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RESEARCH ARTICLE

bFGF and Activin A function to promote survival and proliferation of single iPS cells in conditioned half-exchange mTeSR1 medium

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Abstract Human induced pluripotent stem (iPS) cells can be well maintained by clonal growth. The pluripotent growth of single iPS cells is limited by low survival. To facilitate robust single iPS cells cultured in vitro, half-exchange mTeSR1 medium (HM), whole-exchange medium (WM) and iPS cell-derived conditioned medium (iPS-CM) culture were used. The effects of bFGF and Activin A on the growth of single iPS cells were explored. The dissociation and propagation of single iPS cells also included Accutase enzymatic isolation, Rho-associated protein kinase (ROCK) inhibitor Y27632 protection and high-density single-cell seeding (1×10^6 cells/well). CCK-8 assays demonstrated that the viability of clonal iPS cells in mTeSR1 medium and single iPS cells in HM, iPS-CM or WM supplemented with 100 ng/ml bFGF and 10 ng/ml Activin A was significantly higher than that in WM. Annexin v and propidium iodide (PI) assay, Calcein AM and EthD-III double staining also confirmed the similar results. ELISA assays showed that the levels of bFGF and Activin A of single iPS cells in HM and iPS-CM were higher than single iPS cells in WM. Meanwhile, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), quantitative Polymerase Chain Reaction (qPCR), Western Blotting (WB), Immunofluorescence (IF) and karyotype analysis revealed that HM culture was able to maintain undifferentiated markers of Nanog, Klf4, Sox2, Oct4, and did not affect the karyotype of iPS cells. Undifferentiated single iPS cells in HM displayed homogenized growth. These findings demonstrate that bFGF and Activin A are important for the survival and growth of single iPS cells. HM culture system combined Accutase, Y27632 and high-density single-cell seeding can facilitate short-term growth of single iPS cells in vitro.

Keywords Single iPS cells · Survival · Conditioned medium culture · bFGF · Activin A

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Introduction

Embryonic stem cell-based therapies faced several barriers, including the immunological and ethical challenges for clinical application for a long time. In 2006, Yamanaka et al. [1] published an article in *Cell journal* that opened up a new epoch for stem cell research. They reported that through the retrovirus-mediated transfection of four defined reprogramming transcription factors (Oct4, Klf-4, Sox2, and c-Myc), murine skin fibroblasts were successfully reprogrammed into pluripotent stem cells like embryonic

stem (ES) cells, a type of reprogrammed cells named induced pluripotent stem (iPS) cells. These iPS cells were almost same as ES cells in proliferative abilities, morphology, gene expression, surface antigens, telomerase activity and epigenetic status of pluripotent cell-specific genes [2]. Generation of iPS cells has provided a great promise for studying human diseases with addressing the risk of immune rejection and ethical issues for clinical application. In addition, these cells are able to be utilized for many pharmaceutical and toxicological applications in in vitro disease models. The potential applications of iPS cells, which can be reprogrammed from somatic cells and be induced to differentiate into patient-specific cells for therapy, have provoked enormous research interest within the scientific community. As iPS cells belong to pluripotent stem cells, they may be induced into almost all types of cells in adult body such as pancreatic cells [3], hepatocyte cells [4], neural crest cells [5], corneal epithelial cells [6], retinal pigmented epithelium cells [7], cardiomyocytes [8] and so on.

Usually, undifferentiated iPS cells can be well maintained by clonal growth. To homogeneously differentiate into other specific cells, iPS cell colonies should be dissociated into single cells in most situations [9]. However, the survival and growth of single iPS cells are more difficult than those of iPS cells in clonal growth. Therefore, it is necessary to explore effective procedure to allow the growth of single iPS cells with pluripotent characteristics. In this study, half-exchange medium (HM), whole-exchange medium (WM) and iPS cell-derived conditioned medium (Ips-CM) culture were used for iPS cells by single growth. The dissociation and propagation of single iPS cells also included Accutase enzymatic isolation, Y27632 protection and high-density single-cell seeding. We reported single-cell culture systems based on conditioned medium of HM and iPS-CM for facilitating short-term growth of single iPS cells and the effects of bFGF and Activin A on the growth of single iPS cells.

Materials and methods

Culture of human iPS cells

Human iPS cells were generously provided by South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. These iPS cells at a density of 1×10^6 cells/well were established from the umbilical cord matrix and amniotic membrane mesenchymal cells by transduction of retroviral factors, including Oct4, Sox2, c-Myc, and Klf4 as previously described [10]. Human iPS cells were cultured according to our reported protocol [11]. Briefly, iPS cells were cultured in mTeSR1 medium (StemCell Technologies Inc., Canada) on 1 % Matrigel-coated (BD Biosciences, USA) Petri dishes. Cells were cultured in a 37 °C incubator of 5 % CO_2 with 95 % humidity. Whole-exchange medium (WM) was refreshed daily. To minimize enzymatic damage to cells, iPS cells were passaged every 6 days with mild enzyme Accutase (Sigma, USA) treatment at 37 °C for 3–5 min. When colonies began to roll up the edge and dissociate from the bottom, Accutase solution was removed, and colonies were washed with free-Ca^{2+} PBS. Cells were collected with 1 ml mTeSR1 medium by gently pipetting about 7–10 times and replated onto fresh 1 % Matrigel-coated 6-well culture plates at a ratio of 1:3. 10 μM Y-27632 (Sigma, USA) was added to each well on the first day and then was removed on the second day. For getting single iPS cells, the collected iPS cells were pipetted about 30–50 times, and then colonies were removed by filtration through a 40 μm cell strainer. Then, the remaining single iPS cells (1×10^6 cells/well) were plated onto 1 % Matrigel-coated 6-well culture plates. 10 μM Y-27632 was added to each well on the first day and was removed on the second day. The experiment was divided into five groups as follows: iPS cells cultured by clonal growth in mTeSR1 medium were used as group A. iPS cells cultured by single growth in HM were as group B, in WM as group C, in iPS-CM as group D and in WM supplemented with 100 ng/ml bFGF (Sigma, USA) and 10 ng/ml Activin A (R&D, USA) as group E. iPS-CM was derived by collecting the medium from iPS cells cultured by clonal growth in mTeSR1 medium every 24 h at each sub-passage except for the first day. The collected medium was filtered (0.22 μm) to remove dead cells and stored at −80 °C for at least 2 weeks. The iPS-CM was mixed with mTeSR1 medium at a ratio of 1:2 (CM: mTeSR1).

Live/Dead assay of Calcein AM and EthD-III double staining

Calcein AM and EthD-III double staining (Molecular Probes, USA) was performed as previous report [12]. Briefly, standard working solution containing 4 μM EthD-III and 2 μM Calcein-AM was prepared. Then, cells were incubated with standard working solution for 40 min at room temperature, and then imaged under an inverted microscope (Olympus, Japan). While EthD-III (red) signal was taken as indicating dead cells, Calcein-AM (green) signal was taken as representing living cells. For quantification of cell viability, the acquired images were analyzed using Image J software.

Annexin v and propidium iodide assay

Single iPS cells (1×10^6 cells/well) were plated onto fresh 1 % Matrigel-coated 6-well cell culture cluster with conventional mTeSR1 medium. 10 μM Y-27632 was added to each well on the first day and then was removed on the second day. Then, single iPS cells were, respectively, cultured in HM, WM, iPS-CM and WM with 100 ng/ml bFGF and 10 ng/ml Activin A conditions. At day 3, cells were harvested and washed with cold PBS, and then were resuspended in 200 μl Annexin v binding buffer. After cells were stained with 2 μl of propidium iodide (PI) and 2 μl of FITC-labeled Annexin v, cells were immediately analyzed by flow cytometry analyzer (BD, USA).

CCK-8 assay

Cell counting kit-8 (CCK-8) was used to assay the viability of single iPS cells with different treatment. Briefly, single iPS cells were harvested and seeded in fresh 1 % Matrigel-coated 96-well plate (1×10^4 cells/well) with conventional mTeSR1 medium. 10 μM Y-27632 was added to each well on the first day and then was removed on the second day. Then, single iPS cells were, respectively, cultured in HM, WM, iPS-CM and WM with 100 ng/ml bFGF and 10 ng/ml Activin A conditions. The cell medium was refreshed daily. On the third day, each well was added 10 μl CCK-8 (Bo Seng, China) solutions and incubated at 37 °C for 3 h. At last, the absorbance at 450 nm was immediately measured with a microplate reader. Data analysis was conducted using GraphPad Prism 5 software.

ELISA

To explore the range of bFGF and Activin A presented in fresh mTeSR1 medium (control) and supernatant, we collected control and iPS culture supernatant in group A, group B, group C and group D on the day 3. The samples were treated with enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Chemicon CA, USA). Briefly, 50 μl assay diluent and 200 μl samples were added to precoated wells of 96-well plates. The plates were incubated at room temperature for 2 h, and washed five times with washing buffer. 100 μl peroxidase-conjugated IgG anti-bFGF or anti-Activin A of solution was added to each well at room temperature for 2 h. Then, the plates were washed five times with washing buffer. Then, 100 μl of substrate buffers was added to each well, and incubated in dark at room temperature for 30 min. The enzyme reaction was stopped by 50 μl stop solution. Optical densities were obtained for the quantification of bFGF and Activin A levels by a microplate reader at 550 nm with correction wavelength at 450 nm. Data were conducted by GraphPad Prism 5 software.

Gene expression analysis

Total RNA from single iPS cells and clonal iPS cells was isolated using Tissue RNA Miniprep Kit (Biomiga, USA), and the concentration of RNA was quantified by measuring OD at 260 nm. Total RNA was incubated at 65 °C for 5 min and then on the ice for 5 min. Soon total RNA (1 μg) was reverse transcribed in a 10 μl reaction mixture containing 0.5 μl RT Enzyme Mix, 0.5 μl Primer Mix, 2 μl 5 × RT Buffer, 1–6 μl nuclease-free water at 37 °C for 15 min and at 98 °C for 5 min. Then, cDNA was used for subsequent PCR with 10 μl 2 × Taq-Neo Master Mix, 1 μl forward, 1 μl reverse primers, 1 μg cDNA and 5–7 μl ddH_2O in a total volume of 20 μl, using primers shown in Table 1. The PCR mixture was first denatured at 94 °C for 2 min, and then amplified for 35 cycles (94 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s) using an authorized thermal cycler (Eppendorf, USA). After amplification, 6 μl of each PCR product and 2 μl of loading buffer were mixed and electrophoresed on a 2 % agarose gel containing 1 % nucleic acid fuel. Gels were photographed and scanned by AlphaImager EP (ProteinSimple, USA).

Meanwhile, the part of cDNA was used to perform qPCR to analyze Nanog, Oct4, Sox2, Klf4, c-Myc, Lin28, Nestin and Pax6 gene expression levels (CFX96 Real-Time PCR Detection System, Bio-Rad, USA). The reaction mixture consisted of 10 μl SYB Green Mix, 0.8 μl forward, 0.8 μl reverse primers, 1 μg diluted cDNA and 5–8 μl ddH_2O. The reaction process was 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s. The relative expression of genes was normalized against GAPDH. Melting curves were examined for the quality of PCR amplification of each sample, and quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [13].

Immunofluorescence staining

Immunofluorescence assay was performed in iPS cells after passage 3 times. Briefly, after fixation in 4 % paraformaldehyde for 15 min at room temperature, single iPS cells and clonal iPS cells were permeabilized with 0.1 % Triton-X 100 in Phosphate-Buffered Saline (PBS) for 15 min at room temperature, then washed 3 times with PBS and incubated with 3 % (w/v) BSA in PBS for 30 min at room temperature. Then, cells were incubated with primary antibodies [polyclonal rabbit anti-Nanog antibody (1:500, Cell Signaling, USA), polyclonal rabbit anti-Oct4

Table 1 List of primers

Primers	Sequences (5′–3′)	Product length	Gene bank number
GADPH-F	CCACTAGGCGCTCACTGTTC	180bp	NM_001289746.1
GADPH-R	TTGAGGTCAATGAAGGGGTCA		
Nanog-F	CAAGAACTCTCCAACATCCTGAA	126p	NM_024865.2
Naong-R	CCTGCGTCACACCATTGCTATTC		
Oct4-F	GAAGGATGTGGTCCGAGTGT	183bp	NM_001173531.2
Oct4-R	GTGAAGTGAGGGCTCCCATA		
Sox2-F	CAGGAGTTGTCAAGGCAGAGA	171bp	NM_003106.3
Sox2-R	CCGCCGCCGATGATTGTTA		
Klf4-F	GCCGCTCCATTACCAAGAG	166bp	NM_004235.4
Klf4-R	GTGTGCCTTGAGATGGGAAC		
c-Myc-F	CATCAGCACAACTACGCAGC	120bp	NM_002467.4
c-Myc-R	GCTGGTGCATTTTCGGTTGT		
Lin28-F	CCCATCACTGGGGTGTGTTT	162bp	NM_024674.4
Lin28-R	CAGTTTGCGTACCAATAAGTCTTT		
Nestin-F	AACAGCGACGGAGGTCTCTA	220bp	NM_006617.1
Nestin-R	TTCTCTTGTCCCGCAGACTT		
Pax6-F	TGTTGCGGAGTGATTAGTGGG	196BP	NM_000280.4
Pax6-R	TTGGTGATGGCTCAAGTGTGT		

antibody (1:100, Cell Signaling, USA), monoclonal rabbit anti-Sox2 antibody (1:100, Cell Signaling, USA), monoclonal mouse anti-SSEA-4 antibody (1:500, Cell Signaling, USA), monoclonal mouse anti-TRA-1-60 antibody (1:500, Cell Signaling, USA)] for 60 min, and then with the secondary antibodies [Goat anti-mouse IgM antibody (1:100; Bioss, USA), mouse anti-rabbit IgG antibody (1:100; Santa Cruz, USA), Goat anti-mouse IgG antibody (1:100; Bioss, USA)] for 60 min. Then, the cells were washed with PBS thrice for 5 min each time. Then, samples were incubated in the moist chamber for 15 min with DAPI for nuclear stain. Lastly, samples were rinsed again. Cells were examined by an inverted fluorescence microscope (OLYPAS, Japan).

Western blotting assay

Nanog, Klf4, Sox2, Oct4 and TRA-1-60 were detected by western blotting in single cells and clonal iPS cells. Single iPS cells and clonal iPS cells were washed with PBS and then lysed with radio immunoprecipitation assay (RIPA) buffer (Bocai Biotechnology, China) to obtain total proteins. The protein concentration of sample was measured with BCA^{TM} Protein Assay Kit (Vazyme Biotech, China). 50 μg of proteins was then separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5 % non-fat dry milk (Cell Signaling, USA) in Tris-Buffered Saline Tween-20 (TBST) buffer for 1 h. Then, the membranes were incubated overnight with primary antibodies (Abcam, USA) at 1:1000 dilutions in 5 % non-fat dry milk at 4 °C with continuous agitation. Then, the membranes were incubated with HRP-conjugated anti-rabbit or anti-rabbit IgG secondary antibody (Bioword, USA) at 1:3000 dilutions for 1 h at room temperature and washed 3 times with TBST. After adding enhanced chemiluminescence detection regents (Pierce, USA), the membrane was visualized by scanning immunostaining band (Tanon2500, China). The intensity of band was analyzed using Image J software.

Karyotype analysis

Human iPS cells were incubated with 50 μg/ml of colcemid solution (Invitrogen, USA) for 2.5 h at 37 °C in 5 % CO_2 incubator. Cells were washed with PBS and trypsinized at room temperature for 2 min. Then, the cells were fixed in methanol/glacial acetic acid (3:1) for three times and dropped onto slides for chromosome spreads. At last, the slides were baked at 55 °C for overnight, treated with trypsin for 30 s and stained with Giemsa solution.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical significance was evaluated by a two-tailed Student's t test, and statistical analyses were performed using SPSS 17.0. The $p < 0.05$ was considered statistically significant.

Results

The condition of half-exchange medium facilitated the short-term growth of single iPS cells

Human iPS colonies gradually became larger and increased with time when iPS cells cultured by clonal growth on 1 % Matrigel-coated Petri dishes in conventional mTeSR1 medium culture. Clonal iPS cells grew well and reached 90 % confluence on day 6 when mTeSR1 medium was refreshed daily. We also found that single iPS cells could adherently grow well on 1 % Matrigel-coated Petri dishes in HM culture. Undifferentiated single iPS cells in HM displayed homogenized cellular states on day 6 (Fig. S1). Western blotting manifested that both groups expressed Nanog, Klf4, Sox2, Oct4 and TRA-1-60, and there were no significant differences in protein expressions between two groups (Fig. S2). RT-PCR also displayed the positive gene expressions of Nanog, Oct4, Sox2, Klf4, c-Myc, Lin28, Nestin and negative gene expression of Pax6 in both single iPS cells and clonal iPS cells on day 6 (Fig. S3A). Meanwhile, qPCR analysis was used to quantitatively compare gene expression levels of Nanog, Oct4, Sox2, Klf4, c-Myc, Lin28, Nestin and Pax6 between clonal iPS cells and single iPS cells. The data demonstrated no significant difference between the two groups (Fig. S3B). Chromosome stability of iPS cells in both group A and group B was analyzed by karyotype analysis. Karyotype analysis revealed that both single iPS in HM and clonal iPS cells in mTeSR1 medium maintained a normal chromosome complement of 46XX (Fig. S4). These results implied that HM culture promoted the homogeneous growth of single iPS cells and maintained their characteristics of undifferentiated stem cells. HM culture did not affect protein and gene expressions of stemness markers in single iPS cells. HM culture also did not alter chromosomal stability of single iPS cells.

bFGF and Activin A promoted the growth of single iPS cells based on conditioned medium culture

Human iPS cells cultured by clonal and single growth

Human iPS colonies grew well when mTeSR1 medium was refreshed daily on day 6 (group A, iPS cells cultured by

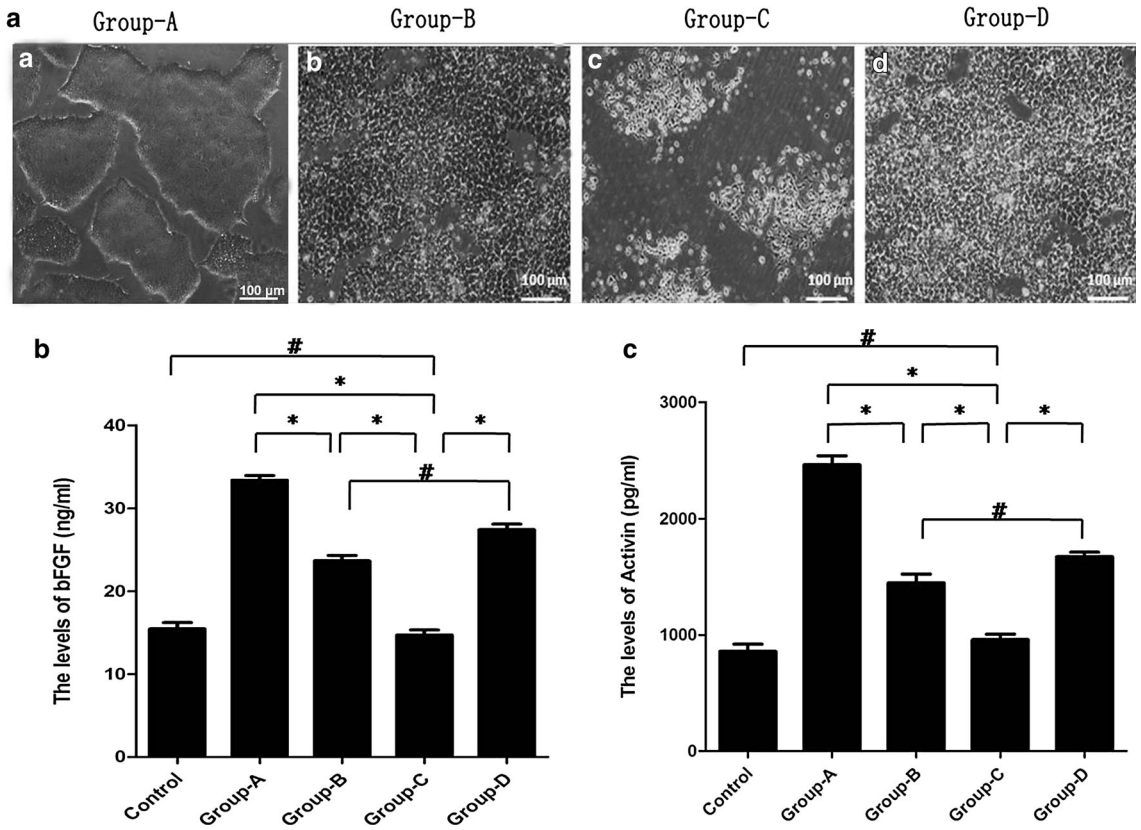

Fig. 1 The growth of iPS cells and levels of bFGF and Activin A in fresh mTeSR1 medium and the supernatant. (**A**a) Clonal iPS cells were cultured in conventional mTeSR1 medium culture. Single iPS cells in HM (Ab), in WM (Ac) and in iPS-CM (Ad). (B) The levels of bFGF in control and supernatant of group A, group B, group C and group D. (C) The levels of Activin A in control and supernatant of group A, group B, group C and group D. Data represented the mean ± SD and obtained from three independent experiments. Difference with $^{*}p < 0.05$ was considered statistically significant. Difference with $^{\#}p > 0.05$ was considered no statistically significant

clonal growth in mTeSR1 medium) (Fig. 1Aa). However, iPS cells cultured by single growth in group B (HM), group C (WM) and group D (iPS–CM) revealed different growing characteristics. A large number of single iPS cells in group B could adherently grow well on 1 % Matrigel-coated Petri dishes and got almost 90 % confluence on day 6 (Fig. 1Ab) when HM was refreshed daily. Single iPS cells in group C first could well attach to 1 % Matrigel-coated Petri dishes on day 1. But when WM was refreshed daily, adherent single iPS cells gradually detached and died. There were only a few adherent single iPS cells retained in dishes on day 6 in group C (Fig. 1Ac). Single iPS cells in group D grew well when iPS-CM was refreshed daily (Fig. 1Ad). These results indicated that the short-term growth of single iPS cells cultured in vitro could be promoted when half-exchange medium or iPS cell-derived conditioned medium was refreshed daily.

The levels of bFGF and Activin A in fresh mTeSR1 medium and supernatant of iPS cells detected by ELISA

To determine whether the levels bFGF and Activin A in supernatant of iPS cells are related to the growth status of iPS cells, we used ELISA to detect the levels of bFGF and Activin A presented in fresh mTeSR1 medium (control) and supernatant of iPS cells in group A, group B, group C and group D cultured on day 3. The levels of bFGF in group A, 33.42 ± 1.23 ng/ml, were the highest among all groups, in group B were 23.21 ± 1.59 ng/ml, in group C were 14.01 ± 1.08 ng/ml, in group D were 25.18 ± 1.43 ng/ml and in control were 16.34 ± 1.15 ng/ml. There was no significant difference for the levels of bFGF between group C and group D (Fig. 1b). Almost the same tendency was gained for Activin A (Fig. 1c). The levels of Activin A in control were (986.31 ± 23.14) pg/ml, while in group A, group B, group C and group D were (2448.89 ± 54.18) pg/ml, (1438.28 ± 23.56) pg/ml, (1001.31 ± 65.57) pg/ml and (1585.48 ± 55.76) pg/ml, respectively. These data showed that the better survival of the short-term growth of single iPS cells with HM and iPS-CM culture could partly relate to bFGF and Activin A. Well-grown iPS cells by clonal and single growth were associated with increased bFGF and Activin A in culture supernatant.

Cell viability and proliferation analysis by CKK-8 assay

CKK-8 assay was first used to explore the optimal concentration of exogenous bFGF and Activin A added into WM for propagation of single iPS cells cultured in vitro. We found that the proliferation of single iPS cells in WM reached optimal viability result when adding 100 ng/ml bFGF and 10 ng/ml Activin A (Fig. 2a).

Meanwhile, CKK-8 assay was also conducted to investigate the proliferation of clonal iPS cells cultured in mTeSR1 medium (group A), and single iPS cells cultured in HM (group B), WM (group C), iPS-CM (group D) or WM supplemented with bFGF and Activin A (group E) on day 3. Group C manifested the lowest viability result. The proliferative cells in group A were more 48.53 % than those in group C ($*p < 0.05$). And group B was more 43.22 % than group C ($*p < 0.05$). Group D was more 44.14 % than group C ($*p < 0.05$). Group E was more 34.41 % than group C ($*p < 0.05$) (Fig. 2b). These results indicated that HM and iPS-CM culture significantly enhanced proliferative cells in short-term growth of single iPS cells, and adding exogenous bFGF and Activin A could effectively promote the growth of single iPS cells in WM culture.

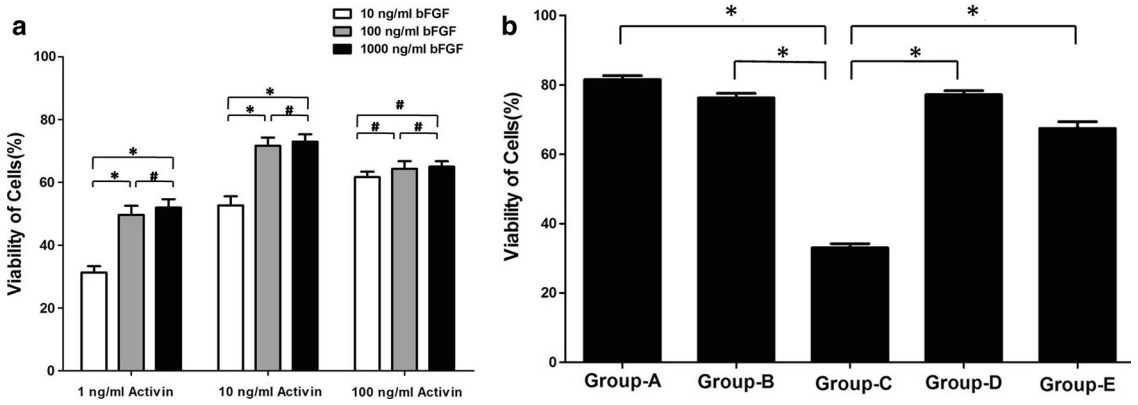

Fig. 2 The viability of iPS cells by CKK-8 assays. **a** 100 ng/ml bFGF and 10 ng/ml Activin A displayed the optimal concentrations for the growth of single iPS cells in WM. **b** The proliferation of iPS cells cultured in group A, group B, group C, group D and group E. Data represented the mean ± SD and obtained from three independent experiments. Difference with $*p < 0.05$ was considered statistically significant. Difference with $^{\#}p > 0.05$ was considered no statistically significant

Cell viability analysis by Annexin v and propidium iodide assay

Annexin v and PI were analyzed by flow cytometry to detect cell viability in cultured iPS cells. To the viable cells, cells typically possess an intact cell membrane that is not stained with PI. However, externalization of phosphatidylserine (membrane phospholipids) can be detected by annexin V. As dead cells, cells have a destroyed membrane and can be stained with PI. The percentage of dead cells was calculated from Q1 and percentage of viable cells was calculated from Q3 areas by flow cytometric analysis. Clonal iPS cells in group A showed (1.51 ± 0.05) % dead cells and (97.5 ± 0.82) % viable cells (Fig. 3a). While single iPS cells in group B had (3.2 ± 0.45) % dead cells and (95.8 ± 1.22) % viable cells (Fig. 3b), (32.4 ± 1.12) % dead cells and (68.3 ± 1.45) % viable cells in group C (Fig. 3c), (4.2 ± 0.43) % dead cells and (89.8 ± 1.23) % viable cells in group D (Fig. 3d), and (10.2 ± 0.43) % dead cells and (88.7 ± 1.23) % viable cells in group E (Fig. 3e). There were significant differences between group A and group C, between group B and group C, between group D and group C, and between group E and group C in percentages of viable cells and dead cells ($^{**}p < 0.01$) (Fig. 3f). The results indicated that HM and iPS-CM culture significantly promoted the survival of short-term growth of single iPS cells, and adding exogenous bFGF and Activin A could effectively recover the survival of the single iPS cells in WM culture.

Live/Dead assay of Calcein AM and EthD-III double staining

To compare the effect of treatment with mTeSR1 medium, HM, WM, iPS–CM, or WM supplemented with 100 ng/ml bFGF and 10 ng/ml Activin A on the viability of iPS cells, cell viability analysis by Calcein AM and EthD-III double staining assays was carried out. The assay demonstrated that the vast majority of clonal iPS cells in group A and single iPS cells in group B, group D and group E were viable cells, which manifested an green fluorescence in the live cytoplasm from Calcein AM stain. However, single iPS cells in group C revealed less viable cells and more dead cells than those in group A, group B, group D and group E (Fig. 4a), showing red fluorescence in dead cell nucleus from EthD-III stain. The percentages of viable cells in group A, group B, group C, group D and group E were (98.42 ± 1.33) %, (94.45 ± 1.54) %, (48.87 ± 1.36) %, (95.88 ± 1.36) % and (90.13 ± 1.23) %, respectively. There were significant differences between group A and group C, between group B and group C, between group D and group C and between

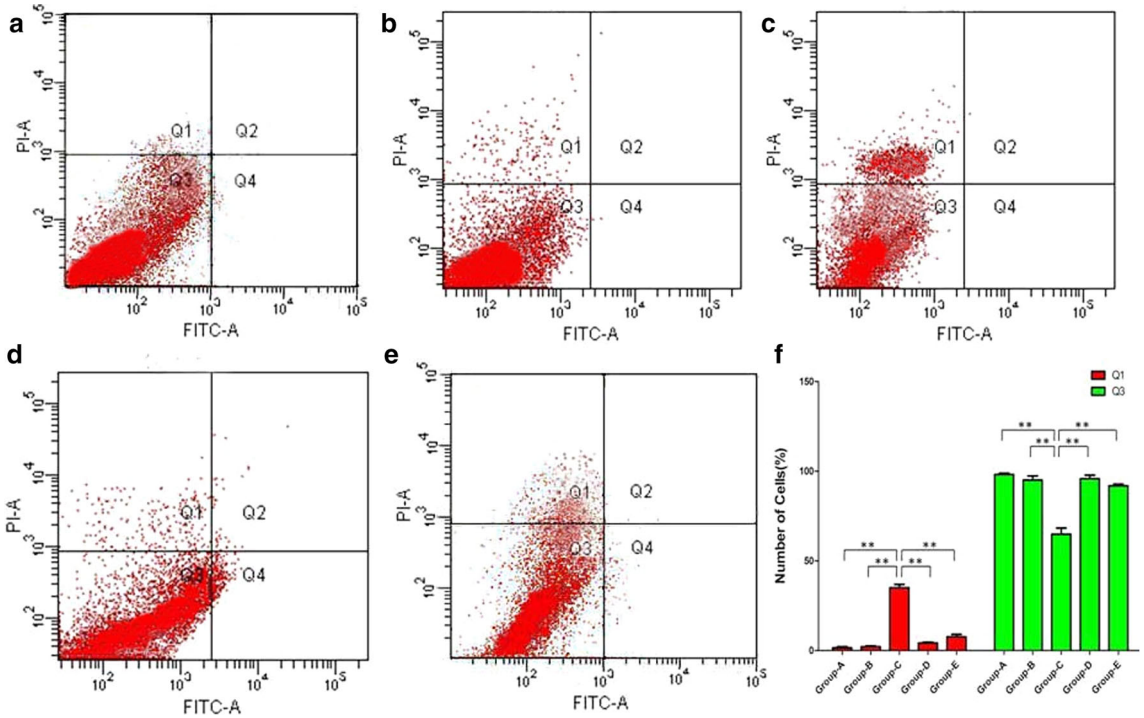

Fig. 3 The viability of iPS cells by Annexin v and propidium iodide assays. Flow cytometric analysis of group A (**a**), group B (**b**), group C (**c**), group D (**d**) and group E (**e**). **f** The quantification percentage of living and dead cells of five groups. Data represented the mean ± SD and obtained from three independent experiments. Difference with $^{**}p < 0.01$ was considered statistically significant. Difference with $^{\#}p > 0.05$ was considered no statistically significant

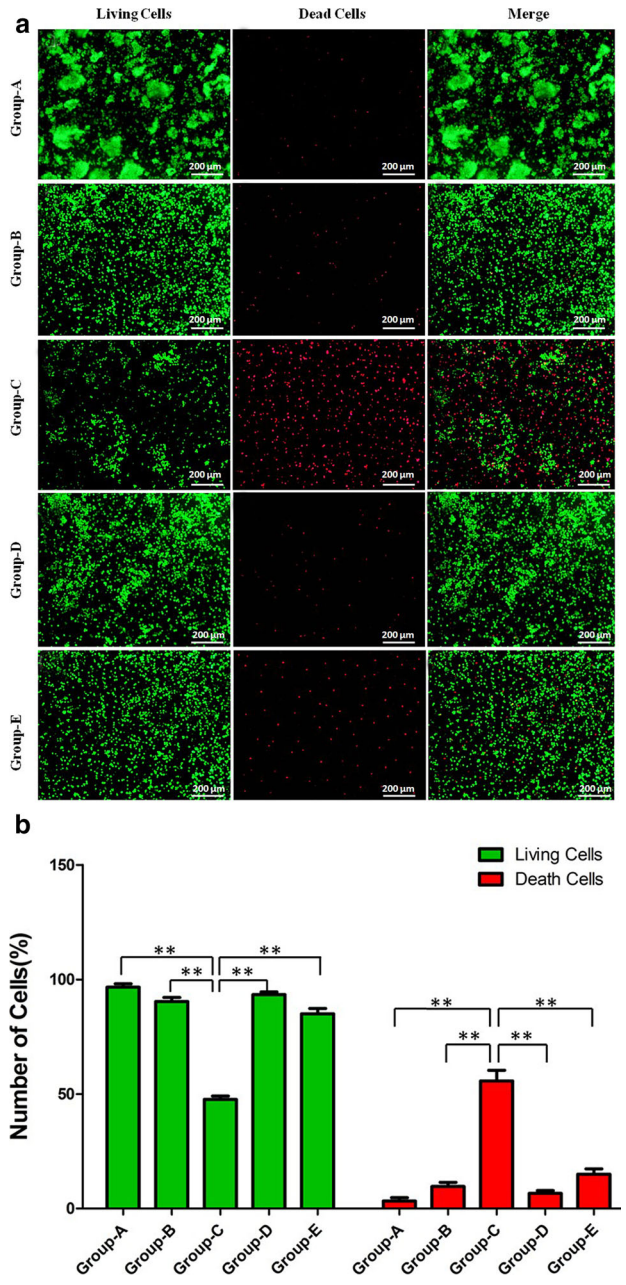

Fig. 4 The viability of iPS cells by Calcein AM and EthD-III double staining. **a** The iPS cells were double stained by Calcein AM and EthD-III in group A, group B, group C, group D and group E. **b** The quantification of cell viability using Image J software. Data represented the mean ± SD and obtained from three independent experiments. Difference with $^{**}p < 0.01$ was considered statistically significant. *Scale bars* 200 μm

group E and group C in percentages of viable cells ($^{**}p < 0.01$). The percentages of dead cells in group A, group B, group C, group D and group E were (1.58 ± 0.42) %, (5.55 ± 0.56) %, (52.13 ± 1.08) %, (4.12 ± 0.57) % and (9.87 ± 0.77) %, respectively (Fig. 4b). There were significant differences between group A and group C, between group B and group C, between group D and group C and between group E and group C in percentages of dead cells ($^{**}p < 0.01$). These results showed that single iPS cells could improve survival and decrease death in HM, iPS-CM or WM with 100 ng/ml bFGF and 10 ng/ml Activin A.

Discussion

The propagation of pluripotent stem cells requires intercellular signals and cell–cell contact. So, ES and iPS cells conventionally grow and propagate as clumps or colony. However, standard colony culture can exhibit slow expansion, often giving rise to heterogeneous cells and frequent chromosomal abnormalities [14, 15]. Therefore, a non-colony type single iPS or ES culture is preferable. But cell colonies dissociated to single cells were plated at a low efficiency. Single-cell dissociation is usually avoided during their passage [16, 17]. Although there have been significant advancements in the technical aspects used to culture iPS cells in recent years, cultured iPS cells are still limited by the low survival that commonly follows enzymatic dissociation and single dissociated growth, which is an obstacle for iPS cells to manipulate and induct in vitro. The dissociation of clonal ES and iPS stem cells to single cells can provide better condition for the purpose of directed differentiation [9]. As an example, mouse ES cell-derived cardiomyocytes alone did not form functional cardiac biosynthetic tissue constructs and required additional input of cardiac fibroblasts, whereas a single-cell source of mouse ES and iPS-derived cardiac progenitors was sufficient to allow the formation of constructs with advanced electromechanical properties [8, 18].

Human iPS cell line derived from umbilical cord mesenchymal cells (UMC) was used in this work. Our previous report displayed that this iPS cell line revealed a normal chromosome complement of 46XX and the characteristics of undifferentiated stem cells. Such iPS cells had the ability to form EBs and further differentiate. The differentiated cells expressed SOX1, PAX6, GATA4, PPAR, FOXA2, SOX17 and NESTIN, which are crucial markers of three embryonic layers [11]. To avoid the low survival of undifferentiated single-cell growth of our iPS cells, in this research, we utilized cell detachment solution of Accutase and Rho-associated protein kinase (ROCK) inhibitor Y27632. In contrast to trypsin dissociation, the enzyme cocktail Accutase usually did not result in massive cell death. Single-cell dissociation of cell colonies with Accutase could trigger continuous cultures of ES and iPS cells that were viable and highly pluripotent [19, 20]. Y27632 was successfully used to support single-cell dissociation and maintain survival of a multitude of kinds of cells which

included ES and iPS stem cells. Accutase-passage human ES cells can be grown at high density as monolayer, with viable single ES cells to be further cultured as pluripotent ES cells [21–25]. It was reported that the addition of Y-27632 to the extracellular matrix (ECM) also increases the plating efficiency and supports more undifferentiated growth of ES cells and iPS cells upon passage [26]. However, in this work, we found that most of the single iPS cells after WM culture gradually detached and died, even though the combination of high-density single-cell seeding (1×10^6 cells/well), Accutase dissociation, Y27632 protection and Matrigel ECM was used.

It was reported that using neurobasal medium which was refreshed by changing half of the volume after 3 days followed by a complete medium change every week was beneficial for culturing primary fetal rat cortical neurons. The cells displayed progressively robust neurite extension in vitro [27]. Accordingly, we also tried to use mTeSR1 medium that was refreshed by changing half of the volume for culturing single iPS cells. Interestingly, iPS cells cultured by single growth in HM could well grow and maintain their characteristics of undifferentiated stem cells when the combination of Accutase dissociation and Y27632 protection was used. HM culture did not affect undifferentiated protein expressions including Nanog, Klf4, Sox2, Oct4, SSEA-4 and TRA-1-60, undifferentiated gene expressions including Nanog, Oct4, Sox2, Klf4, c-Myc, Lin28, Nestin and chromosomal stability in single iPS cells.

We further tried to use iPS-CM for culturing single iPS cells. It was reported that iPS cell-derived CM could reduce apoptosis, oxidative stress and fibrosis, as well as improve cardiac function in streptozotocin-induced diabetic rats [28]. High-tidal-volume-induced ventilator induced lung injury (VILI) also could be suppressed by iPS-CM. The mechanisms involved inhibition of PI3K/Akt pathway and an interferon gamma-induced protein 10 (IP-10)-dependent paracrine regulation [29]. Recently, a culture method based on single-cell passage and non-colony type monolayer (NCM) culture was developed. This method comprises seeding dissociated cells (single cells) at high density in the presence of mouse embryonic fibroblasts (MEFs)-conditioned medium (MEF-CM) and ROCK inhibitors to facilitate the initial 24 h single-cell plating efficiency and to prevent the formation of colonies. The advantages of NCM culture included controllable growth rates, generation of homogeneous human iPS cells, improved cell plating efficiency, robust cell production, and rapid (2- to 4-day) cell recovery from cryopreservation compared with frozen cells from colony type culture, which usually take 1–3 weeks to recover. The iPS cells grown as NCMs were extremely efficient at forming teratomas and were reversible to colony type culture when the cells were plated as clumps [30–32].

We also found that the survival of single iPS cells could be promoted by iPS-CM. At the same time, HM culture enjoyed similar augment effects as iPS-CM on single iPS cells cultured in vitro. We demonstrated that adherent single iPS cells could grow exceedingly well and reach almost 90 % confluence on day 6 by both HM and iPS-CM culture. The viable cells of single iPS cells in HM and iPS-CM were more than that in WM by Live/Dead assay. CCK-8 assays revealed that the viability of single iPS cells in HM and iPS-CM was significantly higher than that in WM. Annexin v and PI assay also had similar outcome. It was known that pluripotent stem cells depend on cell–cell interactions as well as para/autocrine signals [33]. In our research, quite a few soluble factors secreted from iPS cells could exist in both HM and iPS-CM culture. The medium refreshed by exchanging half of the volume everyday could supply sufficient new nutrition for the growth of single iPS cells. We supposed that HM culture might be considered as self-iPS cell-derived CM (self-CM). The retentive half medium contained something similar to that of iPS-CM, which could enhance the proliferative and survival capacity of human single iPS cells. We also found the optimal growth for single iPS cells in self-CM at a ratio of 1:1 (fresh CM: mTeSR1) and in iPS-CM at 1:2 (thawed CM: mTeSR1) after a series of concentration of conditioned medium was compared (data not shown). Such cultures based on conditioned medium of HM and iPS-CM culture guaranteed the robust growth of single iPS cells when the combination of high-density single-cell seeding, Accutase and ROCK inhibitor was used. We also uncovered that the enhanced growth of single iPS cells based on the conditioned medium culture of HM and iPS-CM was associated with increased bFGF and Activin A.

As is known to all, there is variety of soluble paracrine factors released from pluripotent stem cells. Zhang and coworkers reported that rat iPS-CM contained more trophic factors than rat cardiomyoblasts (H9C2 cells)-derived CM. Paracrine factors released from iPS cells prevent stress-induced senescence of H9C2 cells [34]. bFGF is a multifunctional cytokine. Activin A is a member of the transforming growth factor-β superfamily. bFGF and Activin A have been identified in a wide variety of tissues and cells as an autocrine or paracrine regulator of diverse biological functions. Both bFGF and Activin A are mitogens and their signaling pathways are involved in numerous biological systems, and are especially critical in the embryo [35–37] Activin A is a key regulator in the maintenance of self-renewal and pluripotent status as well as the support of feeder and serum-free growth in iPS cells and ES cells. Activin A could induce the expression of other regulators including Oct4, Nanog and bFGF [38, 39]. bFGF was also a key factor in maintenance of undifferentiated growth in pluripotent stem cells. Endogenous bFGF secreted by ES

cells maintained pluripotency gene expression. While the main effect of exogenous bFGF was promoting cell adhesion and survival [40, 41], Activin A and bFGF involved in maintaining pluripotency and self-renewal of ES cells. bFGF is an essential cofactor for Activin A/Nodal to maintain pluripotency in human ES cells [40]. Both of them also cooperated to maintain pluripotency of iPS cells [42]. In this research, iPS cells poorly grown by single growth in WM were associated with decreased bFGF and Activin A in culture supernatant. On the contrary, well-grown single iPS cells in HM and iPS-CM culture were related to increased bFGF and Activin A, which might be secreted from cultured clonal iPS cells into the supernatant. The existence of exogenous bFGF and Activin A promoted the growth of single iPS cells in WM, with similar effect to that of single iPS cells in HM.

In general condition, WM can supply sufficient nutrition for standard colony growth of iPS cells. But WM alone is not enough for the survival of single growth of iPS cells. In this work, culture system based on conditioned medium culture of HM combined the advantages of Accutase, Y27632 and iPS-CM, which could not only generate homogeneous single iPS cells with pluripotency, but also effectively improve the survival of short-term growth of single iPS cells. Such improved growth in single iPS cells was partly related to bFGF and Activin A. In HM culture system, single iPS cells cultured in the microenvironment of more bFGF and Activin A displayed superior viability, and were able to maintain undifferentiated pluripotent state. Lately, Tano and co-workers reported that Essential 8 medium better facilitated the growth of human iPS cells dissociated into single cells on laminin-521 than in mTeSR1 medium. Essential 8 medium allowed robust proliferation of iPS cells on laminin-521 even at low cell density and without a ROCK inhibitor [43]. Therefore, we will next examine HM culture using Essential 8 medium and laminin-521 for the growth of single iPS cells at low cell density. Moreover, we will further explore the mechanism of the effects of bFGF and Activin A on the survival of single iPS cells and their different roles. This study has confirmed that iPS cells cultured by single growth in HM could maintain their pluripotency with undifferentiated protein and gene expressions. Additionally, well-grown iPS cells by single growth are associated with increased bFGF and Activin A in culture supernatant. So we indirectly reasoned that the pluripotency of single iPS cells was partly related to bFGF and Activin A. Although there were some reports demonstrated that bFGF and Activin A were able to maintain undifferentiated growth in pluripotent stem cells [38–41], direct proof of undifferentiated pluripotent characteristics relating to the single iPS cells cultured with bFGF and Activin A will be our next exploration.

Recently, several studies have demonstrated that primed state pluripotent stem cells can be converted to naive state cells by genetic manipulation or even by altering their culture conditions. iPSCs with high quality pluripotent in a naïve-like state could be developed in the human [44–48]. Nishishita and co-workers demonstrated that possible residual exogenous genome (such as Sendai virus genome) in naïve iPSCs could be checked and removed at a single-cell level (not at a cell clump level) by utilizing a single-cell cloning technique. Naive hiPSCs displayed up to 35 % single-cell cloning efficiency after trypsinization and sorting, whereas primed (non-naïve) hiPSCs largely did not survive single-cell cloning. In the presence of ROCK inhibitor, naive hiPSCs had single-cell cloning efficiency of up to 88 %, whereas that of primed cells increased only up to 22 % [49]. Even though single iPSCs derived from the cells established with our method (non-naïve iPSCs) are more useful in the present condition, we believe that iPSCs from naïve state will also be more rewarded by single-cell culture systems.

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