Biomaterials 102 (2016) 148–161

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Substrate-mediated reprogramming of human fibroblasts into neural crest stem-like cells and their applications in neural repair

Ting-Chen Tseng [a], Fu-Yu Hsieh [a], Niann-Tzyy Dai [b], Shan-hui Hsu [a, c, *]

[a] Institute of Polymer Science and Engineering, National Taiwan University, Taipei, Taiwan
[b] Division of Plastic and Reconstructive Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[c] Center of Tissue Engineering and 3D Printing, National Taiwan University, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 1 June 2016
Accepted 7 June 2016
Available online 11 June 2016

Keywords:
Substrate-mediated cell reprogramming
Forkhead box D3 (FOXD3)
Neural crest
Neural repair

ABSTRACT

Cell- and gene-based therapies have emerged as promising strategies for treating neurological diseases. The sources of neural stem cells are limited while the induced pluripotent stem (iPS) cells have risk of tumor formation. Here, we proposed the generation of self-renewable, multipotent, and neural lineage-related neural crest stem-like cells by chitosan substrate-mediated gene transfer of a single factor forkhead box D3 (FOXD3) for the use in neural repair. A simple, non-toxic, substrate-mediated method was applied to deliver the naked FOXD3 plasmid into human fibroblasts. The transfection of FOXD3 increased cell proliferation and up-regulated the neural crest marker genes (FOXD3, SOX2, and CD271), stemness marker genes (OCT4, NANOG, and SOX2), and neural lineage-related genes (Nestin, β-tubulin and GFAP). The expression levels of stemness marker genes and neural crest maker genes in the FOXD3-transfected fibroblasts were maintained until the fifth passage. The FOXD3 reprogrammed fibroblasts based on the new method significantly rescued the neural function of the impaired zebrafish. The chitosan substrate-mediated delivery of naked plasmid showed feasibility in reprogramming somatic cells. Particularly, the FOXD3 reprogrammed fibroblasts hold promise as an easily accessible cellular source with neural crest stem-like behavior for treating neural diseases in the future.

1. Introduction

Neurological disorders are diseases of the central nervous system (CNS) and peripheral nervous system (PNS). CNS disorders including stroke, Alzheimer disease, epilepsy, Parkinson's disease, and Huntington's disease are lack of effective treatments. Cell- and gene-based therapies have emerged and shown potentials in treating neurological disorders and better functional recovery, compared to traditional drug treatments [1,2]. Neural stem cells (NSCs) have been proposed as a promising vehicle for cell-based neural therapies. Many challenges still need to be overcome before clinical applications of NSCs. For example, adult NSCs are difficult to expand to large numbers in culture [3]. Other than adult NSCs, embryonic stem cell (ESC)-derived neuroprogenitors or neurons have debatable ethical issues [4,5]. Mesenchymal stem cells (MSCs) can generate functional neuronal cells by reagents such as retinoic acid but only in low efficiency [6]. The above problems lead to a shortage of cells for potentially treating the neurological disorders.

Somatic cells can be reprogrammed to a pluripotent state by the enforced expression of a few embryonic transcription factors [7–9]. The Yamanaka group first reported the generation of induced pluripotent stem (iPS) cells from mouse skin fibroblasts by the retroviral transduction of Oct4, Sox2, Klf4, and c-Myc genes [10]. The iPS cells could be induced and differentiate into NSCs *in vitro* [11,12]. Meanwhile, induced neural stem cells (iNSCs) could be generated from mouse fibroblasts by direct reprogramming with a combination of transcription factors (Brn4/Pou3f4, Sox2, Klf4, c-Myc, plus E47/Tcf3) [13]. A recent study even reported the generation of iNSCs from mouse or human fibroblasts by direct reprogramming with a single factor, SOX2 [14]. Reprogramming somatic cells by gene transfer may provide the large number of cells required for cell therapy.

* Corresponding author. Institute of Polymer Science and Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan.
E-mail address: shhsu@ntu.edu.tw (S.-h. Hsu).

http://dx.doi.org/10.1016/j.biomaterials.2016.06.020
0142-9612/

Various methods have been developed to improve the efficiency of cell reprogramming, including the use of small molecule compounds and microRNA [15,16]. Biophysical factors such as mechanical properties and substrate topography also play important roles in cellular reprogramming [17]. Topographical cues may affect the direct reprogramming of fibroblasts into induced neurons [18]. The stiffness of hydrogels could regulate the cellular reprogramming efficiency [19]. However, the influence of flat culture substrates on cellular reprogramming has received little attention so far.

Neural crest cells are a transient and migratory cell population that gives rise to diverse cell lineages including melanocytes, peripheral neurons, and etc. Forkhead box D3 (FOXD3) is a transcription factor and one of neural crest markers. FOXD3 transcription factor was required for promoting self-renewal and maintaining pluripotent cells in early mouse embryos [20,21]. FOXD3-deficit ESCs exhibited more apoptosis and extensive differentiation toward multiple lineages [22]. FOXD3 gene was also critical to the spheroid forming process of human gingival fibroblasts because FOXD3-silenced human gingival fibroblasts did not form multicellular spheroids on chitosan and they showed limited neural differentiation *in vitro* [23]. The use of FOXD3 gene as a target gene for cell-based therapy or neurogenesis *in vivo* is rarely seen in literature.

In this study, FOXD3 gene was delivered to human skin fibroblasts by a simple, substrate-mediated method without any viral vector. The transfection efficiency, cell proliferation rate, and neural differentiation capacity of FOXD3 transfected cells were examined *in vitro*. The *in vivo* neurogenesis was further evaluated by injecting the FOXD3-transfected fibroblasts to the neural deficit/injury models of zebrafish embryos as well as adult zebrafish for observing the functional rescue. We sought to determine if FOXD3 delivered by the substrate-mediated method could reprogram human fibroblasts into neural crest-associated cells for potential treatment of neurological disorders.

2. Materials and methods

2.1. Preparation of chitosan membranes and construction of FOXD3 plasmids

Chitosan powder was obtained from Sigma (No. 28191). The molecular weight of the chitosan was 510 kDa and the degree of deacetylation measured by NMR was 77.7%. Chitosan powder was dissolved in 1% acetic acid and stirred at room temperature for 12 h to obtain 1% chitosan solution. The solution was filtered through a 100 μm mesh and then coated on coverslip glass (300 μl of solution on each 15 mm-diameter glass) placed in a petri dish. Substrates after coating were air-dried for one day, then immersed in sodium hydroxide solution (0.5 N) for 3 min, and washed extensively by distilled water. The chitosan substrates were then air-dried for further experiments.

To construct plasmid pCR3.1-FOXD3 (containing the CMV promoter), the FOXD3 cDNA fragment obtained by reverse transcription (RT)-polymerase chain reaction (PCR) with *Eco*RV linkers was inserted into the pCR3.1 vector in frame with a FLAG tag [24].

2.2. Cell culture

The primary human dermal fibroblasts were obtained from human adult foreskin after the surgery of circumcision. The procedures involving human tissues in this study followed the ethical guidelines and were approved by the institutional review board in the Tri-Service General Hospital, Republic of China. Human fibroblasts were isolated and expanded by an established procedure [25]. The tissues were digested by collagenase (No. C6885, Sigma). The cell suspension was then centrifuged and then resuspended in the high-glucose Dulbecco's modified Eagle medium (DMEM) medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Invitrogen). Cells were maintained in 37 °C with 5% CO_2 incubator. After reaching 80% cell confluency, fibroblasts were subcultured using 0.25% trypsin/EDTA solution (Gibco). The medium was refreshed once every 3 days. Cells of the third to the eighth passages were used in all the following experiments.

2.3. FOXD3 transfection

Human fibroblasts were transfected with FOXD3 by the substrate-mediated gene delivery method [26]. The amount of plasmids used for FOXD3 transfection was first optimized (Fig. S1). Human fibroblasts were seeded on chitosan substrate (~2.8×10^4 cells/cm^2) in a 24-well plate, incubated for 12 h, and followed by replacing the culture medium by that (1 ml) containing 1 μg of the FOXD3 plasmids in each well. Cells were then exposed to the plasmid for 24 h. After that, cells were replaced the fresh culture medium and incubated for another 36 h. The FOXD3-transfected fibroblasts were collected by pipetting. To compare the effect of FOXD3 transfection by the substrate-mediated method and the conventional transfection reagent, fibroblasts were transfected by Polyfect (Qiagen). Transfection was followed the manufacturer's protocol. Fibroblasts were cultured on TCPS (~2.8×10^4 cells/cm^2) in a 24-well plate and incubated for 12 h. The culture medium was then replaced by that (1 ml) containing 1 μg of the FOXD3 plasmids and 2.5 μg of Polyfect transfection reagent in each well. Cells were then exposed to the plasmid for 24 h, replaced with fresh cultural medium, and incubated for another 48 h. The transfected fibroblasts were collected by trypsin. These cells were considered as the first passage. To evaluate the long-term stability of the reprogrammed cells, transfected cells were subcultured on chitosan substrates without any FOXD3 plasmid exposure and passaged every 3 days to the fifth passage.

2.4. Analysis of transfection efficiency, cell viability, and proliferation rate

The transfection efficiency of FOXD3 into fibroblasts was assessed by immunofluorescent staining. Anti FLAG antibody is used for the detection of FLAG fusion proteins. Fibroblasts were first fixed in 4% formaldehyde solution for 10 min and permeabilized with 0.1% Triton X-100 made in PBS solution for another 10 min. The samples were blocked with 1% BSA for 1 h and stained with anti FLAG primary antibodies (GTX115043, 1:200, Genetex, USA) overnight at 4 °C. The samples were washed with PBS and incubated with a secondary PE conjugated donkey anti-rabbit IgG antibody (Cat. 406418, 1:200, BioLegend, USA) at room temperature for 1 h. The samples were then sealed and observed under a fluorescence microscope.

Cell viability was determined by the WST-1 assay. Fibroblasts were cultured on TCPS and chitosan substrates, incubated for 12 h, and then exposed to the plasmids for 24 h. The WST-1 reagent was then added to each well and incubated for 1 h. The optical absorbance of the treated samples was measured at 450 nm by a microplate reader (SpectraMax M5, Molecular Devices, USA). The results were expressed as percentages relative to the optical absorbance obtained in the blank control (without FOXD3 transfection).

To evaluate the cell proliferation rate after FOXD3 transfection, cells were collected and measured by the DNA Hoechst 33528 dye stain assay. The fluorescence intensity was determined by the