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Expansion of 3D human induced pluripotent stem cell aggregates in bioreactors with a clinical grade culture medium

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Abstract

Human induced pluripotent stem cells (hiPSCs) are attractive tools for drug screening and disease modeling as well as promising candidates for cell therapy applications. Here we present the development of a defined, feeder-free medium, without human- or animal-derived components. hiPSCs that are cultured in this medium for an extended period of time express expected stem cell markers, remain diploid, and can differentiate into cell types from the three germ layers. Using this complete, clinical-grade culture medium, eight different hiPSC lines that were expanded as a 2D monolayer (2D culture) maintain high expression of pluripotent stem cell markers and lack any expression of differentiation markers over the 12–20 passages tested. In addition, no karyotype abnormalities were reported for any of the tested cell lines. In order to generate clinically relevant quantities of hiPSCs—10^9 and beyond—it is essential to develop efficient, yet robust 3D suspension cultures maintaining the same stability as 2D monolayer cultures. Previous reports in the literature of suspension cultures have typically described a reduced growth rate compared to monolayer cultures with a final cell concentration of 1–2 million cells per milliliter. We demonstrate that our culture system supports large-scale, 3D, non-adherent expansion of hiPSCs in suspension culture in a perfusion bioreactor. Furthermore, by optimizing perfusion rates and dissolved oxygen levels, we were able to expand hiPSCs 1,100-fold within 3 passages over 11 days to a final concentration of 5 million cells per milliliter using our 3D suspension culture system. In summary, our clinical-grade culture system allows for efficient, robust, and scalable production of hiPSCs, thus facilitating the use of hiPSCs for research and large-scale 3D suspension clinical applications.

1 Stable proliferation rates and long-term preservation of genomic stability

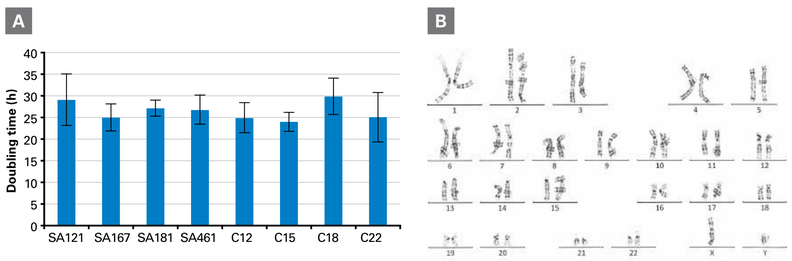

Figure 1. Average doubling time and karyotyping of human pluripotent stem cell (hPSC) lines passaged in Cellartis® DEF-CS™ 500 Xeno-Free Culture Medium without Antibiotics. Four hESC lines (SA121, SA167, SA181, and SA461) and four hiPSC lines (ChiPSC12 [C12], ChiPSC15 [C15], ChiPSC18 [C18], and ChiPSC22 [C22]) were grown as a 2D monolayer up to passage 10. **Panel A.** Proliferation rates are displayed as the population doubling time in hours. Cells were passaged every 3–4 days; at most passages, cells demonstrated a population doubling time of 25–28 hr. **Panel B.** Karyotyping results from hESC line SA121 expanded in the xeno-free culture medium for 30 passages. All hESC and hiPSC lines listed in Panel A displayed normal karyotype over 11–30 passages.

2 Cells maintain their pluripotency without differentiation

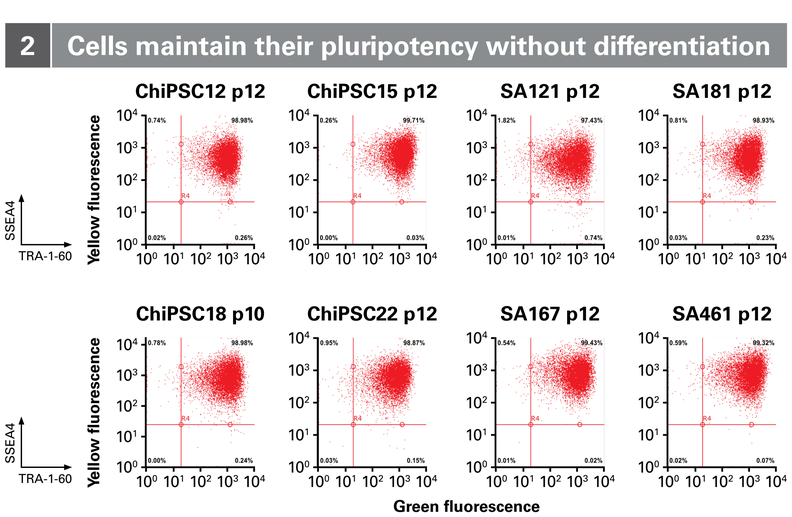

Figure 2. Pluripotency marker expression in cells maintained in Cellartis DEF-CS xeno-free medium. Four hiPSC lines (ChiPSC12, ChiPSC15, ChiPSC18, and ChiPSC22) and four hESC lines (SA121, SA167, SA181, and SA461) were analyzed after 12 passages in conventional 2D monolayer cultures. Flow cytometry analysis shows more than 98% of cells were positive for cell surface markers TRA-1-60 and SSEA4.

3 Cells maintain their differentiation potential into the three germ layers

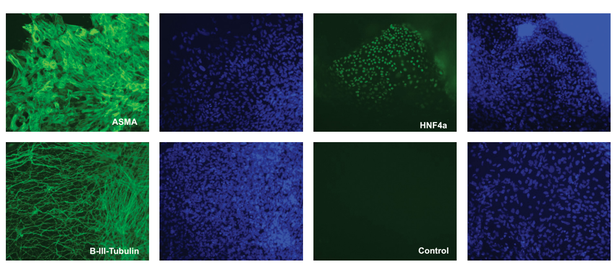

Figure 3. Spontaneous differentiation into the three germ layers. The cell lines described in Figures 1 and 2 were analyzed for their ability to spontaneously differentiate into the three germ layers after 12 consecutive passages using Cellartis DEF-CS xeno-free medium. Undifferentiated cells were centrifuged in 96-well plates to form aggregates. After one week, the aggregates were transferred to cell culture plates for further spontaneous differentiation. Two weeks later, the differentiated cells were fixed and stained for the presence of ASMA (mesodermal marker), β-III-Tubulin (ectodermal marker), and HNF4α (endodermal marker). The images show representative stainings from cell line ChiPSC22.

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4 Expansion workflow for 3D suspension culture

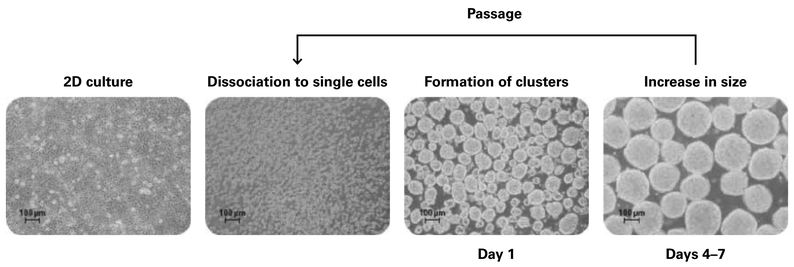


Figure 4. Optimal spheroid generation from hiPSCs using Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics. To create a workflow for scale-up to produce a large number of cells, 3D culture was optimized for the expansion of aggregates of induced pluripotent stem cells. Cells grown as 3D suspension cultures in the culture system formed homogeneous, round spheroids and exhibited a robust proliferation rate. Scale bar = 100 µM.

5 Comparison of dissolved oxygen concentrations for optimized cell growth in 3D culture

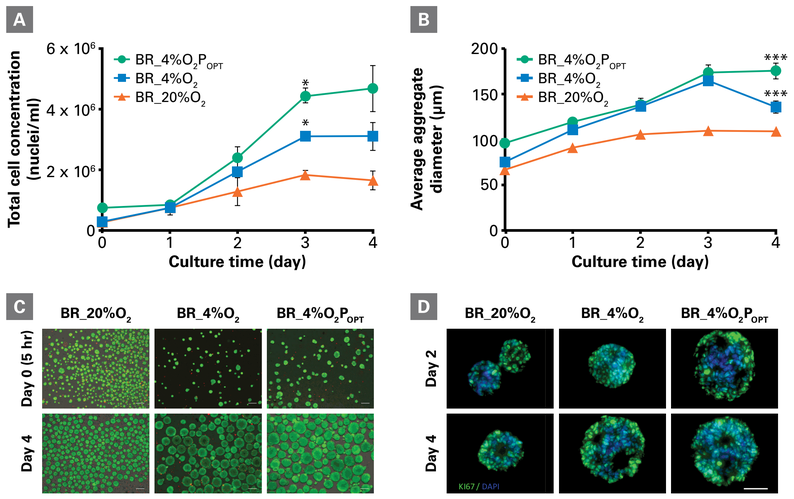


Figure 5. Impact of dissolved oxygen concentration and dilution rate on viability and proliferation capacity of hiPSCs cultured as 3D aggregates in controlled stirred-tank bioreactors. Cells were inoculated at 0.25 x 10^6 cells/ml and cultured in bioreactors operating in perfusion mode with 20% or 4% dissolved oxygen. For the 4% O_2 condition, two dilution ranges were tested: 0.66–0.75 per day ($BR\_4\%O_2$) and 1.3 per day ($BR\_4\%O_2P_{OPT}$). **Panel A.** Total cell concentration and **(Panel B)** average diameter of hiPSC 3D aggregates at the indicated culture times. **Panel C.** Phase contrast and fluorescence images of hiPSC aggregates at Day 0 (5 hours after inoculation) and Day 4 of culture. Scale bar = 200 µm. **Panel D.** Immunofluorescence images of hiPSC aggregate cryosections at Days 2 and 4 of culture; proliferating cells were stained with KI67 (green), dead cells were stained with PI (red), and nuclei were stained with DAPI (blue). Scale bar = 50 µm. For Panel A, *p<0.05 for $BR\_4\%O_2P_{OPT}$ vs. $BR\_20\%O_2$; *p<0.05 for $BR\_4\%O_2$ vs. $BR\_20\%O_2$. For Panel B, ***p<0.001 for $BR\_4\%O_2P_{OPT}$ vs. $BR\_4\%O_2$ and $BR\_20\%O_2$; ***p<0.001 for $BR\_4\%O_2$ vs. $BR\_20\%O_2$.

6 Comparison of dissolved oxygen concentration for optimized pluripotency in 3D culture

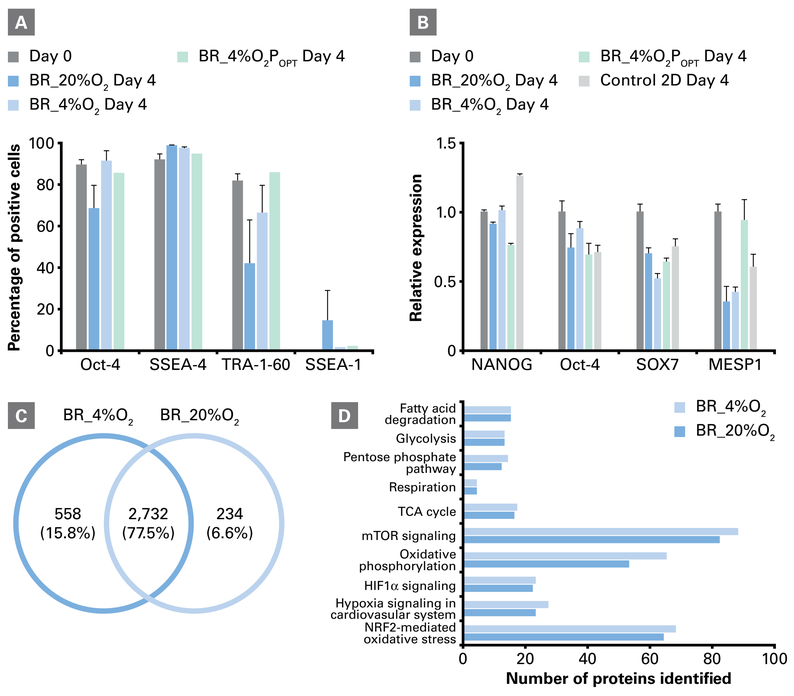


Figure 6. Effect of oxygen concentration and dilution rate on the phenotype of hiPSCs cultured as 3D aggregates in controlled stirred-tank bioreactors. Panel A. Flow cytometry analysis of hiPSC populations at the inoculum (Day 0) and Day 4 of culture; results are expressed as a percentage of positive cells for pluripotency markers (Oct-4, SSEA-4, and TRA-1-60) and early differentiation marker (SSEA1). **Panel B.** Gene expression analysis of hiPSCs by qRT-PCR. Relative expression of pluripotency genes *(NANOG* and *Oct-4)* and early differentiation genes *(SOX7* and *MESP1)* on Day 4 of bioreactor culture and 2D culture (normalized by the expression of each gene at Day 0). **Panels C and D.** Whole proteome analysis of hiPSCs cultured as 3D aggregates in $BR\_20\%O_2$ and $BR\_4\%O_2$ and harvested at Day 4. Panel C shows a Venn-diagram-based comparative analysis of the proteins identified. Panel D shows the number of proteins identified for canonical pathways related to cell metabolism and oxygen signaling, as analyzed by IPA software.

7 Scalable expansion of hiPSCs in bioreactors under optimal O_2 culturing conditions

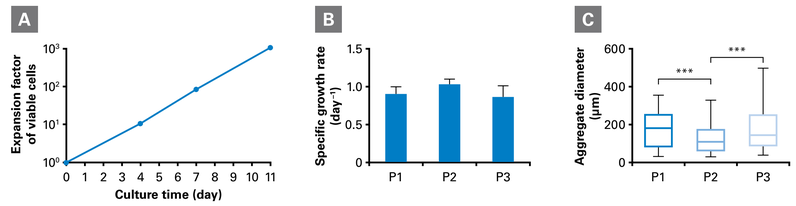


Figure 7. Continuous expansion of 3D hiPSC aggregates in stirred-tank bioreactors. The $BR\_4\%O_2P_{OPT}$ culture strategy was selected and 3D aggregates were mechanically dissociated using nylon meshes with a 70 µm pore size. Three sequential passages were carried out in bioreactors and hiPSCs were subcultured every 3–4 days, as follows. During passage 1 (P1), hiPSCs were initially inoculated as single cells (0.25 x 10^6 cell/ml) in bioreactors and cultured as aggregates for 4 days. During passage 2 (P2), aggregates were harvested at Day 4 and, after mechanical dissociation, re-inoculated as small aggregates (at a concentration of 0.25 x 10^6 cell/ml) in bioreactors. Cells were cultured for an additional 3 days (a total culture time of 7 days). During passage 3 (P3), aggregates were harvested and, after mechanical dissociation, re-inoculated as small aggregates (at a concentration of 0.25 x 10^6 cell/ml) in bioreactors. Cells were cultured for an additional 4 days (a total culture time of 11 days). **Panel A.** Cumulative expansion factor of viable cells obtained in the three sequential passages. **Panel B.** Specific growth rates of viable cells estimated for each passage. **Panel C.** Average diameter of hiPSC aggregates harvested at the end of each passage (corresponding to Days 4, 7, and 11 respectively for P1, P2, and P3); *** indicates p<0.001.

8 Scalable expansion of hiPSCs in bioreactors under optimal culturing conditions maintains pluripotency after multiple passages

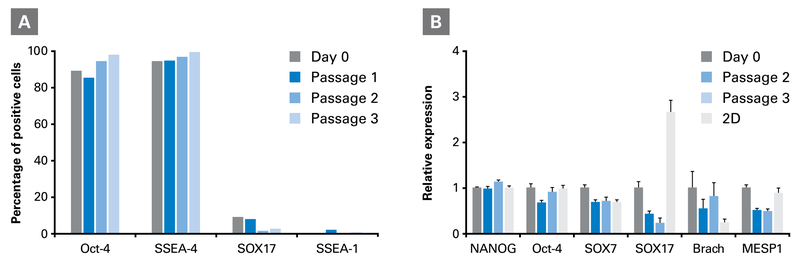
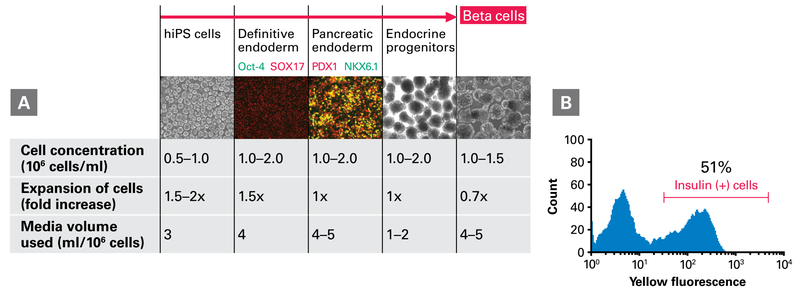


Figure 8. Pluripotency of hiPSCs grown as 3D spheroids under optimal O_2 conditions. Panel A. Flow cytometry analysis of the hiPSC population at the inoculum (Day 0) and after each passage in the bioreactor. Results are expressed as a percentage of cells positive for pluripotency markers (Oct-4 and SSEA-4) and early differentiation markers (SSEA1 and SOX17). **Panel B.** Gene expression analysis of hiPSCs by qRT-PCR: relative expression of pluripotency genes (*NANOG* and *Oct-4*) and early differentiation genes (*SOX7*, *SOX17*, *Brach*, and *MESP1*) on the final days of Passages 2 and 3 of bioreactor culture and after three sequential passages in 2D-monolayer culture. Expression was normalized to the expression of each gene at Day 0.

9 After 3D scale-up, hiPSCs maintain the capacity to differentiate into beta cells



Cell concentration (10^6 cells/ml)	0.5–1.0	1.0–2.0	1.0–2.0	1.0–2.0	1.0–1.5
Expansion of cells (fold increase)	1.5–2x	1.5x	1x	1x	0.7x
Media volume used (ml/10^6 cells)	3	4	4–5	1–2	4–5

Figure 9. Differentiation potential of cells in 3D suspension culture. Panel A. hiPSCs grown in stirred-tank bioreactors can be differentiated into beta cells using a directed differentiation protocol. Undifferentiated cells first assemble from single cells to pluripotent 3D spheres, then cells progress to definitive endoderm (DE; SOX17 positive and Oct-4 negative). Cells then differentiate into pancreatic endoderm (PE; PDX1 and NKX6.1 positive), then to endocrine progenitors, and finally they mature into beta cells. Cells reach their highest concentration and expand during the first steps of differentiation, but stop expanding in the remaining steps of differentiation. More media (16–19 ml) is required to differentiate cells than to maintain undifferentiated cells. DE cells and PE cells were harvested and dissociated into single cells, re-seeded to pre-coated surfaces, and fixed 4 hr post dissociation. The cells were immunostained to detect Oct-4 or SOX17. PE cells were analyzed by immunostaining to detect PDX1/NKX6.1. For hiPSCs, endocrine progenitors, and beta cells, phase contrast images were taken on cells that assembled into 3D spheroids. **Panel B.** Beta cells in 3D suspension were dissociated into single cells, frozen and thawed, then cultured for one week in conventional 2D culture prior to flow cytometry analysis of insulin-positive cells. 51% of the cells were positive for insulin expression, indicating insulin-expressing cells can successfully differentiate in 3D culture.

Conclusions

- Cellartis DEF-CS xeno-free medium is defined and completely free of human- and animal-derived components.
- This culture system is optimized for feeder-free culturing of hPSCs in 2D-monolayer or 3D-suspension-culture formats.
- hPSCs cultured in this system in 2D express the expected stem cell markers, remain diploid, and differentiate into cell types from the three germ layers.
- This culture system has been optimized for the oxygen concentration that facilitates the highest levels of pluripotency in 3D formats.
- The system allows for robust and scalable hiPSC production in a perfusion bioreactor, facilitating the use of hiPSCs for research and large-scale clinical applications.
- Cells grown in 3D suspension can efficiently differentiate into beta cells, although cells only expand through the early progenitor phase of differentiation.

References

Abecasis, B. *et al.* Expansion of 3D human induced pluripotent stem cell aggregates in bioreactors: Bioprocess intensification and scaling-up approaches. *J. Biotechnol.* **246,** 81–93 (2017).