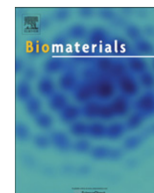
Biomaterials 34 (2013) 3215–3222

Contents lists available at SciVerse ScienceDirect

Biomaterials

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



The effect of forced growth of cells into 3D spheres using low attachment surfaces on the acquisition of stemness properties

Guannan Su [a,b,1], Yannan Zhao [a,1], Jianshu Wei [a,b,1], Jin Han [a], Lei Chen [a], Zhifeng Xiao [a], Bing Chen [a], Jianwu Dai [a,*]

[a] State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[b] Graduate School, Chinese Academy of Sciences, Beijing 100190, China

ARTICLE INFO

Article history:
Received 3 December 2012
Accepted 7 January 2013
Available online 8 February 2013

Keywords:
Stemness
Reprogramming
3D sphere culture
Low attachment surface

ABSTRACT

Embryonic stem cells (ESCs) and neural progenitor cells form three-dimensional (3D) colonies or spheres *in vitro*, and 3D sphere is reported to help maintaining the stemness of stem cells, but the effect of 3D sphere formation on cell reprogramming remains unknown. Here we examined whether 3D sphere culture have any impact on the differentiated cells. We cultured bladder cancer cell RT4 and non-cancerous cell HEK293 on the low attachment dishes coated with soft agarose. When grown on this low attachment dish, cells spontaneously aggregated to form 3D spheres. Data showed that 3D sphere formation promoted the expression of reprogramming factors. Sphere formation of RT4 cells induced cancer stem cell characteristics including higher SP cell percentage, higher metastasis ability and higher tumorigenicity. HEK293 spheres showed upregulation of kidney progenitor cell markers and partially acquired characteristics of ESCs including upregulation of alkaline phosphatase activity, ES cell markers, three germ layer markers and tumorigenicity. The findings suggested that forced growth into 3D spheres by the low attachment surface could induce cells to acquire stemness properties.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Proliferation while maintaining potency and differentiation capacity into specialized cell types endows stem cells with great promise for scientific research and therapeutic applications. Previous works demonstrated that pluripotent stem cells can be generated from somatic cells via overexpression of key transcription factors, creating a new method for cell reprogramming [1,2]. Since then, great efforts have been devoted to designing safer and more efficient methods for cell reprogramming, such as the utilization of proteins [3,4], RNAs [5], microRNAs [6] or defined chemicals [7,8]. Extrinsic cues have recently drawn great attention as accumulating evidences support that microenvironment can play a vital role in determining the properties of stem cells [9], suggesting that stem cell microenvironment might play an important role in cell reprogramming [10].

The microenvironment of cells is mainly composed of soluble growth factors, cell–matrix interactions and cell–cell interactions. Compared to conventional two-dimensional (2D) culture, cells cultured under three dimensional (3D) culture differ considerably in cell morphology, cell–cell contact and cell–matrix interactions [9,11–15]. Many 3D cell culture models have been established including the use of porous scaffolds, hydrogels or polymers. 3D self-organized cellular sphere is another typical 3D cell culture system taking advantage of the natural aggregation tendency of many cell types. Previous studies demonstrated sphere formation may be related to stemness since pluripotent cells tend to exhibit a sphere or colony morphology. For example, embryonic stem cells (ESCs) form colonies on feeder cells, and neural progenitor cells (NPCs) form typical neurospheres in suspension. Sphere-formation assays are also widely used to identify stem cells [16] and 3D sphere helps to maintain the stemness of stem cells. For instance, primary muscle stem cells can be maintained in culture as floating myospheres, retaining the potential to differentiate into muscle cells, adipocytes and osteogenic cells [17]. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell–matrix interactions, rationalizing their enhanced functional potency for myocardial repair [18,19]. Spheroids of mesenchymal stem cells (MSCs) help to maintain the expression of stemness marker genes [20,21].

The close correlation between 3D sphere morphology and stemness promoted us to testify whether 3D sphere culture could induce non-stem cells to acquire stem cell properties. It is known

* Corresponding author. Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 3 Nanyitiao, Zhongguancun, Beijing 100190, China. Tel./fax: +86 (0)10 82614426.
E-mail address: jwdai@genetics.ac.cn (J. Dai).
[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.01.044

that cellular spheroids can be commonly obtained through three approaches. The first one is hanging drop method that spheres are grown in droplets placed on an inverted plate [22]. The second is gyratory rotation technique that trypsinized cells are cultured in a stirred flask to prevent them from adhering to the substrate [23]. The third one is low-attachment cultivation of cells on hydrophobic surface [24]. In this work, we prepared low attachment surface by coating soft agarose on cell culture dish, and performed 3D sphere culture of human urinary bladder papilloma cell line RT4 and human embryonic kidney epithelial cell line HEK293, representing cancer and non-cancerous cells respectively. Compared with conventional 2D culture, 3D sphere culture promoted the expression of reprogramming factors. Sphere formation of RT4 cells induced cancer stem cells characteristics including higher SP cell percentage, higher metastasis ability and higher tumorigenicity. HEK293 spheres showed upregulation of kidney progenitor cell markers and partially acquired characteristics of ESCs including upregulation of alkaline phosphatase activity, ES cell markers, three germ layer markers and tumorigenicity.

2. Materials and methods

2.1. Preparation of low attachment dish

For the preparation of 2× DMEM medium, 13.4 g DMEM high glucose (Powder) (GIBCO) and 3.7 g sodium bicarbonate were dissolved in 500 mL deionized water and sterilized by 0.22 micron filter. 2× DMEM medium was pre-warmed to 37 °C. 1% agarose solution was made with agarose G-10 (BIOWEST) and deionized water and heated until boiling in microwave. Equal volume of pre-warmed 2× DMEM medium and 1% agarose solution was mixed and coated on cell culture dish. When temperature declined, agarose and DMEM mixture will form a layer of 0.5% gel with a thickness of 4 mm.

2.2. Cell culture

HEK293 cell line was purchased from Peking Union cell center (ATCC source) and RT4 cell line was presented by Professor Ying Jin, Shanghai Jiao Tong University.

RT4 and HEK293 cells were cultured in McCOY'5A(HYCLONE) and DMEM(HYCLONE) medium respectively, both supplemented with 10% FBS (GIBCO), 1 mM L-glutamin (Sigma), 1 mM sodium pyruvate (GIBCO), 0.1 mM NEAA (GIBCO) and 50 units of penicillin/streptomycin (HYCLONE). The cells were cultured at 37 °C in 5% CO_2, and the medium was changed every 2 days. When cells have reached 90% confluence, they were trypsinized with 0.25% trypsin (Amresco) and passaged at a ratio of 1:3. For 3D culture, 3×10^6 RT4 or HEK293 cells were transferred to a 60 mm low attachment dish. These cells were cultured at 37 °C with 5% CO_2. Medium was changed every other day.

2.3. Alkaline phosphatase (AP) staining

For AP staining, cells were fixed with 60% acetone (diluted with 1.5M sodium citrate solution) for 1 min. The fixed cells were then washed with PBS for 1 min and then stained with BCIP/NBT solution (Sigma). For BCIP/NBT solution, 330 ul NBT solution and 33 ul BCIP solution were diluted with 10 mL AP working solution (0.1M Tris-base, 100 mM NaCl, 5 mM $MgCl_2$, PH 9.5). The staining process was terminated when purple stainings arised.

2.4. Quantitative RT-PCR

Total RNAs of monolayer and sphere cells were extracted with Trizol reagent (Invitrogen) according to manufacturer's instructions. Total RNA concentration was determined by optical density at 260 nm (OD260) using a spectrophotometer (Amersham Biosciences). After removing residual DNA with Dnase I (Invitrogen), equal amounts of RNA (1 μg) were added to reverse transcriptase reaction mix (SuperScript III First-Strand Synthesis System, Invitrogen) with oligo-dT primers (Invitrogen). Power SYBR Green RT-PCR Kit (Applied Biosystems) was utilized to perform Q-PCR using the Bio-RAD CFX96 Real-Time system (Bio-RAD). The expression level was analyzed and normalized to GAPDH for each cDNA sample. Fold change of gene expression was calculated using the 2-ΔΔCT method. Primer sequences were available in Table S1.

2.5. Nude mice tumor formation assay

All experiments on animals were subjected to Chinese Ministry of Public Health (CMPH) Guide. Cells with or without trypsinization, were suspended in DMEM medium mixed with an equal volume of ECM (Sigma) to a final volume of 200 μl. Cells were injected subcutaneously by 27-gauge needles into the back flanks of 4–5 week old nude mice (8 BALB/c-nu). The mice were monitored twice a week for signs of tumor growth for 2 months. Tumors were fixed in 4% paraformaldehyde and sectioned to 5 μm thickness for hematoxylin-eosin (H&E) staining.

2.6. Western blot

Cells were washed with PBS and lysed in RIPA (Sigma) buffer with Complete Protease Inhibitor Cocktail (Roche) for 30 min on ice to prepare the whole cell lysates. Protein concentrations were measured using Protein Assay against BCA standards (PIERCE). Equivalent quantity of protein lysates were electrophoresed in 12% SDS-ployacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Amershame). The membrane was blocked in TBS containing 10% nonfat milk and 0.05% tween-20 for 2 h at the room temperature. Primary antibodies were incubated for 2 h at the room temperature, and primary antibodies included: NANOG (1:5000, Abcam), SOX2 (1:200, Invitrogen) and beta-Actin (1:500, Santa Cruz). The HRP labeled secondary antibody was incubated for 2 h at the room temperature. Secondary antibodies conjugated with HRP included: Goat-Anti Mouse IgG (1:10,000, SouthernBiotech) and Goat-Anti Rabbit IgG (1:3000, Thermo Scientific).

2.7. Side population analysis using flow cytometry

Monolayer and sphere RT4 cells were trypsinized with accutase (Sigma) and then cells were suspended at 1×10^6 cells/mL in DMEM medium supplemented with 2% FBS. These cells were then incubated at 37 °C for 90 min in darkness with 5 μg/mL Hoechst 33342 (Sigma), either alone or in the presence of 50 μg/mL verapamil (Sigma). At the end of incubation, cells were spun down in the cold and resuspended in ice-cold PBS with 2% FBS. After washing with PBS, the cells were treated with 2 μg/mL propidium iodide to exclude dead cells. SP analysis was done using a FACS-Vantage SE (BD Biosciences).

2.8. Cell migration assay

For measurements of cell migration, RT4 cells under monolayer and 3D culture conditions were pre-treated with 24 h serum hungry. Then the monolayer and sphere cells were trypsinized by 0.25% trypsin/EDTA and resuspended in McCOY'5A media containing no FBS as a density of 5×10^5 cells/mL. 200 μl cells in suspension were plated on the top chamber of 24-well transwell containing 8 μm pores (Corning). 600 μl medium with 10% FBS was added to the bottom chamber. Cells were allowed to migrate in response to FBS. After 24 h, migrated cells on the bottom were stained with 0.1% crystal violet. The number of stained cells on the bottom surface was counted under the microscope and four independent fields were counted for each Transwell.

2.9. Transient transfections and luciferase reporter assays

For DNA transfections, HEK293 (8×10^4) cells were seeded per well of 24-well plates 16 h prior to transfection. The monolayer cells were then transfected with 800 ng DNA plasmids using Lipofectamine 2000 (Invitrogen) according to the protocol supplied. Media was replaced at 6 h post-transfection.

Oct4-Luc vector was constructed by inserting 6W enhancer into PGL3 vector with tk promoter. Nanog-Luc vector was kindly provided by Pro. Duanqing Pei. Sox2/Oct4-Luc vector was kindly provided by Pro. Lisa Dailey. Reporter plasmids were extracted using the Plasmid Mini Kit I (OMEGA) with standard protocol. Transfection was performed according to the manufacturer's instruction. Being cultured for 24 h after transfection, cells were trypsinized and subjected to 2D and 3D cultures respectively. Luciferase assay was carried out according to the standard protocol (Promega).

2.10. Bisulfate sequencing

Genomic DNA was extracted from monolayer and sphere cells using QIAamp DNA Mini Kit (Qiagen). Bisulfite treatment was performed using Epi-Tect Bisulfite kit (Qiagen) according to the manufacturer's recommendations. The transcription regulatory regions of *OCT4*, *NANOG* and *SOX2* were amplified by PCR (Hotstar HiFidelity DNA polymerase, Qiagen). Primer sequences used for PCR amplification were provided in Table S1. The PCR products were subcloned into pMD18-T vector (TaKaRa) and individual clones were then sequenced. Clones were only accepted with more than 90% cytosine conversion. BiQ Analyzer software (Max Planck Society) was used for the quality check and methylation analysis. At least 20 replicates were analyzed for each sequence.

2.11. Statistical analysis

The statistical significance (*P* values) in mean values of two-sample comparison was determined with Students' *t*-test. A value of $P < 0.05$ was considered statistically significant (*) and a value of $P < 0.01$ was considered extremely significant (**). Values shown on graphs represent the means ± s.d.

Download English Version:

https://daneshyari.com/en/article/10229161

Download Persian Version:

https://daneshyari.com/article/10229161

Daneshyari.com