

Neurogenic differentiation of human conjunctiva mesenchymal stem cells on a nanofibrous scaffold

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ABSTRACT The selection of a good quality scaffold is an essential strategy for tissue engineering. Ideally, the scaffold should be a functional and structural biomimetic of the native extracellular matrix and support multiple tissue morphogenesis. However, investigators have previously shown that three-dimensional nanofibrous scaffolds are capable of influencing cellular behavior. In this study, we experimented with a three-dimensional nanofibrous scaffold fabricated from aligned-poly L-lactic acid (PLLA) for its ability to support neurogenic and hinder dopaminergic differentiation of conjunctiva mesenchymal stem cells (CJMSCs) *in vitro*. In this work, CJMSCs were seeded onto nanofibrous scaffolds, and were induced to differentiate along neurogenic lineages by culturing in specific differentiation media. Scanning electron microscopy imaging, RT-PCR and immunocytochemistry were used to analyze cultivated CJMSCs on scaffold and their expression of neurogenic-specific markers. We found a lack of expression of dopaminergic genes in CJMSCs seeded on align PLLA scaffold, while neurocyte-cell markers including Nestin, NSE, MAP-2 and β -Tubulin III were expressed in these cells. On the basis of these experimental results, we conclude that the nanofibrous PLLA scaffold reported herein could be used as a potential cell carrier in neural tissue engineering and that these scaffolds could be useful for the partial inhibition of the dopaminergic differentiation of CJMSCs.

KEY WORDS: *poly (L-lactic acid), PLLA, mesenchymal stem cell, dopaminergic gene, nanofibrous scaffold*

Introduction

Tissue engineering techniques are currently being developed to successfully repair and restore the function of damaged or diseased tissue (Tuan *et al.*, 2003; Caplan, 2000; Cancedda *et al.*, 2003). The basic principle involves the use of an appropriate cell source and a biocompatible and biodegradable scaffold to produce a construct that structurally and functionally mimics the target tissue.

In recent years, Mesenchymal stem cells (MSCs) have been defined as a population of primitive cells with capability of self-renewal and differentiation into multiple cell lineages for treatment of human diseases (Tonti and Mannello, 2008), including models

for osteogenesis imperfecta (Horwitz *et al.*, 2002), spinal cord injury (Sasaki *et al.*, 2001), stroke (Chen *et al.*, 2003), and Parkinsonism (Schwarz *et al.*, 1999). To date, MSCs have been isolated from a variety of human tissue (De Bari *et al.*, 2001; Nakahara *et al.*, 1990; Noth *et al.*, 2002; Miura *et al.*, 2003; Nadri *et al.*, 2008; De Ugarte *et al.*, 2003; Noort *et al.*, 2002; Sarugaser *et al.*, 2005; Bhang *et al.*, 2007). Furthermore, considerable effort has been made to develop scaffolds for tissue engineering. Design and fabrication of scaffolds using appropriate biomaterials is a key for

Abbreviations used in this paper: CJMSC, conjunctiva mesenchymal stem cell; ECM, extracellular matrix; MSC, mesenchymal stem cell; PLLA, poly L-lactic acid.

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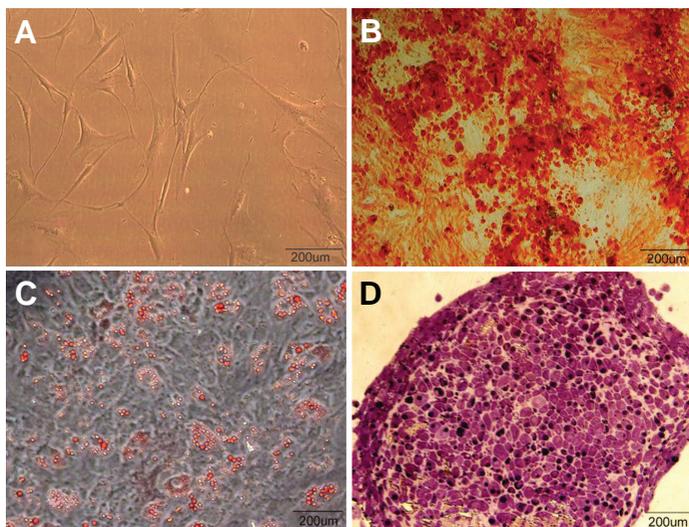


Fig. 1. Morphological characteristics and *in vitro* differentiation of conjunctiva stromal fibroblast-like cells into mesenchymal lineages. (A) Conjunctiva stromal cells (passage 2) have differentiated into (B) mineralizing cells stained with alizarin red. (C) Adipocytes stained with Oil red O. (D) Chondrocytic lineage stained with Toluidin blue. (Magnification = $\times 20$).

the creation of functional engineered tissues and clinical applications (Andrades *et al.*, 1996). The native extracellular matrix (ECM) is comprised of a complex network of structural and regulatory proteins that are arrayed into a fibrous matrix. It also provides resident cells with specific ligands for cell adhesion and migration, and modulates cell proliferation and function (Nishimura *et al.*, 2003; Nguyen and D'Amore, 2001; Shields *et al.*, 2004). However, by combining engineering principles with knowledge of materials science and stem cell biology, investigators seek to create novel constructs that will fully integrate into the host system and restore function of diseases such as central nervous system.

While three-dimensional biomaterial scaffolds have been used to engineer neurocyte cell-type specific constructs, to our knowledge, there has been little report on the successful application of a single three-dimensional scaffold to support cell differentiation into specific types of neurocyte cells. Thus an ideal biomaterial scaffold should be capable of supporting and directing cell differentiation into specific lineages.

Our work focuses on characterizing the response of human CJMSCs to poly L-Lactic acid (PLLA) scaffold, with a view to develop these materials for neuron tissue engineering applications. Previous study indicated that scaffold environments influenced the cellular behavior (Flynn *et al.*, 2008).

Here, we thoroughly examined the conjunctiva mesenchymal stem cells (CJMSCs) differentiation responses in order to widen a deeper perceptive of the influence of the PLLA scaffold on direct differentiation of CJMSCs into neurocyte lineage.

CJMSCs were isolated from human conjunctiva stromal cells (Nadri *et al.*, 2008). The most important properties of conjunctiva stromal-derived MSCs (CJMSCs) were their expression of undifferentiated stem cells markers as Oct-4, Rex-1, and Nanog and lineage-specific markers like cardiac-actin (mesoderm lineage marker) and Keratin (ectoderm lineage marker) (Nadri *et al.*, 2008) (Supplementary Figure 1). Furthermore, These cells were differentiated into dopaminergic neuron (Nadri *et al.*, 2008) and

best candidate for regenerative central nerve system (CNS) injury due to have high proliferation and differentiation capacity into neurocyte cells.

Results

Cell culture of conjunctiva mesenchymal stem cells (CJMSCs)

In this work, the mesenchymal stem cells with spindle-shaped morphology from human conjunctiva stromal cells were isolated by adhesion of these cells onto the surface of the plastic culture dishes. After 2 weeks, fibroblast-like cells with spindle-shape morphology appeared on culture dishes (Fig 1A). The cells were utilized for following experiments.

In vitro differentiation of CJMSCs into mesenchymal lineages

To confirm mesenchymal nature, the fibroblast-like cells were treated with appropriate osteo-, chondro- and adipo-inductive media (Nadri *et al.*, 2008), and their differentiation was confirmed via appropriate staining including, alizarin red (for osteogenic differentiation), alcian blue (for chondrogenic differentiation) and oil red (for adipogenic differentiation) staining (Fig 1 B-C).

Fabrication of electrospun nanofibrous PLLA

To fabricate nanofiber, we used electrospinning techniques. Electrospinning of PLLA-based nanofiber resulted in a scaffold composed of uniform; align oriented fibers of an average diameter of 700-2000 nm, as seen by scanning electron microscopy (SEM; Fig. 2). Following an 8 week incubation in culture medium at 37°C, scaffolds maintained their integrity and three-dimensional structure, while exhibiting no noticeable change in dry weight over the entire culture period (data not shown).

Expression of gene transcripts

To induce neurocyte differentiation, the seeded cells on scaffold and non-seeded cells were cultivated in neuroinductive medium

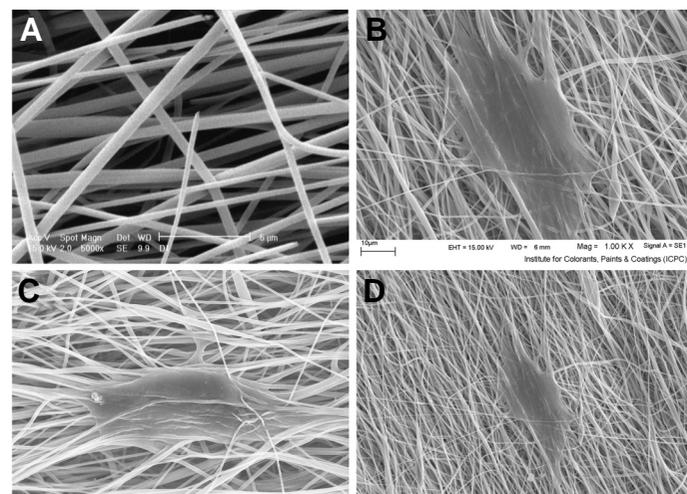


Fig. 2. SEM micrographs of CJMSCs seeded on PLLA nanofibrous scaffolds. (A) The PLLA scaffold contains aligned fibers with uniformly sized fibers of an average diameter of 700-2000 nm fabricated using electrospinning methods. (B,C,D) The attachment of an anchorage-dependent conjunctiva mesenchymal stem cell on the porous structure of PLLA nanofibrous scaffold at different magnifications (100X, 1500X and 500X respectively).

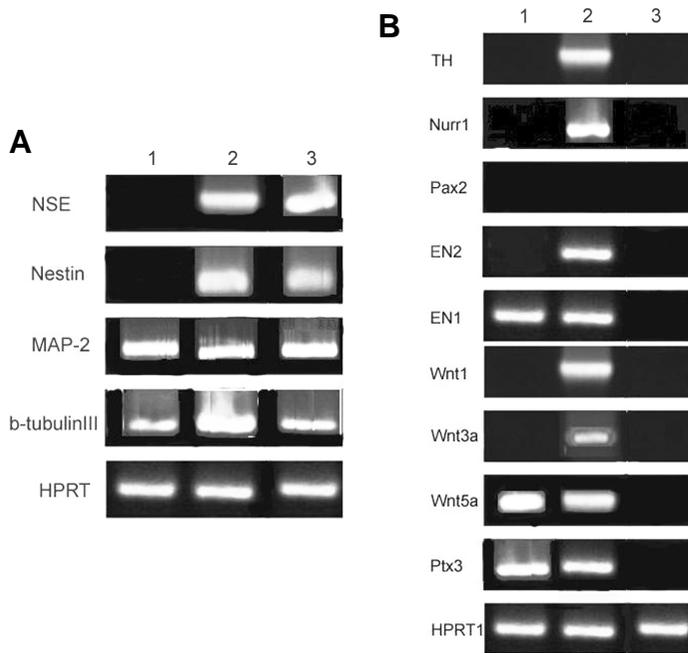


Fig. 3. Investigation of differentiation-specific gene expression. (A) Expression of neuron markers such as neuron-specific enolase (NSE), beta-tubulin III, microtubule-associated protein-2 (MAP-2) and Nestin were analyzed in (1) control (day 0), (2) differentiation culture (day 6) and (3) the seeded cells on scaffold in differentiation culture. (B) Expression of dopaminergic-associated markers were analyzed in (1) control (day 0), (2) differentiation culture (day 6) and (3) the seeded cells on scaffold in differentiation culture (6 days). Hypoxanthine phosphoribosyltransferase-1 (HPRT-1) is used as a housekeeping gene control.

containing RA, IBMX and dbcAMP. After 6 days, we examined with the neural and dopaminergic markers by RT-PCR. RT-PCR analysis was indicative of the expression of Nestin, NSE MAP-2 and b-tubulin III genes in either seeded and non-seeded on scaffold, whereas the dopaminergic genes including TH, Nurr1, Pax2, En1, En2, Wnt1, Wnt3a, Wnt5a and Ptx3 were not expressed in the seeding of CJMSCs on PLLA scaffold (Fig. 3 A,B).

Immunocytochemistry analysis

Immunocytochemistry was used to analyze the intracellular TH and b-Tubulin III proteins. The result showed which B-Tubulin III proteins were detected in the CJFLCs on PLLA nanofiber after treatment in neuroinductive medium for 6 days (Fig. 4).

Discussion

While stem cells have shown promise for tissue engineering applications, it is necessary to understand the scaffold properties that promote the diffusion of nutrient, metabolites and soluble factors until the seeded cell can produce a new functional matrix and regenerate the desired tissue structures (Langer and Vacanti.1993; Nerem.2000; Vacanti and Langer.1999). Within the seeded constructs, cell material interactions may directly influence the lineage commitment process of the multipotent stem cells(Zuk *et al.*, 2002; Rosen and MacDougald.2006). In the present study, we examined the influence of PLLA nanofibrous scaffold on direct differentiation of CJMSCs to neurogenic lineages.

MSCs have been considered as an appropriate source for cell and gene therapy tools for treatment in a number of congenital and degenerative diseases (Baksh *et al.*, 2004). Promising evidences have been reported with the use of cells in a number of animal models for human diseases, including models for spinal cord injury (Sasaki *et al.*, 2001), stroke (Chen *et al.*, 2003), and Parkinsonism (Schwarz *et al.*, 1999). These cells have been isolated from different tissue sources including periosteum (Nakahara *et al.*, 1990), trabecular bone (Noth *et al.*, 2002), adipose tissue (De Ugarte *et al.*, 2003), synovium (De Bari *et al.*, 2001), deciduous teeth (Miura *et al.*, 2003), lung (Noort *et al.*, 2002), and human umbilical cord perivascular cells derived from the Wharton’s jelly (Sarugaser *et al.*, 2005). Recently, we successfully isolated MSCs

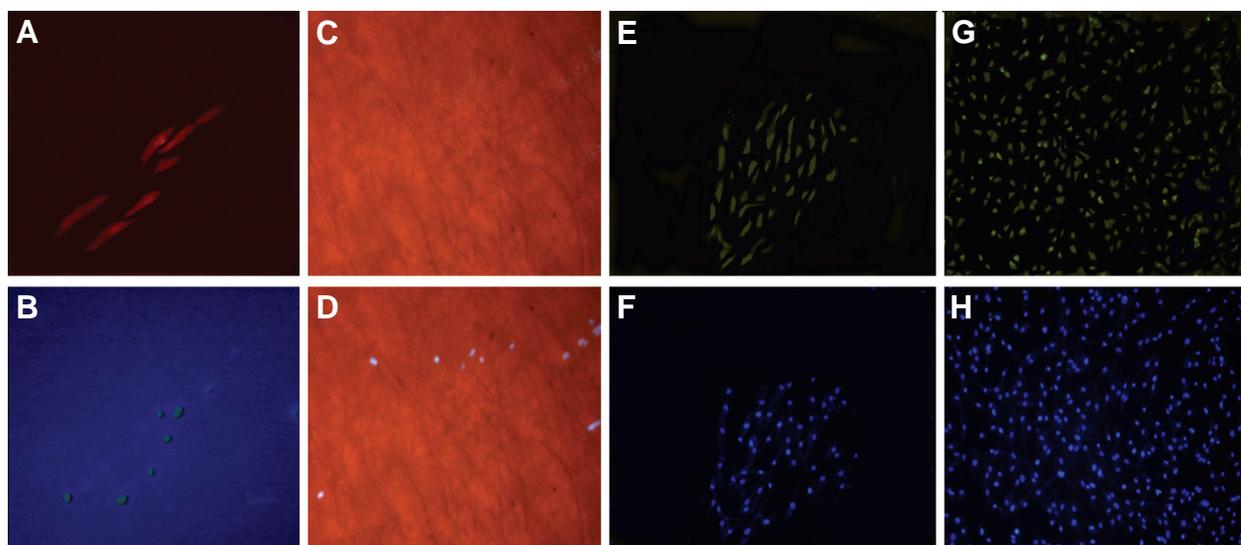


Fig. 4. Immunostaining of cells (seeded and non-seeded on PLLA scaffold) with tyrosine hydroxylase (TH) and beta-tubulin III staining. The seeded cells on PLLA scaffold (A,C) and non-seeded cells (tissue culture polystyrene) (E,G) were maintained in neurogenic medium for 6 days and analyzed for expression of β -Tubulin III (A,E) and TH (C,G) proteins. Cells were co-stained with 4, 6 diamidino-2-phenylindole to visualize nuclei (blue) related to TH (B,D,F,H).

of eye conjunctiva stromal cells. Furthermore, these cells have spindle-shape morphology and were simply differentiated into mesenchymal lineages (osteoadipochondrogenic cells) and neuronal cells. In this work CJMSCs seeded on PLLA scaffold and maintained in neurogenic medium for 6 days.

The results from this research showed that the scaffolds could inhibit the expression of dopaminergic genes in CJMSCs. Bhang *et al.*, indicate that PLLA scaffold which fabricated using phase separation methods, have inhibitory effect on proliferation and neural differentiation of hippocampal progenitors cells due to the hydrophobic surface of its (Bhang *et al.*, 2007). Though this method supplied scaffolds for tissue engineering, there were however difficulties encountered in controlling fiber diameter and alignment (Yang *et al.*, 2005). In present study, PLLA scaffolds were fabricated using electrospinning methods and enhanced hydrophilic surface of polymer using oxygenated gas plasma. Previous researcher reported that modification of surface by gas plasma enhanced cell attachment to polymer (Ghasemi-Mobarakeh *et al.*, 2008). In this work, After exposure to neurogenic media, end point RT-PCR analysis indicated that in despite of the non seeding of CJMSCs on scaffold (tissue culture polystyrene (TCPS) plates, control), specific dopaminergic genes including TH, Nurr1, Ptx3, En1, En2, Pax2, Wnt3a and Wnt1 were not expressed in the seeding of CJMSCs on scaffold while neural stem cell markers expressed in these cells. A TH protein is the initial and rate-limiting enzyme in the catecholamine synthesis pathway, and considered the principal regulator of dopamine biosynthesis in the central nervous system (Kan *et al.*, 2007). It has been reported that Nurr1 and Ptx3 are a nuclear receptor and transcription factor, respectively, essential for differentiation of the dopaminergic precursor neurons (Jankovic *et al.*, 2005; Chu *et al.*, 2006; Martinat *et al.*, 2006). EN1 and EN2 have been shown to have significant roles in controlling the developmental fate of midbrain dopaminergic neurons and the expression of the genes genetically linked to Parkinson's disease (Simon *et al.*, 2001). On the other hand, Wnts are essential regulators for proliferation and differentiation of ventral midbrain DA precursor neurons (Castelo-Branco *et al.*, 2003). RT-PCR and immunocytochemistry analysis indicated that dopaminergic genes were not expressed in CJMSCs seeded on PLLA scaffold. Most importantly, the PLLA-based nanofibrous scaffold inhibited dopaminergic differentiation of the seeded CJMSCs when the constructs were placed under specific, differentiation-promoting culture condition. In this work, to ensure a correct differentiation of the CJMSCs on a nanoscaffold and inhibition of scaffold on dopaminergic-associated genes in our study, we used RT-PCR analysis to compare the expression of dopaminergic genes on seeded and non-seeded CJMSCs on PLLA-scaffold. However, further research is required to elucidate the influence of nanofiber on the biochemical pathways and cellular signaling mechanisms that regulate cell morphology, growth, proliferation, differentiation, motility, and genotype. Insight into how natural ECM components secreted by cells replace the biodegradable polymeric scaffolds is also needed. This complete understanding of cell-nanofiber scaffold interactions will pave the way for successful engineering of various tissues and organs, such as vascular grafts, nerve, skin and bone regeneration, cornea transplants, skeletal and cardiac muscle engineering, gastrointestinal and renal/urinary replacement therapy, and even stem cell expansion and differentiation to specific cells types and organ regeneration.

Both none seeding and seeding of CJMSCs on scaffold express neural stem cell markers such as NSE, Nestin, MAP-2 and β -tubulin III, which are markers of neurogenic differentiation. This finding has brought into question the clinical applicability of CJMSCs cultured on PLLA-nanoscaffold for regeneration of spinal cord injury. This study is a first to report neurogenic differentiation of CJMSCs seeded in a PLLA nanofibrous scaffold. This work represents an important step forward in the optimization of the scaffold design for neuron tissue engineering applications, by helping to elucidate the conditions that support CJMSCs differentiated into neurogenic differentiation.

Collectively, the results in this study indicate that nanofiber scaffolds positively support differentiation of CJMSCs to specific neural lineage. This can be qualified to the ECM-like properties of the nanofiber scaffolds that mimic the natural tissue environment.

Materials and Methods

Isolation and culture of CJMSCs

CJMSCs were isolated according to a protocol modified from Nadri and co-worker (Nadri *et al.*, 2008). In brief, after obtaining informed consent from the individuals, 2–3mm² of conjunctiva biopsies were obtained from patients undergoing pterygium surgery and incubated in supplemented hormonal epithelial medium (SHEM), which is made of Dulbecco's modified Eagle's medium (DMEM)/F-12 (GIBCO-BRL, Grand Island, NY) containing bicarbonate (Sigma Chemical Co, St. Louis, MO), 0.5% dimethyl sulfoxide, 2 ng/ml human epidermal growth factor (EGF; Peptotech, Rocky Hill, NJ), 5 mg/ml insulin (GIBCO-BRL), 5 mg/ml transferrin (GIBCO-BRL), 5 ng/ml sodium selenite (GIBCO-BRL), 0.5 mg/ml hydrocortisone (GIBCO-BRL), 30 ng/ml cholera toxin A subunit, 5% fetal calf serum (FCS; GIBCO-BRL), 50 mg/ml gentamicin (Sigma Chemical Co), and 1.25 mg/ml amphotericin B (Sigma Chemical Co) for 2–5 min. Then, the biopsies were treated in SHEM containing 50 mg/ml dispase II (Sigma Chemical Co) and 100mM sorbitol (España *et al.*, 2003). Under a stereomicroscope, epithelial sheets were separated and the isolated stromal tissue segment was cultured in DMEM/F-12 (1:1) (GIBCO-BRL) supplemented with 10% knockout serum (GIBCO-BRL), 4 ng/ml basic-FGF (Peptotech), 5 mg/ml insulin (Sigma Chemical Co), and 10 ng/ml human LIF (Chemicon; Temecula, CA) (Dravida *et al.*, 2005) and incubated at 37°C with 5% CO₂ in a humidified chamber for 2 weeks. After 2 weeks, biopsy was removed and the mesenchymal stem cells were trypsinized (0.25% Trypsin-ethylenediaminetetraacetic acid [EDTA]; GIBCO-BRL) after confluency. The cells were subsequently expanded by two passages in DMEM (GIBCO-BRL), which was supplemented with 100 IU/ml penicillin (Sigma Chemical Co), 100 mg/ml streptomycin (Sigma Chemical Co), and 15% FCS.

In vitro differentiation of CJMSCs into mesenchymal lineages

To identify MSCs-nature of the isolated cells, the cells were treated with osteogenic (DMEM composed of 50 μ g/ml ascorbic acid 2-phosphate (Sigma Chemical Co), 10nM dexamethasone (Sigma Chemical Co), and 10mM β -glycerophosphate (Sigma Chemical Co), adipogenic (DMEM, supplemented with 50 μ g/ml indomethacine (Sigma Chemical Co) and 100nM dexamethasone (Sigma Chemical Co) and chondrogenic (DMEM supplemented with 10 ng/ml transforming growth factor- β 3 (TGF- β 3; Sigma Chemical Co), 500 ng/ml bone morphogenetic protein-6 (BMP-6), 10⁻⁷M dexamethasone (Sigma Chemical Co), and 50 μ g/ml ascorbate-2-phosphate (Sigma Chemical Co), 50 μ g/ml insulin-transferrin-selenium (ITS; GIBCO-BRL) medium for 21 days (Nadri *et al.*, 2008). At the end of the cultivation period, for alizarin red staining, the cells were fixed with 10% formalin for 10 min and stained with alizarin red (Sigma) for 15 min at room temperature. For oil red staining, the cells were stained with 0.5% oil red O (Sigma) in methanol for 15 min for adipocyte detection and for toluidine blue staining, some pellets were prepared for embedding in Araldite (Sigma).

The pellets were fixed for 1 h using the Karnovsky fixative at room temperature, washed with 0.1 M OsO₄ (Sigma) in 0.1 M phosphate buffer for 1 h at 20°C and then subjected to graded ethanol dehydration before being embedded. Sections of 0.3 µm were made, and stained with toluidine blue.

Fabrication of electrospun nanofibrous PLLA scaffolds

Nanofibrous poly L- lactic acid (PLLA) scaffolds were fabricated and produced by an electrospinning setup according to a protocol modified with Mohammadi *et al.*, (Mohammadi *et al.*, 2007). PLLA was dissolved in chloroform (10% wt/wt) and added directly to the chloroform/N, N-dimethylformamide (DMF) (Sigma, Steinheim, Germany) solution (10:1). PLLA was delivered to metal capillaries with a constant mass flow. For stabilization, the nanofibrous mats were treated with methanol, rinsed with sodium hydroxide solution and deionized water. The nano-structured scaffolds were then freeze-dried and stored in a desiccator at room temperature until use for culturing cells.

Surface modification of nanofiber

A low frequency plasma generator of 40 KHz frequency with a cylindrical quartz reactor (Diener Electronics, Germany) was used. Pure oxygen was introduced into the reaction chamber of system at 0.4 mbar pressure and purged for 10 min before beginning of treatment. Then the glow discharge was ignited for 10 min. After termination of treatment, the generator was switched off, and the system was flushed with oxygen gas for 20 min before venting to atmospheric pressure.

Seeding and differentiation of CJMSCs on PLLA scaffold

Nanofibrous PLLA scaffolds were placed in 70mm² cultures (Corning Glass Works, Corning, NY) and 4×10⁵ of CJMSCs were seeded on it. Cellular scaffolds were incubated at 37 °C or 4 h to allow CJMSCs to diffuse into and adhere to the scaffold before the addition of 20mL of culture medium to each plate. During the 4 h incubation, 20 mL of serum containing culture medium was applied every 30 min to each cellular scaffold to prevent the constructs from drying. For neurogenic differentiation, the cells

were incubated in neurogenic medium consisting of DMEM, supplemented with 0.5mM isobutyl methyl xanthin (IBMX; Sigma Chemical Co), 1mM dibutyryl-cAMP (dbcAMP; Sigma Chemical Co), and 10 mM retinoic acid (Sigma Chemical Co) (Nadri *et al.*, 2008) for 6 days. The seeded cells on scaffolds were maintained for 6 days in a humidified incubator at 37 °C and 5% CO₂ with medium changes every 2 days. The non-seeded cells on scaffold (seeded on tissue culture polystyrene (TCPS) plate) were maintained in neurogenic medium as a control cells.

Scanning electron microscopy (SEM)

The cell-polymer constructs were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, vacuum dried, mounted onto aluminum stubs, and sputter coated with gold. Samples were examined using a scanning electron microscope (S- 4500; Hitachi, Japan) at an accelerating voltage of 20 kV.

RT-PCR analysis

Total RNA was isolated from cells by using the Nucleospin RNAII kit (Macherey-Nagel, Germany). Prior to reverse transcription (RT), RNA samples were digested with DNase I (EN0521; Fermentas) to remove contaminating genomic DNA. DNase I was dissolved in 10X reaction buffer with MgCl₂, and 1 µl of DNase I was added per 1 µg of RNA and incubated for 30 min at 37°C. DNase I activity was arrested following addition of 1µl of 25 mM EDTA and incubated at 65°C for 10 minutes. Standard RT was performed using the RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) and 2 µg total RNA, 0.5 µg oligo (dt18) per reaction, according to the manufacture's instructions. Reaction mixtures for PCR, included 2.5 µl cDNA, 1x PCR buffer (AMS TM, Sinagen, Iran), 200 µM dNTPs, 0.5 µM of each of

Forward and Reverse primers and 1U Taq DNA polymerase (Fermentas, MD, USA). The primers are listed in Table 1. Polymerase chain reactions were performed at 94°C for 1 min, 25-30 cycles 94°C for 30 s, 55-63°C for 30 s, and 72°C for 30 s and 72°C for 10 minutes. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidium bromide (10 µg/ml) and photographed on a UV transilluminator (uvidoc, UK).

Immunocytochemistry analysis

The cells treated with neurogenic medium fixed by incubation in 1% paraformaldehyde/PBS for 3–5 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and post-fixed for an additional 10 min in 4% paraformaldehyde in PBS. The cells were then reacted with primary antibodies (mouse anti-tyrosine hydroxylase (TH), and Beta-tubulin III (b-Tubulin III, Chemicon) at 4°C for 24h, washed with PBS and reacted with the phycoerythrin (PE), fluorescent isothiocyanate (FITC)-conjugated anti mouse IgG as the secondary antibody (Sigma Chemical Co) at room temperature for 3h. Then, the cells were washed with PBS-Tween 0.1% three times and incubated with diaminobenzidine (DAB) solution (Sigma Chemical Co) for 10 min.

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TABLE 1

SPECIFIC PRIMERS USED FOR PCR AMPLIFICATION

Genes	Primer sequences	Size (bp)	Accession Number
Nestin	F:GCCCTGACCACTCCAGTTTA R:GGAGTCCTGGATTTCCCTTCC	199	NM:051373
NSE	F:AAGGACAATACGGCAAGGA R:TGGACCAAGGACGCCAATC	327	NM:22349
MAP2	F:CCATTGCAACAGGAAGACAC R:CAGCTCAAATGCTTGAACATAT	428	NM:002374.3
βTubulinIII	F:ATGTACGAAGACGACGAGGAG R:GTATCCCGAAAATATAAACACA	310	NM:BC:003021
EN1	F:CTGGGTGTACTGCACACGTTAT R:TACTCGCTCTCGTCTTTGTCTT	356	NM:001426.3
EN2	F:GTGGGTCTACTGTACGCGCT R:CCTACTCGCTGTCCGACTTG	358	NM:001427.3
Nurr1	F:GCACCTCGGCAGAGTTGAATGA R:GGTGGCTGTGTTGCTGGTAGTT	491	NM:001457.2
Ptx3	F:TGGGAGTCTGCCTGTTGCAG R:CAGCGAACCGTCTCTGGG	213	NM:005029.3
Pax2	F:ATGTTCCGCTGGGAGATTCG R:GCAAGTGCCTCCGCAAACTG	361	NM:003989.3
Wnt1	F:TAGCCTCCTCCACGAACCTG R:CAGCCTCGGTTGACGATCTTG	239	NM:005430.2
Wnt3a	F:AAGCAGGCTCTGGGACGCTA R:GACGGTGCCTGACAGTTCCA	234	NM:033131.2
Wnt5a	F:ATCCTGACCACTGGAAGCCCTGT R:GGCTCATGGCGTTCCACCAC	358	NM:003392.3
TH	F:GTCCCTGGTTCCCAAGAAAAGT R:TCCAGCTGGGGATATTGTCTTC	333	NM:000360.3
HPRT1	F:CCTGGCGTCTGATTAGTG R:TCAGTCTGTCCATAATTAGTCC	125	NM:000194.1

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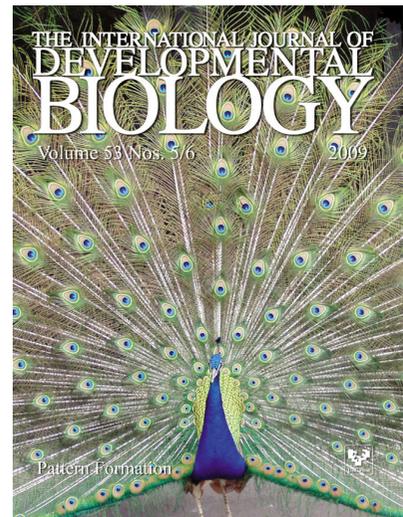
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