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Spheroid cultures promote the stemness of corneal stromal cells

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ABSTRACT

Several culture methods generated spheroids of rabbit and mouse corneal stromal cells (CSCs) in vitro. In this study, rabbit CSC spheroids were positively expressed the mesenchymal and stem cell phenotypes, which contained immunopositive for vimentin (a mesenchymal cell marker) and CD34 (a stem cell marker), as well as mRNA expression of nestin (a neural stem cell marker) and Nanog (a stem cell marker), in suspension or adherent cultures that were induced by methylcellulose, a rotary cell culture system (RCCS) or reprogramming proteins and VPA. Mouse CSCs showed poor growth and hardly formed spheroids after treatment with methylcellulose or reprogramming proteins and VPA. Our work has laid a promising foundation to elucidate CSCs and the further use of CSC spheroids for reprogramming, bioprinting and tissue engineering

1. Introduction

The corneal stroma mainly consists of a dense and regularly packed collagen fibril extracellular matrix (ECM) deposited by corneal stromal cells (CSCs) during late embryonic development (Hassell and Birk, 2010). According to specific environmental condition and signals, CSCs possess at least three different phenotypes, including quiescent dendritic keratocytes, fibroblasts and myofibroblasts (Jester and Ho-Chang, 2003). The cues presented to CSCs are the major determinants of their phenotypes. Moreover, there is a small population of corneal stromal stem cells (CSSCs), progenitors or precursors in the corneal stroma, which is largely located in the peripheral stroma, and CSCs represent the default lineage. These CSSCs have a role in corneal stromal wounds healings (Pinnamaneni and Funderburgh, 2012; Mimura et al., 2008a,b). Isolation of CSSCs by sphere forming assay has been reported (Funderburgh et al., 2005; Yoshida et al., 2005). These cells formed spheres in culture, showed side population characteristics, were multipotent and expressed various adult stem cell markers. Normally, cells in spheroid culture exhibit some properties that are distinct from monolayer cells. For example, they grow with similar characteristics to in vivo tissue and can simulate native tissue behaviors much more accurately than two-dimensional (2-D) cultures. Furthermore, stem cells with self-renewing capacity usually possess spheroid forming capacity (Dontu et al., 2003; Page et al., 2013). Thus, spheroid cultures can be used to isolate stem cells from tissue. When grown as spheres, cells have increased cell viability and functional performance compared with monolayer cultures (Lin and Chang, 2008). Cultured spheroids have advantages provided by the three-dimensional (3-D) microenvironment, which maintains cell-to-cell interactions and allows for engraftment (Hattori et al., 2010). Spheroid cultures also provide favorable conditions for tissue engineering using bioprinting or other reconstruction techniques (Page et al., 2013; Lin and Chang, 2008; Takács et al., 2009; Jo et al., 2014).

The simulated microgravity (SMG) conditions of a rotary cell culture system (RCCS) allow cells to proliferate in under rotating conditions but with low shear stress and in a low turbulence environment. Our previous studies showed that rabbit CSCs in SMG culture tended to aggregate. CSCs under RCCS conditions on scaffolds of decellularized bovine cornea grew in spheres for 19 days, but in static culture, they grew as a 2-D monolayer (Chen et al., 2007). We also found that rabbit CSCs in SMG culture were round in shape with many prominences and were more likely to aggregate and grow into the pores of the decellularized cornea carriers when supplemented with valproic acid (VPA), vitamin C (VC) and

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10% fetal bovine serum (FBS). However, rabbit CSCs in static plastic culture conditions only displayed a spindle shape and were rarely interconnected (Dai et al., 2012).

Growing cells in a 3-D environment generates important differences in cellular characteristics and behavior compared with 2-D environments. 3-D cell culture represents an important bridge for linking our current knowledge of cell structure and metabolism to the extensive complexity of tissues and organs (Page et al., 2013). In this work, we investigated CSC 3-D spheroid culture in suspension and aggregated growth induced by methylcellulose, the recombinant cell-penetrating reprogramming of proteins PTD-Oct4, PTD-Klf4, and PTD-Sox2 (PTD-Oct4/Klf4/Sox2), VPA and RCCS to further understand characteristics in various environmental conditions.

2. Materials and methods

2.1. Materials

Culture reagents were purchased from Gibco (Grand Island, NY, USA). Unless otherwise stated, all the other reagents were from Sigma (St. Louis, MO, USA). VPA was from Energy Chemical (Guangzhou, China), and B27 was purchased from Invitrogen (CA, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Pepro Tech (CA, USA). The monoclonal anti-CD34 antiboby was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China), and the monoclonal anti-vimentin antiboby (NeoMarkers) was from Lab Vision Corp (Fremont, MO, USA). The EZgene TM Tissue RNA Miniprep Kit was from Biomiga (San Diego, CA, USA), and the ReverTra Ace qPCR RT Kit, Blend Taq® and Blend Taq®-Plus were purchased from Toyobo (Osaka, Japan). Primers were synthetized by BGI (Beijing, China).

2.2. Preparation and activity of reprogramming proteins

The reprogramming proteins, including Oct4, Klf4 and Sox2, were expressed and purified as fusion proteins with an N-terminally linked protein transduction domain (PTD) with an amino acid sequence of YGRKKRRQRRR and a 6-His purification tag at the C-terminal, respectively. In brief, the genes encoding the fusion proteins were cloned into the expression vector pKYB to construct the recombinant expression vectors. After the recombinant vectors were transformed into the ER2566 E. coli strain, the fusion proteins, such as PTD-Oct4, PTD-Klf4 and PTD-Sox2, were expressed and purified by Ni-affinity chromatography. The fluoresceinisothiocyanate (FITC) labeled PTD-Oct4, PTD-Klf4 and PTD-Sox2 were used to investigate the penetrating ability of the fusion protein into Chinese hamster ovary (CHO) cells as previously described (Li et al., 2011; Su et al., 2011; Liu et al., 2011). Briefly, the fusion proteins were labeled with FITC (excitation 490 nm, emission 525 nm) using a FITC labeling kit (Xirun. Bio. China). CHO cells were grown to confluence on a 24 well plate, and then, the culture medium was replaced with 200 μL FITC labeled PTD-Oct4, PTD-Klf4 or PTD-Sox2 solution for 1 h. The cells were thoroughly washed with phosphate-buffered saline (PBS) four times and then were imaged using an inverted fluorescence microscope. The FITC labeled fusion protein maxadilan (MAX) was used as control, which has no penetrating ability (Zeng et al., 2009). The rate of proteins labeled with FITC was calculated with the following formula: $F(FITC)/P(proteins) = -3.053 \times OD(495\,nm)/OD(280\,nm) - 0.225 \times OD(495\,nm)$. After 1 h incubation with FITC labeld proteins, CHO cells were treated with RIPA cell lysis buffer. A fluorescence detector was used to observe the penetrating FITC in the cell lysis solution at an OD of 495 nm, and the protein transduction into CHO cells = OD 495 nm/rate of FITC proteins labeled. The transmembrane protein crossing efficiency was calculated as below: protein transduction into CHO cells/total content of FITC labeled proteins in each well.

Table 1
List of the specific sequences of OCT4, Klf4 and Sox2.

Oct4-F: 5′-cy3-ATGCATGCAAATATGCAAAT-3′
Oct4-R: 5′-cy3-CAGT ATTTGCATATTTGCAT-3′
Klf4-F: 5′-cy3-ATGCACCCCAGTCACCCTAGC-3′
Klf4-R: 5′-cy3-TCTAGGGTGATAGGGTGCAT-3′
Sox2-F: 5′-cy3-CAGTCAAACAAAGACAAACAAAGAGCAT-3′
Sox2-R: 5′-cy3-ATGCACTTTGTTTGTCTTTGTTTGACTG-3′

The binding activities of the reprogramming proteins, which have target sequences, were identified using fluorescence resonance energy transfer (FRET) assays. Briefly, two single-stranded oligo nucleotides sequences from Oct4, Klf4 and Sox2 were produced by chemical synthesis, which connected the anthocyan dye (CY3) (excitation wavelength of 550 nm, emission wavelength of 575 nm) at the 5′ end. The specific sequences of Oct4, Klf4 and Sox2 were shown in Table 1. Each double stranded DNA sequence was obtained by annealing two reverse compliment single DNA strands, which were synthesized by Invitrogen (Guangzhou, China). The Cy3-labeled double-stranded target DNA sequences specific binding of Oct4, Klf4 and Sox2 were obtained by denaturing annealing (95 °C 5 min, 37 °C 2 min, 0 °C 2 min). The reprogramming proteins PTD-Oct4, PTD-Klf4 and PTD-Sox2 were labeled with FITC (excitation 490 nm, emission 525 nm) using a FITC labeling kit (Xirun. Bio., China). The binding of the reprogramming proteins with their target sequences Oct4, Klf4 and Sox2 resulted in energy transferring from FITC to Cy3. Fluorescence emission energy scanning from FITC labeled reprogramming proteins following the addition of its Cy3 labeled target DNA sequences was performed with a multiple function scanner (Perkin Elmer, German) using an non-target DNA sequence as negative control. Moreover, the variation in the emission spectrum was measured to confirm the fluorescence resonance energy transfer, which represents the binding of the reprogramming proteins to their target sequences.

2.3. Animals and CSC isolation

Primary cultures were established from the corneas of the New Zealand white rabbit (4 eyes), which were aged 3–4 months with a weight range of 2–2.5 kg and the corneas of C57 mice that were 7–8 weeks old. Animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institute Animal Care and Use Committee of Jinan University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Eyes from New Zealand white rabbits were obtained and corneas were excised for CSCs. Rabbit CSCs were isolated and expanded by explant culture as previously described (Choong et al., 2007). Briefly, the connective tissues and external muscles of eyes were removed. The corneas were rinsed with saline containing an antibiotic solution (prepared with 100 U/mL penicillin G sodium and 100 mg/mL streptomycin sulfate). Corneas stripped of both endothelial and epithelial tissues were rinsed with saline containing an antibiotic solution three times, minced into fine pieces, and explanted onto the tissue culture plates in the CSC culture medium which was composed of DMEM supplemented with 10% FBS. Cells were incubated at 37 °C in a 5% CO_2 incubator. After three days, CSCs emerged from the small explants of corneal stromal tissue. The explants were removed on day 7, and the CSCs were grown under adherent conditions on tissue culture plates until 80% confluent before further passaging further. The culture medium was changed every second day.