Long-term single-cell passaging of human iPSCs fully supports pluripotency and high-efficient trilineage differentiation capacity

Estela Cruvinel[1,¶], Isabella Ogusuku[1,¶], Rosanna Cerioni[1], Jéssica Gonçalves[1], Maria Elisa Góes[2], Anderson Carlos Silva[3], Alexandre Pereira[3], Rafael Dariolli,[1,3] Marcos Valadares[1], Diogo Biagi[1, *]

[1]PluriCell Biotech, São Paulo, 05508-000, Brazil

[2]Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brazil

[3]Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), Medical School, São Paulo, 05403-900, Brazil.

[¶]Co-first author

*Corresponding author

E-mail: diogo.biagi@pluricellbiotech.com

ABSTRACT

In spite of the great advance in human induced pluripotent stem cells knowledge, several non-consensual protocols to cultivate this peculiar cell type can be found in the literature. Laboratories and companies worldwide have been trying to provide equivalent results regarding long-term cultivation of hiPSCs and their derivatives, so it is mandatory the establishment of reproducible pipelines for cell generation, cultivation, differentiation, etc. Here, we validated a straightforward single-cell passaging cultivation method that enabled high-quality maintenance of human induced pluripotent stem cells (hiPSCs) over 50 passages without the appearance of karyotypic abnormalities or loss of pluripotency. Further, the hiPSC clones were able to generate derivatives from the three germ layers at high passages by embryoid body formation and high-efficient direct differentiation into keratinocytes, cardiomyocytes and definitive endoderm (DE). Thus, our findings support the routine of hiPSCs single-cell passaging as a reliable procedure even after long-term cultivation, providing healthy PSCs to be used in high-standard cellular modeling research and therapeutic approaches.

INTRODUCTION

Induced Pluripotent Stem Cells (iPSC) unique features – which include self-renew, indefinite expansion and the potential to generate specialized cells – makes them motive for huge excitement among scientific and medical communities as an alternative to embryonic stem cells. Since their discovery[1], iPSCs have been widely used for research on tissue-specific development, disease modeling, cell-based therapies and drugs discovery. In order to meet both the especial needs of the cells and the rapid demand regarding iPSCs, culture methods have evolved to optimize growth conditions while maintaining pluripotency. However, many laboratories are still following out-of-date procedures to cultivate iPSCs that remain from the discovery of Embryonic Stem Cells (ESC) such as colony passaging,[2] which is an approach that usually follows uneven confluency as well as unpredictable growth rates.[2,3]

To overcome this issue, several groups committed to the search of conditions that could support single-cell passaging[4–8] but, despite the rapid progress, there is still a lack of straightforward standard protocols for iPSC cultivation that discuss the effects of combining different novelty biotechnologies as well as the effects when culturing PSC for a long time. Indeed, several recent reports present lacks of information about hiPSC genetic integrity[9], in special, after long-term cultivation in vitro,[10] even though many studies suggest abnormalities to be progressively favored by suboptimal culture conditions such as single-cell passaging[11] or high-cell density cultures.[12]

Once long-term maintenance in culture seems likely to promote self-renewal[10,13] and limit differentiation through progressive selection of genetic variants,[14] the assessment and validation of PSC cultivation protocols is mandatory to support reproducibility in differentiations. To bypass the inconsistencies of long-term cultivation, many laboratories maintain multiple PSC cell lineages in culture, however it is important to recognize that this approach not only implies high cost and labor demand, but also it does not guarantee success for differentiation protocols as this and other variabilities in PSC cultivation methodologies impairs reproducibility, homogeneity and scalability in differentiations.[15]

In the present work we used hiPSC derived from two distinct primary sources to develop a controlled long-term culture methodology that supports single-cell passaging while maintaining pluripotency markers, genomic integrity and high ability to generate derivatives of all three germ layers by directed differentiation, resulting in high-purity specialized cells.

MATERIALS AND METHODS

Ethics Statement

This investigation conforms to the principles outlined in the Declaration of Helsinki and the study protocol was approved by the Ethics Committee for Medical Research on Human Beings of the Institute of Biomedical Sciences from the University of São Paulo (#2.009.562). Signed informed consent was obtained from all participants.

iPSC Reprograming and Maintenance

Human erythroblasts and skin fibroblast were used to evaluate our culture method. hiPSC lines were derived from erythroblasts by transfection with plasmids pEB-C5 and pEB-Tg using the Human CD34+ nucleofector kit and the Nucleofector II device, following manufacturer's instructions. On the other hand, hiPSC derivation from fibroblast was performed following Epi5™ Episomal iPSC Reprogramming Kit protocol from Invitrogen (Thermo-Fisher) with some modifications.

In brief, to obtain hiPSCs blood and skin samples were collected from four healthy donor ranging in age from 30 to 40 years old: two males (ACP, PC3) and two females (PC2, PC4). From these samples four cell lines were generated being one derived from erythroblasts (ACP) and three derived from skin fibroblasts (PC2, PC3, PC4). After clonal picking and expansion, one clone of ACP (ACP5) and three clones of the lines PC2 (PC2.2, PC2.3, PC2.4), PC3 (PC3.1, PC3.2, PC3.3) and PC4 (PC4.3, PC4.5, PC4.6) were saved. For practical reasons (due to their growth rates and the fact they were obtained by using different sets of plasmids for reprogramming), only ACP5 and PC4 clones were used for the next steps of long-term cultivation, characterization and differentiation.

Doubling time calculation

For doubling time (DT) calculation, hiPSC were counted first when seeding the cells and then when detaching them for passage. At each time point cells were counted twice with Tripan blue using a Neubauer's chamber and mean was used to determine total number of cells. DT calculation was determined by $DT= t. Log10(2)/ [Log10(x) – Log10(x0)]$, whereby t is expressed in hours. Results were then plotted as mean ± standard deviation (SD) for each given day. The significance of differences among passages was analyzed for each clone trough ANOVA. $P <0.05$ was considered statistically significant.

Confluence monitoring – CellCountAnalyser software

We used an ImageJ associated software previously published by Busschots et. al.[16] and upgraded for our team (and so renamed CellCountAnalyser; details can be found in the supplementary material) to monitor and so to work only with cells in logarithmic phase of growing. To do so, 24-96 hours after cell seeding, five photos were recorded from predefined (marked) areas that covered center and borders of cell dishes using EVOS FL Optical microscope (Thermo Fisher, USA). Then using our one-click Python-based platform, cell confluence was individually measured by photo. An excel file containing individual percentage of confluence per photo and mean SD calculations was generated. hiPSC dishes were split every time confluence reached 70-85%.

EB formation

EB were generated as described by Lian and Chen[17] with minor modifications. In brief, hiPSC were resuspended in E8 medium supplemented with PVA and cultivated in non-adherent plates for 24 hours. Next, the medium was changed to Essential 6TM medium carefully to not remove EB in suspension. After 13 days, RNA was extracted for RT-PCR analysis.

Karyotype

Cells were incubated with 10 ug/mL Colcemid (Sigma-Aldrich) for 1 hour and, after washing with DPBS, cells were incubated with 0.075 M KCl for 20 minutes at 37°C. Fixation was performed by using methanol/glacial acetic acid (3:1) solution. Conventional chromosome analysis was performed on iPSC cultures, using GTG banding at a 400-band resolution according to standard protocols with minor modifications.[18] A total of 10 metaphase cells were analyzed. Cell images were captured using the CytoVysion system (Applied Imaging Corporation, USA).

Integration PCR, RT-PCR and RT-qPCR

To check for any episomal integration into host DNA we performed an integration PCR analysis using three set of primers (S1 Table) targeting specific sites of the plasmids DNA as described by

Chou and colleagues.[19] To evaluate gene expression, RT-PCR and quantitative RT-PCR (qPCR) were performed using RNA extracted from all clones at specific passages. Detailed information about the primers can be found in supplementary material (S2 Table). Further, human embryonic stem cells (hESCs) BR1[20] were used as positive control of pluripotency and human skin fibroblasts (the somatic cells of origin for PC4 clones) were used as negative control of pluripotency.

Directed differentiation into keratinocytes

iPSC's were plated with mitomicyn C-inactived 3T3 cells (donation from Monica Mathor's laboratory) in 20% confluency. After 2 days, defined-KSFM medium (Thermo Fisher, USA) supplemented with 10ng/mL of BMP4 (R&D Systems, USA) and 1uM retinoic acid (Sigma Aldrich, USA) as described by Itoh and colleagues. [21] At day 4 cells were cultivated with fresh defined-KSFM medium (Thermo Fisher, USA) for at least another 24 days. Cells were passaged in different plates depending on the experiment. Cells were characterize by immunostaining and flow cytometry. To induce superficial-layer epithelial cells generation by K14 to K10 switch, hiPSC-KCs were subjected to a 1uM CaCl treatment for 5-7 days as previous described by Bikle et. al. 2012. [22]

Directed differentiation into cardiomyocytes

iPSC's were differentiated using a monolayer directed differentiation method modified from previous reports [16,19]. iPSC's were grown in feeder-free conditions until they reached 60%–70% confluence. Cells were singularized, counted and plated (2,5 x 10^5 cells/cm²) with E8 with 5µM of Ri (Cayman Chemical, USA). E8 medium was changed daily until cells reached 100% confluence. This day was considered day 0 and medium was changed to RPMI supplemented with 1X B27 supplement (Thermo Fisher, USA) without insulin (RB-) 4 µM CHIR99021 (Merck Millipore Sigma, USA). 24 hours later, medium was changed to RB- supplemented with 10ng/mL BMP4 (R&D Systems, USA). In day 2, medium was changed to fresh RB- supplemented with

2,5uM KY2111 and XAV939 (both from Cayman Chemical, USA). At day 4 and every two days, medium was changed to fresh RPMI supplemented with 213 µg/ml Ascorbic Acid (Sigma Aldrich, USA), 500 µg/ml DPBS 35% BSA and 2ug/ml Plasmocin (InvivoGen, USA). Cells were cultivated for 30 days when passaged as single-cells to specific experiments.

Directed differentiation into definitive endoderm

Endoderm differentiation was performed following previous reports [23,24] with minor modifications. Cells were plated at 1,5x105 cells/cm² and cultivated with mTeSR1 (Stem Cell Technologies, CA) for 3 days with daily media changes. At day 0, cells were treated with RPMI supplemented with 100 ng/mL Activin A (Peprotech Inc., BR) and 4 uM CHIR99021 (Merck Millipore Sigma, USA) for 5 days with daily medium change. At day 5, RNA was isolated, or cells were passaged as single-cells for flow cytometry.

Flow cytometry and Immunofluorescence

Protein expression was analyzed by Flow Cytometry (FC) and Immunofluorescence (IF). Detailed information about the antibodies can be found in supplementary material (S3 Table). For IF, all images were generated in EVOS FL (Thermo Fisher, USA). As for FC, data was acquired using Canto BD equipment and analyzed by FlowJo Software considering 1-2% of false positive events.

Statistical Analysis

All descriptive data is presented as the mean ± standard deviation (SD), and the significance of differences was analyzed using one-way ANOVA combined with Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

Detailed protocols are described in the Supplementary material.

RESULTS

Single-cell passaging do not affect growth rate of hiPSCs providing predictable conditions for long-term cultivation

To check for any spontaneous integrations of the expression vectors used for reprogramming set of primers specific to each vector [19,25] were used for a PCR analysis (each clone at passages 5 and 11). Only positive control samples (vector DNA) displayed expression of the expected fragments after PCR indicating no DNA integration into hiPSC clones (S2 Fig.).

hiPSCs were cultivated using E8 and E8flex mediums on GELTREX-coated plates, where cells grew in monolayer maintaining an undifferentiated ESC-like morphology (Fig. 1 and Fig. 2). hiPSCs could be passaged as single-cells by enzymatic dissociation and posterior seeding with ROCK inhibitor (Y-27632), which provided a quite controllable weekly routine based on predictable growth rates (Fig. 1A). The confluence of seeded cells was monitored by CellCountAnalyser (Fig. 1C, S8 Fig.). hiPSCs were spread every time they reached 70-85% confluency (logarithmic phase of cellular growth). Thus, cell seeding densities were optimized by tracking the growth rates for each one of the clones and, to attend the proposed workflow, ACP5 was seeded at densities of 40,000 (Thursdays) or 20,000 (Fridays) and PC4 clones were seeded at of 43,000 (Thursdays) or 22,000 (Fridays) cells per cm2 for passages every 3 or 4 days (Monday or Thursday passages respectively; Fig. 1A-B). Moreover, hiPSCs population doubling time (PDT) was calculated for each clone, confirming that single-cell passaging had no significative impact in growth rates over 50 passages. (Fig. 1D) Furthermore, mean PDT for ACP5 (28.3 □ 8.5 h) was statistically different ($p<0.05$) compared to PC4.3 (33.0 □ 11.4 h), PC4.5 (33.9 □ 12.6 h) or PC4.6 (32.8 □ 12.5 h). (Fig. 1E)

Figure 1. Single-cell passaging did not impair hiPSC morphology or colony formation capacity. (A) Scheme of the iPSCs maintenance workflow. **(B)** Cells morphology throughout the days before media changing. Right line scale bar is 1000 uM and left line scale bar is 100 uM. **(C)** Cell confluence was monitored by CellCounterAnalyser. I) Representative scheme of the 5 recorded photos per 6 wells plates, II) quantification of X different passages recorded three 3 days after seeding, III) representative images of the photos recorded for quantification. **(D)** hiPSCs doubling time population of the clones ACP5 (n=8), PC4.3 (n=5), PC4.5 (n=4) and PC4.6 (n=5)

from passage 20 to 60**. (E)** All PDT values obtained for clones ACP5 (n=165), PC4.3 (n=83), PC4.5 (n=100), PC4.6 (n=112). Data is presented as mean ± SD. *P< 0.05 was considered statistically significant for ANOVA.

Long-term single-cell passaging did not affect hiPSCs pluripotency

After successive passages under single-cell passaging, cells were characterized for their pluripotency and potential of differentiation to keep track of the clones features at different passages. Immunostaining showed that all clones expressed expected levels of the pluripotency markers OCT4, NANOG, and TRA-1-60. (Fig. 2A, S3 Fig.). NANOG, OCT4 and SOX2 was also assessed by flow cytometry using clones at passages 10, 30 and 50. (Fig. 2B, S4 Fig.). Results were then analyzed for each marker in two distinctive tests, which showed no statistically significant difference between clones (over 95% NANOG+ cells and OCT4+ cells, and over 85% SOX2+ cells, Fig. 2C) neither between passages (over 70% NANOG+ cells, and over 90% OCT4+ and SOX2+ cells, Fig. 2D) for any of the markers. This result suggests that not only clones are likely to display a very similar profile but also that long-term cultivation had not significant impact on cells pluripotency.

To further characterize the hiPSC, we also measured gene expression of NANOG, OCT4, SOX2, LIN28 and DNMT3B by RT-qPCR (Fig. 2E) and we found no statistical difference of expression when comparing the PSC, whereas fibroblasts were confirmed to be significantly lower from all others for each tested gene (*P < 0,05).

Figure 2. Immunocytochemistry, flow cytometry and RT-qPCR confirmed both protein and genetic expression of pluripotency markers in iPSC clones PC4.3, PC4.5, PC4.6 and ACP5. (A) Immunostaining of ACP5 iPSC clone at passage 20 showing expression of pluripotency markers OCT4, NANOG (both nuclear) and TRA-1-60 (membrane). Experiments were performed for all iPSC clones **(B-D)** Cytometry data of iPSC clones at passages 10, 30 and 50

using NANOG, OCT4 and SOX2 markers. Light and dark grey indicate negative control (fibroblasts) and stained cells (iPSC), respectively. Statistical analysis revealed percentage positivity was not statistically different for any marker when comparing clones in C (n = 3) or passages (n = 4) in D; **(E)** Expression of pluripotency markers NANOG, OCT4, SOX2, DNMT3B and LIN28 in hiPSC clones at high passages, determined by RT-qPCR. The gene expression of the hiPSC was normalized to that of BR1 (ESC). PC4 clones were obtained by reprogramming fibroblast cells (PC fibro), which were used as negative control for pluripotency. *P< 0.05 (mean ± SD, n = 3). All significance of difference was analyzed using ANOVA with Tukey's post hoc test.

Long-term single-cell passaging of hiPSCs did not elicit chromosomal aberration

Chromosomal abnormalities were also checked by performing a G-banding karyotype, which confirmed no aberrations at passages 10, 30, 50 or 70 for clones ACP5, PC 4.3, PC 4.5 and PC 4.6 (Fig. 3). hiPSCs PC3 and PC2 were also tested for chromosomal abnormalities and no karyotypical aberrations were found either (S1 Fig.).

Figure 3. Karyotype confirmed absence of karyotypic abnormalities. G-Banding karyotype of ACP5 and PC4 clones after 10, 30, 50 and 70 single-cell passages. No aneuploidies were detected.

hiPSC clones subjected to single-cell passing preserved their trilineage displaying high-efficiency for differentiation

The plasticity of hiPSCs was tested in vitro. First, we cultured each hiPSC clone under conditions to promote embryoid body (EB) formation. As expected, 20 days EBs revealing positive gene expression for HNF4A, MSX1 and PAX6 (endo, meso and ectoderm representants, respectively) and decreased expression DNMT3B, a pluripotent marker (S5 Fig.). Instead, hiPSC clones at

passage 20 maintained under pluripotent conditions (E8 medium) displayed only high and expected expression of DNMT3B (S5 Fig.).

To further ensure the differentiation potential of long-term single-cell cultivated hiPSCs (up to passage 70), we subjected these cells to directed differentiation protocols to generate cardiomyocytes, keratinocytes and definitive endoderm.

Ectoderm derivative: Keratinocytes

hiPSC clones at high passages (25 to 50) were differentiated into keratinocytes (hiPSC-KC). Differentiation took approximately 30 days to be completed and at the end cells were characterized by immunostaining and flow cytometry. Flow cytometry confirmed high proportion of hiPSC-KCs positive for epithelial marker K14 (over 85%; Fig. 4A) and non-statistical difference was observed between clones (Fig. 4B). Immunostaining of hiPSC-KCs showed that these cells expressed not only epithelial (CD104, CD49f, K10 and K14) but also proliferative cell markers (Ki67, P63), indicating a profile typical of proliferative basal-layer epithelial cells (Fig. 4C). Additionally,

Figure 4. Directed differentiation into keratinocytes using iPSC at high passages yields positive cells for both epithelial and proliferative markers. (A-B) Cytometry data showing expression of K14 marker in keratinocytes obtained from iPSC clones PC4.3, PC4.5, PC4.6 and ACP5. Light and dark grey curves indicate negative control (iPSC) and stained cells (iPSC-KC), respectively. *$P < 0.05$ (mean ± SD, n= 3-6). **(C)** Representative immunostaining of keratinocytes (hiPSC-KC PC4.3) obtained from PC4.3 clone. Ki67 and P63 are characteristic nuclear markers of proliferative cells. Surface markers CD104, CD49f and cytoskeleton markers K10, K14 are all epithelial specific. CD104, CD49f and K14 are expressed especially on proliferative basal layer cells, whereas K10 is mainly expressed on intermediate and outermost layers of epidermis**. (D)** Immunostaining of iPSC-KC PC4.3 after calcium treatment showing higher expression of K10

and lower K14 expression. Results were confirmed by performing independent experiments for PC4.3, PC 4.5, PC4.6 and ACP5 (n = 3-6).

As keratinocytes undergo the process of differentiation, cells from inner layers move up to more superficial levels and switch from producing keratin K14 to produce K10 along with several other metabolic regulations.[26] Being calcium the major regulator of keratinocyte differentiation.[22] After treatment with calcium, hiPSC-KCs were stained using epithelial specific markers K10 and K14. Experiments were performed at least in triplicate using hiPSC-KCs derived from all clones (S7 Fig.). In comparison to the hiPSC-KC without calcium treatment, we observed lower expression of K14, whereas K10 expression showed remarkable increase (Fig. 4D), thus confirming hiPSC clones could generate keratinocytes with the ability to further differentiate into superficial-layer epithelial cells.

Mesoderm derivative: Cardiomyocytes

We have also used hiPSCs up to 70 passages for differentiation into cardiomyocytes. All hiPSC-derived cardiomyocytes (hiPSC-CMs) started to contract approximately at day 7. Thirty days after the beginning of differentiation, hiPSC-CMs showed positive expression of cardiac proteins such as NKX2-5, TNNI1, TNNI3, MYH7, TNNT2 and ACTN2 (Fig. 5A). Interestingly, we have found that hiPSC-CMs exhibited simultaneous expression of TNNI1 and TNNI3, which are especially expressed in fetal and mature cardiomyocytes, respectively. hiPSC-CMs derived from pluripotent cells ranging from passages 30 to 70 were generated with high-efficiency (over 80% ACTN2, TNNT2, TNNI1 and TNNI3 positive cells), in addition, ACP5 and PC4 lineages generated similar amounts of positive cardiac cells (Fig. 5B-C).

Figure 5. Directed differentiation into cardiomyocytes using iPSC at high passages yields positive cells for cardiac markers (A) Immunostaining of cardiomyocytes (iPSC-CM ACP5) obtained from ACP5 clone showing protein expression of cardiac specific markers TNNI1,

TNNI3, MYH7, TNNT2, NKX2.5 and ACTN2. **(B-C)** Cytometry data showing protein expression of cardiac specific markers in hiPSC-CM. Results were confirmed by performing independent experiments using APC5, PC4.3, PC4.5, PC4.6. *P< 0.05 (mean ± SD, n =2-5).

Endoderm derivative: Definitive endoderm

hiPSC clones were differentiated into DE (hiPSC-DE) and then cells were characterized by end-point PCR. Differentiated cells expressed DE-specific markers transcription factor SOX17 (SOX17), C-X-C chemokine receptor type 4 (CXCR4), homeobox protein goosecoid (GSC) and α-hepatocyte nuclear factor 4 (HNF4A), being the last one especially expressed in hepatoblasts.[27] In contrast, expression of pluripotent marker SOX2 was lower in iPSC-DE compared to iPSC clones, suggesting the differentiation procedure generated cells within a definitive endoderm profile that retained potential for further differentiation (Fig.6).

Figure 6. Endpoint RT-PCR of definitive endoderm obtained from clones PC4.3, PC4.5, PC4.6 and ACP5. Analysis showed expression of specific markers of definitive endoderm (SOX17, CXCR4, GSC), derivatives lines from endoderm (HNF4A), whereas lower expression of pluripotent marker SOX2 was observed.

DISCUSSION

In the present study, we have successfully demonstrated that our cultivation method is capable of maintaining hiPSCs obtained from different somatic cells over 50 passages under single-cell conditions. Therefore, hiPSCs were characterized at different timepoints for their expression of pluripotency markers, genetic integrity, growth rates and potential to generate high-efficiently derivatives from the three germ layers.

We have found by flow cytometry that protein expression of pluripotency markers showed no significant alteration neither decrease over time, corroborating permanent reprogramming as suggested by previous studies.[28,29] Interestingly, percentage positivity for OCT4, NANOG and

SOX2 also showed no significant difference between hiPSC clones, even though reprogramming was performed using different set of plasmids and different somatic cells of origin for lineages ACP and PC4. Consistent with this, qPCR also showed no significative difference among clones for mRNA expression of pluripotency markers NANOG, OCT4, SOX2, LIN28 or DNMT3B.

Genomic integrity was assessed by integration PCR and karyotyping as reprogramming and cultivation processes are recognized as the main causes for the alterations found in genome.[9] Because of the potential hazardous effects of reprogramming vectors,[30] several integration-free methods to generate iPSC were developed but, in comparison, the use of episomal plasmid vectors for reprogramming presents to its advantage a much increased efficiency.[19,25] By using plasmids pEB-C5, pEB-TG and Epi5, we were able to successfully generate hiPSC and PCR confirmed no vector integration into host DNA whatsoever. Although integration of transgenes is not the only mechanism that is capable of altering DNA after reprogramming, numerous reports concluded that further changes in the genome related to the vectors are mostly benign and unlikely to be threatening for research or therapy purposes,[31,32] thus, no additional experiments to assess reprogramming-induced genomic alterations were performed. As for culture-induced alterations, one of the main risks of prolonged cultivation is the progressive selection of genetic variants that are better adapted to in vitro culture environment.[13,33] Several studies concerning ESC show that aneuploidies in chromosomes 12, 17 and X are commonly identified after long-term culture.[10,13] As recently reviewed by Assou and colleagues,[9] a karyotyping routine is essential for the quality assessment of PSCs as it can identify many unacceptable genomic abnormalities, which already have been reported to emerge after only 5 passages.[11] We have screened ACP5 and PC4 clones at passages 10, 30, 50 and 70 and found no aberrations trough G-banding karyotype. Importantly, although we are encouraged by this result, we recognize the need for additional screening to exclude potential infra-karyotypic abnormalities such as 20q11.21 amplification[34] or oncogenic mutations[35], which are also unacceptable for studies concerning hiPSCs.

It's well known that PSCs proliferative log phase is required for the best results in many differentiation protocols such as cardiac ones.[36] So, tracking cell doubling rates during their maintenance to avoid predictable high confluence and so cell cycle stuck is strongly desirable and it can be easily performable by PDT calculation and cell confluence monitoring as routine of culturing. Thus, we have assessed population doubling time (PDT) and cell confluence from passage 20 to 60, a time range that corresponded to approximately 120 days of cultivation.9 Statistical analysis of different cultivation timepoints showed no significative impact in PDT for neither ACP5 nor PC4 clones. Conversely, other groups found increasing growth rates related to long-term cultivation.[11,14] This discrepancy may be explained by the fact these findings were linked to aneuploidies found within the studies, as a strong correlation between the proportion of cell lines with abnormal karyotypes and population doubling has been previously reported in an extensive study with both ESC and iPSC.[37] Additionally, our software (CellCountAnalyser) was able to measure cell confluence quicker and more accurately than other software's available [16,38–40], getting us the ability to split cells always in log-phase of growing.

A final important finding was the maintenance of the differentiation potential after long-term cultivation, as all hiPSC clones at high passages were capable of spontaneous differentiation into all three germlines through EB formation or directed differentiation. Expression analysis of characteristic markers showed that directed differentiation consistently yielded cardiomyocytes, keratinocytes and definitive endoderm (DE) cells using hiPSCs at high passages. Characterization of hiPSC-DE by end-point RT-PCR showed an increase in the genetic expression of specific endoderm markers, whereas expression of pluripotent marker SOX2 was notably lower than that of the respective hiPSC clones. Nonetheless, it is important to state that we have also attempted to characterize cells trough immunocytochemistry for markers albumin (ALB), α-fetoprotein (AFP), cytochrome P450 3A4 (CYP3A4), CXCR4 and SOX17, but iPSC-DE showed no immunoreactivity for ALB nor CXCR4 and we have not found antibodies against AFP, CYP3A4 and SOX17 with sufficient specificity on control groups. (Data not shown). In part, this can be explained by the fact that PSC differentiation potential can vary according to the cell type of

origin,[41] a factor which may have been reflected upon a diminished susceptibility for differentiation pathways such as DE. In parallel, keratinocytes and cardiomyocytes (which are our main focus of research) displayed high purity of cardiac- and epithelial- cells independently of the passage numbers or hiPSC lineage, as routinely reported for others.[15]

This brings two very important points of discussion concerning impact of iPSC management on differentiation success. First, low passages should not be used for directed differentiation as early-passage hiPSCs may retain transient epigenetic memory from adult somatic cell sources impairing PSC plasticity.[41] Second, despite being a popular methodology for differentiation in vitro, it is acknowledged that EB formation is a process that shows very low reproducibility and often ends up with low purity of the desired differentiated cells.[42,43] Therefore, directed differentiation approaches tend to be more efficient and reliable to obtain specialized cells. As previously described in literature, single-cell passaging also facilitates hiPSC directed differentiation by promoting seeding homogeneity and the formation of loosely packed clusters, which leads to a more efficient cellular response to signal molecules.[3] Seeding density has been pointed out as one of the major optimizable factors for cardiomyocyte differentiation, for example,[44] and similarly, adaptation into single-cell culture was found to be a crucial step for differentiation into lung epithelia.[45]

On the other hand, it is well recognized that single-cell passaging is usually followed by great loss of cellular viability[7] and hence several reports suggest it promotes rapid selection of genetically abnormal clones that display increased survival rates. The increasing use of ROCK-inhibitor Y-27632[8] to promote PSC viability after enzymatic dissociation also concern scientists because the lack of reported data on its long-term effects. Indeed, we have found only one study that assessed this question, however it found no direct effect of Y-27632 on ESC genomic integrity.[11] While several studies found chromosomes 17 and 12 to be especially sensitive to adaptation into single-cell passaging,[10,11,46] many others did not detect such abnormalities upon long-term culture.[47–49] It has been previously suggested that the conflicting data concerning impact of single-cell passaging may be explained by this generical designation

encompassing different passaging methods, such as EDTA, dispase, TryPLE and trypsin.[11,47] However, as there are multiple different variables among studies, impact of single-cell passaging techniques remains subject for further investigation.

CONCLUSION

Here we presented an easy long-term and single-cell passaging pipeline to cultivate hiPSCs that maintained their characteristics and karyotype under feeder-free conditions that allowed us to follow a robust hiPSC cultivation routine by regulating cell numbers in a density- and time-dependent manner. This method allows hiPSCs spontaneous differentiation into the three germlines trough EB formation and by high-efficient directed differentiation into keratinocytes, cardiomyocytes and DE.

ACKNOWLEDGMENTS

We thank Fundação de Amparo à pesquisa do Estado de São Paulo (FAPESP) for granting this research (#15/50224-8 and #16/50082-1). We also thank professor Alexander Henning Ulrich to allow us to use his FACs equipment and professor Monica Mator for the donation of 3T3 cells.

REFERENCES

1. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006;126: 663–676. doi:10.1016/j.cell.2006.07.024

2. Chen KG, Mallon BS, McKay RDG, Robey PG. Human pluripotent stem cell culture: Considerations for maintenance, expansion, and therapeutics. Cell Stem Cell. 2014. doi:10.1016/j.stem.2013.12.005

3. Chen KG, Mallon BS, Hamilton RS, Kozhich OA, Park K, Hoeppner DJ, et al. Non-colony type monolayer culture of human embryonic stem cells. Stem Cell Res. 2012; doi:10.1016/j.scr.2012.06.003

4. Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, et al. Derivation of novel human ground state naive pluripotent stem cells. Nature. 2013;504: 282–286. doi:10.1038/nature12745

5. Krawetz R, Taiani JT, Liu S, Meng G, Li X, Kallos MS, et al. Large-Scale Expansion of Pluripotent Human Embryonic Stem Cells in Stirred-Suspension Bioreactors. Tissue Eng Part C Methods. 2010;16: 573–582. doi:10.1089/ten.tec.2009.0228

6. Steiner D, Khaner H, Cohen M, Even-Ram S, Gil Y, Itsykson P, et al. Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. Nat Biotechnol. 2010;28: 361–364. doi:10.1038/nbt.1616

7. Miñambres R, Guasch RM, Perez-Aragó A, Guerri C. The RhoA/ROCK-I/MLC pathway is involved in the ethanol-induced apoptosis by anoikis in astrocytes. J Cell Sci. 2006;119: 271–82. doi:10.1242/jcs.02723

8. Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol. 2007;25: 681–686. doi:10.1038/nbt1310

9. Assou S, Bouckenheimer J, De Vos J. Concise Review: Assessing the Genome Integrity of Human Induced Pluripotent Stem Cells: What Quality Control Metrics? Stem Cells. 2018; doi:10.1002/stem.2797

10. Mayshar Y, Ben-David U, Lavon N, Biancotti JC, Yakir B, Clark AT, et al. Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell. 2010; doi:10.1016/j.stem.2010.07.017

11. Bai Q, Ramirez J-M, Becker F, Pantesco V, Lavabre-Bertrand T, Hovatta O, et al. Temporal Analysis of Genome Alterations Induced by Single-Cell Passaging in Human Embryonic Stem Cells. Stem Cells Dev. 2014; doi:10.1089/scd.2014.0292

12. Jacobs K, Zambelli F, Mertzanidou A, Smolders I, Geens M, Nguyen HT, et al. Higher-Density Culture in Human Embryonic Stem Cells Results in DNA Damage and Genome Instability. Stem Cell Reports. 2016; doi:10.1016/j.stemcr.2016.01.015

13. Harrison NJ, Baker D, Andrews PW. Culture adaptation of embryonic stem cells echoes germ cell malignancy. Int J Androl. 2007; doi:10.1111/j.1365-2605.2007.00762.x

14. Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, et al. Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Hum Mol Genet. 2005; doi:10.1093/hmg/ddi345

15. Fujita J, Tohyama S, Kishino Y, Okada M, Morita Y. Concise Review: Genetic and Epigenetic Regulation of Cardiac Differentiation from Human Pluripotent Stem Cells. Stem Cells. 2019; doi:10.1002/stem.3027

16. Busschots S, O'Toole S, O'Leary JJ, Stordal B. Non-invasive and non-destructive measurements of confluence in cultured adherent cell lines. MethodsX. 2015;2: 8–13. doi:10.1016/j.mex.2014.11.002

17. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc. 2013; doi:10.1038/nprot.2012.150

18. Dariolli R, Bassaneze V, Nakamuta JS, Omae SV, Campos LCG, Krieger JE. Porcine Adipose Tissue-Derived Mesenchymal Stem Cells Retain Their Proliferative Characteristics, Senescence, Karyotype and Plasticity after Long-Term Cryopreservation. PLoS One. 2013; doi:10.1371/journal.pone.0067939

19. Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LMS, et al. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res. 2011; doi:10.1038/cr.2011.12

20. Fraga AM, Sukoyan M, Rajan P, Braga DP de AF, Iaconelli A, Franco JG, et al. Establishment of a Brazilian line of human embryonic stem cells in defined medium: Implications for cell therapy in an ethnically diverse population. Cell Transplant. 2011; doi:10.3727/096368910X522261

21. Itoh M, Kiuru M, Cairo MS, Christiano AM. Generation of keratinocytes from normal and recessive dystrophic epidermolysis bullosa-induced pluripotent stem cells. Proc Natl Acad Sci. 2011; doi:10.1073/pnas.1100332108

22. Bikle DD, Xie Z, Tu C-L. Calcium regulation of keratinocyte differentiation. Expert Rev Endocrinol Metab. 2012; doi:10.1586/eem.12.34

23. Illing A, Stockmann M, Swamy Telugu N, Linta L, Russell R, Müller M, et al. Definitive endoderm formation from plucked human hair-derived induced pluripotent stem cells and SK channel regulation. Stem Cells Int. 2013; doi:10.1155/2013/360573

24. Szkolnicka D, Farnworth SL, Lucendo-Villarin B, Hay DC. Deriving functional hepatocytes from pluripotent stem cells. Curr Protoc Stem Cell Biol. 2014; doi:10.1002/9780470151808.sc01g05s30

25. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, et al. A more efficient method to generate integration-free human iPS cells. Nat Methods. 2011; doi:10.1038/nmeth.1591

26. Eichner R, Sun TT, Aebi U. The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. J Cell Biol. 1986; doi:10.1083/jcb.102.5.1767

27. Alder O, Cullum R, Lee S, Kan AC, Wei W, Yi Y, et al. Hippo signaling influences HNF4A and FOXA2 enhancer switching during hepatocyte differentiation. Cell Rep. 2014; doi:10.1016/j.celrep.2014.08.046

28. Bhattacharya B, Miura T, Brandenberger R, Mejido J, Luo Y, Yang AX, et al. Gene expression in human embryonic stem cell lines: Unique molecular signature. Blood. 2004; doi:10.1182/blood-2003-09-3314

29. Abeyta MJ, Clark AT, Rodriguez RT, Bodnar MS, Reijo Pera RA, Firpo MT. Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum Mol Genet. 2004; doi:10.1093/hmg/ddh068

30. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007; doi:10.1038/nature05934

31. Abyzov A, Mariani J, Palejev D, Zhang Y, Haney MS, Tomasini L, et al. Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells. Nature. 2012; doi:10.1038/nature11629

32. Bhutani K, Nazor KL, Williams R, Tran H, Dai H, Dzakula Z, et al. Whole-genome mutational burden analysis of three pluripotency induction methods. Nat Commun. 2016; doi:10.1038/ncomms10536

33. Andrews PW. The selfish stem cell. Nat Biotechnol. 2006; doi:10.1038/nbt0306-325

34. Lefort N, Feyeux M, Bas C, Féraud O, Bennaceur-Griscelli A, Tachdjian G, et al. Human embryonic stem cells reveal recurrent genomic instability at 20q11.21. Nat Biotechnol. 2008; doi:10.1038/nbt.1509

35. Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, et al. COSMIC: Mining complete cancer genomes in the catalogue of somatic mutations in cancer. Nucleic Acids Res. 2011; doi:10.1093/nar/gkq929

36. Laco F, Woo TL, Zhong Q, Szmyd R, Ting S, Khan FJ, et al. Unraveling the Inconsistencies of Cardiac Differentiation Efficiency Induced by the GSK3β Inhibitor CHIR99021 in Human Pluripotent Stem Cells. Stem Cell Reports. 2018;10: 1851–1866. doi:10.1016/j.stemcr.2018.03.023

37. Amps K, Andrews P, Anyfantis G, Armstrong L, Avery S, Baharvand H, et al. Screening a large, ethnically diverse population of human embryonic stem cells identifies a chromosome 20 minimal amplicon that confers a growth advantage. Nat Biotechnol. 2012; doi:10.1038/nbt.2051.Screening

38. Jaccard N, Griffin LD, Keser A, Macown RJ, Super A, Veraitch FS, et al. Automated method for the rapid and precise estimation of adherent cell culture characteristics from phase contrast microscopy images. Biotechnol Bioeng. 2014;111: 504–517. doi:10.1002/bit.25115

39. Georg M, Fernández-Cabada T, Bourguignon N, Karp P, Peñaherrera AB, Helguera G, et al. Development of image analysis software for quantification of viable cells in microchips. Deli MA, editor. PLoS One. 2018;13: e0193605. doi:10.1371/journal.pone.0193605

40. Venter C, Niesler C. Rapid quantification of cellular proliferation and migration using ImageJ. Biotechniques. 2019;66: 99–102. doi:10.2144/btn-2018-0132

41. Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol. 2010; doi:10.1038/nbt.1667

42. James D, Nam H, Seandel M, Nolan D, Janovitz T, Tomishima M, et al. Expansion and maintenance of human embryonic stem cell – derived endothelial cells by TGFb inhibition is Id1 dependent. Nat Biotechnol. 2010; doi:10.1038/nbt1605

43. Spater D, Hansson EM, Zangi L, Chien KR. How to make a cardiomyocyte. Development. 2014; doi:10.1242/dev.091538

44. Burridge PW, Holmström A, Wu JC. Chemically Defined Culture and Cardiomyocyte Differentiation of Human Pluripotent Stem Cells. Curr Protoc Hum Genet. 2015; doi:10.1002/0471142905.hg2103s87

45. Wong AP, Chin S, Xia S, Garner J, Bear CE, Rossant J. Efficient generation of functional CFTR-expressing airway epithelial cells from human pluripotent stem cells. Nat Protoc. 2015; doi:10.1038/nprot.2015.021

46. Maitra A, Arking DE, Shivapurkar N, Ikeda M, Stastny V, Kassauei K, et al. Genomic alterations in cultured human embryonic stem cells. Nat Genet. 2005; doi:10.1038/ng1631

47.Bajpai R, Lesperance J, Kim M, Terskikh A V. Efficient propagation of single cells accutase-dissociated human embryonic stem cells. Mol Reprod Dev. 2008; doi:10.1002/mrd.20809

48.Kibschull M, Mileikovsky M, Michael IP, Lye SJ, Nagy A. Human embryonic fibroblasts support single cell enzymatic expansion of human embryonic stem cells in xeno-free cultures. Stem Cell Res. 2011; doi:10.1016/j.scr.2010.08.002

49.Ellerström C, Strehl R, Noaksson K, Hyllner J, Semb H. Facilitated Expansion of Human Embryonic Stem Cells by Single-Cell Enzymatic Dissociation. Stem Cells. 2007; doi:10.1634/stemcells.2006-0607

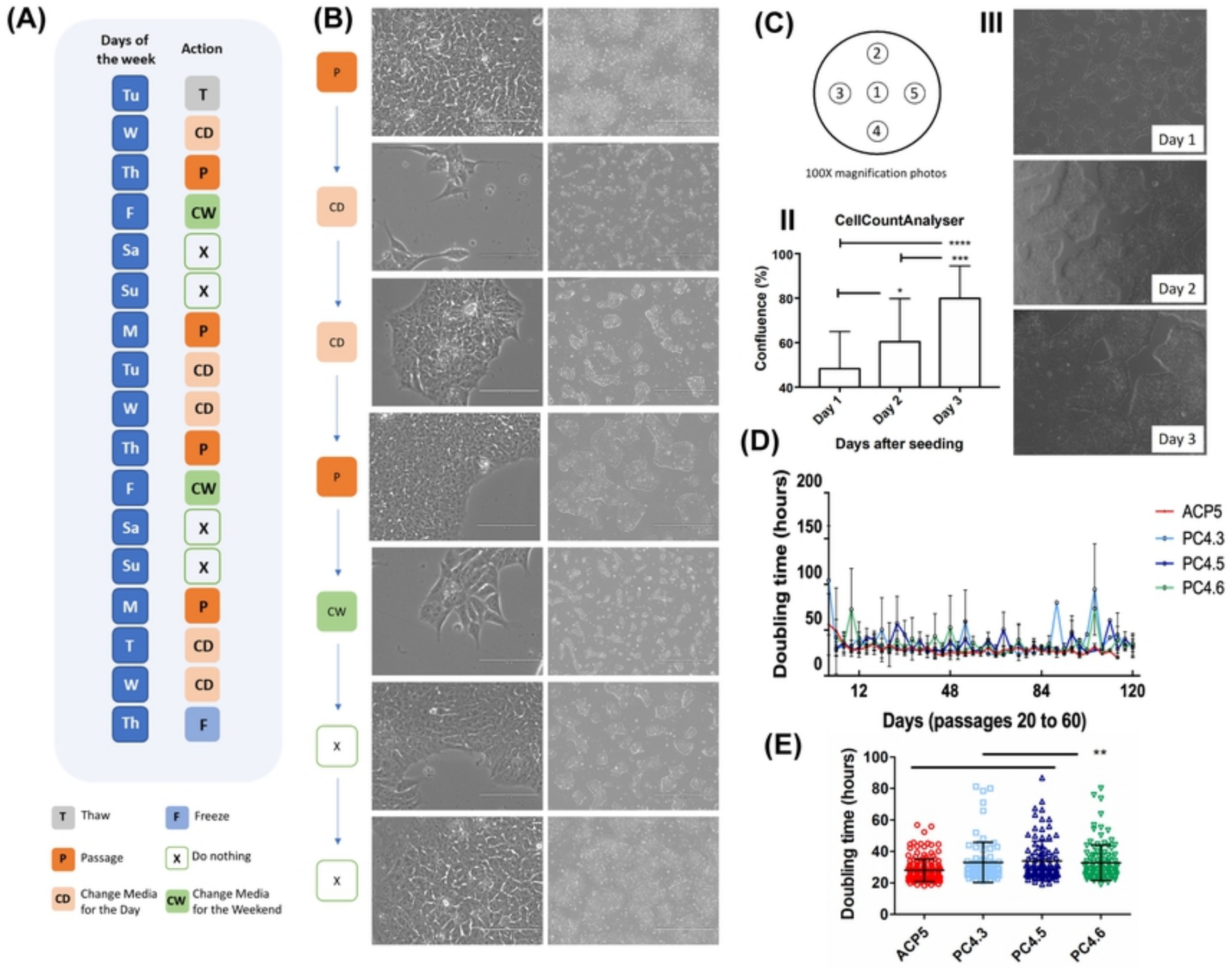
(A)
Days of the week
Action
Tu T
W CD
Th P
F CW
Sa X
Su X
M P
Tu CD
W CD
Th P
F CW
Sa X
Su X
M P
T CD
W CD
Th F
T Thaw
F Freeze
P Passage
X Do nothing
CD Change Media for the Day
CW Change Media for the Weekend
(B)
P
CD
CD
P
CW
X
X
(C)
100X magnification photos
II
CellCountAnalyser
Confluence (%)
Day 1
Day 2
Day 3
Days after seeding
III
Day 1
Day 2
Day 3
(D)
Doubling time (hours)
Days (passages 20 to 60)
ACP5
PC4.3
PC4.5
PC4.6
(E)
Doubling time (hours)
ACP5
PC4.3
PC4.5
PC4.6

Figure 1

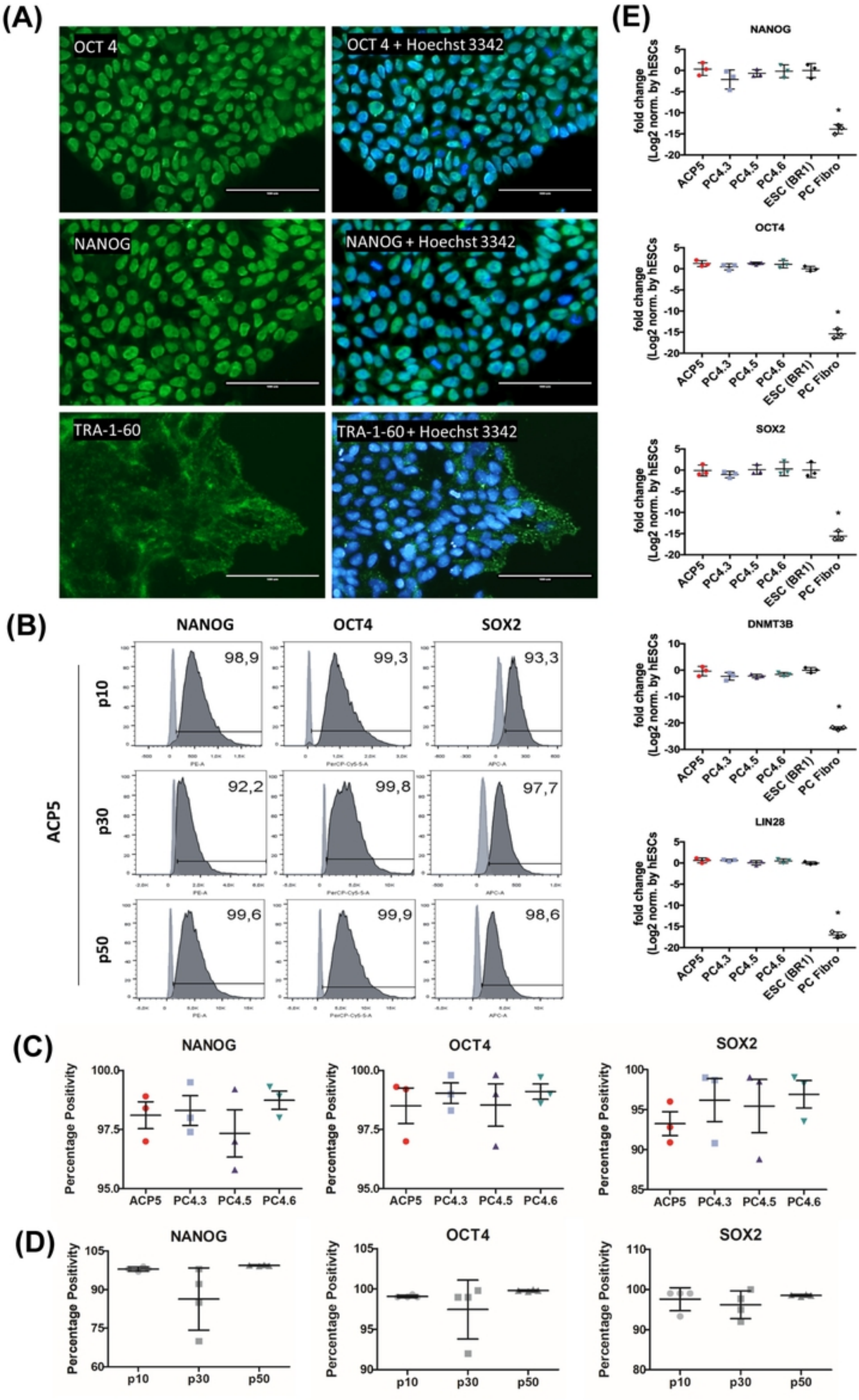
(A)
OCT 4
OCT 4 + Hoechst 3342
NANOG
NANOG + Hoechst 3342
TRA-1-60
TRA-1-60 + Hoechst 3342
(B)
NANOG
OCT4
SOX2
ACP5
p10
p30
p50
98,9
99,3
93,3
92,2
99,8
97,7
99,6
99,9
98,6
(C)
Percentage Positivity
ACP5
PC4.3
PC4.5
PC4.6
(D)
p10
p30
p50
(E)
fold change (Log2 norm. by hESCs)
ACP5
PC4.3
PC4.5
PC4.6
ESC (BR1)
PC Fibro
DNMT3B
LIN28

Figure 2

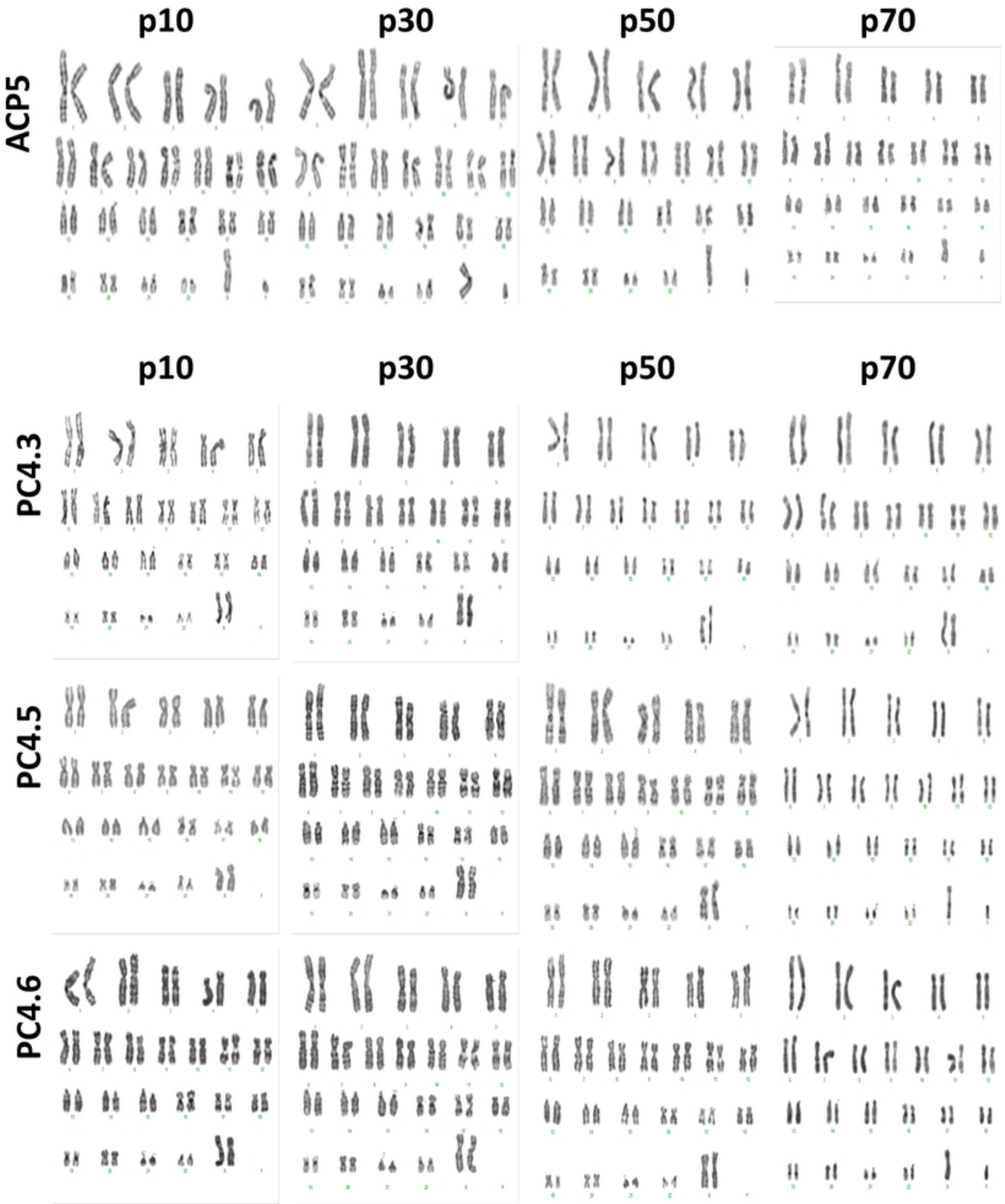

Figure 3

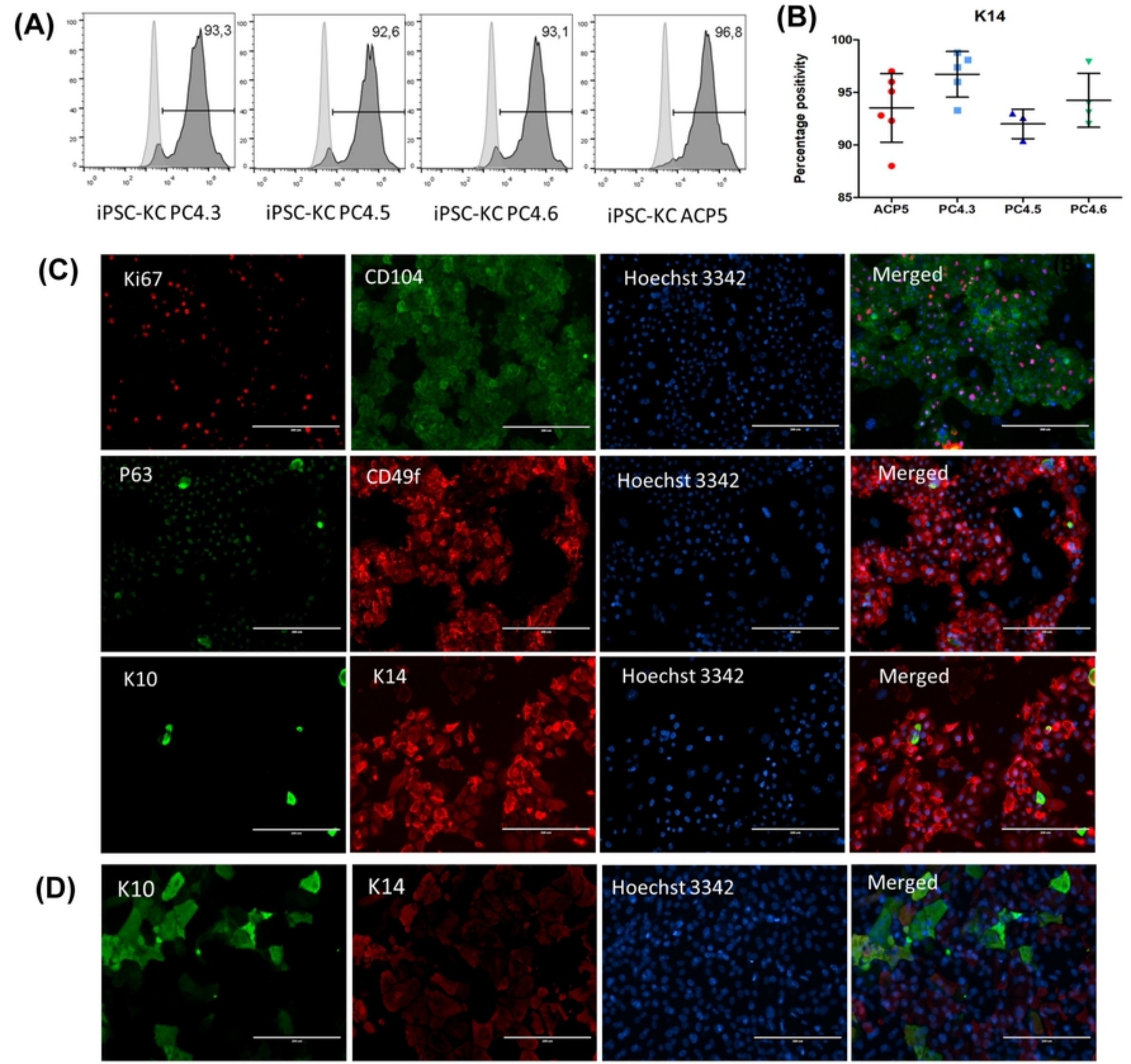
(A)
93,3
92,6
93,1
96,8
iPSC-KC PC4.3
iPSC-KC PC4.5
iPSC-KC PC4.6
iPSC-KC ACP5
(B)
K14
Percentage positivity
100
95
90
85
ACP5
PC4.3
PC4.5
PC4.6
(C)
Ki67
CD104
Hoechst 3342
Merged
P63
CD49f
Hoechst 3342
Merged
K10
K14
Hoechst 3342
Merged
(D)
K10
K14
Hoechst 3342
Merged

Figure 4

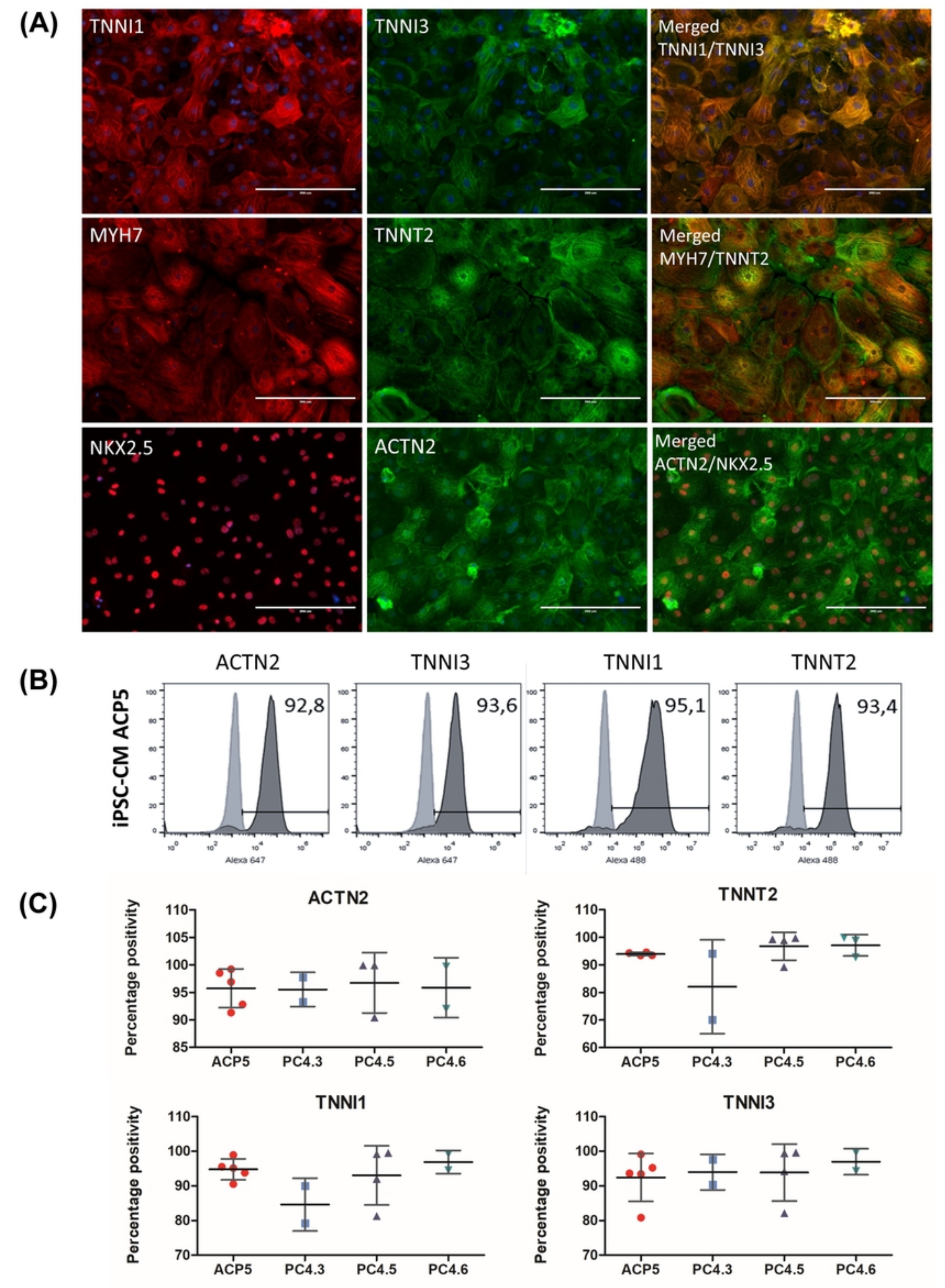
(A)
TNNI1
TNNI3
Merged
TNNI1/TNNI3
MYH7
TNNT2
Merged
MYH7/TNNT2
NKX2.5
ACTN2
Merged
ACTN2/NKX2.5
(B)
ACTN2
TNNI3
TNNI1
TNNT2
iPSC-CM ACP5
92,8
93,6
95,1
93,4
Alexa 647
Alexa 647
Alexa 488
Alexa 488
(C)
ACTN2
TNNT2
TNNI1
TNNI3
Percentage positivity
ACP5
PC4.3
PC4.5
PC4.6

Figure 5

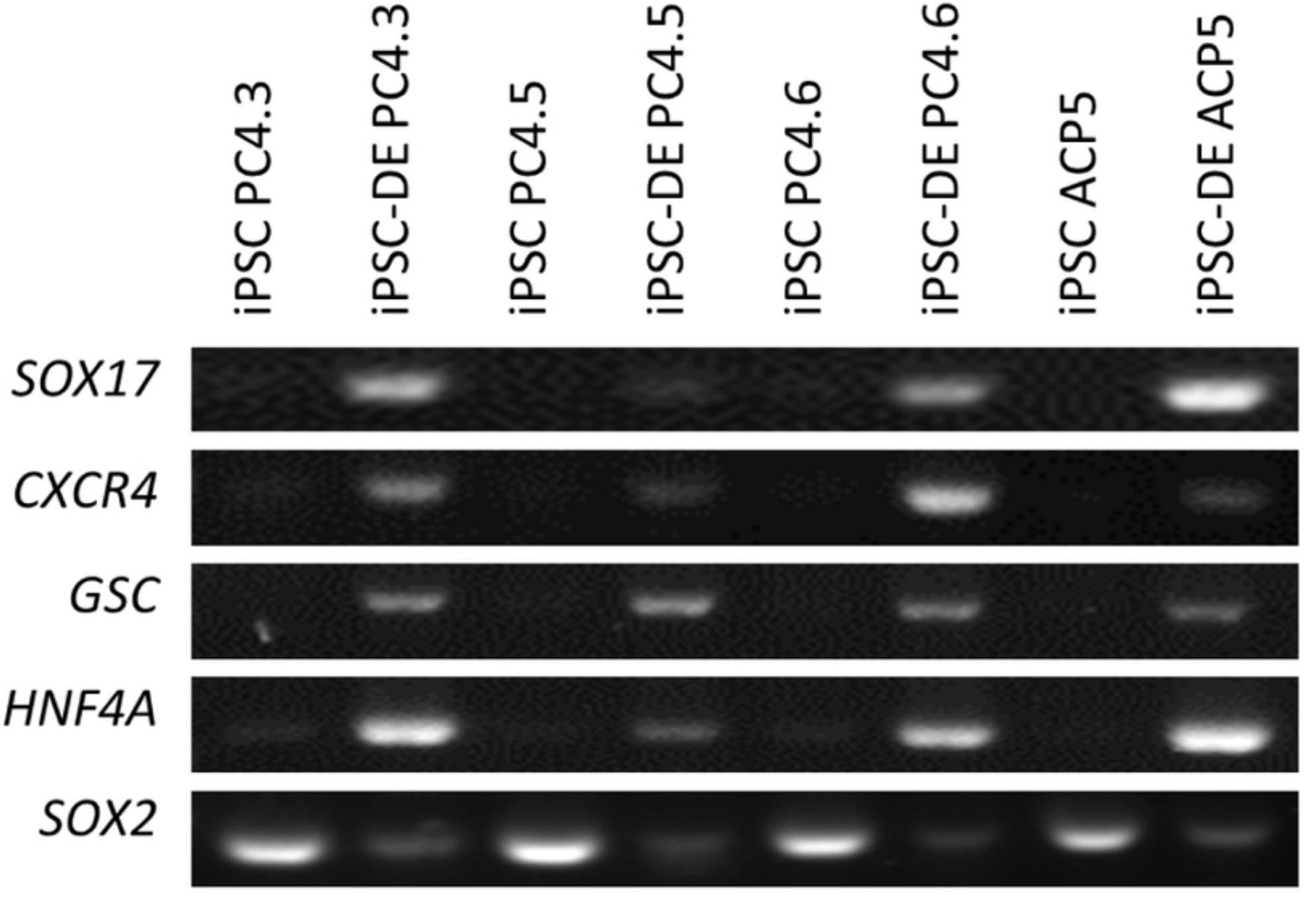
iPSC PC4.3
iPSC-DE PC4.3
iPSC PC4.5
iPSC-DE PC4.5
iPSC PC4.6
iPSC-DE PC4.6
iPSC ACP5
iPSC-DE ACP5
SOX17
CXCR4
GSC
HNF4A
SOX2

Figure 6