Title: Oriented growth and transdifferentiation of mesenchymal stem cells towards a Schwann cell fate on micropatterned substrates

Running title: Transdifferentiating MSCs on micropatterned films

Anup D. Sharma^{1,4}, Svitlana Zbarska², Emma M. Petersen^{2,3}, Mustafa E. Marti¹, Surya K. Mallapragada^{1,4} and Donald S. Sakaguchi^{2,3,4,*}

¹Department of Chemical and Biological Engineering, Iowa State University, Sweeney Hall, Ames, Iowa 50011, USA.

²Department of Genetics, Development, and Cell Biology, Iowa State University, Science II building, Ames, Iowa 50011, USA.

³Biology Program, Iowa State University, Science II building, Ames, Iowa 50011, USA.

⁴Neuroscience Program, Iowa State University, Science II building, Ames, Iowa 50011, USA.

Corresponding Authors:

Donald S. Sakaguchi

Department of Genetics, Development and Cell Biology

503 Science II

Iowa State University, Ames, Iowa 50011, USA.

Voice: 515-294-3112

FAX: 515-294-8457

Email: dssakagu@iastate.edu

Abstract

While Schwann cells (SCs) have a significant role in peripheral nerve regeneration, their use in treatments has been limited because of lack of a readily available source. To address this issue, this study focused on the effect of guidance cues by employing micropatterned polymeric films to influence the alignment, morphology and transdifferentiation of bone marrow-derived rat mesenchymal stem cells (MSCs) towards a Schwann cell (SC) - like fate. Two different types of polymers, biocompatible polystyrene (PS) and biodegradable poly(lactic acid) (PLA) were used to fabricate patterned films. Percentages of transdifferentiated MSCs (tMSCs) immunolabeled with SC markers (α -S100 β and α -p75^{NTR}) were found to be similar on patterned versus smooth PS and PLA substrates. However, patterning had a significant effect on the alignment and elongation of the tMSCs. More than 80% of the tMSCs were oriented in the direction of microgrooves (0°-20°), while cells on the smooth substrates were randomly oriented. The aspect ratio [AR, ratio of length (in direction of microgrooves) and breadth (in direction perpendicular to microgrooves)] of the tMSCs on patterned substrates had a value of approximately five, as compared to cells on smooth substrates where the AR was one. Understanding responses to these cues *in vitro* helps us in understanding the behavior and interaction of the cells with the 3D environment of the scaffolds, facilitating the application of these concepts to designing effective nerve guidance conduits for peripheral nerve regeneration.

Keywords: Micropatterning, Polymeric films, Mesenchymal stem cells, Transdifferentiation, Schwann cells.

Introduction

Schwann cells of the peripheral nervous system (PNS) are glia that form myelin sheaths, and are known to play significant roles during peripheral nerve regeneration (1). Following nerve injury, Schwann cells clear the myelin debris while proliferating and forming bands of Büngner that promote axonal regeneration through the distal nerve stump. In addition, SCs secrete trophic and growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF), all of which promote neural regeneration (2, 3). It has been shown that implantation of SCs supports axonal elongation and regeneration *in vitro* and *in vivo* (4, 5). Furthermore, SCs were also reported to overcome the inhibitory effect of central nervous system (CNS) components following injury (6). These properties enable SCs to be one of the most attractive cell-based therapies for PNS and CNS regeneration.

However, harvesting SCs requires multiple surgeries and sacrifice of a donor nerve that may result in donor site morbidity. These drawbacks have led to alternative approaches, such as transdifferentiation of more readily available cells into SC-like phenotypes. Multipotent MSCs are a versatile cell source for neural repair strategies and can be transdifferentiated into SC-like phenotypes (7, 8). These cells resemble typical SCs morphologically and molecularly (5). MSCs can easily be harvested from several sources, i.e. bone marrow, adipose tissue, and umbilical cord and can differentiate into multiple mesodermal cell lineages (chondrocytes, osteocytes, and adipocytes)(9); and several neuronal and glial phenotypes (10, 11) under certain conditions.

The morphological and phenotypic changes in the transdifferentiated MSCs (tMSCs) were demonstrated using SC markers by immunocytochemistry (ICC), enzyme-linked immunosorbent assay (ELISA) and Western blotting analyses (5, 7, 12). These phenotypes were

shown to enhance axonal growth (13) and remyelination (14) as well as promote functional outcomes (7, 14, 15, 16).

Enhanced neuronal regeneration should be supported with directionality. An oriented axonal regrowth or cell alignment will be superior compared to randomized cellular therapies for nerve regeneration. Specific microarchitectures and topographical cues such as grooves and pores have been shown to promote axonal growth and direct cellular orientation (17, 18). Micropatterning of the polymer scaffold enables building of specific biomaterial architectures and can also influence stem cell differentiation. Previously our lab has reported enhancement in neuronal differentiation and 75% alignment along micropatterned substrates for adult rat hippocampal progenitor cells (AHPCs) (19). Furthermore, the synergistic effects of micropatterned substrates, astrocyte-derived soluble factors and co-culturing with astrocytes, resulted in enhanced neuronal differentiation of AHPCs (20). Nerve regeneration conduits with micropatterned inner lumen pre-seeded with SCs have been shown to promote directed axonal outgrowth and nerve regeneration (21, 22, 23). However, disadvantages associated with SC transplantation have led to use of cellular transplantation therapies along with guidance cues such as micropatterning to enable directional growth and generate a multifunctional nerve guidance conduit which can enhance the regeneration even further. These micropatterned films can easily be rolled into the shape of a conduit and along with transplantation of SC-like phenotypic cells can lead to improved regeneration. But before transplanting SC-like phenotypic cells it is important to characterize growth and morphology of SC-like cells on these patterned surfaces and to the best of our knowledge, no other research group has characterized the effect of micropatterned polymeric substrates on the transdifferentiation of MSCs into SC-like phenotypic

cells and how patterning can collectively control the morphology, alignment and transdifferentiation of the MSCs.

Therefore, in the present study, the transdifferentiation of Brown Norway rat MSCs seeded on micropatterned substrates to SC-like phenotypes was investigated. Biocompatible polymers polystyrene (PS) and biodegradable poly(lactic acid) (PLA) were used for fabricating the micropatterned films to investigate the effect of topographical cues on transdifferentiation, alignment and morphology of the MSCs. In the present study, the transdifferentiation ability of rat MSCs to a SC-like phenotype was tested quantitatively as well as qualitatively on smooth and micropatterned polymer substrates.

Materials and methods

Fabrication of micropatterned polymeric films

Two different types of polymers were chosen for polymeric film fabrication – polystyrene (PS) (M.W. 125,000-250,000) (Cat #00575, Polysciences, Inc., Warrington, PA) and poly(lactic acid) (PLA) (M.W._n. 53,285, Polydispersity 1.6). PS is a widely used biocompatible polymer in tissue culture applications, while PLA is a biodegradable polymer and is frequently used for making transplantable scaffolds for *in vivo* studies. Previously in our group, we have developed a method (19, 24) of producing micropatterned PS and PLA films using micropatterned silicon wafers. Micropatterned silicon wafers were fabricated using conventional lithographic techniques and polymer films were fabricated using solvent casting (19) and detached from the wafers. These films have been shown to support growth of dorsal root ganglion cells, SCs, astrocytes and neural progenitor cells (NPCs) (19, 22, 24, 25).

For PS films, 6% PS solution in toluene was poured on the micropatterned silicon wafer and dried for approximately 4 hours. For PLA films, 10% PLA solution was poured onto a silicon wafer spinning at a speed of 75 rpm using a spin coater (Model PWM32-PS-R790, Headway Research, Inc. Garland, TX). The PLA solution on the wafer was left to dry for 10 minutes on the spin coater before transferring to a chemical hood for an additional 6 hours. Films were carefully peeled off using forceps after submerging the wafer and the dried films in deionized water. Dimensions of the micropatterns obtained on the polymeric films were measured using scanning electron microscopy (SEM, Quant FEG 250, Hillsboro, OR) and were found to be 11-13 μm , 16-18 μm and 3.5-4.5 μm in groove, mesa and depth, respectively for both PS and PLA films. The dried square films were cut to the size of 0.71 cm^2 with a razor blade, with half of the film being patterned and the other half smooth. Silastic medical adhesive (Dow Corning Corp., Midland, MI) was used to attach the square film with a side length of 8.4 mm to a detergent-cleaned 12 mm glass coverslip. The glass coverslips with attached films were sterilized with 70% ethanol and UV light, and coated with 10% poly-L-lysine (PLL, Sigma Aldrich, St. Louis, MO) in water before cell plating. A 40 μl drop of 10% PLL was placed on a sterile Parafilm strip and polymeric films adhered to glass coverslips were placed on top of the PLL drop with film side touching the PLL solution, and left overnight at room temperature. Before cell plating, films were placed in new 35 mm cell culture dishes and the excess PLL was washed away using PBS before adding cell culture media.

Isolation of Brown Norway rat MSCs

All animals were acquired and cared for in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* and all procedures adhered to the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the

Society for Neuroscience and had the approval of the Iowa State University Institutional Animal Care and Use Committee, and were performed in accordance with committee guidelines. Brown Norway rats were obtained from Charles River Labs, and at 6 weeks of age were used for the isolation of bone marrow. The animals were kept in a constant environment (temperature: 22°C; humidity: 20 %; 14/10-hour light-dark cycle) with food and water provided ad libitum until the bone marrow was isolated. The rats were allowed to acclimate after arrival for at least 7 days before harvesting bone marrow.

The rats were euthanized with isoflurane before the dissection of the femora and tibiae. The bones were placed in 50 mL conical tubes with ice-cold maintenance media (MM) composed of alpha minimum essential media (α MEM, Gibco BRL, Gaithersburg, MD), supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 4 mM L-glutamine (Gibco BRL, Gaithersburg, MD), and antibiotic-antimycotic (Invitrogen, Carlsbad, CA). Using a syringe and a 23-gauge needle filled with 3 mL MM, the marrow was forced from the Epiphysis of each bone, into a culture dish. The marrow suspension was dispersed through a 70 μ m nylon cell strainer (BD Falcon, Bedford MA), dampened with MM and transferred into a T75 flask with 20 mL MM. The cell suspension was maintained in a culture incubator (37°C, 5% CO₂/95% humidified air atmosphere). After 48 hours, post-harvest, spindle-shaped cells adhered to the bottom of the flask. The cells were washed with phosphate buffered saline (PBS; Gibco BRL, Gaithersburg, MD) and the media was replaced with fresh MM.

Culturing of Brown Norway rat MSCs

Brown Norway rat MSCs were plated in T75 flasks in MM, consisting of α minimum essential medium (α MEM, Gibco BRL, Gaithersburg, MD), 20% fetal bovine serum (FBS; Atlanta Bio.), 4 mM L-glutamine (Gibco), and antibiotic-antimycotic (Invitrogen, Carlsbad, CA)

and incubated at 37°C/ 5% CO₂/ 95% humidified air atm. Adherence to the flask was observed from flat fibroblast-like cells. When the MSCs were ~80% confluent, media was aspirated and cells were washed with PBS to remove serum before trypsinizing the cells. Cells were collected transferred into 15 ml conical tubes and centrifuged at 500 rpm for 5 minutes. Following centrifugation, the cell pellets were resuspended in MM and the cell suspensions plated out onto various polymeric films following Trypan blue viable cell counts.

***In vitro* transdifferentiation of rat MSCs into SC-like phenotypes**

In vitro chemical induction of the rat MSCs into SC-like phenotypes was performed in three steps following the protocol established by Dezawa and coworkers (7). Rat MSCs were kept in MM for 1-2 days in T75 flasks at 37°C/ 5% CO₂. After reaching 30-40% confluence, the MM media was replaced with the transdifferentiation media-1 (TDM-1) containing α MEM supplemented with 1 mM β -mercaptoethanol (BME; Sigma-Aldrich, St. Louis, MO) and incubated for one day. After a PBS wash, TDM-1 was replaced with the TDM-2 containing α MEM, 10% fetal bovine serum (FBS) and 35 ng/ml all-trans-retinoic acid (ATRA; Sigma, St. Louis, MO). The cells were incubated in TDM-2 for the following 3 days at 37°C/ 5% CO₂. Finally, the cells were incubated in TDM-3 which contained α MEM, 10% FBS, 14 μ L forskolin (FSK; EMD Millipore, Billerica, MA), 5 ng/mL platelet derived growth factor (PDGF; Sigma), 10 ng/mL basic fibroblast growth factor (bFGF, Promega Corporation, Madison, WI) and 200 ng/mL heregulin β 1 (HRG; Calbiochem, EMD Millipore, Billerica, MA) for 8 days *in vitro* (DIV). Prior to the media changes, cells were always washed with PBS. At the transition to TDM-3, the cells were plated onto glass coverslips or half patterned-half smooth polymeric substrates attached to glass coverslips. The polymer film substrates were coated with PLL and placed in a 35 mm petri dish etched into quadrants with a soldering iron. Control or

undifferentiated MSCs (uMSCs; MSCs cultured in MM) were always grown in parallel to the MSCs undergoing transdifferentiation (tMSCs; SC like-cells). Undifferentiated and transdifferentiated MSCs grew at different rates in MM and different TDMs respectively. In order to obtain a comparable cell number after a period of 14 DIV, uMSCs were plated at a density of 1000 cells/cm² while tMSCs were plated at a density of 3000 cells/cm². MSCs from passages 4 to 7 were used for replicate studies of the transdifferentiation experiment. P4-P7 MSCs displayed consistent plasticity and were able to transdifferentiate into SC-like cells. Furthermore, by using P4-P7 cells we minimized the number of animals necessary to generate multiple vials of MSCs for future studies. Immunocytochemical procedures were used to characterize the uMSCs and tMSCs (see below). The transdifferentiation and cell morphology on the patterned half of each substrate was compared with that of the smooth half on the same substrate.

Immunocytochemistry (ICC)

After 8 DIV, the cells in culture dishes were rinsed with 0.1 M PO₄ buffer and fixed for 20 minutes with cold 4% paraformaldehyde (stored at 4°C) prepared in 0.1 M PO₄ buffer. The cells were then rinsed with filtered phosphate buffered saline (PBS; Fisher-Scientific) for 3 times every 7 minutes and incubated in blocking solution of PBS supplemented with 5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA), 0.4% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher Scientific).

Several antibodies were used for ICC analysis for characterization of undifferentiated MSCs (uMSCs) and transdifferentiated MSCs (tMSCs) (Table 1). The primary antibodies were diluted with blocking solution. Samples were incubated with primary antibodies at 4°C overnight. On the following day, the cells were rinsed with PBS for 4 times every 8 minutes, and

198 subsequently incubated in appropriate secondary antibodies diluted in blocking solution. The
199 following secondary antibodies were used: Donkey- α -Mouse-Cy3 (1:500, Jackson
200 ImmunoResearch, West Grove, PA), Donkey- α -Rabbit-Cy3 (1:500, Jackson ImmunoResearch,
201 West Grove, PA). In addition, MSCs were stained with Alexa Fluor 488 Phalloidin (1:1000,
202 Molecular Probes-Life Technologies, Carlsbad, CA), and DAPI (4',6-diamidino-2-phenylindole)
203 (1:2000, Invitrogen, Carlsbad, CA), all diluted in the same blocking solution. The cells were
204 incubated for 60-90 minutes in the dark at room temperature and then rinsed with PBS for 3
205 times every 7 minutes. Immunolabeled control samples were incubated without primary
206 antibodies, and with secondary antibodies to determine any autofluorescence associated with the
207 samples. For the glial cell markers (S100 β , p75^{NTR} and GFAP) cryosectioned retinal tissue was
208 used as a positive control. Samples were mounted by careful removal of the polymeric film from
209 the cover slip with forceps. The film was slid into the middle of a drop of Vectashield, cell side
210 up, on a microscope slide. Cover glass was placed on top of the film and Vectashield, and an air
211 tight seal was formed with nail polish. Preparations were examined and images captured using an
212 upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital
213 camera controlled by QCapture software (QImaging, Surrey, BC, Canada). For image analysis, a
214 minimum of 8 microscope fields from each substrate were taken randomly using a 20X
215 objective. Each field represents 0.17 mm² (476 μ m x 357 μ m). To calculate the percentage of
216 immunoreactive cells on each substrate, the number of cells immunoreactive for each antibody
217 was divided by the total number of cells (DAPI-stained nuclei). Images were uploaded to
218 MetaXpress software (Molecular Devices, Sunnyvale, CA) and with help of multiwavelength
219 cell scoring module; number of cells stained with different antibodies/wavelengths were
220 obtained.

Treatment with bromodeoxyuridine (BrdU)

MSCs were cultured for 3 DIV and 12 hours before fixation, the cells were incubated with MM supplemented with BrdU to a final concentration of 5 μ M. After fixation, the cells were treated with 2N HCl at 37°C for 15 minutes and then replaced with 0.1 M borate neutralization buffer for 15 minutes. The neutralizer was removed and the cells were rinsed with PBS for 3 times every 7 minutes. The rest of the procedure was carried out in accordance with the ICC stated above, starting with the one hour incubation in blocking solution. Primary antibody used was Rat α BrdU (1:200, Abcam, Cambridge, MA).

Morphometric and statistical analysis

Aspect ratio (AR) of an object is defined as the ratio of object's width to its height, when the width of the object is longer than its height. Aspect ratio of MSCs have been shown to affect their lineage commitment (26). Objects such as regular polygons or circular objects have AR equal to one, which means that there is no difference in height and width of the object. Here in this study, we have captured images such that the patterns are always horizontal in direction with respect to the image and the AR was calculated by dividing the length of the cell in the direction of the microgrooves by breadth of the cell in the direction perpendicular to microgrooves. Images were binarized by determining minimum threshold intensity to differentiate between the background and cells stained with phalloidin. Integrated morphometric analysis module of MetaXpress software was used for measuring various morphological parameters such as width, height, cellular area and orientation of the cellular objects obtained after thresholding. Orientation of the cells was measured between -90 to +90 degrees from the horizontal where all the images captured had micropatterns parallel to the horizontal. Thirty cells per condition (PLA pattern, PLA smooth, PS pattern, PS smooth) per experiment were analyzed to obtain the data.

Three independent experiments were analyzed, giving 90 cells per condition. GraphPad prism v6 software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. One way ANOVA with Tukey post-hoc test was used to compare the means across the various conditions. A p-value ≤ 0.05 was considered significant during mean comparisons. Error bars in graphs represent standard error of the mean.

Results

Patterned PS and PLA films were successfully fabricated by solvent casting using micropatterned silicon wafers. Immunocytochemistry and cell staining procedures were used to examine changes in MSCs' transdifferentiation, morphology and alignment. We have demonstrated that MSCs align themselves along the direction of microgrooves when grown on micropatterned substrates. In contrast, MSCs growing on a smooth, non-patterned surface were randomly oriented. Patterning did not affect transdifferentiation of MSCs towards SC-like phenotypic cells as compared to smooth polymeric substrates and a high percentage of cells expressed the SC-marker proteins on both patterned and smooth polymeric films. Results indicate that substrate topography strongly influences the morphology and growth of the MSCs.

Characterization of uMSCs

Undifferentiated MSCs isolated from bone marrows of Brown Norway rats were characterized by immunostaining for various positive and negative rat MSC markers. MSCs were found to be positively immunostained against Fibronectin, Collagen, CD54 (ICAM1, Intracellular adhesion molecule 1), and CD29 (Integrin $\beta 1$). Negative rat MSC markers such as CD14 and CD45 did not stain any of these cells.

Fabrication of Polystyrene and Poly (lactic acid) films

PS and PLA films were successfully fabricated by solvent casting, and scanning electron microscopy (SEM) was used to measure the dimensions of micropatterns. Dimensions of the micropatterns obtained on the polymeric films were found to be 11-13 μm , 16-18 μm and 3.5-4.5 μm in groove, mesa and depth, respectively for both PS and PLA films. These dimensions were chosen based on previous studies where these dimensions were able to orient SCs and selectively differentiate AHPCs (22, 24). Figure 1 shows SEM images of micropatterned PS and PLA films at different magnifications. PLA films were found to have a small taper at the side of mesas as compared to PS films.

Effect of guidance cues on alignment and morphology of MSCs

Undifferentiated MSCs (uMSCs) were grown on PLL coated half patterned –half smooth polymeric substrates in MM for 3DIV (days *in vitro*). While the cells on smooth substrates were found to be fibroblastic in morphology, cells on patterned substrates were found to be elongated and growing in parallel to the direction of the grooves. Cells were found to be growing both within the grooves as well as on the top of the mesas. In order to determine the orientation and morphological changes associated with guidance cues, cells were then stained with Alexa Fluor 488 Phalloidin and DAPI, which stains actin filaments and nuclei, respectively.

Angle of the longest chord through each cell relative to the horizontal axis (i.e. parallel with the direction of the micropatterns) was used for the orientation data and was compared to the cells grown on the smooth part of the substrate. Number of cells was grouped between 10° sectors between 0° to 90° . More than 80% of the cells were found to be oriented in the direction of micropatterns (0° to 20°) (Fig. 2A for PS substrates and 2B for PLA substrates).

The aspect ratio (AR), an indicator of cell elongation, was calculated and used to compare MSCs grown on patterned substrates with cells grown on smooth substrates. Figure 3A showed that cells on patterned substrates were elongated with an AR closer to 5, while cells on the smooth substrates had an AR close to 1, showing the inclination of the cells to have a circular shape in the absence of patterns. Cells grown on PS Pattern and PLA Pattern were found to have AR of 5.03 ± 1.14 and 4.99 ± 1.72 respectively as compared to values of 1.08 ± 0.06 and 1.28 ± 0.19 for PS Smooth and PLA Smooth, respectively. Cellular area was also compared between the cells grown on smooth versus patterned substrates. While the uMSCs seem to be elongated on patterned substrates, the average area of the cells on pattern substrates was found to be less as compared to cells grown on smooth substrates (Fig. 3B). Cellular area for cells grown on Pattern PS, Pattern PLA, Smooth PS and Smooth PLA was found to be 821.10 ± 194.02 , 594.29 ± 73.34 , and 1196.26 ± 147.60 and $957.43 \pm 67.56 \mu\text{m}^2$, respectively.

Aspect ratio of the cells growing on patterned PS (PS Pa) and patterned PLA (PLA Pa) was found to be significantly higher than smooth polystyrene (PS Sm) and smooth PLA (PLA Sm) substrates at $p \leq 0.05$. Cellular area was found to be greater for the cells growing on smooth substrates. However, no significant differences were observed between cellular areas of the cells growing on patterned and smooth parts of the same polymer film at $p \leq 0.05$.

Effect of guidance cues on proliferation of MSCs

In addition to quantifying the morphological changes of the cells associated with micropatterning, we also investigated whether patterning affects the proliferation of uMSCs as compared to the smooth control part of the polymeric film. BrdU was added to the cells 12 hours prior (at 2.5DIV) to fixation of the cells (3DIV). No significant differences (at $p \leq 0.05$) were observed in the proliferation of uMSCs on the basis of BrdU immunostained cells across all 4

conditions (PS Pa, PS Sm, PLA Pa and PLA Sm). Percentage of BrdU-stained uMSCs grown on PS Pa, PS Sm, PLA Pa and PLA Sm was found to be 33.62 ± 8.01 , 39.24 ± 8.62 , 42.44 ± 14.87 and $36.53 \pm 10.24\%$ respectively (Figure 4). Proliferation was found to be slightly higher on PLA substrates as compared to PS substrates, suggesting that the biodegradability of substrates may have some influence on proliferation of uMSCs. Proliferation was found to be least for glass substrates (data not shown). Glass substrates were used for initial characterization and were no longer used for comparison for their inability to support cellular proliferation. Culturing of the cells on micropatterned substrates seems to cause no obvious reduction in proliferation. Supplemental Figure 3 shows percentage of BrdU immunostained uMSCs data for both smooth and pattern PS and PLA substrates.

These results show that micropatterning of polymeric substrates can be used as a strong tool to influence the morphology and alignment of the cells without causing any unexpected or deleterious changes in the elementary natural characteristics of the MSCs.

Effect of guidance cues on transdifferentiation of MSCs toward a SC-like phenotype.

Identification of the transdifferentiated MSCs (tMSCs) was based upon immunolabeling with antibody markers used to identify SCs, anti-S100 β and anti-p75^{NTR} (27). The MSCs were plated on the polymeric films attached to a glass coverslip. MSCs subjected to transdifferentiation conditions grew at a slower rate compared to uMSCs. Based on an initial study, the control undifferentiated MSCs (uMSCs) were plated at 1000 cells/cm² and the MSCs subjected to the transdifferentiation conditions at 3000 cells/cm². The results, obtained by fluorescent microscopy, revealed that both anti-S100 β (Fig. 5 and 6) and anti-p75^{NTR} (Supplemental Fig. 1 and 2) antibodies immunolabeled very few uMSCs compared to tMSCs.

333 While no anti-S100 β immunostaining was detected for the uMSCs, anti-p75^{NTR} sometimes
334 stained the nuclei of the uMSCs. However, anti-p75^{NTR} staining for tMSCs was found to be
335 cytoplasmic as opposed to occasional nuclear staining of uMSCs.

336 During data quantification, tMSCs and uMSCs were counted only when cytoplasmic staining of
337 anti-p75^{NTR} was observed. Figures 7A and 7B graphically show the comparison of data across
338 various substrate conditions for immunostaining of anti-S100 β and anti-p75^{NTR}, respectively for
339 both tMSCs and uMSCs. These results demonstrated that a greater proportion of MSCs subjected
340 to the transdifferentiation protocol were anti-S100 β and anti-p75^{NTR} immunoreactive in
341 comparison to the MSCs cultured under control conditions (cultured in maintenance media
342 (MM)). It was also observed that MSCs transdifferentiated on polymeric (PS and PLA)
343 substrates have a higher percentage of cells immunoreactive for S100 β and p75^{NTR} compared to
344 glass coverslips (data not shown). Neurotrophin receptor p75^{NTR} immunostaining also followed a
345 similar trend as S100 β , where very few uMSCs were found to be immunolabeled for p75^{NTR} as
346 compared to tMSCs where more than 90% cells showed presence of p75^{NTR} protein. For both PS
347 and PLA patterned and smooth substrates, the percentage of S100 β and p75^{NTR} immunolabeled
348 cells were found to be significantly higher in cells subjected to the transdifferentiation protocol
349 (tMSCs) as compared to cells grown just in MM (control cells: uMSCs) at $p \leq 0.05$.

350 As shown before for uMSCs, more than 80% of tMSCs were found to be oriented in the direction
351 of the micropatterns for both PS (Figure 8A) and PLA (Figure 8B) substrates. These results
352 illustrate that micropatterning did not cause any decrease in the level of transdifferentiation, and
353 yet facilitated an oriented cell alignment, which is likely to be advantageous for enhanced neural
354 regeneration. Aspect ratio (Figure 9A) was found to be significantly higher on patterned
355 polymeric substrates as compared to their smooth counterpart at $p \leq 0.05$. Average cellular area

(Figure 9B) was found to be greater on smooth substrates as compared to pattern substrates, but no significant differences were observed at $p \leq 0.05$.

Discussion

In this study, we have successfully shown that micropatterned polymeric substrates act as very useful tools in providing orientation to the growing uMSCs and tMSCs. Mesenchymal stem cells grown on patterned substrates grew in the direction of the microgrooves, were highly oriented and showed elongation along the grooves. More than 80% of the cells were oriented parallel with the grooves on both PS and PLA patterned substrates. Cells were profoundly elongated ($AR > 4$) in the direction of the patterned substrates compared to their smooth counterparts ($AR \sim 1$). On the other hand, cells on the smooth substrates were found to have higher average cellular areas than cells on micropatterned substrates, but no significant differences were observed. While we were able to control the morphological characteristics of the cells with the help of these guidance cues, we found no obvious deleterious effect of patterning on the proliferation and transdifferentiation of the cells. Biodegradable PLA substrates supported the growth and directionality of the cells in a similar fashion to control biocompatible PS substrates. Transdifferentiation seemed to occur more on polymeric substrates as opposed to glass coverslips. Transdifferentiation of MSCs towards a SC fate on the smooth substrates were found to have a variety of morphologies from flat, fibroblast to multipolar in nature as opposed to mostly flat, fibroblast looking shape of undifferentiated control MSCs. The transdifferentiated cells also immunolabeled for SC-specific protein markers such as α -S100 β and α -p75^{NTR}. Micropatterned substrates did not affect transdifferentiation of cells in any way as compared to smooth substrates and tMSCs on patterned substrates were elongated, oriented along direction of microgrooves and expressed SC marker proteins.

Transdifferentiation of MSCs

Autologous nerve grafts are considered as gold standards for nerve regeneration. However, disadvantages associated with autografts such as donor site morbidity and limited length of graft tissue, have refocused strategies to neural regeneration conduits/scaffolds (NRCs). Along with NRCs, SCs have been the foremost choice for cellular transplantation at the site of nerve injuries. However, the only source of SCs is to sacrifice a healthy nerve (analogous to an autograft) and SCs do not seem to proliferate well under cell culture environments (28, 29). In seeking alternatives, researchers are investigating other cell types for nerve regeneration strategies such as olfactory ensheathing cells (30, 31, 32, 33), neural stem cells (34, 35, 36, 37) and MSCs derived from various sources (bone marrow (38, 39), adipose derived (38, 40), umbilical cord derived (41), dental pulp (42)). Both bone marrow and adipose derived MSCs are of considerable interest since their potential to transdifferentiate into SC-like cells has been documented (5, 7, 8, 10, 13, 16, 43). Furthermore, MSCs possess unique properties and appear to be capable of mediating neurorepair via transdifferentiation and cell replacement, as well as via their paracrine function and immunomodulatory properties(44).

In the present study, we have used bone marrow derived MSCs because, in a clinical setting, they can easily be isolated by performing a routine surgical procedure under general anesthesia. MSCs are multipotent cells capable of differentiating into adipocytes, osteoblasts and chondrocytes. MSCs grown in large numbers under cell culture conditions and then transdifferentiated into SC-like cells (tMSCs), can be used as a great alternative source of SCs. These tMSCs were characterized and compared with undifferentiated MSCs (uMSCs) by immunostaining using glial markers such as α -S100 β , α -p75^{NTR} and α -GFAP (8, 13, 45). Ladak et.al.(45) and Zaminy et.al.(8) have reported the S100 β and p75^{NTR} expression in the tMSCs to

be about 50% and 75% respectively. In our experiments, on polymeric substrates, we found that S100 β expression was about 70-80% and p75^{NTR} about 90-95% in the transdifferentiated cells. Also, we did not see any expression of GFAP protein on tMSCs unlike some of the previous transdifferentiation studies. Possible reasons for this difference in percentages could be attributed to the fact that we used MSCs from a different rodent model (Brown Norway rat) and polymeric films fabricated in this manner may have a positive effect on transdifferentiation as compared to the transdifferentiation performed on glass coverslips in the earlier studies. In the experiments where we transdifferentiated the cells on glass coverslips in parallel to polymeric films, the percentage of glial marker-stained cells was found to be lower on glass coverslips as compared to polymeric films (data not shown). One of the differences between the uMSCs and tMSCs is the change in the morphology of the cells. Transdifferentiated cells have been shown to express a bipolar, spindle shape (5, 7) while uMSCs showed a flat-fibroblast-like morphology. In our experiments, the tMSCs population was a heterogeneous one and consisted of multipolar, spindle as well as fibroblastic cells (on the smooth substrates) while uMSCs were found to be mostly flat-fibroblast like cells. Also, tMSCs were found to be proliferating more slowly than uMSCs. Different concentrations of FBS in transdifferentiation media (10%) and maintenance media (20%) may have produced the difference in proliferation rates. In order to ensure a similar number of cells at the end of experiment, we plated uMSCs (1000 cells/cm²) and tMSCs (3000 cells/cm²) at different initial seeding densities.

Scaffolding and MSCs

Controlling the behavior and morphology of the cells using biomaterial scaffolds has become an important area of research in the past 20 years for tissue regeneration purposes (46, 47, 48). Both natural and synthetic biomaterials have been used for fabrication of these scaffolds.

425 Here we have used two common synthetic polymers – biocompatible polystyrene (PS) and
426 biodegradable poly (lactic acid) (PLA) for fabrication of micropatterned films using solvent
427 casting. Our group has previously produced patterned PS films combining novel patterning
428 techniques via reactive ion etching and solvent casting to study the effect of physical guidance
429 cues on alignment and differentiation of glial cells (19, 20, 22) and neural stem cells (24, 25).
430 These micropatterned films significantly enhanced the alignment of the cells in all the previous
431 studies conducted using these polymeric films. We are able to achieve similar results while we
432 grew bone marrow derived MSCs on these films. Both PS and PLA films supported the growth
433 of MSCs on both smooth and patterned regions of the substrates. MSCs were successfully
434 transdifferentiated on these films and no significant differences were observed by percentages of
435 α -S100 β and α -p75^{NTR} immunostained tMSCs (>70%) between smooth and patterned substrates.
436 Undifferentiated MSCs grown in parallel to tMSCs on these smooth and patterned substrates had
437 a lower percentage of cells immunostained with the glial markers (<10%), showing that only
438 transdifferentiation reagents were responsible for the transdifferentiation of the MSCs and
439 patterning has no effect on transdifferentiation. Previously in our research group we have shown
440 that similar PS films were able to cause selective differentiation in AHPCs(24) but in case of
441 transdifferentiation we saw no impact of the micropatterned substrates which shows that
442 different cells types behave differently on patterned substrates. However, patterning was still
443 able to control the morphology and alignment of the MSCs without negatively affecting their
444 transdifferentiation potential. Physical guidance provided by the microgrooves greatly increased
445 the alignment of the cells in the direction of the patterns for both PS and PLA substrates. More
446 than 80% of the uMSCs as well as tMSCs were found to be aligned in the direction of the

micropatterns (0° - 20°) while the cells growing on smooth substrates were found to be randomly oriented.

In order to quantify the morphological changes of uMSCs and tMSCs associated with micropatterns, we calculated the AR, a parameter which takes into account both the length (in direction of microgrooves) and breadth (in direction perpendicular to microgrooves) of the cells. Transdifferentiated MSCs and uMSCs growing on patterned PS and PLA substrates were found to have AR of about 5 showing that cells were highly elongated while growing on the patterned substrates. Both tMSCs and uMSCs had an AR of about 1 when they were grown on smooth polymeric films displaying that cells were, in general, circular in shape (same length and width) or they do not have inclination to elongate in one particular direction. Changes in AR of cells on patterned substrates were significantly different from the cells on smooth substrates at $p \leq 0.05$. Also when we compared the cellular areas of the tMSCs and uMSCs between the smooth and patterned substrate we found that cells growing on smooth substrates had a somewhat larger surface area than cells growing on patterned substrates but no significant differences were observed at $p \leq 0.05$. Differences in surface area of the cells may be due to the 3D nature of the patterned substrates, as the method we used for calculating the surface area gives us a 2D projection of the surface area and does not take into account the surface area of the part of the cells growing along the vertical walls of the grooves. In order to compare the biodegradable nature of the two polymers, we found that mesas of the micropatterns of PLA substrate were considerably degraded and was difficult to visualize under differential interface contrast (DIC) microscopy as compared to the mesas of the PS substrates which were visualized easily by DIC imaging. A somewhat higher number of cells immunolabeled with glial markers (S100 β and p75^{NTR}) on PLA substrate as compared to PS substrate points towards a possible effect of

degrading PLA on the differentiation of MSCs. Degradation of the mesas of PLA films was found to have no effect on alignment and elongation of both uMSCs and tMSCs as compared to the PS substrate. Thus, our results have shown that these patterned films can be used as a means to control the alignment and morphology of the cells without causing any negative effect on their differentiation potential.

Our research group has previously shown that nerve regeneration conduits with rolled biodegradable micropatterned PLA films in the inner lumens, after seeding with SCs significantly improve the sciatic nerve regeneration in a rodent model of peripheral nerve injury (23). Our future studies will include rolling these biodegradable films to fabricate a nerve guidance conduit along with transdifferentiated MSCs transplantation to test the synergistic potential of physical and biological cues on peripheral nerve repair in a rodent model.

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Figure legends

Figure 1. Scanning Electron Microscopy (SEM) images of micropatterned polystyrene (PS) and poly (lactic acid) (PLA) films. Dimensions of the micropatterns obtained on the polymeric films were found to be 11-13 μm , 16-18 μm and 3.5-4.5 μm in groove, mesa and depth, respectively for both PS and PLA films. (A) PS film at 300X magnification. (B) PS film at 1500X magnification. (C) PLA film at 300X magnification. (D) PLA film at 3000X magnification.

Figure 2. The distribution of uMSCs alignment on micropatterned and smooth PS and PLA substrates. It was demonstrated that an oriented cell alignment was obtained with micropatterning for uMSCs. The data was grouped in 10° sectors from 0° to 90° . Over 80% of the cells were found to be in range of 0° - 20° for patterned substrates while cells were found to be randomly oriented on smooth substrates. Thirty cells per experiment (Total 90 cells/condition) were analyzed. (A) uMSCs alignment on Polystyrene films. (B) uMSCs alignment on PLA films. N=3 independent experiments and error bars represents standard error of the mean.

Figure 3. The comparison of aspect ratios and cellular areas of uMSCs grown on micropatterned vs. smooth PS and PLA substrates. (A) Aspect ratio of uMSCs on various substrates. **Aspect ratio is the ratio of length (in direction of microgrooves) of cell and breadth (in direction**

perpendicular to microgrooves) of cell. Aspect ratios greater than one implies elongation of the object in one particular direction. (B) Cellular area of uMSCs on various substrates. Aspect ratios were found to be significantly different while cellular areas were not.

Figure 4. Graph showing percentage of BrdU-labeled uMSCs for various polymeric substrate conditions. Error bar represents standard error of the mean. N=3 independent experiments. No significant differences were observed between any of the substrate types and patterning does not seem to influence proliferation of uMSCs.

Figure 5. Transdifferentiated MSCs (tMSCs) on micropatterned PLA and PS films have a higher percentage of cells labeling with anti-S100 β antibody than uMSCs. A, A' and A'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of tMSCs on patterned PLA substrate. B, B' and B'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of uMSCs on patterned PLA substrate. C, C' and C'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of tMSCs on patterned PS substrate. D, D' and D'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of uMSCs on patterned PS substrate. Abbreviations: uMSCs, undifferentiated mesenchymal stem cells; tMSCs, transdifferentiated mesenchymal stem cells; S100 β , anti- S100 β antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100 β , Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100 μ m

Figure 6. Transdifferentiated MSCs (tMSCs) on non-patterned (smooth) PLA and PS films have a higher percentage of cells labeling with anti-S100 β antibody than uMSCs. A, A' and A'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image

of tMSCs on smooth PLA substrate. B, B' and B'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of uMSCs on smooth PLA substrate. C, C' and C'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of tMSCs on smooth PS substrate. D, D' and D'' represents the corresponding anti-S100 β , actin filaments (phalloidin) staining and merged image of uMSCs on smooth PS substrate. Abbreviations: uMSCs, undifferentiated mesenchymal stem cells; tMSCs, transdifferentiated mesenchymal stem cells; S100 β , anti- S100 β antibody; PLA, poly(lactic acid); PS, polystyrene; Merged, merged image of S100 β , Alexa-488 Phalloidin, DAPI and DIC (Differential interference contrast). Scale bar = 100 μ m

Figure 7. Graphs showing percentage of S100 β and p75^{NTR} labeled cells for various polymeric substrate conditions. (A) S100 β labeling of the tMSCs were found to significantly different ($p \leq 0.05$) than the uMSCs for all substrate types. (B) p75^{NTR} labeling of the tMSCs was significantly greater ($p \leq 0.05$) than the uMSCs for all substrate. Patterning caused no deleterious effect on the transdifferentiation of MSCs into SC-like phenotypic cells. Both polymers were found to have more S100 β immunostaining as compared to glass coverslips (data not shown). Error bar represents standard error of the mean. N=3 independent transdifferentiation experiments were carried out. *Significantly different from smooth substrates at $p \leq 0.05$.

Figure 8. The distribution of tMSCs alignment on micropatterned and smooth PS and PLA substrates. It was demonstrated that an oriented cell alignment was obtained with micropatterning for tMSCs. The data was grouped in 10° sectors from 0° to 90°. Over 80% of the cells were found to be in range of 0°-20° for patterned substrates while cells were found to be randomly oriented on smooth substrates. Thirty cells per experiment (Total 90 cells) were

700 analyzed. (A) tMSCs alignment on Polystyrene films. (B) tMSCs alignment on PLA films. N=3
701 independent experiments and error bars represents standard error of the mean.

702 **Figure 9.** The comparison of aspect ratio and cellular area of tMSCs grown on micropatterned
703 vs. smooth PS and PLA substrates. (A) Aspect ratio of tMSCs on various substrates. Aspect ratio
704 is ratio of width to the height of the cell. Aspect ratio is the ratio of length (in direction of
705 microgrooves) of cell and breadth (in direction perpendicular to microgrooves) of cell. (B)
706 Cellular area of tMSCs on various substrates. Aspect ratios were found to be significantly
707 different while cellular areas were not. Thirty cells per experiment (Total 90 cells) were
708 analyzed. N=3 independent experiments and error bars represents standard error of the mean.
709 Abbreviations: PS, polystyrene; PLA, poly (lactic acid), Pa, patterned substrate; Sm, smooth
710 substrate. *Significantly different from smooth substrate at $p \leq 0.05$.

Figure 2

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Figure 9

Table 1. Antibody markers used to characterize undifferentiated and transdifferentiated MSCs.

Primary Antibody	Dilution	Poly or monoclonal	Pre-treatment	Incubation time	Reference Company (Catalog number)
Mouse α CD54	1:100	monoclonal	None	Overnight	Millipore (MAB2130)
Rabbit α CD29	1:1000	polyclonal	None	Overnight	Millipore (AB1952)
Rabbit α Collagen type I	1:200	polyclonal	None	Overnight	Millipore (2003605)
Rabbit α Fibronectin	1:1000	polyclonal	None	Overnight	Millipore (2003606)
Mouse α CD14	1:100	monoclonal	None	Overnight	Millipore (2003608)
Mouse α CD45	1:200	monoclonal	None	Overnight	Millipore (2003607)
Mo α S100 β	1:1000	monoclonal	None	Overnight	Abcam (ab11178)
Mo α GFAP	1:1000	monoclonal	None	Overnight	Millipore (MAB360)
Rab α p75 ^{NTR}	1:1000	polyclonal	None	Overnight	Promega (G3231)
Rat α BrdU	1:200	monoclonal	2N HCl/0.1 M borate buffer	Overnight	Abcam (ab6326)