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Collagen-graft mixed cellulose esters membrane maintains undifferentiated morphology and markers of potential pluripotency in feeder-free culture of induced pluripotent stem cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are unique and unlimited clinical sources of stem cell therapy for the regenerative medicine. Feeder layer preparation is an important step for iPSCs production, which is expensive, time-consuming and requires conversance. In the present study, we investigated the maintenance of pluripotency, and stemness of the iPSCs through feeder-free culture on a collagen-grafted Mixed Cellulose Esters membrane (MCE-COL) after three passages during twelve days. Results have demonstrated that the iPSCs cultured on MCE-COL membrane had a fine, typical undifferentiated morphology, increased proliferation rate and significant multi-lineage differentiation potential. Alkaline phosphatase (ALP) staining and pluripotency associated gene markers expression further confirmed that iPSCs cultured on the surface of MCE-COL had more ALP positive colonies and enhanced expression of Oct-4, Nanog, Sox-2 and ALP in comparison with MCE and control groups. Since MCE-COL membrane has three dimensional structure and bioactivity, it has the potential for usage in the feeder-free culture of iPSCs, and could be a suitable candidate to use as a feeder layer in stem cells preparation.

1. Introduction

Embryonic stem cells (ESCs) were first discovered in 1981 when isolated from mouse blastocysts [1].These cells were discovered with special abilities to self-renew, pluripotency and ability to differentiate into various cell type [2,3]. The factors that play the main role in identifying ESCs can also play a critical role on pluripotency induced by reprogramming of several types of somatic cells called induced pluripotent stem cells (iPSCs) [2,4,5].

iPSCs were first prepared from mouse fibroblasts in 2006 [2,6]. These cells are similar to ESCs in all characteristics such as morphology, proliferation, gene expression, epigenetic status of pluripotent cell-specific genes [6,7]. In recent years, stem cell therapies have been increasing around the world and researchers are trying to improve stem cell usage methods in medical stem cell therapies, i.e., immunomodulatory effects, cancer therapies, tissue engineering and etc. [8–10]. Stem cells and scaffolds are two essential components for improving tissue engineering within different protocols [11–13]. Advent of new modified scaffolds increases the application of tissue engineering in treating many hospitalized patients around the world [10,14]. The best tissue-engineered products would mimic characteristic properties of damaged tissues [15]. These characteristic properties involve are

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biocompatibility, biodegradability and resemblance to the host tissue in order to prevent the rejection of grafted cells or tissues [10,16]. In recent years, scientists have been trying to find scaffolds to achieve the best results with least incompatibility and more advantages. Generation of human iPSCs from dermal fibroblasts by reprogramming is ethically acceptable, and it creates great hope for progression of regenerative medicine [17].One of the advantages of iPSCs compared to ESCs is that iPSCs are derived from patient-specific somatic cells which decreases the potential immune concerns. Recently, Human iPSCs (hiPSCs) or Mouse iPSCs (miPSCs) have been used for tissue engineering [14,18–20]. Creating optimum feeder for culturing the iPSCs is a critical for preparing iPSCs for the next steps of experiments. iPSCs were cultured on mouse embryonic fibroblast (MEF) feeder cells in serum-containing media, which can supply the required matrix and synthesize inhibitors of differentiation [21]. It has been recently proposed that nanofibrous structure developed by electrospinning technology provides the closest form of *in vivo* situation for culturing and differentiation of stem cells in *in vitro* [22]. Thus, significant efforts have been focused on developing 3D nanofibrous scaffolds to boost tissue engineering at laboratory level [14,22–24]. An ideal iPSCs feeder must provide biophysical and biochemical cell properties similar to 3D micro-environments [25]. Although MEF used as the routine cells' feeder layers provides these properties for iPSC culturing, concerns about the immune responses against these heterogeneous cells has hindered the advances in improved usage of these new advanced methods. Thus, to reduce xeno-components, several new feeder-free culture systems have been previously been reported by using matrigel-matrix, MEF-conditioned media, and different growth factors for maintenance, expansion and therapeutics of human ESCs [26,27]. This study is aimed to investigate the possible effect of Mixed Cellulose Esters membrane (MCE) as xeno-free feeder for hiPSCs-3D culturing.

2. Materials and methods

2.1. Membrane filter

MCE membrane is biologically inert mixture of cellulose acetate and cellulose nitrate with 1.2 μm pore size, 47 mm diameter plain and hydrophilic (cat number: RAWP04700,Merck Millipore, USA).

2.1.1. Sterilization of MCE membrane

The membranes were cut out in 1 cm^2 in diameters. In order to sterilize, the membranes were incubated in 70% ethanol for 2 h, then washed twice with phosphate-buffered saline (PBS) and then incubated in Dulbecco's modified Eagle's medium (DMEM) mixed with 1: 1 Ham's nutrient mixture F-12 (DMEM/F12) supplemented with 15% knockout serum replacement (KSR) penicillin, streptomycin, and amphotericin (all from Invitrogen Co. USA) overnight to prevent bacteria, fungi and yeast growth.

2.1.2. Membrane filter preparation and surface treatment

After sterilization, the membrane filters were immersed in sterilized 1 mg/ml collagen type I solution (Nutacon BV, Netherlands) overnight. All experiments were carried out in four main groups: MCE with collagen-grafted (MCE-COL) and without collagen grafted (MCE). Tissue culture polystyrene with gelatin-coated (TCPS-Gel) and without gelatin grafted (inactivated MEF on TCPS) was used as control group.

2.2. Cell culture

hiPSCs lines were gifted from Stem Cells Technology Research Center Cell Bank (Tehran, Iran). These cells were previously characterized and published [28]. hiPSCs cultured on mitomycin-C (Invitrogen, USA) inactivated MEF under DMEM/F12 medium supplemented with 15% KSR, 0.1 mmol/L non-essential amino acids, 1 mmol/L L-glutamine (all from Invitrogen), 0.1 mmol/L β-mercaptoethanol (Sigma), penicillin/streptomycin (sigma) and 4 ng/ml of human fibroblast growth factor 2 (Invitrogen), and about 60% of the medium was replaced every days. After that hiPS colonies were detached with 0.1% Collagenase IV and transferred on to MCE-COL, MCE, TCPS-Gel and inactivated MEF on TCPS for 12 days (3 passages, every 4 days) and the medium was replaced every days.

Table 1
Primers used in the qRT-PCR technique.

Genes	Primers 5′→3′	Product size (bp)
Nanog	F: TGATTTGGTTGGTGTCTTG R: TGTGATGGCGAGGGAAG	175
Oct	F: GGATGGCATACTGTGGAC R: CTTGGCAAACTGTTCTAGC	233
Sox-2	F:GGACTGAGAGAAAGAAGAGGAG R: GAAAATCAGGCGAAGAATAAT	196
ALP	F:CCTAAAAGGGCAGAAGAAGGAC R: TCCACCTAGGATCACATCAATG	444
β2-M	F: TTCAGTCGCGGTCGCTTCAGTC R:CAATGTGAGGCGGGTGGAACTG	196

2.3. MTT assay

For biocompatibility evaluation of membranes, the proliferative rate of hiPSCs seeded on MCE, MCS-Col, TCPS-Gel and inactivated MEF on TCPS was evaluated via MTT assay. Cells were seeded with an initial cell density of 1×10^4 cells per cm^2 in a 96-well culture plate and incubated at 37 °C, 5% CO_2 condition. After 24, 48, 72 and 96 h of cell seeding, 50 μl of MTT solution (5 mg/ml in DMEM) was added to each well ($n = 3$). For conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells, the plate was incubated at 37 °C, 5% CO_2 for 3.5 h. The supernatant was removed and constant amount of Dimethyl sulfoxide (DMSO) solvent was added. The optical density was read at a wavelength of 570 nm in a micro-plate reader (BioTek Instruments, USA).

2.4. Evaluation of potency

2.4.1. Adipogenic and osteogenic differentiation

After 12 days (3 passages, every 4 days), the hiPSCs were detached with 0.1% Collagenase IV and cultured at a density of 5×10^4 cells/cm^2 on six-well plate under the osteogenic and adipogenic differentiation media. To induce adipocyte differentiation, hiPSCs were cultured under DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO), 1 mM dexamethasone, 10 mg/mL insulin, 200 μM indomethacin, 1.7 μM insulin, 500 lM 0.05 U/mL penicillin, and 0.05 μg/mL streptomycin for 14 days. For adipogenic differentiation, Oil red-O-staining was used to display intracellular lipid accumulation. Cells were fixed in cold 4% paraformaldehyde for 30 min at 4 °C, washed with PBS and stained with Oil red-O-solution for 5–10 min at 37 °C, followed by repeated washing with PBS three times and Oil red staining was detected by light microscopy. For osteogenic differentiation, hiPSCs were cultured under DMEM supplemented with 10% FBS, 3 mM β-glycerophosphate (βGP), 50 μg/mL ascorbic acid (AA) and 10^{-9} M dexamethasone. The medium was replaced every two days. After 2 weeks, to evaluate the mineralized matrix, Alizarin red staining was performed, by washing cells with PBS and then fixed in cold 4% paraformaldehyde for 30 min at 4 °C. These cells were then washed again with PBS. The fixed samples were stained with 2% Alizarin red S at pH 7.2 (Sigma). After 5–10 min at