Feeder-Free Derivation of Naive Human Pluripotent Stem Cells

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HUMAN PLURIPOTENT STEM CELLS (HPSCs) cultured in conditions that maintain pluripotency through fibroblast growth factor and transforming growth factor beta signaling have been described as being in a primed state. These cells have been shown to exhibit characteristics more closely related to mouse epiblast-derived stem cells than to so-called naive mouse pluripotent stem cells (PSCs) said to possess a more ground state pluripotency that mimics the early mouse embryo inner cell mass [1,2]. Initial attempts to create culture conditions favorable for generation of naive HPSCs from primed HPSCs have required the use of mouse embryonic fibroblasts as a feeder layer or a mixture of truncated vitronectin mixed with gelatin to support this transition [3,4]. A protocol for the routine derivation and maintenance of naive HPSCs in completely defined xeno-free conditions is highly desirable for stem cell researchers to enhance the study and clinical translation of naive HPSCs. In this study, we describe a standard protocol for transitioning primed HPSCs to a naive state using commercial RSeT media and xeno-free recombinant full-length vitronectin.

Primed HPSCs maintained in defined conditions on a recombinant vitronectin substrate have a stereotypical morphology characterized by small cells with a large nuclear:cytoplasmic ratio that form flat monolayer colonies (Fig. 1A). After transition into the naive state, the HPSCs assumed a tightly packed cell morphology forming rounded three-dimensional colonies with distinct phase bright edges, which could be passaged routinely using TrypLE-mediated single-cell dissociation (Fig. 1B). Critically, in contrast to other published protocols for feeder-free naive HPSC derivation, the cells transitioned to the naive state using our protocol exhibit a normal karyotype (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). When cultured in 10 nM extracellular signal-regulated kinase (ERK) inhibitor (PD0325901), primed HPSCs are not able to maintain pluripotency and display immediate differentiation (Fig. 1C). However, ERK signaling has been reported as dispensable for naive HPSCs to maintain a pluripotent state, and naive cells transitioned from the primed state maintain their undifferentiated state in RSeT media supplemented with an additional 10 nM PD325901 (Fig. 1D). Our transitioned naive HPSCs maintain marker expression characteristic of HPSCs and can differentiate into three germ-layer derivatives in vitro (Supplementary Fig. S1). Expression of the pluripotent markers *CDH1* and *OCT4* is changed less than

twofold in naive HPSCs from primed HPSCs, while expression of *NANOG* is increased (Supplementary Fig. S2). These cells were stably maintained in the naive state culture conditions for more than 30 passages.

The culture matrix may be the limiting factor defining a successful transition to a naive pluripotent state in the feeder-free conditions described in this study. The use of a full-length recombinant vitronectin in the manner described with our specific manipulation of the cells during transition supports the successful transition between pluripotent states, whereas the use of Matrigel did not (Supplementary Fig. S3). Although the HPSCs transitioned to the naive state on Matrigel had many of the hallmarks of being characteristic of naive pluripotent stem cells, karyotype analysis revealed clonal abnormalities in all transitioned cell lines (data not shown). It is possible that vitronectin has a unique ability to allow HPSCs to compensate for transition stresses without accumulating these abnormalities. This is the first description of a protocol for the routine successful transition of primed HPSCs to a naive state in completely defined conditions. This process has been repeated with multiple primed HPSC lines from various genetic backgrounds. This technology is a critically important step for research and translation of HPSCs cultured in the naive ground state.

Materials and Methods

Feeder-free naive pluripotent stem cell derivation

Primed induced pluripotent stem cells (UMN PCBC16iPS) [5] to be used for naive cell derivation were cultured for at least two passages in mTeSR1 medium (STEMCELL Technologies) in either normoxic or hypoxic conditions. Prime induced pluripotent stem cells (iPSCs) were treated with Gentle Cell Dissociation Buffer (STEMCELL Technologies) for 5 min at room temperature and removed from the plate with a 5 mL pipette in 1 mL TeSR1 medium. Aggregates of 250 prime iPSCs were plated in 1 mL mTeSR1 (STEMCELL Technologies) per well of a 12-well tissue culture treated plate (Corning) coated with 5 µg/mL full-length vitronectin (PeproTech) [6]. The next day, cells were transferred to hypoxic conditions and switched to RSeT medium (STEMCELL Technologies) culture conditions. Cells were monitored for compaction and defined colony edges. When naive iPSC colonies reached 250 mm in diameter, the well was passaged. For passaging, cells were washed 1× with

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FIG. 1. Naive pluripotent stem cells derived on vitronectin are distinct from primed pluripotent stem cells. **(A)** Morphology of a primed HPSC colony. **(B)** Morphology of a transitioned naive HPSC colony (passage 10). ERK inhibitor PD0325901 induces differentiation in primed HPSCs **(C)** but not in naive HPSCs **(D)**. Expression of NANOG **(E, F)**, SSEA-3 **(G, H)**, and SSEA-4 **(I, J)** in primed and transitioned naive HPSCs. Scale bar = 50 μ m. ERK, extracellular signal-regulated kinase; HPSC, human pluripotent stem cell.

phosphate-buffered saline (PBS)–Ca²⁺/–Mg²⁺ followed by addition of 250 μ L TrypLE Express (Thermo Scientific) for 3 min at 37°C in hypoxic conditions. TrypLE was neutralized with 750 μ L RSeT supplemented with 10 μ M Rho-associated kinase inhibitor Y-27632 (BD Biosciences). Cells were agitated 3 $\times$ using a p1000 pipet tip to achieve 10–12 cell clumps. After collection, cells were spun at 300 g for 5 min. Cells were resuspended in 120 μ L of RSeT medium supplemented with 10 μ M Y-27632 by pipetting 6 $\times$ with a p200 pipet tip to break cells into clumps of 2–3 cells. Cells were plated in dilutions of 1:3, 1:4, 1:8, and 1:10 to ensure optimal plating density.

ERK inhibitor assay

One day after passaging, 10 nM ERK inhibitor PD0325901 (PZ0162; Sigma-Aldrich) was added to naive or primed

PSCs. Cells were imaged each day for 4 days to follow morphology changes.

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then made permeable with 0.2% Triton X-100 in PBS for 30 min. Cells were blocked with 3% bovine serum albumin (BSA) in PBS for 2 h then incubated overnight at 4°C with primary antibodies diluted in 3% BSA. The antibodies used were SSEA-3 (1 μ g/mL; MAB4303; Millipore) and SSEA-4 (1 μ g/mL; MAB4304; Millipore). Cells were washed in PBS for 5 min 3 $\times$ and incubated with 0.5 μ g/mL respective secondary antibody (A21042, Alexa Fluor anti-goat 488; A21432, Alexa Fluor anti-goat 555; A21434, Alexa Fluor anti-rat 555; A31572, Alexa Fluor anti-rabbit 555; A11001, and Alexa Fluor anti-goat 488, all from Life Technologies) for 1 h at room temperature and washed with PBS. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μ g/mL; Invitrogen) diluted in PBS for 10 min at room temperature. Images were processed using Adobe Photoshop to optimize brightness and contrast, with all control and experimental images being treated identically. For NANOG, wells were fixed for 10 min at room temperature using formalin. The cells were permeabilized with a mixture of 1% BSA and 0.2% Triton X-1000 in PBS for 10 min. The cells were blocked with Block (1% BSA + 0.1% Tween) for 30 min at room temperature. The cells were incubated overnight at 4°C with the primary antibody against NANOG (5 μ g/mL; AF1997; R&D Systems) diluted in block. The primary antibody was washed off using block, and the secondary (0.5 μ g/mL, A21432, Alexa Fluor anti-goat 555) was added for 1 h at room temperature. The wells were washed 3 $\times$ with PBS, and 4,6-diamidino-2-phenylindole (DAPI, 1 μ g/mL; Invitrogen) diluted in PBS was added for 5 min at room temperature. The imaging was performed the same way described above.

Pluripotency assay

Differentiation potential of naive PSCs into the three germ layers was performed using STEMdiff Trilineage Differentiation Kit (05230; STEMCELL Technologies). Briefly, cells were plated onto Matrigel (Corning) and treated with endoderm or mesoderm differentiation media for 5 days or ectoderm differentiation media for 7 days. Cells were then fixed, stained, and imaged as described.

Karyotype

Naive PSCs were examined by high-resolution G banding after 10 passages in RSeT medium.

Reverse transcription-quantitative polymerase chain reaction

RNA was isolated from primed HPSCs at passage 18 and naive HPSCs at passage 7, passage 10, and passage 12 using the RNeasy RNA Isolation Kit (Qiagen), and cDNA was prepared using SuperScript IV (Invitrogen). RT-qPCR was run on the Mastercycler ep gradient S (Eppendorf) and analyzed using ep realplex software (Eppendorf). Prime-Time assays for *CDH1* (Hs.PT.58.3324071), *POU5F1* (Hs.PT.58.14648152.g), *NANOG* (Hs.PT.58.21480849), and

GAPDH (Hs.PT.39a.22214836) were obtained from Integrated DNA Technologies.

Author Disclosure Statement

No competing financial interests exist.

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