Biomaterials 35 (2014) 7724–7733

Contents lists available at ScienceDirect

Biomaterials

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

The importance of three-dimensional scaffold structure on stemness maintenance of mouse embryonic stem cells

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ARTICLE INFO

Article history:
Received 13 March 2014
Accepted 21 May 2014
Available online 12 June 2014

Keywords:
Three-dimensional scaffold
mES cells
Stemness
Geometry

ABSTRACT

Revealing the mechanisms of cell fate regulation is important for scientific research and stem cell-based therapy. The traditional two-dimensional (2D) cultured mES cells are in a very different 2D niche from the *in vivo* equivalent-inner cell mass (ICM). Because the cell fate decision could be regulated by many cues which could be impacted by geometry, the traditional 2D culture system would hamper us from understanding the *in vivo* situations correctly. Three-dimensional (3D) scaffold was believed to provide a 3D environment closed to the *in vivo* one. In this work, three different scaffolds were prepared for cell culture. Several characters of mES cells were changed under 3D scaffolds culture compared to 2D, and these changes were mainly due to the alteration in geometry but not the matrix. The self-renewal of mES cells was promoted by the introducing of dimensionality. The stemness maintenance of mES was supported by all three 3D scaffolds without feeder cells in the long-time culture. Our findings demonstrated that the stemness maintenance of mES cells was promoted by the 3D geometry of scaffolds and this would provide a promising platform for ES cell research.

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1. Introduction

ES cells, isolated from blastocysts have the ability to self-renew and differentiate into all cell types [1,2]. These two characters make them holding great promise in stem cell based therapy, drug discovery and developmental research. In 2006, Yamanaka and his colleagues showed that induced pluripotent stem (iPS) cells, which have the similar pluripotency as mES cells, could be generated from adult differentiated cells [3]. These findings greatly facilitated the clinical application of ES cells.

The major goal for mES cell research is to reveal the mechanisms of self-renewal and differentiation regulation, which would be beneficial for embryogenesis study and stem cell based therapy. Traditionally, mES cells or iPS cells were cultured on two-dimensional (2D) surfaces with mouse embryonic fibroblast (MEF) feeder cells in medium containing serum and leukemia inhibitory factor (LIF) [4] or other factors to inhibit the spontaneous differentiation. The 2D culture system is a convenient platform for mES cell study, and many important regulating mechanisms have been revealed based on the 2D culture system [5–10].

As we know, mES cells are originated from ICM in which the pluripotent cells do not self-renew for a long time. As the *in vivo* equivalent of mES cells, ICM cells are in a different 3D niche compared to mES cells cultured on 2D surfaces *in vitro* [11,12]. Many cues have been shown to regulate the fate decision, and many of them are impacted by the geometry, for example, the oxygen distribution [13–16], cell–cell and cell-extracellular matrix (ECM) associations [17–20]. The geometry was also able to impact ES cells behavior directly. For example, the topography could influence the adhesion, migration, self-renew and differentiation of ES cells [21–23]. And specific topography was proved to maintain the stemness of ES cells for a long time through inhibiting cell spreading, which would make the cells less flat and then increase the clone integrity [24]. As a result, the 2D culture system is considered to be limited to study the real situations *in vivo*.

A 3D scaffold culture system which could provide 3D geometry resemble that *in vivo* is subsequently suggested. Cells will become less flat when they were cultured in 3D scaffolds [25]. As a result, the 3D scaffold was believed to promote stemness maintenance

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http://dx.doi.org/10.1016/j.biomaterials.2014.05.060
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through specific 3D geometry. Until now, many scaffolds have been used for the mES 3D culture, however, there is not a consolidated opinion on what influence the scaffold structure has on mES cells [19,26–30]. Moreover, these results were made based on experiments carried out on one specific scaffold. For example, the scaffolds they used were made of different matrix, for example collagen, fibrin, polyethylene glycol (PEG), polylactic acid (PLA), hyaluronic acid (HA). chitosan and so on. Because different matrixes have different and important regulation functions on mES cells [18,31,32], the influence on mES cell fate decision can hardly be ascribed to changes in special matrix or geometry of the scaffold. Therefore, we suggest that studies across different scaffolds would be useful to find out the common functions of 3D scaffold geometry on mES cell fate regulation.

Three different 3D porous scaffolds were prepared for the 3D culture of mES cells. The goal of this work was to study the importance of geometry of 3D scaffolds on mES cells behavior regulation, and the possibility of supporting mES cells long-time culture in different 3D scaffolds was also probed.

2. Materials and methods

2.1. Scaffolds

Collagen scaffolds were prepared as previously described [33]. Briefly, the collagen membranes were immersed in 0.5 M acetic acid solution for 8 h at 4 °C. And then the solution was mixed in a blender for 15 min to obtain homogeneous collagen solution and neutralized by 4 M NaOH. The homogeneous solution was dialyzed in deionized water for 5 days and lyophilized. The obtained porous collagen scaffolds were cut into 0.1 × 0.5 × 0.5 cm pellets and cross linked by 1 mg/ml 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 0.6 mg/ml N-hydroxysuccinimide (NHS). After crosslinking, the pellets were lyophilized again, sterilized by Co 60 and stored at 4 °C for utilization.

Chitosan solutions with concentrations of 2 wt% were prepared by dissolution in 0.2 M acetic acid on ice overnight. The samples were stored at −20 °C and then lyophilized until dry. The samples were immersed in 95% ethylalcohol at 10 °C for 2 h, and ethylalcohol was changed twice. Then, the samples were immersed in 0.5 M NaOH solution dissolved for 4 h. After wash by 70% ethylalcohol solution, the samples were lyophilized again, sterilized by Co 60 and stored at 4 °C for utilization.

PLGA scaffolds were purchased from Daigang Biotechnology Corporation, Jinan, Shandong, China.

Before the experiments, these scaffolds were cut into slices with desired size. The thickness was about 1 mm, and the average length and width were both about 7 mm.

2.2. Scanning electron microscopy analysis

The morphology of scaffolds and cells seeded in 3D collagen scaffolds was shown by Scanning Electron Microscopy (SEM, S-3000N; Hitachi, Tokyo, Japan). Firstly, samples were washed with cold DPBS for 3 times and fixed with cold 2% glutaraldehyde solution for 12 h at 4 °C. And then, dehydration of the samples was followed in a series of ethanol (50%, 75%, 85%, 95%, 100% and 100%). Samples were critical point dried and sputter coated with gold platinum prior to SEM imaging.

2.3. Cell culture

The mES cells named DM2, which was obtained from 129/sv mouse blastocyst, were kindly presented by Professor Shaorong Gao from National Institute of Biological Sciences.

For conditional 2D mES cell culture, the cells were cultured according to standard protocol. Briefly, the mES cells were cultured on feeder cells coated cell culture plates in mES culture medium at 37 °C with 5% CO_2. mES medium was DMEM (Gibco) supplemented with 15% FBS (Gibco), 1 mM L-glutamin (Sigma), 1 mM sodium pyruvate (Gibco), 0.1 mM NEAA (Gibco), 50 units of penicillin/streptomycin (Hyclone), 0.1 mM β-mercaptoethanol (GIBCO) and 1000 units/ml LIF (Millipore). The medium was changed every day. After 2 or 3 days culture, the cells were trypsinized with 0.25% trypsin (Sigma) with 1 mM EDTA solution at 37 °C for 5 min. After trypsinization, an equal amount of DMEM with 10% FBS was added and the pieces were pipetted up and down for a few times. Cells were collected by centrifugation (250 g for 5 min at room temperature) and resuspended in fresh mES medium. The cells were then implanted on cell culture plates or scaffolds.

For 3D scaffolds culture, the scaffolds were immersed in mES medium to strip away the gas. After trypsinization, the mES cells cultured on plates were collected by centrifugation (250 g for 5 min at room temperature) and resuspended in fresh mES medium. The cell suspension was implanted on cell culture plates and allowed to attach to the surfaces for 45 min. After 45 min, the suspension was then collected to eliminate the feeder cells. Approximately 1×10^5 cells in 10 μl medium were seeded in scaffolds. After 4–6 h, mES medium was added to cover the scaffolds. The scaffolds were kept static overnight and then transferred to shakers with 60 rpm. The medium was changed every day.

2.4. Cell proliferation analysis

The proliferation rates of cells cultured in 3D scaffolds were assayed using hemocytometer. For cell trypsinization, 0.25% trypsin was added and incubated in 37 °C for 6 min. Trypsinization was terminated by DMEM with 10% FBS. The cell number was calculated on hemocytometer at indicated time points. 1×10^5 cells were seeded in one scaffolds on day 0. The cells were detached from the scaffolds and the cells numbers were counted on day 1, which were the cells truly attached on the scaffolds. The folds of cell numbers were calculated by dividing the cell numbers on day 1.

2.5. Cell cycle analysis

For each sample, approximately 1×10^6 cells were collected and fixed with 300 μl PBS plus 700 μl 70% ethanol. After being fixed at 4 °C overnight, the cells were resuspended with 500 μl PBS containing 50 μg/ml PI and 100 μg/ml RNase A. The cells were incubated at 37 °C for 30 min and then analyzed on a FACS-LSR (BD Biosciences) equipped with Celluest (BD Biosciences) software.

For the mES cells cultured with feeders, the feeders were removed by differential attachment method.

2.6. Microarray

Gene expression profiles were assessed by the Affymetrix Mouse 430 2.0 expression microarrays. Total RNA was extracted using TRIZOL Reagent (Life technologies) following the manufacturer's instructions and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies). Qualified total RNA was further purified by RNeasy mini kit (QIAGEN) and RNase-Free DNase Set (QIAGEN). Total RNA were amplified, labeled and purified by using GeneChip 3′IVT Express Kit (Affymetrix) followed the manufacturer's instructions. Array hybridization and wash was performed using GeneChip Hybridization, Wash and Stain Kit (Affymetrix) in Hybridization Oven 645 (Affymetrix) and Fluidics Station 450 (Affymetrix) followed the manufacturer's instructions. Slides were scanned by GeneChip Scanner 3000 (Affymetrix) and Command Console Software 3.1 (Santa Clara) with default settings. Raw data were normalized by MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent technologies).

2.7. Alkaline phosphatase (AP) staining

600 cells were seeded on one well of 48-well plate coated with feeder cells. AP staining was carried out after 2-day culture in mES medium. For AP staining, cells were fixed with 60% acetone (diluted with 1.5 M 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 μl NBT solution and 33 μl BCIP solution were diluted with 10 mL AP working solution (0.1 M Tris-base, 100 mM NaCl, 5 mM $MgCl_2$, PH 9.5). The staining process was terminated when staining arose purple. The AP positive clones were counted.

2.8. Quantitative RT-PCR

Total RNA was isolated with Trizol LS Reagent (Life Technologies). After removing residual DNA with Dnase I (Life Technologies), equal amounts of RNA (1 μg) were added to reverse transcriptase reaction mix (SuperScript III First-Strand Synthesis System, Life Technologies) with oligo-dT primers (Life Technologies). Quantitative PCR analysis was performed in triplicate ($n = 3$) using Power SYBR Green RT-PCR Kit (Life Technologies). *GAPDH* was used as a normalizer to determine the other gene relative transcripts (ΔΔCt). Primer sequences were available in Table S2.

2.9. Immunocytochemistry

For immunocytochemistry, cells were fixed with 4% paraformaldehyde. After permeabilized with 0.1% TritonX-100, cells were incubated with PBS containing 10% normal horse serum. Then the cells were incubated with primary antibody including Oct4 (1:500, Santa Cruz), Nanog (1:200, Abcam), SSEA-1 (1:50, eBioscience), Tuj1 (1:500, Millipore), a-SMA (1:50, Abcam) and Gata4 (1:50, Santa Cruz). Secondary antibodies included Dylight 488-conjugated AffiniPure Donkey Anti-Mouse IgG (1:400, Jackson ImmunoResearch) and Alexa Fluor 594 donkey anti-Rabbit IgG (1:400, Jackson ImmunoResearch). Then nuclei were stained with Hoechst 33342 (Life Technologies).

2.10. EB differentiation

1×10^6 cells in scaffolds were harvested by trypsinization and transferred to 10 cm bacterial culture dishes in the mES medium without LIF. Medium was changed at day-1 and day-3. After 5 days, aggregated cells were plated on gelatin-coated cell culture plates and incubated for another 5 days in mES medium without LIF. The cells were stained with different antibodies.

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