Journal of the Taiwan Institute of Chemical Engineers 45 (2014) 295–301

Contents lists available at SciVerse ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

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

# Preparation of induced pluripotent stem cells on dishes grafted on oligopeptide under feeder-free conditions

Akon Higuchi [a,b,c,1,*], Feng-ling Lin [d,1], Yu-Kai Cheng [a], Ta-Chun Kao [a], S. Suresh Kumar [a,e], Qing-Dong Ling [c,f], Chun-Han Hou [g], Da-Chung Chen [h], Shih-Tien Hsu [i], Gwo-Jang Wu [j]

[a] Department of Chemical and Materials Engineering, National Central University, Taoyuan 32001, Taiwan
[b] Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan
[c] Cathay Medical Research Institute, Cathay General Hospital, Taipei 10630, Taiwan
[d] Department of Dermatology, Cathay General Hospital Sijhih Branch, Taipei 22174, Taiwan
[e] Department of Medical Microbiology and Parasitology, Universities Putra Malaysia, Slangor, Malaysia
[f] Institute of Systems Biology and Bioinformatics, National Central University, Taoyuan 32001, Taiwan
[g] Department of Orthopedic Surgery, School of Medicine, National Taiwan University, Taipei 10051, Taiwan
[h] Department of Obsterics and Gynecology, Taiwan Landseed Hospital, Taoyuan 32405, Taiwan
[i] Department of Community Medicine, Taiwan Landseed Hospital, Taoyuan 32405, Taiwan
[j] Graduate Institute of Medical Sciences and Dept. of Obstetrics & Gynecology, Tri-Service General Hospital, National Defense Medical Center, Taipei 11490, Taiwan

ARTICLE INFO

*Article history:*
Received 26 January 2013
Received in revised form 7 June 2013
Accepted 23 June 2013
Available online 24 July 2013

*Keywords:*
Induced pluripotent stem cell
Feeder-free culture
Adipose-derived stem cell
Vitronectin
Oligopeptide
Biomaterial

ABSTRACT

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have potentially therapeutic applications in the treatment of many diseases, due to their unique ability to differentiate into any type of somatic cell. However, the clinical potential of hESCs and hiPSCs is restricted by the use of mouse embryonic fibroblasts (MEFs) as a feeder layer for these cells. We report that hiPSCs can be successfully generated without the use of a feeder layer of MEFs. We generated hiPSCs by transducing human adipose-derived stem cells (hADSCs) with a retrovirus containing pluripotency genes, and the hiPSCs were cultured on synthetic dishes grafted with an oligopeptide derived from vitronectin (VN-dish). On the fourth day after transduction, the hADSCs transduced with pluripotency genes were transferred to a MEF layer for culturing as a control condition or to VN-dishes for culture. The hiPSC colonies in the MEF-cultures were clearly observed at day 14 after transduction, whereas hiPSC colonies were detected on the VN-dishes after the cells were passaged. When $10^5$ hADSCs were seeded on the dishes, the number of colonies generated on the MEFs was $120 \pm 28$, while the number of colonies generated on VN-dishes was $25 \pm 8$. Thus, the efficiency of hiPSC generation on the VN-dishes under feeder-free conditions was lower than hiPSCs cultured on MEFs. However, the hiPSC colonies from VN-dishes demonstrated alkaline phosphatase activity, and immunohistochemistry suggested that the hiPSCs generated on VN-dishes expressed the pluripotency protein, stage-specific embryonic antigen-4 (SSEA-4), under feeder-free conditions.



## 1. Introduction

Human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2,3] have potentially therapeutic applications in the treatment of many diseases, due to their unique ability to differentiate into any type of somatic cell [4]. For example, hESCs and hiPSCs have been differentiated into nerve cells that secrete dopamine and β cells that secrete insulin, and these cells can be transplanted for the treatment of Parkinson's disease [5,6] and diabetes [6,7], respectively. The pluripotent nature of these cells could permit the development of a wide range of stem cell-based regenerative therapies and drug discovery platforms [4].

However, the tentative clinical potential of hESCs and hiPSCs is restricted by the use of mouse embryonic fibroblasts (MEFs) as a feeder layer in the culture of these cells. While the addition of leukemia inhibitory factor (LIF) to the culture medium can allow mouse ESCs to proliferate and remain undifferentiated in the absence of a feeder layer of MEFs, this method is not effective for the culture of hESCs or hiPSCs [1,8]. The addition of LIF to the culture medium is insufficient to maintain the pluripotency and

* Corresponding author at: Department of Chemical and Materials Engineering, National Central University, Taoyuan 32001, Taiwan. Tel.: +886 3 4227151 34257; fax: +886 3 4252296.
*E-mail addresses:* higuchi@ncu.edu.tw, akon.higuchi@gmail.com, higuchilb@yahoo.co.jp (A. Higuchi).
[1] These authors contributed equally to the work.

1876-1070/$ – see front matter 
http://dx.doi.org/10.1016/j.jtice.2013.06.022

self-renewal of hESCs and hiPSCs in a feeder-free culture [4]. The possibility of xenogenic contamination during culture with MEFs restricts the clinical use of transplanted hESCs and hiPSCs [9,10]. Furthermore, the process of culturing hESCs and hiPSCs using feeder layers is elaborate and costly, thereby limiting the large-scale culture of these cells. The variability of MEFs between laboratories and across batches also affects the characteristics and the pluripotency of hESCs and hiPSCs [4]. Feeder-free cultures that use synthetic polymers or biomacromolecules as stem cell culture materials offer more reproducible culture conditions and lower the cost of production without introducing xenogenic contaminants. These improvements could increase the potential clinical applications of differentiated hESCs and hiPSCs [4].

Several feeder-free cultures of hESCs and hiPSCs have been reported. Swistowski *et al.* investigated hESC culture on human albumin- and fibronectin-coated dishes (Cellstart™ coating dishes) [11,12]. The pluripotency of the hESCs was maintained for over 25 passages in their study. Rodin *et al.* reported hESC and hiPSC culture on laminin-511-coated dishes, maintaining pluripotency for 4 months (20 passages) under xeno-free and feeder-free conditions [13]. The adhesion of hESCs was found to be dependent on integrin α6β1 [13,14], which binds to laminin-511 [13,15]. Melkoumian *et al.* developed acrylate dishes grafted with synthetic oligopeptides that were derived from bone sialoprotein (KGGNGEP**RGD**TYRAY) or vitronectin (KGGPQVT**RGD**VFTMP), and these surfaces supported the pluripotency of hESCs and hiPSCs for more than 10 passages [16]. Oligopeptides and glycosaminoglycans, such as heparin, play an important role in supporting the pluripotency of hESCs and hiPSCs. Klim reported that hESCs could maintain their pluripotency on surface-immobilized heparin-binding peptides (GKKQRFRHRNRKG) for three months (17 passages) [17].

Fully chemically synthetic materials have been reported as coating materials for cell culture dishes that sustain the pluripotency of long-term hESC cultures [18–20]. Villa-Diaz developed a zwitterionic polymer, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH) and showed that the polymer-coated dishes supported the pluripotency of H9 hESCs for ten passages in a defined serum-free medium [18,19]. However, the PMEDSAH-coated dishes did not support the culture of other hESC cell lines such as BG01 [18].

Although hiPSCs and hESCs can retain their pluripotency under feeder-free conditions, in most cases the reprogramming of somatic cells into hiPSCs has been performed using MEF [2,3] or human somatic cells such as fibroblasts [21] and mesenchymal stem cells [22,23] as feeder layers.

Here, we report the reprogramming of human adipose-derived stem cells (hADSCs) into hiPSCs without the use of feeder layers (*e.g.*, MEFs) using gene transduction by a retrovirus containing pluripotent genes during cell culture on synthetic dishes grafted with an oligopeptide derived from vitronectin (KGGPQVT**RGD**VFTMP).

## 2. Materials and methods

### 2.1. Preparation of hADSCs

The experiments performed for this research were approved by the ethics committee and institutional review board (IRB) of National Central University, Taiwan Landseed Hospital, and Cathay General Hospital. Adipose tissue from the intestinal omentum of a 53-year-old male was carefully dissected and washed with phosphate-buffered saline (PBS) to remove blood and impurities. The adipose tissue was minced into small pieces (approximately 2 $mm^3$) and digested with 2.5 mg/mL of collagenase type-IV (Gibco™, Invitrogen, Grand Island, NY) at 37 °C for 60 min [24,25]. Enzymatic activity was neutralized with Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS, Biological Industries). The digested solution was centrifuged at 1200 × *g* for 6 min [24,25]. The resulting cells were suspended in erythrocyte lysis buffer (154 mM $NH_4Cl$, 20 mM Tris, pH 7.4) for 2 min to remove red blood cells, followed by neutralization with DMEM containing 10% FBS [24,25]. The cell solution was centrifuged at 1200 × *g* for 6 min. The cells were resuspended in DMEM containing 10% FBS to obtain a suspension of adipose tissue cells (adipose tissue-derived stromal vascular fraction [SVF]). The total number of cells in this suspension was counted by flow cytometry. The adipose-tissue cells were then seeded into polystyrene tissue culture dishes (TCPs) and cultured in DMEM containing 10% FBS for 21 days.

The number of hADSCs in the SVF was counted by using flow cytometry with antibodies to CD34 (IM1870U, FITC mouse anti-human CD34, Beckman Coulter, Marseille, France), CD44 (IM1219U, FITC mouse anti-human CD44, Beckman Coulter, Marseille, France), CD73 (550257, PE mouse anti-human CD73, BD Biosciences, San Jose, CA), CD90 (IM1840U, PE mouse anti-human CD90, Beckman Coulter, Marseille, France), and their isotype controls (733179, PE mouse anti-human IgG1 and 41116015, FITC mouse anti-human IgG1, Beckman Coulter, Marseille, France). The total cell number in the primary suspension of adipose tissue cells was also counted by flow cytometry (Coulter EPICS™ XL, Beckman Coulter, Marseille, France) after staining with 7-AAD (A07704, Beckman Coulter, Marseille, France).

After culture for 5–7 days, more than 85% of the cells exhibited the mesenchymal stem cell markers CD73 and CD90, which indicated that the cells were primarily hADSCs. These hADSCs were cultured and passaged using conventional culture techniques [26]. The hADSCs purified by the culture method were used to generate hiPSCs by transduction with pluripotent genes using a retrovirus, as described below.

### 2.2. Establishment of hiPSCs using a retrovirus

hiPSCs were generated using the procedures described by Yamanaka and colleagues [3], with some modifications. A brief schematic of the protocol is shown in Fig. 1.

First, 293T cells (ATCC) were plated in OPTI-MEM (Invitrogen, Grand Island, NY) containing 10% FBS at 2 × $10^6$ cells per 100 mm tissue culture polystyrene dish and incubated overnight. Cells were transfected with retrovirus vectors (5 μg of phOct4, phSox2, phcMyc, and phklf4, Addgene) and virus-producing vectors (3.3 μg of pCMV-GagPol and 1.7 μg of pCMV-VSV-G, Cell Biolabs, Inc.) by Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Three days after transfection, the supernatant of the transfectant was collected, filtered through a cellulose acetate filter with 0.45 μm pores (OE67, Whatman, Kent, UK), and centrifuged at 8000 × *g* at 4 °C to obtain the virus pellet. One mL of serum-free DMEM containing penicillin/streptomycin (Invitrogen, Grand Island, NY) was added to the virus pellet, and this retrovirus solution was shaken overnight. The retrovirus solution was stored at −135 °C or used for the transduction of hADSCs as follows.

hADSCs were seeded at 1 × $10^5$ cells per well in tissue culture polystyrene plates (6 wells per plate). The cell medium was replaced with the retrovirus solution containing 4 μg/ml of polybrene (Sigma–Aldrich, St. Louis, MO), and cells were incubated with this solution for two days. Half of the medium was exchanged with fresh DMEM containing 10% FBS after the first 8 h and subsequently every day.