Journal of Bioscience and Bioengineering
VOL. 121 No. 3, 325–335, 2016

www.elsevier.com/locate/jbiosc

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

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Received 27 May 2015; accepted 14 July 2015
Available online 11 September 2015

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-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-S100β 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.

[**Key words:** Micropatterning; Polymeric films; Mesenchymal stem cells; Transdifferentiation; Schwann cells]

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, Schwann cells (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 mesenchymal stem cells (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 (7,14–16).

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

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.07.006

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 (21–23). However, disadvantages associated with SC transplantation have led to use of alternative 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. However, 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 no. 00575, Polysciences, Inc., Warrington, PA, USA) 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 h. 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, USA). The PLA solution on the wafer was left to dry for 10 min on the spin coater before transferring to a chemical hood for an additional 6 h. 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, USA) 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, USA) 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, USA) 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-h 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 (αMEM, Gibco BRL, Gaithersburg, MD, USA), supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine (Gibco BRL), and antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA). 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 μm nylon cell strainer (BD Falcon, Bedford, MA, USA), 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% CO_2/95% humidified air atmosphere). After 48 h, post-harvest, spindle-shaped cells adhered to the bottom of the flask. The cells were washed with phosphate buffered saline (PBS; Gibco BRL) 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 (αMEM, Gibco BRL), 20% fetal bovine serum (FBS; Atlanta Biologicals), 4 mM L-glutamine (Gibco), and antibiotic–antimycotic (Invitrogen) and incubated at 37°C/5% CO_2/95% 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 min. 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% CO_2. After reaching 30–40% confluence, the MM media was replaced with the transdifferentiation media-1 (TDM-1) containing αMEM supplemented with 1 mM β-mercaptoethanol (BME; Sigma–Aldrich, St. Louis, MO, USA) and incubated for one day. After a PBS wash, TDM-1 was replaced with the TDM-2 containing αMEM, 10% fetal bovine serum (FBS) and 35 ng/mL all-trans-retinoic acid (ATRA; Sigma). The cells were incubated in TDM-2 for the following 3 days at 37°C/5% CO_2. Finally, the cells were incubated in TDM-3 which contained αMEM, 10% FBS, 14 μL forskolin (FSK; EMD Millipore, Billerica, MA, USA), 5 ng/mL platelet derived growth factor (PDGF; Sigma), 10 ng/mL basic fibroblast growth factor (bFGF, Promega Corporation, Madison, WI, USA) and 200 ng/mL heregulin β1 (HRG; Calbiochem, EMD Millipore) 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/cm^2 while tMSCs were plated at a density of 3000 cells/cm^2. 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 After 8 DIV, the cells in culture dishes were rinsed with 0.1 M PO_4 buffer and fixed for 20 min with cold 4% paraformaldehyde (stored at 4°C) prepared in 0.1 M PO_4 buffer. The cells were then rinsed with filtered phosphate buffered saline (PBS; Fisher-Scientific) for 3 times every 7 min and incubated in blocking solution of PBS supplemented with 5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA, USA), 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 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 min, and subsequently incubated in appropriate secondary antibodies diluted in blocking solution. The following secondary antibodies were used: Donkey-α-Mouse-Cy3 (1:500, Jackson ImmunoResearch), Donkey-α-Rabbit-Cy3 (1:500, Jackson