Cell-Imprinted Substrates Direct the Fate of Stem Cells

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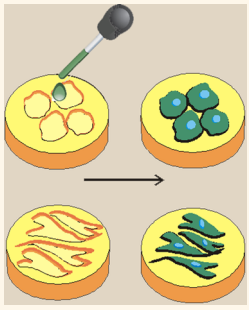
ABSTRACT Smart nanoenvironments were obtained by cell-imprinted substrates based on mature and dedifferentiated chondrocytes as templates. Rabbit adipose derived mesenchymal stem cells (ADSCs) seeded on these cell-imprinted substrates were driven to adopt the specific shape (as determined in terms of cell morphology) and molecular characteristics (as determined in terms of gene expression) of the cell types which had been used as template for the cell-imprinting. This method might pave the way for a reliable, efficient, and cheap way of controlling stem cell differentiation. Data also suggest that besides residual cellular fragments, which are presented on the template surface, the imprinted topography of the templates plays a role in the differentiation of the stem cells.



KEYWORDS: stem cells · biomimicking · cell morphology · cell fate · differentiation · smart substrates

Stem cells have enormous potential therapeutic effects in catastrophic diseases such as cancer and neurodegenerative diseases.[1,2] In this case, controlling of the behavior of stem cells cultured in the laboratory is a crucial issue. Growth factors have been used as a conventional method for the control of stem cell fate.[3] However, disappointing clinical results with some of the growth factors (*e.g.*, angiogenic factors) demonstrated the emerging need for the development of alternative strategies to induce stem cells differentiation.[4−7] As an example in this direction, engineering of the cell culture substrates was proposed.[8−13] It is now recognized that stem cells can sense and produce an appropriate response to the physicochemical properties (*e.g.*, surface composition, surface adhesive ligands and their local densities, surface topography, surface smoothness/roughness, and surface flexibility/rigidity) of their extracellular matrix (ECM) *via* the regulation of their complex signaling pathways.[14−18] By activation of these specific signaling pathways, stem cells can control their crucial future function/strategy such as their gene expression profile and differentiation.[1,2,19,20] For instance, culturing of single human epidermal stem cells on polydimethylsiloxane (PDMS) and polyacrylamide (PAAm) hydrogel surfaces (with a stiffness of 0.1 kPa and 2.3 MPa, respectively) has been demonstrated to cause different cellular responses.[21] Stem cells on PAAm hereby could not form stable and differentiated focal adhesions (*i.e.*, as a result of decreased activation of the extracellular-signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway), whereas their spreading and differentiation were unaffected by PDMS.[21] In addition to substrate stiffness, it has been also shown that the substrate pattern can affect the response of stem cells.[22,23] In this case, substrates with various micro- and nanotopographies have been intensively used to control the differentiations of stem cells.[8,24−30] For example, embryonic stem cells were cultured on nanopatterned PDMS and the results revealed that nanopatterning of the culture substrate can promote the self-renewal of stem cells.[22] Because of these studies, it is recognized that cells grown on optimized patterned substrates behave more similarly to ones within tissue than individual cells do. Besides the fact that a certain critical threshold of cell density is required to induce differentiation,[31]

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also the geometry of the printed nano- and micropatterns (*e.g.*, triangle, square, pentagon, hexagon, and circular shapes) plays a crucial role in cytoskeletal tension of the cultured stem cells, which in turn has significant influence on their differentiation.[32−34] Despite the intense scientific efforts to determine the fate of stem cells with engineered patterned substrates, reliable, high yield, safe, and cheap control of stem cell behavior outside the body is still a great challenge.

Motivated by previous reports,[35−37] in the present work we report a potentially reliable, reproducible, and cheap method for controlling the fate of stem cells by using nano/micropatterned substrates that biomimic cell shapes. Patterns were obtained by employing cells as a template on which a silicone mold was cast, leaving the cells' topography imprinted on the cured substrates. Substrates, thus, resemble the specific topography of the cellular plasma membranes of the cells which had been used as template and, consequently, may emulate the surface of cells.[38] The possibility to control the differentiation of stem cells seeded on these templates was investigated.

RESULTS AND DISCUSSION

Manufacturing of Templates and Their Characterization. Substrates were based on the imprinted surface topography of chondrocyte cells, which are the only cells found in cartilage. These cells were isolated from cartilage slices of New Zealand white rabbits (see Materials and Methods for details). The isolated chondrocytes were dedifferentiated to fibroblast-like cells by growing them as cell monolayer on tissue culture polystyrene plates over the period of weeks. One week after isolation, mature chondrocytes had spherical cellular shapes, whereas three weeks after isolation, chondrocytes were dedifferentiated to fibroblasts with spindle cellular shapes. Cell-imprinted substrates were made from both spherically (matured) and spindle shaped (dedifferentiated) chondrocyte cells. The imprinted substrates were derived from living chondrocytes as template, without the use of fixation materials (*e.g.*, aldehydes). Silicone (*i.e.*, PDMS) was used as the shaping material due to its transparency, capability of molding nano/microstructures, similar density as the cell culture medium, rubbery properties, and ease of handling. The prepared silicone solution was poured on top of the chondrocyte cells. Upon completion of the curing process, the silicone layer was removed from the polystyrene plates on which chondrocytes had been grown, followed by an extensive washing with 1 M NaOH solution with the aim to remove attached cells and chemical/biomolecular residues. In addition to the extensive washing, the substrates were autoclaved at 120 °C for 10 min in order to deactivate possibly remaining membrane fragments. In this way,

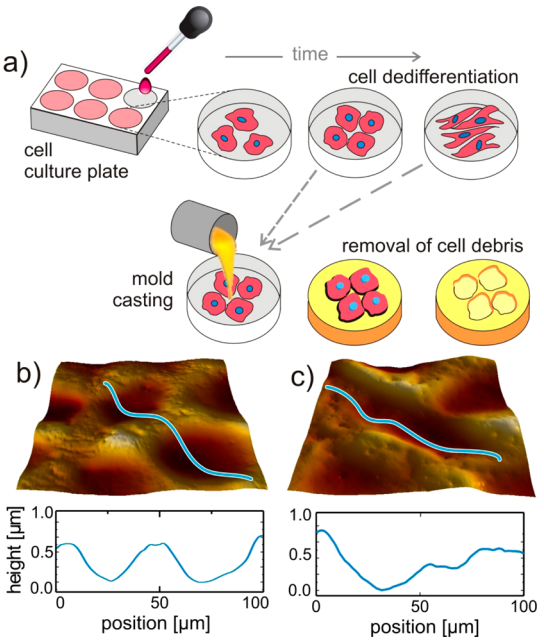

Figure 1. (a) Scheme of the cell-imprinting method: chondrocyte cells were grown on a polystyrene culture plate and their cell morphologies at different stages of dedifferentiation were transferred to a silicone replica by mold casting. After a curing step, the cell debris was removed and the silicone cast acted as negative replica with an imprinted pattern of the cell surfaces. AFM images show the morphology obtained on cell-imprinted replicas for (b) matured chondrocytes (spherically shape) and (c) dedifferentiated chondrocytes (elongated shape).

substrates with imprinted replicas of differently shaped (matured as well as dedifferentiated) chondrocytes were obtained, *cf.* Figure 1a. We clearly need to point out that curing conditions are critical, and so far reduce the yield of obtaining usable substrates. In a first quality control *via* optical microscopy, improperly formed substrates (*e.g.*, no indentations due to cells visible) were discarded. For "good" substrates, atomic force microscopy (AFM) and scanning electron microscopy (SEM) images of the imprinted substrate demonstrate the formation of cellular 3D shapes on the silicone substrates, *cf.* Figure 1b,c. The cultured cells used as template had impressed some distinct groove casts on the surface of the silicone mold, which were not found on replicas taken from bare polystyrene plates (*i.e.*, the control silicone surfaces were approximately smooth and only a negligible roughness of a few nanometers was detectable, see the Supporting Information for details). It is noteworthy to mention that the observed height of the imprinted substrate is less than a micrometer and thus lower than the height of the cells which had been used as template (*cf.* Figure 1b,c). This may be explained by the fact that the silicone rubber could compress the cells and make cells slightly flattened.

Culture of Stem Cells on Top of Cell-Imprinted Substrates. The ADSCs were cultured (for one week) on both types of cell-imprinted silicone substrates, *cf.* Figure 2a.

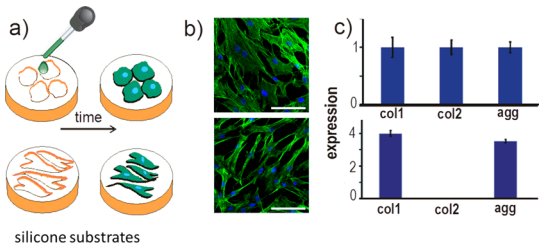

Figure 2. Morphological evolution of ADSC stem cells that were grown (a) on cell-imprinted silicone substrates derived from matured (upper row) or dedifferentiated chondrocytes (lower row). Confocal fluorescence micrographs (b) show that cells adopted different morphologies dependent on the substrate (data obtained with commercial ADSCs) and (c) expressed (relative expression) different genes (type I collagen, type II collagen, aggrecan; data obtained with freshly prepared ADSCs). The scale bars correspond to 10 μm.

According to AFM, fluorescence- and optical-microscopy imaging, the morphology of the cultured ADSCs on the different substrates is notably different, *cf.* Figure 2b. The cultured ADSCs adopt a fibroblast-like phenotype with spindle cellular shape if grown on the dedifferentiated chondrocyte shaped matrix, while their morphology becomes round shaped on the matured chondrocyte shaped matrix. To probe the fate of stem cells on a molecular level, the polymerase chain reaction (PCR) method was employed for probing gene expression associated with the differently differentiated cells[39,40] (type I collagen, type II collagen, aggrecan). ADSCs were cultured on the imprinted substrates derived from mature and dedifferentiated chondrocytes followed by analyzing their gene expressions. For reasons of reproducibility and reliability, several batches (*i.e.*, different substrates, freshly prepared ADSCs from rabbits and commercial ADSCs, *etc.*) were used. PCR results show that ADSCs cultured on the silicone substrates with imprinted patterns of mature chondrocytes expressed specific chondrocyte gene markers such as collagen type II and aggrecan (*cf.* Figure 2c). In contrast, ADSCs cultured on the silicone substrates with imprinted patterns of spindle-shaped fibroblast-like cells did not express collagen II at all, and showed a decreased expression of aggrecan ratio to collagen I. This demonstrates that the underlying substrate also changed gene expression of cells. According to the obtained results, one can conclude that the fate of stem cells in terms of their shape and gene expression pattern can be modulated with the cell-imprinted substrates as presented in this work.

Origin for Cell Differentiation on Cell-Imprinted Substrates. While experiments shown in Figure 2 demonstrate that cell-imprinted substrates can direct the fate of ADSCs, these data do not provide information about the mechanism. In an elegant experiment, Ren *et al.*[35] demonstrated that while bacteria can recognize cell-imprinted substrates, this ability is lost once the substrates are overcoated with a thin siloxane layer. While this siloxane layer had no effect on the topography of the cell-imprinted substrates, it covered their chemical signature. Thus, the authors concluded that attachment of bacteria to cell-imprinted substrates originates from chemical recognition by cellular compounds residing in the cell-imprinted substrate, and that topography effects are negligible.[35] However, their work deals with the binding of cells within a flow channel to the cell-imprinted substrates, which involves only a short time contact between cells and substrates (cell-selective attachment). In our case, however, cells are directly grown for much longer time on the cell-imprinted substrates. Optical microscopy and AFM images confirmed that ADSCs fitted themselves to the imprinted specific cellular shape of the substrate. We, therefore, speculate that in our case besides chemical recognition also recognition of the topography of the cell-imprinted substrates can play a significant role. While surely adhesion forces of cells to surfaces mediated by molecular (chemical) recognition will be stronger, upon extended contact over time eventually also weaker attachment forces mediated by topography might come into play. Cells have a great tendency to be attached to rough instead of smooth surfaces.[41] We, thus, hypothesize that the membrane of cells might fill into the topographic pattern of the cell-imprinted substrates, and that the specifically induced membrane shapes, which are finger-printed according to the respective mature cell types used as templates, could control the selective activations of genes of the printed matured cells, followed by auto-activation of specific complex cell signaling and metabolomic pathways. To test this hypothesis, chemical and topographical recognition had to be separated. Corresponding high resolution AFM data for cell-imprinted replicas of different cells (see the Supporting Information) essentially yield the same root-mean-square (RMS) surface roughness of less than 2 nm. Though this shows high quality of the mold surfaces, the remaining roughness prevents conclusive statements about the presence of residual molecular messengers which may affect the cell differentiation.

Despite attempts to purify the cell-imprinted substrates from remaining cellular fragments of the template cells, immunostaining of the cell-imprinted substrates with fluorescently labeled wheat germ agglutinin (WGA) indicated the presence of some cellular debris, which can comprise residual molecular messengers. Consequently, differentiation of seeded ADSCs as demonstrated in Figure 2 might only originate from chemical recognition of the constituents of template cells. We note that a simple cleaning of the replica surfaces by means of heating is not sufficient as it might cause partial decomposition or cross-linking of cellular debris or fragments of molecular messengers so that their complete desorption cannot be assured. Thus, to get larger areas of cell-imprinted substrates free from cellular debris, still the imprinting method

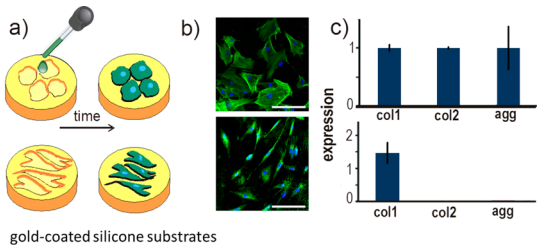

Figure 3. Morphological evolution of ADSC stem cells that were grown (a) on gold overcoated cell-imprinted silicone substrates derived from matured (upper row) or dedifferentiated chondrocytes (lower row). (b) Confocal fluorescence micrographs reveal that different cell morphologies are adopted dependent on the substrate (data obtained with commercial ADSCs) and (c) different genes are expressed (type I collagen, type II collagen, aggrecan; data obtained with freshly prepared ADSCs). The scale bars correspond to 100 μm.

would need to be optimized. Following the idea of Ren *et al.*, [35] we overcoated the cell-imprinted substrates with a thin gold film of about 15 nm deposited by sputtering. To avoid heating of the molds and minimize dewetting of the gold adlayer and cluster formation, the sputtering was performed in pulses. This procedure yielded rather smooth coatings which exhibited only slightly increased roughness (RMS about 2.5 nm), hence providing a camouflage of the possible chemical signature of the surface. Although we were never able to obtain gold films without any fractures, the overcoating still should have camouflaged the chemical signature of the surface. Similar to the bare cell-imprinted substrates, we also seeded ADSCs on the surface of the gold-coated cell-imprinted substrates. Our data indicate that, again, morphology together with gene expression of ADSCs is modulated by the type of cell-imprinted substrate (*cf.* Figure 3). This indicates that in our case also topography effects of the cell-imprinted substrate play a role in the differentiation of ADSCs. Further insight might be obtained in the future by avoiding contamination of the cell-imprinted substrates with cellular fragments on first place. This could be accomplished, for example, by using "double replicated surfaces" (*i.e.*, surfaces that were created using one master originally obtained by actual cell replication), or by creating templates by first imaging the topography of cells, and then creating the template based on these data by 3D printing.

CONCLUSIONS

We introduced a biomimicking substrate for inducing stem cell differentiation based on cell-imprinted templates. Regardless of the detailed mechanism (chemical *versus* topographical recognition), cell-imprinted substrates have been used to differentiate ADSCs in 2D (*in vitro*) cell cultures. Our technique, however, might be expanded for mimicking also 3D *in vivo* cell shapes for enhancing 3D stem cell technologies. Capturing the complexity of organs (*e.g.*, bone and cartilage) in three-dimensional *in vitro* models remains one of the most important challenges in the field of tissue engineering, and thus, cell-imprinted templates may help in this direction. For instance, it was shown that bioscaffolds with cellular pattern of heart tissue (obtained from an *in vivo* organ by coronary perfusion followed by decellularization, which preserved the underlying extracellular matrix) could mimic cardiac cell composition.[42] In this case, reseeding of these constructs with cardiac or endothelial cells could build a new functional heart. Concerning the mechanism, we suggest that also topographical recognition can have an effect, in particular concerning long-term culture.

MATERIALS AND METHODS

Fabrication of Cell-Imprinted Substrates. For the fabrication of the cell-imprinted substrates, polydimethyl siloxane (PDMS, Sylgard 184 , RTV, Dow Corning) was used. The silicone resin and curing agent were mixed with a ratio of 10:1, and the mixture was heated for 30 min at 45 °C (the solution temperature as tested with a thermometer). A total of 30×10^3 cells were seeded on a polystyrene cell culture plate for 24 h in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Scotland)/Ham's F12 supplemented with 10% fetal bovine serum (FBS, Seromed, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). The preheated elastomer solution was cooled to 37 °C, cast on cultured cells, and incubated at 37 °C for 24 h. Subsequently, the cured silicone was peeled off the cell culture plates (*cf.* Figure 1a). This step was followed by extensive washing of the silicone substrates using 1 M NaOH solution in order to remove remaining cells and other chemicals from the substrates as much as possible, leading to the final cell-imprinted substrates. Remaining cellular debris was visualized with immunostaining with fluorescently labeled WGA. To obtain a precise imprinting of the 3D structure of the cells, the amount and the curing time of silicone had to be optimized. The curing time and the total mass of the silicone (lower thickness is preferred, best thinner than 2 mm) are crucial for getting efficient results. Nonproper curing time and thicknesses of the cast elastomer may affect the cellular shapes. For instance, for too short curing times (*i.e.*, less than a minute), the cell shape could not be formed on the shaping material and entrapment of the cells in the shaping material happened. On the other hand, the curing time should not be too long (more than 40 min) as cell migration during this time may change the shape of the cell-imprint.

Gene Expression of Stem Cells Cultured on Cell-Imprinted Substrates. A RNeasy MiniKit (QIAGEN, 74104) was utilized for RNA extraction of the cultured ADSCs (directly applied to the cultured cells on the substrates) according to the manufacturer's instructions. The concentration of cellular RNA was quantified by determining the absorbance maximum at the wavelength of 260 nm in a UV−visible spectrophotometer (Eppendorf, Germany). Complementary DNA was obtained by mixing 1 μg of total RNA and 20 μL of reaction mixture including 4 μL of PCR buffer (15×), 2 μL of dNTPs (20 mM, Roche, Germany), 1 μL of 10 pmol/μL random hexamer (N6; Roche, Germany), 2 μL of deionized sterile H_2O, and 1 μL of reverse transcriptase (200 U/ μL; Fermentase, Russia). Finally, the mixture was kept at 42 °C for 45 min and then incubated at 90 °C for 5 min. The primer sequences specific for the target genes used for PCR analysis are given in the Supporting Information. The real-time PCR was performed in an

ABI 7300 real-time PCR system (Applied Biosystems) with the SYBR Green PCR master mix (Applied Biosystems).

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Full methodology and additional data are provided. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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