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Dynamic culture improves cell reprogramming efficiency

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ABSTRACT

Cell reprogramming to pluripotency is an inefficient process and various approaches have been devised to improve the yield of induced pluripotent stem cells. However, the effect of biophysical factors on cell reprogramming is not well understood. Here we showed that, for the first time, dynamic culture with orbital shaking significantly improved the reprogramming efficiency in adherent cells. Manipulating the viscosity of the culture medium suggested that the improved efficiency is mainly attributed to convective mixing rather than hydrodynamic shear stress. Temporal studies demonstrated that the enhancement of reprogramming efficiency required the dynamic culture in the middle but not early phase. In the early phase, fibroblasts had a high proliferation rate, but as the culture became over-confluent in the middle phase, expression of p57 was upregulated to inhibit cell proliferation and consequently, cell reprogramming. Subjecting the over confluent culture to orbital shaking prevented the upregulation of p57, thus improving reprogramming efficiency. Seeding cells at low densities to avoid over-confluency resulted in a lower efficiency, and optimal reprogramming efficiency was attained at a high seeding density with dynamic culture. Our findings provide insight into the underlying mechanisms of how dynamic culture condition regulate cell reprogramming, and will have broad impact on cell engineering for regenerative medicine and disease modeling.

1. Introduction

Cell reprogramming is a major advancement in the field of cell biology and cell engineering. The forced expression of the transcription factors Oct4, Klf4, Myc, and Sox2 (OSKM) reprograms somatic cells into induced pluripotent stem cells (iPSCs) [1,2] which can be used for disease modeling and regenerative medicine applications. The efficiency of this process is relatively low, ranging between 0.1% and 10% for most somatic cell types [3]. To date, extensive work has been done to improve efficiency through further genetic manipulation [4–7] or addition of chemicals [8–12].

Manipulating the biophysical environment of the cells can also improve reprogramming efficiency. Culturing fibroblasts on microtopography can modulate their epigenetic states to improve reprogramming efficiency by as much as 4 folds [13]. Subjecting the culture to hypoxic condition can also improve efficiency, although the underlying mechanism is not yet elucidated [14]. By using a cell suspension culture, higher yield of iPSCs can be obtained over time [15]. Culturing fibroblasts on soft hydrogel can also improve efficiency by promoting mesenchymal to epithelial transition during the early phase of reprogramming [16]. Such biophysical approaches demonstrate the potential of adopting this paradigm in improving reprogramming efficiency. However, in most cases, the underlying mechanisms are not well understood.

When we investigated the effects of dynamic culture conditions on adherent reprogramming cells, we found a significant increase in reprogramming efficiency. We further demonstrated that the dynamic culture condition prevented the cell cycle arrest in the middle phase of reprogramming by suppressing the expression of cell cycle inhibitor p57.

2. Materials and methods

2.1. Fibroblast isolation, cell culture and reprogramming

Skin fibroblasts were derived from transgenic mice that carried doxycycline-inducible OSKM genes (stock no 011011, Jackson Lab).

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The four reprogramming factors, OSKM, were expressed from the collagen type 1 gene locus upon induction with doxycycline [17]. 1 day post-partum mice were sacrificed by decapitation with a pair of sharp scissors. The skin was peeled off and floated on 0.05% freshly thawed trypsin overnight and the dermis was separated from the epidermis next day. The dermis was cut up and digested in 200 U/ml collagenase II and 0.1% Trypsin at 37 °C for 30 min. The digesting mixture was mixed with fetal bovine serum (FBS) to quench the digestion enzyme activity and spun down. The pellet was plated on gelatin coated dish and expanded for 2 days in MEF medium (DMEM with 10% FBS and 1% penicillin/streptomycin). Thereafter, the culture was trpysinized, passed through a 40 μm filter and frozen down into aliquots in a medium containing 10% dimethyl sulfoxide (DMSO) and 90% FBS.

For cell reprogramming experiment, fibroblasts were seeded into 6 well plates in MEF medium. Unless otherwise stated, cells were seeded at a density of 3000 cells/cm^2. The next day, medium was replaced with reprogramming medium (Knockout™ DMEM, 10% Knockout Serum Replacement™, 5% FBS, 1% penicillin/streptomycin, 1% Glutamax, 1000 U/ml LIF and 2 μg/ml doxycycline) to induce the expression of OSKM.

For dynamic culture, the culture plates were placed on an orbital shaker in the incubator and, unless noted otherwise, agitated at 100 rounds per minute (rpm). At the indicated times, doxycycline was removed from the reprogramming medium to stop the exogenous expression of OSKM, with all other medium components retained. For all experiments, media was changed once every 2–3 days.

Reprogrammed iPSC colonies with three dimensional dome-like morphology and clear boundary were picked on day 20 and expanded in reprogramming medium for 4 passages. To form embryoid bodies (EBs), the iPSCs were suspended in 20 μl hanging drops at 2000 cells per drop for 3 days in EB medium (same as reprogramming medium but with LIF removed). The embryoid bodies were then plated on gelatin coated plate and cultured for an additional 9 days in EB medium to allow further spontaneous differentiation before fixation and staining.

2.2. Immunofluorescence staining and microscopy

For immunostaining, cells were fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton-X 100 for 15 min. Cells were stained with the respective primary and secondary antibodies, and imaged with a Zeiss AxioObserver epi-fluorescent microscope. Refer to Supplementary Table 1 for list of primary antibodies. Secondary antibodies were Alexa Fluor 488 or Alexa Fluor 546 (ThermoFisher Scientific).

To determine cell proliferation rate, EDU staining was performed by using Click-iT® EdU Alexa Fluor® 488 Imaging Kit (ThermoFisher Scientific). Samples were pulsed with 10 μM EDU for 30 min. Subsequent steps were performed according to manufacturer's instructions.

2.3. Quantification of reprogramming efficiency

At the indicated time, culture was fixed and immunostained for iPSC markers Nanog and SSEA1. Stained samples were kept in phosphate buffered saline (PBS) and loaded onto ImageXpress Micro High-Content Analysis System (Molecular Devices) for automated whole well imaging at 4× magnification. The images were stitched using the MetaXpress Analysis Software (Molecular Devices) and loaded in Photoshop for colony counting. A transparent layer was created and positive colonies were marked and covered with a circular brush. The layer was then saved as a separate image and the number of circular marks were counted using ImageJ. Reprogramming efficiency was obtained by dividing the number of Nanog + positive colonies by the total number of cells seeded at the beginning of the experiments.

For flow cytometry analysis, the culture was first treated with 500 U/ml collagenase II dissolved in plain Knockout™ DMEM for 15 min to partially digest the ECM, followed by trypsin treatment. The detached cells was triturated with progressively smaller needles to obtain a single cell suspension before incubation with StainAlive™ SSEA1 antibody (Stemgent) for 30 min. The suspension was spun down and washed with PBS two times before being analyzed with Guava (Merck Millipore).

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

At the indicated time, culture was lysed with Trizol (ThermoFisher Scientific) and RNA was extracted following the manufacturer's instruction. RNA concentration was quantified with Nanodrop 1000 (Thermo Scientific) and equal amount was loaded for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). cDNA was then loaded into 96 well PCR plate with primers and Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific). Primer information is listed in Supplementary Table 2. Thermal cycling and data acquisition was performed on iQ5 system (Biorad). GAPDH used as housekeeping gene for nomalization. Data was analyzed with ΔΔCt method.

2.5. Western blotting analysis

To determine p57 expression, cells were lysed with a lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM NaF along with protease inhibitors (phenylmethyl sulphonyl fluoride, Na_3VO_4 and leupeptin). Lysates were centrifuged and the supernatants removed and quantified by Direct Detect® Infrared Spectrometer (Merck Millipore). For analysis of YAP/TAZ and β-Catenin localization, nuclear and cytoplasmic lysates were prepared with NucBuster™ Protein Extraction Kit (Merck Millipore) according to the manufacturer's instructions.

Equal amounts of proteins were separated with SDS–PAGE and then transferred to polyvinylidene fluoride membranes. Membranes were blocked in either denatured 5% BSA (when staining for phosphorylated proteins) or 3% non-fat milk and then incubated with primary antibodies. Refer to Supplementary Table 1 for list of antibodies. Next, membranes were incubated with HRP-conjugated IgG secondary antibodies (Santa Cruz Biotechnologies) for 1 h. Protein bands were visualized by using Western Lightning Plus-Enhanced Chemiluminescence Substrate (Perkin Elmer Life & Analytical Sciences).

2.6. Measurement of viscosity, shear stress and mixing rate

Dextran was added to manipulate the viscosity of the culture medium. Measured amount of dextran was sterilized by incubation in boiling water bath for 30 min. The viscosity of medium with and without dextran at 37 °C was measured with rheometer (MCR300, Anton Paar, Ashland, VA). 50 mm parallel plate at a gap height of 0.5 mm was used. A humidity chamber was placed around the sample to prevent dehydration. The lower plate temperature was regulated with a Peltier heating element connected to a recirculating water bath. Medium was sheared at different rates and the torque measurement was used to calculate viscosity by the equation: