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Microfluidic isolation of highly pure embryonic stem cells using feeder-separated co-culture system

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Engineered artificial tissues from stem cells show great potential in regenerative medicine, disease therapies and organ transplantation. To date, stem cells are typically co-cultured with inactivated feeder layers to maintain their undifferentiated state, and to ensure reliable cell purity. Herein, we propose a novel microfabricated approach for feeder-separated coculture of mouse embryonic stem (mES) cells on polydimethylsiloxane (PDMS) porous membrane-assembled 3D-microdevice. Normal mouse embryonic fibroblasts (mEFs) without inactivation were specifically co-cultured with mES cells, resulting in the formation of mES cell colonies on spatially controlled co-culture with feeder layers. An excellent undifferentiated state was confirmed by the expressions of Nanog, octamer binding protein 4 (Oct-4) and alkaline phosphatase (ALP) after 5 days culture. As a result, with the significant advantages of efficiency and simplicity, pure mES cell populations (a purity of 89.2%) from mEFs co-cultures were easily collected without any further purification or separation.

Stem cell engineering is an emerging field that shows great promises in healing damaged tissues and replacing non-functional organs[1–3]. Effective expansion and separation of pure stem cells are of greatly significant in providing cell sources for the regeneration of aged, injured and diseased tissues[4,5]. For example, feeder layers (mouse embryonic fibroblasts (mEFs)) are traditionally co-cultured with mouse embryonic stem (mES) cells to supply the essential intrinsic regulators and environmental cues[6,7], which are particularly important for regulating stem cell growth, self-renewal and differentiation[8]. To obtain pure stem cells, the feeder layers are commonly inactivated by γ-irradiation or mitomycin C. However, there are three issues associated with inactivating the feeder layers: i) it is time-consuming and expensive; ii) residual mitomycin C and mEFs apoptosis may have cytotoxicological effects on stem cell fate[9]; iii) most importantly, further experiments on stem cells require removal of mitotically inactivated mEFs, because the inactivated mEFs will still survive for several days. Although the feeder layer-free culture method has been developed for stem cell culture[10], the feeder layers are still required for maintaining the embryonic stem cells, especially for human embryonic stem cells. For these reasons, an isolation-free strategy for recovering stem cells is of great significance in basic and applied research in stem cell tissue engineering[11].

Microfluidics is increasingly emerging as a powerful tool for cell culture[12,13], metabolism[14,15], isolation[16,17], as well as stem cell differentiation[18] and tissue engineering[19] owing to its unique advantages of low reagent consumption, ease of integration, high throughputs, and excellent reproducibility[20–24]. Recently, microfluidic-based stem cell studies have attracted much interests because microfabricated technologies provide novel and improved methods of mimicking the complexity of spatially and temporally controlled cellular microenvironment through constructing well-defined architectures[25–27]. For example, to quantitative culture of embryonic stem cells, Kamei and his coworkers designed and fabricated an integrated microfluidic platform that allowed co-culture of ES cells with growth-arrested mEFs feeder layers[28]. In such method, the use of γ-irradiated mEFs will bring effects on separating stem cells for subsequent experiments. Alternatively, Lecault *et al.* reported a microfluidic platform array for hematopoietic stem cell proliferation containing thousands of nanoliter-scale chambers, resulting in 90% cell recovery under feeder-free condition[29]. However, the co-culture of mEFs is still significant to long-term genetic stability of stem cells[9]. Thus, the use of normal mEFs layer, which allows direct separation of pure stem cells for further stem cells experiments, is much attractive and will prove to be beneficial in stem cell studies.

The porous membrane allows free exchange of signal molecules (such as proteins, carbohydrates and other small molecules)[15,20,30]. Engineering the complex cell-cell interactions in a spatially and temporally regulated

manner is an alternative approach to mimic stem cell 3D-microenvironment in tissue culture processes. Thus, in this work, we present a simple and versatile microfluidic stem cell-coculture design that allows the use of normal mEF feeder layers (without chemical or physical treatment) to maintain the stem cells in an undifferentiated state and recover a high purity of mES cells for further application. To achieve this, co-culture of mES cells was technically separated with mEFs feeder layers on the designed PDMS porous membrane-assembled 3D-microdevice. Then, the mEFs were demonstrated to proliferate with high viability and survival for more than 3 weeks, and to maintain the stem cells in an undifferentiated state by the expressions of Nanog, octamer binding protein 4 (Oct-4) and alkaline phosphatase (ALP). As a result, our method serves two advantages: 1) the mEFs can grow and maintain normal viability for several days on-chip, avoiding to apoptosis because of no chemical treatment or γ-irradiation for inactivated conditions; 2) further experiments on stem cells can be performed without additional purification. Thus, our methods will be an excellent strategy for stem cells culture, which will be greatly potential in generating artificial tissues for biomedical applications.

Results

Design and operation of co-culture microdevice. The stem cell co-culture microdevices were designed and fabricated with alignment and permanent bonding of a thick porous PDMS membrane between two PDMS layers with microchannels. As shown in Fig. 1A, the mES cells and mEFs can be introduced into the top and bottom channels, respectively. After that, these two types of cells were adhered to the both sides of PDMS porous membrane and grew into spatially patterned cell co-culture (Fig. 1B). As reported previously[20,29,31], PDMS provides high gas permeability for the efficient exchange of carbon dioxide, oxygen, and some small molecules[32]. Thus, with the effective separation of mES cells from mEFs, the PDMS porous membrane also allows cytokine diffusion from bottom channels to top channels, enabling maintenance of the mES cells at an undifferentiated state, which is comparable to conventional co-culture method. Importantly, the PDMS porous membrane-integrated microfabricated device enabled recovery of pure mES cells by direct trypsin digestion in a simplified process (Fig. 1C). In this work, the PDMS porous membrane was fabricated by using standard soft lithography and replica molding techniques (Fig. 1G). SU-8 posts with controllable diameters were fabricated on a silicon wafer for tunable porous pore diameter. A thin film of PDMS was then spin-coated on the wafer such that its thickness was less than the height of the SU-8 posts. The resulting thin porous PDMS films were easily peeled off with a thickness of 10 μm and a porous diameter of 11.2 μm using a specially designed cured PDMS frame (Fig. 1D–F). This microfabricated platform will be used for co-culture of mES cells in high purity.

Proliferation and viability of normal mEFs on 3D-microdevice. Mitotically inactivated mEFs (with γ-irradiation or mitomycin C treatment) are traditionally used for co-culture of mES cells by providing stem cell microenvironment for expansion and undifferentiation[4]. Inactivated mEFs commonly lose the ability of mitosis and have lower viability (apoptosis & death). To provide the proof of principle for a novel and versatile approach, normal

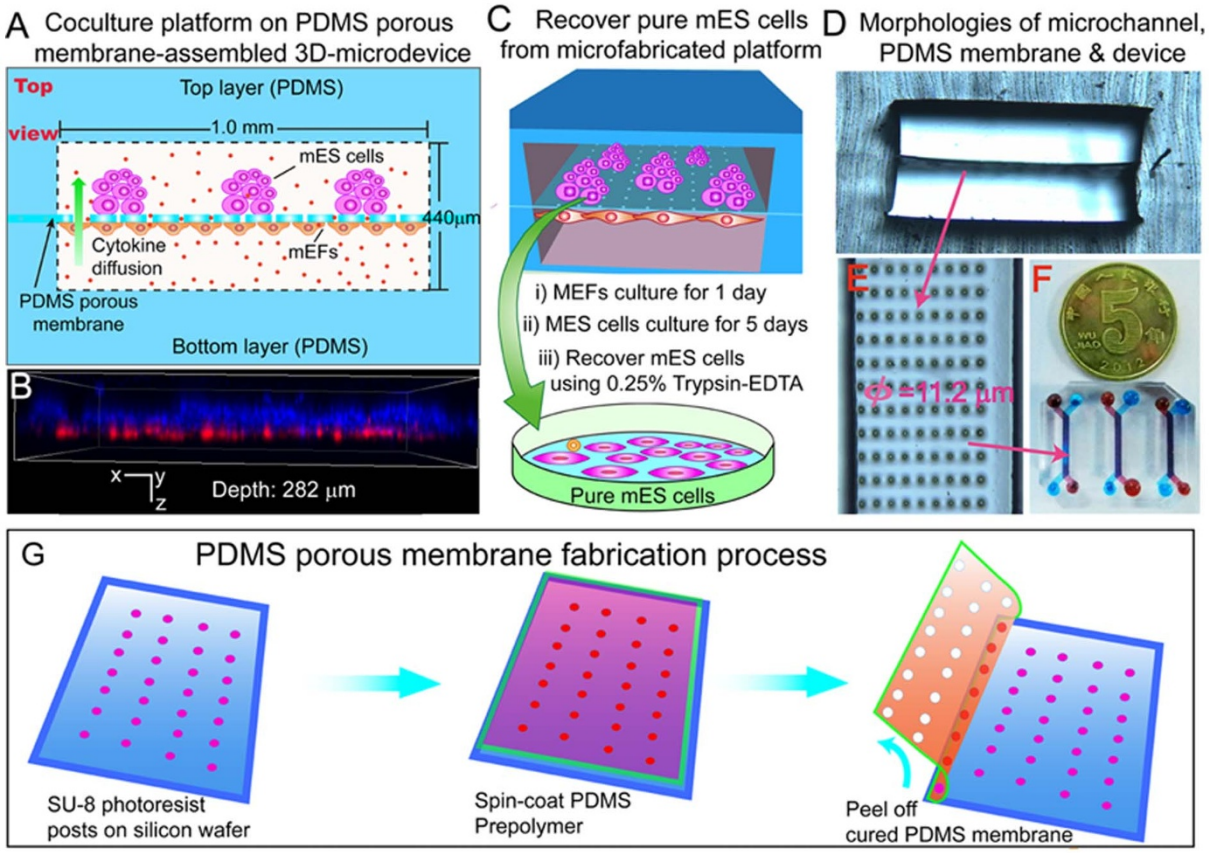

Figure 1 | Schematic of PDMS porous membrane-assembled microfluidic co-culture platform. (A) Top view illustration of the device dimensions and mES cells/mEFs co-culture on microchannels. (B) Confocal morphology showing the mES cells/mEFs coculture (depth 282 μm); stem cells layer strained with Hoechst 33342) (blue fluorescence) and feeder layer with RFP-mEFs. (C) Recover pure mES cells using 0.25% Tryspin-EDTA after 5 days culture. (D, E and F) Morphologies and structure of assembled microchannels, PDMS porous membrane (thickness: 10 μm; pore diameter: 11.2 μm) and fabricated microdevice. (G) Fabrication of PDMS porous membrane using standard soft lithography and replica molding techniques.

mEFs were conceptually used to co-culture with mES cells on a PDMS membrane-integrated 3D microdevice. As shown in Fig. 2A and 2B, the growth of mEFs was observed by monitoring the expression of RFP. The mitosis of mEFs was observed by long-term monitoring the expression of RFP from day 1 to day 7 (Fig. S4 in supporting information). Fig. 2C showed the fluorescence intensity curve of mEFs proliferation (viable more than 3 weeks) with average RSDs of 5.7% (N = 8). The mitosis-enabled normal mEFs will effectively support the mES cells for its expansion and undifferentiation. To further assess the cell viabilities, γ-irradiated mEFs and mitotically active normal mEFs were individually cultured on two different 3D-microdevices and then stained with live/dead kit (Calcein AM/EthD-1) after 5 days culture (Fig. 2D and 2E). Obvious dead cells from inactivated mEFs were observed with viabilities of 67% ± 9.6% (N = 8) (Fig. 2F), which may interfere with the downstream applications of stem cells. In contrast, the nomal P3 mEFs showed a high viability of 97%. The co-culture of mES cells and normal mEFs is more suitable for mimicking the complexity cellular architectures that are particularly important for regulating stem cell growth and self-renewal. We believe that the use of normal mEFs for stem cells co-culture will be beneficial for the studies of stem cells tissue engineering.

Microfabricated platform for co-culture of mEFs and mES cells. To verify the co-culture platform on PDMS porous membrane-based 3D microdevice, RFP expressing mEFs and mES cells were introduced into their respective microchannels, attaching to the opposite surfaces of the PDMS porous membrane. The 3D morphology of mES cells and mEFs was characterized after 5 days by laser confocal fluorescence microscopy (see Fig. 2G and Movie S1 in supporting information). The mES cells were effectively separated with mEFs as a feeder layer by integrated PDMS porous membrane as described above, forming many mES cell-colonies on spatially controlled co-culture with feeder layers. In Fig. 2H, the high cell viability of co-cultures after 5 days also indicates the significant advantages of efficiency and simplicity by this method. In comparison, as shown in Fig. 2I, the co-cultures of the inactivated mEFs and mES cells have high ratio of dead cells, which may affect the signaling pathways of stem cells during stem cell differentiation and stability during co-culture[9]. Thus, using mitotically active normal mEFs for mES cell co-culture will be a novel and promising method for stem cell application, which is different from conventional methods.

Identification of mES cells pluripotent status by co-cultures. As a microfabricated approach for co-cultures, it is critical to maintain the mES cells in an undifferentiated state. To examine the stem cell undifferentiated potentiality, expressions of Oct-4 and ALP, two well-known biomarkers of undifferentiated mES cells[33], were characterized after 5 days culture on 3D-microdevice co-culture platform. The Oct-4 expression levels of mES cells from feeder-separated co-cultures and direct contact co-cultures were respectively showed in Fig. 3A and Fig. S5 in supporting information, which was higher compared to the absence of mEFs (Fig. 3B). The level expression of Oct-4 by qualitative comparison was shown in Fig. 3C. The Oct-4 expression of stem cells on feeder-separated co-culture was approaching 100% (RSD = 8.2%; N = 8) compared with that on direct contact co-cultures, whereas only 54.2% (RSD = 11.5%; N = 8) Oct-4 expression level of stem cells without co-culture of mEFs. These results implied that the mitotically active mEFs was important for the mES cell co-culture. Additionally, the

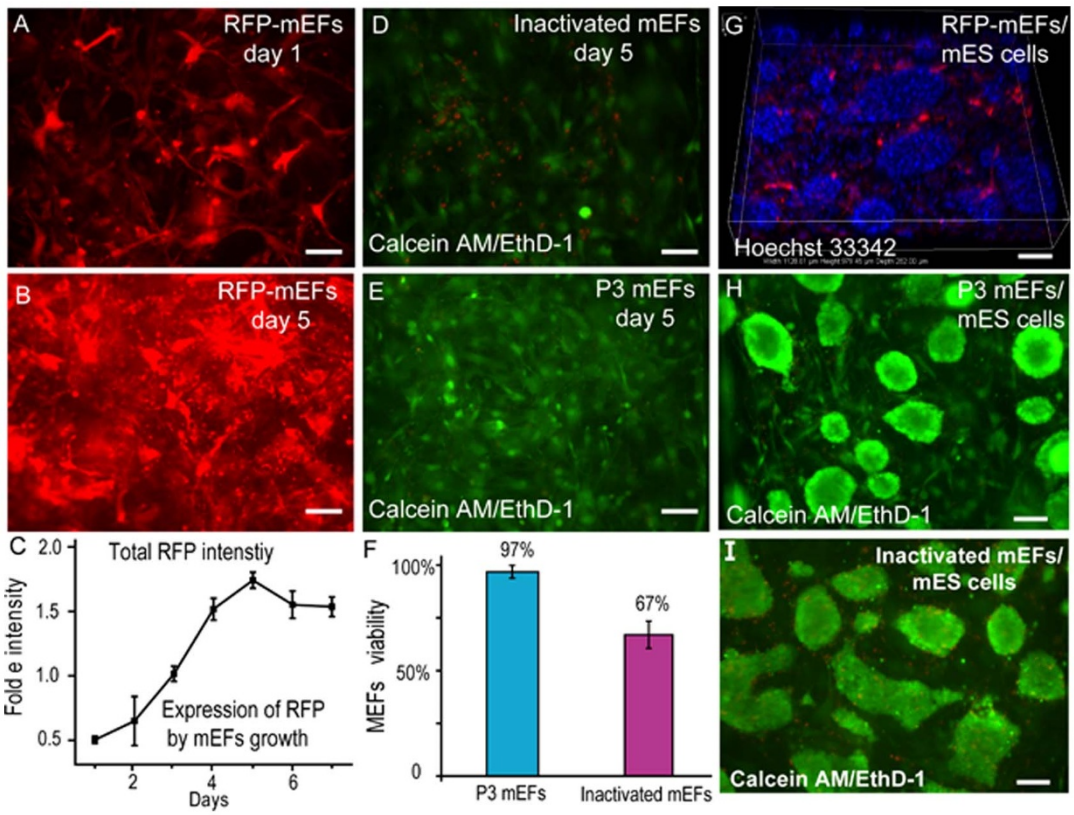

Figure 2 | Proliferation and viabilities of mEFs and mES cells on the co-culture microdevice. (A, B) The fluorescence profile of RFP-mEFs at day 1 and 5 showing the cell proliferation, scale bar: 100 μm. (C) The curve of fluorescence intensity folds from day 1 to day 7. (D, E. F) The viabilities of inactivated mEFs and P3 mEFs were characterized with Calcein AM/EthD-1 (10 μL, 10 mg/mL) at day 5 and imaged by the fluorescence microscopy. (G) Confocal morphology of RFP-mEFs/mES cells, stained with Hoechst 33342. (H, I) Viabilities of P3 mEFs/mES cells and inactivated mEFs/mES cells on 3D-device, stained with cell live/dead kit (Calcein AM/EthD-1). Scale bar: 100 μm.

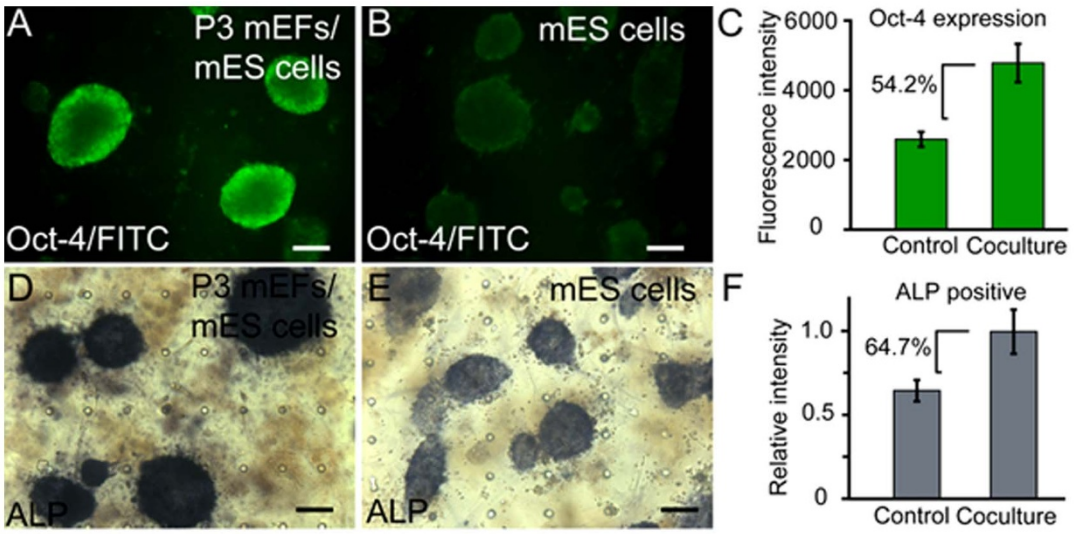

Figure 3 | Expressions of undifferentiating markers including Oct-4 and alkaline phosphatase (ALP) on mES cells. (A, B) indicate the expression of Oct-4 and (D, E) indicate the ALP positive. (C, F) compare the Oct-4 expression and ALP positive intensity of mES cells between co-cultured with P3 mEFs on 3D-device and no feeder layer as a control after 5 days. Scale bar: 100 μm.

ALP positive mES cells were also showed in Fig. 3D and 3E, and qualitative comparison was showed in Fig. 3F. We found that the intensities of ALP positive on feeder-separated co-culture were comparable to that on direct contact co-cultures (approaching 100%, RSD = 9.7%, N = 8), whereas only 64.7% (RSD = 13.2%; N = 8) ALP positive for stem cells without co-culture of feeders. These results were similar to the Oct-4 expression level as described, suggesting that the feeders-separated co-culture can maintain the mES cells in a pluripotent status. Furthermore, the staining of Nanog biomarkers on mES cells is critical to further characterize their self-renewing pluripotencin[34]. As shown in Fig. S6, the obvious fluorescence of Nanog-IgG antibody/IgG-FITC staining indicates that the mES cells still behaved the self-renewing pluripotency. Those phenomena are justifiable because mEFs support the pluripotency and robust expansion of mES cells. Thus, the co-culture of mEFs and mES cells on 3D-microdevice platform was demonstrated to be suitable for stem cell studies.

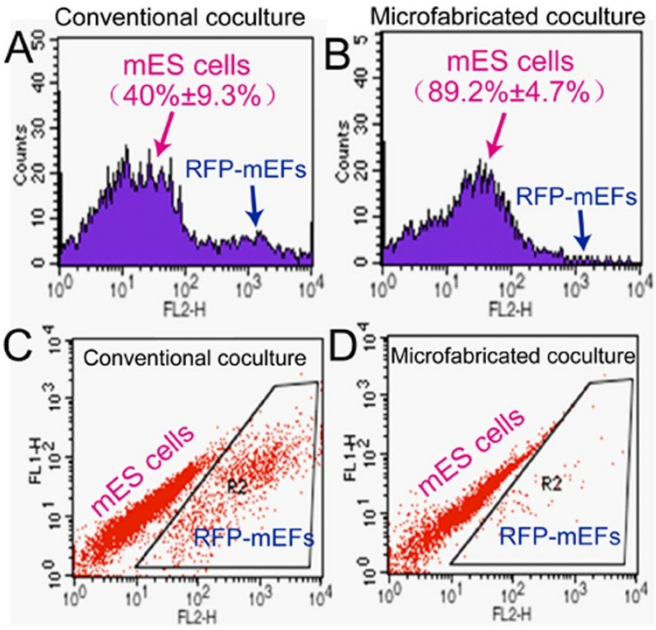

Figure 4 | Quantitative analysis of the mES cells purities from conventional co-culture and microfabricated co-culture methods. (A, C) shows the flow cytometric analysis of mES cells purities from conventional co-culture methods. (B, D) shows the flow cytometric analysis of mES cells purities from 3D-microfabricated co-culture methods. The mES cells were distinguished by red fluorescence of RFP-mEFs.

Assessment of cell purity in recovered mES cell populations. In stem cell differentiation and tissue engineering, the co-cultured mEFs will interfere with the downstream applications of stem cells (Fig. S3). Thus, the purity of isolated stem cells is greatly important. Using our method, it is very easy to recover pure mES cells without further purification base on this microfabricated co-culture technique. After co-culturing with feeder layers for several days, the mES cells on top-layer were trypsin-digested for 5.0 min. Flow cytometric analysis shows a purity of 89.2% ± 4.7% mES cells (top layers) were recovered from 3D-microdevice co-culture platform (Fig. 4A and 4C). This superior method was demonstrated to obtain pure populations of mES cells from mEFs co-culture within 5.0 min, with the significant advantages of efficiency and simplicity. As a control, the conventional co-culture of mES cells and RFP-expressing mEFs on culture dishes contained approximately 40% ± 9.3% of mES cells in the recovered cell mixtures (Fig. 4B and 4D), which may greatly affect further stem cell experiments. Most importantly, the microfabricated co-culture approach avoided using the inactivated mEFs, not only cutting down the time- and reagent-consumption, but also leading to higher viability of mEFs, which enabled robust expansion of mES cells and maintained an undifferentiated state. Thus, the developed porous membrane-integrated microfabricated system for feeder-isolated co-culture of mES cells will serve as a promising method in the stem cell culture and tissue engineering applications.

Discussion

A number of approaches for co-culture of embryonic stem cells with feeder cells have been reported to regulate stem cell growth, self-renewal and differentiation, most of which rely on using inactivated feeder layer for long-term genetic stability of stem cells[8]. Though these methods are commonly utilized for stem cell studies, the feeder layers for co-culture with stem cells usually need to be treated with γ-irradiation or mitomycin C. These types of stem cell expansions

require lots of time and reagent, as well as further purification because of the mixture with surviving feeder cells. Our method described above is more amenable to high-throughput stem cell culture but still enables cell-to-cell interactions for mimicking the complexity of cellular microenvironment through constructing well-defined architectures. The feeder cells were allowed to keep proliferating for several days or to survive for more than 3 weeks. The normal mEFs mitosis can secrete continuous cytokine to effectively support the mES cells for its expansion and undifferentiation. The mES cells were also effectively separated with mEFs as a feeder layer by integrated PDMS porous membrane, forming many mES cell-colonies on spatially controlled co-culture with feeder layers.

Investigation of stem cell pluripotency was confirmed by molecular expressions of Nanog, Oct-4 and ALP of stem cells grown with the co-culture with feeder layers. The pluripotency and robust expansion of mES cells were successfully achieved on feeder-separated system because the normal mEFs (P3) could secret extracellular matrix molecules and cytokines for mES cells. Of great interest is the use of mitotically active mEFs, which is able to maintain long-term genetic stability of stem cells; this will provide a novel strategy for stem cell culture and expansion, and also lead to simple and effective method to obtain pure stem cells without further purification. Our advantages of this superior method therefore serve to avoid additional chemical or physical treatments for inactivating mEFs and further purification of stem cells. For these reasons, it will be served as a promising method in the stem cells culture and tissue engineering applications.

In conclusion, the novel and simple microfabricated approach was successfully developed for feeder-isolated co-culture of stem cells that allows the use of normal mEF as a feeder layer, as well as pure mES cells without further purification. With the significant advantages of efficiency and simplicity by this method, the mES cells and feeder layers were spatially adhered to the PDMS porous membranes and forming 3D cell colonies with high viabilities. The self-renewal and pluripotency of mES cells were comfirmed by the expressions of ALP, Oct-4 and Nanog. As a result, this microfabricated approach is different from conventional methods that use the inactivated feeder layer and require additional purification for pure stem cells to downstream applications. Furthermore, if one combines the throughput and automation, this system will also allow for culturing large numbers of mES cells for detailed studies of colony growth and differentiation by removing the feeder layers. Consequently, the designed microfabricated system supporting simple and robust stem cell co-cultures shows considerable promises for basic and applied stem cell research.

Methods

Culture of mEFs and mES cells. Murine embryonic fibroblasts (mEFs, P0) were kindly provided by Professor Dr. Kehkooi Kee (School of Life Science in Tsinghua University) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (FBS). The RFP (red fluorescene protein) -expressing mEFs were purchased from SiDan-Sai Biotechnology Co., Ltd, Shanghai, China. The OriCell™ strain mouse embryonic stem (mES, Cyagen Biosciences Inc., Guangzhou, China) cells were cultured in 6-well plate coated with 0.1% gelatin with DMEM containing 10% FBS, 1000 U ml^{-1} leukemia inhibitory factor (LIF, Millipore), 1% nonessential amino acid solution, 1.0 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified incubator (37°C in an atmosphere of 5% CO_2). The mES cells were grown on an inactivated mEFs (Cyagen Biosciences Inc., Guangzhou, China) feeder layer and passaged with a ratio of 1 : 4 every 2 days.

Fabrication of the 3D microfluidic cell culture chamber. The PDMS microfluidic chip was fabricated by standard soft lithographic and replica molding techniques as described in our previous works[14]. First, the top and bottom layers of the microfluidic chip with three parallel microchannels were produced by casting the mixture of PDMS and curing agent (10 : 1) from a prepared mold on silicon wafer. The mold was fabricated by spin-coating photoresist (SU-8 2050) twice at 1000 rpm for 60 s. The central cell culture microchambers are 10 mm (length) × 1.0 mm (width) × 220 μm (height, single layer). Further, the PDMS porous membrane was produced by spin-coating of PDMS prepolymer (10 : 1) on a silanized wafer 40 μm-tall posts. The array with 40 μm-tall posts was fabricated by spin coating SU-8 2015 at 2500 rpm for 60 s with UV exposure for 30 s, and then spin-coating SU-8 2050 at 3000 rpm for 60 s with UV exposure for 50 s under a designed photomask. Then, a mixture of PDMS prepolymer and cyclohexane was spin-coated at 2100 rpm for 60 s and a 10-μm thick PDMS membrane with circlewise through-holes was produced. After curing at 65°C for 3.0 min, the bottom PDMS layer with the three microchnnels was put on the membrane. The PDMS porous membrane was peeled from the wafer with the bottom PDMS layer after curing at 65°C for 3 hour. Finally, the top layer was bonded with bottom layer for 3D-microdevice (see Fig. S1 in Supporting Information).

Co-culture of mEFs and mES cells on 3D microfluidic chip. Prior to mEFs and mES cell cultures on-chip, the microchannels were coated with gelatin (0.1% in H_2O) at room temperature and sterilized under UV light for 0.5 hour. After that, the gelatin solution was replaced with DMEM media with 10% FBS. Then, the inlet 1 was shut down with PE tubing to make sure that only one type of cells was kept in specific microchannels. To seed the mEFs on the side of porous membrane, the mEFs were trypsinzed and suspended with mEFs culture media. 10 μL of 10^7 cells/ml suspension was injected into microchannels by transpitter and then the microchips were upturn to grow the mEFs onto PDMS porous membrane. After 24 hours of culture, the mEFs were adhered to the porous membrane and the microchip was upturn. The mEFs culture media was introduced into microchannels to maintain the cellular proliferation with high viability. After that, the PE tubing was transferred to inlets 1 and the mES cells were introduced into top layers of microchannels from inlet 2. The cell medium was supplied to the microchannels every 12 hours. This process was illustrated in Fig. S2.

Cell labeling and fluorescence staining. Lentiviral transduction of mEFs expressing red fluorescence protein (RFP) was utilized to monitor the cell proliferation and behavior for several days. When RFP-mEFs and mES cells were co-cultured on the 3D-microdevice after 5 days, 10 μL of Hoechst 33342 (100 μM) was introduced into the microchannels and incubated with these two types of cells for 10 min at 37°C. The images for 3D co-culture were collected by laser confocal fluorescence microscopy (A1RSi, Nikon). Cell viabilities of P3 mEFs and mES cells were determined using molecular probes of calcein AM and ethidium homodimer (live/dead kit). The fluorescence images were collected under a fluorescence microscope (Leica DMI 4000 B, Germany). For Oct-4 and Nanog immunostaining, mES cells were fixed with 4% (w/v) paraformaldehyde (PFA) solution for 15 min. After blocking with 5% (w/v) bovine serum albumin (BSA) solution for 30 min, the mES cells were incubated with Nanog (M-149) and rabbit anti-Oct-3/4 (H65) polyclonal antibody (200 μg/ml, Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG-FITC (200 μg/ml, Santa Cruz Biotechnology, Inc.) for 1 hour and 0.5 hour, respectively. After rinsing with PBS solution three times, the mES cells were imaged by a fluorescence microscope. For alkaline phosphatase (ALP, SiDan-Sai Biotechnology Co., Ltd, Shanghai, China) identification, the mES cells were fixed with 4% PFA solution for 5 min and then rinsed with PBS and TBST solution, sequentially. The ALP detection kit solution (solution A : B : C = 50 μl : 50 μl : 400 μl) was introduced into microchannels and incubated with mES cells for 15 min at 37°C. After rinsing with PBS solution, the ALP positive mES cells were detected by an optical microscope.

Recovery of mES cells and purity analysis by flow cytometry. After co-culturing on the 3D-microdevice, the recoveries of mES cells were conducted by injecting 0.25% trypsin EDTA into microchannels. These microdevices were then incubated for 5 min in a 5% CO_2-humidified air atmosphere at 37°C, leading to cell detachment from the PDMS porous membrane. For cell recoveries, pipettes were inserted into the one side of the microchannels, and then 200 μl of cell media was injected to dissociate the mES cells and RFP-mEFs using a transfer pipette several times. For flow cytometric analysis, the collected cells were centrifuged for 5.0 min at 1200 rpm after being washed with PBS. The cells were resuspended in PBS at single cells. In flow cytometric analysis, the mES cells and RFP-mEFs were analyzed using CY5 channel on a BD FACSAria II instrument.

Statistics. The quantifications were calculated by integration of fluorescence intensities base on the same area from images using OriginPro 8.5.1. And the RSDs were calculated by ANOVA. The "N" represented the numbers of microchannels in the different experiments, and the error bar represented average ± s.d.

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Author contributions

Q.C. and J.M.L. conceived, designed and directed the project. Q.C., J.W. and Z.Q. made and operated the PDMS devices. Q.C., J.W., X.L. and J.Z. performed the stem cell experiments, measurements and analysis. Q.C. and J.M.L. co-wrote the manuscript draft. All authors discussed the results and commented on the manuscript.

Additional information

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