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Kursad Turksen *Editor*

Stem Cell Nanotechnology

Methods and Protocols

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Preface

As approaches utilizing nanotechnologies continue to grow and permeate many aspects of science, so too does their applicability to cell biology and more specifically stem cell biology. The emerging interest in nanotechnology as relates to stem cell biology was the driving force behind putting together this volume of protocols. Although it was not possible to approach the topic in an encyclopedic fashion so as to collect all the different protocols in one volume, I have attempted to select a subset of representative protocols that will provide both a flavor of the field as it currently stands and hopefully stimulate new approaches and methodologies to advance the utility of nanotechnologies in stem cell biology.

The protocols gathered here are faithful to the mission statement of the *Methods in Molecular Biology* series: In brief, they are well established and described in an easy-to-follow step-by-step fashion so as to be valuable for not only experts but also novices in the stem cell field. That goal is achieved because of the generosity of the contributors who have carefully described their protocols in this volume, and I thank them for their efforts.

My thanks as well go to Dr. John Walker, the Editor in Chief of the *Methods in Molecular Biology* series, for his guidance and support not just for this volume but through the years.

I am also grateful to Patrick Marton, the Editor of *Methods in Molecular Biology* and Springer Protocols series, for his continuous support from idea to completion of this volume.

Finally, I would like to thank David Casey for his outstanding help during the production of this volume.

Ottawa, ON, Canada ***Kursad Turksen***

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Propagation and Differentiation of Human Wharton's Jelly Stem Cells on Three-Dimensional Nanofibrous Scaffolds

Kalamegam Gauthaman, Chui-Yee Fong, Jayarama Reddy Venugopal, Arijit Biswas, Seeram Ramakrishna, and Ariff Bongso

Abstract

Stem cells isolated from the Wharton's jelly of the human umbilical cord (hWJSCs) are unique compared to other stem cell types as they lie in between embryonic stem cells (ESCs) and adult mesenchymal stem cells (MSCs) on the developmental map and share stemness markers of ESCs and MSCs. Yet, they do not induce tumorigenesis and are hypoimmunogenic and proliferative and fresh cell numbers can be harvested painlessly in abundance from discarded umbilical cords. Additionally, they secrete important soluble bioactive molecules from the interleukin and cell adhesion family, hyaluronic acid, collagen, glycosoaminoglycans, and chondroitin sulfate. Many of these molecules are involved in bone, cartilage, and joint repair. It has also been shown that hWJSCs attach, proliferate, and differentiate efficiently in the stem cell niches of three-dimensional matrices, particularly nanofibrous scaffolds. Thus, tissue constructs made up of hWJSCs and biodegradable nanofibrous scaffolds will facilitate clinical translation and improved functional outcome for arthritis, bone, and cartilage diseases. When applied in vivo, the hWJSCs from the tissue construct may improve repair either by differentiating into new chondrocytes or osteocytes and/or release of important factors that favor repair through paracrine functions. The nanofibrous scaffold is expected to provide the architecture and niches for the hWJSCs to perform and will later biodegrade encouraging engraftment. This chapter provides a step-by-step protocol for the preparation of such tissue constructs involving hWJSCs and nanofibrous scaffolds. The methodology also includes the targeted in vitro differentiation of the hWJSCs to osteogenic and chondrogenic lineages when attached to the nanofibrous scaffolds.

Keywords: Human Wharton's jelly stem cells, Nanofibrous scaffolds, Osteocytes, Chondrocytes, Mineralization, Immunohistochemistry, Real-time polymerase chain reaction

1 Introduction

The power of regenerative medicine is being realized much more in this decade than ever before due to the tremendous advancements witnessed in two main disciplines, namely, "stem cells" and "tissue engineering." Human embryonic stem cells (hESCs) remain the most versatile with hopes of treatments for many incurable diseases but their clinical hurdles of immunorejection and tumorigenesis

have deferred their use in clinical settings (1). Attempts to bypass the problem of immunorejection by personalizing tissues to patients have led to the development of human-induced pluripotent stem cells (hiPSCs) but the potential problem of tumorigenesis still exists because even hiPSCs induce teratoma formation in immunodeficient mice (2). Furthermore, epigenetic changes occur in hiPSCs with continuous passaging leading to the accumulation of various chromosomal anomalies (3). One possible reason for these disadvantages may be that the derivation and growth of hiPSCs do not follow the established paradigms of human development (4) and defects may originate from the matured differentiated somatic cell being reprogrammed, at the point of reprogramming and during the passaging process (5). Mesenchymal stem cells (MSCs) derived from fetuses, adult organs, and bone marrow also have their concerns. Fetal MSCs are controversial as they are derived from abortuses and MSCs derived from bone marrow and organs require painful harvest and are limited in terms of cell numbers. Thus, the search for an alternative and useful stem cell type led to the isolation of human umbilical cord Wharton's jelly stem cells (hWJSCs) that appear to have several advantages over embryonic stem cells (ESCs) and MSCs (6–9).

Since hWJSCs are derived from birth-associated tissues they lie in an intermediate position between ESCs and adult MSCs on the human developmental map and as such share some of the benefits of both ESCs and adult MSCs. They possess high-level expression of the CD markers of MSCs (CD29+, CD73+, CD90+, CD146+, CD14−, CD34−) and low-level expression of some ESC markers (SSEA3+, SSEA4+, TRA-1-81+, OCT4, SOX2, and NANOG). Even though they probably inherit some of these pluripotent ESC markers they do not induce tumor formation in vivo as demonstrated in both laboratory (10) and nonhuman primate animal models (11). Also, unlike bone marrow MSCs harvest is painless since they are isolated from discarded umbilical cords and have no issues of donor site morbidity. They are hypoimmunogenic (6), have longer telomeres and hence prolonged self-renewal, and could be differentiated into many desirable tissue types (7, 8, 12–14). Recently, it was also shown that they may not participate in cancer formation when in the tumor microenvironment as they do not generate tumor-associated fibroblasts (TAFs) unlike bone marrow MSCs (15). It was also reported that hWJSCs secrete in abundance specific bioactive molecules that are the building blocks of cartilage, bone, and joint repair and that may also act as anticancer agents (16). These molecules include cytokines, hyaluronic acid, collagen, glycosoaminoglycans (GAGs), cell adhesion, and anti-inflammatory factors (10, 17, 18). Such advantages of hWJSCs make them an attractive stem cell for use in tissue engineering.

Tissue engineering is an interdisciplinary field that embraces both the "principles of engineering" and "life sciences" to develop biological substitutes using either biological or synthetic materials for cell support (19). Choosing the most appropriate biomaterial and design is important as the structural and physio-chemical properties will ultimately dictate the interaction, attachment, growth, differentiation, and function of the cells within the polymer matrix. Most conventional biomaterials are of micron scale (μm) with larger surface properties. However in vivo, cells are known to interact and function effectively at the nano level (nm) (20). The natural extracellular matrix (ECM) proteins are in the range of 50–500 nm and with the emergence of nanotechnology it is now possible to devise various polymeric scaffolds at nanoscale levels that mimic the in vivo state. The ideal scaffold should be biocompatible and biodegradable, have large surface area to provide better cell attachment, provide stem cell niches, and possess high porosity to facilitate exchange of nutrients and cell movement (21–23). Today, there are several different techniques of scaffold fabrication such as electrospinning, phase separation, particulate leaching, fiber bonding, and gas foaming (24–27).

Recent studies have married the advantages of hWJSCs with nanofibrous scaffolds in preparing the architecture of three-dimensional tissue constructs for transplantation therapy of cartilage, bone, and joint defects (12, 13). In this chapter we describe the step-by-step protocol of the fabrication of nanofibrous scaffolds using electrospinning of various polymers and also describe their successful interaction with hWJSCs in terms of cell attachment, expansion, and differentiation into osteogenic and chondrogenic lineages for the production of tissue constructs for future bone, cartilage, and joint repair.

2 Materials

2.1 Cell Derivation and Culture

1. Human umbilical cords (see Note 1)
2. hWJSCs
3. hWJSC medium, comprising Dulbecco's modified Eagle's medium [high glucose, 20% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 16 ng/ml basic fibroblast growth factor (bFGF) (Millipore Bioscience Research Agents, Temecula, CA), and antibiotic/antimycotic mixture of penicillin (50 IU), streptomycin (50 mg/ml), and amphotericin B (25 μg/ml) (Invitrogen Life Technologies, Carlsbad, CA)]
4. T25, T75 tissue culture flasks (Nunclon, Copenhagen, Denmark)
5. 6-well, 24-well tissue culture plates (Nunclon, Copenhagen, Denmark)

6. 60 mm tissue culture dishes (Nunclon, Copenhagen, Denmark)
7. Trypsin (TrypLE™ Express, Invitrogen, Carlsbad, CA)
8. Centrifuge (Eppendorf, Hamburg, Germany)
9. Carbon dioxide Incubator (5% CO_2 in air) (Binder, Tuttlingen, Germany)
10. Sterile glass Pasteur pipettes (Lab IVF Asia Pte Ltd, Singapore)
11. Sterile plastic serological pipettes (Becton Dickinson, Franklin Lakes, NJ)

2.2 Fabrication of Nanofibrous Scaffolds (Polycaprolactone/ Collagen and Polycaprolactone/ Collagen/ Hydroxyapatite)

1. Polycaprolactone (PCL) (Mol Wt, 80,000) (Sigma, St. Louis, MO, USA)
2. 1,1,1,3,3,3-hexafluoropropanol (HFP) (Sigma, MO, USA)
3. 2,2,2-trifluroethanol (Sigma, MO, USA)
4. Crystalline hydroxyapatite (HAp) (gift from the Department of Metallurgical and Materials Engineering, Indian Institute of Technology, Chennai, India) (see Note 2)
5. Bovine collagen type I (Koken Company Ltd, Tokushima-ku, Tokyo, Japan)
6. Methanol (Sigma, MO, USA)
7. Chloroform (Sigma, MO, USA)
8. Fourier Transform Infrared spectroscopy (Thermo Nicolet, Waltham, MA, USA)
9. Electrospinning machine (ELECTROSPUNRA, Singapore)
10. High voltage power supply (Gamma High Voltage Research, USA)
11. Glass coverslips (diameter 150 mm)
12. Ground collection plate (aluminum foil)
13. Vacuum machine (KNF Neuberger Inc, Trenton, NJ, USA)
14. Gold sputter-coating machine (JEOL JFC-1600 Auto Fine Coater, Japan)
15. Field Emission Scanning Electron Microscope (FESEM) (FEI-QUANTA 200F, The Czech Republic)
16. Image J Software (National Institutes of Health, NIH, USA)

2.3 CD Marker Analysis of hWJSCs

1. Trypsin (TrypLE™ Express, Invitrogen)
2. Phosphate buffered saline (PBS−) (Invitrogen)
3. 10 % normal goat serum (NGS) (Invitrogen)
4. Primary antibodies (1:100) for the following CD markers, viz., CD13, CD14, CD29, CD 34, CD44, CD73, CD90, CD 105, and CD117 (Biolegend, San Diego, CA)
5. Secondary antibody Alexa Fluor® 488 (1:750) (Invitrogen)

6. 60 μm nylon strainer (BD, Franklin Lakes, NJ, USA)
7. CyAn™ ADP Analyzer (Beckman Coulter, Fullerton, CA, USA)

2.4 Cell Attachment and Proliferation: Phase Contrast and Scanning Electron Microscopy

1. Inverted microscope (Nikon Instruments, Tokyo, Japan)
2. PBS (Invitrogen)
3. 3% Glutaraldehyde (Sigma Chemical Co, MO, USA)
4. Ethanol (70, 80, and 95%) (Fisher Scientific, Singapore)
5. Hexamethyldisilazane (Sigma Chemical Co, MO, USA)
6. Gold sputter-coating machine
7. FESEM (FEI-OUANTA 200F, The Czech Republic)
8. MTT assay kit (Sigma Chemical Co, MO, USA)
9. Microplate ELISA reader (μQuant, BioTek, and Winooski, VT, USA)

2.5 Osteogenic and Chondrogenic Differentiation

1. Nanofibrous scaffolds (PCL/Coll/HA) on glass coverslips
2. 24-well tissue culture plates (BD, NJ, USA)
3. Sterilized steel rings
4. hWJSCs (primary cultures and early passages)
5. Osteogenic medium [hWJSC medium supplemented with 100 nM dexamethasone, 50 mM ascorbic acid 2-phosphate, and 10 mM b-glycerophosphate (Sigma)]
6. Chondrogenic medium: Comprising (1) differentiation basal medium and (2) individual singlequots of dexamethasone, ascorbate, insulin–transferrin–selenium (ITS), penicillin–streptomycin, sodium pyruvate, proline, and glutamine (LONZA, Basel, Switzerland)

2.6 Immunohistochemistry

1. 100 % cold ethanol
2. PBS (Invitrogen)
3. 10 % NGS (Invitrogen)
4. Primary antibodies [mouse monoclonal osteocalcin (OCN); mouse monoclonal collagen type II; mouse monoclonal SOX 9 (1:100, Abcam, Cambridge, MA, USA)]
5. Goat antimouse fluorescent secondary antibody (Alexa Fluor 488; 2 μg/ml)
6. 4′-6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) (Molecular probes, Invitrogen)
7. Fluorescence microscope (Leica, Germany)

2.7 Mineralization of hWJSCs: Alizarin Red-S Staining

1. PBS
2. Ice-cold ethanol
3. Alizarin red-S (ARS)
4. Cetylpyridinium chloride (Sigma)
5. Optical microscope (Olympus)
6. Spectrophotometer

2.8 Von Kossa Staining

1. PBS
2. 3 % Formaldehyde solution
3. 1 % Silver nitrate solution (Sigma Chemical Co, MO, USA)
4. Ultraviolet light source
5. 3 % sodium thiosulfate (Sigma Chemical Co, MO, USA)
6. 1 % nuclear fast red (Sigma Chemical Co, MO, USA)
7. Optical microscope (Olympus)

2.9 Alcian Blue Staining

1. 10% neutral buffered formalin (Sigma, Chemical Co, MO, USA)
2. 0.5 % Alcian Blue (Sigma Chemical Co, MO, USA)
3. 0.1 % Nuclear Fast Red (Sigma Chemical Co, MO, USA)
4. Trypsin (TrypLE™ Express, Invitrogen)
5. Cytospinning machine [Cyotospin™ (Thermo Scientific, Barrington, IL, USA)]
6. Glass coverslips
7. Permount

2.10 Glycosaminoglycan Assay

1. hWJSC conditioned medium (hWJSC-CM)
2. 1.5 ml Eppendorf tubes (Axygen, Inc., CA, USA)
3. Blyscan sulfated GAG assay kit (Biocolor, County Antrim, UK)
4. Centrifuge
5. Vortex machine
6. Incubator (5 % CO_2 in air)
7. Spectrophotometer
8. Microplate ELISA reader (μQuant, BioTek, and Winooski, VT, USA)

2.11 Hyaluronic Acid Assay

1. 24 h hWJSC-CM
2. 96-well plate (Nunclon, Copenhagen, Denmark)
3. DuoSet ELISA Development Kit (R&D System, Minneapolis, USA)
4. Incubator (5 % CO_2 in air)
5. Spectrophotometer
6. Microplate ELISA reader (μQuant, BioTek, Winooski, VT, USA)

Table 1
The osteogenic related genes and primer sequences used for quantitative real-time PCR

	Osteogenic related gene primers
GAPDH	F: 5′-ACCACAGTCCATGCCATCAC -3′ R: 5′-TCCACCACCCTGTTGCTGTA -3′
ALP	F: 5′-GGACCATTCCCACGTCTTCAC-3′ R: 5′-CCTTGTAGCCAGGCCCATTG-3′
CBFA1	F: 5′-CACTGGCGCTGCAACAAGA-3′ R: 5′-CATTCCGGAGCTCAGCAGAATAA-3′
RUNX2	F: 5′-GAGGTACCAGATGGGACTGTG-3′ R: 5′-TCGTTGAACCTTGCTACTTGG-3′
ALP	F: 5′-GGACCATTCCCACGTCTTCAC-3′ R: 5′-CCTTGTAGCCAGGCCCATTG-3′
OCN	F: 5′-CCCAGGCGCTACCTGTATCAA-3′ R: 5′-GGTCAGCCAACTCGTCACAGTC-3′
OPN	F: 5′-ACAGCCACAAGCAGTCCAGATT-3′ R: 5′-TGCTCATTGCTCTCATCATTGG-3′

F forward primer, *R* reverse primer, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, ALP alkaline phosphatase, *CBFA1* core binding factor alpha 1, *RUNX2* runt-related transcription factor, *OCN* osteocalcin, *OPN* osteopontin

2.12 Quantitative Real-Time Polymerase Chain Reaction

1. TRIzol™ reagent (Invitrogen)
2. Nanodrop™ (Nanodrop Technologies, Wilmington, DW, USA)
3. SuperScript™ first strand synthesis system (Invitrogen)
4. SYBR green (Applied Biosystems, California, USA)
5. Primers: Osteogenic related genes (Table 1), chondrogenic related genes (Table 2)
6. ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)

3 Methods

3.1 Cell Culture

hWJSCs were derived directly from the Wharton's jelly of human umbilical cords according to a method developed in our laboratory [7, 8].

1. The hWJSCs are cultured in sterile T75 tissue culture flasks in hWJSC medium.
2. Early passages of hWJSCs are used for all experiments (see Note 3).

Table 2
The chondrogenic related genes and primer sequences used for quantitative real-time PCR

	Chondrogenic related gene primers
GAPDH	F: 5′-GCACCGTCAAGGCTGAGAAC-3′ R: 5′-GGATCTCGCTCCTGGAAGATG-3′
COL2A1	F: 5′-GTGACAAAGGAGAGGCTGGA-3′ R: 5′- CCTCTAGGGCCAGAAGGAC-3′
COMP	F: 5′- GGAGATCGTGCAGACAATGA-3′ R: 5′- GAGCTGTCCTGGTAGCCAAA-3′
SOX9	F: 5′- GTACCCGCACTTGCACAAC-3′ R: 5′- GTAATCCGGGTGGTCCTTCT-3′

F forward primer, *R* reverse primer, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *Col2A1* collagen type II A1, *COMP* cartilage oligomeric matrix protein, *SOX9* sex-determining region Y-box 9

3.2 Fabrication of Nanofibrous Scaffolds (Polycaprolactone/Collagen and Polycaprolactone/Collagen/Hydroxyapatite)

The polycaprolactone/collagen (PCL/Coll) and polycaprolactone/collagen/hydroxyapatite (PCL/Coll/HAp) nanofibrous scaffolds were fabricated using the electrospinning technique.

1. Dissolve PCL (10 % w/w) in methanol and chloroform (1:3) and collagen type I (Coll, 80 mg/ml) in HFP by stirring overnight.
2. Mix PCL/Coll polymers at the ratio of 1:1 and PCL/Coll/HAp at the ratio of 10:60:30.
3. Fabricate the nanofibers from the polymer solutions by electrospinning using a flow rate of 1 ml/h at a voltage of 13 kV from a high-voltage power supply.
4. Collect the nanofibers on glass coverslips (diameter 15 mm) placed on a ground collection plate (aluminum foil) located around 13 cm from the electrospinning needle tip.
5. Dry the electrosprayed nanofibers on coverslips in a vacuum at room temperature.
6. Sputter-coat the nanofibers with gold for visualization and characterization using FESEM at an accelerating voltage of 10 kV.
7. Measure the diameters of nanofibers with scanning electron microscopic (SEM) images using Image J Software (see Note 4).

3.3 CD Marker Analysis of hWJSCs

The stemness properties of the hWJSCs were evaluated using various MSC CD marker surface antigens.

1. Dissociate the hWJSCs using TrypLE™ Express for 2 min, wash with PBS without calcium and magnesium [PBS (−)], and then block with 10 % NGS to avoid nonspecific binding.
2. Incubate the cells with primary antibodies (1:100) for 30 min with MSC-related CD markers, viz., CD13, CD14, CD29, CD 34, CD44, CD73, CD90, CD 105, and CD117.
3. Wash the cells to remove the primary antibodies and then incubate with secondary antibodies [Alexa Fluor-488 (1:750)] for 30 min.
4. Finally wash the cells with PBS (−), filter using a 60 μm nylon strainer to remove the cell clumps, and analyze using Cy-An™ADP Analyzer (see Note 5).

3.4 Osteogenic and Chondrogenic Differentiation

The hWJSCs cultured on PCL/Coll and PCL/Coll/HAp nanofibrous scaffolds were differentiated along the osteogenic and chondrogenic lineages.

1. Sterilize the PCL/Coll and PCL/Coll/HAp nanofibrous scaffolds on glass coverslips (see Note 6), place in 24-well tissue culture plates, and prime with hWJSC medium.
2. Sterilize the steel rings and place them on top of the nanofibrous scaffolds to prevent the nanofibers from lifting off from the glass coverslips.
3. Seed early passages (P4–P7) of hWJSCs (2×10^4 cells/ml) on the PCL/Coll/HAp nanofibrous scaffolds (osteogenic differentiation) or 1×10^4 cells/ml on PCL/Coll nanofibrous scaffolds (chondrogenic differentiation) and culture in hWJSC medium for 24 h to enable the cells to attach for proliferation and differentiation.
4. Subsequently change the medium to either osteogenic medium (see item 5 of Section 2.5) or chondrogenic medium (see item 6 of Section 2.5) and continue cell culture for 21 days.

3.5 Cell Attachment and Proliferation: Phase Contrast and Scanning Electron Microscopy

The hWJSCs cultured on PCL/Coll and PCL/Coll/HAp nanofibrous scaffolds in chondrogenic and osteogenic differentiation medium, respectively, were analyzed for their morphology and proliferation.

1. Sterilize the PCL/Coll and PCL/Coll/HAp nanofibrous scaffolds on glass coverslips (see Note 6), place in 24-well tissue culture plates, and prime with hWJSC medium.
2. Place sterilized steel rings on top of the nanofibrous scaffolds to prevent the nanofibers from lifting off from the glass coverslips.
3. Culture the hWJSCs (2×10^4 cells/well) on the nanofibrous scaffolds in 24-well tissue culture plates (BD), monitor them daily for their attachment and morphological changes during culture, and photograph using inverted phase-contrast optics.

4. Scaffold–cell interaction can be observed in more detail with SEM. For SEM, gently wash the nanofibrous scaffolds containing the attached hWJSCs with PBS to remove dead cells and then fix in 3 % glutaraldehyde for 3 h at room temperature.
5. Dehydrate through a series of graded alcohol solutions and finally dry overnight in hexamethyldisilazane.
6. Sputter-coat the dried cellular constructs with gold and examine cell attachment on the nanofibrous scaffolds under SEM at an accelerating voltage of 10 kV (see Note 7).
7. Proliferation rates of the hWJSCs grown on the nanofibrous scaffolds can be evaluated using the cell proliferation MTT assay.
8. For the MTT assay, add 10 μl MTT reagent (0.5 mg/ml) to 100 μl of fresh medium in the culture dishes and incubate the cells for 3–4h.
9. Remove the medium, add 100 μl of the detergent reagent to the cells, and incubate in the dark for 2h.
10. Measure absorbance at 570 nm spectrophotometrically using a microplate ELISA reader with a reference wavelength of 650 nm and calculate the cell proliferation (see Note 8).

3.6 Immunohistochemistry

The hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in the osteogenic medium for 21 days were analyzed for OCN and the hWJSCs cultured on PCL/Coll nanofibrous scaffolds in the chondrogenic medium were analyzed for collagen type II and SOX 9, respectively, by immunohistochemistry.

1. Fix the cells with 100 % cold ethanol for 5 min, wash with PBS, and block with 10 % NGS for 15–20 min at room temperature (RT).
2. Incubate the cells with mouse monoclonal primary antibody (1:100) for collagen type II, SOX9, and OCN for 1 h.
3. Subsequently incubate the cells with goat anti-mouse secondary antibody (Alexa Fluor 488; 2 mg/ml) for 30 min, stain with DAPI (0.5 μg/ml) for 5 min at room temperature, and then analyze using fluorescence microscopy (see Note 9).

3.7 Von Kossa Staining

The hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in the osteogenic medium for 21 days can be evaluated for cell mineralization by Von Kossa staining.

1. Wash the cells with PBS thrice and fix them in 3.7 % formaldehyde solution for 10 min at RT.
2. Wash the cells with distilled water thrice and stain with 1 % silver nitrate solution under ultraviolet light for 60 min.

3. Wash the cells thrice with distilled water, treat the cells with 3 % sodium thiosulfate for 5 min, and counterstain with 1 % nuclear fast red for 5 min.
4. Wash the cells thrice with distilled water and take photographs under inverted phase-contrast optics (Nikon Instruments) (see Note 10).

3.8 Mineralization of hWJSCs: Alizarin Red-S Staining

The hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in osteogenic medium for 21 days can be evaluated for cell mineralization using ARS staining.

1. Wash the cells on the scaffolds gently with PBS three times.
2. Fix the cells in ice-cold ethanol for 1 h, and then gently wash thrice in distilled water before staining with ARS (40 mM) for 15 min at room temperature.
3. Observe the stained cells under an optical microscope and photograph them.
4. Plate the supernatant and read the absorbance at 570 nm using a spectrophotometer (see Note 11).

3.9 Alcian Blue Staining

The hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days can be analyzed for chondrogenic differentiation by Alcian blue staining.

1. Dissociate the hWJSCs grown on nanofibrous scaffolds with trypsin.
2. Wash with PBS (−) and plate the cells directly onto glass slides using Cyotospin™ at 500 rpm for 5 min.
3. Stain the cells with 0.5 % Alcian blue for 30 min at RT and then rinse with tap water.
4. Counterstain with 0.1 % Nuclear Fast Red for 5 min (see Note 12).

3.10 Glycosaminoglycan Assay

The hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days can be analyzed for GAG.

1. Harvest the 24-h conditioned medium (CM) of the hWJSCs on day 21 and analyze for the GAGs (GAG assay) using a Blyscan sulfated GAG assay kit according to the manufacturer's instructions.
2. Add 1 ml of the Blyscan dye reagent to 100 μl of CM in 1.5 ml microcentrifuge tubes and gently mix for 30 min at 5-min intervals followed by centrifugation at 10,000 × *g* for 10 min.
3. Remove the unbound dye by careful draining of the tubes.
4. Add 1 ml of the dissociation reagent to each of the tubes, mix well by vortexing, and then incubate for 1 h at RT.
5. Calculate GAG values from the absorbance obtained at 656 nm using a microplate ELISA reader (see Note 13).

3.11 Hyaluronic Acid Assay

The hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days can be analyzed for HA.

1. Harvest the 24-h CM of the hWJSCs on day 21 and analyze for HA using the DuoSet ELISA development kit according to the manufacturer's instructions.
2. Coat the 96-well plate with capture reagent 0.5 μg/ml in diluent (5 % Tween 20 in PBS; 100 μl/well) and incubate overnight at RT.
3. Block the nonspecific reactions by incubation with block buffer (5 % Tween 20 in PBS with 0.05 % NaN_3; 300 μl/well) for 1 h at RT.
4. Add 100 μl/well of the CM sample and incubate for 2 h at RT.
5. Add detection reagent (0.3 g/ml in diluent; 100 μl/well) and incubate for a further 2 h at RT.
6. Add streptavidin–horseradish peroxidase conjugate (100 μl/well) and incubate in the dark for 20 min.
7. Add the substrate solution [1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine)] and incubate for 20 min.
8. Stop the reaction by addition of the stop solution (2 N H_2SO_4; 50 μl/well).
9. Calculate HA values from the absorbance obtained at 450 nm using a microplate ELISA reader.
10. Between each step wash the 96-well plates thrice with wash buffer (0.05 % Tween 20 in PBS) (see Note 14).

3.12 Quantitative Real-Time Polymerase Chain Reaction

1. The total RNA from the hWJSCs cultured on PCL/Coll nanofibrous scaffolds (in chondrogenic medium) for 21 days can be analyzed for chondrogenic related gene expression using quantitative real-time polymerase chain reaction (qRT-PCR).
2. Isolate the RNA from hWJSCs at 21 days following differentiation using TRIzol™ and measure the quality and quantity of RNA using Nanodrop™.
3. Prepare cDNA with random hexamers using the SuperScript™ first-strand synthesis system.
4. Design primers as needed or use published primer sequences.
5. Run the real-time PCR (qRT-PCR) analysis. Perform qRT-PCR analysis with the ABI PRISM 7500 Fast Real-Time PCR System using SYBR green and carry out relative quantification using the comparative CT (2-ΔΔCT) method (see Note 15).

4 Notes

1. Umbilical cords were donated by patients after informed consent and ethical approval from the Ministry of Health Institutional Domain Specific Review Board (DSRB), Singapore.
2. Bone matrix is composed mainly of hydroxyapatite, type I collagen, calcium, and phosphorous in a crystalline form (28) together with other components such as GAGs, OCN, and osteonectin (29).
3. hWJSCs can be derived in large numbers with no risks of patient morbidity, have high proliferation rates with short population doubling times (PDTs), are extraembryonic, have long telomeres and prolonged stemness properties for many passages in vitro, are widely multipotent and hypoimmunogenic, and do not induce teratoma formation in immunodeficient mice (7, 8, 10). Moreover, these primitive cells are known to harbor less genetic mutations compared to adult cells.
4. Many different scaffolds have been explored including natural decellularized matrix, synthetic polymers, microporous scaffolds, nanofibrous scaffolds, and even combinations of microporous and nanofibrous scaffolds fabricated using different methods to promote cellular adhesion and differentiation (24–27). Electrospinning appears to be the best method for fabrication of nanofibrous scaffolds and of the various polymers (viz., PCL, gelatin, collagen, hydoxyapatite, and their various combinations), and PCL/Coll/HAp and PCL/Coll nanofibrous scaffolds appear to be the most efficient for the differentiation of hWJSCs along the osteogenic and chondrogenic lineages, respectively. The electrospun nanofibers of the PCL/Coll and nanofibrous scaffold examined under FESEM were without breaks, free of beads, and interwoven in a three-dimensional random topography. The diameters of the PCL/Coll nanofibers were 255 ± 0.94 nm and the pore size ranged between 2 and 15 μm. The diameters of the PCL/Coll/HAp nanofibers were 450 ± 0.14 nm and the pore size ranged between 5 and 20 μm. The porosity of both nanofibrous scaffolds ranged between 75 and 95 % (Fig. 1a, b). The porosity facilitates cell migration, free exchange of nutrients, and release of waste products. PCL is a nontoxic, biodegradable, and cost-effective polymer that has been used as a bone substitute (30). Additionally, the mineral composition of HA closely resembles natural bone and has osteo-conductive properties (31). Collagen type I is the main organic component of the bone ECM that aids in mineralization (32) and also collagen-based scaffolds together with the addition of HA improve early-stage chondrogenesis (33). Of note is the fact that hWJSCs naturally secrete more GAGs including HA (17).

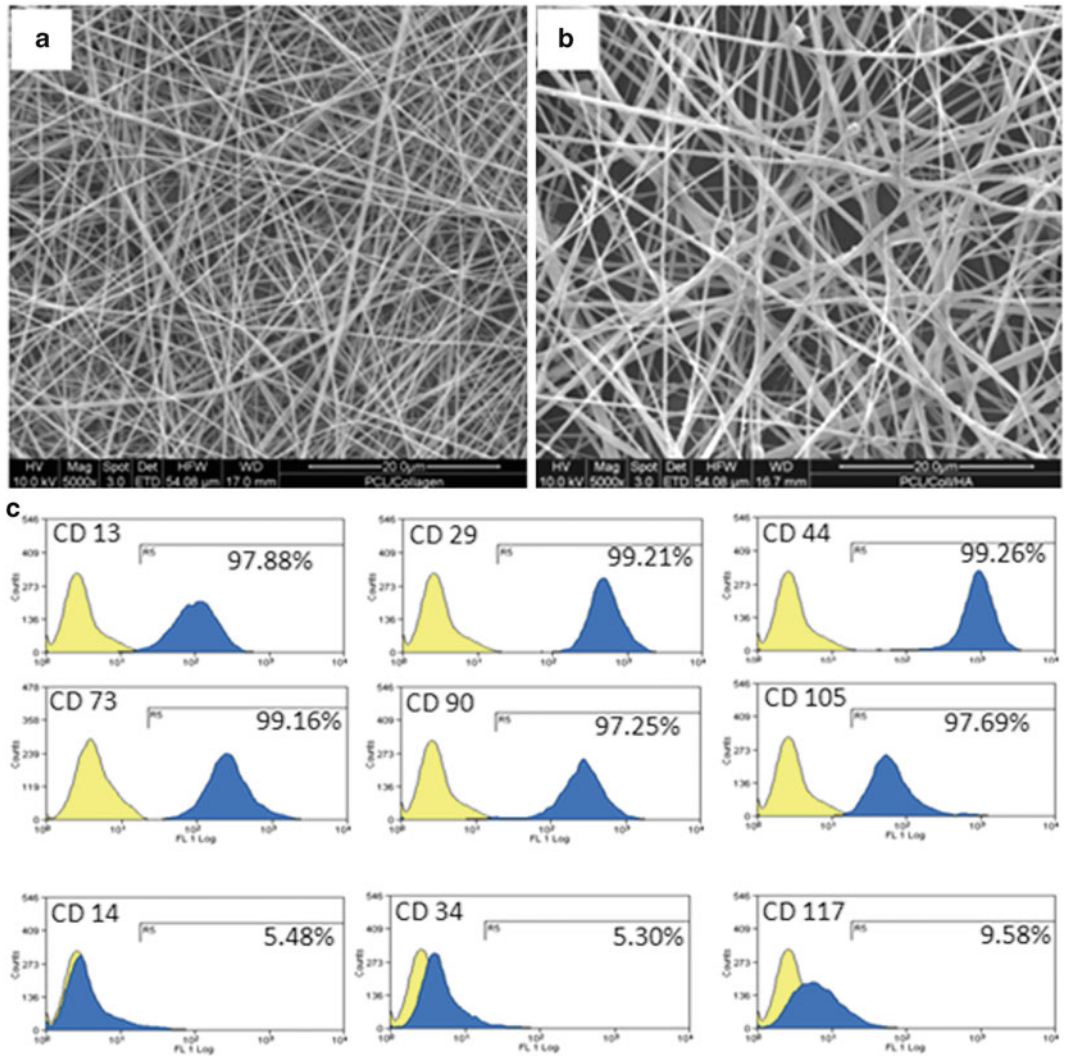

Fig. 1 (**a**, **b**) Scanning electron microscopic images of the electrospun polycaprolactone/collagen nanofibrous scaffolds (PCL/Coll) and polycaprolactone/collagen/hydroxyapatite (PCL/Coll/HAp) nanofibrous scaffolds. The diameters of the PCL/Coll nanofibers were 255 ± 0.94 nm and the pore size ranged between 2 and 15 μm. The diameters of the PCL/Coll/HAp nanofibers were 450 ± 0.14 nm and the pore size ranged between 5 and 20 μm (magnification 5,000×). (**c**) CD surface marker characterization of human Wharton's jelly stem cells analyzed using fluorescence-activated cell sorting (FACS) analysis

5. Flow cytometric analysis shows that hWJSCs are positive for several MSC markers, namely, CD13, CD29, CD44, CD73, CD90, and CD105 (Fig. 1c). Of these markers, CD13, CD29, CD44, CD73, CD90 and CD105 are highly expressed (97.88, 99.21, 99.26, 99.16, 97.25, and 97.69 %, respectively). The hWJSCs are negative for CD14, CD34, and CD117 (Fig. 1c). The Mesenchymal and Tissue Stem Cell Committee of the

International Society for Cellular Therapy proposes minimal criteria to define human MSCs (34) and hWJSCs satisfy all these criteria.

6. It is important that the stainless steel rings and the nanofibrous scaffolds are sterilized well before being used for these studies. The nanofibrous scaffolds and the steel rings are first sterilized using ultraviolet light for 2 h. The scaffolds on glass coverslips are then placed in the wells of 24-well tissue culture plates and the stainless steel rings then placed on top of the coverslips. Each well containing the scaffold and steel ring is soaked in 100 % ethanol for 15 min. This is followed by PBS washes (thrice) for 15 min each time. The scaffolds are then primed in the culture media and incubated overnight in a 5 % CO_2 in air incubator before use in experiments.
7. SEM of hWJSCs cultured on PCL/Coll and PC/Coll/HAp nanofibrous scaffolds shows attachment, proliferation, and cell sheets covering the entire scaffold with mineralization nodules indicative of chondrogenic and osteogenic differentiation (Fig 2a, b). The presence of HAp increases bioactivity and mineralization in the presence of osteogenic growth factors (21, 35, 36). Of note is the fact that culture of hWJSCs could be prolonged until 21 days in the presence of 3D nanofibrous scaffolds which otherwise is not possible as hWJSCs cultured on 2D plastic tissue culture dishes tend to detach and lift off when confluent. Additionally, the nanofibrous scaffolds because of their intrinsic properties of enhanced adsorption of adhesion molecules and presence of stem cell niches encourage cell attachment, differentiation, efficient diffusion of nutrients and waste products, and integration with the surrounding tissue upon transplantation in vivo. Also, their biodegradability and excretion without harmful side effects will enable their beneficial use in regenerative medicine (30, 37, 38).
8. hWJSCs cultured on the PCL/Coll and PC/Coll/HAp nanofibrous scaffolds show increased proliferation when cultured in chondrogenic and osteogenic media, respectively, until the first few days and thereafter the cell numbers decrease or form a plateau around 21 (Fig 2c, d). The cell numbers do not increase significantly with time when cultured on nanofibrous scaffolds in differentiation medium perhaps because the hWJSCs may have already entered the differentiation phase of chondrogenesis and osteogenesis. Increased cell proliferation in the early culture period (7 days) and decrease with extended culture have been reported by several groups (39, 40).
9. hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium show positive staining at 21 days for the chondrogenic related proteins, viz., collagen type II (Fig. 3a)

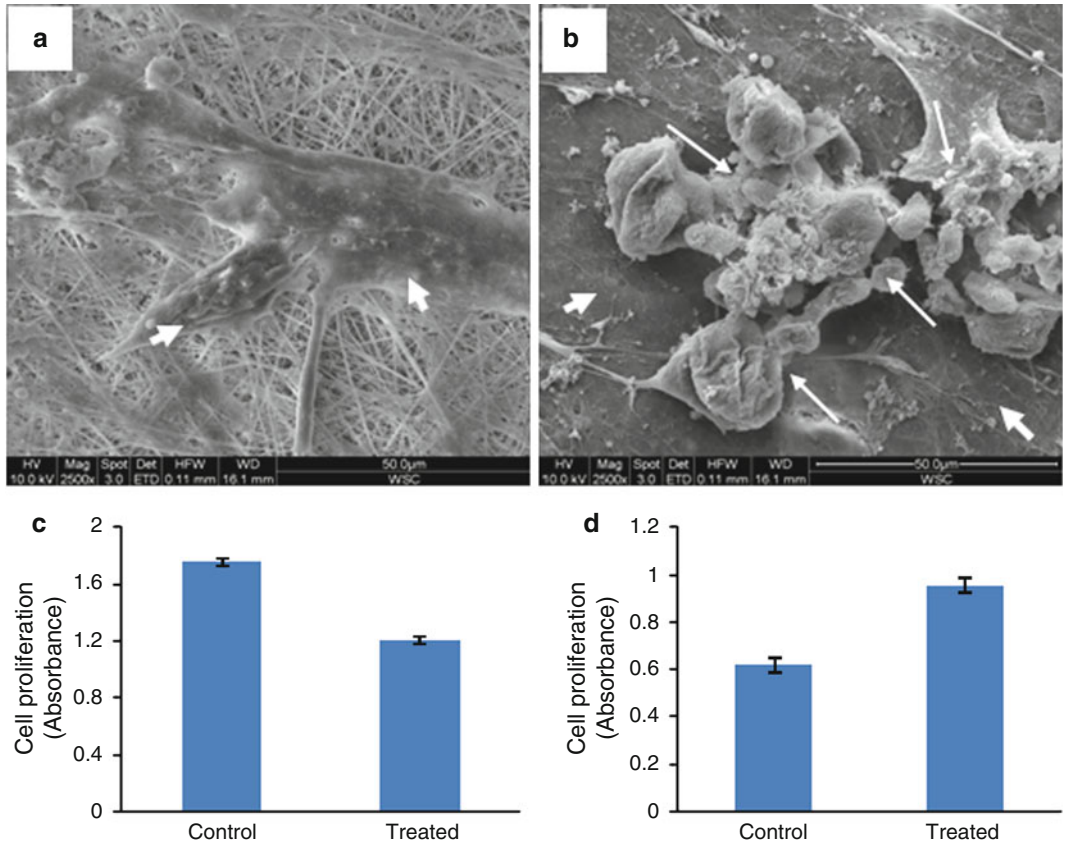

Fig. 2 (**a**, **b**) Scanning electron microscopic images of hWJSCs grown on electrospun polycaprolactone/collagen (PCL/Coll) nanofibrous scaffolds in chondrogenic medium and on polycaprolactone/collagen/hydroxyapatite (PCL/Coll/HAp) nanofibrous scaffolds in osteogenic medium for 21 days. The cell sheets are indicated by *short thick arrows* and mineralization nodules are indicated by *thin long arrows*. (**c**, **d**) hWJSC proliferation on the PCL/Coll nanofibrous scaffolds in chondrogenic at 21 days and on PCL/Coll/HAp nanofibrous scaffolds in osteogenic medium in early culture analyzed by MTT assay

and SOX9 (Fig. 3b). Collagen type II fibril is known to interact with various GAGs and increase the tensile strength of ECM (41). SOX9 is a transcription factor of the SRY family that regulates sex determination, cartilage development, and numerous other developmental events (42, 43). Similarly, hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in osteogenic medium for 21 days show positive staining for OCN by immunohistochemistry (Fig. 3c). The bone Gla protein or OCN is found exclusively in bone tissue forming 10–20 % of the non-collagenous protein in bone and is thought to play a role in ossification and mineralization (44). It is a late marker of osteogenesis and is also involved in bone resorption after bone mineralization (45). OCN expression confirms the differentiation of hWJSCs into osteocytes.

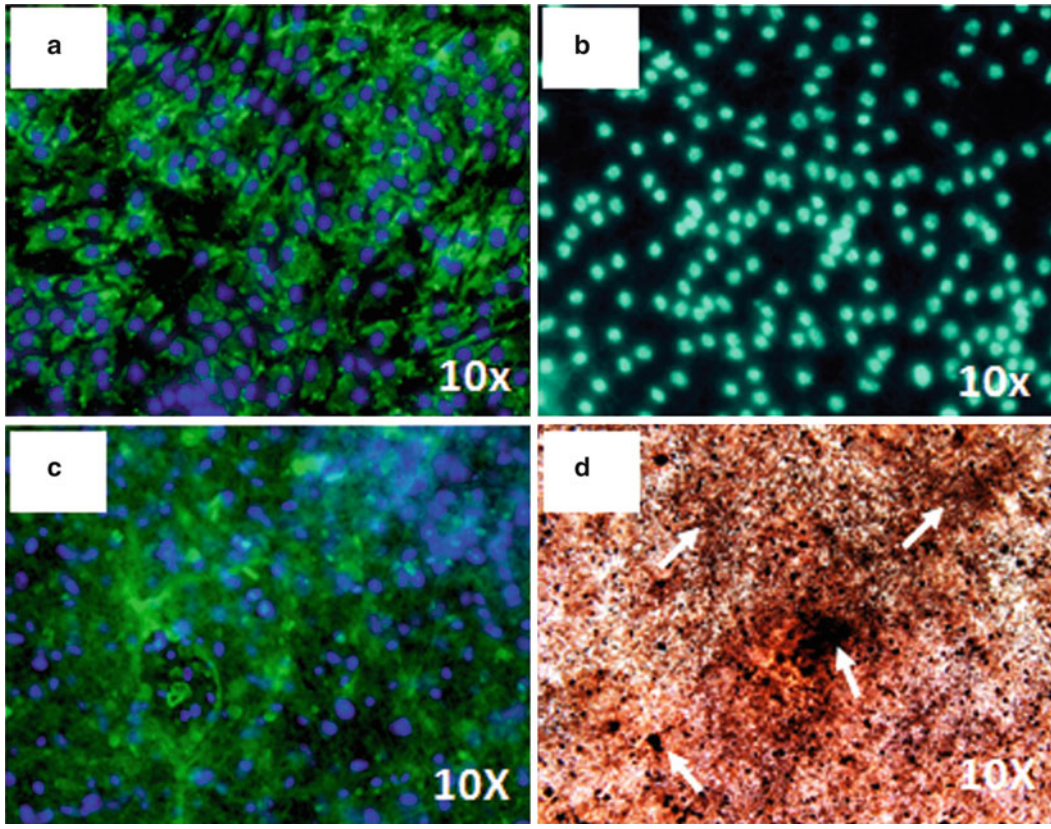

Fig. 3 (**a**, **b**) Immunohistochemistry images of hWJSCs grown on electrospun polycaprolactone/collagen nanofibrous scaffolds (PCL/Coll) in chondrogenic medium for 21 days showing positive staining for collagen type II and the transcription factor SOX9, respectively. (**c**, **d**) hWJSCs grown on polycaprolactone/collagen/hydroxyapatite (PCL/Coll/HAp) nanofibrous scaffolds in osteogenic medium for 21 days showing positive staining by immunohistochemistry for bone-related osteocalcin (**c**), and calcium mineralization by Von Kossa staining (**d**). *White arrows* in (**d**) indicate the mineralization nodules (magnification 10×). *Source*: From Reference (12) with kind permission from Mary Ann Liebert, Inc, and from Reference (13) with kind permission from Springer Science+Business Media

10. hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in osteogenic medium for 21 days show intense Von Kossa staining of the nodules that are indicative of mineralization (Fig. 3d). Although Von Kossa staining is known to react with phosphate in the presence of acidic material, it has been routinely used as an indirect index of calcium mineralization (46). It is therefore necessary to complement Von Kossa staining with other markers of bone differentiation using other techniques such as immunohistochemistry and gene expression.
11. hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in osteogenic medium for 21 days show many large nodules on the scaffold surfaces that stain strongly with ARS suggestive of

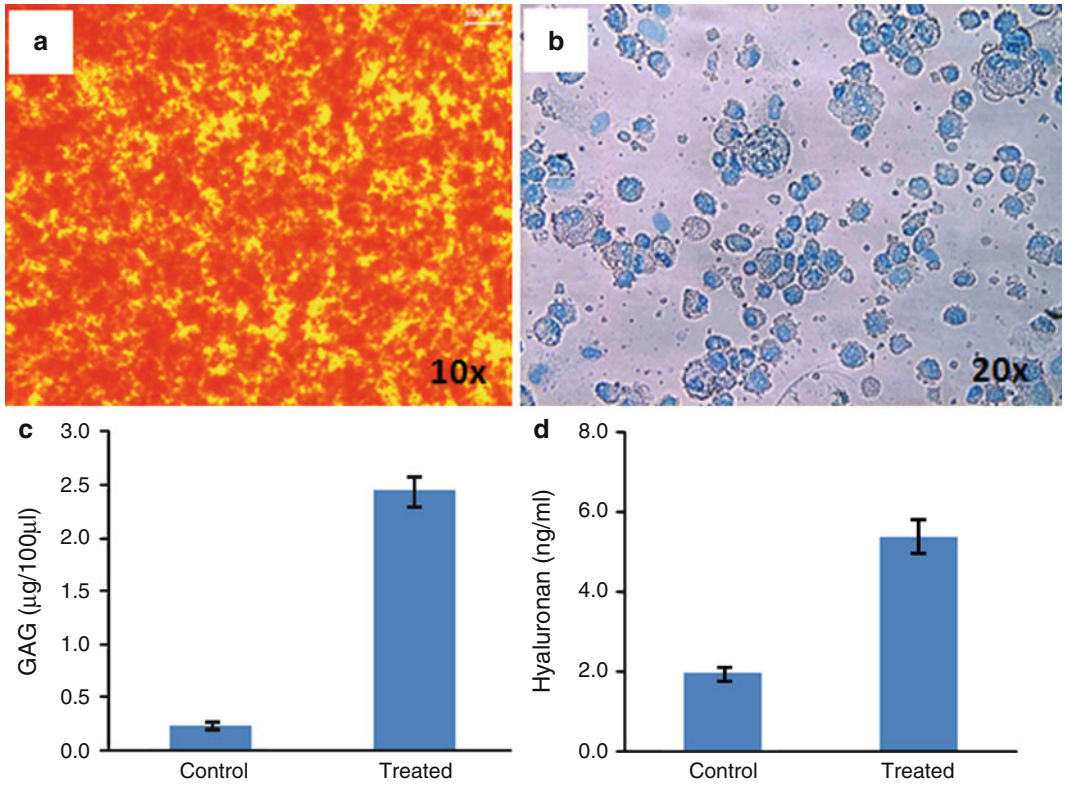

Fig. 4 (**a**) Optical microscopic image of hWJSCs grown on polycaprolactone/collagen/hydroxyapatite (PCL/Coll/HAp) nanofibrous scaffolds in osteogenic medium for 21 days showing intense Alizarin Red S (ARS) staining indicative of mineralization (magnification 10×). (**b**) Alcian blue staining of hWJSCs grown on polycaprolactone/collagen nanofibrous scaffold (PCL/Coll) in chondrogenic medium for 21 days showing positive staining (magnification 20×). (**c**, **d**) Concentrations of glycosaminoglycans and hyaluronic acid (HA) in 24-h conditioned medium harvested from hWJSCs grown on PCL/Coll nanofibrous scaffold in chondrogenic medium for 21 days. *Source*: From Reference (12) with kind permission from Mary Ann Liebert, Inc, and from Reference (13) with kind permission from Springer Science+Business Media

mineralization (Fig. 4a). ARS is an anthraquinone derivative that forms an ARS–calcium complex in a chelation process and is a simple and rapid method that is used to identify microcrystalline or noncrystalline calcium by ordinary light microscopy. Although ARS is not highly specific for calcium, the staining may not be affected by the presence of magnesium or iron which might show some interference (47, 48).

12. hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days show positive Alcian blue staining for chondrogenesis (Fig. 4b). Chondrogenesis is accompanied by a lower number of cells per section area and this is also reflected by the decrease in cell proliferation upon differentiation by MTT assay.

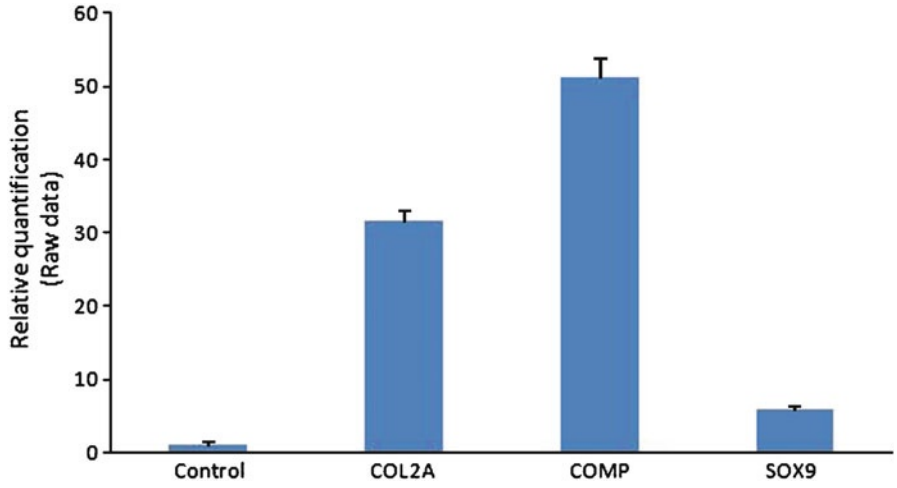

Fig. 5 Real-time polymerase chain reaction (qRT-PCR) analysis for chondrogenic related genes from the hWJSCs grown on polycaprolactone/collagen (PCL/Coll) nanofibrous scaffold in chondrogenic medium for 21 days. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal control. Data analysis and relative quantitation were done using the comparative Ct (ΔΔCT) method

13. hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days show increased GAG levels (Fig. 4c). hWJSCs also have the inherent property of secreting GAGs in the absence of chondrogenic supplements (17, 49) and therefore hWJSCs are more suitable stem cell types for cartilage tissue engineering.
14. hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days show increased HA levels (Fig. 4d). Hyaluronic acid (HA) and GAGs are the two most important building blocks for cartilage and bone repair. HA is also incorporated as a constituent in biomaterials as it mimics the function of ECM and chondrogenic related protein and gene expression has been reported from cells cultured on HA-based hydrogels (50). hWJSCs naturally secrete large amounts of HA (17) and this inherent property makes them an ideal stem cell for chondrocytic differentiation for cartilage repair.
15. qRT-PCR analysis of hWJSCs cultured on PCL/Coll nanofibrous scaffolds in chondrogenic medium for 21 days shows increased expression of chondrogenic related genes [cartilage oligomeric matrix protein (COMP), collagen type II, sex-determining region Y-box 9 (SOX 9)] (Fig. 5). Expression of COMP, collagen type II fibromodulin, and SOX 9 by hWJSCs cultured on PCL/Coll nanofibrous scaffolds indicates that the hWJSCs are undergoing chondrogenesis. Earlier studies of MSCs in three-dimensional cultures have showed distinct chondrogenesis with expression of cartilage-specific genes, including COMP, collage type II, and aggrecan when stimulated with transforming growth factor-

beta (51). In vivo chondrogenesis involves aggregation and condensation of loose MSCs that express various ECM components including collagen type II, aggrecan, COMP, and the important transcription factor SOX 9 (52, 53). Similarly, qRT-PCR analysis of hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds in chondrogenic and osteogenic medium, respectively, for 21 days shows increased expression of osteogenic related genes [alkaline phosphatase (ALP), core binding factor alpha 1 (CBFA1), OCN, osteopontin (OPN), and runt-related transcription factor 2 (RUNX2)] (12). Expression of the ALP, RUNX2, and CBFA1 genes by hWJSCs cultured on PCL/Coll/HAp nanofibrous scaffolds indicates that the hWJSCs undergo osteogenesis. CBFA1 belongs to the runt-domain gene family and is known to induce osteoblast differentiation of non-osteoblastic cells although it mainly regulates expression of other osteoblast-related genes such as OCN, OPN, and BSP during embryonic development (54). Activated RUNX2 induces downstream osteoblast-specific markers OCN and OPN (55). OPN is thought to regulate the size and shape of the mineral crystals (56) and bone Gla protein or OCN is thought to play a role in ossification and mineralization (44). Considering the several advantages of hWJSCs over other stem cell types, they may be the ideal stem cell for development of tissue-engineered constructs using nanofibrous scaffolds that may provide the necessary niches for the hWJSCs to attach, proliferate, and differentiate in vivo into useful tissues for future clinical application.

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Composite Electrospun Nanofibers for Influencing Stem Cell Fate

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Abstract

The design of new bioactive materials, provided with "instructive properties" and able to regulate stem cell behavior, is the goal for several research groups involved in tissue engineering. This new function, commonly reserved for growth factors, can lead to the development of a new class of implantable scaffolds, useful for accelerating tissue regeneration in a controlled manner. In this scenario, the likely most versatile and effective tools for the realization of such scaffolds are based on nano- and microtechnology. Here, we show how exploiting the electrostatic spinning (ES) technique for producing a nanofibrillar composite structure, by mimicking topographically the extracellular matrix environment, can influence the fate of human bone marrow mesenchymal stem cells, inducing osteogenic differentiation in the absence of chemical treatments or cellular reprogramming. Basic cues on the choice of the materials and useful experimental instructions for realizing composite nanofibrous scaffolds will be given as well as fundamental tips.

Keywords: Tissue engineering, Regenerative medicine, Cell fate, Stem cells, Cell differentiation, Osteogenic differentiation, Nanofibers, Electrostatic spinning, Electrospinning, Composite materials

1 Introduction

Adult stem cells, including bone marrow mesenchymal stem cells (MSCs), have demonstrated their multipotency leading to the formation of cartilage, bone, muscle, connective tissue, or fat (1). The fine control of the differentiation lineage in vitro is commonly obtained by using defined inductive media. Autologous MSCs are routinely used in conventional tissue engineering ex vivo in combination with biomaterials to create a cell-based scaffold in order to replace or improve the tissue regeneration in vivo (2–4). In addition, endogenous MSCs can locate in cell-free implanted scaffolds through a non-completely understood mechanism, named

homing, contributing to the regeneration of the injured tissue and demonstrating their crucial role in the regenerative process (5).

In all these cases, the host response to the implanted scaffold depends on the cell/material interface that affects MSC function and differentiation. For this reason, the design of smart strategies to finely influence the differentiation of MSCs towards specific lineages with a fully material-driven control (i.e., in the absence of chemical treatments or cellular reprogramming) is a challenge in the development of new performing implantable scaffolds. A very few studies report on the control of the differentiation for MSCs in basal medium condition, exploiting micropatterned fibronectin (6–8), silane- or amino-modified surfaces (9, 10), matrix stiffness (11), and nanotopography (12, 13). Similar results can be obtained with a composite extracellular matrix (ECM)-like structure, realized by electrostatic spinning (ES) technique (14).

Employing polymer solutions of both natural and synthetic polymers, ES is gaining great attention as a simple, low-cost, continuous, high-throughput, and versatile technology for realizing polymer nanofibers due to its capability to produce fibers in the submicron range consistently that is otherwise difficult to achieve by using standard fiber-drawing techniques (15, 16). With smaller and ultimately tunable pore size and higher surface-to-volume ratio than regular fibers, electrospun fibers show very peculiar properties and are effectively applied in several research areas, from optoelectronics to biological applications (17–20). In tissue engineering, electrospun scaffolds aim to topologically mimic the physiological ECM structures that are indeed three-dimensional architectures mainly made by nanofibrous architectures of proteins (21). In this field, the huge versatility of ES, in terms of chemical composition, morphology, and possibility of surface modification of realized fibers, embraces tissue engineering requirements for accomplishing specific cellular requirements (14, 22–24).

This technique is based on the uniaxial stretch of a viscoelastic polymer jet under an intense applied electric field. The experimental setup is extremely simple (Fig. 1): a syringe pump allows to pull out a drop of a polymer solution at the end of a syringe metal needle; a high-voltage supply connected to the needle provides a bias (commonly, in the range 5–50 kV); a metal surface, placed at a distance in the range 5–50 cm and negatively biased, collects solid polymer nanofibers (for modifications to this standard setup, see ref. 20, 25). On the other hand, the spinning physical mechanism is rather complicated. Based on experimental observations and electrohydrodynamic theories, mathematical models have been developed by several groups to describe the ES process (15, 26, 27). From a simplistic point of view, the bias generates a distribution of electrostatic charges on the surface of the droplet at the capillary edge, and when the electric field overcomes a critical value, at which the electrostatic force intensity is larger than the surface tension of the solution,

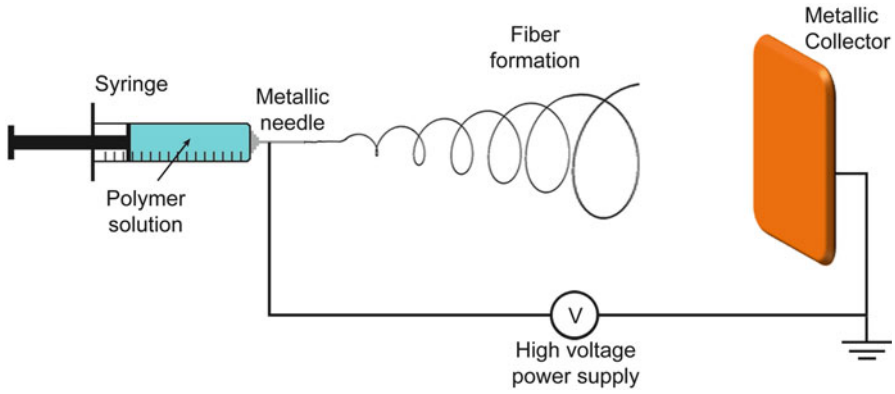

Fig. 1 Schematic drawing of the ES process in a horizontal setup. In the case of a vertical setup, the collector is placed on the hood bench and the needle in a higher position

the droplet becomes more and more unstable and a jet is formed from the needle to the collector. During the needle–collector path, the solvent evaporates, and the resulting polymer fiber is collected onto a substrate. Three different types of processing parameters affect the morphology and diameter of electrospun fibers: (a) the intrinsic properties of the solution, including the type of polymer, its chain conformation, viscosity (or concentration), electrical conductivity, and the solvent polarity and surface tension; (b) the operational conditions, including the strength of applied electric field, the needle–collector distance, and the feeding rate for the polymer solution; and (c) the environmental conditions, such as temperature and relative humidity (28).

In this chapter, we show how the ES technique can be applied for the realization of an ECM-like composite structure, able to influence the differentiation of human bone marrow mesenchymal stem cells (hMSCs) towards the osteogenic lineage in vitro, in the absence of inductive chemical treatments or cellular reprogramming. Specific guidelines and fundamental tips are described in order to overcome typical experimental issues.

2 Materials

2.1 Polymer Solution for ES

1. Organic solvent: Hexafluoroisopropanol (HFIP, >99 %; Carlo Erba), see Note 1.
2. Polymer: Polycaprolactone (PCL) powder (M_w 70,000–90,000; Sigma-Aldrich), see Note 2.
3. Ceramic nanocrystals (NCs: β-tricalcium phosphate, TCP), commercially obtained (Berkeley Advanced Biomaterials) or lab-made by sol–gel method.

4. Oleic acid (>99 %; Sigma-Aldrich).
5. Glass vials and stirring bars.
6. Magnetic stir plate.

2.2 Scaffold Realization

1. Conventional aluminum foil, copper grids with 300 mesh (TAAB Laboratories Equipment), and round borosilicate coverslips (Carlo Erba) as substrate for collecting the fibers, in addition to a 10 × 10 cm^2 metal collector, and carbon tape if needed. See Note 3.
2. Plastic syringes, metal needles (27 gauge), and syringe infusion pump (Harvard Apparatus).
3. High Voltage power supply (EL60R0.6–22, Glassman High Voltage). See Note 4.
4. A chemical fume hood, where the ES setup is placed. The air relative humidity and temperature conditions should be kept constant at about 40 % and 23 °C, respectively.
5. Spin coating apparatus or similar film deposition apparatus for realizing polymer film as reference material for in vitro studies. See Note 5.
6. A vacuum desiccator for samples storage.

2.3 Scaffold Characterization

1. Scanning electron microscopy (SEM) apparatus (Raith 150 system) and an imaging analysis software (WSxM from Nanotech Electronica or ImageJ from NIH).
2. Transmission electron microscopy (TEM) apparatus (Jeol Jem 1011).
3. Fourier-transform infrared (FTIR) spectroscopy system (Spectrum 100, Perkin Elmer). See Note 6.

2.4 Cell Culture

2.4.1 MSC Expansion

1. Human MSCs (Lonza). See Note 7.
2. Basal medium (BM): Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). Store at 4 °C.
3. Osteogenic medium (OM): Differentiation BulletKit, Lonza. Store at 4 °C.
4. Phosphate-buffered saline (PBS), without magnesium and calcium, pH 7.4 (Euroclone).
5. Trypsin 0.05 %/0.2 % EDTA in PBS without Phenol Red, magnesium, and calcium (Euroclone). Store at −20°C. When ready to use, store at 4 °C.
6. 100-mm round cell culture dishes.
7. Pasteur pipettes.
8. A humidified 5 % CO_2, 37 °C incubator.

2.4.2 Cell Counting

1. 0.4 % (w/v) Trypan Blue solution (Sigma Aldrich).
2. Hemacytometer.
3. PBS (Euroclone).

2.4.3 Cell Adhesion

1. Sodium cacodylate buffer, at pH 7.2: 50 ml of 0.2 M anhydrous sodium cacodylate and 4.2 ml of 0.2 M HCl in a volume of 200 ml of distilled water.
2. Glutaraldehyde 2.5 % in cacodylate buffer. CAUTION: Toxic irritant. Prepare in fume hood and wear appropriate skin and eye protection.
3. 70 % Ethanol. CAUTION: Flammable. Stored in a flammable safe cabinet.

2.4.4 Cell Proliferation and Early Differentiation

1. AlamarBlue (Invitrogen). Store at 4 °C.
2. 15- and 50-ml centrifuge tubes.
3. ALP staining kit (Sigma kit 86-R, Sigma-Aldrich).

2.4.5 RNA Extraction

1. RNAeasy micro kit (Qiagen).
2. Deoxyribonuclease I (Invitrogen).
3. Eppendorf Mastercycler ep Realplex2 (Eppendorf).
4. RealMasterMix SYBR Green (5prime).
5. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen).
6. Ethanol 70 %.
7. DNase I stock solution.
8. Primers and probe for human RUNX2, Coll I, and BSP were designed as reported in Table 1. GAPDH was used as reference gene.

Table 1
Primer sequences and annealing temperature for real-time RT-PCR

	Primer forward	Primer reverse	T_m (°C)
GAPDH	5′-ACC CAC TCC TCC ACC TTT GA-3′	5′-CTG TTG CTG TAG CCA AAT TCG T-3′	61
RUNX-2	5′-CTT CAT TCG CCT CAC AAA CA-3′	5′-TTG ATG CCA TAG TCC CTC CT-3′	58
Col-I	5′-TTG CTC CCC AGC TGT CTT AT-3′	5′-TCC CCA TCA TCT CCA TTC TT-3′	58
BSP	5′-GAA GAA GAG GAG ACT TCA AAT G-3′	5′-TAT CCC CAG CCT TCT TGG GA-3′	61

3 Methods

3.1 Preparation of the Polymer Solution

1. For polymer nanofibrous scaffolds, prepare a 3.5 % PCL solution (w/w) using HFIP as solvent, by weighing accurately the polymer content in a glass vial. Add a magnetic bar and the required HFIP. Place the vial on a stir plate for a few hours. See Fig. 2 and Note 8.
2. For composite nanofibrous scaffolds, prepare a 3.5 % PCL–2 % NCs–0.05 % oleic acid solution (w/w) using HFIP as solvent, by weighing the polymer and the NCs in a glass vial as first step. Add a magnetic bar, the required oleic acid, and HFIP. Place the vial on a stir plate for 1 week at moderate stirring speed (≈500 rpm, at room temperature). See Fig. 3 and Note 9.

3.2 Scaffold Production

1. Place the substrate (Al foil, TEM copper grids, and/or round borosilicate coverslips) onto the metal collector placed 20 cm far from the metal needle, using carbon tape if needed. See Note 10.

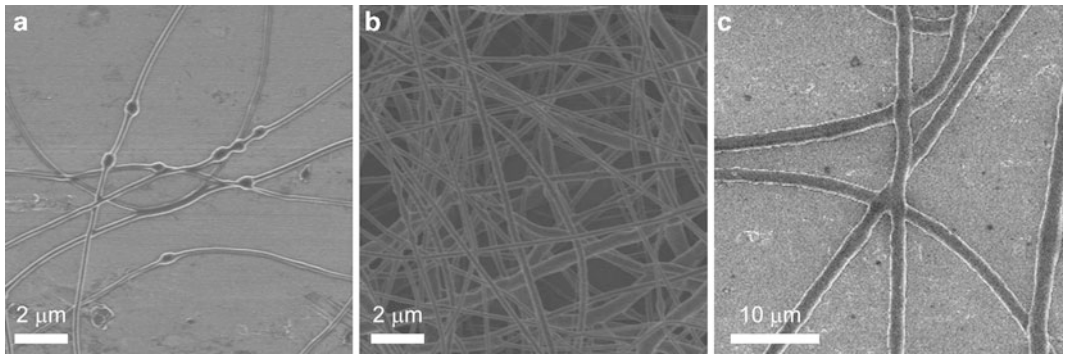

Fig. 2 SEM micrographs of PCL NFs electrospun from 2 % (**a**), 3.5 % (**b**), 7 % (**c**) w/w HFIP solutions

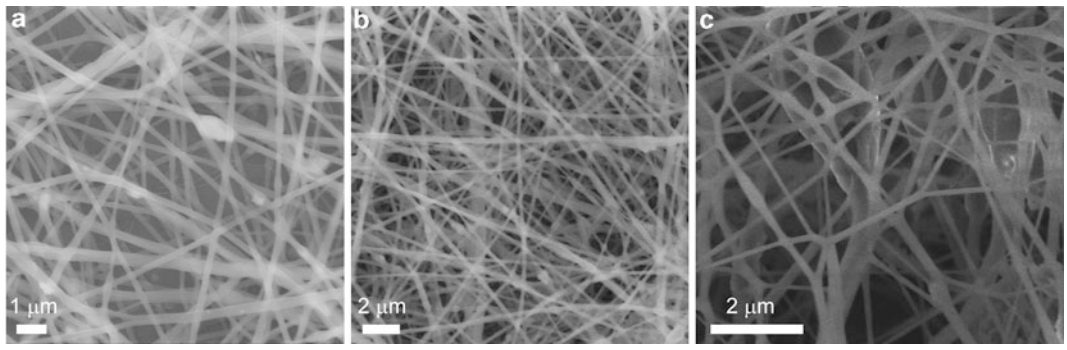

Fig. 3 SEM micrographs of PCL-NC NFs, electrospun from 3.5 to 0.5 % (**a**), 3.5 to 2 % (**b**), 3.5 to 3 % (**c**) w/w HFIP solutions

2. Fill the syringe with the polymer solution and connect it to the syringe infusion pump (0.5 ml/h).
3. Connect the power supply to the needle, increasing the applied voltage gradually up to 4.5 kV.
4. Switch on the pump: At the tip of the needle a cone should appear from the meniscus of the polymer solution, and a white circle should be notable on the collector. See Note 11.
5. Wait for 2 h to obtain a robust sheet of NFs on your substrate. If a TEM grid is used, few seconds are required for having enough NFs to investigate (the presence of too many fibers can dramatically decrease the signal intensity during TEM analysis).
6. Switch off the pump and power supply, decreasing the applied voltage gradually to 0 kV. Store the samples in a vacuum desiccator until use, to allow the complete evaporation of residual solvent molecules (even few hours are enough).
7. For producing reference film materials, use the same ES solution, round glass coverslips, and a spin coating apparatus (5,000 rpm for 60 s).

3.3 Scaffold Characterization

1. SEM (morphology): Cut the Al foil sheet with the NFs into 1 cm × 1 cm pieces and analyze them using an accelerating voltage of 5 kV and an aperture size of 20 mm. Note that if the analysis is difficult due to the low conductivity of the sample, a sputter coating (10 nm) can be employed. Collect several images, using different magnitude, and analyze 100 fibers, at least, by an imaging software for calculating the mean diameter.
2. TEM (morphology): Analyze the TEM grids operating at an accelerating voltage of 100 kV.
3. FTIR (chemical composition): Lift up the NF layer from the Al foil substrates using a razor blade or tweezers, in order to have a 2 cm × 2 cm stand-alone NF sheet. Analyze it in transmission mode, recording spectra in the 450–4,000 cm^{-1} range, with 256 scans at spectral resolution of 2 cm^{-1}. See Note 12.

3.4 In Vitro Studies

MSC isolation and culture steps should be performed in a laminar flow hood using sterile technique and sterile reagents.

3.4.1 MSC Culture and Seeding on Scaffolds

1. Resuspend a pool of five healthy donors of hMSC in MSCGM.
2. Plate cells on round 100-mm tissue culture dishes at a density of $1.7 \times 10^6/cm^2$ and culture them in incubator.
3. After 48 h of incubation, remove nonadherent cells by aspirating away the old media using a sterile Pasteur pipette.
4. Add fresh MSCGM to adherent cells in dishes and feed cells with MSCGM every 2–3 days.

5. Grow cells in vitro to 80 % confluency (10,000 cells/cm^2) before subculturing.
6. Aspirate old media using a sterile Pasteur pipette from the cell culture dishes.
7. Wash cells with sterile PBS. Aspirate PBS.
8. Add 3 ml of 0.5 % trypsin/EDTA to 100-mm round dishes.
9. Incubate at 37 °C for 3 min or longer until about 90 % of the cells detach.
10. Add 5 ml of MSCGM to deactivate trypsin activity; see Note 13.
11. Collect cells and centrifuge at 500 × *g* for 5 min.
12. Aspirate the supernatant, being careful not to disturb cell pellet.
13. Resuspend cell pellet in appropriate amount of MSCGM and break up cell pellet by pipetting up and down.
14. Plate cells onto new 100-mm dishes. Cells are generally subcultured at a dilution ratio of 1:3 to give a density of 3,000–4,000 cells/cm^2.
15. Grow cells in vitro to 80 % confluency (passage P1) and feed cells with MSCGM every 2–3 days.

3.4.2 Cell Counting

1. Trypsinize cells (refer to Section 3.4.1, steps 6–13).
2. Mix thoroughly and draw up 10 μl of cells in media using a micropipette. Mix with 10 μl of Trypan Blue solution (1:2 dilution of cells).
3. Place 20 μl of Trypan Blue-stained cells under a glass coverslip on a hemacytometer.
4. Count the total cells in each corner (one quadrant) of the hemacytometer. Also keep a running tally of the number of Trypan Blue-positive cells (these cells will appear blue).
5. Calculate the average number of cells per quadrant that are Trypan Blue negative. Multiply this number by 2 × 10^4. This number represents the number of cells present per milliliter.

3.4.3 Cell Seeding onto Scaffolds

1. Resuspend cells onto a proper volume of MSCGM, at a density of 0.5–0.8 × 10^6 cells/ml.
2. Pipette 100 μl of cell suspension onto both nanofibrous and film scaffolds. Seed cells also on plastic surface as control, at the same cell density.
3. Check cell seeding under the microscope to make sure that seeding density and distribution are correct. See Note 14.
4. Transfer scaffolds to incubator and let cells attach for 1–2 h.

5. Add either basal (MSCGM) or osteogenic (Differentiation BulletKit) media (hereafter called BM and OM, respectively) onto the scaffolds (both nanofibrous and film scaffolds) so that each 100-mm dish hosting scaffold holds 10 ml media.
6. Culture cell constructs for 2 weeks.

3.4.4 Cell Adhesion onto Scaffolds

At day 7 of culture, stop cell culture onto a sample of nanofibrous and film scaffolds, and process them for SEM analysis.

1. Wash samples in sodium cacodylate buffer for 10 min at 4 °C.
2. Fix in 2.5 % glutaraldehyde buffer for 30 min at 4 °C.
3. Rinse them twice in cacodylate buffer solution at 4 °C for an overall time of 45 min.
4. Dehydrate scaffolds in increasing concentrations of ethanol and air-dry.
5. Sputter-coat with gold samples for 150 s at 60 mA current and below 10^{-1} mbar vacuum. Sputter-coating deposits a conductive metal on the scaffold to enable imaging using the electron beam current.
6. Prepare SEM images of the scaffolds and determine fiber diameter using NIH Image J software available freely at http://rsbweb.nih.gov/ij/.

3.4.5 Cell Proliferation

Cell proliferation assay was performed according to AlamarBlue instructions, every 24 h for a week, in proliferation medium (BM).

1. Add 1/10th volume of AlamarBlue reagent directly to cells in culture medium.
2. Incubate the scaffolds for 3 h in a humidified chamber at 37 °C, protected from direct light.
3. Transfer 250 μl of the incubated solution from each scaffold into a single well of a 96-well plate.
4. Measure the absorbance at 570 nm, using 600 nm as a reference wavelength for normalization.
5. Create a standard curve using samples with known numbers of hMSC to correlate absorbance to cell number.

3.4.6 ALP Activity

ALP enzyme activity of hMSCs, cultured either on NFs or control film, was assessed after 7 days of cell culture in either basal or osteogenic medium. Cell culture on plastic surface was used as control. See Note 15.

1. Use two samples for each experimental condition to assess the ALP activity of the cells using an alkaline phosphatase kit following the manufacturer's instructions.
2. Aspirate media and wash cells with PBS twice.

3. Fix cells with fixative solution for 5 min.
4. Rinse cells briefly with distilled water. Do not allow cells to dry out.
5. Stain cells with alkaline phosphatase staining solution for 30 min at room temperature, according to the ALP staining kit following the manufacturer's instructions.
6. Aspirate staining solution and rinse briefly with water.
7. Take photos under a light microscope of the resulting red, insoluble, granular dye deposit that indicates sites of ALP activity.
8. Convert the images in gray scale (0–255 bit), and select a region of interest (ROI) of 41516 px.
9. Determine the mean intensity value and its standard deviation for each image.

3.4.7 RNA Extraction and RT-PCR

mRNA was isolated and analyzed after 1–2 weeks of culture on plastic or on nanofibrous and film materials, either under BM or OM. See Note 16. RNA extraction was performed following the manufacturer's instructions (RNeasy Micro Kit):

1. Pipette 350 μl of buffer RLT onto the cell-seeded scaffolds, mix well by pipetting up and down, and homogenize the lysate.
2. Add a volume of 70 % (vol/vol) ethanol to the lysate and mix well by pipetting up and down.
3. Take an RNeasy MinElute spin column fitted to a 2 ml collection tube and transfer the whole sample volume onto its membrane.
4. Centrifuge for 15 s at 8,000 × *g* at RT and discard the flow-through.
5. Add 350 μl buffer RW1 to the RNeasy MinElute spin column. Centrifuge for 15 s at 8,000 × *g* at RT and carefully remove the supernatant.
6. Add 10 μl of DNase I stock solution to 70 μl Buffer RDD and mix gently.
7. Add the DNase I Incubator mix (80 μl) directly to the RNeasy MinElute spin column and incubate for 15 min at RT.
8. Add 350 μl of buffer RW1 to the RNeasy MinElute spin column and centrifuge for 15 s at 8,000 × *g* at RT.
9. Place the RNeasy MinElute spin column in a new 2 ml collection tube; add 500 μl of buffer RPE to the spin column, centrifuge for 15 s at 8,000 × *g* at RT, and discard the flow-through.
10. Add 500 μl of 80 % (vol/vol) ethanol onto the RNeasy MinElute spin column, centrifuge for 2 min at 8,000 × *g* at RT, and discard the flow-through.

11. Put the RNeasy MinElute spin column into a new 2 ml collection tube and centrifuge it at full speed for 5 min with an open lid. The opened lid allows the ethanol to flow through the column completely.
12. Put the RNeasy MinElute spin column into a new 1.5 ml collection tube and add 14 μl of RNase-free water directly to the center of the spin column membrane. Centrifuge for 1 min at full speed at RT.
13. Assess the purity of the RNA with a spectrophotometer measuring the 260 nm/280 nm and 260 nm/230 nm optical density (OD) ratios. High RNA quality has a 260 nm/280 nm ratio of >2 and a 260 nm/230 nm ratio of >1.8. As the absorption maximum of proteins is 280 nm, a low 260/280 ratio is an indicator of protein contamination.
14. Treat all mRNA samples with deoxyribonuclease I prior to reverse transcription.
15. Perform first-strand cDNA synthesis using equal amount of RNA samples (2 μl), according to M-MLV RT instructions.
16. Perform real-time PCR reactions, using the Eppendorf Mastercycler ep Realplex2.
17. Analyze by real-time PCR gene expression levels of core binding factor alpha 1 (CBFA1/RUNX-2), collagen type 1 (Col-I), and bone sialoprotein (BSP). Use as housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
18. Use RealMasterMix SYBR Green (5prime, Hamburg, Germany) in a total volume of 13 μl for each PCR reaction performed.
19. Assess each sample at least in duplicate.

4 Notes

1. HFIP is an organic solvent with high solubilizing potential for peptides and peptide intermediates, often used as NMR solvent. Its properties, such as electric constant ($\varepsilon = 16.7$, at 25 °C) and boiling point (bp = 59 °C), fulfill the experimental requirements of optimal ES solvents ($\varepsilon > 5$, bp < 100 °C). In ES, it is commonly employed for solubilizing biopolymers as well as synthetic polymers. As alternative, chloroform, dichloromethane, 2,2,2-trifluoroethanol, and formic acid are the most used.
2. Though we focus on the osteoinductive properties of composite PCL-based nanofibers, similar effects could be hopefully achieved by using different biodegradable polymers (natural or synthetic) easily processable by ES. Since the M_w affects

both the rheological and electrical properties of the solution, i.e., viscoelasticity, surface tension, conductivity, and dielectric strength (29), it should be carefully chosen. A low M_w polymer solution generally tends to form beads rather than fibers and a high M_w solution usually gives fibers with larger average diameters (30).

3. An Al foil, placed on the collector, is commonly used as substrate for collecting nanofibers. For in vitro studies, round borosilicate coverslips are preferable as substrate since (a) glass is biologically inert and transparent in the visible range, and (b) it is often difficult to have stand-alone, uniform nanofibrous sheets after the ES process (the fibers are very sticky and easily tend to fold). The diameter of the coverslips should be chosen taking into account the plastic multiwell culture plates used for in vitro studies (e.g., a diameter of 15 mm is suitable for 24-well plates). The TEM copper grids (with or without a carbon film) are necessary for TEM investigation.
4. Two HV power supplies can be occasionally necessary, when the fiber collection is not selective towards the collector. This issue can be due to an electrostatically charged ambient in which the ES apparatus is placed. In this case an HV power supply with positive polarity should be connected to the needle of the syringe and another one with negative polarity to the collector or vice versa (see Fig. 1 for clarity).
5. The choice of the reference material for in vitro studies is important. A polymer film, using the same ES solution, is useful for studying the effect of topography on cell behavior, removing the material chemistry as variable. Here, the use of composite materials requires the realization of fully polymer materials (i.e., without NCs) for studying the effect of the material chemistry. Other reference materials should be bare glass coverslips and plastic treated cell culture plates, with or without a polylysine coating.
6. Other apparatus such as a dynamic mechanical analyzer (i.e., DMA Q800, TA Instruments) and a water contact angle analysis system (i.e., CAM-200 KSV Instruments) can be used for studying the mechanical and wettability properties of nanofibrous mats, respectively. Though these tests offer the possibility to characterize these kinds of materials more in depth, they are not present in all the nanofiber-based studies for tissue engineering applications.
7. In place of performing bone marrow isolation, isolated MSCs are commercially available from various companies.
8. The here reported polymer concentration has been optimized for obtaining almost all of the fibers with a sub-500 nm diameter and very few beads (Fig. 2). The concentration is one of the

most important ES parameters that affects the fiber diameter and the presence/absence of beads. A minimum solution concentration is required. At low solution concentration, both beads and fibers are obtained. As the solution concentration increases, uniform fibers with increased diameters are produced because of the higher viscosity resistance (29). Higher solution concentrations generally lead to fibers with higher diameters.

9. In our experimental approach, firstly the polymer concentration was optimized for obtaining a sub-500 nm fiber diameter, i.e., the same scale of the ECM architectures (21), supposing that the subsequent introduction of NCs has no detrimental effects on the diameter. We then added NCs at the highest concentration (2 % w/w) suitable for electrospinning beadless NFs (higher values lead to the formation of very large clusters of NCs along the fibers, Fig. 3). Moreover, both the introduction of a surfactant (oleic acid), playing as mediator in the interaction between the hydrophilic ceramic and the hydrophobic polymer, and a long stirring time (a week) were found essential for avoiding the bead formation and obtaining a homogeneous composite solution.
10. If a horizontal ES setup is used the use of carbon tape can be necessary. In the vertical setup, the substrates can be just placed onto the metal collector without tape. In terms of the ES process, the two setups lead to identical results.
11. When more than one cone is present, the voltage is probably too high. A high voltage leads sometimes to an increase of the local temperature and an early evaporation of the solvent at the needle edge. In contrast, when a solution drop is continuously formed at the needle tip, the flow rate should be decreased.
12. A baseline and average correction after the spectra collection are usually required for NFs, since the samples can be slightly different in terms of thickness. If a saturated signal is obtained, consider to decrease the ES time in order to have thinner sheets.
13. Commercially available media for MSCs is specifically designed with prescreened FBS to keep cells undifferentiated. MSCGM should be used when expanding cells over multiple passages to prevent spontaneous differentiation. MSCGM consists of a basal media with supplements. Supplements are stored at −20 °C and added to basal media, which is stored at 4 °C. When the supplements are added to the basal media to complete the MSCGM formulation, the media is stored at 4 °C and must be used within a month.
14. Be careful to spread cell solution all over scaffold, but not to let the solution fall off the slide. Important: It is critical that the scaffolds be dried correctly so that the cell solution remains on the scaffold layer. Once the solution falls off, it is extremely

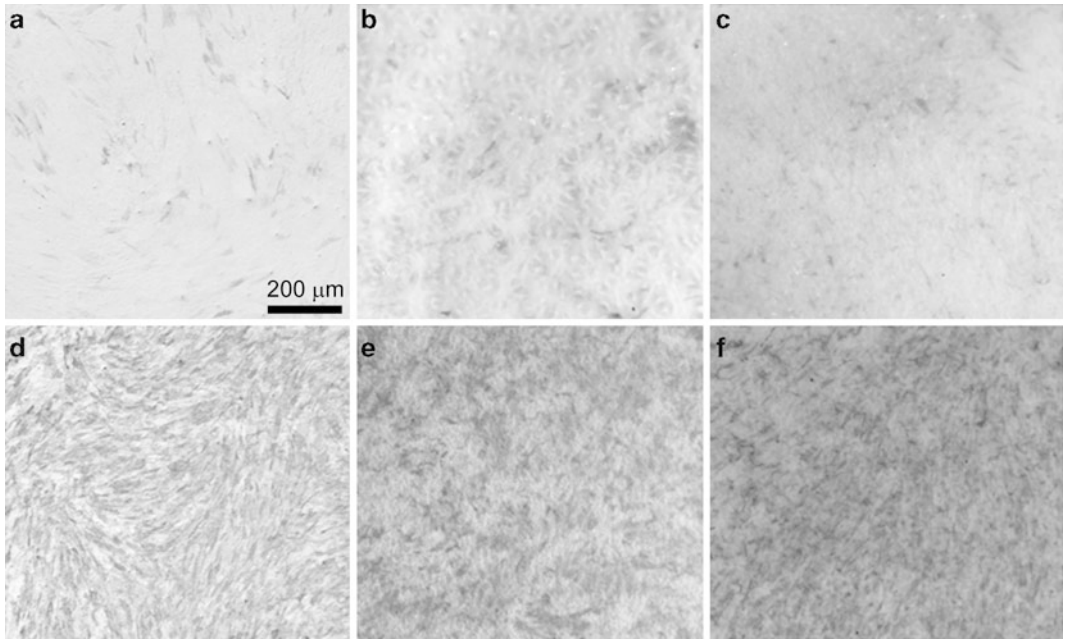

Fig. 4 ALP activity evaluated at day 7 of cellular culture onto PCL NFs (**b**, **e**), PCL-NC NF (**c**, **f**) samples, and control PCL-NC films (**a**, **d**), either in control medium (**a**–**c**) or in osteogenic medium (**d**–**f**)

difficult to put the cells back on and there may be cells that cannot be retrieved from the dish, making seeding uneven.

15. During the osteoblast differentiation, cells initially proliferate up to 7–14 days and then start to produce early differentiation markers and secrete ECM proteins. ALP is an enzyme involved in the pathway resulting in the deposition of minerals on ECM molecules. Therefore, we immuno-histochemically evaluated its activity for hMSCs cultured for 1 week onto samples either in osteogenic or in basal growth medium (Fig. 4).
16. We performed a quantitative analysis of mRNA expression levels, focusing on runt-related transcription factor 2 (Runx-2), BSP, and type I collagen (Col-1) genes, since Runx2 strongly influences the differentiation process of hMSCs into osteogenesis in the early stage, regulating bone development by G protein-coupled signaling pathway and promoting an up-regulation of ALP, osteopontin, osteocalcin, and BSP (31); Col-1 is fundamental for the development of the bone cell phenotype, being correlated to the formation of the ECM; and BSP is a highly sulfated and glycosylated phosphoprotein found in bone matrix, considered one of the late markers of mineralized tissue differentiation (32), and also associated with the capacity for bone formation by MSCs (33). However, this molecular analysis could also be extended to other types of genes, following the same procedural scheme.

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In Vitro Nanoparticle-Mediated Intracellular Delivery into Human Adipose-Derived Stem Cells

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Abstract

Adipose-derived stem cells (ASCs) are multipotent cells that are emerging as an extremely promising therapeutic agent for tissue regeneration. The ability to manipulate ASC phenotypes by the delivery of biologically active cargoes is essential to understand their role and to design novel therapeutic strategies based on the use of ASCs. Here we describe a simple and efficient protocol for the conjugation and efficient delivery of biological materials into ASCs based on the use of polystyrene nanoparticles as carrier system.

Keywords: Polystyrene nanoparticles, Adipose-derived stem cells, Bioactive cargoes, Stem cell transfection, Nanospheres

1 Introduction

Mesenchymal stem cells are a prototypical adult stem cell with capacity for self-renewal and differentiation with a broad tissue distribution. From a therapeutic perspective, and facilitated by the ease of preparation and immunologic privilege, adult stem cells are emerging as an extremely promising therapeutic agent in the field of regenerative medicine. Adipose tissue is a rich source of mesenchymal stem cells, known as adipose-derived stem cells (ASCs) that provide a clear advantage over other stem cell sources. Adipose tissue can be obtained by a minimally invasive procedure and cells can be harvested with a relatively simple method. Furthermore ASCs show a remarkable plasticity and can be directed to differentiate towards different cell types, such as cardiomyocytes, endothelial cells, and chondrocytes (1–3). The ability to manipulate ASC phenotypes by the delivery of biologically active cargoes is

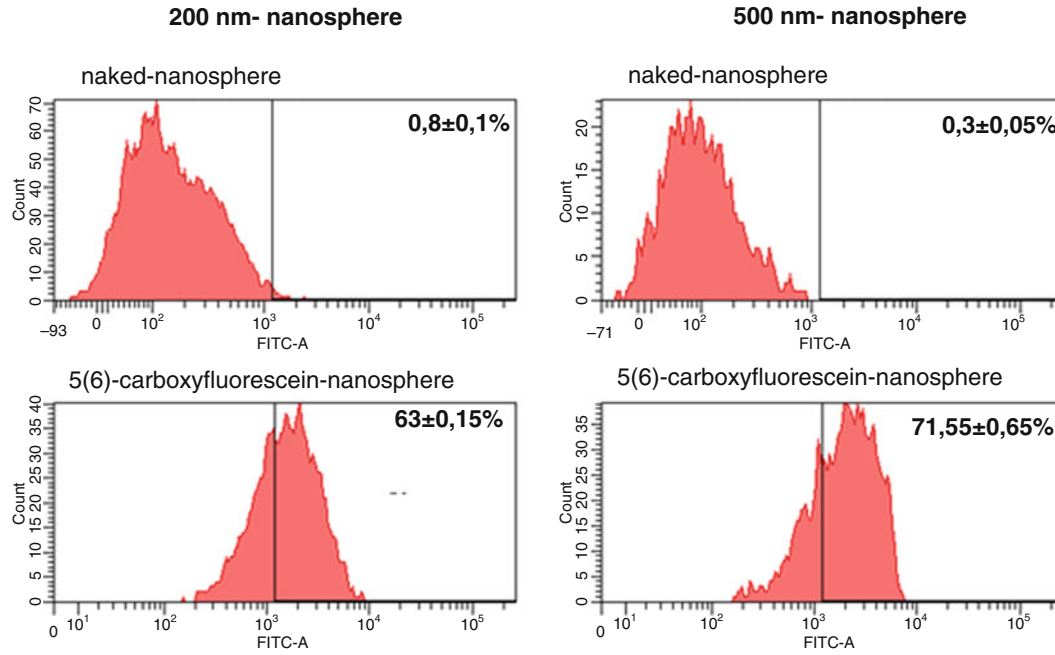

Fig. 1 Flow cytometry histograms of ASCs cultured with 200 nm (*left*) and 500 nm (*right*) 5(6)-carboxyfluorescein-nanosphere during 24 h. Naked-nanospheres were used as control. All data were obtained from three independent experiments performed in triplicate and are expressed as mean ± SE

essential to understand their role and to design novel therapeutic strategies based on the use of ASCs. Most standard transfection methods yield poor transfection efficiencies for ASCs (<1 %). Previous work shows that in-house synthesized polystyrene nanoparticles with a range of bioactive cargoes can be effectively taken up by different types of cells including mouse embryonic stem cells (mESC) and human neuronal stem cells (NSCs) without affecting ES pluripotency. We demonstrate that the nanospheres can efficiently deliver various dye molecules, proteins, and small interfering RNAs (siRNAs) into murine ES cells. We also show that their internalization does not affect ES cell pluripotency as bead-treated ES cells could be used to generate high-contribution chimeric embryos (4–9). In addition to standard ES cell cultures, these nanoparticles can be used on other embryo-derived stem cell cultures such as real-time differentiating ES cells, and TS and NS cells.

The efficacy of the polystyrene nanoparticles to deliver their cargoes into ASCs was demonstrated by flow cytometry. As can be appreciated in Fig. 1, around 60 % of the cells showed intracellular fluorescence after culturing the cells for 24 h with 200 nm nanospheres; the delivery efficacy of the nanoparticles increased up to 70 % when 500 nm nanospheres were used. Present data

demonstrate the potential utility of polystyrene nanoparticles to transfect ASCs with high efficiency.

Here we report in detail an efficient and easy protocol to transfect ASCs efficiently. This technique is based on the conjugation of the bioactive cargo or biomarker to amino-functionalized cross-linked polystyrene nanoparticles and their efficient internalization into ASCs.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Labeling of Nanoparticles with 5(6)-Carboxyfluorescein

1. Amino-functionalized cross-linked polystyrene nanoparticles were prepared following the protocol previously described (10).
2. Dimethylformamide (peptide synthesis grade DMF, 68-12-2, Scharlab).
3. 5(6)-Carboxyfluorescein (72088-94-9, Sigma Aldrich).
4. *N*,*N*′-Diisopropylcarbodiimide (DIC, 693-13-0, Sigma Aldrich).
5. Oxyma Pure (3849-21-6, NovaBiochem).

2.2 Cell Isolation and Expansion: Beadfection

1. Human subcutaneous adipose tissue (it should be obtained after informed consent from the patients and approval from the Ethics Committee of each institution).
2. Type IA collagenase (cat. no. C9891 Sigma).
3. Hank's Balanced Salt Solution (HBSS; cat. no. 14170–088, Gibco-BRL).
4. Bovine serum albumin (BSA, Fraction V, 7.5 % (w/v) solution; Gibco, cat. no. 15260–037).
5. Ammonium Chloride Solution, NH_4Cl 160 mmol/L, 500 mL (cat. no. 07850, Stem Cell).
6. Dulbecco's modified Eagle's medium (DMEM, D6429, Sigma).
7. Fetal bovine serum (FBS, cat. No. DE 14-801F, Lonza).
8. Trypsin–EDTA Solution 0.25 %, 100 mL (cat. no. T4049, Sigma).
9. 1 % Penicillin/streptomycin (P/S, P-0781, Sigma).
10. Cell culture plastic consumables (Corning).

3 Methods

3.1 Labeling of Nanoparticles with 5(6)-Carboxyfluorescein (Note 1)

1. Place 1 mL of the water dispersion of amino-functionalized polystyrene nanoparticles in a centrifuge tube.
2. Centrifuge (15,000 × *g*, 20 min, RT) and remove supernatant, 20 min for 500 nm and 30 min for 200 nm.
3. Wash them with DMF (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 10 min, RT).
4. In a separate glass vial, dissolve 5(6)-carboxyfluorescein (5 eq) in DMF and add a magnetic bar.
5. Add Oxyma (5 eq) and stir for 5 min using a magnetic stirrer.
6. Add DIC (5 eq) and stir for 10 min at room temperature.
7. Add the solution of 5(6)-carboxyfluorescein, oxyma, and DIC to the nanoparticles (Note 2).
8. Stir in a thermo-stirrer (Thermomixer comfort, Eppendorf) at 1,000 × *g* for 2 h at 60 °C.
9. Allow to cool down to room temperature.
10. Centrifuge (15,000 × *g*, 5 min, RT) and remove supernatant.
11. Wash them with DMF (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 10 min, RT).
12. Wash them with methanol (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 5 min, RT).
13. Wash them with deionized water (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 20 min, RT).
14. Store in deionized water at 4 °C.

3.2 Sterilization of Nanoparticles

1. Centrifuge (15,000 × *g*, 5 min, RT) and remove supernatant.
2. Wash them with 70 % ethanol in water (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 10 min, RT).
3. Wash them with sterile water (3 × 1 mL) by dispersing them with sonication and sedimenting them by centrifugation (15,000 × *g*, 20 min, RT).
4. Expose to UV light for 20 min.

3.3 ASC Isolation

1. Prepare fresh a solution of 1 g/L type IA collagenase and 1 % BSA in HBSS. The volume of the collagenase solution should equal the volume of lipoaspirate to be processed (usually 100–200 mL) (Notes 3 and 4).

2. Warm the PBS, collagenase solution, and medium to 37 °C.
3. Transfer 25 mL of the lipoaspirate to a sterile 50 mL plastic centrifuge tube and add 25 mL of sterile PBS.
4. Gently invert the tube five times to mix the lipoaspirate and the PBS.
5. Centrifuge at 400 × *g* at 21 °C for 7 min.
6. After centrifugation a viscous yellow fatty layer lay on top of the PBS phase. Transfer the fat layer from top, with the help of a Pasteur pipette, to a fresh 50 mL plastic centrifuge tube and add 25 mL of sterile PBS (Note 5).
7. Wash the fat layer with PBS (1:1 volume) up to four times.
8. After the fat layer has been thoroughly washed, mix it 1:1 (v:v) with the warm collagenase solution. Place it in the 37 °C shaker water bath and gently shake for 1 h.
9. Centrifuge at 400 × *g* at 21 °C for 10 min.
10. Resuspend cell pellets in 2 mL of 160 mM NH_4Cl. Incubate at 4 °C for 7 min (Note 6).
11. Centrifuge at 400 × *g* at 21 °C for 10 min.
12. Resuspend pellet in 1 mL DMEM-CM (10 % SBF–1 % P/S).
13. Plate 25,000 cells per 75 mm culture flasks with 12 mL of DMEM (10 % SBF–1 % P/S).
14. After 24 h, change the media on the cells to wash away any non-adherent cell.
15. Keep cells in the incubator at 37 °C and 5 % CO_2.

3.4 ASC Expansion

1. Feed the cells every 3 days for up to 7 days by replacing 100 % of the media in each flask.
2. Harvest cells when they are 80 % confluent by trypsinization.
3. Aspirate off expansion medium. Wash the flasks gently with 20 mL of PBS warmed to 37 °C.
4. Add 3 mL of trypsin/T75 flask, and incubate for 5 min at 37 °C (Note 6). Inactivate trypsin with a 10 mL of 10 % BFS-DMEM.
5. Centrifuge at 400 × *g* at 21 °C for 5 min to pellet cells. Discard medium.
6. Resuspend cells in 1 mL of media, count, and plate in T75 flask. Cells can be frozen at this point.
7. To freeze cells, resuspend pellet in 1 mL of 7 % DMSO-SBF; place in a cryotube, and keep in −80 °C for 1 week. After 1 week cells are transferred to a liquid nitrogen tank.

3.5 Beadfection Protocol: Cellular Uptake of nanoparticles by hACS In Vitro

1. The day before the assay, harvest cells by trypsinization and dilute them in DMEM-CM to obtain a desired cell density (Note 7) (50,000 cells/mL).
2. Plate 50,000 cells/mL (1 mL/well) in a 12-well plate (or a 6-well plate containing polylysine-coated glass coverslips for confocal microscopy studies) and incubate at 37 °C and 5 % CO_2 for 24 h.
3. Remove old media from the wells and add 1 mL of a freshly prepared DMEM-CM dispersion of fluorescently labeled nanoparticles (20–40–60 ng of nanoparticles/mL DMEM-CM) to each well and incubate at 37 °C and 5 % CO_2 for 24 or 48 h (Note 8).
4. Remove the old media and wash carefully with PBS (1 mL) three times to eliminate all extracellular nanoparticles (Note 9).
5. Add 100 μL of trypsin–EDTA solution and incubate at 37 °C and 5 % CO_2 for 5 min.
6. Add 1 mL of DMEM-CM and resuspend.
7. Place each well sample in a centrifuge tube, centrifuge at 900 × *g* for 5 min, and remove supernatant.
8. Add 400 μL of 2 % FBS in PBS and collect the cells into an FACS tube.
9. Analyze cell fluorescence by flow cytometry using a FACSAria flow cytometer (Becton Dickinson) to evaluate the internalization of labeled nanoparticles. A total of 10,000 events per sample were analyzed.

4 Notes

1. All reactions must be carried out in a suitable fume hood, with appropriate safety protection and assessments of all chemical hazards.
2. It is important to sonicate till a homogeneous suspension is obtained to ensure high reaction efficiency.
3. Cell isolations are routinely performed on the day of receipt or performed the next day, after leaving the tissue at room temperature overnight.
4. CAUTION: Procedures must be performed in accordance with Institutional Review Board policies for obtaining human tissue including informed consent by personnel certified and trained to work with blood-borne pathogens. All procedures involving the human tissue should be performed at Biosafety Level 2 with appropriate personal protection.
5. Fat phase is very dense.

6. Verify that the cells have been dislodged from the cell culture plate using a microscope.
7. Count cells.
8. It is very important to sonicate sample of nanoparticles in the media to make sure that they get homogeneously distributed to increase efficiency of uptake and ensure reproducible results.
9. CRITICAL STEP: It is important that all extracellular nanoparticles are removed.

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Screening of Nanoparticle Embryotoxicity Using Embryonic Stem Cells

Luisa Campagnolo, Ivana Fenoglio, Micol Massimiani, Andrea Magrini, and Antonio Pietroiusti

Abstract

Due to the increasing use of engineered nanoparticles in many consumer products, rapid and economic tests for evaluating possible adverse effects on human health are urgently needed. In the present chapter the use of mouse embryonic stem cells as a valuable tool to in vitro screen nanoparticle toxicity on embryonic tissues is described. This in vitro method is a modification of the embryonic stem cell test, which has been widely used to screen soluble chemical compounds for their embryotoxic potential. The test offers an alternative to animal experimentation, reducing experimental costs and ethical issues.

Keywords: Nanoparticles, ENP, Engineered nanoparticles, Nanomaterials, Embryo, In vitro test, Mouse embryonic stem cells

Electronic supplementary material

The online version of this chapter (doi:10.1007/7651_2013_11) contains supplementary material, which is available to authorized users.

1 Introduction

Over the last two decades the introduction of nanotechnology and the use of engineered nanomaterials (ENMs) have brought considerable progress in a number of industry, medicine, and basic research fields. Developing new nanomaterials with enhanced physicochemical properties has thus attracted great interest from the industrial and scientific community. In this respect, identification of challenges that nanomaterials may pose to public health and the environment has become a concern. Many data have been published demonstrating adverse effects of engineered nanoparticles (ENPs) on different cell types in vitro and in vivo (1–3). Most of the studies have focused on the respiratory and immune systems and only very recently possible adverse effects on mammalian embryonic development

have been investigated for a limited number of ENPs. Indications that certain nanoparticles might negatively interfere with embryonic development may be inferred from studies on the development of the zebrafish embryo (4, 5), a useful model to study molecular mechanisms underlying embryonic development; fish and mammal development, however, can only be compared for limited aspects. The need for information on embryotoxic effects of ENPs in mammals has stimulated studies to identify in vitro model systems to rapidly screen different ENPs, and also to identify physicochemical properties that might be modulated to limit such effect.

A few years ago, an in vitro test using mouse embryonic stem (mES) cells was developed to evaluate embryotoxicity of chemical compounds (6, 7). Such method has been validated by the European Committee for the Validation of Alternative Methods (ECVAM) and is currently used in the pharmaceutical industry. By using two stable cell lines (NIH3T3 and mES cells, representing differentiated and embryonic tissue, respectively) the embryonic stem cell test (EST) aims to identify the concentrations of a tested compound that inhibit by 50 % the proliferation of the two cell lines and the differentiation of mES cells. An algorithm eventually integrates the three values, called IC_{503T3}, IC_{50mES}, and ID_{50}, to infer an evaluation of embryotoxicity for the compound.

Only few attempts have been reported in recent years to in part apply the EST to the evaluation of silica, cobalt ferrite, and gold nanoparticles (8, 9). We have recently published data on the use of the complete EST protocol to evaluate the embryotoxic potential of single-walled carbon nanotubes (SWCNTs). An essential difference with the two above-mentioned studies was that to validate the EST as a test that might be used to reliably predict in vivo effects of ENPs, we additionally performed parallel in vivo experiments on pregnant mouse females that did demonstrate a correspondence between the in vitro and in vivo results (10).

Few issues need to be addressed when applying the EST to nanomaterials. For any tested material, determination of elemental composition (including trace elements, size, shape, solubility, surface coating, and charge) is of primary importance. The techniques used for such physicochemical characterization largely depend on the kind of nanoparticle and therefore a detailed description of methods cannot be done here. This information is however essential for the correct interpretation of the EST results.

A crucial concern is the preparation of nanoparticle suspensions. Nanoparticles need to be uniformly dispersed in the medium and the suspension needs to be stable enough to allow dosage. Adsorption of medium components, in particular proteins, at the surface of nanoparticles may alter the repulsive forces existing among particles, thus causing either particle agglomeration or stabilization of the suspension (11–13). The formation of agglomerates has to be possibly avoided. Thus, analysis of nanoparticle dispersion in culture medium and sonication prior to use are key

factors. In addition, interaction of nanoparticles with the cell medium components might alter medium composition due to (1) partial dissolution of the nanoparticles or (2) adsorption of small molecules or proteins at the surface of the nanomaterials. In this respect, since among the different components of cell culture medium, proteins are those having the largest affinity for the surface, depletion of proteins, leading to possible cell toxicity, needs to be also controlled.

Here we report a protocol for the assessment of ENP embryotoxicity using the EST, including the basic chemical determination, which is needed prior to the biological experiments.

2 Materials

All media and medium supplements are purchased from Lonza (Basel, Switzerland) and are endotoxin free. To guarantee that all tests are performed with the same batch of serum, thus reducing experimental variability and allowing comparison between results, serum batches—prescreened for supporting either stemness or differentiation—are purchased in amounts sufficient to cover the prospected experiments and stored at −80 °C until use, to guarantee that all tests are performed with the same batch of serum, reducing experimental variability and allowing comparison of the results. Leukemia-inhibiting factor (LIF) is purchased from Immunological Sciences (Rome, Italy).

All disposable cell culture supplies (plates, flasks, pipettes, tubes) are purchased from Corning Inc. (NY, USA) and have been tested for supporting proliferation of undifferentiated mES cells.

2.1 Cell Culture Equipment and Preparation of Media

1. Cell lines: mES D3 clone, representing the embryonic tissue, and the fibroblast cell line NIH3T3, representing the differentiated tissue, are purchased from the American Type Culture Collection (Manassas, VA, USA) and are stored in liquid nitrogen upon arrival.
2. Preparation of primary mouse embryonic fibroblasts (MEFs): Stereomicroscope; tissue culture hood; benchtop centrifuge; mouse embryos at day 13 or 14 of gestation; PBS; watchmaker forceps; trypsin–EDTA solution; scalpels.
3. MEF medium: For 500 ml of medium mix 435 ml DMEM with 50 ml heat-inactivated fetal calf serum (FCS), 10 ml 1 M Hepes, and 5 ml of a 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.
4. mES media:
   (a) mES pluripotency medium: For 500 ml of medium, mix 400 ml of DMEM with 75 ml of heat-inactivated FCS, 10 ml of 1 M Hepes, 5 ml of 10 mM NEAA, 1 ml of 55 mM β-mercaptoethanol, 5 ml of 200 mM L-glutamine,

and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C. Before use, transfer 10 ml of medium in a conical tube and add 10 μl of LIF (100 U/μl), to make the ES cell medium.

(b) mES proliferation medium: It is the same as the pluripotency medium without the final addition of LIF.

(c) mES differentiation medium: For 500 ml of differentiation medium, mix 424 ml DMEM with 50 ml heat-inactivated FCS, 10 ml 1 M Hepes, 5 ml 10 mM NEAA, 1 ml 55 mM β-mercaptoethanol, 5 ml 200 mM L-glutamine, and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.

5. NIH3T3 cell medium: For 500 ml mix 430 ml DMEM with 50 ml heat-inactivated FCS, 10 ml of 1 M Hepes, 5 ml of 200 mM L-glutamine, and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.
6. Gelatine solution: To make 500 ml of 0.7 % gelatine, weigh 3.5 g of cell culture-tested gelatine type A (C1890, Sigma Chemical Company, St. Louis, MO, USA) and add the powder to a clean bottle containing 500 ml of freshly made 18 MΩ distilled water; immediately autoclave and store at room temperature.

2.2 Proliferation Assays

Cell Proliferation Reagent WST-1 (Roche Diagnostics, Indianapolis, IN, USA). ELISA reader.

3 Methods

3.1 Preparation of Nanoparticle Suspensions

1. Weight 4 mg of nanoparticles and add 2 ml of a suitable solvent (stock solution) which needs to be determined depending upon the kind of material. In general, for hydrophilic nanoparticles (uncoated metal oxides or nanoparticles coated with charged or hydrophilic coatings) water or cell medium (DMEM) containing FCS (between 5 and 10 %) may be used. PBS generally decreases the stability of the suspension and therefore should be avoided. In the case of hydrophobic nanoparticles (unfunctionalized carbon-based or polymeric nanoparticles, nanoparticles coated with hydrophobic materials) medium containing FCS gives in some cases good results. Ethanol or a mixture of water/ethanol, DMSO, or surfactant like Tween 80 may also be used to improve dispersion.
2. Sonicate the stock solution for 10–30 min (bath sonicator, 40 W). Alternatively, a probe sonicator (100 W) may be used. In this case sonicate at a potency of 50 % for 1–2 min by maintaining the suspension on ice.

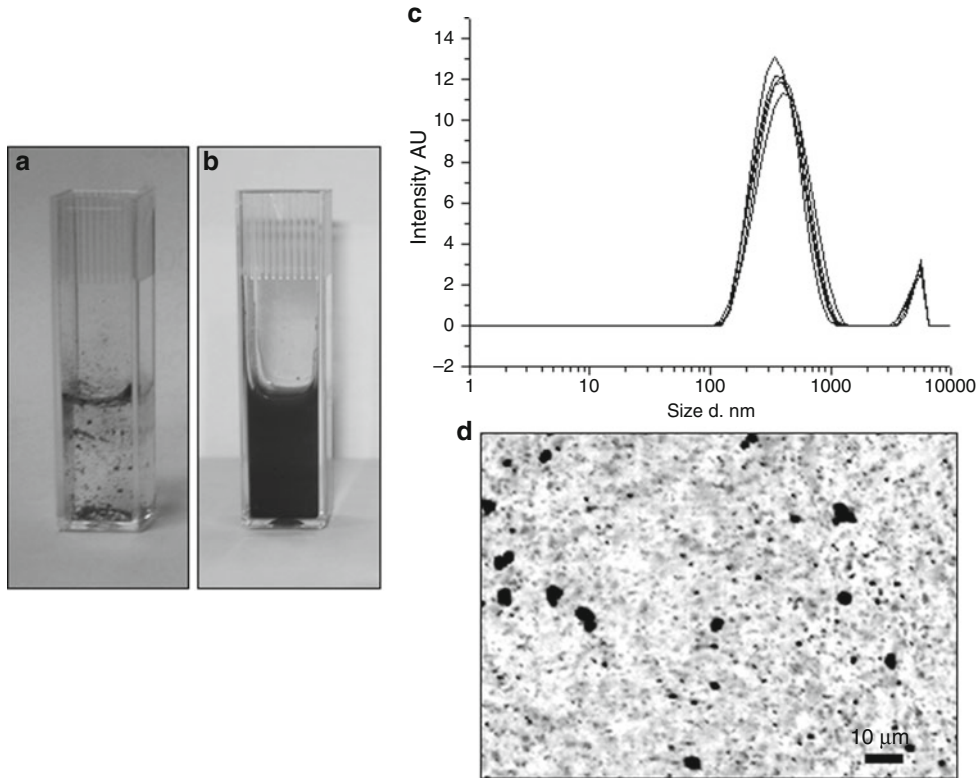

Fig. 1 Example of a nanoparticle suspension: Dispersion of carbon nanotubes. (**a**) Carbon nanotubes in PBS; (**b**) carbon nanotubes in culture medium + 10 % FCS; (**c**) measurements of agglomerate size (DLS) in the suspension shown in (**b**). The *curves* correspond to five measurements over a period of 15 min; (**d**) optical micrographs of the suspension shown in (**b**)

3. Add 10 ml of ES or NIH3T3 medium to 1, 0.5, 0.05, 0.005, and 0.0005 ml of stock solution (final concentrations 200, 100, 10, 1, and 0.1 µg/ml) and repeat the sonication. Use the prepared suspensions within few minutes.

3.2 Analysis of Nanoparticle Dispersion in Culture Media

1. Transfer 1.5 ml of the suspension in a disposable cuvette and analyze the size of aggregates (upper limit resolution of 1 µm) by dynamic light scattering (DLS) technique (see Note 1). Perform several measurements on the same suspension to evaluate its stability during the time needed for seeding the cells (Fig. 1).

3.2.1 Dissolution of the Nanoparticles

1. Incubate 2 ml of the suspension prepared as in Section 3.1 at 37 °C.
2. After 10 days, centrifugate the suspension at >13,000 × *g* to remove nanoparticles from the medium.
3. Collect the supernatant and pass it through a 100 nm acetate cellulose disposable filter.

4. Add to the collected supernatants 10 ml of a mixture 1:1 of H_2SO_4/HNO_3 and heat by using a water bath at 100 °C until the solutions become transparent.
5. Analyze the solutions diluted up to 100 ml with doubly distilled water by inductively coupled plasma atomic emission spectrometry (AE-ICP).

3.2.2 Adsorption of Proteins

1. Incubate nanoparticles in ES or NIH3T3 medium and collect supernatant as reported in Section 3.2.1.
2. Measure total protein content in the medium before and after incubation with nanoparticles using the bicinchoninic acid (BCA) assay (biuret method).

3.3 Preparation of γ-Irradiated Mouse Embryonic Fibroblasts

1. Isolate embryos, place them in a culture dish filled with PBS, and wash them. Perform all the following steps in a tissue culture hood.
2. Transfer embryos in a clean dish. Under the dissection microscope, use forceps to remove head and internal organs from embryos, leaving only the carcasses.
3. Wash carcasses twice with PBS, and place them in a clean dish with a few milliliters of 0.5 % trypsin–EDTA (enough to cover the embryos).
4. Using a scalpel, mince carcasses into small pieces; for ten embryos this step should take approximately 15 min. Add 2 ml more of trypsin–EDTA, mix with the tissue, and incubate for 30 min at 37 °C.
5. In the meanwhile prepare MEF medium.
6. Remove the dish from the incubator and add 10 ml of MEF medium. Transfer the suspension to a 50 ml conical tube and dissociate the tissue by vigorous pipetting using a 10 ml serological pipette.
7. Allow large fragments to settle down by gravity and gently transfer the supernatant to a new tube.
8. To the tube containing the fragments add 10 ml of MEF medium and pipette again. Repeat this three more times.
9. Combine all supernatants so that at the end of the procedure, a tube containing about 40 ml of cell suspension should be obtained. Plate cells in T165 flasks considering one embryo per flask.
10. After 2 days cells should reach confluence and can be frozen with DMSO (two vials from each flask). Store cells in liquid nitrogen. These vials are the stock of MEF cells and are used to prepare the γ-irradiated feeder layer.

11. To make γ-irradiated MEFs, thaw one vial of primary MEFs from liquid nitrogen, transfer the suspension to a 15 ml conical tube, and centrifuge for 10 min at 120 × *g* to pellet the cells.
12. Remove supernatant, resuspend the pellet in 6 ml MEF medium, split the volume in three T165 flasks containing 30 ml of MEF medium, and incubate.
13. When at confluence (it usually takes 3 days) harvest cells and plate them in nine T165 flasks.
14. After an additional 3–4 days (or when at confluence) harvest once more the cells and plate them in twenty-seven 150 mm maxiplates.
15. Wait for cells to reach the confluence and then trypsinize each dish with 2 ml of 0.25 % trypsin–EDTA. Combine cell suspensions from all dishes in a 50 ml tube and centrifuge for 10 min at 120 × *g*.
16. Remove supernatant and resuspend pellet in 45 ml MEF medium. Irradiate cells with 30 Gy using a γ-irradiation source.
17. Add 5 ml DMSO, split in 0.5 ml aliquots in freezing vials, and store at −80 °C. Each vial is sufficient to make a feeder layer for a T75 flask.

3.4 Culture of mES Cells

All the following procedures are carried in a cell culture hood.

1. Gelatinize a T25 flask with 3 ml of a 0.7 % gelatine solution and incubate it for 15 min at 37 °C.
2. In the meanwhile, thaw at 37 °C an aliquot of γ-irradiated mouse fibroblasts and transfer to a 15 ml conical tube containing 4.5 ml of DMEM; centrifuge at 120 × *g* for 5 min, discard the supernatant, and resuspend the pellet in 1 ml MEF medium.
3. Remove excess gelatine from the flask and add 4.5 ml MEF medium. Transfer 0.5 ml of the MEF cell suspension to the flask. Place the flask in the incubator (37 °C, 5 % CO_2) for 30 min.
4. After about 20 min, quickly thaw mES cells from liquid nitrogen; transfer the suspension to a 15 ml conical tube prefilled with 4 ml DMEM. Centrifuge for 5 min at 120 × *g*. Remove supernatant and suspend the pellet in 5 ml ES medium.
5. Remove from the incubator the T25 flask and check for the presence of adhered MEF cells under an inverted microscope. At this time most MEFs should appear as small round, dark gray cells with a central nucleus attached to the flask, and it generally takes a day for them to spread completely and assume the typical fibroblast phenotype.

6. Aspirate the medium from the flask and add the ES cell suspension. Incubate at 37 °C in an atmosphere of 5 % CO_2. ES cells are usually subcultured every 2–3 days; medium is replaced everyday.

3.5 Cell Proliferation Experiments

3.5.1 Proliferation of mES Cells

1. When mES colonies are about 80 % confluent, trypsin–EDTA the culture in order to obtain a single-cell suspension.
2. Plate the cells on a gelatinized 10 cm culture plate containing 7 ml of mES medium and place it in the incubator for 20–30 min, to remove the more adherent MEFs.
3. Collect the culture supernatant, centrifuge it at 120 × *g* for 5 min, and resuspend the pellet in 2 ml of mES proliferation medium. Carefully count cells with a hemocytometer, ignoring possible contaminating MEFs that appear much larger than mES.
4. Using mES proliferation medium, dilute the cell suspension to 10,000 mES/ml, pipette 50 µl per well of a 96-well plate that has been previously gelatinized, and place in the incubator to allow cells to adhere to the plate (see Note 2).
5. After 2 h, add to each well 150 µl of control medium or medium containing the nanoparticles under evaluation that have been previously sonicated as reported in Section 3.1. Do so following the scheme in Fig. 2: In each well at the periphery of the plate add mES proliferation medium. In columns 2, 6,

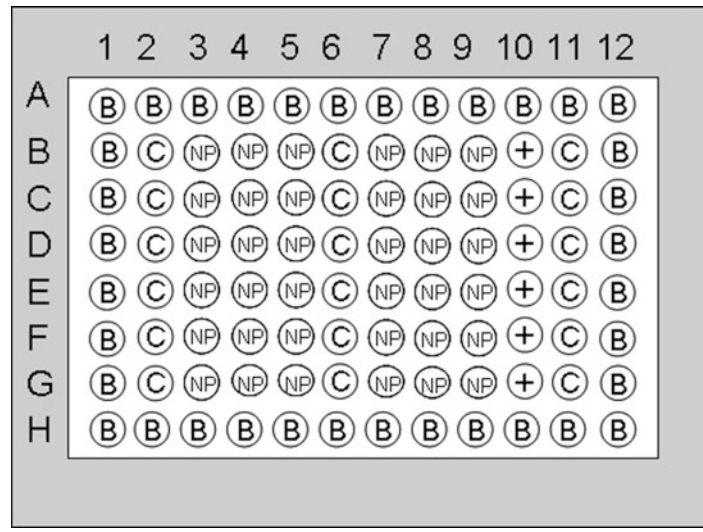

Fig. 2 Preparation of a 96-well plate for the proliferation assay. (*B*) Blank made with the culture medium; (*C*) cells grown in the presence of culture medium and the ENP suspension medium (vehicle control); (*+*) cells grown in the presence of a proven embryotoxic compound (positive control); (*NP*) cells grown in the presence of ENPs

and 11 add proliferation medium containing the nanoparticle suspension vehicle (use the amount present in the highest ENP concentration tested). In columns 3–5 and 7–9 add proliferation medium containing ENP at different concentrations. In a first pilot experiment use a range of concentrations between 0.1 and 100 μg/ml, from which the test concentrations for the following experiments can be extrapolated. In column 10 add mES proliferation medium containing a known embryotoxic compound, e.g., 0.086 μg/ml 5-Fluorouracil.

6. Following the scheme previously used, replace medium at days 3 and 7 of culture. After 10 days of culture, perform the WST-1 colorimetric assay.

3.5.2 Proliferation of NIH3T3 Cells

1. Plate 500 NIH3T3 cells suspended in 50 μl 3T3 medium in each well of a 96-well plate and let them adhere for 2 h.
2. Add the ENPs under study diluted in 150 μl of the same medium at the desired concentration. Initially test a concentration range between 1 and 200 μg/ml (see Note 3).
3. On day 6 of culture, perform the colorimetric assay, using the cell proliferation reagent WST-1, as detailed in the following section.

3.5.3 Cell Viability Tests

1. Thaw the WST-1 reagent (see Note 4). Mix in a tube 9.8 ml of culture medium and 980 μl of the WST-1 reagent.
2. Remove medium from the 96-well plate.
3. Wash wells twice with 150 μl PBS.
4. Dispense 110 μl of the diluted WST-1 solution in each well.
5. Place the plate in the incubator for 2 h.
6. Remove the plate from the incubator, shake it for 1 min on a shaker, and read the absorbance against a background control using a microplate reader (Bio Rad Microplate Reader 3550) at 450 nm, with reference wavelength at 655 nm.
7. Analyze data and present them as percentages relative to control (mean ± standard error).

3.6 mES Cell Differentiation Experiments

1. Harvest mES cells (obtained as in Section 3.5.1) and plate them on a gelatinized 10 cm plate containing 7 ml of mES medium; incubate at 37 °C for 20–30 min.
2. Collect and count cells in the supernatant, and centrifuge the suspension at 120 × *g* for 5 min. Discard supernatant and suspend the pellet in differentiation medium in order to have 37,500 cells in 20 μl.
3. Fill one 1 ml tube for every ENP concentration to be tested with 950 ml differentiation medium; add 30 μl of ENP suspension at the appropriate concentrations (test the same

concentration range used for the mES cell proliferation assays). Add 20 μl of the cell suspension and mix by pipetting, and then aliquot the obtained suspension on the lid of a 10 cm plate making forty to fifty 20 μl drops. With a gentle but firm movement invert the lid with the drops and place it to cover the bottom part of the plate containing 10 ml of deionized water. Place the plate in the incubator.

4. After 3 days prepare a number of 6 cm Petri dishes equivalent to the number of 1 ml tubes previously prepared, and fill each of them with 5 ml differentiation medium containing each of the ENP concentration under test.
5. Remove from the incubator one plate with a given concentration of ENPs, gently remove and invert the lid, and with the help of a dissection microscope, collect all the drops, which now contain small spheres of aggregated cells, the embryoid bodies (EBs). Transfer all the EBs in a 6 cm plate containing differentiation medium and the correspondent ENP concentration. Put in the incubator. Repeat the same procedure for all dishes.
6. After 2 days, prepare two 24-well plates for each 6 cm dish by putting in each well 1 ml of differentiation medium containing the correspondent concentration of ENPs. Transfer one EB per well.
7. After 5 additional days of culture, using an inverted microscope evaluate the presence of beating cell areas in each EB, reflecting the differentiation of contractile cardiomyocytes (see Video 1 in supplementary material). Identify by a dose–response curve the concentration of ENPs that inhibits by 50 % the formation of contracting EBs (ID_{50}) (see Note 5).

EST algorithm

The obtained IC_{50} and ID_{50} values are introduced in the following algorithm:

Function I: $5.92\ \lg(IC_{50 3T3}) + 3.50\ \lg(IC_{50mES}) - 5.31(IC_{50 3T3} - ID_{50}/IC_{50 3T3}) - 15.27$

Function II: $3.65\ \lg(IC_{50 3T3}) + 2.39\ \lg(IC_{50mES}) - 2.03(IC_{50 3T3} - ID_{50}/IC_{50 3T3}) - 6.85$

Function III: $-0.125\ \lg(IC_{50 3T3}) - 1.92\ \lg(IC_{50mES}) + 1.50\ (IC_{50 3T3} - ID_{50}/IC_{50 3T3}) - 2.67$

According to the standard classification applied to chemical compounds from EST data, ENPs are classified into three classes:

Class 1, non-embryotoxic, if I > II and I > III.

Class 2, weakly embryotoxic, if II > I and II > III.

Class 3, strongly embryotoxic, if III > I and III > II.

4 Notes

1. DLS measurements, although give a good estimation of nanoparticle dispersion, underestimate the amount of small aggregates.
2. Accuracy of pipetting when performing the proliferation assays is fundamental to reduce variability. In proliferation assays always prepare suspensions and solutions for at least two additional wells, to correct for pipetting errors and avoid ending up without enough solution for the last wells. We have experimented that the use of a p100 instead of a multichannel pipette gives better results. The multichannel pipette helps during the PBS washing steps.
3. Consider that 3T3 cells are generally less sensitive to factors perturbing culture conditions than mES; thus, when planning experiments with 3T3, it is advisable to test a concentration range ten times higher than that used for mES cells.
4. To evaluate cell viability and proliferation, several cell proliferation reagents can be used. As a general rule, when investigating the cytotoxic effect of nanoparticles, possible interference of the nanoparticles themselves with the colorimetric assay used needs to be assessed.
5. Results from the differentiation experiments are to be discarded if in the control plate less than 75 % of EBs acquire a contractile phenotype.

Electronic Supplementary Material

Below is the link to the electronic supplementary material.

7651_2013_MOESM1_ESM.avi
Video 1. Visualization of contracting EBs at the end of the differentiation experiments (AVI 15,590 kb)

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Peptide Nanofiber Scaffolds for Multipotent Stromal Cell Culturing

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Abstract

Self-assembled peptide nanofibers are versatile materials providing suitable platforms for regenerative medicine applications. This chapter describes the use of peptide nanofibers as extracellular matrix mimetic scaffolds for two-dimensional (2D) and three-dimensional (3D) multipotent stromal cell culture systems and procedures for in vitro experiments using these scaffolds. Preparation of 2D and 3D peptide nanofiber scaffolds and cell culturing procedures are presented as part of in vitro experiments including cell adhesion, viability, and spreading analysis. Analysis of cellular differentiation on peptide nanofiber scaffolds is described through immunocytochemistry, qRT-PCR, and other biochemical experiments towards osteogenic and chondrogenic lineage.

Keywords: Peptide nanofibers, Self-assembly, Hydrogels, Multipotent stem cells, Nanofiber networks

1 Introduction

Multipotent stromal cells (MSCs) hold great potential for regenerative medicine applications due to their ease of isolation from bone marrow and other tissues such as adipose, periosteum, synovial membrane, and synovial fluid (1). These cells are more prone to generate particular tissue types such as cartilage, bone, and adipose, and have potential to escape from immune system (2). Due to these reasons, use of MSCs is highly preferable in regenerative medicine applications.

MSC fate decision can be fine-tuned by a number of signaling molecules residing in the microenvironment of these cells. The dynamic interactions of cell–cell, cell–extracellular matrix, and cell–soluble factors that take place in stem cell niche determine the differentiation patterns of stem cells. In regenerative medicine studies, artificial scaffolds are engineered by exploiting these interactions to mimic stem cell milieu. By this way engineered scaffold system on which MSCs reside provides control over cell fate. Therefore,

mechanical, chemical, and biological signals incorporated within artificial scaffold systems must be precisely controlled in a spatio-temporal fashion (3).

Self-assembled peptide nanofibers are versatile nanostructures that can trigger and control cellular behaviors such as adhesion, proliferation, migration, and differentiation through functional epitopes. These nanofibers are usually composed of peptide molecules that have bioactive groups and β-sheet-forming peptide segments in addition to hydrophobic groups. Hydrophobic part of the peptide molecules triggers self-assembly process through hydrophobic collapse in water. Hydrogen bonding between the β-sheet-forming peptide segments induces formation of well-defined and long cylindrical fibers instead of spherical micelles. Self-assembly through charge screening can also be triggered by addition of charged amino acids in peptide sequence followed by mixing with oppositely charged peptides or electrolytes (4). Peptide nanofibers are versatile structures so that many bioactive peptide epitopes can be presented on the design in high density and this characteristic provides a variety of functional properties that can be utilized in nanotechnology and regenerative medicine applications. This characteristic is especially important for mimicking extracellular matrix components, which are highly important in regenerative medicine applications. Figure 1 illustrates the nanofiber formation through self-assembly of

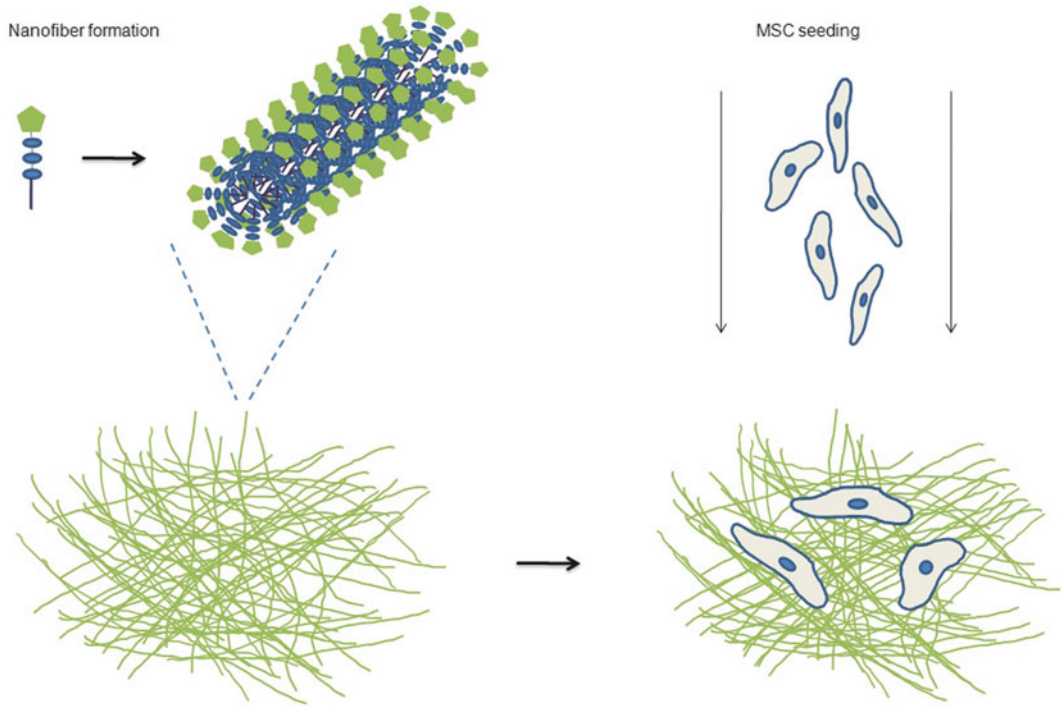

Fig. 1 Schematic representation of nanofiber formation through self-assembly and MSC seeding on the peptide nanofiber scaffolds

monomers and MSC seeding on the scaffolds. So far, researchers have used a number of bioactive epitopes on the peptide nanofiber systems to alter cellular behaviors by mimicking functional ligands found in the extracellular matrix that interact with cell surface components, such as growth factor receptors, integrins, and glycosaminoglycans. For example, cellular adhesion can be triggered through commonly used peptide ligands such as RGDS (5), REDV (6), or KRSR (7) and differentiation can be induced by IKVAV (8) epitope for neural cells and DGEA (9) for osteogenesis.

In this chapter, we describe protocols used to develop 2D and 3D self-assembled peptide nanofiber scaffolds and their use for MSC culture and differentiation in detail. Specifically, MSC culturing on 2D and 3D peptide nanofibers including analysis of cellular characteristics such as adhesion, spreading, viability, and differentiation by using immunocytochemistry, qRT-PCR, and osteogenic or chondrogenic specific biochemical assays is discussed (see Note 1).

2 Materials

Materials used in peptide synthesis, purification, and characterization were previously described (6–8).

2.1 Cell Culture

1. Pipettes and tissue culture plates (Corning).
2. 1× PBS.
3. Expansion medium: Dulbecco's Modified Eagle's Medium-Glutamax, Low (1.5 g/L) Glucose (Invitrogen) supplemented with 10% MSC qualified FBS and 1% penicillin–streptomycin (Invitrogen).
4. 0.25% Trypsin–EDTA (Invitrogen).
5. Osteogenic differentiation medium: Dulbecco's Modified Eagle's Medium-Glutamax, Low (1.5 g/L) Glucose (Invitrogen) supplemented with 10% MSC qualified FBS and 1% penicillin–streptomycin (Invitrogen), 10 mM β-glycerophosphate (Alfa Aesar), 50 μg/mL L-ascorbic acid (Alfa Aesar), and 10 nM dexamethasone (Sigma).
6. Chondrogenic differentiation medium: Dulbecco's Modified Eagle's Medium, High (4.5 g/L) Glucose (Invitrogen) supplemented with 10 ng/mL TGF-β1 (Invitrogen), 1×10^{-7} M dexamethasone (Sigma), 100 μM L-ascorbic acid (Alfa Aesar), 1% sodium pyruvate (Invitrogen), 0.5 mg/mL BSA, 10 μg/mL insulin, 6 μg/mL transferrin, and 3×10^{-8} M sodium selenite.

2.2 Adhesion, Spreading, Viability, and Proliferation

1. Adhesion medium: Dulbecco's Eagle modified medium with Low (1.5 g/L) Glucose supplemented with 4 mg/mL BSA (Invitrogen), 50 μg/mL cyclohexamide (AppliChem), and 1% penicillin–streptomycin.
2. FITC-conjugated phalloidin (Sigma).
3. 2% glutaraldehyde (Sigma) and 4% osmium tetroxide (Sigma).
4. Increasing concentrations of EtOH.
5. Calcein AM (Invitrogen) diluted to 1 μM in PBS.

2.3 ALP Activity

1. M-PER Protein Extraction Solution (Thermo)
2. Protease inhibitor cocktail
3. BCA Protein Assay Kit (Thermo)
4. NaOH (0.25 M)
5. *p*-nitrophenol (Sigma)
6. *p*-nitrophenylphosphate substrate solution (Sigma)

2.4 Alizarin Red Staining

1. Alizarin Red-S solution: Prepare 40 mM Alizarin Red-S solution by dissolving appropriate amount of Alizarin Red-S (Sigma) in ddH_2O and adjust pH to 4.2 by titrating with 1 M NH_4OH.
2. Ethanol (70%).
3. Cetylpyridinium chloride (CPC) solution: Prepare 10% (w/v) CPC solution by dissolving appropriate amount of CPC (Merck) in Na_3PO_4 buffer (10 mM, pH 7).

2.5 Papain Digestion

1. PBE buffer: First prepare 100 mL 500 mM Na_2EDTA by adding 18.6 g Na_2EDTA to 80 mL ddH_2O. Adjust pH to 8. Then prepare 100 mM sodium phosphate buffer by dissolving 6.53 g Na_2HPO_4 and 6.48 g NaH_2PO_4 in 900 mL and add 10 mL of 500 mM Na_2EDTA. Adjust pH to 6.5, and then complete volume to 1 L. Sterilize with 0.22 μm filter.
2. Papain digestion buffer: Prepare first 10 mM L-cysteine solution by dissolving 47.25 mg L-cysteine hyrdrochloride in 30 mL PBE buffer. Then transfer 20 mL of PBE–cysteine solution to a sterile tube and add papain (Sigma) to get 125 μg/mL concentration of papain solution (see Note 2).

2.6 Dimethylmethylene Blue Assay

1. 1 L DMB solution: Prepare 40 mM Glycine (Sigma), 40 mM NaCl by dissolving 2.37 g NaCl and 3.04 g glycine in 900 mL ddH_2O. Then dissolve 16 mg dimethylmethylene blue (DMMB) (Sigma) in 5 mL absolute EtOH for 16 h with a magnetic stir bar. Add dissolved DMMB to NaCl–glycine solution. Then adjust pH to 3. Complete to 1 L with ddH_2O. Then sterilize with 0.22 μm filter (see Note 3).
2. Papain digests.
3. Chondroitin sulfate salt standard solutions.

2.7 RNA Isolation and qRT-PCR

RNA Isolation:

1. Trizol Reagent (Invitrogen)
2. DNAse/RNase free water (Gibco)
3. DNAse/RNase free Eppendorf tubes
4. Temperature-controlled centrifuge
5. RNase-free solvents: Chloroform, isopropanol, and increasing concentrations of EtOH

qRT-PCR:

1. SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen)
2. PCR thermal cycler (Bio-Rad)
3. Quick spin
4. Vortex
5. RNA samples

2.8 Immunocytochemistry

1. 1× PBS
2. Fixative; 4% paraformaldehyde/PBS
3. Blocking solution: 1% BSA–PBS
4. 0.1% Triton X-100
5. Prolong Gold antifade reagent (Invitrogen)

2.9 Fixation, Dehydration, Embedding, and Sectioning of 3D Cultures

1. Fixative; 4% paraformaldehyde/PBS
2. Coverslips
3. Glass slides
4. Xylene (Sigma)
5. Graded EtOH: 70, 80, 95, 100%
6. Paraffin
7. Embedding cassettes
8. Embedding molds
9. Microtome (Leica SM 2010 R)
10. Hybridization oven

3 Methods

3.1 Peptide Synthesis, Purification, and Characterization

Peptide synthesis is performed by using a standard Fmoc-protected solid-phase peptide synthesis method. Purification and characterization steps are carried out as described previously (6–8). Alternative peptide synthesis protocols can be accessed from here (10).

1. After collection of peptide from high-performance liquid chromatography (HPLC) purification, remove the organic solvent

by using rotary evaporator. Then, freeze the peptide samples at 80°C for 4–5 h and lyophilize to obtain peptide powder.

2. Dissolve lyophilized peptide samples in ultrapure water. In order to completely dissolve peptide samples, add appropriate amount of HCl or NaOH according to the p*K*a values of peptides. Sonication might be required to prevent the small peptide aggregate formations in the solution by using ultrasonicator.
3. Carry out circular dichroism (CD) experiment to reveal the secondary structure of peptides. Dissolve peptides in ultrapure water at 10^{-5} M concentration separately. (Peptide concentrations can be prepared in the range of 1×10^{-6} and 5×10^{-4}). The details of the measurement procedure can be found here (6–8, 11). Analysis of the secondary structure of peptide nanofibers is important for determining the effects of structural properties of the nanofiber system on the cells.
4. In order to analyze mechanical characteristics of peptide nanofiber hydrogel system, perform rheology experiments by using a rheometer. According to the plate diameter and gap size, mix peptides in a way that the gap is full with peptide mixture and wait for gelation for 10 min. Then, perform the analysis by using various measurements depending on the purpose, such as frequency sweep or time-dependent measurements. Gel formation is described with the equation $G' > G''$. Further details can be found here (6–8, 11).
5. Characterize self-assembled peptide nanofibers and their network formation by using various microscopy techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described here (12).
6. After characterizing the peptide molecules and self-assembled peptide nanofibers, cell culture experiments on peptide nanofibers are performed.

3.2 Cell Culture

3.2.1 Cell Culture on 2D Scaffold

Self-assembly of two oppositely charged peptide molecules at neutral pH has been previously defined (13). Peptide gels have the ability to mimic the native extracellular matrix due to their nanofibrous structure and hydrogel characteristics. In order to construct 2D scaffolds for MSC culturing applications, negatively charged peptides and positively charged peptides are used to form gels on tissue culture plates. Figure 2 illustrates gel formation and sterilization steps before cell seeding.

1. Dissolve the peptides in water to obtain 0.1 mM peptide solution and adjust pH to 7.4 using 1 mM HCl or NaOH, depending on the sequence and the p*K*a of peptide (see Note 4).
2. Sonicate peptide solutions in ultrasound bath for 10–30 min, depending on the volume of your peptide solution.

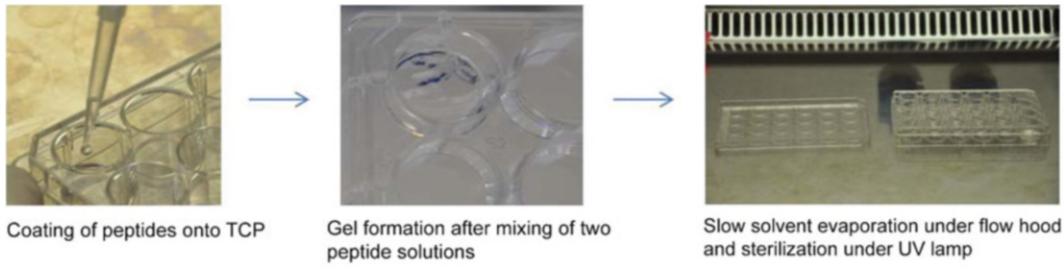

Fig. 2 Steps of 2D scaffold preparation before cell culture. *Left*: Peptide solutions are added one by one. *Middle*: After mixing gel formation immediately occurs. *Right*: Peptide gels are kept in fume hood for drying overnight and sterilized under UV afterwards

3. Add negatively and positively charged peptide solutions at a final concentration of 200 μL/cm^2. After adding the first peptide solution, drip second peptide solution onto the first one and stir for mixing.
4. Incubate at 37°C for 30 min.
5. To get 2D coating, leave gels under laminar flow hood overnight in order to evaporate the solvent.
6. Sterilize coated plates under UV lamp for 30 min to 1 h. After sterilization, keep coated plates sterile.
7. Prepare MSC suspension in expansion medium.
8. Seed 500 μL of cell suspensions onto each coating. In order to distribute cells homogeneously gently tap well plate. Place the plates in a 37°C humidified incubator with 5% CO_2. Change media every 3–4 days until the date of analysis.

3.2.2 Cell Culture on 3D Scaffold

1. Dissolve peptides in HEPES buffer to obtain 10 mM peptide solution (see Note 5).
2. Sonicate peptide solutions in ultrasound bath for 10–30 min, depending on the volume of your peptide solution.
3. Sterilize peptide solutions under UV lamp for 30 min to 1 h. After sterilization keep peptide solutions sterile.
4. Prepare MSC suspension at 2 × 10^4 cells/μL concentration in differentiation medium.
5. Place coverslips into each well of 24-well plate.
6. Add the first peptide solution onto coverslip.
7. Add 10–20 μL of cell solution onto first peptide solution by mixing slightly. Be careful not to disturb the convex shape of peptide solution drop while adding cell suspension.
8. Then add the second peptide solution onto the mixture of cell and first peptide solution. Be careful not to disturb the convex shape of the cell–first peptide mixture while adding the second peptide solution. This method is called the sandwich method

and provides a powerful tool, especially if you would like to analyze the migration or spreading patterns of cells.
Alternatively, you can first mix the first peptide solution with cell suspension in an Eppendorf tube prior to placing the mixture in wells. Then place the mixture onto coverslip and add the second peptide solution onto that. Be careful not to disturb the convex shape of mixture. This method results in a more homogenous cell–scaffold mixture.

9. Incubate gel at 37°C for 30 min.
10. Add 750 μL of culture medium onto gel carefully. Change culture medium every other day.
11. The cells should be cultured for at least 3 weeks.

3.2.3 Fixation, Dehydration, Embedding, and Sectioning of 3D Cultures

1. At determined time points remove the medium from the 3D cultures and wash with 1× PBS, taking care not to disturb constructs.
2. Fix 3D constructs in 4% paraformaldehyde for 24–36 h in the same culture plates.
3. Then first aspirate out paraformaldehyde, dehydrate samples in graded EtOH solutions, and clear them in xylene as follows:
   (a) 70% EtOH—two changes—1 h each
   (b) 80% EtOH—two changes—1 h each
   (c) 95% EtOH—two changes—1 h each
   (d) 100% EtOH—two changes—1 h each
   (e) Xylene—two changes—1 h each
4. Place embedding cassettes into molds and label them.
5. Place 3D constructs onto embedding cassettes and pour warm paraffin gently. Use forceps to hold the glass coverslips that 3D constructs are placed on while transferring them (see Note 6).
6. Cool paraffin blocks at RT for 30 min to 1 h, and then trim and place blocks to microtome.
7. Cut 5 μm sections of paraffin-embedded constructs using microtome. Transfer paraffin ribbon to water bath at 40°C for 2–3 s. Take sections on glass slides.
8. Leave slides at RT to dry and then bake them in 45–50°C oven overnight.
9. Deparaffinize and rehydrate sections in graded EtOH solutions as follows:
   (a) Xylene—2 changes—10 min each
   (b) 100% EtOH—two changes—3 min each
   (c) 95% EtOH—1 min
   (d) 80% EtOH—1 min
10. Then rinse in distilled water.

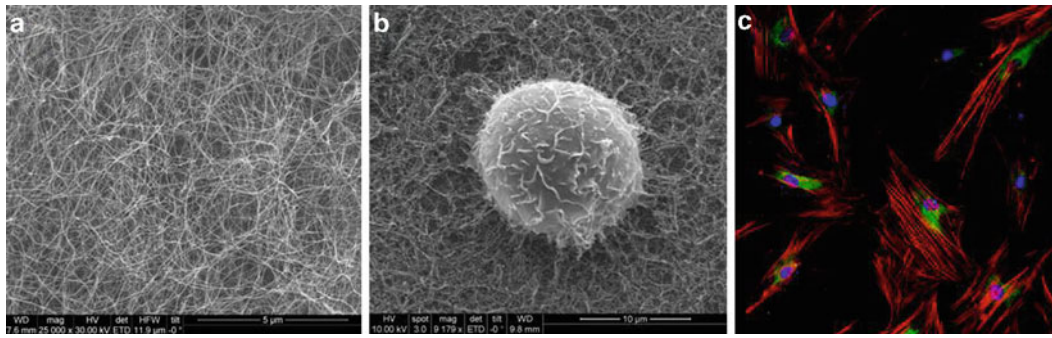

Fig. 3 ECM-mimetic peptide nanonetwork provides mechanical support to the MSCs in addition to bioactive cues presented by the nanofibers. (**a**) Peptide nanofiber scaffold imaged by SEM. (**b**) After 2-h seeding of cells, cells start to adhere on scaffold and extend their protrusions. (**c**) After 24 h, cells spread and gain their native morphology (*Red* : actin filaments; *green*: CD44, a stem cell marker; *blue* : cell nuclei)

3.3 Biological Characterizations

3.3.1 Cell Adhesion Tests

1. Prior to the experiment, replace the medium of stem cells in the flask with serum-free DMEM supplemented with 4 mg/mL BSA and 50 μg/mL cyclohexamide for 1 h at standard cell culture conditions (37°C humidified chamber with 5% CO_2).
2. After trypsinization and resuspension of cells in serum-free medium, count the cell number with hemocytometer.
3. Seed cells on peptide nanofiber-coated surfaces at 5×10^4 cells/cm^2 density.
4. After 1-h incubation remove medium containing unbound cells and wash the wells twice with PBS.
5. Add 200 μL of 2 μM calcein AM in PBS per well and incubate cells for 30 min at standard culture conditions.
6. After incubation, take images of stained cells from at least five random points per well by using fluorescence microscope.
7. Count adhered cells using Image J and normalize data to polystyrene tissue culture plate surface.

3.3.2 Spreading

One of the early cellular responses upon seeding on a material is cell spreading. In order to monitor spreading characteristics of MSCs after seeding on scaffold systems, cells are stained with TRITC-conjugated phalloidin dye that binds to F-actin stoichiometrically. Moreover, spreading characteristics of cells are also analyzed via SEM imaging. Figure 3 illustrates the adhesion and spreading of cells that are cultured on peptide nanofiber scaffolds.

1. For phalloidin staining, remove medium from cultures 3 and 48 h after seeding cells, and wash wells with 1× PBS.
2. Fix cells in 4% paraformaldehyde/PBS for 10 min at room temperature, and then wash extensively with 1× PBS.

3. In order to permeabilize cells, treat cells with 0.1% Triton X-100 in PBS, and wash again with PBS.
4. Stain cells with 50 μg/mL phalloidin solution/PBS for 20 min and wash with PBS extensively.
5. Mount cover glasses with Prolong Gold Antifade Reagent (Invitrogen), seal with nail polish, and then analyze.
6. For SEM imaging, after 3 and 48 h of incubation, remove the medium from cultures and wash cells with 1× PBS.
7. Fix attached cells with 2% glutaraldehyde/PBS solution for 2 h and wash with 1× PBS briefly.
8. Then treat fixed cells with 4% osmium tetroxide for 30 min and wash briefly with 1× PBS.
9. Dehydrate cells in graded EtOH solutions starting with 20% and continuing up to absolute EtOH for 10 min at each step.
10. Dry samples using critical point dryer.
11. Coat samples with 6 nm Au/Pd using a sputter coater.
12. Analyze samples under scanning electron microscope and take images by using an ETD detector at high vacuum mode at 10 keV beam energy.

3.3.3 Viability

Viability of cells on scaffolds is determined using calcein AM staining. Calcein is a membrane-permeable dye. When it enters the cells, intracellular esterases cleave the acetoxymethyl (AM) ester group resulting in the membrane-impermeable calcein fluorescent dye. Dead cells cannot retain calcein dye due to their impaired membrane integrity.

1. On days 1, 2, and 3, remove medium from the cultures and wash wells with PBS in order to remove dead cells.
2. Add 200 μL of 2 μM calcein AM per well and incubate cells for 30 min at standard culture conditions.
3. After incubation, take images of viable cells from at least five random points per well by using fluorescence microscope.
4. Count viable cells using Image J and normalize data to polystyrene tissue culture plate surface.

3.4 Differentiation Studies

3.4.1 Osteogenic Differentiation

Seed MSCs in growth medium (DMEM-Glutamax/10% FBS/1% penicillin) to the peptide nanofiber-coated wells at 10^4 cells/cm^2 ratio. After reaching confluency (1–2 days), exchange growth medium with osteogenic medium as described in Section 2.

ALP Activity

Alkaline phosphatase (ALP) is an enzyme that hydrolyzes inorganic pyrophosphate to provide inorganic phosphate source and its activity is used as an early marker of osteogenic differentiation.

1. At predetermined time intervals (3, 7, 10, 14, 21, and 28 days) remove the osteogenic medium from wells.
2. Wash with 1× PBS once.
3. Add 100 μL of protein extraction solution to each well and incubate on shaker for 30 min at room temperature.
4. Collect the protein from each well and transfer them into chilled 1.5 mL sterile Eppendorf tubes. Pipetting and scratching the surfaces could be beneficial to gather proteins efficiently.
5. Centrifuge the tubes at 14,000 × *g* for 10 min at 4°C.
6. Transfer the supernatants (including proteins) from each tube into clean chilled tubes. Take care not to take pellet, which is composed of cellular debris.
7. Perform protein assay in 96-well plates in order to measure protein concentration of each sample by using protein assay kit.
8. Prepare *p*-nitrophenol standards by serial dilution with 0.25 M NaOH. Use 0.25 M NaOH alone for blank.
9. Add 50 μL of protein solution for each sample to the wells of 96-well plate. Bring the total volume up to 200 μL by adding 150 μL *p*-nitrophenylphosphate substrate solution.
10. After incubating for 30 min on shaker at RT, measure absorbance at 405 nm by using microplate reader.

Alizarin Red-S Staining

Alizarin Red-S staining is used to determine calcium deposition and mineralization of cells through binding of Alizarin Red-S to calcium ions.

1. At predetermined time points (7, 14, 21, and 28 days) discard osteogenic medium from the wells.
2. Wash the cells with 1× PBS once.
3. Add 200 μL of ice-cold ethanol (70%) to each well and incubate them for 1 h at room temperature.
4. Wash with ddH_2O twice (5–10 min).
5. Add 200 μL of Alizarin Red-S solution on top of the cells and incubate them on shaker for 30 min at room temperature.
6. Wash with ddH_2O four to five times to get rid of nonspecific Alizarin Red-S binding.
7. Add enough PBS to cover wells and use optical microscope for imaging.
8. Discard PBS and add 200 μL CPC solution to extract Alizarin Red-S/Ca^{2+} complex from the surface by incubating on shaker for 30 min.
9. Measure absorbance at 562 nm by using microplate reader.

RNA Isolation and qRT-PCR

All tubes and tips should be RNase free and RNA isolation is performed under Class I airflow hood.

RNA Isolation

1. At least 1.5×10^5 cells are required for sufficient RNA isolation per experimental group.
2. Remove the medium from the wells and wash briefly with PBS.
3. Add 500 μL Trizol reagent on cells, mix extensively, and then transfer cell lysates into 2 mL RNase-free Eppendorf tubes. At this step, cell lysates could be stored in a −80°C freezer.
4. Add 300 μL of chloroform onto cell lysates, shake tubes vigorously to dissolve lipids, and leave at RT for 2–3 min.
5. Centrifuge samples at 15,000 rpm for 15 min at 4°C. Transfer 600–700 μL of upper clear phase into a clean tube. Be careful not to disturb bottom layer; otherwise RNA will be contaminated by DNA and phenol extracts.
6. Add equal volume of isopropanol to the clear solution and mix. Incubate for 10 min at RT. In order to increase RNA yield you can incubate samples at −20°C.
7. Centrifuge samples in order to precipitate RNA at 15,000 rpm for 12 min at 4°C.
8. After centrifugation discard supernatant. Add 1 mL of 70% EtOH. Be careful not to dissolve the pellet; just detach it from the tube wall.
9. Centrifuge samples at 8,000 rpm for 8 min at 4°C.
10. Discard all supernatant and air-dry the pellet until there is no EtOH left. Add 30 μL DNase/RNase-free water and dissolve the pellet.
11. Assess yield and purity of RNA using NanoDrop.

qRT-PCR

Osteogenic and chondrogenic differentiation are indexed by analysis of bone- or cartilage-specific gene expression, respectively. For gene expression analysis, comparative Ct method with primer efficiency correction is used.

1. The cDNA synthesis from RNA and qRT-PCR reactions are performed using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit according to manufacturer's instructions.
2. Reaction conditions are as follows: a cDNA synthesis step at 55°C for 5 min and 95°C for 5 min and 40 cycles of 95°C for 15 s, 60°C for 30 s, and 40°C for 1 min, followed by a melting curve to confirm product specificity.
3. Before assessment of target genes, determine reaction efficiencies of each primer set. Prepare fivefold serial dilution of total RNA and generate a standard curve with slope that defines primer efficiency.

4. The efficiency of the reaction can be calculated by the following equation: Eff $= 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be 90–110%.
5. After determining primer efficiencies, perform gene expression analysis using different primers corresponding to different target genes.
6. Normalize resulting gene expression data to the expression level of a housekeeping gene.

Immunofluorescence

1. At predetermined time intervals (1, 14, and 28 days) discard the differentiation medium from the wells.
2. In the fume hood, fix the cells with 4% paraformaldehyde in PBS for 15 min.
3. Rinse three times with PBS.
4. Permeabilize cells with 0.1% Triton X-100 in PBS for 10–12 min.
5. Rinse three times with PBS.
6. Block the cells with 1% BSA solution (0.05% Tween) for 2 h at room temperature.
7. Rinse three times with PBS.
8. Dilute primary antibodies in an appropriate concentration in blocking solution (see Note 7).
9. Add primary antibody in blocking solution at 150 μL/well concentration. Incubate for 2 h at room temperature or overnight at 4°C.
10. Rinse three times with PBS.
11. Add secondary antibody in blocking solution at 150 μL/well concentration. Incubate for 1 h at room temperature. Keep the samples away from light.
12. Rinse three times with PBS.
13. To stain nucleus, add To-PRO in PBS (1:1,000 dilution) at 150 μL/well concentration and incubate for 15 min.
14. Rinse three times with PBS.
15. Mount on slides by using mounting media.

3.4.2 Chondrogenic Differentiation

In the presence of TGFβ1 and small molecules such as insulin, dexamethasone MSCs undergo chondrogenic differentiation gaining chondrogenic morphology and producing cartilage-specific extracellular matrix molecules.

For this purpose, seed MSCs in growth medium (DMEM-Glutamax/10% FBS/1% penicillin) to the peptide nanofiber-coated wells at 5×10^4 cells/cm^2 density. After 24 h of incubation,

exchange the growth medium with chondrogenic medium as described in Section 2.

Safranin-O Staining

Safranin-O staining is used to show spatial organization of sulfated glycosaminoglycans. It is a cationic dye that binds to tissue glycosaminoglycans. The intensity of staining is proportional to the amount of glycosaminoglycans.

1. Remove the medium and wash cells with pre-warmed PBS.
2. Fix the cells with 4% PFA for 15 min followed by washing three times with PBS at room temperature.
3. In order to eliminate nonspecific binding, treat cells on scaffolds with 2% BSA/PBS for 30 min.
4. Treat cells with 0.1% (w/v) Safranin-O in 0.1% (v/v) in acetic acid for 5 min at room temperature.
5. Wash cells with 0.1% acetic acid extensively.

Glycosaminoglycan Assay

DMMB assay is one of the accepted protocols for rapid quantification of sulfated glycosaminoglycans in tissue.

1. Remove the medium and wash cells with pre-warmed PBS.
2. Add 500 μL papain solution on cells and wait for 5 min at RT.
3. Then collect cells after extensive pipetting into 1.5 mL Eppendorf tubes. Seal caps of tubes with parafilm.
4. Place the tubes on a hot plate at 65°C for 16–18 h. The digests are used for both sulfated glycosaminoglycan quantitation by DMMB assay and DNA quantitation. Total DNA content will be used to normalize sulfated glycosaminoglycan content.
5. Quick spin Eppendorf tubes after 16–18 h. You can store papain digests in a −20°C freezer. In order to increase the total DNA extracted from tissue, apply repeated freeze–thaw cycle.
6. Determine total DNA per experimental group by using Qubit dsDNA-HS quantitation kit according to manufacturer's instructions.
7. For DMMB assay, prepare serial dilutions of chondroitin sulfate standards in papain to generate a standard curve (Table 1).
8. Then aliquot 50 μL of sample, controls, and standards in a clear bottom 96-well plate.
9. Add 100 μL of DMMB dye solution on each well using a multichannel pipette.
10. Read optical density of samples at 590 and 530 nm values. Subtract the absorbance values of cell-free control groups from the absorbance values of experimental groups.

Table 1
Volumes for chondroitin sulfate standard curve

Concentration of CS (μg mL^{-1})	Volume of 1 mg mL^{-1} CS (μL)	Volume of papain (μL)
0	0	1,000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975
30	30	970
35	35	965

4 Notes

1. In our laboratory, we utilize MSCs from different origins like human, rat, and mouse. We have observed that using stem cells between passage 4 and 10 gives the most reliable and reproducible results.
2. Papain enzyme and cysteine are highly unstable. For this reason prepare Cys/PBE buffer and papain solution fresh and sterile.
3. DMMB dye solution is stable for 3 months. Check optical density and discard if it decreases appreciably.
4. Mechanical properties and ligand density of peptide gels can be tuned by their concentration. For this reason, molarity and volume of peptide solutions can be changed depending on the purpose of the study.
5. Alternatively, PBS and HEPES buffer can also be used to dissolve peptides. Depending on the sequence of each peptide and p*K*a, adjust pH to 7.4 by using 1 M NaOH or 1 M HCl.
6. Because xylene dissolves plastics, transfer 3D constructs from TCP to glass dishes before xylene changes.
7. Carry out the immunocytochemistry protocol without adding primary antibody to detect nonspecific binding of secondary antibodies.

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Patterned Polymeric Surfaces to Study the Influence of Nanotopography on the Growth and Differentiation of Mesenchymal Stem Cells

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Abstract

The implementation of micro- and nanotechnologies to biomaterials constitutes a unique platform to improve our understanding on microenvironmental regulation of stem cell functions. In the recent years, various methods have been developed for the fabrication of micro- and nanopatterned polymeric culture substrates, and many of these novel surfaces are opening possibilities for new applications. Here, we provide procedures for creating nanoscale topographic features on films of poly(lactic acid), a biodegradable polymer frequently used for the fabrication of tissue engineering scaffolds. In addition, we provide methods to assess the growth and differentiation of mesenchymal stem cells cultured on the substrates.

Keywords: Substrate topography, Biodegradable polymers, Poly(lactic acid), Cell growth, Cell differentiation, Mesenchymal stem cells, Adipose-derived stem cells

1 Introduction

In the fields of tissue engineering and regenerative medicine there is a significant interest in understanding the mechanisms through which the biomaterial surface controls behavior of stem cells. In particular, approaches that allow the fabrication of stem cell microenvironments with controlled nanotopography are highly relevant to investigate how topography at the nanoscale affects primary stem cell processes such as growth, migration, and differentiation (1–3).

Of the different types of materials available for the fabrication of stem cell microenvironments, polymeric biodegradable materials such as those prepared from lactic and glycolic acid appear to be particularly attractive (4–6). One of the main advantages of using these polymers is the ability to easily tailor the chemical composition, porosity, and other physical properties during fabrication to effectively modulate the cell responses.

Previously, electron beam (e-beam) technology has successfully been used to pattern these bioresorbable polymers (7, 8). However, this technique suffers from a few drawbacks such as high cost, low throughput, and use of potentially hazardous chemicals. In order to overcome these problems, various alternative methodologies have emerged during recent years, as for instance those relying on self-templating. Although the pattern regularity cannot be controlled with high precision, these methods allow fabrication of large-scale surfaces, in a wide range of pore sizes, and using various polymeric materials (9).

One of these self-templating approaches relies on the condensation of water vapor into the nonpolar solvent during its evaporation from a surface. The condensed water forms droplets in the drying cast film and pushes the polymer aside, forming circular indentations around the droplets (10). Such an arrangement of densely packed water droplets on the surfaces leads to a hexagonal patterned polymer resembling the structure of a honeycomb lattice. To aid this water droplet formation and their stabilization in the drying cast polymer film, a surfactant like dioleoylphosphatidylethanolamine (DOPE) could be used, which forms reverse micelles around the water droplets, stabilizing the single droplets and at the same time forming the interface between the water droplet and the polymer wall (11). After evaporation of the solvent, the water also evaporates leaving a dry surface patterned by the size of the water droplets. The surfactant remains on the polymer walls of the honeycomb pattern and can be removed by simply washing the surface with a polar solvent such as ethanol. When the films are fabricated under conditions of high relative humidity (80–90 %), the patterns display typical pore diameters in the order of 1–10 μm (11, 12). However, as we have recently shown, under conditions of low relative humidity (30 %) pore diameter is significantly reduced (100–300 nm) (13). This scale range is particularly relevant for an effective modulation of stem cell functions, in view that nanoscale topography plays a fundamental role in controlling primary cell processes through modulation of integrin clustering in focal adhesion formation (14–16).

Here, we describe the use of the water-droplet templating method to fabricate poly(lactic acid) (PLA) films suitable for stem cell culture. In addition, we describe methods to assess the interaction of mesenchymal stem cells with the surfaces by means of immunofluorescence staining and cell growth analysis. Furthermore, we provide methods to investigate the substrate-induced cell differentiation at the transcriptional level using real-time RT-PCR. The aim here is to employ these surfaces as the only cue for stem cell specification towards any particular lineage. The use of other signals of chemical, biochemical, mechanical, or other physical nature may also be used but is not contemplated in the present work.

2 Materials

2.1 Fabrication of the Patterned Substrates

1. PLA, MW = 60,000 (Sigma-Aldrich).
2. Anhydrous chloroform (Sigma-Aldrich).
3. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), >99 % (Avanti Polar Lipids).
4. Ethanol 70 %.
5. Clean glass containers/tubes and microscopy slides (26 × 76 mm).

2.2 Cell Culture

1. Adipose-derived stem cells, which can be either isolated according to established protocols (17, 18) or purchased from commercial sources, as for instance ATCC cat. nr. PCS-500-011.
2. Dulbecco's phosphate-buffered saline (PBS), without calcium and magnesium (Gibco, Life Technologies).
3. Trypsin/ethylene diamine tetraacetic acid (EDTA) mixture. Trypsin (2.5 %, without Phenol red, Life Technologies) is diluted ten times in PBS and sterile filtered. EDTA (anhydrous crystalline, Sigma-Aldrich) is dissolved in PBS at a concentration of 0.02 % and sterile filtered. The mixture is prepared by mixing the trypsin and EDTA solutions 1:1, giving a 0.125 % trypsin/0.01 % EDTA blend.
4. Growth medium, consisting of Minimum Essential Medium alpha (α-MEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml) (all components from Life Technologies) (see Note 1).
5. Trypan blue solution (0.4 %), sterile filtered (Sigma-Aldrich).
6. Tissue culture flasks (Greiner Bio-One).
7. Sterile serological pipettes (2, 5, 10, and 25 ml, Corning) and pipettor.
8. Micropipettes and sterile tips (Eppendorf).
9. Tissue culture dishes (100-mm, BD Falcon).
10. Hemocytometer, counting chamber, and lab counter.
11. Class II laminar flow bench, CO_2 incubator, centrifuge, and inverted phase contrast microscope.

2.3 Cell Proliferation Assay

1. Alamar Blue®, cell viability reagent (Life Technologies).
2. Standard 96-well microtiter plates, white.
3. Fluorescence plate reader. The instrument has to be capable to provide a fluorescence excitation wavelength of 540–570 nm and read fluorescence emission at 580–610 nm.

2.4 Immunofluorescence Assay

1. Formaldehyde (formalin solution, 10 % neutral buffered).
2. Triton X-100 in PBS solution (T-PBS): 0.1 % (w/v) of Triton X-100 in PBS.
3. Bovine serum albumin (BSA) in PBS solution (B-PBS): 1 % (w/v) of BSA in PBS.
4. Primary antibody solution: Rabbit anti-vinculin antibody (V4139, Sigma-Aldrich) diluted 1:200 in a solution of 2 % FBS in PBS.
5. Secondary antibody solution: FITC goat anti-rabbit IgG (F-2765, Life Technologies) diluted 1:150 in a solution of 2 % FBS in PBS.
6. Bodipy 558/568 Phalloidin (Molecular Probes/Life Technologies).
7. Hoechst 33342 (Molecular Probes/Life Technologies).

2.5 Real-Time RT-PCR

1. Aurum total RNA mini kit (Bio-Rad).
2. iScript cDNA synthesis kit (Bio-Rad).
3. SYBR green Supermix (Bio-Rad).
4. PCR primers for tissue-specific markers: Endothelial (VWF and VEGF2), adipogenic (PPARG2 and FABP4), osteogenic (RUNX2 and ONN), chondrogenic (COL2A2 and SOX9), cardiomyogenic (MEF2C and GATA4), skeletal myogenic (MYOD1 and MYF5), and neurogenic (NES and NGFR). Reference genes are YWHAZ, GAPDH, and PPIA (19). The primer sequences are listed below.
   (a) VWF forward: 5′-CGGCTTGCACCATTCAGC-3′; VWF reverse: 5′-GATGAGACGCTCCAGGATGG-3′.
   (b) VEGFR2 forward: 5′-CAGGATGGCAAAGACTACA TTG′; VEGFR2 reverse: 5′-GAGGATTCTG GACTCTCTCTGCC-3′.
   (c) FABP4 forward: 5′-ATGGGATGGAAAATCAACCA- 3′; FABP4 reverse: 5′-GTGGAAGTGACGCCTTT CAT-3′.
   (d) PPARG2 forward: 5′-CCACAGGCCGAGAAGGA-GAAGC-3′; PPARG2 reverse: 5′-GCCAGGGCCCG-GAGGAGGTCAG-3′.
   (e) RUNX2 forward: 5′-GGCAGCACGCTATTAAATCC 3′; RUNX2 reverse: 5′-GTCGCCAAACAGATT CATCC-3′.
   (f) ONN forward: 5′-GGCCTGGATCTTCTTTCTCC-3′; ONN reverse: 5′-CCTCTGCCACAGTTTCTTCC-3′.
   (g) COL2A1 forward: 5′-GGCAATAGCAGGTTCACG TACA-3′; COL2A1 reverse: 5′-CGATAACAGTCTTG CCCCACTT-3′.

(h) SOX9 forward: 5′-CACACAGCTCACTCGACCTTG-3′; SOX9 reverse: 5′-TTCGGTTATTTTTAGGATCATCTCG-3′.

(i) MEF2C forward: 5′-CCCTGCCTTCTACTCAAAGC-3′; MEF2C reverse: 5′-CGTGTGTTGTGGGTATCTCG-3′.

(j) GATA4 forward: 5′-GCCTGGCCTGTCATCTCACT-3′; GATA4 reverse: 5′-ACATCGCACTGACTGAGAACG-3′.

(k) MYF5 forward: 5′-TGATTGAGGGTAGCTTGTTGC-3′; MYF5 reverse: 5′-CACGAGAGACATTTTGATGAGC-3′.

(l) MYOD1 forward: 5′-AACGGACGACTTCTATGACG-3′; MYOD1 reverse: 5′-AGTGCTCTTCGGGTTTCAGG-3′.

(m) NGFR forward: 5′-CCCTGTCTATTGCTCCATCC-3′; NGFR reverse: 5′-CCTTGCTTGTTCTGCTTGC-3′.

(n) NES forward: 5′-TAAGGTGAAAAGGGGTGTGG-3′; NES reverse: 5′-CCTACAGCCTCCATTCTTGG-3′.

(o) PPIA forward: 5′-TCCTGGCATCTTGTCCATG-3′; PPIA reverse: 5′-CCATCCAACCACTCAGTCTTG-3′.

(p) YWHAZ forward: 5′-ACTTTTGGTACATTGTGGCTTCAA-3′; YWHAZ reverse: 5′-CCGCCAGGACAAACCAGTAT-3′.

(q) GADPH forward: 5′-GAATCTCCCCTCCTCACAGTTG-3′; GADPH reverse: 5′-GGCCCCTCCCCTCTTCA-3′.

5. Nuclease-free pipette tips and tubes.
6. 96-Well optically clear microwell plates and optical plate cover (Bio-Rad).
7. Spectrophotometer with capability of measuring microvolumes of sample, such as the NanoDrop (Thermo Scientific).
8. qPCR instrument, such as the MyCycler (Bio-Rad).

3 Methods

3.1 Fabrication of the Culture Substrates

An overview of the fabrication methodology is depicted in Fig. 1.

1. In a clean glass container, prepare a mix of PLA and DOPE (99:1, w:w) in chloroform, at a concentration of 10 mg/ml. Let the mixture dissolve overnight (see Note 2).
2. Cast a small volume (500 μl) of the PLA/DOPE mixture on the glass microscopy slide (see Note 3).
3. Allow the cast film to air-dry at room conditions (see Note 4).
4. Rinse the slide in 70 % ethanol for 5 min at room temperature, to remove the DOPE residuals.

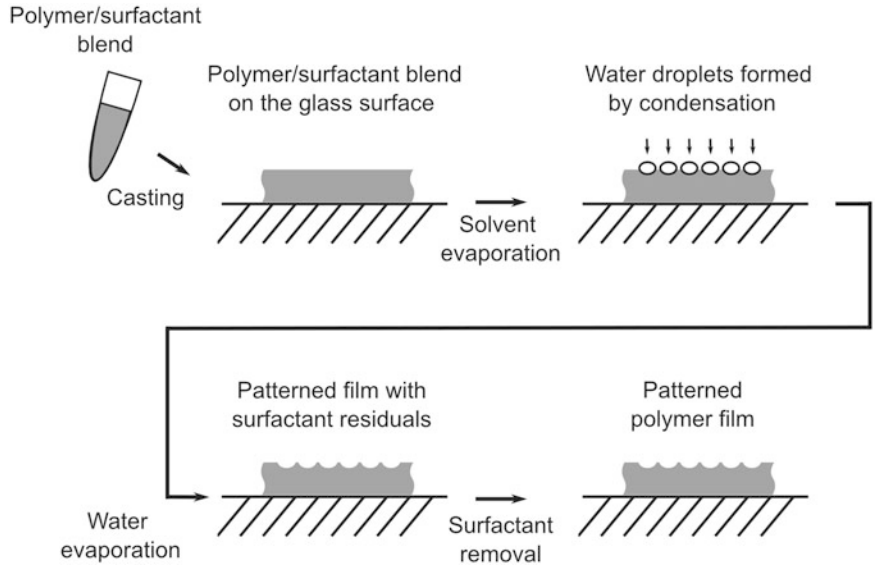

Fig. 1 Steps comprising the fabrication of polymeric patterned films by water-droplet templating. Drawings are not to scale

Alternatively, without using the surfactant:

1. In a clean glass container, dissolve PLA in chloroform, overnight at a concentration of 10 mg/ml.
2. Add 20 μl of deionized water and vigorously mix on a vortex mixer, for 4 min, until a visual homogenous suspension has been formed.
3. Immediately, cast a small volume (500 μl) on the microscope slide and gently dry over a stream of nitrogen gas (see Note 5).

3.2 Culture of ASCs on the Substrates

1. After thawing, cells are expanded in culture flasks for at least one passage.
2. When cells are more than 90 % confluent, aspirate medium from culture bottle and rinse twice with PBS.
3. Add enough trypsin/EDTA mixture to cover the cells. Gently rock the bottle to allow uniform coverage.
4. Incubate in a CO_2 incubator until cells under light microscope are visibly detached. To avoid damage due to prolonged exposure to the enzyme, cells should be checked every 2 min until significant detachment is observed.
5. After cell detachment, add a volume of growth medium that is at least three times the volume of trypsin/EDTA in order to stop the enzymatic reaction. Homogenize the cell suspension by repetitive pipetting.

6. Withdraw a 10 μl aliquot of cell suspension and mix it 1:1 with trypan blue.
7. Determine the cell concentration by using the hemocytometer under the inverted microscope.
8. Resuspend the cells in growth medium to a concentration that will depend on the subsequent assay.
9. Place the substrates on the 100-mm culture dishes and seed them with the cell suspension prepared in step 8. The volume of cell suspension per dish should be approximately 30 ml, which is enough to cover the substrates.
10. Leave the culture plates in the flow bench for 20 min to allow cells to settle before moving the plates to the incubator.

3.3 Immunofluorescence Staining (see Note 6)

1. Seed cells at a density of 2,500/cm^2 and culture them on the substrates for 1–3 days (see Note 7). A representative phase contrast image of ASCs seeded on the substrates for 24 h is shown in Fig. 2a.
2. Aspirate the medium and rinse twice with PBS, prewarmed at 37 °C (see Note 8).
3. Incubate with formalin for 15 min (see Note 9).
4. Aspirate the formalin and rinse three times with PBS.
5. Permeabilize the cells in T-PBS for 15 min.
6. Aspirate the T-PBS and rinse twice with PBS.
7. Block with BSA 1 % for 30 min.
8. Aspirate and incubate with the primary antibody for 1 h at 37 °C (see Note 10).
9. Aspirate and rinse twice.
10. Incubate with the secondary antibody solution for 1 h under mild shaking (see Note 10).
11. Aspirate the stain and rinse twice with PBS.

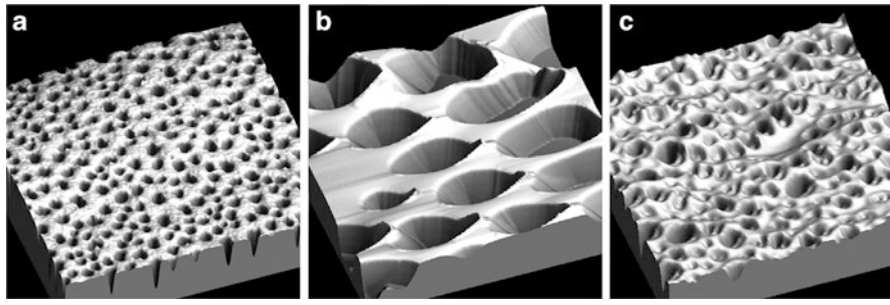

Fig. 2 AFM images displaying patterned PLA films obtained under different conditions. (**a**) Relative humidity of 33 %, (**b**) relative humidity of 80 %, and (**c**) without DOPE. Size of the images is 10 × 10 μm

12. Incubate with the Hoechst and Phalloidin stains for 30 min.
13. Rinse twice and keep in PBS at 4 °C until observation.

Representative photomicrographs of ASCs that have been stained following this protocol are shown in Fig. 2b.

3.4 Cell Proliferation Assay

1. Seed cells at a density of 1,500 cells/cm^2 and culture them for up to 10 days on the PLA substrates in normal growth medium.
2. At each desired time point, add Alamar Blue® in a volume equivalent to 10 % of the total medium in the well. Culture at 37 °C for 4 h. Include three negative controls (media with no cells) (see Note 11).
3. Take 100 μl aliquots and transfer them to the microtiter plate in duplicates.
4. Read fluorescence at 540–570 nm with an excitation of 580–610 nm on the plate reader.
5. Calculate the mean fluorescence intensity by averaging the values obtained at each time point and subtracting the average value of the negative control.
6. Calculate the cell doubling time (t_d) using a spreadsheet software package, such as Microsoft Excel. Firstly, fit the average values obtained in step 5 with an exponential trendline of the form $y = a \times e^{kt}$, where y represents the mean fluorescence intensity at time t, a is the initial number of cells, and k is the frequency of cell cycles per unit time. Using the estimated k value, obtain t_d as $t_d = (\ln 2)/k$ (see Note 12).

3.5 Real-Time RT-PCR

1. Seed cells at a density of 5,000 cells/cm^2 and culture them for 14 days on the PLA substrates in normal growth medium. Replace the medium with fresh medium every third day.
2. Harvest the RNA following the manufacturer's instructions (see Note 13).
3. Measure RNA quality using the spectrophotometer. With the NanoDrop, set up the system for RNA measurement (260/280 nm), blank with 1 μl of distilled water, place 1 μl of sample onto the pedestal, and measure the RNA concentration.
4. Synthesize cDNA as per manufacturer's instructions. cDNA now can be used as a template for amplification in PCR (see Notes 14 and 15).
5. For each of the primers, prepare a reaction mix consisting of 10 pmol of the primer, 0.25 μl of cDNA, and SYBR Supermix.
6. Mix the components by gently pipetting up and down, and spin briefly.

7. Perform the amplification in the qPCR instrument using the following protocol: An initial activation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing/extension step at the annealing temperature (AT) for 30 s.

4 Notes

1. Although α-MEM is recommended here, in our experience, DMEM/F12 and F12 media also appear well suited to support MSC growth and differentiation (20). In addition, we have recently shown that expansion of ASCs is improved by using a defined xeno-free and hypoxic environment (21).
2. The amount of DOPE in the blend has to be at least 1 %. Although higher concentrations may be used, in our experience, this has little influence on the size and distribution of the surface indentations.
3. The absolute thickness of the formed film for this purpose is not of importance, but it is important to note that the indentations formed in the cast polymer film leading to the surface patterning are important. The density and homogenous distribution of the water droplets in the film affect the wall thickness of the honeycomb pattern formed. The depth of the indentation is usually several orders of magnitude smaller than the whole film thickness. In addition, it is noteworthy that the remaining surface of the honeycomb is smooth and flat.
4. The room conditions are crucial in this step, namely, the humidity. In our experience, formation of nano-scaled patterns is favored by drying at low humidity (33 % RH), while micro-sized patterns are obtained by increasing the relative humidity. Figure 3 displays AFM images of films obtained in either low (33 %) or high (80 %) relative humidity conditions.
5. In the case of the surfactant-free patterned surface, the patterning is clearly visible, but the average pore diameter is visibly more irregular than for the DOPE-assisted honeycomb surface (see Fig. 3c). For the DOPE-free honeycomb pattern formation, the homogenization of the added water drop into the chloroform solution is of importance, as is the fast casting of the film after the homogenization and fast evaporation, which can be achieved by a stream of dry nitrogen gas. The accelerated chloroform evaporation is crucial, since the droplets are not stabilized by a surfactant and will coalesce during slow solvent evaporation.
6. In this assay, unless specified, the procedures are carried out at room temperature.

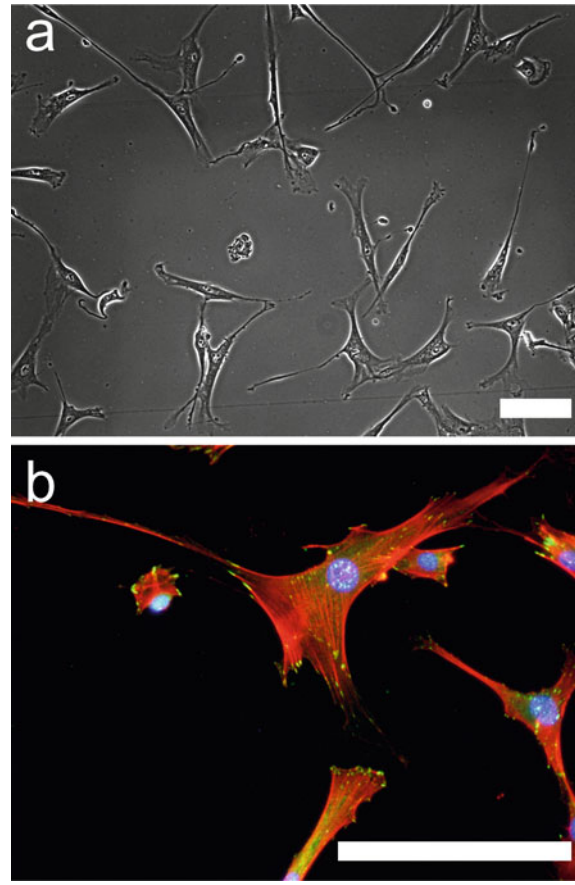

Fig. 3 Representative photomicrographs of cells on the substrates. (**a**) Phase contrast image displaying the typical fusiform, fibroblast-like morphology of the cells growing on the films. (**b**) Fluorescence image of cells, which displays the actin organization and focal adhesions. The focal adhesions are clearly localized at the end of the actin bundles. The image displays the nuclei in *blue*, actin in *red*, and focal adhesions in *green*. Scale bars represent 100 μm

7. For a proper assessment of morphology, cytoskeletal development, and focal adhesion maturation, cells should be seeded at a relatively low density on the substrates. We recommend a seeding density between 2,000 and 4,000/cm^2. In addition, the observation period should be between 1 and 3 days, to avoid cells becoming confluent on the substrates.
8. In our experience, washing and fixation at 37 °C help to better preserve the actin filaments and, in general, the cytoskeletal structure. In addition, cells usually tend to detach in response to cold solutions, which may represent a substantial cell loss when analyzing cells that form relatively weak focal adhesions.
9. Aldehyde fixatives as formalin react with amines and proteins to generate fluorescent products that will produce high

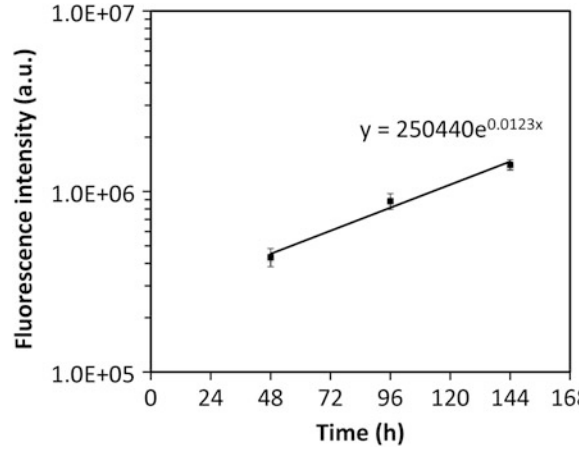

Fig. 4 Graph displaying typical proliferation data used to estimate the doubling rate. The regression results are displayed on the graph

background and may obscure small details, such as focal adhesions. To enhance vinculin staining, aldehyde blocking with sodium borohydride may be performed, which acts by reducing the –CHO groups to –OH. This is usually done by incubating the cells twice for 5 min in 0.1 % sodium borohydride (diluted in PBS) on ice before the step of cell permeabilization.

10. Due to the reduced incubation volume, the samples should be covered with a glass coverslip during incubation to avoid evaporation. Addition of PBS facilitates removal of the coverslip at the end of each step.
11. In our experience, the results obtained with Alamar Blue® are optimal when incubation times are between 3 and 4 h for the cell density recommended here. For lower cell densities, the incubation time should be increased.
12. An example of growth data is shown in Fig. 4, in which the fluorescence measurements were fit using $y = a \times e^{kt}$. The k-value was then used to calculate the doubling time, which resulted in $t_d = 2.3$ days.
13. At this step, the lysates can be stored at −20 °C for up to 6 months.
14. For cDNA synthesis, we usually normalize the amount of RNA template to the sample with the lowest amount of RNA. The maximum amount of RNA template that is recommended is 1 μg.
15. At this step, the cDNA samples can be stored at −20 °C for up to 6 months.

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Transduction of Murine Embryonic Stem Cells by Magnetic Nanoparticle-Assisted Lentiviral Gene Transfer

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Abstract

Genetic modification of embryonic stem (ES) cells is a valuable technique when combined with cell replacement strategies. Obtaining stable transgene expression and low-cytotoxicity lentiviral transduction of ES cells is advantageous. It has been shown that the efficiency of transfection and transduction approaches can be increased by magnetic nanoparticles (MNPs). Here, we present a protocol for MNP-assisted lentiviral transduction of adherent mouse ES cells. The application of MNPs increased transduction efficiency and provided the opportunity of cell positioning by a magnetic field.

Keywords: Mouse embryonic stem cells, Lentivirus, Transduction, Magnetic nanoparticles

1 Introduction

Cell replacement strategies using ES cells are a promising option for the treatment of various diseases caused by cell damage or degradation processes (e.g., cardiac disorders, neurodegenerative diseases, or diabetes) (1–3). Moreover, the therapeutic potential of ES cell-derived cell transplantation can be enhanced by prior genetic manipulation of the cells.

Gene transfer into ES cells has been described applying various methods but all of them have special disadvantages (4): Lipofection and electroporation, the most common gene delivery methods, provide low transfection efficiencies and/or have cytotoxic effects (5–7). Nucleofection enabling direct delivery of plasmid DNA to the nucleus yields a tenfold higher transfection efficiency than conventional electroporation (8, 9), but can also result in severe cell death (7).

Apart from chemical and mechanical transfection strategies, viral transduction has been demonstrated to be especially promising for genetic modification of ES cells because viruses infect a broad range of host cells. Adenoviruses enable a very efficient transduc-

tion (10, 11). Unfortunately, they can evoke a strong host immune response and gene expression is only transient (12, 13). In contrast, retroviruses exhibit a low-cytotoxicity and allow for a stable integration of their genetic material into the host genome; however gene expression can be compromised by strong gene silencing. Lentiviruses that also belong to the family of Retroviridae show a lower extent of gene silencing than other retroviruses and are therefore well suited for the transduction of ES cells (14–18).

Magnetic nanoparticles (MNPs) of the core-shell type consist of small iron oxide cores and different coatings that determine their stability and uptake by cells. In previous studies we could demonstrate that MNPs are versatile tools to enhance lentiviral transduction efficiency in vitro and ex vivo (19, 20). Here, we present a protocol for the combined use of lentivirus and MNPs for the transduction of murine ES cells. Transduction of adherent ES cells with lentiviral vectors alone displays only low transduction efficiency due to the small cell size and colony formation of ES cells. By use of MNPs we achieved a fourfold increase of the number of transduced cells. Additionally, ES cells were magnetically labeled, providing the opportunity for cell positioning by a magnetic field.

2 Materials

1. D3 murine ES cells, strain 129S2/SvPas (ATCC, Manassas, USA, cat. no. CRL-1934).
2. D3 medium: DMEM (Life technologies, Darmstadt, Germany, cat. no. 41965–039), 15 % (v/v) fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany, cat. no. 1902), 1 % (v/v) non-essential amino acids (Life technologies, cat. no. 11140–035), 1 % (v/v) penicillin/streptomycin (Life technologies, cat. no. 15140–122), 0.1 % (v/v) β-mercaptoethanol (Sigma Aldrich, Munich, Germany, cat. no. M7522), 1,000 U/ml leukemia inhibitory factor (LIF, Millipore, Schwalbach, Germany, cat. no. ESG 1107) (see Note 1).
3. 0.05 % Trypsin–EDTA (Life technologies, cat. no. 25300–054).
4. DPBS (Life technologies, cat. no. 14190–094).
5. HBSS (with calcium and magnesium; Life technologies, cat. no. 14025–050).
6. Lentivirus: Experiments were performed with self-inactivating, HIV-1-based lentiviral vectors of the third generation that were obtained by ultracentrifugation of virus containing cell culture supernatant from transiently transfected HEK293T virus producer cells (21). The lentiviral vectors contained the CAG promoter and an eGFP reporter gene cassette (see Note 2).

The biological and physical titers were 2.3×10^9 infectious particles (IPs)/ml and 2.4×10^{10} viral particles (VPs)/ml, respectively (see Note 3). The biological titer was determined as previously described (21), the physical titer was analyzed by measuring the concentration of active reverse transcriptase, and VPs/ml were calculated as described elsewhere (22).

7. MNPs: The nanoparticles applied here (SO-Mag5, kindly provided by O. Mykhaylyk, TU München) were composed of a paramagnetic iron oxide core with a diameter of 7 nm and a surface coating of silicon oxide with phosphonate groups (23). Stock concentration was 15.8 mg Fe/ml.
8. Magnetic plate (Chemicell, Berlin, Germany, cat. no. 9009).
9. 0.1 % (w/v) Gelatine (Sigma Aldrich, cat. no. G2500) in DPBS.
10. 4 % (w/v) PFA (Sigma Aldrich, cat. no. P6148) in DPBS.
11. 0.02 % (v/v) Triton X-100 (Sigma Aldrich, cat. no. 93418) in DPBS.

3 Methods

Pre-warm all buffers and media needed for cell culture work at 37 °C in a water bath. Keep all cell culture materials sterile. Plate D3 ES cells on a feeder layer unless specified otherwise (see Note 4).

3.1 Cultivation of D3 ES Cells

1. Thaw D3 ES cells (see Note 5) in a 37 °C water bath for 3 min (see Note 6).
2. Add D3 medium to the cryotube and transfer the cell suspension from the cryotube to a reaction tube containing 5 ml of D3 medium with LIF.
3. Plate ES cell suspension homogeneously on a 25 cm^2 cell culture flask.
4. Allow ES cells to grow for 2 days (see Note 7).
5. Passage ES cells. Therefore remove the medium, wash once with DPBS, add 1 ml 0.05 % trypsin–EDTA per flask, rock the flask gently to ensure coverage of whole growing surface, and incubate for 3–5 min at 37 °C until the cells detach. Tap the flask for complete cell detachment. Add 5 ml of D3 medium without LIF to inactivate trypsin and aspirate cells several times to produce a single-cell suspension (see Note 8). Centrifuge the cell suspension for 5 min at 1,000 rpm. Remove the supernatant and resuspend the cell pellet in 1 ml D3 medium without LIF. Determine the cell number and seed 150,000 cells per 25 cm^2 flask containing D3 medium with LIF.
6. Next passage is required after 2 days (see Note 7).

3.2 Preparation of D3 ES Cells for Transduction

1. Cultivate D3 ES cells as described in Section 3.1.
2. Passage ES cells once before transduction experiment.
3. Seed 10,000 ES cells per well in 24-well plate.
4. Perform MNP-assisted lentiviral transduction 12–16 h after seeding.

3.3 MNP-Assisted Lentiviral Transduction

1. Calculate the required amount of lentivirus and MNPs. To determine the required number of IPs, multiply cell number per well by desired multiplicity of infection (MOI). In our case 110,000 cells (100,000 feeder cells and 10,000 D3 ES cells) and MOI of 80 were applied resulting in 8.8×10^6 IPs needed per well. Multiply this number by the ratio of physical to biological virus titer to calculate the number of VPs: 8.8×10^6 IPs $\times$ 10.4 VPs/IPs $= 9.2 \times 10^7$ VPs (see Note 9). The required amount of MNPs is obtained by multiplication of VPs (9.2×10^7 VPs) by the desired iron amount per VPs (here, 300 fg Fe/VP) resulting in the total iron amount per well (27.6 μg Fe).
2. Add the required volumes of lentivirus and MNPs to 0.5 ml HBSS and incubate this suspension for 20 min at room temperature.
3. Remove medium from ES cells and wash once with HBSS.
4. Apply the lentivirus/MNP solution to the cells, place the 24-well plate on the magnetic plate, and transfer both to the incubator for the 30-min transduction step (see Notes 10 and 11).
5. Remove the lentivirus/MNP solution and add 0.5 ml D3 medium with LIF.
6. Depending on how transduction efficiency will be analyzed transfer D3 ES cells to a 25 cm^2 flask (for flow cytometry) or seed them on coverslips coated with 0.1 % gelatine (for fluorescence microscopy) 24 h after transduction (see Note 12).
7. Analyze eGFP expression 72 h after transduction by flow cytometry or fluorescence microscopy.

3.4 Analysis of eGFP Expression

3.4.1 Flow Cytometry

1. Trypsinize and harvest transduced ES cells from a 25 cm^2 flask as described in step 5 of Section 3.1.
2. After centrifugation remove the supernatant, add 1 ml of DPBS, and centrifuge again for 5 min at 1,000 rpm.
3. Remove DPBS, add 1 ml 4 % PFA, resuspend cells, and incubate them for 15 min on ice.
4. Centrifuge again for 5 min at 1,000 rpm and replace PFA with 1 ml DPBS.
5. Store fixated cells at 4 °C until flow cytometry analysis is performed.

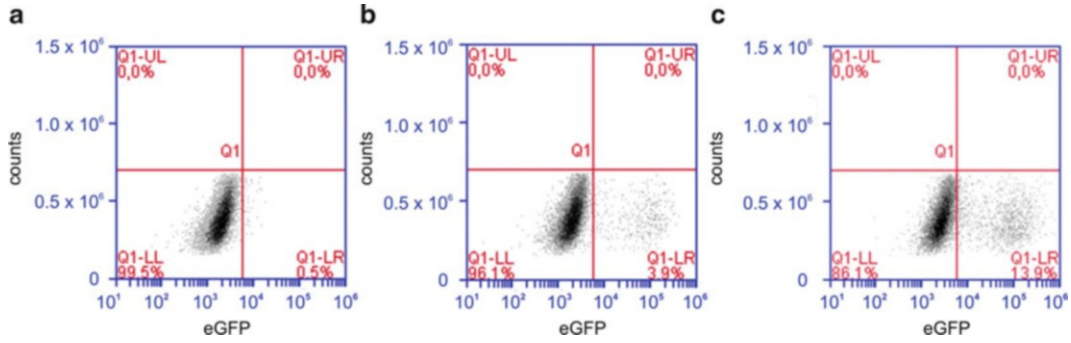

Fig. 1 Flow cytometry analysis of native and lentivirally transduced ES cells 72 h after transduction. (**a**) Untransduced D3 ES cells. (**b**) D3 ES cells transduced with lentivirus carrying a CAG–eGFP cassette overnight (MOI 80). (**c**) D3 ES cells transduced with CAG–eGFP and 300 fg Fe SO-Mag5/VP for 30 min (MOI 80) on a magnetic plate

Flow cytometry analysis revealed a fourfold higher number of eGFP-positive cells after transduction with lentivirus and MNPs compared to the control with virus overnight (see Fig. 1).

3.4.2 Fluorescence Microscopy

1. Remove medium from transduced cells grown on coverslips and wash twice with DPBS.
2. Add 4 % PFA and incubate for 15 min at room temperature.
3. Remove PFA and wash once with DPBS.
4. Add 0.02 % Triton X-100 in DPBS and incubate for 10 min at room temperature.
5. Wash once with DPBS and perform a nuclear staining with Hoechst.
6. Mount the stained cells using an aqueous mounting medium.

Microscopy images depicted a higher amount of eGFP-positive colonies when MNPs were used for transduction (see Fig. 2).

4 Notes

1. We always prepared D3 medium without LIF and added required amounts shortly before using the medium.
2. Different promoters lead to variable transgene expression especially in ES cells (24–26).
3. Depending on the protocols used for lentivirus production and analysis of biological and physical titers differences in the titers can occur.

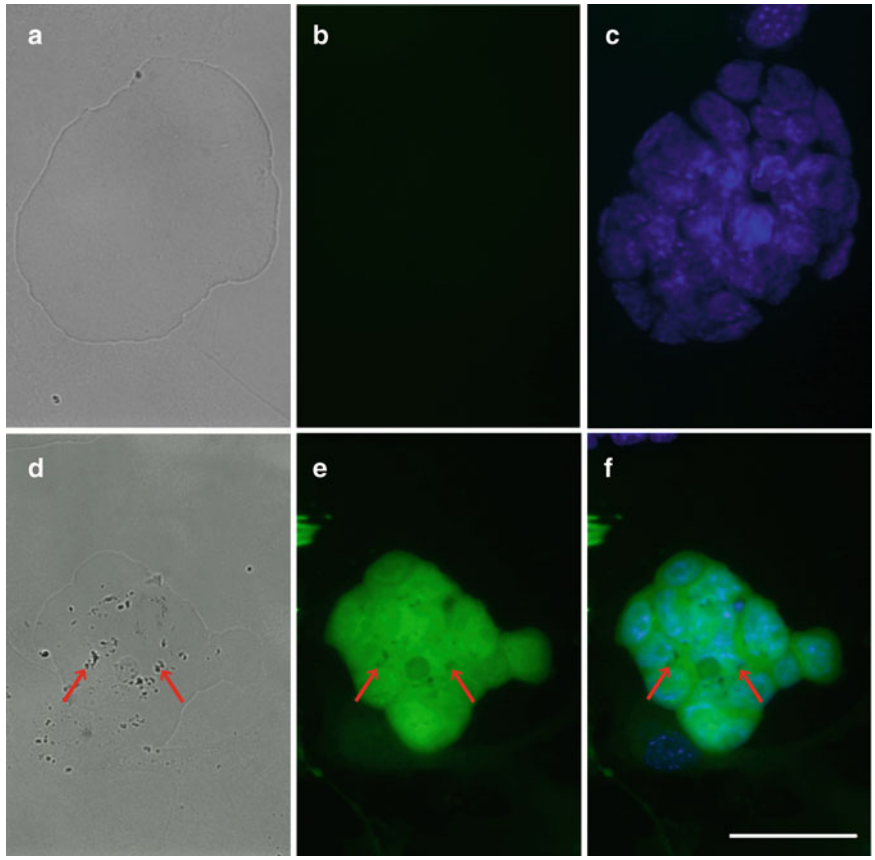

Fig. 2 Fluorescence microscopy of eGFP expression in ES cells 72 h after transduction. (**a**–**f**) Brightfield (**a, d**) and fluorescence (**b**–**c, e**–**f**) images of a representative D3 ES cell colony after lentiviral transduction (MOI 80) with a CAG–eGFP cassette; *blue* = Hoechst, *green* = eGFP, bar = 50 μm. (**a**–**c**) Transduction with lentivirus overnight. (**d**–**f**) Transduction with lentivirus and SO-Mag5 for 30 min on a magnetic plate. *Arrows* indicate incorporated MNPs

4. We used primary mouse embryo fibroblast strain FVB (Millipore, cat. no. PMEF-NL) at the following cell densities: 1.2×10^6 feeder cells per 25 cm^2 flask and 1.0×10^5 feeder cells per well in a 24-well plate.
5. D3 ES cells were frozen as a single-cell suspension in a 1:1 mixture of 20 % DMSO (v/v) (Sigma Aldrich, cat. no. D4540) in FCS and D3 medium. One aliquot contained 1.0×10^6 cells.
6. Remove the cryotube from the water bath before the aliquot is completely thawed. This procedure will preserve cell viability.
7. Check D3 ES cells daily. They should form small colonies with distinct borders. Avoid the development of large colonies that interfere with each other. If seeded at a density of 150,000 cells

per 25 cm^2 flask D3 ES cells usually need to be passaged every 2 days.

8. To prevent foam formation pipette tips with a large opening are recommended.
9. To reduce the required amount of lentivirus/MNP complexes ES cells could be cultured on feeder-free, gelatine-coated plates.
10. This method also works at 4 °C with a similar transduction efficiency (19).
11. Recommendable controls are lentivirus application overnight or for 30 min (without MNPs). Overnight transduction needs to be performed in D3 medium with LIF.
12. To reduce the number of feeder cells, you can perform a pre-plating step. Therefore add the cell suspension to a cell culture dish and wait for 10 min. Feeder cells should sediment and attach faster than D3 cells. Carefully remove the supernatant containing the D3 cells and seed them as described in step 6 of Section 3.3.

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Lanthanide-Based Upconversion Nanoparticles for Connexin-Targeted Imaging in Co-cultures

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Abstract

From the perspective of deep tissue imaging, it is required that the excitation light can penetrate deep enough to excite the sample of interest and the fluorescence emission is strong enough to be detected. The longer wavelengths like near infrared are absorbed less by the tissue and are scattered less implying deeper penetration. This has drawn interest to the class of nanoparticles called upconversion nanoparticles (UCNs) which has an excitation in the near-infrared wavelength and the emission is in the visible/near-infrared wavelength (depending on the doped ions). Here, we discuss surface modification of the UCNs to make them hydrophilic allowing dispersion in physiological buffers and enabling conjugation of antibody to their surface. It was of interest to use connexin 43 gap junction protein-specific antibody on UCNs to target cardiac cell such as H9c2 and co-culture of bone marrow stem cells and H9c2.

Keywords: Upconversion nanoparticles, Connexin 43, Targeted imaging

1 Introduction

Upconversion nanoparticles (UCNs) have gained attention as fluorescent probes in the recent years due to their optical properties and advantages thereof (1–8). The primary advantage is excitation in the near-infrared wavelength and emission in the visible region (e.g., ytterbium (Yb^{3+}), erbium (Er^{3+}) doping) or near-infrared region (e.g., ytterbium (Yb^{3+}), thulium (Tm^{3+})). The NIR excitation and emission would allow noninvasive in-depth imaging as the longer wavelengths are absorbed/scattered less by biological tissue (9–12). These particles also do not show blinking and have negligible photobleaching when compared to organic fluorophores (13, 14). This would allow time-based imaging using these nanoparticles. A system that would highlight the potential of these nanoparticles in imaging was chosen. In case of heart arrhythmia, a cell transplant is one of the economical and viable

options (15–17). Recent reports suggest that the compatibility of the transplanted cells would depend on the expression of the protein connexin 43 (18, 19). Thus it was of interest to use cardiac cells and bone marrow stem cell co-culture as a model and target UCNs conjugated to the connexin 43-specific antibody. Since the nanoparticles do not photobleach over time it would be possible to image the cells over different time points or days (9). Here we discuss the synthesis and modification of the surface of the nanoparticles using silica to obtain core/shell structure and further using a covalent coupling method to conjugate the antibody to the surface of the nanoparticles. The characterization of antibody conjugation, covalent coupling, and antibody activity testing was done prior to using them for imaging cells.

2 Materials

2.1 Synthesis of UCNs

All chemicals were obtained from Sigma unless and otherwise stated.

1. Erbium chloride, ytterbium chloride, yttrium chloride
2. Ammonium fluoride
3. Sodium hydroxide pellets
4. Methanol
5. Ethanol
6. Oleic acid
7. 1-Octadecene
8. Cyclohexane
9. Acetone
10. Centrifuge (Sartorius Sigma 3-18 k)
11. Transmission Electron Microscope (TEM) (JEOL 1220 100 KV)
12. Fluorescence Spectrometer (Acton Pro)

2.2 Silica Coating of UCNs

1. Igepal CO-520
2. Ammonium hydroxide solution
3. Tetra ethyl ortho silicate solution
4. Shaker (Orbit 300, Labnet Inc)

2.3 Antibody Conjugation

1. 3-Amino propyl tri ethoxy silane
2. Trinitrobenzene sulfonic acid
3. Tween-20
4. Glutaraldehyde solution

5. Anti-connexin 43
6. Ethanol amine
7. 10× Phosphate Buffered Saline (PBS) (Invitrogen)
8. Horse Serum (Invitrogen)
9. Zeta Sizer (Malvern)

2.4 Targeted Imaging

1. Dulbecco's Modified Eagles Medium (DMEM) low glucose (Invitrogen)
2. Fetal Bovine Serum (Invitrogen)
3. Penicillin–Streptomycin (Invitrogen)
4. H9c2 cells Rat derived cardiomyoblast/myocytes (ATCC)
5. Confocal Microscope (Nikon TE2000)

3 Methods

3.1 Synthesis of UCNs

1. The UCNs were synthesized by a one-pot synthesis as described by Li et al. (20). Chloride salts of the lanthanides were dissolved in deionized (DI) water and stoichiometric ratios of this solution are stirred in a three-neck round-bottomed flask. The donor ion was ytterbium (Yb^{3+}) and the emitter ion was Er^{3+} to obtain nanoparticles with emission in the visible region.
2. The water was evaporated by heating to 100 °C. 6 ml of oleic acid and 15 ml of octadecene were added to the flask and the temperature increased to 130 °C. The temperature was maintained with constant stirring till the salt precipitate dissolved completely.
3. The temperature was then reduced to room temperature.
4. 0.4 g of sodium hydroxide was dispersed in 10 ml of methanol by sonication and to this solution 4 mmol of ammonium fluoride was added. Mild sonication was done after the addition of ammonium fluoride to enable dissolution of the fluoride salt. This mixture in methanol was added to the solution that was cooled down to room temperature under vigorous stirring.
5. The solution was allowed to stand for 30 min. The temperature was then increased to 60°C to remove the methanol solvent and then to 100 °C to remove any water present.
6. To ensure complete removal of water a vacuum pump was used for 15 min. The solution is then heated to 300 °C under an inert atmosphere of argon for 1.5 h.
7. The UCNs were precipitated from the solution using acetone. Equal volumes of the solution and acetone were centrifuged at 23,000 rcf for 15 min.

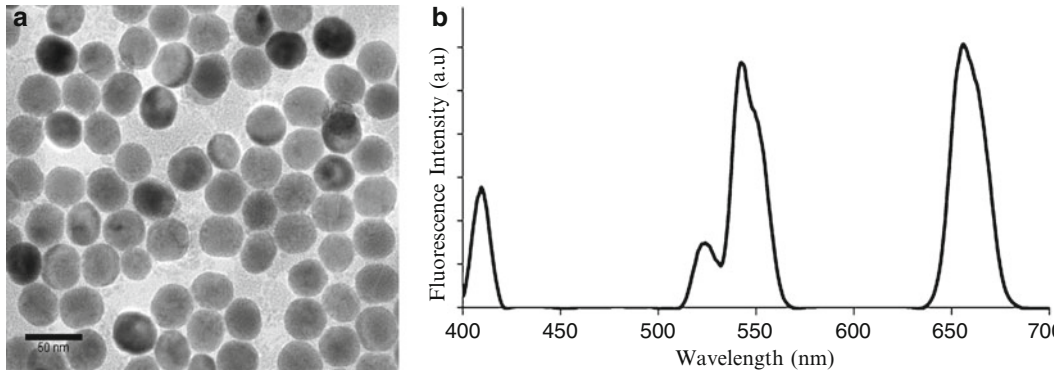
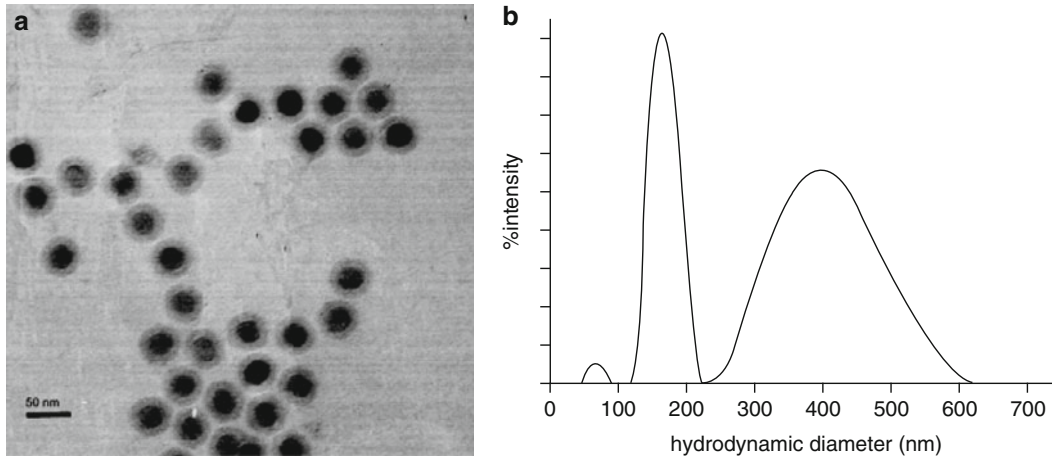


Fig. 1 TEM image of UCNs dispersed in cyclohexane (**a**) and fluorescence spectra recorded with 980 nm excitation (**b**) (9) (reprinted with permission from Springer Science+Business Media)

8. The precipitate was then redispersed in the same volume of cyclohexane as the initial solution. Mild sonication was required to ensure uniform dispersion of precipitate in cyclohexane.
9. To the precipitate in cyclohexane equal volume of 50 % ethanol was added. The solution was gently mixed by hand and allowed to stand. Impurities were found to settle between the two phases. Once the layer was distinct the cyclohexane top layer was carefully pipetted to a clean centrifuge tube. This was repeated until the cyclohexane layer did not show any turbidity.
10. The cyclohexane layer was centrifuged with acetone once prior to dispersion in cyclohexane. Nanoparticles from 1 mmol of the lanthanide salts used were dispersed in 50 ml of cyclohexane. This solution hereafter shall be referred to as stock solution.
11. The UCNs were characterized using a TEM and the fluorescence spectra of these particles were measured at an excitation of 980 nm (see Note 1) (Fig. 1).

3.2 Silica Coating of UCNs

1. The UCNs were silica coated by a modified Stober method (21). 1 ml of the stock was diluted with 4 ml of cyclohexane (see Note 2).
2. To this solution 200 µl of Igepal-CO520 (surfactant to prevent aggregation) and 400 µl of 33% ammonia were added.
3. 150 µl of 10% tetra ethyl ortho silicate in cyclohexane was added and the solution kept under continuous shaking at 700 rpm for 48 h.
4. The modified UCNs (UCN/SiO_2) were precipitated with equal volume of ethanol and centrifuged at 23,000 rcf for 10 min. This was repeated twice and the UCN/SiO_2 were redispersed in 50 % ethanol.



c

UCN	Zeta Potential
UCN:Yb,Er Silica	–41 mV

Fig. 2 TEM image of the UCN/SiO_2 (**a**) (9) (reprinted with permission from Springer Science+Business Media), DLS data of UCNs before (*light gray*) and after silica coating (*black*) (**b**), and zeta potential of UCN/SiO_2 dispersed in DI water (**c**)

5. The modified particles were characterized using TEM, dynamic light scattering (DLS), and zeta potential. The DLS and zeta potential measurements were obtained on a Zetasizer. Fluorescence spectrum of the UCN/SiO_2 was measured at an excitation of 980 nm (see Notes 1 and 3) (Fig. 2).

3.3 Antibody Conjugation to Modified Nanoparticles

3.3.1 Amine Modification of UCN/SiO_2

1. Amine groups were introduced on the surface of UCN/SiO_2 to enable conjugation of biomolecules to the surface (22).
2. 5 ml of UCN/SiO_2 was dispersed in 2.5 ml of ethanol.
3. To this 5 μl of a mixture of 50 mol% 3-aminopropyltriethoxysilane and TEOS was added under constant stirring. 330 μl of 33 % ammonia was added. The reaction was continued with constant stirring for 12 h.
4. The samples were purified by centrifuging the modified UCN/SiO_2 with equal volume of DI water at 18,000 rcf for 10 min. The samples were washed until the precipitate obtained could be readily dispersed in water with mild sonication.
5. The zeta potential of the amine-modified UCN/SiO_2 was measured and a trinitrobenzene sulfonic acid assay was used to confirm the presence of primary amine groups on the surface of the UCN/SiO_2 (23). TNBS forms adduct with primary

amine groups and shows an absorbance at 410 nm. The zeta potential of the sample was measured to be +4 mV after amine modification (see Note 4). Ethanol amine was used as reference for the TNBS assay and an amine concentration of 0.16 M was detected in 5 ml of the amine-modified UCN/SiO_2 (see Note 5).

3.3.2 Antibody Conjugation

1. 1 ml of the amine-modified UCN/SiO_2 was suspended in 4 ml of 1× PBS with 10 % glutaraldehyde. The solution was stirred for 2 h and the modified particles were centrifuged at 18,000 rcf for 10 min.
2. The samples were washed thrice and resuspended in 5 ml of PBS. 1 mg of anti-connexin 43 was added to 5 ml of glutaraldehyde-activated amine-modified UCN/SiO_2 in PBS.
3. The solution was kept under continuous stirring for 4 h. To saturate any activated glutaraldehyde still present, 100 μl of ethanol amine and horse serum each were added.
4. The solution was then centrifuged at 13,000 rcf for 10 min to remove the antibody-conjugated UCNs. The presence of excess antibody in the supernatant was detected by UV–vis spectroscopy (data not shown) and the centrifugation was continued until the antibody presence in the supernatant was too low to be detected.
5. The fluorescence spectrum at an excitation of 980 nm was recorded and the UV–vis spectrum of the antibody-modified UCN/SiO_2 was also obtained. The intrinsic fluorescence from the antibody was also used to identify the optimal concentration of the antibody used for conjugation. This was recorded on a microplate reader with 285 nm excitation (see Note 6). The covalent coupling of the antibody was also checked using a surfactant to leach out the antibody. The intrinsic fluorescence of the antibody from antibody-modified UCN/SiO_2 was measured before and after centrifuging them with 1 % Tween 20. Reference samples of antibody-adsorbed UCN/SiO_2 were prepared by mixing amine-modified UCNs with the antibody without the use of crosslinker. The samples were purified by centrifugation till the supernatant did not show any antibody peaks when checked on UV–vis spectrometer. The intrinsic fluorescence from the adsorbed sample was also measured before and after centrifuging them with 1% Tween 20 (see Note 7) (Fig. 3).

3.4 Targeted Imaging

Bone marrow stem cells (BMSC) have been used in the transplantation of cells in the cardiac environment in case of an arrhythmia. Recent reports suggest that the adaptability of these BMSC in the cardiac environment might be attributed to gap junctions they form with the cardiac cells. These gap junctions in the cardiac

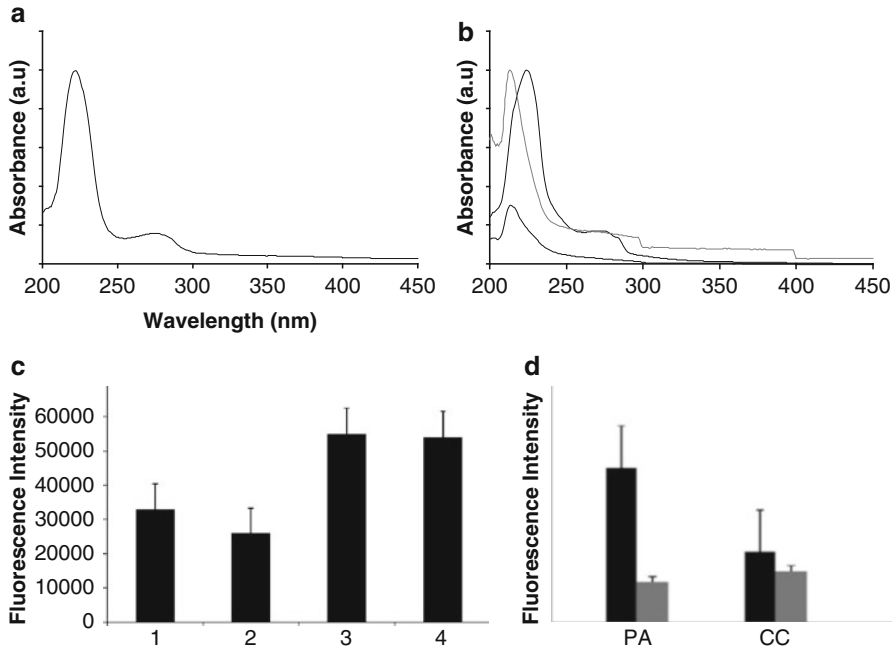


Fig. 3 UV–vis spectra of the antibody dispersed in PBS (**a**), UV–vis spectra of the antibody-modified UCNs with antibody concentration of 0.025 mg/ml (*black*) and 0.05 mg/ml (*gray*) and reference sample of amine-modified UCNs with peak at 210 nm (**b**), intrinsic fluorescence of amine-modified UCNs (1), antibody alone (2), antibody-modified UCNs with antibody concentration of 0.025 mg/ml and 0.05 mg/ml (3, 4) (**c**), intrinsic fluorescence of the antibody-adsorbed UCNs (PA) and antibody-modified UCNs (CC) before (*black*) and after (*gray*) leaching with Tween 20 (**d**) (9) (reprinted with permission from Springer Science+Business Media)

environment are predominantly composed of connexin 43 proteins. Thus we used a model system consisting of H9c2 cells and BMSC (extracted from rat femur). H9c2 cells are a mixture of cardiomyoblast and cardiomyocytes and they show connexin 43 expression.

1. BMSC and the H9c2 cells were grown in DMEM with low glucose, 10 % fetal bovine serum, and 1 % penicillin/streptomycin in a T75 flask.
2. The H9c2 cells and BMSC cells were cultured in 40:1 ratio and allowed to reach 80 % confluence.
3. To this co-culture 20 μl of the antibody-conjugated UCNs was added and the cells incubated at 37 °C for 8 h.
4. The cells were imaged under a widefield microscope with 980 nm laser excitation source after replacement with fresh medium. Skeletal myoblast cells which show negligible expression of connexin 43 were used as control sample (see Note 8) (Fig. 4).

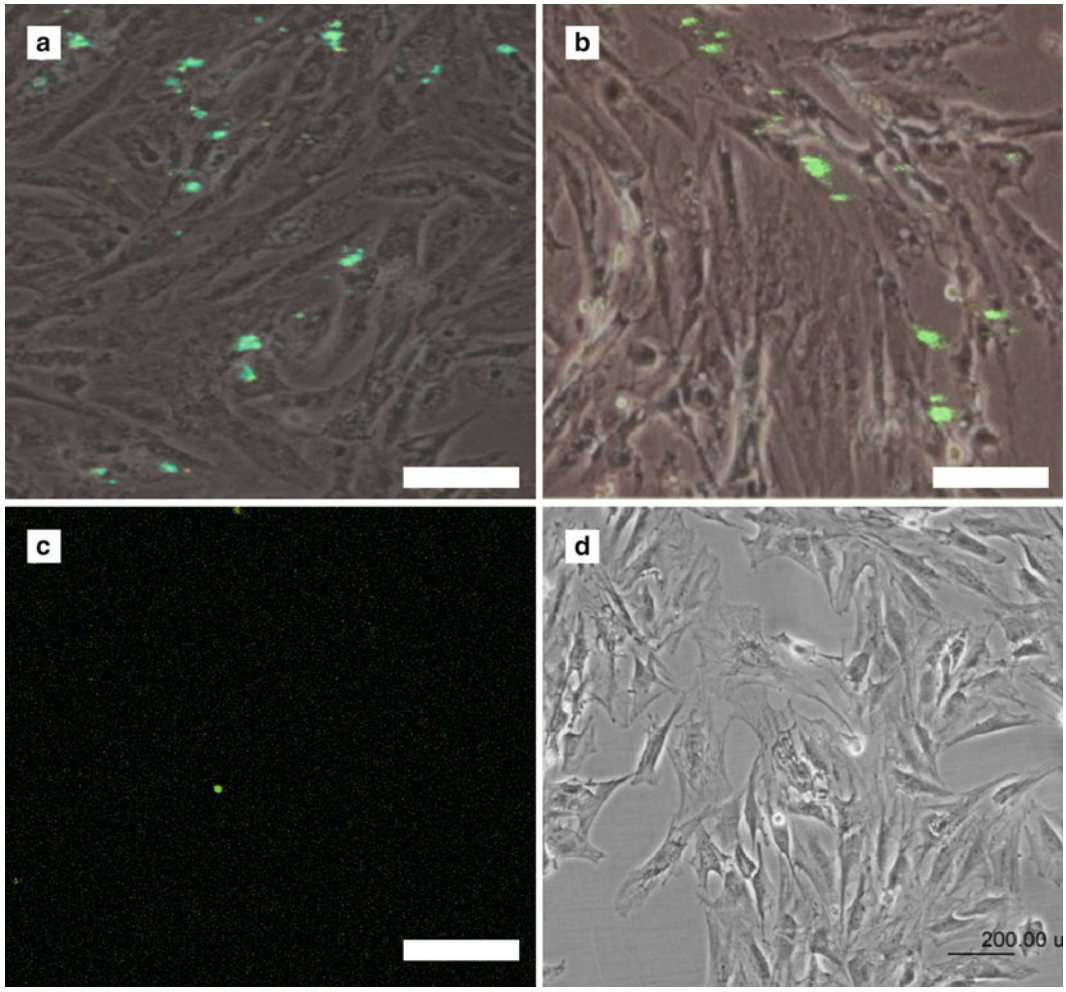


Fig. 4 Overlapped fluorescence and bright field image of antibody-modified UCNs (*pseudocolor green*) labeled H9c2 cells (**a**), H9c2/BMSC co-culture (**b**), and fluorescence image of antibody-modified UCNs incubated with skeletal myoblast control cells (**c**) and bright field image of skeletal myoblasts (**d**) (9) (reprinted with permission from Springer Science+Business Media)

3.5 Conclusion

UCNs were suitably modified with a silica shell to enable conjugation of antibody. The silica surface was further functionalized with amine groups to covalently couple a connexin 43 antibody. These antibody-modified UCNs were used in targeting the extracellular region of the gap junctions that form between H9c2 and BMSC cells. A simple widefield microscope set up with 980 nm excitation source has been used to image the labeled live cells. The UCNs were not found to be toxic to even sensitive cells like BMSC in the concentration used. Since the UCNs show negligible photobleaching it could be possible to do time-based imaging of the labeled gap junctions with a high-speed microscope and this could be used to study disease progression (24–26).

4 Notes

1. The UCNs obtained by the synthesis were found to be monodisperse and the average size was found to be 30 nm and after silica coating it is 50 nm. The size distribution was obtained from TEM images over at least two different batches of synthesis.
2. There is no method to determine the concentration/number of UCNs in a given solution; thus for experiments the fluorescence intensity of the UCNs was measured and maintained the same so as to compare the results between different batches of synthesis.
3. The DLS data estimates the hydrodynamic diameter and this could be affected by the solvent used. Hence, the size of UCNs does not match the TEM data. Thus DLS has mainly been used to determine if there is a significant change in the size, suggesting successful surface modification of the nanoparticles.
4. The zeta potential of the UCN/SiO_2 in DI water was measured to be −41 mV; this suggests that the samples obtained are stable. The change in the zeta potential to +4 mV upon treatment of the samples with APTES suggests the presence of amine group on the surface. Thus the zeta potential has been used to monitor the amine modification of the UCN/SiO_2. Since the zeta potential value after amine modification is less than 30 mV the sample is not stable and the modification is done prior to antibody conjugation.
5. Further, the presence of the amine group was confirmed by TNBS assay. Calibration curve was obtained using known concentrations of ethanol amine and the absorbance of the adduct formed with the amine-modified UCN/SiO_2 was measured. The concentration was estimated to be 0.16 M in the 5 ml of the amine-modified UCN/SiO_2 prepared.
6. Amine-modified UCN/SiO_2 was activated with a glutaraldehyde crosslinker and antibody anti-Cx43 was conjugated to it. A range of concentration was tested as described and the UV–vis spectrum of the antibody-modified samples was measured. The intrinsic fluorescence from the antibody-modified UCN/SiO_2 was also measured. The optimum concentration was determined as 0.05 mg/ml as the intrinsic fluorescence and the absorbance peaks did not shift drastically beyond this concentration. For lower concentrations used, the UV–vis spectrum showed noisy peaks and the data has not been shown.
7. It was also necessary to confirm the covalent coupling of the antibody to the amine-modified UCN/SiO_2. A reference sample was prepared with the antibody adsorbed on the surface of

amine-modified UCN/SiO_2. The intrinsic fluorescence of both the samples were measured before and after centrifuging with Tween 20 surfactant. In the case of physical adsorption the intrinsic fluorescence of the sample decreases more as the antibody is leached out but in case of covalent coupling, the intrinsic fluorescence decrease is less. The slight decrease in the intrinsic fluorescence in the case of covalent coupling could be attributed to some adsorption on the surface of the particles.

8. The antibody-modified UCNs were used to target the gap junctions that are formed between the cells. Targeted imaging was done using a co-culture of H9c2 and BMSC cells. It was ensured that the UCNs were not endocytosed by the cells by optimizing the time period of incubation. Thus only the connexin protein recruited to the membrane or the ones exposed on the extracellular portion of the gap junction could be targeted. It was observed that skeletal myoblasts with negligible expression of connexin 43 showed negligible fluorescence labeling.

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Basic Protocols to Investigate hMSC Behavior onto Electrospun Fibers

Marco A. Alvarez-Perez, Vincenzo Guarino, Valentina Cirillo, and Luigi Ambrosio

Abstract

Human mesenchymal stem cells (hMSC) currently represent a major cell resource in the research laboratory, to study differentiated-cell behavior in 3D scaffolds during the regeneration processes. Adhesion and differentiation of stem cells to a specific phenotype are achieved by culturing them in apposite culture media under precise conditions. Meanwhile, hydrolytic degradation of polymeric scaffolds allows implanted cells to synthesize their own extracellular matrix in situ after implantation so that the degeneration of the foreign scaffold is temporally matched by creation of the new innate one. In this context, structural properties and biochemical signals may concur to influence the cell response to the environmental stimuli during the culture. So, it becomes mandatory to introduce robust protocols to treat hMSC alone—before the culture—and in combination with the scaffolds for the next investigation by scanning electron microscopy. Here, we describe the protocols used to manage hMSC before and during the culture in order to obtain more detailed information on cell mechanisms mediated by polymeric scaffolds.

Keywords: Human MSC, Cell morphology, Nanofibers, Scanning electron microscopy, Tissue engineering

1 Introduction

Tissue engineering approach mainly involves the use of isolated cells or mesenchymal stem cells (MSCs) with three-dimensional (3D) polymer-based scaffolds to mimic the native microenvironment conditions, required to drive the biological mechanisms which regulate the regeneration processes of the most part of tissues and organs including cartilage, bone, skin, blood vessels, and heart valves (1, 2). By using porous scaffolds, the spaces occupied by the biodegradable polymers are progressively filled by cells during the culture, and then, by a large amount of extracellular matter deposited by cells themselves (3–6). So, a growing interest is leading to the implementation of innovative technologies such as

the electrospinning strategies which offer alternatives to conventional process methodologies for the development of micro/nanostructured scaffolds (7). Indeed, electrospinning allows to design nanofibrous platforms which mimic the morphological organization of collagen fibers in the extracellular matrix (8), also providing the high surface area, flexibility, and excellent mechanical strengths which affect the cell response during the advance of tissue formation (9).

In order to understand these processes, it is crucial to define experimental procedures to assure the reproducibility of the biological response and the evaluation of the cell features (i.e., morphology) by microscopy technologies. In particular, the most critical phase certainly concerns the cell adhesion on the scaffold surface, able to greatly influence the biological activity in the later stage, i.e., proliferation, differentiation, and maturation (10). In this context, the introduction of robust protocols to treat hMSC during seeding and culture onto electrospun scaffolds and the optimization of sample treatments (i.e., cell fixation, sputtering of cell-loaded surfaces) before electronic microscopy analyses contribute to obtain more detailed information on cell mechanisms mediated by polymeric scaffolds.

2 Materials

2.1 Materials and Storage Conditions

1. Cryopreserved human mesenchymal stem cells (hMSC) from LONZA (catalog # PT-2501), straight after arrival, are stored in liquid nitrogen (see Note 1).
2. Eagle's alpha minimum essential medium (α-MEM) should be stored at 2–8 °C; it is stable until expiry date as indicated on the label of fabricant. Avoid extended exposure to room temperature or higher temperature.
3. Fetal bovine serum (FBS) for hMSC should be stored at −20 °C; it is stable until expiry date as indicated on the label. Storing at 2–8 °C is not recommended. FBS can be thawed under water bath, aliquoted into smaller volumes, and refrozen to facilitate the preparation of small volumes of complete mesenchymal basal medium (see Note 2).
4. Antibiotic Solution (penicillin/streptomycin, Sigma-Aldrich, Italy) should be stored at −20 °C; it is stable until expiry data as indicated on the label of fabricant. Storing at 2–8 °C is not recommended. Antibiotic solution can be thawed, aliquoted into smaller volumes, and refrozen to facilitate the preparation of small volumes of complete mesenchymal medium (see Note 3).
5. Once the complete mesenchymal basal medium has been prepared, store at 2–8 °C for up to 1 month (see Note 4).

2.2 Equipment and Supplies Required

1. Biohazard safety cabinet certified level II handling of biological materials.
2. 37 °C Incubator with humidity and gas control to maintain >95 % humidity and atmosphere of 5 % CO_2, in air.
3. Benchtop, low-speed centrifuge with option to enable slow deceleration with options to accommodate 15 and 50 mL conical tubes.
4. Vacuum pump and trap containing disinfectant.
5. Water bath with temperature controller.
6. Inverted microscope with phase contrast optics for checking cells and cell count.
7. Routine light microscope for hemocytometer cell count.
8. Hemocytometer.
9. Tissue culture-treated 35 mm dishes (Corning Catalog #3506) or T-25 cm^2 flasks (Falcon Catalog #353109). Always use tissue culture-treated plates, dishes, or flasks for MSC culture.
10. Sterile disposable 5 and 10 mL pipettes.
11. Pipette-aid (e.g., Drummond Scientific).
12. Falcon-style 15 and 50 mL conical polypropylene tubes.
13. Trypsin–EDTA solution (Catalog #07901).
14. Phosphate Buffered Saline (PBS), pH 7.4, (Catalog #37350).
15. Distilled water.
16. Paraformaldehyde. Dilute to a 4 % solution in PBS.
17. Ethanol (SIGMA).
18. Gilson pipettes or equivalent of 20, 200, and 1,000 μL.

3 Methods

3.1 Preparation of Complete Mesenchymal Stem Cell Basal Medium

1. α-MEM medium should be thawed under the biohazard safety cabinet. Meanwhile, FBS should be thawed at 25–37 °C prior to usage in a water bath. Do not thaw the FBS in a 56 °C water bath. Antibiotic solution should be thawed at 8–10 °C prior to usage.
2. Before preparing complete basal medium, FBS and antibiotic solution have to be cleaned with small 75 % of ethanol to avoid contamination after thawing and let under the biohazard safety cabinet.
3. To prepare 500 mL of complete mesenchymal stem cell basal medium: Add 50 mL of the aliquot of thawing FBS, and add 5 mL of antibiotic solution and 200 mM of L-glutamine solution. Once prepared and all components are mixed, the complete basal

medium should be filtered by putting the medium on bottle of 500 mL Corning Incorporated filter system and used vacuum for filter under the biohazard safety cabinet.

4. Close with cap and label with complete basal medium and date of preparation.
5. Complete basal medium should be prepared in volumes that can be used within 1 month.

3.2 Cell Culture of Human Mesenchymal Stem Cells

1. Prewarm complete basal medium in biohazard safety cabinet.
2. Take the tissue culture-treated 35 mm dishes or T-25 cm^2 flasks for seeding cells under biohazard safety cabinet and label with name of cells, in our case hMSC, date of seeding, and passage of culture.

3.2.1 Protocol for Cryopreserved MSC Cells

1. Remove the cryovial from the liquid nitrogen container and immediately place it on dry ice even for short transportation.
2. Submerge the vial into a water bath at 37 °C and continuously agitate for 90 s until ice in the cryovial is no longer visible.
3. After thawing the cells, thoroughly rinse the cryovial with 75 % ethanol to avoid microbial contamination. Then wipe the vial with a tissue.
4. Open the vial under biohazard safety cabinet and resuspend the cells by carefully pipetting up and down.
5. Transfer the cells to a 10 mL of prewarmed medium onto 15 mL conical tube and then pipetting up and down.
6. Centrifuge the suspended cell at 200 × *g* for 10 min.
7. Decant the medium and gently resuspend the pellet in 10 mL of complete basal medium.
8. Transfer to a tissue culture-treated plate or a T-25 (25 cm^2) culture flask.
9. Place the tissue culture flask in an incubator at 37 °C with 5 % of CO_2 and 90 % humidity for cell attachment.
10. Replace the medium after 16–24 h. The cells will be ready to pass between 3 and 7 days and should be subcultured, according to the subcultivation protocol, once they have reached 70–90 % confluency.

3.2.2 Subcultivation Protocol

1. After reaching 70–90 % of confluency cells are ready for passage (11–13).
2. Count the number of cells using hemocytometer and plate the cells at density of 5,000–10,000 cells/cm^2 or desired plating density.
3. The passage and date should be labeled on the new tissue culture plate or a T-25 (25 cm^2) culture flask.

4. Place the complete basal medium and PBS sterile solution under biohazard safety cabinet for prewarming.
5. Take out from freezer the trypsin–EDTA aliquot and place at room temperature for at least 30 min to adjust the temperature of the reagent.
6. In the biohazard safety cabinet, carefully aspirate the medium from cell monolayer to remove and discard from the cell culture plate.
7. Add 5–10 mL of PBS solution to wash the cell monolayer and pipette up and down being careful not to disturb the cell monolayer.
8. Aspirate carefully the PBS solution from the culture plate and add 3–5 mL of trypsin–EDTA/cm^2 of the culture plate surface to ensure that the entire monolayer is covered with the solution (see Note 4).
9. Add 5 mL of complete basal medium for neutralization of trypsin–EDTA and gently pipette the cells up and down until the cells are dispersed into a single suspension.
10. Carefully aspirate the cell suspension and transfer it to a centrifugation tube. Spin down the cells for no more than 5 min at $200 \times g$.
11. Discard the supernatant, add 1 mL of complete basal medium, and resuspend the cells by carefully pipetting up and down.
12. Plate the cells according to the recommended seeding density in a new cell culture flask containing complete basal medium for MSCs.
13. Place the tissue culture flask in an incubator at 37 °C with 5 % of CO_2 for cell attachment.
14. Replace the medium after 16–24 h (see Note 5).

3.3 Preparation of Electrospun Membranes

Polymer-based solutions are processed by electrospinning to create 3D scaffold for cell culture (14).

1. The used homemade apparatus mainly consists of three components: (1) a syringe pump system (Genie Plus k600, Kent Scientific) able to control the mass flow of the solution as the process goes on; (2) two metal electrodes connected to; (3) a single polarity high voltage power supply (Gamma High Voltage Research, mod. ES30) capable of generating DC voltage in a range of 0–30 kV.
2. The apparatus is further enclosed in a Plexiglass box to insulate the system from external fields and to restrain sudden changes of environmental conditions during the electrospinning process.

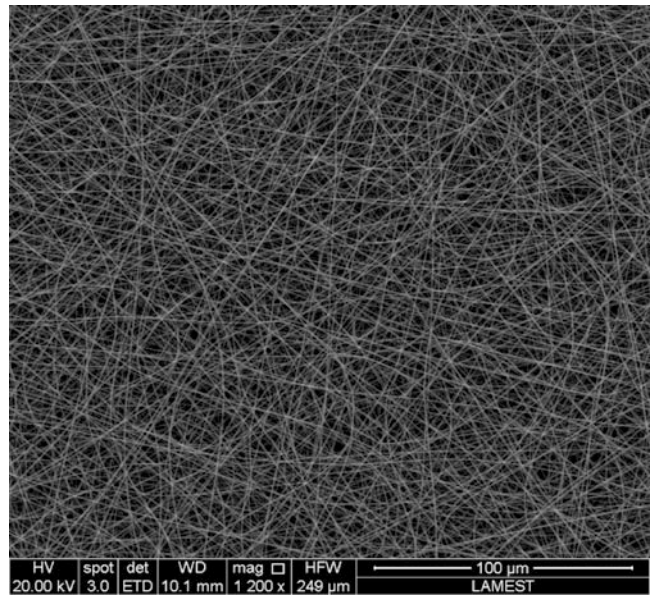

Fig. 1 SEM image of PCL and gelatin electrospun scaffold

3. The polymer solution is placed in a 5 mL syringe (BD Plastipack, Italy), fixed on the pump system and joined to a stainless steel needle with an inner diameter of 0.8 mm or less, connected to the positive polarity.
4. A grounded metallic plate covered by aluminum foil is used as collector for fiber deposition. An important parameter is the distance between the tip of the needle and the collector, which can be set between 5 and 15 cm. The process is carried out in a vertical configuration by selecting an adequate deposition time until the required thickness of the nanofibers is deposited on the grid.
5. Different fibers are realized by controlling the process parameters including the applied voltage—ranging from 1 to 30 kV—and the feed rate—typically ranging from 0.1 to 5 mL/h.
6. An example of polymer-based electrospun fibers obtained by the apparatus, previously described, is reported in Fig. 1.

3.4 hMSC Seeding onto Electrospun Membranes

To initiate the cell seeding onto electrospun membranes, follow the subcultivation protocol—see Section 3.2.2—for obtaining final cell suspension of desired passage of hMSC. Here, experimental procedure passages between 2 and 6 of hMSC are recommended.

1. Count the number of hMSC using a hemocytometer at a concentration of 5×10^5 cells/mL in complete basal medium.
2. Electrospun membranes with micro- or nanofibers are cut into 6 mm disc shape, sterilized (see Note 6), and placed

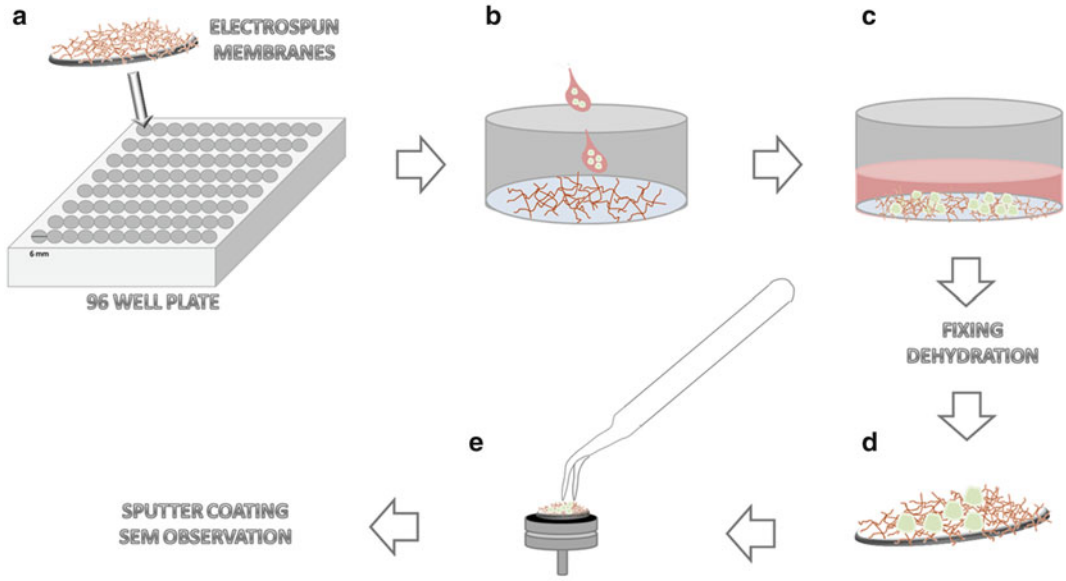

Fig. 2 Scheme of scaffold seeding, culture, and stub preparationz

into each well of suitably sized tissue culture plate (96-well plate) (Fig. 2a).

3. Passage of hMSC as described until the final cell suspension is added onto the electrospun membranes approximately at 1×10^4 cells per well in complete basal medium (Fig. 2b).
4. Place the tissue culture plate in an incubator at 37 °C with 5 % of CO_2 for cell attachment and grown for 24 h (Fig. 2c).
5. After the end of the incubation time, take out the tissue culture plate and let on the biohazard cabinet.
6. In the biohazard safety cabinet, carefully aspirate the medium from the electrospun membranes to remove and discard the cell culture from the plate.
7. Rinse three times for removing nonattached cells with sterile PBS solution pH 7.4.

3.5 hMSC Cell Morphology by Electron Microscopy

1. Samples are preliminarily fixet to freeze cell body shapes by incubation in 4 % paraformaldehyde PBS solution at 4 °C.
2. After time, wash with PBS three times, samples are ready for the dehydration process.
3. Cell-fixed samples are dehydrated at least 5 min in graded series of ethanol (25–100 %).
4. Ethanol traces are completely removed by fast drying under clean environmental conditions (i.e., biological hood).
5. Samples are mounted onto metal stubs (Fig. 2e).

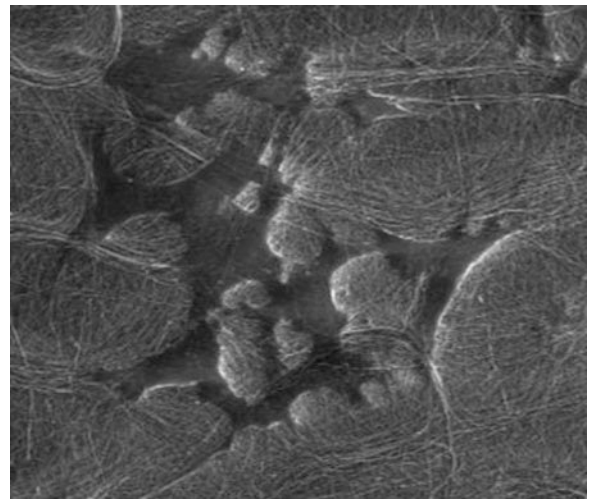

Fig. 3 SEM of hMSC onto PCL electrospun fibers after 24 h: Spot value 5.5

6. Sample surfaces are preliminarily treated by metal coating (i.e., gold/palladium) by vacuum controlled sputtering equipment (Emitech, Italy) in order to improve the electronic conductivity of the surface. The setting of sputtering process parameters, voltage and current, allows for the deposition of a thin layer of about 19 nm which helps to accurately scan the sample without altering the surface properties.
7. Samples are examined by Fields Emission Scanning Electron Microscopy (FESEM) (Quanta-FEG 200, FEI, The Netherlands) under high vacuum conditions.
8. SEM investigation is adapted to the specific properties of cell-loaded samples. In particular, voltage and spot are set to avoid any cell destroy ascribable to high local concentration of the beam energy onto the sample. Below, SEM images of cell-loaded samples evaluated for high spot (see Note 7) values (Fig. 3).

4 Notes

1. Storage at −80 °C is not sufficient for cell preservation and causes irreversible cell damage.
2. Do not freeze–thaw more than twice. Do not thaw at temperature greater than 37 °C.
3. Do not freeze–thaw more than twice. Do not thaw at temperatures greater than 37 °C; thawing at 8–10 °C is recommended.
4. We recommend detaching the cells incubating for no more than 5 min at 37 °C and always examining the cells under a microscope.

When the cells start to detach, gently tap the side of the culture flask to loosen the remaining cells.

5. The cells should be treated as subcultivation protocol for seeding the MSCs onto the electrospun membranes, once they have reached 70–90 % confluency (11–13).
6. For sterilization submerge the electrospun membrane onto 70 % ethanol in PBS with 5 % antibiotic solution (penicillin/streptomycin) and air dried under biohazard cabinet.
7. During SEM observation we recommend not to set high spot (Fig. 3) and high voltage values, which can produce an overcharge of the cell matter, thus altering the morphology of the cell bodies, and also drastically reducing the quality of the image.

Acknowledgements

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General Protocol for the Culture of Cells on Plasma-Coated Electrospun Scaffolds

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Abstract

As opposed to culture on standard tissue-treated plastic, cell culture on three-dimensional scaffolds impedes additional challenges with respect to substrate preparation, cell seeding, culture maintenance, and analysis. We herewith present a general route for the culture of primary cells, differentiated cells, or stem cells on plasma-coated, electrospun scaffolds. We describe a method to prepare and fix the scaffolds in culture wells and discuss a convenient method for cell seeding and subsequent analysis by scanning electron microscopy or immunohistology.

Keywords: Tissue engineering, Electrospinning, Plasma coating, Cell culture, Scanning electron microscopy, Fluorescence microscopy

1 Introduction

Cell culture in three-dimensional systems, the so-called scaffolds, has gained increasing interest during the last decade and found wide application in different fields of basic research and tissue engineering. General cell culture protocols and analytical approaches, however, are optimized for cell culture of adhesion-dependent cells on tissue culture-treated polystyrene (TCPS) and can only marginally be translated to polymeric, porous systems. The culture of cells on porous, three-dimensional scaffolds demands for further consideration of scaffold preparation and sterilization as well as cell seeding procedures and analytical strategies. Particularly, the non-transparent nature of the scaffold, its solubility in organic solvents, and enhanced thickness impede major challenges with respect to immunohistological approaches and microscopy. Exemplary, we herewith present the seeding and microscopical analysis of cells on electrospun, plasma-coated scaffolds of poly(ε-caprolactone) (PCL).

PCL is an FDA-approved polymer that found wide application in the field of medical devices such as sutures, drug delivery vehicles, or scaffolds (1, 2). PCL is biocompatible and biodegradable and can easily be processed into porous scaffolds by various techniques, among them, film casting, freeze drying, phase separation, gas foaming, particle leaching, rapid prototyping, or, most importantly, electrospinning (3–7).

Electrospinning was initially invented and patented in the 1930s by Anton Formhals (8). In brief, an electrical field is generated between a collector and an extrusion electrode or spinneret. A syringe pump provides constant flow of a polymer solution through the needle. The drop of the polymer solution at the orifice will become electrically charged, forming a Taylor cone and emerging as a jet once the electrical field strength overcomes the surface tension of the solution. The jet dries up and is directed towards a grounded or a charged collector where fibers are assembled as a nonwoven mesh. The small fibers display a high surface-to-volume ratio, allowing for enhanced protein adsorption (9) whereas the porous structure of fibrous substrates allows for nutrient diffusion. Electrospinning parameters can be tailored to generate substrates that meet application-specific architectures regarding fiber diameter and orientation, substrate thickness, and shape (10–12). Since the 1990s, electrospinning has been considered as a versatile method for the fabrication of fibers and substrates in a broader context and, among others, employed for biomedical applications and tissue engineering (2, 13–15).

Synthetic polymers are readily available and easily processed, but do not necessarily provide chemical surface properties to facilitate cell adhesion. Scaffold surface functionalization became an interesting subject to combine polymer bulk properties with an adequate biointerface. Oxygen functional groups, such as carboxyl, carbonyl, or hydroxyl groups, are known to enhance cell adhesion (16). In recent years, plasma treatment or plasma polymerization processes evolved as interesting strategies to introduce these functional groups onto synthetic polymers. Plasma is a gas of excited particles, be it ions, electrons, or radicals. Collisions between these highly energetic particles cause chemical reactions to occur and new bonds to be generated. Film growing species are then deposited at the surface, forming functional plasma polymer layers. Depending on process parameters such as gas flow, gas ratio, and power input, layer thickness, cross-linking properties, and degree of functionalization can be closely adjusted (17–20). We applied plasma polymerization processes to superimpose an oxygen functional hydrocarbon layer on the electrospun PCL fibers and found enhanced cell adhesion (21).

Despite multiple approaches in various fields of tissue engineering, cell seeding on scaffolds and microscopical analysis thereon have not been described in detail. Protocols vary from group to group and researchers of different areas are confronted with similar problems. Therefore, we report on a general seeding procedure of primary cells, stem cells, or cell lines on functionalized scaffolds and provide some hints on how to prepare samples for microscopical analysis. Electrospun and plasma-coated scaffolds are blanked using biopsy punches, fixed on silicone-coated well plates, and seeded with a drop of a highly concentrated cell suspension. The herewith presented protocol has been established and evaluated with murine skeletal myoblasts (cell line C_2C_{12} and primary myoblasts), rat mesenchymal stem cells (MSC), mouse embryonic stem cells (ECS), and human umbilical vein endothelial cells (HUVEC) and was found to be appropriate for all evaluated cell types.

The protocol addresses scientists that are already familiar with or use an electrospinning device and a plasma reactor. We do not provide detailed steps for how to build these setups. We do, however, provide information regarding our setup and the utilized components.

2 Materials

2.1 Electrospinning

1. High voltage power supply (Aip Wild AG, Switzerland).
2. Stainless steel collector (manufactured in-house).
3. Grounded Faraday cage.
4. Syringe Pump (KD scientific, USA).
5. Blunt Needle, inner diameter of 0.8 mm (Unimed S.A., Switzerland).
6. Syringes, 5 mL (BD, USA).
7. Solvents: Chloroform ($CHCl_3$, $\geq$ 99 %) and methanol (CH_3OH, $\geq$ 99 %) (Sigma-Aldrich, Switzerland).
8. PCL, 70,000–90,000 g/mol (Sigma-Aldrich, Switzerland).

2.2 Plasma Polymerization Process

1. Reaction chamber: Custom-built batch reactor with symmetric, plane parallel electrodes (size of 30 cm in diameter, gap of 5 cm).
2. 13.56 MHz Radio Frequency generator (Dressler Cesar 133, Germany), capacitively coupled to the reactor chamber.
3. Gas supply via a showerhead in the upper electrode controlled by mass flow controllers.
4. Gases: Ethene (C_2H_4), carbon dioxide (CO_2) and argon (Ar) ($>$ 99 % purity, Carbagas, Switzerland).
5. Substrates are placed on the lower electrode opposite to gas inlet.

2.3 Cell Culture

1. TCPS Dishes (TPP Omnilab Switzerland and BD, Biosciences USA).
2. Sylgard-184 two component silicone (Sylgard, Dow Corning Corporation, USA).
3. Biopsy Punches, 6 mm diameter (Miltex, Germany).
4. Minutiae insect pins (EntoSPHINX, The Czech Republic).
5. Cell culture medium and culture medium supplements depending on the cell type.
6. Phosphate-buffered saline (PBS).
7. CM-Dil fluorescent marker (Invitrogen, USA).

2.4 Analysis

1. 4 % Formaldehyde.
2. Ethanol: 100, 94, 80, and 70 %.
3. Hexamethyldisiloxane (HMDSO) (Sigma-Aldrich, Switzerland).
4. Carbon conductive double sided adhesive tape (SPI Suppliers, USA).
5. Gold sputtering: Polaron Equipment, SEM coating Unit E5100 (Kontron AG, Switzerland).
6. Scanning electron microscope Hitachi S-4800 (Hitachi High-Technologies, Canada).
7. Antibodies according to the experiment.
8. Aqueous mounting medium (Glycergel, Dako, USA).
9. Microscopy slides and coverslips.
10. Fluorescent microscope, Nikon Eclipse 2000 (Nikon, Japan).

3 Methods

3.1 Electrospinning of Micron-Scaled Fibers

1. Add 3 g PCL to 20 mL solvent mixture (18 mL chloroform, 2 mL methanol) and shake overnight in a well-sealed glass container (see Note 1).
2. Electrostatic spinning of the solution: We applied a voltage of 10 kV on the needle and −2 kV on the collector (see Note 2). The polymer solution was provided by the syringe pump at a flow rate of 30 μL/min.
3. To produce scaffolds of 200–300 μm, spin for 20–30 min (see Notes 3 and 4).
4. Cut out a small piece of the prepared patch to image by SEM (see Notes 5 and 6). Add the scaffolds to carbon-conductive double-sided adhesive tape and SEM sample holders. Prior to imaging, gold sputter the constructs to add a gold layer of approximately 5–7 nm for better conductivity.

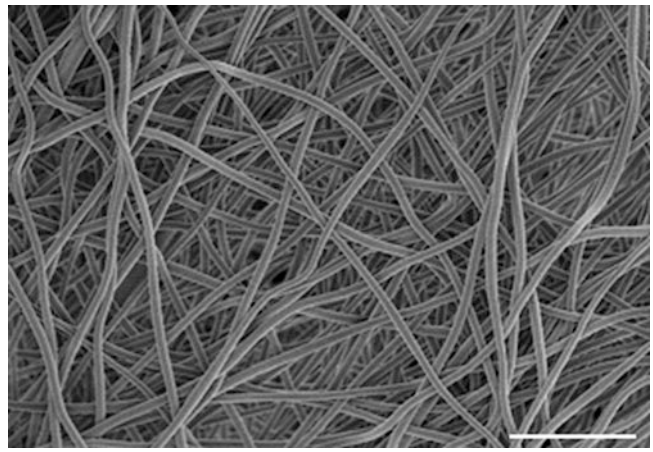

Fig. 1 SEM image of electrospun fibers. Randomly oriented fibers, displaying diameters in the micrometer range (2–3 μm), were assembled on a planar, static steel plate. Scale bar: 50 μm

5. Images were acquired at an accelerating voltage of 2 kV and a current of 10 μA. Higher voltage can melt the fibers (see Note 7 and Fig. 1).

3.2 Plasma Coating—Application of an Oxygen Functional Hydrocarbon Layer

1. Clean the batch reactor with 70 % ethanol prior to any use (see Note 8).
2. Put the spun patches on the lower electrode and fix the corners with tape (see Note 9).
3. Evacuate the plasma reactor (see Note 10) and start the plasma process when the chamber pressure is below 10^{-3} mbar.
4. Set the gas ratio to 4 sccm ethene (C_2H_4), 24 sccm carbon dioxide (CO_2), and 50 sccm argon (Ar). Set the power input to 34 W. Start the plasma process and coat the electrospun patches for 15 min (see Note 11).
5. Stop the plasma process, float the chamber, turn the patches, and proceed similarly for the second side of the patch (see Note 12).

3.3 Cell Seeding

1. Prepare the two-component silicone according to the manufacturer (10:1 ratio) and coat the bottom of 48-well culture dish. Fill the wells up to one-third and let the silicone dry for 48 h (see Note 13).
2. Prepare the culture substrates by blanking scaffolds of 6 mm diameter from the electrospun patches. Put the scaffolds on the dried silicone and fix them with insect pins in the middle (see Note 14). Do not place patches in all wells, but leave one row of empty wells around the patches. Sterilize the dishes under UV light (cell culture hood) for at least 5 h (see Note 15).

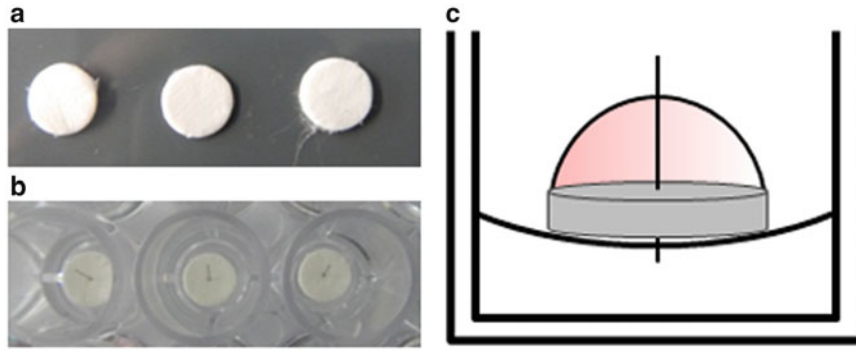

Fig. 2 Scaffold preparation for cell culture. (**a**) Scaffolds of 6 mm diameter were blanked using biopsy punches. (**b**) Scaffolds were then placed in silicone-coated 48-well dishes and fixed with insect pins. A 50 μL drop of cell suspension was then added to the scaffolds. (**c**) Schematic illustration of the setup

3. Culture and expand cells according to your general protocol. Cell seeding and culture must be accomplished under aseptic conditions.
4. To seed cells on the 6 mm scaffolds, a cell suspension volume of 50 μL is appropriate. Prepare the cell solution concentration according to your experiment. We recommend a cell concentration of 100,000–250,000 cells per scaffold, depending on the experiment (see Note 16). Gently depose a drop of 50 μL on the scaffold (see Note 17 and Fig. 2). Fill the empty wells around with sterile PBS and let the cells adhere for 3 h (in the incubator) prior to medium addition.
5. Gently add 250–500 μL culture medium to the wells (see Notes 18 and 19).
6. Incubate the cells under classic culture conditions, at 37 °C and 5 % CO_2.
7. Change medium regularly by aspirating the medium and gently adding new medium. Do not let the scaffolds dry upon medium change.
8. To visualize cells during the culture period, cells can be stained with a fluorescent cell-tracking agent. Prepare a stock solution of 1–2 mg/mL CM-Dil in 100 % ethanol and dilute it in culture medium to a final concentration of 1–2 μg/mL. Incubate the cells for 15 min at 37 °C, followed by incubation at 4 °C for 15 min. Aspirate the CM-Dil solution, rinse constructs with fresh medium, and continue the cell culture. Cells can be imaged under a fluorescent microscope at 549 nm excitation and 565 nm emission wavelength (see Note 20).

3.4 Analysis

1. For SEM imaging, aspirate the medium and wash the constructs gently in PBS. Fix the cells in formaldehyde. Remove the formaldehyde and dry the constructs in an ascending ethanol series (70, 80, 94, and 100 %). Incubate the patch under gentle shaking for 30 min in each ethanol solution. Incubate in 100 % ethanol for 1 h, by refreshing the solution twice. Aspirate the ethanol, add HMDSO, and incubate for 5 min under a chemical hood. Aspirate the HMDSO and depose it in the solvent waste bin. Let the constructs air-dry, close the dishes, and seal with parafilm (see Note 21).
2. Add the scaffolds to carbon-conductive double-sided adhesive tape and SEM sample holders (see Note 6). Acquire images as soon as possible, preferentially on the same day.
3. Prior to imaging, gold sputter the constructs to add a gold layer of approximately 10–20 nm. Acquire images at an accelerating voltage of < 2 kV and a current of 10 μA (see Note 7 and Fig. 3).

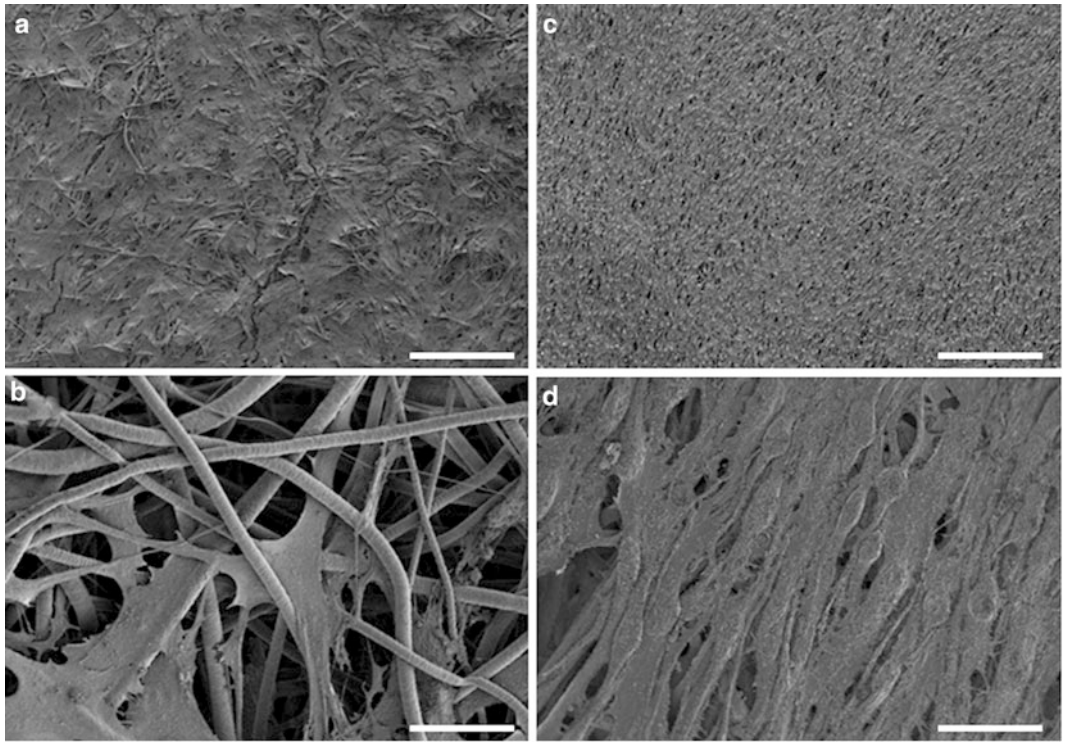

Fig. 3 SEM images of cells cultured on electrospun fibers. (**a**, **b**) Skeletal myoblasts (C_2C_{12}) on electrospun fibers at lower and higher magnification. (**c**, **d**) Endothelial cells (HUVEC) on electrospun fibers. SEM imaging allows to visualize cell–fiber interactions and clearly shows the distinct morphology of different cell types. Skeletal myoblasts spread and attach between the fibers, whereas endothelial cells assemble into parallel oriented patterns. Scale bars: (**a**, **c**) 250 μm, (**b**, **d**) 25 μm

4. For immunohistological analysis, general protocols can be used. Constructs need to be fixed (4 % formaldehyde), permeabilized (triton X-100), and blocked in serum (3 % BSA in PBS). Constructs can then be incubated with the primary antibody, followed by the secondary antibody. 100 µL per patch are enough. See further recommendations in the Notes section especially on washing and mounting (see Notes 22 and 23).

4 Notes

1. For stable fiber production, it is crucial to have a homogeneous spinning solution. Let the PCL dissolve long enough (overnight) and under constant shaking to achieve an appropriate spinning solution.
2. An electrospinning setup can also be built-up without a voltage supply connected to the collector, but by use of a grounded collector. However, the application of a negative voltage to the collector directs the jet in a more efficient manner. An increasing thickness of the spun patch results in increasing insulation of the collector and shielding of the electrostatic field. The negative voltage can be adjusted stepwise to compensate for the insulating effect of the deposed fibers. Different types of collectors can be used. A rotating drum for instance allows the production of parallel oriented fibers (21, 22). Further, by using acetic acid and pyridine as a solvent, nanoscaled fibers, displaying diameters of 100–300 nm, can be produced (21, 23).
3. The spinning duration and consequently the thickness of the patch strongly depend on the spinning parameters, in particular on the flow rate and the voltage. It needs to be adapted accordingly. We found a spinning duration of less than 30 min to be appropriate at the given parameters.
4. We recommend a patch thickness of 200–300 µm. Thinner patches are more difficult to handle and to further process, whereas thicker patches impede problems for mounting with coverslips on microscopy slides.
5. We experienced that even spinning at constant parameters can lead to fibers of varying diameters. We therefore strongly recommend to check for fiber morphology by SEM prior to all experiments. For fiber diameter assessment, we used Image J (http://rsbweb.nih.gov/ij/download.html) and measured 100 individual fibers on at least five different pictures.
6. Commercially available carbon-conductive adhesives for SEM imaging are covered with a thin layer of glue that potentially dissolves the fibers. Take care to evaluate the SEM sample

adhesives with a test sample prior to imaging your real scaffolds. Press only gently on the scaffolds to fix them to the support.

7. Acquisition parameters, i.e., voltage and current, highly depend on the used SEM and need to be adjusted accordingly. We acquired images with collectors in the mixed mode (M), detecting backscattered and secondary electrons. The working distance was set to 8.6 mm. The chamber was under high vacuum, i.e., < 10E-5 mbar.
8. Plasma reactors are often used for various plasma or sputtering processes. We therefore recommend to clean the reactor prior to any use and make sure that no particles or other contaminations are found within the reactor. Remaining particles and desorbing gases could induce impurities in the plasma layer. Depending on the history of the plasma reactor (remaining depositions on the wall containing detrimental elements) further cleaning steps might be required.
9. We found it easiest to fix the patches on the electrode with simple tape (e.g., made of polypropylene or polyimide). Make sure you only use small amounts of tape, avoiding strong outgassing. Potential drawbacks are some impurities that could have an influence on the generated plasma layer. However, we always found reproducible, homogeneous plasma layers on all substrates. Other fixation methods (as for example by manually adding one or two staples) are also possible.
10. The sudden vacuum during chamber evacuation can cause some folding or moving of the patches. Ensure that the patches are flat and well adherent prior to the process. Wrinkles or folds will result in heterogeneous plasma deposition. Using a butterfly valve towards the pumping unit, the evacuation process might be slowed down.
11. The gas ratio and flow rate as well as the power input have a significant impact on the plasma layer formation, the crosslinking, and the degree of functionalization (18–20). Further, the power input has an impact on the mechanical properties of the fibers and needs to be adapted accordingly. We suggest rather low power inputs (around 30 W) for the used reactor size and thus lower monomer gas flow rates yielding longer plasma process durations. Note that nominal power supplied to the plasma reactor is typically higher than the absorbed power in the plasma (due to power losses). We typically achieved 80–90 % absorbed power as measured by V/I probe. Plasma process parameters need to be optimized according to the user's setup.
12. We recommend to coat both sides of the patch. During handling and cell culture, it is likely that the patches will be turned. A homogeneous coating on both sides reduces mistakes.

13. We found it very convenient to prepare several silicone-coated culture dishes in advance. They can be stored at room temperature until further use. We also find it convenient to prepare several petri-dishes of different sizes to transfer patches or to perform later analysis.

    The silicone will form many bubbles. Degasing, however, is not necessary, since the bubbles will disappear after a while and the silicone will dry. 50 mL two-component silicone is usually enough to coat two 48-well dishes and some additional petri-dishes. Do not fill the wells too high (only up to 1/3), since it will reduce the amount of medium that can be added during cell culture.
14. Insect minutiae pins are available in different sizes. We used insect pins of 0.15 mm in diameter. The pins do easily bound when inserted into the silicone. Place them carefully and with sharp tweezers. We recommend placing them exactly in the middle of the patches since it will facilitate subsequent cell seeding. For convenience, thicker insect pins can also be used, especially for training and optimization purposes.
15. Sterilization depends on the intensity of the UV lamp. 5 h seemed appropriate in our settings and we did not encounter problems of contamination. Sterilization overnight is also possible. The UV light, however, induces cross-linking and chain scission processes and changes the mechanical properties of the electrospun fibers. Make sure that the sterilization under UV light does not exceed 12 h.
16. Cell concentration largely depends on the planned experiment. For imaging purposes (i.e., immunohistology and SEM) we used smaller quantities of cells or less per patch. A higher cell number is usually too dense to distinguish between individual cells. For tissue formation and cell differentiation, we used a cell density of 250,000 per patch. For reproducible results, it is crucial to prepare a highly homogeneous cell suspension of single cells. We recommend to use a 2 mL pipette and homogenize the cell clots after centrifugation by pipetting up and down in fresh medium for at least 25 times. For smaller cell suspension volumes, 1 mL Eppendorf pipettes are also recommended. Make sure not to produce any foam or bubbles since this will have an impact on the final concentration.
17. Despite the plasma coating, the patches are still relatively hydrophobic and a drop is easily formed on the surface. Take care to gently depose the drop on the patch. Stable deposition can be achieved by directly adding the drop to the insect pin in the middle. Once the drop touches the wells or the bottom of the dish, it is very difficult to bring it back into the correct position. Gentle and careful seeding is highly recommended.

Seeding can be trained with PBS or water and on the bench without cells.

18. Add the medium very gently by touching the well with your pipette tip and slowly releasing the medium to the wall. Ensure that the whole patch is covered. We recommend a minimal medium volume of 250 μL. Depending on the volume, medium change must be accomplished more regularly. Keep in mind that the cell density and resulting nutrient consumption on the patch are much higher than it would be for cells adherent to the bottom of the 48-well plate (lower cell number).
19. Experimental studies with the culture of C_2C_{12} murine skeletal muscle cells in our group indicated that the amount of medium (i.e., 250 μL vs. 500 μL) has a significant effect on myotube formation. Namely, we found that a reduction in volume resulted in increased myotube formation. We recommend to optimize culture medium volume according to the experimental settings.
20. CM-Dil labelling of cells on the scaffold does not result in clearly stained cells and well-visualized contours. It, however, allows to have an idea of the cell density on the patches. In particular, the differentiation of stem cells into contractile skeletal or cardiac muscle cells and their movement can be visualized with this approach—without harvesting the cells and terminating the culture.
21. A frequent method to dehydrate materials for subsequent analysis by SEM is critical point drying. The method often involves acetone as a solvent and a temperature higher than 60 °C. PCL presents a melting point of 60 °C and is partially soluble in acetone. Critical point drying is therefore not an adequate method and we suggest dehydration with ethanol and HMDSO.
22. For immunohistology, general protocols of fixation, permeabilization, and antibody incubation can be followed. Incubation under gentle shaking is recommended. Ensure to increase the time of all washing steps. We recommend at least three washings of 5 min (under gentle shaking) after every step. Chromophores of HRP systems or fluorescently labelled secondary antibodies tend to adhere to the fibers and result in nonspecific background staining. To reduce background, make sure to wash the patches excessively after the secondary antibody incubation. A washing of 30 min under gentle shaking is recommended. Background staining (especially due to light diffraction of the fibers) can also be reduced by incubation with Sudan black (24).
23. PCL is soluble in most organic solvents. Use aqueous mounting media for mounting after immunohistology. Eukitt or comparable mounting media are not recommended and can dissolve

the fibrous structure of the scaffold. We recommend mounting media that solidify and do not need to be sealed with nail varnish. The increased thickness of the patch, as compared to glass slides, requires a solid mounting medium. Place a drop of mounting medium on the microscopy slide, add the patch (cell-side facing upwards), place a drop of mounting medium on the scaffold, and gently add the coverslip. Ensure that no bubbles are formed and that the whole patch is well embedded. Constructs can then be imaged with a fluorescent or a light microscope.

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Mesenchymal Stem Cells and Nano-structured Surfaces

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Abstract

Mesenchymal stem cells (MSCs) represent multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). Their multi-potency provides a great promise as a cell source for tissue engineering and cell-based therapy for many diseases, particularly bone diseases and bone formation. To be able to direct and modulate the differentiation of MSCs into the desired cell types in situ in the tissue, nanotechnology is introduced and used to facilitate or promote cell growth and differentiation. These nano-materials can provide a fine structure and tuneable surface in nanoscales to help the cell adhesion and promote the cell growth and differentiation of MSCs. This could be a dominant direction in future for stem cells based therapy or tissue engineering for various diseases. Therefore, the isolation, manipulation, and differentiation of MSCs are very important steps to make meaningful use of MSCs for disease treatments. In this chapter, we have described a method of isolating MSC from human bone marrow, and how to culture and differentiate them in vitro. We have also provided research methods on how to use MSCs in an in vitro model and how to observe MSC biological response on the surface of nano-scaled materials.

Keywords: MSCs, Cell differentiation, Bone formation, Scaffold, Nano-structured surfaces

1 Introduction

Stem cells are cells characterized by their abilities of self-renewal and pluripotency. The discovery of stem cells provides a great opportunity in medical application to use them for tissue engineering and treating diseases. This is revolutionizing our daily life and really starts the personalized medicine. Regardless of their originating sources, stem cells share two characteristic properties; firstly, they have the capacity for prolonged or unlimited self-renewal under certain conditions, and secondly they retain the potential to differentiate into a variety of more specialized cell types (1). Undoubtedly, human embryonic stem cells (hESCs) are able to differentiate down different lineages in an efficient manner and maintain an adequate proliferative capacity post-harvest as well (2). However, the political and ethical issues associated with hESCs have cast a shadow of doubt over their

realistic potential for implementation in tissue engineering strategies (3). Thus, mesenchymal stem cells/multipotent stromal cells (MSCs) have emerged as a promising cell source in the ongoing research of bone tissue engineering (4). Numerous studies have demonstrated that MSCs derived from various origins, including skin, hair follicle, periosteum, bone marrow, adipose tissue, umbilical cord blood, and so on, are able to differentiate into various phenotypic lineages, such as cartilage, bone, fat, and nerve in vitro and in vivo (5–15).

The development of nanotechnology has advanced our life in many aspects including these related to energy, environment, and medicine. In medical area, nano-materials provide us new tools to look into the diseases (such as imaging and diagnostic applications) and deliver drugs or treatments. In addition, nano-materials revolutionize tissue engineering by providing a fine structure (scaffold) conductive for tissue regeneration. Indeed, the interaction between MSCs and the fine scaffold surface (or nanosurface) is crucial for the properly directed differentiation of MSCs in tissue regeneration.

Nanosurfaces may be defined as surfaces that have been modified using nanotechnological concepts thereby transforming them to include delicate "nano" scale structures in the range of 1–100 nm. Nanotechnology has the capability to manipulate matter at atomic and molecular levels. This enables us to utilize nanotechnological maneuvers to guide MSCs towards differentiation to mature cell types (16). Rapid improvements in technological advancements have enabled us to incorporate different kinds of nanostructures onto implant surfaces. Such surfaces are widely used for clinical purpose in the fields of orthopedics, dentistry, and tissue engineering. Such surfaces are also used to create scaffolds, medical devices, and drug delivery systems (17, 18). With rapid development of nanotextured surfaces for use as implant surfaces, it becomes necessary for us to understand the interactions of such surfaces with stem cells before they can be used for in vivo and clinical studies eventually.

In this chapter, we will describe a method of how to isolate MSCs from human bone marrow and how to use these cells to differentiate into bone forming cells in vitro and in vivo. The influence of nanosurface on MSC differentiation will also be described.

2 Materials

2.1 Isolation and Expansion of Human MSCs from Bone Marrow

1. Lymphoprep™ (Axis-shield PoC AS, Norway).
2. Low glucose Dulbecco's Modified Eagle's Medium (DMEM-LG; Gibco®, Life Technologies Pty Ltd., Australia).
3. Fetal bovine serum (FBS; In Vitro Technologies, Australia).

4. Penicillin/Streptomycin (P/S; Gibco®, Life Technologies Pty Ltd., Australia).
5. 10 % Heparin (Gibco®, Life Technologies Pty Ltd., Australia).
6. 0.05 % Trypsin–EDTA (Gibco®, Life Technologies Pty Ltd., Australia).
7. Phosphate-buffered saline (PBS; Gibco®, Life Technologies Pty Ltd., Australia).
8. Hank's balanced salt solution (HBSS; Gibco®, Life Technologies Pty Ltd., Australia).
9. 20 mL syringes.
10. Jamshidi® bone marrow biopsy/aspiration needles.
11. 100 μm cell strainer (BD Bioscience, USA).
12. 15 and 50 mL Falcon™ tubes (BD Bioscience, USA).
13. 25 and 75 cm^2 tissue culture flasks (BD Bioscience, USA).
14. 5, 10, and 25 mL Serological pipettes (BD Bioscience, USA).

2.2 Characterization

1. MSC surface marker antibodies or their antibody conjugates with fluorescence materials from different companies.
2. Flow cytometry facility.

2.3 In Vitro Differentiation

1. High glucose Dulbecco's Modified Eagle's Medium (DMEM-HG; Gibco®, Life Technologies Pty Ltd., Australia).
2. Dexamethasone (Dex; Sigma-Aldrich, Australia).
3. β-Glycerophosphate (βGP; Sigma-Aldrich, Australia).
4. L-Ascorbic acid-2-phosphate (AsAP; Sigma-Aldrich, Australia).
5. Sodium pyruvate (Sigma-Aldrich, Australia).
6. Proline (Sigma-Aldrich, Australia).
7. Insulin transferin selenimn-plus (ITS-plus; consisting of 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenious acid, 5.33 μg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin; Sigma-Aldrich, Australia).
8. Transforming Growth Factor beta-3 (TGF-β3; Sigma-Aldrich, Australia).
9. Chondrogenic base medium (CBM), consisting of DMEM-HG supplemented with 1 % P/S, 100 nM Dex, 50 μg/mL AsAP, 100 μg/mL sodium pyruvate, 40 μg/mL proline, and ITS-plus.
10. Chondrogenic induction medium (CIM), consisting of chondrogenic base medium supplemented with 10 ng/mL TGF-β3.
11. Adipogenic induction medium (AIM), consisting of DMEM-HG supplemented with 10 % FBS, 1 % P/S, 1 μM dexamethasone, 100 μM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/mL insulin.

12. Adipogenic maintenance medium (AMM), consisting of DMEM-HG supplemented with 10 % FBS, 1 % P/S, and 10 μg/mL insulin.
13. Tissue culture flasks or multiwell cell culture plates (BD Bioscience, USA).

2.4 In Vivo Application

1. Cell-scaffold constructs.
2. Ketamine hydrochloride (Troy Laboratories, Australia).
3. Xylazine (Troy Laboratories, Australia).
4. 0.9 % physiological saline.
5. 1-mL syringes.
6. Needles, 25–27 gauge.
7. Surgical instruments, e.g., blade, fine scissors, blunt forceps, sharp forceps, staple sutures.
8. Betadine and alcohol swabs.

2.5 MSCs on Nanosurfaces

1. Expansion and cell differentiation medium as described in Sects. 2.1 and 2.3.
2. Methylthiazolyldiphenyl-tetrazolium (MTT) (Sigma-Aldrich, Australia).
3. Dimethyl sulfoxide (DMSO) (Univar, Ajax Finechem Pty Ltd, Australia).
4. Triton® X-100 (Merck, Germany).
5. PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Germany).
6. Complete ULTRA tablets, Mini, EDTA-free Protease Inhibitor cocktail (Roche, Germany).
7. TRIzol® Reagent (Ambion, Life Technologies, USA).
8. Chloroform–isoamyl alcohol (24:1) (Sigma-Aldrich, USA).
9. Isopropanol (Sigma-Aldrich, USA).
10. Ethanol (Sigma-Aldrich, USA).
11. UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Life Technologies, USA).

3 Methods

3.1 Isolation and Expansion of Human MSCs from Bone Marrow

The first MSC population to gain significant attention from researchers, and provide the largest contribution to the current knowledge regarding the multilineage differentiation potential of MSCs, was derived from the bone marrow cavity in tight contact with and supporting the hematopoietic compartment (19). Bone marrow-derived MSCs (BMSCs) have been identified as a population of organized hierarchical postnatal stem cells with the potential to undergo osteogenic, as well as chondrogenetic and adipogenic differentiation when

exposed to appropriate microenvironmental cues (20). Here in this chapter, human BMSCs are taken as an example to detail the isolation and expansion protocol currently used in our lab.

3.1.1 Aspiration of Human Bone Marrow

Bone marrow aspirates were collected from patients undergoing elective orthopedic surgeries or samples donated for research after receiving informed consent from donors and Institutional Ethical Clearance. The protocol for bone marrow aspiration during elective knee replacement surgeries by orthopedic surgeons has been described here. All experiments related to human samples were approved by Queensland University of Technology Human Ethics Committee and the Prince Charles Hospital Human Ethics Committee.

1. Locate the femoral canal during standard knee replacement surgery.
2. Make a femoral drill hole through the distal femur and pass an intramedullary rod into the femoral canal.
3. Insert the aspiration needle with stylet into marrow cavity. Once the needle is anchored, remove the stylet.
4. Draw a small amount (1–2 mL) of 10 % heparin into a 20-mL syringe. Wet the interior of the syringe and expel all heparin.
5. Attach the heparinized syringe to the needle, then apply strong and quick suction to obtain approximately 2–3 mL bone marrow contents (Note 1).
6. Remove the needle with syringe attached with slight twisting motion.
7. Immediately remove the syringe from the needle and bring back the bone marrow samples to the laboratory.

3.1.2 Isolation and Expansion of BMSCs

Isolation and Seeding of BMSCs

1. The bone marrow sample is placed in 50-mL Falcon™ tube containing 5 mL PBS supplemented with 200 U/mL heparin.
2. After straining through a 100-μm cell strainer, the sample is mixed with HBSS to make a total volume of 15 mL in a 50-mL Falcon™ tube.
3. 10 mL of Lymphoprep™ is gently layered under the sample (Note 2).
4. The sample is then centrifuged at 400 × *g* without acceleration or brake for 20 min at 20 °C.
5. The buffy coat layer located at the interface between the HBSS and Lymphoprep™ is collected and further resuspended in 1 mL DMEM-LG supplemented with batch-tested 10 % (v/v) FBS and 1 % (v/v) P/S.
6. Plate the cell suspension into a 75 cm^2 tissue culture flask and place it in a 37 °C humidified tissue culture incubator containing 5 % CO_2.
7. The medium is unchanged for the initial 5 days, and then changed every 3 days.

Expansion of BMSCs

The primary cell culture contains a mixture of cells, including erythrocytes and leukocytes, which are not capable of attaching to the culture flaks, and eventually removed during the course of routine changes of culture medium. Adherent BMSCs are then passaged once grown to 70–80 % confluence (Notes 3–5).

1. Remove the cell culture medium.
2. Rinse the cell layer with PBS to wash out excess serum that inhibits the function of trypsin–EDTA.
3. Add 0.05 % trypsin–EDTA (approximately 1 mL for T25 culture flask and 3 mL for T75 culture flask) and gently swirl the culture flask to cover the entire surface with trypsin–EDTA. Return the flask to the incubator and incubate for 2–5 min (Note 6).
4. When the majority of the cells have become rounded or detached from the culture flask, stop the reaction by adding equal or greater volume of culture medium.
5. Draw up the cell suspension with a pipette and gently wash the remaining cells from the culture flask.
6. Transfer the cell suspension to a labelled Falcon™ tube.
7. Centrifuge at 1,000 rpm for 10 min.
8. Discard supernatant carefully and resuspend in a suitable volume of culture medium (Note 7).
9. Count the number of the cells with a hemocytometer.
10. Adjust the volume of culture medium as necessary and seed the cells at a density of 1,000–3,000 cells/cm^2.
11. Return the flasks into incubator and change the culture medium every 3 days.
12. Further subculture of BMSCs is conducted in essentially the same manner.

3.2 Characterization

According to different researchers, MSCs stained positive for a long list of cell surface markers, such as CD29, CD44, CD73, CD90 (Thy-1), CD105, CD117, CD133, CD166 (activated leukocyte-cell adhesion molecule, ALCAM), CD186, and so on (21, 22). Other markers including Sca-1 (stem cell antigen-1), SCF R/c-kit (stem cell factor), SH2, SH3, SH4, STRO-1, and HLA Class I have also been identified (23–25). Furthermore, it is generally agreed that the immunodepletion of the hematopoietic cells using anti-CD11b, CD34, and CD45, forms the fundamental concept for negative selection method of MSCs purification (26, 27).

In addition, the systemic analysis of cell surface molecules of MSCs has revealed that MSCs also express receptors for numerous extracellular matrix (ECM) proteins including collagen

(α1β1-, α2β1-integrin), laminin (α6β1-, α6β4-integrin), fibronectin (α3β1-, α5β1-integrin), and vitronectin (αvβ1-, αvβ3-integrin) (28–31). These specific expression patterns of adhesion molecules suggest the potential interactions between MSCs and other cell types in vivo (32–34). Owing to the complexity of the stem cell niche, the study of dynamic interactions between stem cells and differentiated neighboring cells in their physiological microenvironment has only recently been explored (35–38). Depending on the ECM and signal molecules in the microenvironment, MSCs can develop into adipocytes, osteoblasts, chondrocytes, myoblasts, or other phenotypes (39–42). However, no single marker has yet been identified that definitively delineates in vivo MSCs and hence there is a lack of thorough understanding of the mechanisms underlying mesenchymal stem cell renewal and its functional differentiation characteristics. For detecting these cell markers described above, antibodies or antibody conjugates can be purchased from different companies to stain the expanded BMSCs and flow cytometry (FACS) would be normally used to analyze the staining.

3.3 In Vitro Differentiation

3.3.1 Osteogenic Induction

1. Subculture BMSCs as described in Sect. 3.1.2.
2. Prepare osteogenic induction medium consisting of DMEM-LG supplemented with 10 % FBS, 1 % P/S, 100 nM Dex, 0.05 mM AsAP, and 10 mM βGP.
3. Osteogenic differentiation medium is changed twice weekly for up to 3 weeks.

3.3.2 Chodrogenic Induction

1. Expand the cells as described in Sect. 3.1.2.
2. Trypsinize the cells and transfer to 15-mL Falcon™ tubes.
3. Centrifuge at 200 × *g* for 5 min.
4. Aspirate the supernatant and resuspend the cell pellet in 1 mL CBM.
5. Count the number of the cells with a hemocytometer.
6. Adjust the volume of medium as necessary and aliquot 1 mL of cell suspension with 3–5 × 10^5 BMSCs in a 15-mL polypropylene Falcon™ tube (Note 8).
7. Centrifuge at 200 × *g* for 5 min.
8. Remove CBM and resuspend the pellet in 500 μL CIM.
9. The cell pellets are formed by immediately centrifuging at 500 × *g* for 15 min (Note 9).
10. Loosen the cap of the tube to allow gas exchange and incubate upright at 37 °C in a humidified atmosphere of 5 % CO_2. DO NOT disturb the pellets for 48–72 h.

11. Feed the cell pellets every 2–3 days by replacing the medium in each tube with 500 μL of pre-warmed CIM (Note 10).
12. Chondrogenic pellets can be harvested after 3 weeks in culture.

3.3.3 Adipogenic Induction

1. Expand the cells as described in Sect. 3.1.2.
2. Remove normal culture medium and replace with AIM.
3. After 72 h, change to AMM.
4. Change back to AIM after 24 h.
5. After three inductions are completed, maintain the cell culture in AMM for 2–3 weeks.

3.3.4 Nano-surfaces Effect on MSC Differentiation

The native niche of MSCs is full of natural nanostructures and nanoscale components in the form of extracellular matrix proteins and structural elements. Nanoscale modifications to the in vitro or in vivo environment are hence responsible for conditioning cells towards different biological processes like proliferation, maturation, differentiation, and mobility. Mammalian cell growth and differentiation requires diverse components that play important roles during the process of cell proliferation and maturation. The native niche in the first place should have the capability to provide a proper base for cellular attachment. Cellular attachment of anchorage-dependent cells is dependent on the expression of transmembrane receptor molecules like integrins and membrane-cytoskeletal proteins like vinculin and focal adhesion kinase. The arrangement and localization of these proteins in cells have been shown to be important for their consequent differentiation patterns (16). Surfaces potentiating differentiation of cells are usually seen to have reduced proliferative capacity. The clinically successful modified titanium dental implant surface: modSLA (chemically modified hydrophilic sand-blasted, large grit and acid-etched surface), that has recently been reported to have nanostructures (43, 44) has been typically observed to have reduced proliferative capability (45). The modSLA surfaces have been demonstrated to have better osteogenic properties (45–47) and higher expression of integrins (47, 48) compared to its predecessor, the SLA surface and smooth polished titanium surfaces. Experimentally induced changes in cell shapes and morphology have been shown to be able to predict the fate of MSCs upon differentiation (49, 50).

The presence of nanostructures on surfaces allows them to have large surface areas. Greater surface areas of nanosurfaces compared to their "non-nano" counterparts facilitate them to adsorb and retain growth factors, nutrients, and proteins necessary for cellular process. In response to the interaction of cells with the surrounding, they secrete several signalling proteins that eventually guide the process of cell development and differentiation. The ability of

surfaces to retain these molecules is important. Hydrophilic surfaces with nanostructures, by virtue of their affinity to fluids can bind to essential nutrients when they come in contact with blood and thereby result in better osseointegration properties (51, 52). During the process of their growth, cells secrete extracellular matrix in their surrounding which is important in providing support and help in regulating intercellular communication for further growth and maturation of cells. The interaction of nano-surfaces with the extracellular matrix deposited is important for lineage commitment and maintenance of differentiation of MSCs.

Studying the interactions of stem cells with their surroundings and surfaces are essential to understand the potential of such surfaces to be able to support cell maturation and differentiation. Biocompatibility of materials is one of the most important criterions before such substances can be taken up for further testing. Stem cell behavior is dependent on the substrate they grow on, and understanding such interactions and changes is essential before substrates can be considered for in vivo applications.

3.4 MSCs on Nano-surfaces

3.4.1 Cell Attachment and Growth on Nano-surfaces

Cell Viability/Proliferation Assessment Using Thiazolyl Blue Tetrazolium (MTT) Assay

1. Shape nano-surfaces to fit appropriate tissue culture multi-well plate (24-wells/48-wells).
2. Sterilize nano-surfaces by autoclaving. In cases of sensitive nano-surfaces that cannot be sterilized by autoclaving, see below.
3. Sterilize using ultraviolet (UV) radiation. Irradiate surfaces with UV (using transilluminator for at least 1 h prior to seeding). For UV degradable surfaces, soak them in 70 % ethanol (Note 11) for at least 1 h prior to seeding. Wash the surfaces with sterilized PBS before seeding cells.
4. Expand the cells as described in Sect. 3.1.2.
5. Seed MSCs on the nano-surfaces.
6. Allow 45 min to 1 h for cells to attach to the surfaces before adding more media.
7. Incubate the cells at 37 °C in humidified tissue culture incubator containing 5 % CO_2.
8. For cell viability assay, add MTT solution (5 mg/mL in PBS) to the medium (10 % final concentration) after 24 h of culture. For cell proliferation assay, decide the time-points and add MTT solution as above. Incubate for a further 3–4 h.
9. Discard the medium and add 200 μL of DMSO (Note 12).
10. Cover the plate with aluminum foil and swirl it for at least 5 min to thoroughly mix with the formazan generated (Note 13).
11. Measure absorbance at 570 nm (reference wavelength >650 nm).

Preparing Samples for Morphological Observation Using Scanning Electron Microscopy (SEM)

1. Prepare the nano-surfaces and seed MSCs on them as described in Sect. 3.4.1 and incubate them at 37 °C in humidified tissue culture incubator containing 5 % CO_2. Decide on the time-points.
2. At the determined time-points, discard the medium and wash the cells with PBS. Add 3 % glutaraldehyde in 0.1 M cacodylate buffer to fix the cells (Note 14). Keep the samples in fixative at 4 °C until further processing.
3. Discard the aldehyde fixative (appropriate container in fume hood) and put the samples in 0.1 M sodium cacodylate buffer for 10 min and discard (repeat this two more times).
4. Add osmium tetroxide to the samples for 1 h.
5. Discard osmium tetroxide (appropriate container in fume hood) and perform two washes (10 min each) with distilled water (discard solution in appropriate container in fume hood).
6. Dehydrate the samples progressively using increasing ethanol concentration (50, 70 and 90 %) (two times in each ethanol concentration, 10 min each time). Finally place the samples in 100 % for 15 min. Discard and replace with 100 % ethanol before proceeding for critical point drying.
7. Critical point dry using the critical point drying apparatus (as per the instructions).
8. Gold coat the samples using sputter coating device.
9. Store the samples in a desiccated chamber until imaged under SEM.

3.4.2 Preparing Samples for Protein and Gene Expression Studies

1. Prepare the nano-surfaces and seed MSCs on them as described in Sect. 3.4.1 and incubate them at 37 °C in humidified tissue culture incubator containing 5 % CO_2. Decide on the time-points.
2. Prepare protein lysis buffer: final concentrations—0.01 M Tris–HCl (pH = 8), 0.15 M NaCl, 1 % Triton X-100, and 0.05 M EDTA in double distilled water. Add PhosSTOP Phosphatase Inhibitor Cocktail and cOmplete ULTRA tablets, Mini, EDTA-free Protease Inhibitor Cocktail as per the manufacturer's protocol.
3. At the determined time-points, discard the medium and wash the cells with PBS. The subsequent steps should be carried out on ice (4 °C).
4. For protein expression studies, lyse cells with freshly prepared lysis buffer as described above. Usually 10 μL/cm^2 of culture area achieves workable concentrations (Note 15). Centrifuge

the collected samples at a very high speed at 4 °C. Collect the solution (discard the debris) in fresh tubes. The samples are now ready for protein expression studies and can be stored at −80 °C for long time.

5. For RNA isolation, lyse cells with TRIzol® Reagent (inside chemical fume hood) (1 mL of reagent/cm^2) and collect them in microcentrifuge tubes. The samples can be stored at −80 °C for long time before proceeding for RNA isolation.
6. Stabilize the samples for 5 min at 4 °C and then add 200 μL of chloroform–isoamyl alcohol (24:1) (inside fume hood). Shake vigorously for 15 s and then allow them to stay on ice (4 °C) for 5 min.
7. Centrifuge at 4 °C at 12,000 × *g* for 15 min. Observe for an aqueous phase separation.
8. Transfer the upper aqueous phase carefully, without touching the interface to a fresh tube (inside chemical fume hood). Check the amount of the aqueous phase and add an equal amount of isopropanol. Store at 4 °C for 10 min (Note 16).
9. Centrifuge at 4 °C at 12,000 × *g* for 10 min. Keenly observe for a tiny pellet (contains total RNA) in the tube and carefully discard the supernatant without removing the pellet.
10. Wash the RNA pellet with 1 mL of ice-cold 75 % ethanol. Centrifuge the samples at 7,500 × *g* (4 °C) and discard the ethanol. Repeat this step one more time.
11. Dry the RNA pellet under vacuum for 10 min.
12. Resuspend the pellet using 20 μL of UltraPure™ DNase/RNase-Free Distilled Water. Check the RNA concentration using Nanodrop (ratios 260/280 and 260/230 ≥1.8, are considered good quality RNA). The RNA samples can be stored at −80 °C for a long time.
13. The samples obtained above will be analyzed by Western blotting and real-time PCR for osteogenic differentiation markers like alkaline phosphatase, osteocalcin, and osteopontin.

3.4.3 In Vitro Differentiation of MSCs on Nano-surfaces

1. Prepare the nano-surfaces described in Sect. 3.4.1.
2. Expand the cells as described in Sect. 3.1.2.
3. Seed MSCs on the nano-surfaces and induce differentiation as described in Sect. 3.3.1 of osteogenic differentiation.
4. Allow 45 min to 1 h for cells to attach to the surfaces before adding more medium.
5. Follow steps as described in Sect. 3.3.1.

3.5 In Vivo Application

3.5.1 Cell Seeding

1. Remove the cell culture medium.
2. Rinse cell cultures with PBS and remove liquid and add 0.05 % trypsin–EDTA.
3. Return the flask to the incubator and incubate for 2–5 min.
4. When the majority of the cells have become rounded or detached from the culture flask, stop the reaction by adding equal or greater volume of culture medium.
5. Draw up the cell suspension with a pipette and gently rinse remaining cells off plate.
6. Transfer the cell suspension to a labelled Falcon™ tube.
7. Centrifuge at 1,000 rpm for 10 min.
8. Discard supernatant carefully and resuspend in a suitable volume of culture medium.
9. Determine the number of cells with a hemocytometer and calculate the cell concentration needed for seeding onto the scaffold.
10. Add approximately 2×10^4 to 1×10^5 cells in 50 μL of culture medium to the scaffold.
11. Incubate the seeded scaffold for 1 h and then supplement with an additional 150 μL of culture medium (e.g., 96-well plate).

3.5.2 Implantation of Cell-Scaffold Constructs into Severe Combined Immunodeficient (SCID) Mice

1. Get the mouse cages from the holding room (Note 17).
2. Spray the entire working area with 70 % ethanol. Cover the operating field with a sterile towel and place the sterilized instruments on it.
3. SCID mouse is anesthetized with intraperioneal injection of a 1:1:8 solutions of ketamine hydrochloride, xylazine, and 0.9 % physiological saline at a dose of 0.1 mL per 25 g body weight.
4. Place the anesthetized mouse on the heating pad after skin preparation. Wipe the mouse's back with Betadine and alcohol swabs.
5. Open skin with scissors and create subcutaneous pockets with blunt forceps.
6. Insert cell-scaffold constructs.
7. Close skin with staples.
8. Wipe ear with alcohol and make ear notch for identification if necessary. Fill out the cage card with the scaffold location as planned.

4 Notes

1. More volume than this may dilute the marrow with sinusoidal blood.
2. Make sure that the Lymphoprep™ does not mix with the bone marrow sample.
3. In order to conduct this procedure safely we recommend that proper personal protective equipment including lab coat, goggles, and gloves are worn during the operation.
4. Process all the cell culture work in a Class II biological safety cabinet.
5. All the reagents must be sterilized and warmed in the water bath at 37 °C before use so that cells are not shocked by cold liquid.
6. Keep the time of exposure as brief as possible. Prolonged exposure of the cells to trypsin may result in damage to cell surface markers and reduce cell viability.
7. Be sure the cells are well dispersed, otherwise cell clumps or aggregates will be present.
8. Polypropylene tubes are used so that the cells do not adhere to the tube.
9. DO NOT aspirate the supernatant or resuspend the pellet.
10. After replacing the medium, gently flick the bottom of each tube to ensure the pellet is free-floating.
11. Ensure that the nano-surfaces do not cross-react with ethanol.
12. Use more amount of DMSO if large number of cells are seeded or if observed color is too dark/spectrophotometric readings are too high.
13. Mix for longer time in case the color generated is too low/spectrophotometric readings are too low.
14. All steps for preparation of samples for SEM before drying must be performed under chemical fume hood.
15. Using 10 μL of protein lysis buffer to lyse cells may be difficult to handle, therefore it is suggested to pool few samples together (samples obtained from same nano-surface and same cell type). If 6 wells of a 24-well plate are pooled, use 120 μL of the lysis buffer to lyse cells from first well and then transfer this buffer with lysed cells to the second well, and so on. Try to restrict frothing of the sample.
16. The aqueous phase–isopropanol mix obtained during the process of RNA isolation can be stored at −20 °C overnight.
17. Before entering the procedure room, put on shoe covers, cap, mask, goggle, sterile gown, and gloves.

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Chondrogenic Differentiation of Menstrual Blood-Derived Stem Cells on Nanofibrous Scaffolds

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Abstract

Cartilage tissue engineering is a promising technology to restore and repair cartilage lesions in the body. In recent years, significant advances have been made using stem cells as a cell source for clinical goals of cartilage tissue engineering. Menstrual blood-derived stem cells (MenSCs) is a novel population of stem cells that demonstrate the potential and differentiate into a wide range of tissues including the chondrogenic lineage. Incorporation of cell culture with extracellular matrix (ECM) like substratum plays an important role in cartilage tissue regeneration by providing attachment sites as well as bioactive signals for cells to grow and differentiate into chondrogenic lineage. The electrospun nanofibers are a class of polymer-based biomaterials that have been extensively utilized in tissue engineering as ECM-like scaffold. This chapter discusses potential of electrospun nanofibers for cell-based cartilage tissue engineering and presents detailed protocols on immunophenotyping characterization and chondrogenic differentiation of MenSCs seeded in poly-ε-caprolactone (PCL) nanofibers. The isolated MenSCs are characterized using flow cytometry, seeded on the nanofibers, imaged using scanning electron microscopy, and subsequently differentiated into chondrogenic lineage in culture medium containing specific growth factors and cytokines. Immunofluorescence and alcian blue staining are used to evaluate the development of seeded MenSCs in PCL nanofibrous scaffold into chondrogenic lineage.

Keywords: Stem cell, Chondrogenic, Differentiation, Nanofiber, Menstrual blood

1 Introduction

Cartilage is composed of sparsely distributed chondrocytes embedded within extracellular matrix (ECM). The ECM is composed of mostly type II collagen and proteoglycans that provide the cartilage tissue with sufficient mechanical properties for in vivo function (1). Cartilage lesions have limited self-repair capacity due to the intrinsic biology of cartilage tissue, such as lack of vascular supply and low matrix turnover. Cell-based cartilage tissue engineering is a novel

and promising treatment option to restore cartilage inability (2, 3). However, one of the major obstacles in engineering tissues for clinical use is the large cell numbers that are often required for forming new tissues. The obvious advantage of using stem cells is that these cells can potentially provide an unlimited supply of differentiated chondrocytes for transplantation. At present, the interest in adult stem cells has particularly been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in the therapeutic approaches (4). Adult stem cells also known as multipotent mesenchymal stromal cells have been derived from different tissues such as bone marrow, cord blood, adipose tissue, and amniotic fluid (5–8). However, problems such as invasive techniques for sample collection, limited availability, and minimal proliferation ability of adult stem cells in comparison to embryonic stem cells decrease their applicability for research and clinical use (4). Although great efforts have been accomplished to utilize induced pluripotent stem cells for clinical trial, some limitations such as tumor formation risk have been unsolved (9, 10). Thus, alternative resources of stem cells suitable for clinical application of different diseases are still challengeable.

Menstrual blood contains a unique population of cells, referred to as menstrual blood-derived stem cells (MenSCs), with properties similar to adult stem cells (11–13). High regenerative capacity of human endometrium through cyclic processes of cellular proliferation, differentiation, and detaching is a robust evidence to support the assumption (14). The adherent fraction of MenSCs has a mesenchymal like morphology, but possesses some markers of embryonic stem cells such as OCT-4 in addition to mesenchymal stem cell (MSC) markers (15). The proliferation rate of MenSCs is much higher than umbilical cord- and bone marrow-derived MSCs (BMSCs) (14, 15). Indeed, the MenSCs could inhibit proliferative responses of lymphocytes in allogeneic mixed lymphocyte reaction indicating that this cell population has immunomodulatory activity and could be further investigated and potentially used in future cell therapy-based approaches (16). These characteristics, as well as ease of access and no specific limitations in sample collection, make menstrual blood an appropriate stem cell resource for tissue engineering and regenerative medicine.

Chondrogenic differentiation of stem cells is generally controlled by numerous cues in their microenvironment. The use of exogenous cytokines and growth factors is one step forward in the development of a defined culture milieu for directing the chondrogenic differentiation of stem cells. However, the in vitro culture milieu for directing

chondrogenic differentiation should also incorporate naturally or artificially synthesized ECM substratum to optimize cell attachment, proliferation, and differentiation (1, 17, 18).

The three-dimensional synthetic biodegradable scaffolds designed using nanofibers due to their morphological and mechanical similarities to the fibrillar ECM serve as an excellent framework for cell adhesion, proliferation, and differentiation and are a popular choice to repair and regenerate various soft tissues such as cartilage (19–21).

Currently, there are three techniques available for the synthesis of nanofibers: electrospinning (22), self-assembly (23), and phase separation (24). Of these techniques, electrospinning is the most widely used technique that has demonstrated promising results in tissue engineering applications (25). Electrospinning offers great flexibility in terms of the choice of scaffold material and fiber diameter. Electrospun polymeric fibrous meshes also present a higher surface area for cell attachment. Indeed, fabrication of electrospun nanofibers is easy, inexpensive, scale-up, and relatively reproducible (25–28).

The availability of a wide range of natural and synthetic biomaterials has broadened the scope for development of nanofibrous scaffolds (29). Synthetic polymer-based systems offer additional advantages with their adjustable mechanical properties, as well as ease of surface modification via protein coatings, or conjugation of specific signaling molecules (1, 20, 21). The most common electrospun nanofibers designed for cartilage tissue engineering are made of poly (α-hydroxy esters) (Table 1). These meshes have been used since the early 1990s for cartilage regeneration. Poly-ε-caprolactone (PCL) is one member of the poly (α-hydroxy ester) family that has been electrospun to form nanofibrous scaffolds capable of supporting chondrocyte proliferation and function (32–34, 36, 37, 44). Furthermore, supporting role of these scaffolds in chondrogenesis of MSCs has been reported in different studies (19, 30, 31, 35–43, 45).

This chapter presents detailed protocols about characterization and chondrogenic differentiation of isolated MenSCs on a nanofibrous scaffold fabricated from PCL. After immunophenotyping characterization of isolated MenSCs using flow cytometric analysis, the cells are seeded on fabricated PCL nanofibers. Cell attachment and infiltration in nanofibers are evaluated by scanning electron microscopy (SEM). The seeded MenSCs on the nanofibers are differentiated in chondrogenic culture medium and subsequently evaluated using immunofluorescence and alcian blue staining.

Table 1
Cartilage tissue engineering using nanofibrous scaffolds

Species	Cell source	Cell type	Nanofiber composite	Culture media	References
Human	Bone marrow	Mesenchymal stem cells (MSCs)	Poly(ε-caprolactone) (PCL)	DMEM, TGF-β1, dexamethasone, L-ascorbic acid (AA), ITS + 1, sodium pyruvate, and L-proline	(30, 31)
Porcine	Articular cartilage	Chondrocytes	Poly(D,L-lactide-co-glycolide) (PLGA)	DMEM/F-12, fetal bovine serum (FBS), and AA	(32)
Porcine and rabbit	Articular cartilage	Chondrocytes	Polyesters poly(L-lactide) (PLLA) and PLGA (50:50) modified by porcine type II collagen and an Arg-Gly-Asp (RGD)-containing protein	DMEM and fetal calf serum (FCS)	(33)
Bovine	Articular	Chondrocytes	PLGA with hyaluronic acid (HA)	DMEM, FBS, AA, L-proline, and insulin + transferrin + selenium (ITS)	(34)
Rabbit	Bone marrow	MSCs	PLGA	Eagle's MEM and FBS	(35)
I. Swine II. Human	I. Articular cartilage II. Bone marrow	I. Chondrocytes II. MSCs	PCL	DMEM and FBS	(36)
I. Bovine II. Human	I. Articular II. Bone marrow	I. Chondrocytes II. MSCs	Poly(glycolic acid) (PGA), PLLA, poly (D,L-lactic acid) (PDLLA), PLGA, and PCL	I. DMEM, FBS, and AA II. DMEM and FBS	(37)
Human	Bone marrow	MSCs	PCL	DMEM, sodium pyruvate, TGF-β1, dexamethasone, AA, L-proline, and insulin + transferrin + selenium + linoleic acid + BSA (ITS + 1)	(38)

Rabbit	Bone marrow	MSCs	Poly(vinyl alcohol)/PCL (PVA/PCL)	DMEM, dexamethasone, AA, ITS, TGF-β1, and b-FGF	(39)
Calves	Femurs and tibiae	MSCs	PCL	DMEM, dexamethasone, L-proline, sodium pyruvate, ITS, and TGF-β1	(40)
Human	Bone marrow	MSCs	PCL	α-MEM, ITS, dexamethasone, sodium pyruvate, AA, L-proline, and TGF-β3	(41)
Human	Umbilical cord	MSCs	PLGA and PCL	DMEM, ITS, AA, dexamethasone, TGF-β1, TGF-β3, IGF-1, BMP6, and L-proline	(42)
Rabbit	Bone marrow	MSCs	Nanofibrous articular cartilage extracellular matrix (ACECM) with synthetic PLGA	DMEM and FBS	(43)
Calves	Femurs and tibiae	Chondrocytes	PLLA	DMEM, AA, dexamethasone, L-proline, sodium pyruvate, ITS, IGF-1, and TGF-β1	(44)
Human	Bone marrow	MSCs	PCL	DMEM, sodium pyruvate, TGF-β1, dexamethasone, AA, L-proline, and ITS + 1	(45)
Human	Adipose tissue	Adult adipose-derived stem cells (ASCs)	PLGA	DMEM, sodium pyruvate, pyridoxine hydrochloride, AA, ITS, and TGF-β1	(46)
Goat	Bone marrow	MSCs	PVA-methacrylate (PVA-MA)	DMEM, dexamethasone, AA, L-proline, sodium pyruvate, ITS + 1, and TGF-β1	(19)
Human	Menstrual blood	MenSCs	PCL	DMEM-F12, FBS, sodium pyruvate, TGF-β3, IGF-1, dexamethasone, ITS + 1, and AA	(47)

2 Materials

2.1 Study Population

Menstrual blood is obtained from healthy female volunteers, 20–45 years old, after menstrual flow is initiated (see Note 1). Donors should have regular menstrual cycle with no sign of apparent vaginal infection. Due to considerable effects of hormonal therapy on the morphology and function of MenSCs, such treatments should be considered as exclusion criteria. Donors undergo a standard medical history evaluation and physical examination for ruling out malignancy, diabetes, and autoimmune diseases. It is wise to exclude donors with positive test for such blood-borne viruses as HIV, HCV, and HBV. All procedures for collection, processing, and usage of menstrual blood should be performed under approval of Institutional Review Board (IRB).

2.2 Collecting Menstrual Blood

1. Diva cup (Diva International Co., Lunette, Finland) (see Note 2).
2. Phosphate-buffered saline (PBS; 0.15 M, pH 7.2): Add about 150 mL deionized water (DW) to a 1-L graduated cylinder. Weight 0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, and 1.15 g Na_2HPO_4 and transfer to the cylinder. Place a magnetic stir bar into the cylinder and dissolve the solid materials by mixing the solution over a stirrer at room temperature (RT). Add DW to about 950 mL. Mix well and adjust pH with HCl. Make the final volume to 1 L with DW. Filter through 0.2 μm filter unit and store at 4 °C.
3. EDTA stock solution (0.5 M, pH 8): Add 18.61 g disodium ethylenediaminetetra-acetate·$2H_2O$ ($Na_2EDTA{\cdot}2H_2O$) (Merck, product number: 1084211000, Darmstadt, Germany) to a glass beaker containing about 80 mL DW. Mix the solution over a magnetic stirrer. Adjust pH to 8 using about 2.02 g NaOH pellet. Make the final volume to 100 mL with DW. Filter through 0.2 μm filter unit and store at 4 °C.
4. Transfer buffer: To prepare 100 mL transfer buffer, mix 1 mL EDTA 0.5 M, 2 mL 100× penicillin/streptomycin (Sigma, product number: P4456, MO, USA), 0.2 mL fungizone 250 μg/mL (Gibco, product number: 15290–018, Scotland, UK) (see Note 3), and 96.8 mL PBS (see Note 4).

2.3 Isolation and Culture of Menstrual Blood-Derived Stromal Cells

1. Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) (Sigma, Product Number: D8900): Measure out 90 % of final required volume of water. Water temperature should be 15–20 °C. While gently stirring the water, add 15.6 g powdered medium and 1.2 g sodium bicarbonate for each liter and stir until dissolved. While stirring, adjust the pH of the medium to 0.1–0.3 pH units below the desired pH, since it may rise during filtration. The use of 1 N HCl or 1 N NaOH is

recommended. Add additional water to bring the solution to final volume. Sterilize immediately by filtration using a membrane with a porosity of 0.22 μm. Aseptically dispense medium into sterile container and store at 4 °C.

2. Complete DMEM/F-12 (cDMEM/F-12): Prepare cDMEM/F-12 medium by supplementation of DMEM/F-12 with 15 % (v/v) Fetal Bovine Serum (FBS) (Gibco, product number: 10270–106, Scotland, UK), 1× non-essential amino acids (Gibco, product number: 11140, Scotland, UK), 2 mM L-glutamine (Gibco, product number: 25030, Scotland, UK), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone.
3. Ficoll paque™ PLUS (GE Healthcare, product number: 17-1440-03 Uppsala, Sweden).
4. Trypsin–EDTA 10× (Gibco, product number: 15090046, Scotland, UK).
5. Cell culture plastic wares including T-75 flasks (SPL LIFE SCINCES, Korea), 15 falcon tubes (Greiner Bio-One, product number: 188271, Frickenhausen, Germany), 50 mL falcon tubes (Greiner Bio-One, product number: 210261, Frickenhausen, Germany), and Pasteur pipette 230 mm (ISO LAB, product number: 108.03.002, Wertheim, Germany).
6. Sterile PBS (see above).

2.4 Phenotypic Characteristics

1. Stain buffer: PBS containing 2 % (v/v) FBS (PBS–FBS) for diluting antibodies.
2. Fixation buffer (paraformaldehyde 4 %): 0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4, 4 % (w/v) paraformaldehyde (SERVA, product number: 31628, Heidelberg, Germany). For making 500 mL fixation buffer, dissolve 6.7 g $Na_2HPO_4{\cdot}7H_2O$ and 3.45 g $NaH_2PO_4{\cdot}7H_2O$ in about 450 mL DW using a magnetic stirrer. Add 20 g paraformaldehyde. Stir for about half an hour at 70 °C using a heater stirrer. Allow to cool, adjust pH to 7.0 by adding 1 M NaOH, and bring DW to 500 mL. Divide into aliquots in 15-mL tubes and store up to 1 year at −20 °C.
3. Permeabilization buffer: PBS containing 0.1 % (w/v) saponin (Merck, product number: 558255, Darmstadt, Germany), 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.05 % (w/v) NaN_3, 0.1 % (w/v) BSA, 10 mM HEPES. Adjust pH to 7.4 using 1 M NaOH, and store up to 6 months at 4 °C.
4. Antibodies: Dilute antibodies for 2×10^5 cells using stain buffer (v/v) as follows:
   (a) FITC-conjugated monoclonal antibodies against CD34 (5:100) (clone 581, BD Pharmingen, product number: 55582, California, USA), CD38 (5:100) (clone HIT2, BD Pharmingen, product number: 555459), CD44

(5:100) (clone 515, BD Pharmingen, product number: 550989), and CD45 (5:100) (clone HI30, BD Pharmingen, product number: 555482).

(b) PE-conjugated monoclonal antibodies against CD10 (5:100) (clone HI10a, BD Pharmingen, product number: 555375), CD29 (5:100) (clone MAR4, BD Pharmingen, product number: 555443), CD73 (5:100) (clone AD2, BD Pharmingen, product number: 550257), CD105 (10:100) (clone 166707, R&D, product number: FAB10971P, USA), CD133 (5:100) (clone TMP4, eBioscience, product number: 12–1338, San Diego, USA), CD146 (5:100) (clone P1H12, BD Pharmingen, product number: 550315), and SSEA-4 (10:100) (clone MC-813-70, eBioscience, product number: 12–8843).

(c) Unconjugated monoclonal antibody against STRO-1 (1:100) (clone STRO, R&D, product number: MAB1038, USA).

(d) Unconjugated rabbit antibodies against OCT-4 (1:100) (Abcam; product number: ab19857, Cambridge, USA) and Nanog (1:500) (clone NNG-811; Sigma; product number: N3038).

(e) Rabbit polyclonal anti c-Kit (4:100) (Abcam; product number: ab5506, Cambridge, USA).

(f) FITC-conjugated sheep anti-mouse Ig (2:100) and FITC-conjugated sheep anti-rabbit Ig (1:100) (Avicenna Research Institute, Tehran, Iran).

(g) Isotype-matched FITC-conjugated antibody (5:100) (clone MOPC-21, BD Pharmingen, product number: 555748) or isotype-matched PE-conjugated antibody (5:100) (clone MOPC-21, BD Pharmingen, product number: 5559320).

2.5 Preparation of Nanofibrous PCL Scaffolds

1. Synthetic polymer: PCL (Mn: 70,000–90,000 kDa) (Sigma).
2. Organic solvents: Chloroform ($CHCl_3$).
3. Syringe—with blunted tip needle—10 mL, 18 gauge, 1.0 in. length.
4. Electrospinning machine (ANSTCO, Iran).
5. Rotating cylindrical drum (50 mm).
6. Heavy aluminum foil.
7. Microwave plasma generator (DienerElectronics, Germany).
8. Stainless steel sharp punch.

2.6 Scanning Electron Microscopy

1. Double-sided carbon tape (8 mm × 20 mm).
2. Aluminum SEM specimen mount stubs.

3. Sharp blade or scalpel.
4. Sputter coater with gold foil.

2.7 Chondrogenic Differentiation

1. Recombinant fibroblast growth factor basic (b-FGF) (Sigma, product number: F0291).
2. Recombinant Transforming growth factor-β3 (TGF-β3) (Sigma, product number: T5425).
3. Recombinant Insulin like growth factor-1 (IGF-1) (Sigma, product number: I3769).
4. Dexamethasone (Sigma, product number: D8893): To prepare 5 mM stock solution, add 1 mL absolute ethanol per milligram dexamethasone; gently swirl to dissolve. Add 49 mL sterile medium per milliliter of ethanol added, while mixing, to achieve final concentration of 5 mM. The stock solution may be diluted further using medium. Store at −20 °C.
5. Ascorbic acid (Sigma; product number: A4544): To prepare 5 mg/mL stock solution add 50 mg L-ascorbic acid in 10 mL water and then sterilize by filtration using a membrane with a porosity of 0.22 μm. Store in aliquots at −20 °C.
6. Chondrogenic differentiation medium (CDM): DMEM/F-12 supplemented with 2 % FBS, 100 μg/mL sodium pyruvate (Gibco, product number: 11360), 20 ng/mL TGF-β3, 10 ng/mL IGF-1, 100 nM dexamethasone, 1× Insulin– Transferrin–Selenium + linoleic acid and bovine serum albumin (BSA) (ITS + 1) premix (Sigma, product number: I3146), and 50 μg/mL L-ascorbic acid (see Note 5).

2.8 Evaluation of Differentiated Cells

1. 3 % Glutaraldehyde (Sigma, product number: G5882).
2. 0.5 % Triton X-100 (Sigma, product number: T8787): Add 500 μL Triton X-100 to 100 mL PBS and then vortex properly until dissolved.
3. 4 % BSA (Sigma, product number: A2153): Add 4 g BSA to 100 mL PBS and then vortex properly until dissolved.
4. Primary antibodies: Dilute Anti-collagen II antibody (1:100) (clone 5B2.5; Abcam; product number: ab3092, Cambridge, USA) and Anti-collagen I antibody (1:500) (Abcam; product number ab6308, Cambridge, USA) using 1 % BSA.
5. Secondary antibody: Dilute FITC-conjugated sheep anti-mouse Ig (1:50) and FITC-conjugated sheep anti-rabbit Ig (1:100) using 1 % BSA.
6. Lab-Tek 2-well glass chamber slide (Thermo Scientific Nunc, Denmark).
7. 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, product number: 32670): Dissolve 10 mg powder in 2 mL of deionized water.

For long-term storage the stock solution can be aliquoted and stored at –20 °C in the dark. For using dilute DAPI (1:1,000) by PBS (see Note 6).

8. 3 % Acetic acid (Merck, product number: 1000562500, Darmstadt, Germany): Add 3 mL glacial acetic acid in 97 mL DW.
9. Alcian blue (Sigma, product number: A3157): Add 1 g alcian blue in 100 mL acetic acid, 3 % solution, then mix, and adjust pH to 2.5 using acetic acid.
10. 0.1 % Nuclear fast red (Sigma, product number: N8002): Dissolve 5 g aluminum sulfate in DW. Add 0.1 g nuclear fast red and slowly heat to boil and cool. Filter and add grain of thymol as a preservative.

3 Methods

3.1 Collecting Menstrual Blood

1. Instruct the donors how to use Diva cup according to the manufacturer's instruction and ask them to collect menstrual blood in Diva cup on day 1, 2, or 3 of menstrual cycle (see Notes 7 and 8).
2. Upon collection, pour the sample (5 mL) into a sterile 50 mL falcon tube containing 5 mL transfer buffer and immediately transport it to the laboratory in cold chain.

3.2 Isolation and Culture of Menstrual Blood-Derived Stem Cells

1. Under sterile condition, top the collection up to about 50 mL with sterile cold PBS and centrifuge at 400 × *g*, 4 °C, for 10 min. Aspirate the supernatant and discard. Repeat washing step once more.
2. Resuspend and dilute the cell pellet with two volumes of cold PBS and slowly overlay the cell suspension in a 15 mL falcon tube containing one volume of Ficoll-Hypaque (see Note 9). Centrifuge the tube at 400 × *g*, RT, for 20 min.
3. Collect mononuclear cell layer containing MenSCs (see Note 10) by Pasteur pipette, transfer them to 15 mL falcon tube, and wash the cells three times with 10 mL cold PBS at 400 × *g*, 4 °C, for 10 min. Prior to final washing step, resuspend cell pellet in about 2 mL cold PBS by gentle pipetting and perform a cell count and viability analysis using trypan blue dye exclusion test (see Note 11).
4. Resuspend cell pellet in 15 mL cDMEM/F-12 and seed cell suspension into a T-75 flask (see Note 12). Incubate flask for 24 h at 37 °C, 5 % CO_2, in a fully humidified incubator. During this period, stem cells will adhere firmly while non-adherent cells remain floating.

5. Using warm DMEM/F-12 gently wash out non-adherent cells and allow adherent cells to propagate (see Note 13). Change the medium twice a week.
6. After about 7–10 days, when the cells become 70 % confluent (see Note 14), remove culture medium and detach cells by treatment with about 5 mL prewarmed 1× trypsin–EDTA solution for 2–3 min in CO_2 incubator.
7. Immediately neutralize trypsin by adding 10 mL medium containing 20 % FBS to culture flask and collect cells by gentle pipetting.
8. Centrifuge cell suspension at 400 × *g*, RT, for 10 min, wash the pellet twice with cold PBS, and determine cell count and viability.
9. Passage the cells into two T-75 flasks (passage 1). After 2–3 passages, sufficient cell number will be available for differentiation experiment.
10. Detach the cells as above and proceed with phenotypic characterization and chondrogenic differentiation.

3.3 Phenotypic Characterization

1. For cell surface staining, resuspend a fraction of cells obtained in step 10 in PBS–FBS and adjust cell concentration to 2×10^6/mL.
2. Aliquot 100 μL of cell suspension in flow cytometry tubes and wash the cells two times with PBS.
3. Dilute specific antibodies against cell surface markers in stain buffer to reach the concentrations mentioned before. Then add diluted antibodies to cell suspension in darkroom for 30–45 min at 4 °C.
4. Wash the cells two times with cold PBS, and analyze expression of the markers by a flow cytometer (Partec GmbH, Munster, Germany). If flow cytometric analysis is not to be done immediately, fix the cells by adding equal volume of PFA 4 % and place the tube at 4 °C for not more than 48 h.
5. For indirect staining, as in the case of STRO-1, Nanog, and c-kit, incubate the cells with unconjugated primary antibodies for the time specified above and after three washes add conjugated secondary antibodies and continue incubation for 30 min in the dark. Wash the cells and proceed as above for signal monitoring.
6. For intracellular staining (in the case of OCT-4), resuspend the cells in 0.1 mL stain buffer and add equal volume of fixation buffer. Mix well and incubate tube for 15 min at room temperature.

7. Wash the cells twice with stain buffer by centrifugation at 400 × *g*, for 10 min each time.
8. Resuspend the cells in permeabilization buffer and incubate at cold temperature for 10 min. Centrifuge tube and aspirate supernatant.
9. Add 100 μL anti-OCT-4 antibody optimally diluted in permeabilization buffer as mentioned in materials section and incubate for 40 min at 4 °C.
10. Wash the cells twice with permeabilization buffer and incubate in the dark with optimal concentration of FITC-goat anti-rabbit antibody diluted in permeabilization buffer for 30 min at 4 °C.
11. Proceed with two washing steps with permeabilization buffer and analyze the cells by a flow cytometer (see Note 15). For each subclass of specific antibodies, an isotype-matched control tube should be used.

3.4 Scaffold Preparation

1. Dissolve PCL in chloroform in glass beaker at room temperature just before the electrospinning process (see Notes 16–18), while the overall concentration of PCL in the solution is maintained at 12 % (w/v). Desolation is following by solvent evaporation; therefore cap the glass beaker tightly.
2. Enfold cylindrical drum with heavy aluminum foil and place it in rotator inside the electrospinning chamber.
3. Fill the 10 mL syringe with 5 mL of the polymer solution, remove all air bubbles, and then attach blunted needle to it.
4. Place the syringe–needle assembly containing the polymer solution onto the syringe pump.
5. Program the machine with these parameters:
   (a) Flow rate: 0.5 mL/h.
   (b) High voltage: 18 kV.
   (c) Distance of cylindrical drum from needle: 15 cm.
   (d) Rotating speed: 65 × *g*.
6. Remove the aluminum foil containing the electrospun scaffold and place it in macrowave plasma generator with a cylindrical quartz reactor for oxygen plasma treatment (see Note 19). Use pure oxygen in reaction chamber at 0.4 mbar pressure and apply frequency of 2.45 GHz for 10 min.
7. Punch plasma-treated sheets into 15 mm diameter, place in 24-well plate, and then press with a stainless steel ring to ensure complete contact of the scaffolds with the wells.
8. Sterilize the samples using UV sterilization in a cell culture hood for 30 min each for both and then through filtrated 70 % ethanol for 2 h.

9. Soak the sterile scaffolds overnight in DMEM/F-12 at 37 °C in a humidified incubator. Incubation overnight in medium will wet the scaffolds and allow the medium to infiltrate into the porous structure (see Note 20).

3.5 SEM

1. Place double-sided carbon tape onto the aluminum specimen mount stubs.
2. Cut the electrospun scaffolds using a sharp blade into squares of side 0.5 cm. Attach the scaffolds to the carbon tape and sputter-coat with gold for 150 s at 60 mA current and below 10^{-1} mbar vacuum. Sputter-coating deposits a conductive metal on the scaffold to enable imaging using the electron beam current.
3. Prepare images of the scaffolds using an SEM.

3.6 MenSC Seeding on the Nanofibers

1. Ensure that the cells have reached 70 % confluence in the T-75 flasks. Rinse the flasks gently with warmed PBS and lift the monolayer of cells using 0.25 % trypsin/1 mM EDTA for 3 min. Neutralize trypsin activity with cDMEM/F-12 and gently pellet the cells for 10 min at 400 × *g*.
2. Resuspend the cell pellet in cDMEM/F-12 and dilute using medium to 2.5×10^5 cells/40 μL (see Note 21).
3. Place the wetted scaffolds into wells of a 24-well plate.
4. Place 40 μL of the cell suspension onto each scaffold and incubate for 2 h at 37 °C in a humidified chamber.
5. Add 0.5 mL of cDMEM/F-12 into each well and incubate overnight at 37 °C in a humidified chamber (see Notes 22–24).

3.7 Chondrogenic Differentiation

1. After overnight incubation of cell-seeded scaffolds in cDMEM/F-12, replace medium with fresh cDMEM/F-12 supplemented with 20 ng/mL b-FGF and culture the cells for 2 days prior to differentiation (see Note 25).
2. Induce differentiation by treating cells in 0.5 mL of CDM (see Note 26).
3. Replace medium every second day for 3–4 weeks.

3.8 Evaluation of Differentiated Cells

3.8.1 Immunofluorescence Analysis of Cell–Scaffold Constructs

1. At the end of differentiation, rinse twice the cell–scaffold constructs with warm PBS and fix them in 3 % glutaraldehyde for 1.5 h at RT (see Note 27).
2. Permeabilize cells with 0.5 % Triton X-100 for 20 min and then incubate cell constructs in 4 % BSA for 30 min to block unspecific binding of the antibodies.

3. Incubate washed cells overnight at 4 °C with primary antibodies, including monoclonal mouse anti-human collagen type I, and mouse anti-human collagen type II.
4. Subsequently, wash the cells three times with PBS and incubate them with FITC-labeled goat anti-mouse IgG at RT for 1 h in the dark (see Note 28).
5. After washing with PBS, incubate cells for 10 min with DAPI for nuclear staining.
6. Wash the scaffolds three times with warm PBS and transfer to clean glass chamber slide. Protect the samples from light (see Note 29).
7. View the constructs using a confocal microscopy (see Notes 30 and 31).

3.8.2 Alcian Blue Staining

1. Fix the cell–scaffold constructs in 3 % glutaraldehyde solution as described above.
2. After washing with PBS, incubate the constructs in 3 % acetic acid for 5 min, and then rinse the acid using PBS.
3. Stain the samples with alcian blue solution for 20 min.
4. Wash three times the samples with PBS on a shaker.
5. Stain the nuclei with nuclear fast red for 5 min.
6. Wash two times the samples with PBS on a shaker and then check them with an inverted microscope (Note 32).

4 Notes

1. Since menstrual blood-collecting cups should be placed inside the vagina, virgin females are not recommended to use it. In such cases, using mens cups may be painful and may tear the hymen.
2. Diva cup is available in two sizes, Model 1 and Model 2. There is a small difference between the two sizes (Model 1 is smaller), but it is important to use the recommended sizing to prevent leakage. Smaller size is for women under 30 years old with no history of vaginal or caesarean delivery. Model 2 is recommended for women over 30 years old and/or for women who have delivered vaginally or by caesarean section.
3. In most cases, penicillin/streptomycin is sufficient to preclude cell culture contamination and there is no need to supplement the transfer buffer with fungizone. However, if vaginal yeast

infection is suspected or when the first sample from a donor is contaminated with yeast, supplementation of medium with fungizone is recommended. After the first passage and when no contamination with yeast is documented, fungizone could be omitted.

4. By mixing 5 mL of transfer buffer with 5 mL of menstrual blood, the final concentration of constituents would be as follows: 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 mM EDTA.
5. TGF-β3 can be substituted by TGF-β1.
6. The less water-soluble DAPI dihydro-chloride may take some time to completely dissolve in water and sonication may be necessary.
7. Typical menstruation period lasts from 3 to 5 days. During menstruation, blood flow can be light, moderate, or heavy. It is important that menstrual blood is collected on the day in which blood flow is the heaviest. This will shorten the collection time to less than 3 h, and usually occurs during the second day of menstruation. A volume of about 5 mL blood is collected during this period which provides sufficient stromal cells for most experiments. In case of light blood flow, collection time might exceed to an overnight period. If possible, collect the sample in the morning to be able to isolate MenSCs on the same day of sample collection.
8. Diva cup is reusable and can be washed up with warm water and a mild, water-based (oil-free) soap. As needed, Diva cup could be further cleaned by boiling in an open pot of sufficient boiling water for 5–10 min.
9. Ficoll separation step is optional and could be skipped. In this case, collected menstrual blood is washed first with cold PBS containing 5 mM EDTA and then two times with cold PBS. Pellet is resuspended in cDMEM/F-12 and cultured in two T-75 flasks. In the latter method, cultured cells is a cocktail of all cells present in menstrual blood including red blood cells and hence more washing steps are required to remove non-adherent contaminating cells (see below). Generally, inclusion of Ficoll separation as a pre-enrichment step will increase purity of cultured cells but at the same time may lead to a lower yield.
10. Shedding epithelial glands have high buoyant density and sediment along with red blood cells to the bottom of Ficoll density gradient medium. This will potentially reduce the likelihood of subsequent contamination of MenSCs with loosely adherent

epithelial cells. Single-cell epithelial cells are placed on a mononuclear layer of Ficoll medium and should then be separated from firmly adherent MenSCs.

11. About 1–3 $\times$ 10^6 nucleated cells with more than 90 % viability could be procured from each milliliter of menstrual blood. When Ficoll separation step is omitted, about 3 $\times$ 10^6 nucleated cells per each milliliter of menstrual blood are obtained.
12. The number of T-75 flasks used for cell culture depends upon the total volume of menstrual blood collected and hence the number of mononuclear cells procured upon Ficoll separation. As a general rule, seed cells obtained from each 5 mL of menstrual blood in one T-75 flask.
13. In spite of sample treatment with a cocktail of antibiotics and shipment in cold chain, a small proportion of cell cultures might still become contaminated, especially with fungi. In such cases discard the culture and repeat sampling in the next menstrual cycle.
14. Although doubling time of cultured MenSCs is less than 20 h, this rate is much lower at initial culture periods. Later on, when the number of cells increases and cells become more confluent, the growth slope becomes steeper.
15. Typically MenSCs are positive for stromal cell/MSC markers including CD10, CD29, CD44, CD73, CD146, CD105, as well as OCT-4 (an embryonic stem cell marker), while CD34, CD38, CD45, CD133, and STRO-1, SSEA-4, Nanog, and c-kit are lacking (Fig. 1).
16. Chloroform is a neurotoxic chemical. Therefore, all work by chloroform must be conducted in the hood and safety glasses are needed at all times.
17. Chloroform will dissolve most plastics, so everything that contacts chloroform must be glass. For cleaning the glass syringe, screw clean needle on glass syringe. Draw up 1–2 mL chloroform through the needle into the glass syringe. Draw up an additional 1–2 mL air so that the syringe contains both chloroform and air. Remove the needle carefully. Rinse syringe by swishing chloroform around by gently rolling or rocking syringe. Do not shake syringe. Squirt chloroform out into waste chloroform container. Repeat this procedure two more times, then remove plunger from syringe base, and allow to air-dry.
18. Most commonly used solvent for electrospinning PCL is chloroform; however a variety of solvents such as methylene chloride, dichloroethane, dimethylformamide (DMF), *n*-hexane, and methanol can be used to dissolve PCL. Some of the solvents are used in combination as a solvent system; for instance, dichloromethane and *N*,*N*-dimethylformamide were used as solvents for PCL. In the other surveys, the innovative solvent

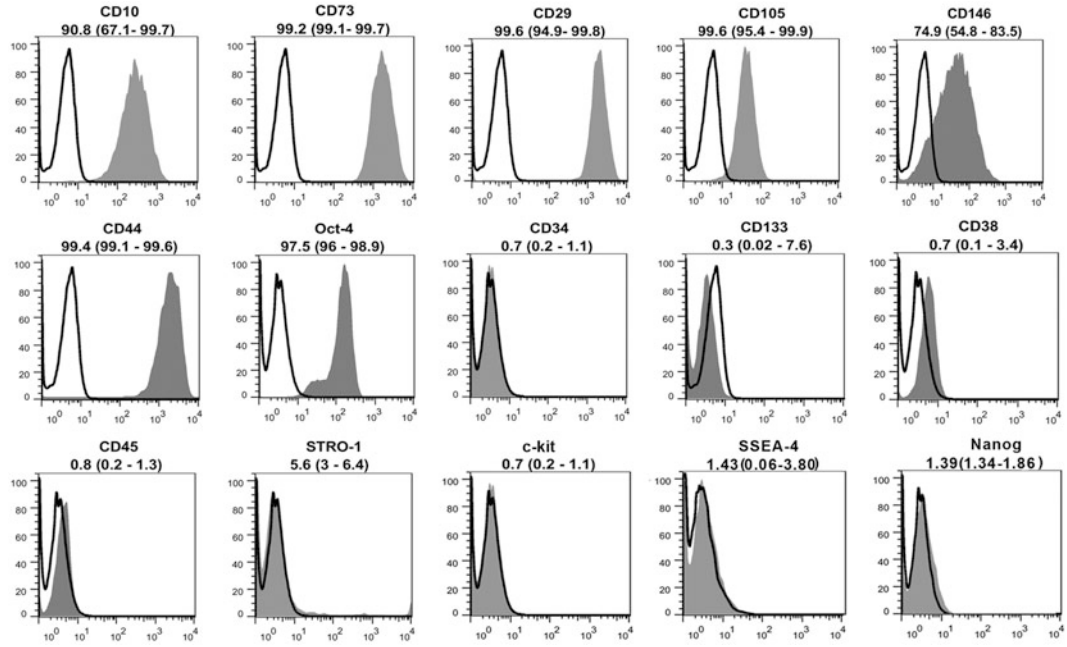

Fig. 1 Immunophenotyping of MenSCs by flow cytometery. CD markers are demonstrated in *gray* and the respective isotype control is shown as *colorless*. The results are presented as median (range) of 3–5 independent experiments

mixture of formic acid and acetic acid was found to produce uniform PCL fibers in the nanoscale range. Glacial acetic acid, 90 % acetic acid, methylene chloride/DMF, glacial formic acid, and formic acid/acetone are the other possible combinations which have been investigated as a solvent system for PCL electrospinning (48).

19. Plasma surface modification can improve biocompatibility and bio-functionality. It can efficiently promote cell adhesion, and reduce surface friction as well as tackiness. The plasma is effective at near-ambient temperature without damage for most heat-sensitive biomaterials. Plasma treatment modifies only the near surface of treated substrates and does not change the bulk material properties. There are, however, some disadvantages, including the need for a vacuum environment, relatively high cost, and poorly defined chemistry. However, on the whole, the advantages of plasma surface treatment outweigh any disadvantage because it allows many types of modifications that cannot be generated by other methods (49).
20. Immersion in ethanol may cause shrinkage of some electrospun scaffolds. Thicker and bigger scaffolds may accommodate these dimensional changes.
21. Higher cell density reduces the transdifferentiation efficiency.

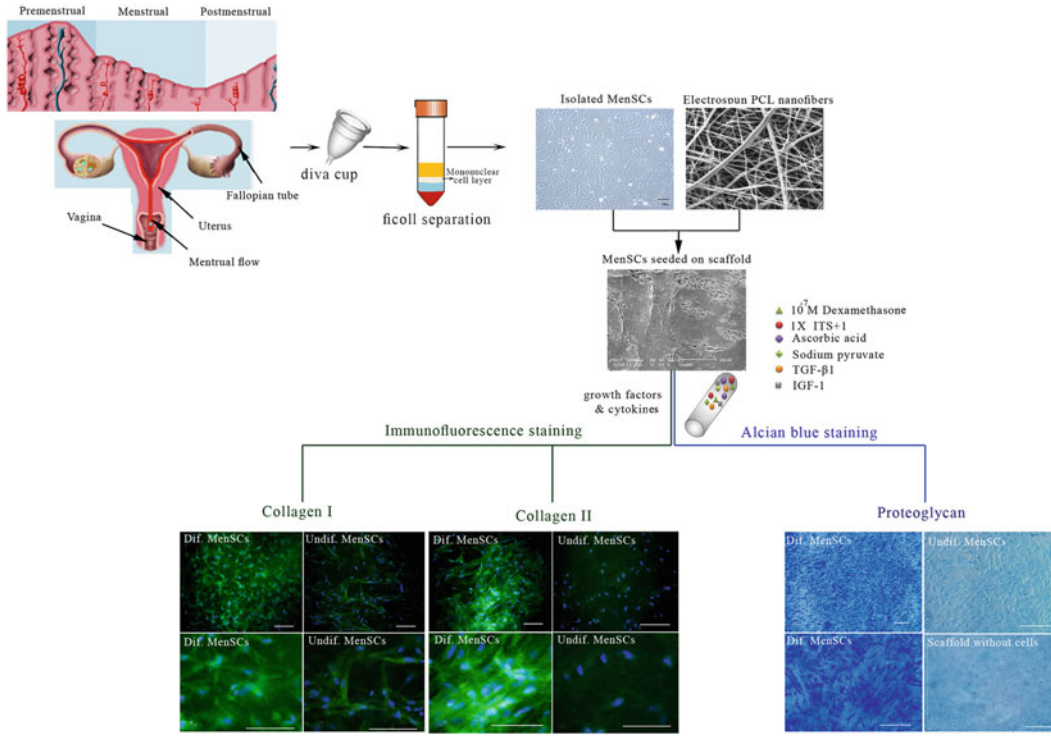

Fig. 2 Schematic diagram of isolation, culture, and chondrogenic differentiation of MenSCs on nanofibrous scaffold. MenSCs are isolated from menstrual blood and cultured at optimal conditions. The isolated MenSCs have fibroblastic shape and conserve their morphology up to passage 10. The cultured cells at passage 2 are seeded on nanofibrous PCL scaffold. In this nonwoven, randomly oriented nanofiber, the region of diameter distribution of PCL ranges from 200 to 1,500 nm. The image analyses of the SEM micrographs of cells seeded on the scaffold show that cells penetrated and adhered well on the surface of the web. Development of cartilage-like tissue in cultured constructs has been examined histologically with respect to the presence of proteoglycan (*alcian blue*) and collagen type II/I (immunostain)

22. Medium should be added gently along the sides of the well following incubation of the wetted scaffold with the cell suspension.
23. During the initial 2 h of incubation, apply 50 μL of serum-containing culture medium to each cellular scaffold every 30 min to prevent drying out of the constructs.
24. For the first day at least, avoid moving the plate as much as possible.
25. Prior to differentiation culture in medium containing b-FGF has a significant effect on the ability of MSCs to undergo chondrogenesis (50, 51).
26. The seeded cells on scaffold without chondrogenic induction are served as control group.
27. Wash the cell–scaffold constructs with warm PBS and transfer them to clean well. Keep the scaffolds wet using PBS for future use.

28. In this step, rinsing the cell–scaffold constructs is too important for removing background stain. Washing should be lasted for at least 5 min in 10–15 mL PBS on a shaker.
29. Keep the scaffolds wet using PBS.
30. The results of prepared sections of the constructs with ≤ 5 μm thickness can be followed using an epifluorescence microscopy.
31. Unlike undifferentiated MenSCs, cells differentiated on scaffold have strong immunoreactivity with monoclonal antibody against collagen type II, the hallmark of chondrogenic differentiation. Moreover, the ratio of collagen type II:I would increase during differentiation of cultured MenSCs (Fig. 2).
32. An abundant accumulation of proteoglycan, the ECM material produced by differentiated cells, is shown in newly formed construct (Fig. 2).

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Engineering of a 3D Nanostructured Scaffold Made of Functionalized Self-Assembling Peptides and Encapsulated Neural Stem Cells

Carla Cunha, Silvia Panseri, and Fabrizio Gelain

Abstract

Three-dimensional (3D) in vitro models of cell culture aim to fill the gap between the standard 2D cell studies and the in vivo environment. Especially for neural tissue regeneration approaches where there is little regenerative capacity, such models must rely on scaffolds that mimic the extracellular matrix in providing support; allowing the natural flow of oxygen, nutrients, and growth factors; and possibly favoring neural cell regrowth. Their combined use with stem cells has many potentialities for tissue engineering applications. Here, we describe a new 3D model of stem cell culture, using a nanostructured biomaterial, made of self-assembling peptides, where adult neural stem cells are completely embedded. This new 3D cell culture system takes advantage of the nano- and microfiber assembling process of these biomaterials under physiological conditions. The assembled scaffold forms an intricate and biologically active matrix able to display specifically designed functional motifs such as RGD, BMHP1, and BMHP2. Such model has the potential to be tailored to develop ad hoc designed peptides for specific cell lines.

Keywords: 3D scaffold, Biomaterials, Self-assembling peptides, Neural stem cells

1 Introduction

It is now accepted that 3D models of cell culture have superior potential for tissue engineering applications; still, for the peripheral and central nervous systems, few 3D approaches have been proposed. The nature of the scaffold is a key concern since it will provide cell support and allow exchanges of oxygen, nutrients, growth factors, and cytokines. In trying to reproduce the extracellular matrix (ECM) conditions, such biomimetic scaffolds need to display a series of characteristics: biodegradability and negligible immune response by the host tissue, tailored mechanical properties, appropriate porosity and permeability, and, finally, they need to be produced in a large scale and in a reproducible way (1, 2). One of

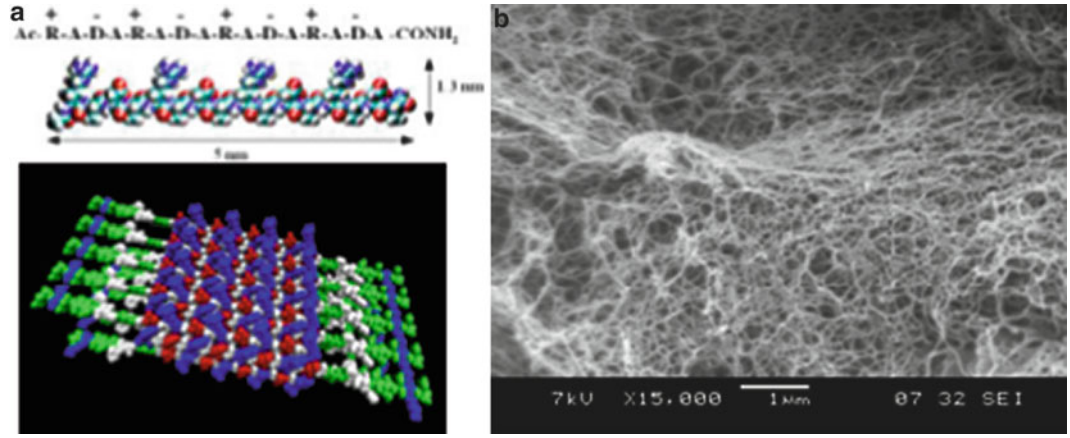

Fig. 1 RADA16 is a 16-residue peptide composed of alternating hydrophilic arginine, hydrophobic alanine, and hydrophilic aspartic acid. It self-assembles forming antiparallel β-sheets at physiological pH (**a**). It forms an intricate nanofiber matrix that closely mimics the architecture of the ECM and that is able to supply environment and support to neural stem cells (**b**)

the main difficulties in many 3D culture systems is obtaining the appropriate internal organization of the scaffold. In order to have cells in a truly 3D microenvironment, the dimensions of the scaffold fibers and pores must be substantially smaller than the cells so that cells are fully surrounded by the scaffolds, much like the in situ cytoarchitecture. Moreover, most of the scaffolds used so far are made from either synthetic polymers, which present very limited cellular recognition motifs therefore hindering cell–scaffold interaction, or naturally derived polymers that often present residual undefined or non-quantified elements (2). New classes of biomaterials are needed, which comply with the biological requirements and that may overcome most of these problems. RADA16-like self-assembling peptides (SAPs) are a recent class of biosynthetic materials with potential use in the development of scaffolds for 3D cell cultures (3). They are composed of natural amino acids that spontaneously self-assemble under physiological conditions into double-layered antiparallel β-sheets forming a scaffold with nano- and microfibers and pores with dimensions 10–100 times smaller than those of cells so that cells enclosed within are in a true 3D environment that closely mimics the architecture of the ECM (see Fig. 1) (4, 5). The most used SAP for scope of neural cell culture is RADA16-I, a 16-residue peptide composed of alternating hydrophilic arginine, hydrophobic alanine, and hydrophilic aspartic acid (RADARADARADARADA). They consist of greater than 99% water content and are made of peptide molecules that can break down into natural amino acids, which can potentially be used by the cells. RADA16-I can be synthesized commercially with high purity and, importantly, it can be custom-tailored in

order to incorporate functional motifs for specific cell culture applications.

While most of the research on biomaterial scaffold for tissue engineering resides on the fabrications of the biomaterial itself, few has analyzed them as a whole with a cellular component. In the last decades neural stem cells (NSCs) have been the focus of much attention for tissue engineering purposes due to their capacity to potentially regenerate all the major cellular phenotypes in a site of neural tissue injury without inducing an immune response due to their inherent undifferentiated state. Moreover, they are able to replicate indefinitely in vitro, maintaining a stable profile; so they are a potential renewable source of cell lineages from the CNS (6, 7).

In this chapter, we describe a 3D cell culture assembling protocol, making use of RADA16-I-based SAPs functionalized with motifs from collagen VI (RADA16-RGD) and two bone marrow homing peptides (RADA16-BMHP1 and RADA16-BMHP2). These SAPs are soluble in aqueous solutions and assemble into nanofiber scaffolds upon exposure to physiological pH solutions such as the NSC culture medium. Cells are mixed with the SAPs and immediately placed in contact with culture medium in order to allow assembling of the scaffolds with cell embedded within. This 3D system has demonstrated high biocompatibility, NSC proliferation, and differentiation into the three major neural cellular phenotypes: neurons, astrocytes, and oligodendrocytes and rheological features within the range of native brain tissue (8).

We will describe three separate procedures: (1) biomaterial synthesis, purification, and characterization; (2) subculturing and long-term expansion of adult mouse NSCs; and (3) 3D scaffold preparation and NSC encapsulation. Such 3D model system has the potential to be further developed and improved for use with different cell lines, with a low batch-to-batch variability and on a large scale, for different tissue engineering strategies and to meet clinical application standards in the near future.

2 Materials

2.1 Components for Biomaterial Synthesis, Purification, and Characterization

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

1. Amino acids, protected form (Novabiochem).
2. Rink amide MBHA resin (Sigma).
3. Acetic anhydride (Sigma).
4. Reagent K (82.5% TFA/5% phenol/5% water/5% thioanisole/2.5% ethylene dithiol).

5. Liberty Microwave Peptide Synthesizer (CEM).
6. MALDI-TOF mass spectrometry (Applied Biosystems).
7. Reverse phase HPLC (Waters).
8. Analytical and semi-preparative BioBasic C4 300 Å (Thermo Scientific).
9. HCl (Sigma).
10. Methanol (Sigma).
11. Ethanol (Sigma).
12. Glutaraldehyde (Sigma).
13. Diethyl ether (Sigma).
14. H_2O/CH_3CN (60/40 v/v) (Sigma).
15. Lyophilizer (Labconco).
16. Polaron Range sputter coater (DentonVaccum).
17. CO_2 critical point dryer (Tousimis).
18. Scanning Electron Microscope, JSM-6060 (JEOL).

2.2 Components for Subculturing and Long-Term Expansion of Adult Mouse NSC

2.2.1 Cell Culture Medium and Solutions

These cell cultures are extremely sensitive to contaminants present in water or glassware. Distilled sterile apyrogenic water should be used (before use filter sterilize in sterile disposable plastic bottles). Otherwise you can purchase ultrapure cell culture-grade water.

1. *EGF*: Recombinant human epidermal growth factor. Peprotech. Aliquot stock: Reconstitute EGF in order to have a 500 ng/ml stock. Aliquot into sterile tubes and store at −20°C.
2. *FGF2*: Recombinant human fibroblast growth factor (FGF2) basic. Peprotech. Aliquot stock: Reconstitute FGF2 in order to have a 500 ng/ml stock. Aliquot into sterile tubes and store at −20°C.
3. Neurocult® NS-A Basal Medium (Mouse). StemCell Technologies. To 450 ml of NS-A add 50 ml of proliferation or differentiation supplement, 40 μl EGF-stock (final concentration 20 ng/ml), and 20 μl FGF2-stock (final concentration 10 ng/ml).
4. NeuroCult® NSC Proliferation Supplement (Mouse) (StemCell Technologies).
5. NeuroCult® NSC Differentiation Supplement (Mouse) (StemCell Technologies).
6. 1× DPBS, no calcium, no magnesium (Gibco).
7. Penicillin/streptomycin (Gibco).
8. Trypan blue (Sigma).
9. Matrigel, growth factor-reduced. BD Biosciences. 100× matrigel stock solution: Thaw a vial of matrigel overnight at 4°C. Aliquot into sterile tubes (0.5 ml/aliquot) using refrigerated plastic pipettes and store at −20°C.

2.2.2 Plasticware and Instruments

All plasticware must be sterile. A set of glassware to be used only for cell culture should be prepared. Bottles, cylinders, beakers, etc. should be accurately rinsed several times with distilled water before being sterilized in autoclave that is used for cell culture purposes only. We suggest that medium and all stock solutions be prepared in sterile disposable tubes/bottles.

1. Flasks: 75 cm^2, 0.2 mm vented filter cap (Corning).
2. Bottles (Costar Incorporated).
3. Syringe filters, cellulose acetate 25-mm, 0.22 mm (Nalgene).
4. 10 ml plastic pipettes (Costar Incorporated).
5. 15 ml plastic conical tubes (Falcon).
6. Pipette set: 2, 20, 200, 1,000 μl (Gilson).
7. Sterile pipette tips: 10, 20, 200, 1,000 μl (Corning).
8. 24-Multiwell plates (Corning).
9. Bench centrifuge (Thermo).
10. 37°C waterbath (F.lli Galli).
11. Cell culture incubator set at 37°C, 5% CO_2 (Binder).
12. Laminar flow hood (Olympia).
13. Routine light microscope (Axiovert, Zeiss).

2.3 Components for 3D Scaffold Preparation and NSC Encapsulation

1. Distilled H_2O (GIBCO).
2. Cell culture insert, PET track-etched membrane, 1.0 μm pore size (BD Biosciences).
3. 24-Multiwell plates (BD Biosciences).
4. 0.5 ml conical tubes (Eppendorf).
5. Glucose (Sigma). 8% glucose solution: Mix 8 g glucose in 100 ml water. Filter sterilize. Store at 4°C.
6. Cryoscopic osmometer, osmomat 030 (Gonotec).
7. Sonicator, S30H Elmasonic (ELMA).
8. Cell culture incubator set at 37°C, 5% CO_2 (Binder).
9. Laminar flow hood (Olympia).

3 Methods

3.1 Biomaterial Synthesis, Purification, and Characterization

Carry out all procedures at room temperature unless otherwise specified.

1. Synthesize the functionalized SAPs by Fmoc solid-phase method, using a Liberty Microwave Peptide Synthesizer. Purchase all amino acids in their protected forms and use a Rink amide MBHA resin.

2. Acetylate the N-terminal of each peptide by acetic anhydride and perform cleavage with reagent K.
3. Verify the molecular weight of each peptide by MALDI-TOF mass spectrometry.
4. Verify the purity of each peptide by analytical high-performance liquid chromatography (HPLC) (see note 1).
5. Precipitate the cleaved product and wash it with diethyl ether multiple times.
6. Dissolve the product in H_2O/CH_3CN (60/40 v/v) and lyophilize. Replace trifluoroacetic acid salts, arising from synthesis and purification of SAPs, with chloride salts via dissolution in HCl.
7. Verify the molecular weight of the peptide by MALDI-TOF mass spectrometry.
8. Purify the crude peptide in a CH_3CN/water gradient, using a reverse-phase Waters HPLC equipped with an analytical and semi-preparative BioBasic C4 300 Å.
9. Lyophilize the purified product (see note 2).
10. Upon incorporation of functional motifs to the RADA16 peptide, one concern is that the appended portion of the peptides could inhibit nanofiber formation. To address this concern, different characterization techniques may be used: scanning electron microscopy (SEM) (see note 3), rheological analysis (see note 4), circular dichroism spectroscopy (CD) (see note 5), and atomic force microscopy (AFM) (see note 6).

3.2 Subculturing and Long-Term Expansion of Adult Mouse NSC

The procedure for adult mouse NSC isolation from the subventricular zone (SVZ) and subsequent expansion in a fully chemically defined, serum-free medium has been described previously (6, 7, 9, 10). For in vivo experimentation, each researcher must follow his or her country's legislation and guidelines for the care and use of laboratory animals. Carry out all in vitro procedures under a sterile laminar flow hood.

1. For in vitro culture and expansion, culture NSCs in Neurocult NS-A Basal Medium supplemented with NeuroCult® NSC Proliferation Supplement and containing 20 ng/ml of epidermal growth factor (EGF) and 10 ng/ml of FGF2, seeded in 75 cm^2 cell culture flasks at a concentration of 10^4 cells/cm^2 (see note 7).
2. For NSC subculturing by mechanical dissociation, tap the flask to dislodge neurospheres and transfer its content to a 15 ml plastic conical tube using a 10 ml plastic pipette. Rinse the flask with 5 ml of fresh medium and add it to the tube (see note 8).
3. Pellet the cell suspension by centrifugation at 900 rpm for 10 min (see note 9).

4. Remove the supernatant leaving behind approximately 150 μl.
5. Use a 200 μl pipette set at a volume of 180 μl to gently dissociate the pellet 50–60 times by passing the cells through the tip (see notes 10 and 11).
6. Count the number of viable cells using the trypan blue dye exclusion test and reseed cells at the density of 10^4 cells/cm^2 in culture medium in untreated cell culture flasks (see notes 12 and 13).
7. Feed cells with fresh medium every 3 days (see note 14).
8. After 4/5 days, when neurospheres reach diameters of near 200 μm, proceed with a new subculturing step (see notes 15 and 16).
9. For verification of NSC staminality, culture NSCs in Neurocult NS-A Basal Medium supplemented with NeuroCult® NSC Differentiation Supplement, seeded in 24-multiwell plates coated with Matrigel, at a concentration of 10^4 cells/cm^2 (see notes 17 and 18).

3.3 3D Scaffold Preparation and NSC Encapsulation

Carry out all procedures under a sterile laminar flow hood.

1. The day before NSC encapsulation, dissolve all functionalized SAPs and RADA16-I in sterile dH_2O to the concentration of 1% (w/v).
2. The day of the experiment, sonicate SAPs for 30 min, immediately prior to use.
3. Dilute 12 μl of each SAP with 12 μl of glucose 8%, in order to cope with the cellular osmolarity (~260–320 mOsm/l), to the final concentration of 0.5% (w/v).
4. Keep the final 24 μl of each SAP in a 0.5 ml conical tube.
5. Slowly add 8 μl of serum-free culture medium (see note 19) containing a cell density of 4,000 cells/μl, in a total of 3.2×10^4 cells, to the SAP and gently mix using a 200 μl pipette (see notes 20–22).
6. Place the NSC/SAP mixture directly over the membrane of a cell culture insert, already inserted into a 24-multiwell plate containing appropriate cell culture medium for either proliferation or differentiation assays (see Fig. 3 and note 23).
7. Allow SAP to self-assemble at 37°C, 5% CO_2 for 30 min (see-note 24).
8. Replace culture medium every 3 days (see note 25).
9. Evaluation of cell proliferation may be made by MTT assay and evaluation of cell differentiation may be made by immunofluorescence against neurons, astrocytes, and oligodendrocytes (see note 26).

4 Notes

1. In HPLC purification, an acid elution solution is recommended to prevent peptide self-assembling in the analytical and semi-preparative columns. Usually 0.1% of trifluoroacetic acid will suffice.
2. An acceptable purity of the final product must be >95%.
3. For SEM characterization, dissolve SAPs in water and allow to self-assemble at 37°C. Soak samples in 5% glutaraldehyde at 4°C for 2 h, wash in MilliQ water, slowly dehydrate in 10% increment steps of ethanol for 5 min each, and place in a pressurized liquid CO_2/siphon for 1 h using a CO_2 critical point dryer. Sputter-coat scaffolds using a Polaron Range sputter coater and mount on a copper grid to be examined by SEM.
4. Mesoscopic mechanical properties of the assembled scaffolds could be assessed with a TA Instruments AR2000 rheometer. Storage moduli should be measured at frequencies from 1 to 10 rad/s using a 20 mm, 0.5° stainless steel cone with a truncation gap of 9 μm while the strain is held constant at 1%. Each measurement should be performed with 35 μl of gel assembled with 70 μl of 1× PBS solution after allowing 1 h for assembly.
5. CD is recommended to assess the secondary structure of the SAPs in solution. Solutions for analysis are made by diluting 1% (w/v) stock peptide solution to a concentration of 20 μM using Milli-Q water. Spectral deconvolution via CDNN software is a valid option for quantification of β-sheet structure components.
6. AFM images may be performed with a silicon scanning probe (FESP, Veeco Probe Inc., California), with a resonance frequency of 75 kHz, spring constant 2.8 N/m, tip curvature radius 10 nm, and 225 μm length. Images are obtained with a multimode AFM microscope (Nanoscope IIIa, Veeco, Santa Barbara, California) operating in tapping mode. Typical scanning parameters are as follows: RMS amplitude before engage 1–1.2 V, set point 0.7–0.9 V, integral and proportional gains of 0.2–0.6 and 0.4–1.0, respectively, and scan rate 1.51 Hz. Images are subsequently flattened and graphically sliced to measure fiber height and width. Because of tip convolution the measured widths and lengths should be mathematically deconvolved according to tip radius and fiber height.
7. Serum-free CNS stem cell cultures represent a selective system in which most primary differentiated CNS cells are eliminated soon after having been put in culture while the undifferentiated stem cells enter into an active proliferation state. Four conditions should be met for the NSCs to become the main cell type in

these cultures: (1) low cell density (approximately 5×10^4 cells/cm^2), (2) absence of serum, (3) addition of the appropriate growth factors (i.e., EGF and/or FGF2), and (4) absence of a strong cell adhesion substrate.

8. To prevent cells from sticking to the walls of the Pasteur pipette rinse the pipette several times with medium before every dissociation step, or when a new pipette is used.
9. The centrifugation speed depends on the dimension of the spheres.
10. The aim is to obtain a single-cell suspension to be replated. Rinse tip with medium first, to avoid cell sticking inside the tip. Rinse down the walls of the tube periodically to dislodge undissociated spheres. Slightly tilt the pipette and press tip against the bottom of the tube to generate a fair amount of resistance.
11. During dissociation always avoid foaming and bubbles.
12. Viability after dissociation should never fall below 50–60% of the total cell number.
13. If dissociation has been efficient almost the totality of the cells plated after subculturing should be single cells (see Fig. 2a).
14. Feed cells with fresh medium, replacing about 50% of the total volume in the flask.
15. Subculture when the neurospheres start to lift off and float in suspension. This will require approximately 4/5 days for adult murine cultures; at this time they normally reach a diameter of approximately 100–200 μm. As a general rule, neurospheres should be passaged when they are sufficiently large (see

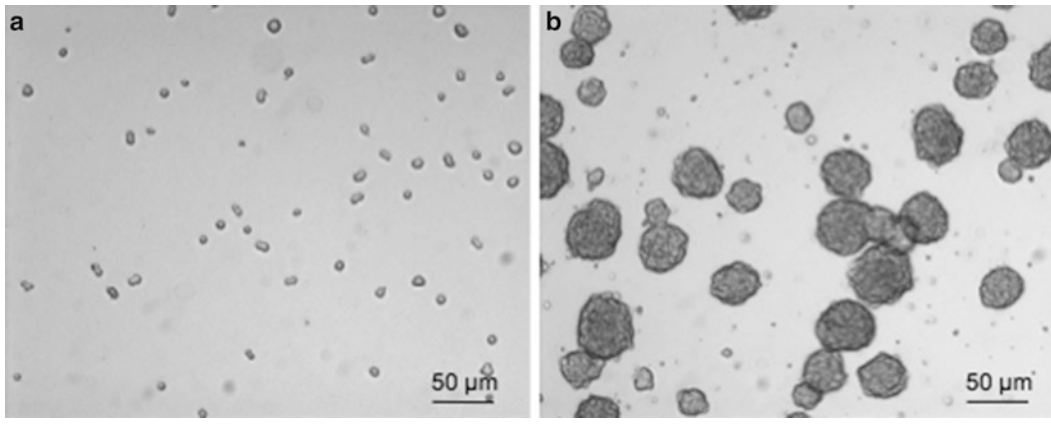

Fig. 2 Adult mouse NSCs cultured in Neurocult NS-A Basal Medium supplemented with NeuroCult® NSC Proliferation Supplement and containing 20 ng/ml of epidermal growth factor (EGF) and 10 ng/ml of fibroblast growth factor (FGF2). (**a**) Single cells, immediately after mechanical dissociation; (**b**) neurospheres can be seen after 4/5 days in culture, ready to be mechanically dissociated

Fig. 2b). If neurospheres are too small the yield will be low; if they are too large the number of dead cells will be high, dissociation will be difficult, and viability of the culture will be low. Should this situation be protracted for some subculturing steps, the total number of cells in the culture will progressively decrease and the culture will be lost.

16. Each subculturing step should result in an increase of the total cell number from two to five times, depending on the culture conditions and operator's skills. The overall cell number increase is due to the simultaneous expansion of the NSC pool as well as due to an increase in the number of more mature progenitors and differentiating cells. The latter two will be lost at each subculturing step.
17. For determination of staminality, quantify the relative proportion of neurons, astrocytes, and oligodendrocytes. Generally, an increase in subculturing passages does not affect the proportion of the different cell types produced by stem cell progeny upon differentiation.
18. Adhesion to a substrate and removal of growth factors may result in up to 50% cell loss, so use an initial large number of cells for plating.
19. It is important to use serum-free cell culture media to avoid adsorption of random serum proteins within the scaffolds.
20. NSCs used for SAP encapsulation should be used 2 days after the last mechanical dissociation in order to obtain the maximum percentage of NSCs.
21. Use NSCs that have been subcultured at least twice so that in your working cultures short-term dividing precursors are absent.
22. The mixing of NSCs with SAP must be done gently but quickly in order to avoid self-assembling and prolonged exposure to low-pH environment at the tip of the pipette and, moreover, to guarantee that the whole amount is transferred to the cell culture insert.
23. Place the 24 μl of SAP/NSC mixture in the center of the membrane so that it will not touch the walls of the cell culture inserts. In this way, such volume will form a drop and not distribute throughout the membrane and will assemble a scaffold with thickness that reaches 300 μm.
24. Scaffold self-assembling proceeds gradually as culture medium crosses the cell culture insert porous membrane. Upon assembly, a hydrogel consisting of greater than 99% water (peptide content 1–10 mg/ml) is produced.
25. Feed cells with fresh medium, replacing about 50% of the total volume in the well.

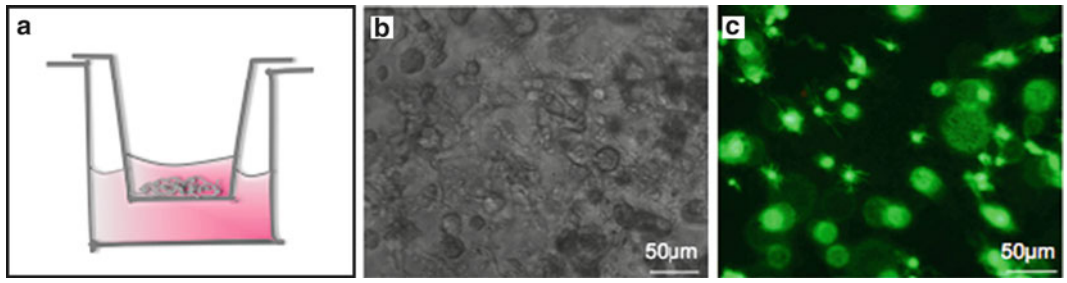

Fig. 3 (**a**) Schematic representation of the 3D culture model set up for NSC encapsulation within SAP scaffolds. SAPs were mixed with NSCs and placed within a cell culture insert, which is then immerged in the culture medium. Scaffold assembling proceeds gradually as culture medium crosses the cell culture insert porous membrane. (**b**) Adult mouse NSCs cultured for 5 days within the 3D scaffold made of RADA-BMHP2. (**c**) Adult mouse NSCs cultured for 7 days within the 3D scaffold made of RADA-BMHP2. Cells were incubated with calcein AM

26. Differentiation within the scaffold is extremely difficult to quantify due to possible binding of the antibodies to the scaffold, but mostly due to the high number of scattering z-stacks in the samples. This poses a challenge to fluorescence microscopy; suitable imaging techniques are needed, able to systematically analyze systems that are much more challenging than cell monolayers. In Fig. 3, we can see an example of differentiated NSCs marked with calcein AM for live cells.

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