of culture on TCP for Col II (green) and DNA (with DAPI, blue). (c1) Immunostaining of ADSCs at 14 days of culture on silicon wafer for Col I (green) and DNA (with DAPI, blue). (c2) Immunostaining of ADSCs at 14 days of culture on silicon wafer for Col II (green) and DNA (with DAPI, blue). (d1) Immunostaining of ADSCs at 14

days of culture on C28/I2 imprinted substrate for Col I (green) and DNA (with DAPI, blue). (d2) Immunostaining of ADSCs at 14 days of culture on C28/I2 imprinted substrate for Col II (green) and DNA (with DAPI, blue)

4. Discussion

Regeneration of cartilage tissue and chondrogenic differentiation of stem cells is one of the challenging issues due to the complexity of hyaline cartilage properties³⁶. Since stem cells such as adipose-derived MSCs or bone-marrow-derived MSCs are among the primary sources for cartilage tissue regeneration, controlling stem cell behavior with different biophysical or biochemical properties of the scaffolds is crucial^{37,38}. It has been demonstrated that the materials included in cartilage ECM, such as collagen, hyaluronic acid, and chondroitin sulfate, can effectively alter stem cell behavior^{12,39}. Varghese et al. cultured bone-marrow-derived MSCs on a three-dimensional poly (ethylene glycol) (PEG) hydrogel scaffold composited with chondroitin sulfate. They have demonstrated that culturing the cells on the scaffold could result in cartilaginous tissue production and enhancement of chondrogenic gene expression compared to the PEG scaffold without chondroitin sulfate⁴⁰. Biophysical properties of the ECM also play a key role in regulating stem cell behavior^{41,42}. Substrate biophysical properties such as topography and stiffness can direct the differentiation of stem cells toward a specific lineage or maintain cells in the desired phenotype^{12,43–45}. Advances in substrate patterning technologies have enabled the production of various topographies in the micro and nanoscale, such as grooves, pillars, and pits, which can induce behavioral changes in cells such as adhesion, migration, and differentiation due to interactions at the cell-substrate interface^{18,46,47}. Joergensen et al. used microstructures to enhance the proliferation of chondrocytes. They showed that the micropillars sizes in the range of 2 and 4 μm and 1 μm gap between them could efficiently increase cell proliferation and affect cell behavior⁴⁸. Previously, it was shown that cell imprinted substrates induce signals for differentiation of stem cells towards the phenotype of the replicated cells^{30,49,50}. In the conventional imprinting process, the nano/microtopography of a cell membrane is transferred to the PDMS substrate. Indeed, the imprinted PDMS can be introduced as a new substrate, a highly effective substitute for conventional tissue culture plates for directing stem cell fate. In this article, different patterns were produced and compared to investigate the effect of micro and nano topography of the cell's surface

1
2
3 in inducing the chondrogenic differentiation of stem cells. In the first step, the
4 morphology of normal chondrocytes was analyzed by microscopy observations. The
5 SEM images showed the spherical morphology of freshly isolated chondrocytes,
6 approximately in the size of 16 to 18 micro-meters depending on the stage of cell
7 proliferation. The AFM images confirm the presence of nanoscale patterns on
8 substrates. Kamguyan et al. showed that each cell type has its specific nano-
9 topography on imprinted substrates, which can be used as a fingerprint for
10 identification³¹.
11

12 Based on these findings, we assume two different zones for simulation of the
13 chondrocyte membrane, including a micro-scale shape in the range of 5-30 μm and
14 nanoscale features of 1-500 nm. However, precise fabrication of these zones
15 separately or together using conventional photo/electron beam lithography
16 techniques is very difficult, expensive, and time-consuming. For this purpose, we
17 have tested if the spherical morphology of chondrocytes were sufficient for the
18 successful induction of chondrocyte-specific markers in the imprinting process.
19 Therefore, two different cell types (normal chondrocyte and C28/I2 cell line) with
20 different morphologies (spherical and spindle) and similar expressions (collagen
21 type II) were selected. Also, we made spherical pits with a similar diameter of
22 chondrocytes with the lithography technique to compare the effect of micro and nano
23 topography on stem cell differentiation.
24

25 The gene expression analysis confirmed the expression of collagen type II for stem
26 cells cultured on chondrocyte imprinted and C28/I2 imprinted substrates. At the
27 same time, there was no significant expression of collagen type II for stem cells
28 cultured on fabricated pits. The same results were obtained by immunostaining of
29 collagen type II.
30

31 This means that the expression of collagen type II in stem cells is not solely
32 related to the microscopic morphology of replicated cells. Regardless of the whole
33 cell shape topography, the presence of antigens related topography on the cell
34 membrane induces the expression of collagen type II in stem cells.
35

36 Based on these results, we have successfully shown the effectiveness of the
37 nanometric features of the cell surface in directing stem cell fate. Therefore we
38 suggest that the cell imprinting technique, due to its ability to precise transferring
39 micro and nanostructures of the cell surface, can count as a cost-effective and
40 straightforward tool for the differentiation of stem cells toward specific lineage
41 without using complex lithography methods
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

5. Conclusions

Although designing patterns by conventional lithography methods has been shown to enhance differentiation toward specific lineage, simulating such ECM properties is hardly achievable. Because these methods cannot easily imitate nanostructures of the cell membranes, which is an essential factor for controlling cell behavior, here, we showed that two chondrocyte imprinted substrates (C28/I2 cell line and primary chondrocyte) could induce chondrogenic differentiation in ADSCs while pits with microscale features similar to spherical chondrocyte shape could not. Our results suggest that the cell-imprinting method can effectively transfer nano and microstructures of the cell surface, which is necessary for preferable surface topography simulation for inducing stem cell differentiation.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to appreciate Iran National Science Foundation grant No. 94017533 and Pasteur Institute of Iran grant No. 1793 for their financial support. We also would like to thank the CMi-EPFL (Lausanne, Switzerland), particularly for the fabrication of wafers.

References:

1. Dolatshahi-Pirouz A, Nikkhah M, Kolind K, Dokmeci MR, Khademhosseini A. Micro- and Nanoengineering Approaches to Control Stem Cell-Biomaterial Interactions. *J Funct Biomater*. 2011;2(3):88-106. doi:10.3390/jfb2030088
2. Wasyleczko M, Sikorska W, Chwojnowski A. Review of synthetic and hybrid scaffolds in cartilage tissue engineering. *Membranes (Basel)*. 2020;10(11):1-28. doi:10.3390/membranes10110348
3. Mohajeri M, Eskandari M, Ghazali ZS, Ghazali HS. Cell encapsulation in alginate-based microgels using droplet microfluidics; A review on gelation methods and applications. *Biomed Phys Eng Express*. 2022;8(2):22001. doi:10.1088/2057-1976/ac4e2d
4. Camarero-Espinosa S, Rothen-Rutishauser B, Foster EJ, Weder C. Articular cartilage: From formation to tissue engineering. *Biomater Sci*. 2016;4(5):734-767. doi:10.1039/c6bm00068a
5. Walter SG, Ossendorff R, Schildberg FA. Articular cartilage regeneration and tissue engineering models: a systematic review. *Arch Orthop Trauma Surg*. 2019;139(3):305-316. doi:10.1007/s00402-018-3057-z
6. Han HS, Lee S, Kim JH, Seong SC, Lee MC. Changes in chondrogenic phenotype and gene expression profiles associated with the in vitro expansion of human synovium-derived cells. *J Orthop Res*. 2010;28(10):1283-1291. doi:10.1002/jor.21129
7. Binette OIS. Differential Expression of Multiple Genes During Articular Chondrocyte Redifferentiation. 2001;98(December 2000):91-98.
8. Lach MS, Wroblewska J, Kulcenty K, Richter M, Trzeciak T, Suchorska WM. Chondrogenic differentiation of pluripotent stem cells under controllable serum-free conditions. *Int J Mol Sci*. 2019;20(11). doi:10.3390/ijms20112711
9. Cell D, Kit V, Blue A, Red A, Cm CH. Mesenchymal Stem Cell-Based Cartilage is Unstable in Very Long Term In-Vitro Culture. 2013:104-105.
10. Vyas C, Mishbak H, Cooper G, Peach C, Pereira RF, Bartolo P. Biological perspectives and current biofabrication strategies in osteochondral tissue engineering. *Biomanufacturing Rev*. 2020;5(1):1-24. doi:10.1007/s40898-020-00008-y

11. Zhang L, Hu J, Athanasiou KA. The role of tissue engineering in articular cartilage repair and regeneration. *Crit Rev Biomed Eng.* 2009;37(1-2):1-57. doi:10.1615/CritRevBiomedEng.v37.i1-2.10
12. Wu YN, Law JBK, He AY, et al. Substrate topography determines the fate of chondrogenesis from human mesenchymal stem cells resulting in specific cartilage phenotype formation. *Nanomedicine Nanotechnology, Biol Med.* 2014;10(7):1507-1516. doi:10.1016/j.nano.2014.04.002
13. Thorp H, Kim K, Kondo M, Maak T, Grainger DW, Okano T. Trends in Articular Cartilage Tissue Engineering: 3D Mesenchymal Stem Cell Sheets as Candidates for Engineered Hyaline-Like Cartilage. *Cells.* 2021;10(3). doi:10.3390/cells10030643
14. Rosadi I, Karina K, Rosliana I, et al. In vitro study of cartilage tissue engineering using human adipose-derived stem cells induced by platelet-rich plasma and cultured on silk fibroin scaffold. *Stem Cell Res Ther.* 2019;10(1):1-15. doi:10.1186/s13287-019-1443-2
15. Cun X, Hosta-rigau L. Topography : A Biophysical Approach to Direct the Fate of Mesenchymal Stem Cells in Tissue Engineering Applications. 2020.
16. Li J, Liu Y, Zhang Y, Yao B, Li Z. Biophysical and Biochemical Cues of Biomaterials Guide Mesenchymal Stem Cell Behaviors. 2021;9(March):1-13. doi:10.3389/fcell.2021.640388
17. Curtis A, Wilkinson C. Topographical control of cells. *Biomaterials.* 1997;18(24):1573-1583. doi:10.1016/S0142-9612(97)00144-0
18. Ermis M, Antmen E, Hasirci V. Micro and Nanofabrication methods to control cell-substrate interactions and cell behavior: A review from the tissue engineering perspective. *Bioact Mater.* 2018;3(3):355-369. doi:10.1016/j.bioactmat.2018.05.005
19. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues - State of the art and future perspectives. *J Biomater Sci Polym Ed.* 2001;12(1):107-124. doi:10.1163/156856201744489
20. Chemistry A. Micropatterning strategies to engineer controlled IS IN. 2015. doi:10.2144/000114245
21. Hasirci V, Kenar H. Novel surface patterning approaches for tissue engineering and their effect on cell behavior. *Nanomedicine.* 2006;1(1):73-90. doi:10.2217/17435889.1.1.73

22. Liu X, Tang R. Biological responses to nanomaterials : understanding nano-bio effects on cell behaviors. *Drug Deliv*. 2017;0(0):1-15. doi:10.1080/10717544.2017.1375577
23. Curtis ASG, Dolby M, Gadegaand N. Cell signaling arising from nanotopography: Implications for nanomedical devices. *Nanomedicine*. 2006;1(1):67-72. doi:10.2217/17435889.1.1.67
24. Nguyen AT, Sathe SR, Yim EKF. From nano to micro: Topographical scale and its impact on cell adhesion, morphology and contact guidance. *J Phys Condens Matter*. 2016;28(18):183001. doi:10.1088/0953-8984/28/18/183001
25. Ghazali ZS, Eskandari M, Bonakdar S, et al. Neural priming of adipose-derived stem cells by cell-imprinted substrates. 2021;13.
26. Lee EA, Im SG, Hwang NS. Efficient myogenic commitment of human mesenchymal stem cells on biomimetic materials replicating myoblast topography. *Biotechnol J*. 2014. doi:10.1002/biot.201400020
27. Bruder JM, Monu NC, Harrison MW, Hoffman-Kim D. Fabrication of polymeric replicas of cell surfaces with nanoscale resolution. *Langmuir*. 2006. doi:10.1021/la0608563
28. Mahmoudi M, Bonakdar S, Shokrgozar MA, et al. Cell-Imprinted Substrates Direct the Fate of Stem Cells Cell-Imprinted Substrates Direct the Fate of Stem Cells. National Cell Bank , Pasteur Institute of Iran , Tehran , Iran CIC Biomagune , San Sebastian , Spain. 2013. doi:10.1021/nn403844q
29. Bonakdar S, Mahmoudi M, Montazeri L, et al. Cell-Imprinted Substrates Modulate Differentiation, Redifferentiation, and Transdifferentiation. *ACS Appl Mater Interfaces*. 2016;8(22):13777-13784. doi:10.1021/acsami.6b03302
30. Kamguyan K, Katbab AA, Mahmoudi M, et al. An engineered cell-imprinted substrate directs osteogenic differentiation in stem cells. *Biomater Sci*. 2018;6(1):189-199. doi:10.1039/c7bm00733g
31. Mashinchian O, Bonakdar S, Taghinejad H, et al. Cell-imprinted substrates act as an artificial niche for skin regeneration. *ACS Appl Mater Interfaces*. 2014;6(15):13280-13292. doi:10.1021/am503045b
32. Kamguyan K, Moghaddam SZ, Nazbar A, et al. Cell-imprinted substrates: In search of nanotopographical fingerprints that guide stem cell differentiation. *Nanoscale Adv*. 2021;3(2):333-338. doi:10.1039/d0na00692k

33. Bonakdar S, Emami SH, Shokrgozar MA, Farhadi A, Ahmadi SAH, Amanzadeh A. Preparation and characterization of polyvinyl alcohol hydrogels crosslinked by biodegradable polyurethane for tissue engineering of cartilage. *Mater Sci Eng C*. 2010;30(4):636-643. doi:10.1016/j.msec.2010.02.017
34. Yazdian Kashani S, Keshavarz Moraveji M, Taghipoor M, et al. An integrated microfluidic device for stem cell differentiation based on cell-imprinted substrate designed for cartilage regeneration in a rabbit model. *Mater Sci Eng C*. 2021;121:111794. doi:10.1016/j.msec.2020.111794
35. Şenol MS, Özer H. Architecture of cartilage tissue and its adaptation to pathological conditions. In: *Comparative Kinesiology of the Human Body*. ; 2020. doi:10.1016/b978-0-12-812162-7.00007-2
36. Baugé C, Boumédiène K. Use of Adult Stem Cells for Cartilage Tissue Engineering : Current Status and Future Developments. 2015;2015.
37. Muzzio N, Moya S, Romero G. Multifunctional Scaffolds and Synergistic Strategies in Tissue Engineering and Regenerative Medicine. 2021.
38. Murchison AC, Xie Y, Sutrisno L, et al. Three-dimensional culture and chondrogenic differentiation of mesenchymal stem cells in interconnected collagen scaffolds Three-dimensional culture and chondrogenic differentiation of mesenchymal stem cells in interconnected collagen scaffolds.
39. Wu YN, Yang Z, Hui JHP, Ouyang HW, Lee EH. Cartilaginous ECM component-modification of the micro-bead culture system for chondrogenic differentiation of mesenchymal stem cells. *Biomaterials*. 2007;28(28):4056-4067. doi:10.1016/j.biomaterials.2007.05.039
40. Varghese S, Hwang NS, Canver AC, Theprungsirikul P, Lin DW, Elisseeff J. Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biol*. 2008;27(1):12-21. doi:10.1016/j.matbio.2007.07.002
41. Metavarayuth K, Sitasuwan P, Zhao X, Lin Y, Wang Q. Influence of Surface Topographical Cues on the Differentiation of Mesenchymal Stem Cells in Vitro. *ACS Biomater Sci Eng*. 2016;2(2):142-151. doi:10.1021/acsbiomaterials.5b00377
42. Griffin MF. Control of stem cell fate by engineering their micro and nanoenvironment. *World J Stem Cells*. 2015;7(1):37.

doi:10.4252/wjsc.v7.i1.37

43. Lim JYUL, Donahue HJ. Review Cell Sensing and Response to Micro- and Nanostructured Surfaces Produced by Chemical and Topographic Patterning. 2007;13(8). doi:10.1089/ten.2006.0154
44. Costa E, González-garcía C, Luis J, Ribelles G, Salmerón-sánchez M. Maintenance of chondrocyte phenotype during expansion on PLLA microtopographies. 2018. doi:10.1177/2041731418789829
45. Abagnale G, Sechi A, Steger M, et al. Surface Topography Guides Morphology and Spatial Patterning of Induced Pluripotent Stem Cell Colonies. *Stem Cell Reports*. 2017;9(2):654-666. doi:10.1016/j.stemcr.2017.06.016
46. Norman JJ, Desai TA. Methods for fabrication of nanoscale topography for tissue engineering scaffolds. *Ann Biomed Eng*. 2006;34(1):89-101. doi:10.1007/s10439-005-9005-4
47. Tay CY, Irvine SA, Boey FYC, Tan LP, Venkatraman S. Micro-/nano-engineered cellular responses for soft tissue engineering and biomedical applications. *Small*. 2011;7(10):1361-1378. doi:10.1002/smll.201100046
48. Joergensen NL, Le DQS, Andersen OZ, et al. Topography-Guided Proliferation: Distinct Surface Microtopography Increases Proliferation of Chondrocytes In Vitro. *Tissue Eng - Part A*. 2015;21(21-22):2757-2765. doi:10.1089/ten.tea.2014.0697
49. Moosazadeh Moghaddam M, Bonakdar S, Shokrgozar MA, Zaminy A, Vali H, Faghihi S. Engineered substrates with imprinted cell-like topographies induce direct differentiation of adipose-derived mesenchymal stem cells into Schwann cells. *Artif Cells, Nanomedicine Biotechnol*. 2019;47(1):1022-1035. doi:10.1080/21691401.2019.1586718
50. Abadi PPSS, Garbern JC, Behzadi S, et al. Engineering of Mature Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Using Substrates with Multiscale Topography. *Adv Funct Mater*. 2018;28(19). doi:10.1002/adfm.201707378