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Neural priming of adipose-derived stem cells by cell-imprinted substrates

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Abstract: Cell-imprinting technology is a novel method for directing stem cell fate using substrates molded from target cells. Here, we fabricated and studied cell-imprinted substrates for neural priming in human adipose-derived stem cells in the absence of chemical cues. We molded polydimethylsiloxane (PDMS) silicone substrates on fixed differentiated neural progenitor cells (ReNcellTM VM). The ReNcellTM cell line consists of immortalized human neural progenitor cells that are capable to differentiate into neural cells. The fabricated cell-imprinted silicone substrates represent the geometrical micro- and nanotopology of the target cell morphology. During the molding procedure, no transfer of cellular proteins was detectable. In the first test with undifferentiated ReNcellTM VM cells, the cell-imprinted substrates could accelerate neural differentiation. With adipose-derived stem cells cultivated on the imprinted substrates, we observed modifications of cell morphology, shifting from spread to elongated shape. Both immunofluorescence and quantitative gene expression analysis showed upregulation of neural stem cell and early neuronal markers. Our study, for the first time, demonstrated the effectiveness of cell-imprinted substrates for neural priming of adipose-derived stem cells for regenerative medicine applications.

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Neural Priming of Adipose-Derived Stem Cells by Cell-Imprinted Substrates†

Zahra Sadat Ghazali^a, Mahnaz Eskandari^{a*}, Shahin Bonakdar^b, Philippe Renaud^c, Omid Mashinchian^{d,e}, Shahriar Shalileh^f, Fabien Bonini^g, Ilker Uckay^h, Olivier Preynat-Seauveⁱ, Thomas Braschler^{g*}

* Corresponding authors:

M. Eskandari (eskandarim@aut.ac.ir)

T. Braschler (Thomas.Braschler@unige.ch)

Abstract

Cell-imprinting technology is a recent method for directing stem cell fate using substrates molded from target cells. Here, we fabricate and study cell-imprinted substrates for neural priming in human adipose-derived stem cells in the absence of chemical cues. We mold polydimethylsiloxane (PDMS) silicone substrates on fixed differentiated neural progenitor cells (ReNcell^{TM} VM). The fabricated cell-imprinted silicone substrates represent the geometrical micro- and nanotopology of the target cell morphology. During the molding procedure, no transfer of cellular proteins was detectable. In a first test with undifferentiated ReNcell^{TM} VM cells, the cell-imprinted substrates were capable of accelerating neural differentiation. With adipose-derived stem cells cultivated on the imprinted substrates, we observed modifications of cell morphology, shifting from spread to elongated shape. Both immunofluorescence and quantitative gene expression analysis showed upregulation of neural stem cell and early neuronal markers. Our study demonstrates the effectiveness of cell-imprinted substrates for neural priming of adipose-derived stem cells for regenerative medicine applications.

Keywords: neural tissue engineering, topography, differentiation, PDMS, cell-imprinted substrate

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^a Department of Biomedical Engineering, Amirkabir University of Technology, Tehran, Iran

^b National Cell Bank Department, Iran Pasteur Institute, Tehran, Iran

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

^d Nestlé Research, École Polytechnique Fédérale de Lausanne Innovation Park, 1015 Lausanne, Switzerland

^e School of Life Sciences, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

^f School of Electrical and computer engineering, University of Tehran, Iran

^g Department of Pathology and Immunology, University of Geneva, Switzerland

^h Orthopedic Surgery Service, Geneva University Hospitals, Switzerland

ⁱ Laboratory of Therapy and Stem Cells, Geneva University Hospitals, Switzerland

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Introduction

The irreversible loss of nerve cells in the brain or spinal cord associated with neurodegenerative diseases as well as stroke and traumatic injury is a major cause of morbidity and mortality.¹⁻³ For example, about 15 million annual stroke cases world-wide lead to the death of 5 million people and cause permanent disability in another 5 million.² Therefore, there is a tremendous need for restoration of neural tissue structure and function. Recent studies suggest that neural stem cell (NSC) transplantation is a promising option for neural regeneration, by direct cell replacement or transiently secreted favorable factors.^{4,5} However, very limited availability of NSCs *in vivo*, technical issues in isolation, low purity and not least ethical concerns in the case of harvest from embryonic tissues limit progression in the domain of NSC-based cell therapy.⁶

A major goal of recent research is therefore to derive cells providing NSC functionality from more accessible sources such as embryonic or induced pluripotent stem cells,⁷ but also mesenchymal stem cells (MSCs).⁶ The ease of isolation and multipotency of MSCs indeed make them a particularly valuable cell source in regenerative medicine.⁸ MSCs further have advantageous properties such as reduction of inflammation, prolonging graft survival, reduction of the release of free radicals, and reduction of apoptosis.^{6,9} Neuroprotective effects can be specifically enhanced by pre-differentiation of MSCs to neural stem-like cells.¹⁰ Such neurotrophic stem cells¹¹ are for example under clinical trial for slowing the progression of amyotrophic lateral sclerosis.¹²

Adipose-derived MSCs, also referred to as adipose-derived stem cells (ADSCs), are superior to bone marrow mesenchymal stem cells in some aspects such as ease of culture, rapid growth, and longevity.¹³ In chemical differentiation, ADSCs have additionally been demonstrated to exhibit greater neuronal differentiation potential than bone-marrow derived MSC.¹⁴ This concerns structural markers such as the stem cell marker nestin (“neuroepithelial stem cell protein”¹⁵) and neural markers such as β -III tubulin (Tuj1), but also associated secretion of neurotrophic factors such as NGF, NT-3 or BDNF.¹⁴ Furthermore adipose tissue is abundant and easy to obtain. It can therefore be considered as one of the more promising resources in cell transplantation for neurological disorders.¹³ Yet, at present, effective differentiation of ADSCs into relevant neurosupportive neural-stem cell like cells is still a major challenge. The design of differentiation protocols based on small molecules and growth factors is not only complex, but the necessary chemical and biological factors are expensive and often rather toxic.^{14,16}

This has motivated recent investigations into the use of purely physical factors such as topography or substrate elasticity to induce the desired differentiation.^{17,18} For example, cells are known to respond to extracellular topography through contact guidance. Through topographically triggered changes in cell adhesion, alignment and cell morphology, gene and protein expression^{19,20} are affected, making topography a valuable physical tool to induce a desired biological response. In the specific case of neurons, both topographical features on the micron scale (size of axon and growth cone) and nanometric scale (size of subcellular structures of cells such as filopodia) are known to provide contact guidance.²¹

Based on the importance of topography for cell culture substrates, various artificial micro and nano topographies such as grooves, gratings, and parallel fibers have been used to investigate

different stem cells' behavior.²² For example, Qi et al. studied the effect of linear and circular grooves, as well as regular dot micro patterns on adult neural stem cells.²³ This study showed that all the patterns could suppress proliferation, but the groove patterns additionally enhanced neuronal over astrocytic differentiation, with higher efficiency at smaller feature size (2µm vs. 10µm).²³ 350 nm grooves fabricated in polyurethane acrylate were shown to be suitable for neural differentiation of human embryonic stem cells.²⁴ In this system, the neural stem cell marker nestin was expressed on both patterned and un-patterned surfaces, but the patterns were necessary to obtain expression of the neural marker Tuj1 (β-III tubulin).^{24,25} Hierarchically patterned substrate (HPS) with both microgrooves and nanopores were further shown to enhance hNSCs differentiation beyond the levels achieved with either type of patterns alone.²⁶

Despite the advances in pattern design and fabrication, optimizing artificial topography to direct a given stem cell towards a desired lineage remains a formidable challenge. Cell-imprinting has emerged as a possible generic bio-inspired approach to this challenge.²⁷ Cell-imprinting involves precision molding of cultured cells of a target cell type, to produce a substrate with cells in negative relief.²⁷ Such cell-imprinted molds would then contain appropriate multi-scale topographic pattern for the differentiation of more or less closely related stem cells towards the target cell type. Cell-imprinting has been used previously to obtain differentiation of mesenchymal stem cells along a variety of mesenchymal (chondrogenic,²⁷ tenogenic,²⁸ and osteogenic²⁹), but also epithelial lineages (keratinogenic³⁰ and Schwann cell differentiation³¹), and finally to enhance cardiomyocyte differentiation from human induced pluripotent stem cells iPSC.³² Although the mechanisms are not known with certitude, cell guidance and ensuing specific changes in cell morphology are thought to be the primary contributors to gene and protein expression changes observed in these experiments.^{27,28,30}

Because of the challenges in nerve tissue engineering, we evaluate the ability of cell-imprinted substrates to enhance neural differentiation. We first do so in a committed neural stem cell model; given the promising role of adipose-derived stem cells as a readily available cell source for regenerative medicine, we then assess the capacity of cell-imprinted substrates to induce a pro-neural state in these cells.

Materials and Methods

For safety precautions, see SI-4.

2.1. Neural stem cell culture conditions

The human immortalized cell line ReNcell™ VM obtained from EMD Millipore (Catalog Number SCC008) were grown on laminin-coated plates (Laminin: Sigma L2020, coated at 2 µg/cm², diluted in DMEM/F12, 4h at 37°C and 5% CO₂). Maintenance medium was prepared using NeuroBasal (Gibco, 21103049) supplemented with B-27 supplements (Gibco, 17504044), basic fibroblast growth factor (bFGF, 20ng/mL, Prospeg, Catalog Number CYT-218), epidermal growth factor (EGF, 20ng/mL, Cell Guidance, GFH26-1000) and penicillin/streptomycin (100 UI/mL, 100 µg/mL respectively, Gibco, 15140122). To initiate differentiation, cells are grown to about 70% confluency, followed by withdrawal of the growth factors from the medium. During differentiation, the medium was changed every two days until day 14. All cells were used between passages 10 to 20.

2.2. Fabrication procedure of cell-imprinted substrates

ReNcell™ VM cells were fixed after 14 days of differentiation with 4% glutaraldehyde (GLA, Sigma) in a phosphate buffered solution (PBS) at room temperature for 45 minutes. The fixed substrates were then washed with DI and dried at room temperature. Silicon elastomer kit (polydimethylsiloxane, PDMS, SYLGARD 184, RTV, Dow Corning, United States) was used according to the manufacturer's protocol to prepare the cell-imprinted substrates. For this, the Sylgard 184 polymer and the curing agent were mixed at the prescribed ratio of 10:1, degassed for one hour under vacuum, and poured onto the fixed, dried cells. The PDMS was then cured for 24 hours at 37°C. After curing, the PDMS was peeled from the cells and washed in a NaOH solution (1 M) at 80 °C for 30 minutes. Un-patterned PDMS was obtained by polymerization in tissue cultures plates for 1h at 80°C. To avoid possible imprinting from the tissue culture substrates, we used the upper surface, exposed to air only, as unstructured controls. Structured and unstructured samples were cut to fit into 24-well plates, and autoclaved prior to cell culture.

2.3. X-ray Photoelectron Spectroscopy

X-Ray Photoelectron Spectroscopy (XPS) was performed using a PHI VersaProbe II scanning XPS microprobe (Physical Instruments AG, Germany). X-rays generated from an Al K α X-ray source at a power of 45.7W were monochromatized and focused onto the sample in a 200micrometer spot. Photoelectrons were collected and analysed with a hemispherical capacitor analyser at 45° take-off angle, with a pass energy of 187.85 eV. Curve fitting was performed using the PHI Multipak software.

2.4. Adipose-derived stem cell seeding and culturing

Mesenchymal stem cells of adult fat tissues, called Adipose-Derived Stem Cells (ADSCs) were derived from healthy surgical adipose tissue excision samples (Hôpitaux Universitaires de Genève, Switzerland, Ethical Committee of Geneva approval NAC 14-183 with informed consent obtained from the patients). Cells were isolated as described previously.³³ All cells were used between passages 3 to 6. The cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), and penicillin (100 IU/mL)-streptomycin (100 µg/mL) (Sigma). About 10⁴ cells in 500

μL of culture medium were seeded on each imprinted sample, un-patterned PDMS, and tissue culture polystyrene (controls) and incubated at 37 °C overnight. The day after, 500 μL of culture medium was added to further cover the cells, followed by incubation for two weeks with medium exchange every three days.

2.5. Culture of human ReNcell^{TM} VM cells on cell imprinted surface

To investigate the effect of cell-imprinted substrates on neural stem cell differentiation in committed cells, we also seeded ReNcell^{TM} VM cells on cell-imprinted substrates, control PDMS substrates and tissue culture polystyrene (TCP) controls. For this purpose, firstly, we placed patterned and un-patterned PDMS substrates in 24 well plates. We then proceeded with the coating of the samples, and reference empty 24 well plates (TCP control) with laminin as outlined in section 2.1 above. For cell seeding on these samples, we used the same number of cells and procedures as for the ADSCs. Cells were maintained in NSC Medium without growth factors for two weeks.

2.6. Microscopic observation

For scanning electron microscopy (SEM) samples were fixed with 1.25% fresh glutaraldehyde in phosphate buffer followed by washing them in cacodylate buffer. The samples were post-fixed in 0.2% osmium tetroxide in cacodylate buffer. After washing with deionized water, dehydration was performed in graded ethanol series (30% to 99.9%), 3 minutes in each bath. Samples were sputter-coated with gold-palladium and scanning electron microscopy was done in high vacuum at different magnifications (SEM Zeiss Merlin). Imprinted substrates were also observed by atomic force microscopy AFM (Bruker) to investigate the surface topography of the substrate (height sensor).

2.7. Immunofluorescence staining

Cells were fixed for 30 minutes with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. This was followed by blocking with 10% goat serum (Merck Millipore) for 2 hours. The samples incubated overnight at 4 °C with primary antibodies diluted in 1% goat serum followed by incubation with secondary antibodies for 1 hour at room temperature. The primary antibodies and the dilutions used in this study are as follows: mouse monoclonal anti β -III tubulin antibody (Clone Tuj1, 1:1000, Sigma Aldrich T8660) and rabbit polyclonal anti-nestin antibody (1:400, Millipore, ABD69). The secondary antibodies used were Alexa Fluor 555 and Alexa Fluor 488 (both at 1:1000, Invitrogen). Cells were counterstained with DAPI (Sigma Aldrich). Imaging was performed with the DMI6000 inverted microscope (Leica).

2.8. Gene expression analysis

RNeasy MiniKit (QIAGEN, 74104) was used for RNA isolation from the cultured ADSCs and ReNcell^{TM} VM cells on the different substrates, according to the manufacturer's instructions. The quality of RNA was examined with a NanoDrop 2000c spectrophotometer (Thermo Scientific). cDNA was synthesized from 500 ng of total RNA using a mix of random hexamers – oligo d(T) primers and PrimerScript reverse transcriptase enzyme (Takara bio Inc, Kit) following the supplier's instructions. SYBR green assays were designed using the program Primer Express v 2.0 (Applied Biosystems) with default parameters. PCR cycles were run on a SDS 7900 HT instrument (Applied Biosystems) with the following parameters: 50°C for two minutes, 95°C for ten minutes, and 45 cycles of 95°C 15 seconds-60°C one minute. Each reaction was performed

in three replicates on a 384-well plate. Gene expression levels were obtained from C_t values averaged over the technical replicates, assuming 100% replication efficiency, and normalized to GAPDH as a house-keeping gene. For the purpose of comparison to TCP control conditions, the expression levels were further normalized to the corresponding TCP value.

2.9. Quantitative Image analysis

Total cell numbers in each picture related to a substrate and cells that show positive reactions for β -III tubulin (Tuj1) and nestin antibodies were counted using MATLAB image processing toolbox (Supplementary information SI-2). Thresholds were set manually, and identically for all images being compared. Neurite analysis was performed by assessing the length of cellular processes on binarized and skeletonized β -III tubulin images, with removal of the nuclear area. To sensibly outline the shape of the cells, we implemented a custom algorithm based on step-wise growth of the regions initiated by the nuclei to eventually fill out the cellular contours (Supplementary information SI-3, <https://github.com/tbgitoo/growRois>). The algorithm, intermediate between ballooning of individual regions of interest³⁴ and pixel-based watershedding³⁵ provides a reasonable compromise between following fine greyscale structure³⁵ and the overall geometry of the regions of interest³⁴ (SI-3, user manual on <https://github.com/tbgitoo/growRois>). From the individual cell shapes, Fiji's centroid measurement was used to define the long (major) and short (minor) axis, and eccentricity as the ratio of the two.

2.10. Statistical analysis

Student's t-test (unpaired, unequal variance) was used for comparison of two experimental conditions. For the comparison of several conditions of interest to a given reference condition (control) we used Dunnett's test procedure (default parameters in Graphpad). Multiple comparisons among various conditions used Tukey's procedure. In the case of normalized data, where the reference values are by definition identical to 1 and therefore display an artificial variance of 0, Tukey's or Dunnett's procedures were modified by calculating the relevant variance from the experimental conditions only, in order not to artificially decrease the variance due to the normalization. For comparison of absolute qPCR expression values (normalized only to the housekeeping gene GAPDH), the log of the expression was used for statistical testing to ensure homoscedasticity. The figures report the mean to indicate central tendency and standard deviation of the individual measurements (images for fraction of positive cells; cells for geometric analysis of cell shape; samples in qPCR). Data analysis was carried out in Graphpad Prism 8.0 for default analysis (one-way ANOVA with Tukey's or Dunnett's *post-hoc* tests), or in R-Cran 3.2.3 for adaptation of Dunnett's and Tukey's procedure in the case of normalization, where gene expression in control conditions was considered as equal to 1.0 per definition, and where the reference condition therefore had to be excluded from variance calculation. P values of < 0.05 were considered statistically significant. Multiple testing was performed as inherent in Dunnett's and Tukey's procedure, otherwise the Bonferroni correction was applied.

Results

Our aim was to evaluate patterned polydimethylsiloxane (PDMS) substrates for their capacity of physically inducing or enhancing neural differentiation. For this purpose, we used a cell-imprinting approach, as schemed in **Fig. 1**. We obtained the cell-imprinted substrates by molding a silicone polymer (PDMS) on the neural target cells (**Fig. 1a**). Our ultimate goal is to use this system in autologous cell therapy to replenish non-replicating cells such as neurons from readily available cell sources such as adipose-derived stem cells. In this report, we assessed the capacity of the cell-imprinted substrates to enhance differentiation of already committed neural stem cells and to induce expression of neural markers in ADSCs.

Fig. 2. shows the production and characterization of the cell-imprinted substrates. We obtained neural cells by differentiating ReNcell^{TM} VM cells *in-vitro*. The ReNcell^{TM} VM cells are human immortalized cells from the fetal ventral mesencephalon region; in the presence of the growth factors EGF and bFGF, these cells show typical neural stem cell morphology and proliferation (**Fig. 2a**).³⁶ Upon withdrawal of growth factors, they differentiate and assume a progressively more elaborate neural architecture (**Fig. 2b**) with finely branched neurites. The differentiation of the ReNcell^{TM} VM cells remained nevertheless somewhat incomplete up to 14 days, as despite striking morphological changes, the stem cell marker nestin remained largely detectable (**Fig. 2c**, large neurites nestin-positive, with actin staining outlining the fine neural network). This particularity of the ReNcell^{TM} VM line to retain some stem-cell features throughout differentiation is known,³⁷ and is consistent with our aim to obtain a neurally specified, but not fully differentiated phenotype.

We proceeded with the fabrication of the cell-imprinted substrates. For this, we fixed the ReNcell^{TM} VM cells after 14 days of differentiation, and we prepared cell-imprinted substrates by molding of liquid PDMS precursor (**Fig. 2d**). SEM (**Fig. 2e**) and AFM (**Fig. 2f**) imaging confirm high-resolution, negative replication of the neurite shapes (depth of fine neurites between 20 to 60 nm). At lower resolution (*c.f.* **Fig. 5b**), SEM analysis confirms that not only the nanometric neurite structures, but also larger features such as the overall cell architecture with the cell body, axons and larger neurites are successfully replicated, indicating multiscale feature transfer from the nanometric to micrometric domain. Elemental analysis of the cell-imprinted surfaces (**Fig. 2g**, **Fig. SI-1**) by X-ray photoelectron spectroscopy (XPS) confirm the absence of detectable nitrogen on our cell imprinted substrate, allowing to state that there is no detectable protein transfer. This excludes massive presence of cell debris.^{38,39}

As a first test of the capacity of the cell-imprinted substrates to direct cells cultured on them towards an early neural phenotype, we analysed the differentiation of ReNcell^{TM} VM cells on cell-imprinted substrates as compared to regular tissue culture plates. At 7 days of culture, we found enhancement of neurite outgrowth on the cell-imprinted substrate (**Fig. 3b**) as compared to control conditions (**Fig. 3a**). Neurite length quantification (**Fig. 3c**) indicated that the difference is significant (t-test, $P = 0.0076$). This provides an indication that the cell-imprinted substrates enhance neural differentiation.

Encouraged by these morphological results, we examined the differentiation process of the ReNcell^{TM} VM cells in further detail by qPCR (**Fig. 3d**). In this experiment, we also included

unstructured PDMS substrates to dissect the influence of material chemistry and elasticity from the specific effects of topography. The analysis of the qPCR results is shown in **Fig. 3d**. The cell-imprinted substrate indeed significantly enhanced the expression of the early neural marker Tuj1³⁷ as well as the maturity markers MAP2 and synapsin,⁴⁰ as compared to the TCP control. Consistent with the early neural phenotype of the master cells,³⁷ we also found that nestin expression is enhanced, albeit to a lesser extent (**Fig. 3d**).

The inclusion of unstructured PDMS controls allows to address a critical question: to what extent is the effect due to the chemical nature of the PDMS substrate (as compared to regular tissue culture plates), as opposed to being due to the imprinted topography? For our ReNcell^{TM} VM cells experiments, we found that most of the effect was explained by the use of PDMS as a substrate material, as an overall linear model over the four genes analyzed showed highly significant differences between unstructured PDMS and TCP (Tukey's multiple comparison, $P=7.6 \times 10^{-6}$) as well as imprinted substrates and TCP ($P=3.3 \times 10^{-9}$), whereas there was only a marginal difference between patterned substrates and un-patterned PDMS ($P=0.053$). It should be said that in this experiment, we had to coat all substrates with laminin since otherwise the ReNcell^{TM} VM cells would simply not adhere. This specific coating may have in part masked nanoscopic features of the cell-imprinted substrate. In any case, our findings show that the material choice itself can be very important and should ideally synergize with cell-imprinting features.

Our main aim was to characterize the effect of the cell-imprinted substrate on ADSCs. Therefore, we seeded ADSCs on cell-imprinted substrates, as well as un-patterned PDMS and TCP controls (**Fig. 4a**). After 14 days of culture, we observed the morphological and protein expression changes (**Fig. 4b**, **Fig. 4c**). On the cell-imprinted substrates, we observed more frequently elongated, spindle-like cell shapes, with enhanced nestin and Tuj1 expression (Dunnett's test for positive cell fraction against TCP control, $P=0.0015$ for nestin and $P=2 \times 10^{-4}$ for Tuj1). On the PDMS substrates, we observed enhanced nestin ($P=0.04$ vs. TCP), but not Tuj1 ($P=0.99$) expression. Furthermore, nestin was often found in a more perinuclear pattern on bare PDMS as opposed to the wider somatic localization on the cell-imprinted substrates (**Fig. 4b**). Gene expression analysis by qPCR confirmed a significant increase of nestin and Tuj1 expression on cell-imprinted substrates as compared to tissue culture plate controls. Possible changes on PDMS were too weak to reach statistical significance, and so were possible changes in the mature neuron marker MAP2 regardless of the substrate. Further, there seemed to be no changes in collagen I expression. Altogether, these changes suggest a neural priming effect on the ADSCs, however without interruption of the typical mesenchymal collagen synthesis program.

We next aimed to better understand the mechanisms involved in this neural priming effect of the cell-imprinted substrate on the ADSCs. For this we proceeded with high resolution SEM imaging of ADSCs on both glass cover-slides and cell-imprinted substrates. Visual analysis of the resulting images confirmed gross morphological changes with increased cell elongation on the cell-imprinted substrates (**Fig. 5b**) vs. more spread-out morphology under control conditions (**Fig. 5a**), as suggested by the immunohistological imaging (**Fig. 4b** and **4c**). However, contrary to what has been observed in chondrogenic cell-imprinted substrates,²⁷ the ADSCs do not seem to conform to the hollow cell shapes. The cell bodies of the ADSCs were indeed much bigger than the ones of the imprinted ReNcell^{TM} VM-derived neurons, and so it seems rather difficult

for the cells to physically fit into the cavities left by the cell bodies of the ReNcell^{TM} VM cells. The ADSCs also do not generally conform to the fine imprinted network of neurites (**Fig. 5b**). Rather, the imprinted structures seem to locally direct and favor adhesion of the lamelli- and sometimes filopodia of ADSCs, while providing more limited adhesion to the central areas of the cell body (**Fig. 5b**).

A likely consequence in changes of adhesion pattern is a change in cell shape. We therefore quantified the overall cell shapes of ADSCs grown on various substrates. For this, we made use of the immunohistochemical analysis already carried out for nestin and Tuj1 along with actin and DAPI and defined the overall cell area by a low threshold on the overall fluorescence intensity (**Fig. 5c, SI-3**). We segmented the images by a modified watershed algorithm that uses the nucleus of each cell as a starting point for segmentation (³⁵, SI-3). This allowed us to outline the areas belonging to each cell, and to obtain the major and minor axis of the associated centroid (**Fig. 5c**). From the ratio of the major to the minor axis length, we obtained the eccentricity of each cell – a gross measure of cell elongation. Fig. 5d shows the distribution of the cell eccentricities for the cell-imprinted, bare PDMS and TCP control substrates. In addition to ADSCs population present on the TCP with low eccentricity values mainly between 1 and 2, we found more long cells with eccentricity values up to about 3 and higher on the PDMS and particularly on the cell-imprinted substrates. Statistical analysis of the eccentricity distributions by χ^2 testing clearly sets the three substrates apart: ($P=6.7 \cdot 10^{-8}$ for imprinted vs. TCP, $P=8.3 \cdot 10^{-8}$ for PDMS vs. TCP and $P=4.8 \cdot 10^{-4}$ for imprinted vs. PDMS). Hence, we can conclude that the cell-imprinted substrates have a specific effect on cell elongation in ADSCs, aided by an independent contribution due to the PDMS.

In **Fig. 5e**, we further assessed cellular growth rates over the 14 days culture periods used. With the profound gene and protein expression and morphology changes observed on the various substrates, one might also expect changes in proliferation rate. As shown in Fig. 5e, we do find such changes: calculated over the 14 days of culture, we found an overall significant influence of the substrate on ADSCs cell growth (linear regression with substrate, ordered TCP – PDMS - imprinted, $P=0.021$); in post-hoc analysis, the difference between TCP control and cell-imprinted substrate was significant ($P=0.03$). The result shows that the cell-imprinted substrate leads to slower cell growth.

Overall, our ADSCs culture results show that the cell-imprinted substrates have a pro-neural effect as quantified by increased gene and protein expression of nestin and Tuj1; this is associated with increased cell elongation and decreased cell proliferation. Part of the effect, namely cell elongation and Tuj1 expression is apparently associated with the topographical pattern, while another part, as evidenced by the increased nestin protein expression, seems mostly due to the PDMS substrate.

Our next aim was to quantitatively assess the efficiency of the cell-imprinted substrate to induce a pro-neural phenotype in the ADSCs. For this, we comparatively assessed gene expression level on the two early neural differentiation markers nestin and Tuj1, and the mature neuron marker MAP2. The results of the comparative gene expression analysis between ADSCs as negative control, ReNcell^{TM} VM cells as positive control, and ADSCs differentiated on cell-imprinted substrates as the test item of interest are shown in Fig. 5f. MAP2 was expressed at very low

levels for all three cases; furthermore, there was no significant contrast between the negative and positive control. As outlined before, this indicates that the master ReNcell^{TM} VM cells exhibited an early neural phenotype, and so we consider the cell-imprinting process to have produced a cell-imprinted substrate reflecting an early, rather than fully mature neural differentiation stage.

We therefore focused on the nestin and Tuj1 markers, which did display a significant contrast between negative and positive control ($P=0.0028$ respectively $P=0.047$, Tukey test on log expression). Regarding the test group with ADSCs cultured on cell-imprinted substrates, the gene expression levels of the target markers nestin and Tuj1 rose, and achieved levels between the original ADSCs cell population (negative control) and the levels observed in the master ReNcell^{TM} VM cells (positive control). Indeed, in linear effect modelling, there is both a significant increase due to the use of the cell-imprinted substrates ($P=0.0095$), and a smaller, yet significant difference to the master ReNcell^{TM} VM cells ($P=0.047$, Dunnett's test against the imprinted substrates in both cases). This completes the picture of significant, yet partial conversion also suggested by constant collagen I expression (**Fig. 4f**) and significant changes in cell morphology that nevertheless suggest subsisting differences between the transdifferentiated ADSCs and the ReNcell^{TM} VM cells (**Fig. 5a, 5b, 5d; 2b and 2c**).

Overall, we can conclude that the cell-imprinted substrates accelerate differentiation in the committed ReNcell^{TM} VM cells (**Fig. 3**) and have a pro-neural effect on ADSCs (**Fig. 4** and **Fig. 5**). In the latter case, there are significant changes in neural marker expression (nestin and Tuj1), cell morphology and proliferation; there is nevertheless room for further improvement regarding both marker expression and cell morphology.

Discussion

Our study demonstrates that cell-imprinted substrates, obtained by PDMS molding on differentiated neural cells have pro-neural activity on both undifferentiated, but committed ReNcell™ VM cells and adipose-derived stem cells (ADSCs). To the best of our knowledge, this is the first report of application of the cell-imprinting technique to neural induction.

We obtain the cell-imprinted substrates by using PDMS molding. This raises the immediate question of the contribution of the PDMS itself to pro-neural phenotypes, regardless of surface topography. To address this question, we included bare PDMS controls in both the experiments regarding the differentiation of the committed ReNcell™ VM cells and the neural induction in ADSCs. The results obtained are contrasting and instructive. Bare PDMS is nearly as efficient as the cell-imprinted molds at enhancing neural differentiation of the committed ReNcell™ VM neural stem cells. The pro-neural effect of PDMS is documented in literature reports,⁴¹ although it is not clear whether this effect is primarily due to lower cell adhesion,⁴¹ softer substrate,⁴² release of chemicals⁴³ or on the contrary elimination of some lipophilic constituents such as estrogens from the cell culture media.⁴³

For the induction of a pro-neural phenotype in ADSCs, our results are more contrasted. The topographic features imparted by the cell-imprinting technique were necessary to induce efficient Tuj1 expression and cell elongation. Yet, we found that both the bare PDMS and the cell-imprinted substrates were able to significantly induce nestin protein expression in the ADSCs, albeit with subtle differences in cellular localization. Regarding the ADSCs, we therefore conclude that the observed phenotype change arose from synergistic interaction between the PDMS and the topographical features arising through cell-imprinting. Hence, in order to obtain optimal results with cell-imprinting techniques, it seems advisable to choose a substrate that is by itself favourable to the desired change in phenotype.

Moreover, our results highlight the change in cell morphology as part of the neural induction process. Cytoskeleton-mediated mechano-transduction arising through imposed cell morphology changes is well-known, and has been demonstrated to induce change of cell fate in various systems,⁴⁴ including cell-imprinting.^{31,32} While in our particular system, we do not find a 1:1 match between the negative imprints of the molded cells and the seeded ADSCs, the nanopatterning afforded by the cell-imprinting substrates nevertheless has a distinct, quantitatively important effect on cell elongation. By using lithographically designed parallel nanometric grooves, cell elongation and expression of neural markers including nestin and Tuj1 has been demonstrated in bone-marrow derived mesenchymal stem cells.⁴⁵ In comparison to the neuronal induction by designed nanopatterns, the cell-imprinted substrates are relatively unordered, and alignment of cells is at best a local phenomenon. The similarity in morphological, gene expression and protein expression observed on the highly ordered nanopatterns⁴⁵ and the cell-imprinted substrates used here therefore suggest that mechanical elongation, rather than alignment to a specific pattern, is associated with the observed phenotype switch.

Intriguingly, we observed a significant increase in neural expression markers in the ADSCs cultured on the cell-imprinted substrates, along with undiminished collagen I expression. Some expression of neural stem cell markers, including nestin and Tuj1 used here, is often found in a

subset of cells of mesenchymal origin, although it is debated whether this should be attributed to stem cell plasticity or possibly a lack of marker specificity.^{46,47} Our results suggest that there might indeed be a certain independence of cellular programs, such that inducing a neural-stem cell-like phenotype does not necessarily imply shutting down pro-fibroblastic gene expression, particularly in the presence of serum. This is in line with recent evidence from trans-differentiation experiments: to complete neural induction in fibroblasts, it is not sufficient to induce a neural program with small molecules, it is additionally necessary to inhibit the fibroblast program.⁴⁸

The ability to induce the neuroepithelial stem cell marker nestin in the ADSCs is of interest by itself: the small fraction of nestin-positive cells found among mesenchymal stem cell isolates from various organs is thought to provide the majority of their typical proliferative and direct tissue regeneration potential.⁴⁹ Additionally, this subset is also thought to efficiently secrete anti-inflammatory, angiogenic and neuroprotective factors, making it a desirable target for neuroprotection and neural support.^{47,49,50} Tuj1 as an immature neuron marker is of further interest, as it suggests that beyond enhancing a nestin-positive stem cell fraction, the cell-imprinted substrates developed here provide for early neural priming.⁵¹ The cell-imprinting technology is therefore a cost-effective and accessible tool to produce nanoscale features capable of inducing neural-stem cell-like features in ADSCs. For autologous transplantation *in-vivo*, the tool maybe invaluable, as it may permit purely physical induction with a high safety profile. Whether the induced partial pro-neural state in ADSCs is sufficient to provide neurotrophic microenvironments or possibly neural cell replacement by complete reprogramming *in-vivo* will be the subject of our future studies.

Conclusions

Our results suggest that the cell-imprinting method is capable to induce and improve neural differentiation. The patterned nanostructures obtained by the cell-molding technique lead to cell elongation in adipose-derived stem cells (ADSCs), while exact cell-shape matching does not occur. Gene and protein expression analysis shows induction of neural stem cell and early neural markers expression due to the cell-imprinted substrates. This study demonstrates that cell-imprinting procedure is a highly cost-effective and safe protocol for the enhancement of neuronal differentiation in committed progenitor cells and steering of a pro-neural state in adipose-derived stem cells. The physical cues offered by the cell-imprinted substrates therefore provide an interesting, cost-effective and simple pathway for neural priming in light of cell therapy applications.

Conflicts of interest

The authors declare no conflicts of interest.

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Figures

Figure 1: Schematic representation of cell imprinting procedure. (a) Human neurons (derived by differentiation of ReNcell™ VM neural stem cells) are fixed and molded using polydimethylsiloxane (PDMS). After curing of the PDMS, this yields a cell-imprinted silicone stamp where the micro- and nanomorphology of the cells is present in negative. (b) The cell-imprinted stamps are then used as substrate for culture of adipose-derived autologous stem cells ADSCs. We examine here the capacity of the cell-imprinted physical micro- and

nanoenvironment to induce a neural expression program in the ADSCs. The ultimate aim is to use the neurally primed cells to aid in recovery of central nervous system lesions, for example traumatic spinal cord injury by secretion of neurotrophic factors or completion of the differentiation process *in-vivo*.

Figure 2: Preparation of the cell-imprinted substrates. (a) ReNcell^{TM} VM cells in proliferation medium (undifferentiated state). (b) Differentiation of ReNcell^{TM} VM cells (10 days). (c) Differentiated ReNcell^{TM} VM cells at 14 days, stained for nestin (green), actin (phalloidin, red) and nuclei (DAPI, blue, intensities adjusted linearly to use full range). (d) Preparation of the PDMS stamp after fixation of the cells. (e) Scanning electron microscope SEM image of the molded PDMS, showing molded neurites. (f) AFM height image of the PDMS showing that neurites are imprinted in negative relief (contrast enhanced). (g) XPS elemental analysis of the surface of the stamp, confirming absence of detectable protein transfer by absence of detectable nitrogen.

Figure 3: Acceleration of differentiation of ReNcell™ VM cells by means of cell-imprinted substrates. (a) Morphology of ReNcell™ VM cells cultured on the Tissue culture plates (TCP), stained for Tuj1 at day 7. (b) ReNcell™ VM cells cultured on the cell-imprinted substrates, also stained for Tuj1 at day 7. (c) Average neurite length per neural cell based on Tuj1 staining, at 7 days. (d) Comparative gene-expression (qPCR) analysis of ReNcell™ VM cells cultured for 14 days on cell-imprinted substrates. In addition to the tissue culture plates, non-structured PDMS was included as well to control for the effect of a PDMS rather than polystyrene TCP substrate. All substrates coated with laminin. a) and b): luminosity enhanced proportionally and identically.

Figure 4: Differentiation of human adipose-derived stem cells (ADSCs) on cell imprinted substrates. (a) Schematic representation of experimental design. Human ADSCs are cultured under identical conditions (DMEM, 10% FBS) on cell-imprinted substrates, on unstructured PDMS to assess the effect of the chemical composition of the substrate, and on regular tissue culture plates for negative control. (b) Immunostaining at 14 days of culture for actin (with phalloidin, red), nestin (green) and DNA (with DAPI, blue). (c) Immunostaining under similar conditions as for b) but for β -III tubulin (Tuj1 antibody), actin and DNA. (d) Quantification of the percentage of nestin positive cells for all three different substrates. (e) Quantification of the percentage of β -III tubulin positive cells for all three different substrates. Statistical testing by Dunnett's test against TCP for d) and e). (f) qPCR quantification of gene expression for the three conditions at 14 days of culture. Gene expression normalized first to GAPDH (housekeeping gene), and then expressed relative to control conditions (culture on tissue culture plastic TCP). The scale bar represents 50 μ m, all pictures are taken at identical magnification and identical exposure for corresponding channels. Proportional (and identical per marker) adjustment of brightness for lisibility.

Figure 5: Morphological modifications and neural priming efficiency by absolute gene expression analysis. (a) Scanning electron microscope (SEM) image of adipose-derived stem cells (ADSCs) on flat glass surfaces. (b) Morphology of ADSCs on cell-imprinted substrates. Negative imprints of the molded differentiated ReNcell^{TM} VM cells can be distinguished, including both neurites and cell bodies (holes). (c) Segmentation of cell shapes on immunohistological images (here, nestin/phalloidin). We use the nuclei as a starting point for a watershed algorithm^(35, SI-3), restricting segmentation to bright areas regardless of the immunohistochemical label. The major and minor axis of the elliptic fit (centroid) for each cell identified are shown (major axis thick, minor axis thin white line, cell boundaries yellow). Image: ADSCs differentiated for 14 days on a cell-imprinted substrate. (d) Cell eccentricity as the ratio of major to minor axis: distribution of the cell eccentricity values after 14 days of culture of ADSCs on tissue culture plate (TCP), unstructured PDMS and cell-imprinted substrates. For the imprinted substrate, an additional high eccentricity population appears, corresponding to very elongated cells. (e) Cell growth rates over 14 days of culture of ADSCs on the three different substrates. (f) Comparison of gene expression levels between ADSCs grown on tissue culture plastic (TCP) as negative control, ADSCs grown on cell-imprinted substrates as the item of interest, and ReNcell^{TM} VM cells differentiated on TCP as positive control. Gene expression levels by qPCR, normalization only to GAPDH as a housekeeping gene, but not to any particular condition. Mixed effect analysis by multivariate linear regression of the log of expression with gene identity (using nestin and Tuj1 only, MAP2 remains low even in the positive control) and culture condition (negative control, test condition on imprinted substrates, positive control) as explanatory factors.