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Research Article

A reproducible and versatile system for the dynamic expansion of human pluripotent stem cells in suspension

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Reprogramming of patient cells to human induced pluripotent stem cells (hiPSC) has facilitated in vitro disease modeling studies aiming at deciphering the molecular and cellular mechanisms that contribute to disease pathogenesis and progression. To fully exploit the potential of hiPSC for biomedical applications, technologies that enable the standardized generation and expansion of hiPSC from large numbers of donors are required. Paralleled automated processes for the expansion of hiPSC could provide an opportunity to maximize the generation of hiPSC collections from patient cohorts while minimizing hands-on time and costs. In order to develop a simple method for the parallel expansion of human pluripotent stem cells (hPSC) we established a protocol for their cultivation as undifferentiated aggregates in a bench-top bioreactor system (BioLevitator™). We show that long-term expansion (10 passages) of hPSCs either in mTeSR or E8 medium preserved a normal karyotype, three-germ-layer differentiation potential and high expression of pluripotency-associated markers. The system enables the expansion from low inoculation densities (0.3×10^5 cells/mL) and provides a simplified, cost-efficient and time-saving method for the provision of hiPSC at midi-scale. Implementation of this protocol in cell production schemes has the potential to advance cell manufacturing in many areas of hiPSC-based medical research.

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1 Introduction

The development and broad implementation of human pluripotent stem cells as tools for disease modeling and compound screening would greatly benefit from cell culture technologies that enable automation, parallelization, simplification and cost reduction of stem cell cultivation. To meet those specifications, the manufacturing field of automated cell cultivation often uses highly specialized bioreactors. Recently, an increasing number of publications described the expansion of hPSC in suspension under dynamic conditions [1]. Initially, cultivation of hPSC on microcarriers was necessary to support adherent growth and self-renewal in a suspension culture system [2–4]. The use of microcarriers was mainly due to the fact that culture conditions were not defined and insufficient to promote self-renewal under non-adherent culture conditions. Since then, an increasing number of defined culture systems have been developed that eventually led to major improvements in terms of standardization, scalability and robustness. Currently, hPSC can be routinely expanded as single cell suspensions under feeder-free conditions using a variety of commercially available undefined (e.g. Matrigel®, Geltrex®) or defined (e.g.

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Abbreviations: E8, essential-8 medium; **hESC**, human embryonic stem cells; **hiPSC**, human induced pluripotent stem cells; **hPSC**, human pluripotent stem cells; **SNP**, single nucleotide polymorphism

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Laminin, Vitronectin, CELLstart™) matrix proteins as well as serum-free, defined media (e.g. mTeSR™1, TeSR™2, NutriStem™, StemPro®) [5–11]. Quite recently, Chen and colleagues formulated a cell culture system in which all protein reagents for media, attachment surfaces, and splitting are chemically defined. The so-called Essential-8 (E8) medium can facilitate research use and clinical applications of hPSC [12, 13]. However, broad implementation of such cell culture systems is often slowed down due to high costs or lack of validation studies. Nevertheless, these technical improvements and the desire for large-scale and cost efficient expansion methodologies have propelled the development of suspension culture systems for hPSCs. As early as 2010, Steiner et al. and Amit et al. independently published aggregate-based expansion of hPSC in suspension based on proprietary media compositions [14, 15]. Also in 2010, Zweigerdt and colleagues described a straightforward culture protocol for hPSC in suspension based on mTeSR-1 medium [16–18]. This was followed by a number of publications moving hPSC cultures from two-dimensional (2D) to three-dimensional (3D) systems as cell aggregates, alginate microencapsulates or encapsulated in thermoresponsive hydrogels [19–23].

The bioreactor systems used include shaking flasks [24], rocking 1 L or 20 L perfusion bag bioreactors (WAVE™), shaken Erlenmeyer flasks [25] and different spinner flask systems ranging from modular set-up (DASGIP®, [26]) of 100 mL to several liter sized bioreactors (Brunswick™, [24]). Most of those systems required large media volumes, experienced operators, advanced software training or tedious and cumbersome priming and preparation of bioreactor equipment or sampling. Furthermore, they are predominantly suited for large scale production in a clinically relevant context, whereas generation of multiple patient-specific hiPSC lines for biobanking and disease modeling requires primarily parallelized small scale production. These applications would greatly benefit from a midi-scalable bioreactor approach that enables the implementation of hiPSC expansion and differentiation protocols at low costs and in parallel experimental settings.

Here, we present a protocol for the robust aggregate-based expansion of hPSC from single cells in a novel bench-top incubator and bioreactor hybrid with no impellers (BioLevitator, Hamilton). The BioLevitator is a benchtop incubator for microcarrier and suspension cell culture. It handles up to four independent 50 mL single use culture vessels in parallel that are located in a fully temperature and CO_2 controlled culture chamber. Microcarriers or cells are kept in suspension by gentle tube rotation, thereby providing optimal nutrient supply. Cell culture parameters including temperature, CO_2 partial pressure and tube rotation can be configured by the user via touch screen and saved for each individual vessel. Robust and reproducible high expansion rates (>5) for hiPSC and hESC were achieved in mTeSR-1 and E8 conditions. We were able to show serial passaging (10 passages), maintenance of pluripotency marker expression, differentiation potential and karyotypic stability. We expect this approach to serve as versatile and simple system for scalable, 3D-based expansion of hPSC for hiPSC-related research and biobanking.

2 Materials and methods

2.1 Human pluripotent stem cells

Experiments were performed using iLB-C-31f-r1 hiPSC generated from dermal fibroblasts and H9 hESC. Prior to suspension culture cells were maintained in mTeSR medium on matrigel or using standard feeder co-cultures. Cells were cultured in NUNC six-well plates and daily media changes were performed (2 mL/well). Cultures were passaged as single cells in the presence of 10 µM ROCK inhibitor (RI; Y-27632, Merck) using Accutase™ (PAA) treatment. Feeder co-cultivation of hESC was performed as described previously [27]. HESC were cultured in Knockout DMEM medium containing 20% serum replacement, 100 µM non-essential amino acids, 0.1 mM 2-β-Mercaptoethanol, 1 mM L-glutamine and 4 ng/mL human bFGF (all from Life Technologies). Culture media were changed every day, and hESC were passaged every four to five days by 1 mg/mL collagenase type IV (Life Technologies) treatment.

2.2 3D hPSC culture

HPSC were harvested from adherent mTeSR or E8 cultures by Accutase (1 mg/mL) treatment for 5–10 min. Cell culture dishes were rinsed with DMEM/F12 and cells were centrifuged at 235 ×*g* for 4 min. Subsequently, cells were resuspended in an appropriate volume of mTeSR or E8 medium in the presence of 10 µM Rock inhibitor and inoculated (day 0) in the bioreactor. Inoculation was performed in a final volume of 10 mL with an inoculation density of 0.33 to 2.0 × 10^5 cells/mL. Subsequently, the vessel was transferred to the BioLevitator bioreactor system. Cultivation was started in a volume of 10 mL with daily additions of either 5 mL mTeSR (day 1–3) or E8 (day 1–3). For harvesting and passaging of hPSC suspension cultures, the vessels were transferred under a laminar flow hood and aggregates were allowed to settle down for 5–10 min. The supernatant was aspirated and the remaining cell suspension was transferred to a 15 mL conical tube and centrifuged at 104 ×*g* for 3 min. After aspiration of the supernatant, the cell pellet was incubated with 1 mL Accutase (1 mg/mL) for 10 min at 37°C. Subsequently, cells were triturated to a single cell suspension. Reaction was toned by the addition of 5 mL DMEM/F12. Cells were pelleted (235 ×*g* for 4 min) and used for 2D or

3D inoculation in the desired cell culture medium. In case bioreactor cultures were initiated from feeder co-cultures, hESC were initially detached by collagenase IV treatment, centrifugation at 104 ×*g* for 3 min and resuspended in Accutase in order to obtain a single cell suspension as described.

2.3 Immunofluorescence analysis

For immunofluorescence cells were fixed with 4% PFA. Subsequently cells were incubated with the following primary antibodies in the presence of 0.1% Triton X-100: monoclonal mouse IgG anti TUBB3 (BioLegend; MMS-435P; 1:1000), monoclonal mouse IgG anti SMA (Covance; 1:200), mouse anti IgG AFP (BD Bioscience; 1:200), polyclonal rabbit IgG anti AFP (Hiss Diagnostics, 1:500), polyclonal rabbit IgG anti Pax-6 (Hiss Diagnostics), polyclonal rabbit or rabbit IgG anti Oct-4 (Santa Cruz; 1:400). For detection of cell surface markers, cells were incubated with monoclonal mouse IgM Tra1-60 (R&D Systems; 1:2000) and mouse anti SSEA-1 (ESTOOLS; 1:300). Antigens were visualized using appropriate fluorophore-conjugated secondary antibodies: Goat anti-mouse IgG Alexa 488 (Life Technologies; A11001; 1:1000), goat anti-rabbit IgG Alexa 488 (Life Technologies; A11008; 1:1000) and Goat anti-mouse IgG Alexa 555 (Life Technologies; A21424; 1:1000), goat anti-rabbit IgG Alexa 555 (Life Technologies; A21429; 1:1000). Nuclei were counterstained with DAPI (Sigma; 1:10 000) in 10 mM sodium hydrogen carbonate, pH 8.2 for 2 min. Images were acquired using an epifluorescence microscope (Observer Z1) equipped with the ApoTome technology from Zeiss.

2.4 Aggregate sectioning

Aggregates were fixed in 4% PFA for 20 min at RT and washed twice with PBS. Subsequently, aggregates were incubated in 30% sucrose (Fisher Scientific) overnight at 4–8°C. Immediately before cutting, sucrose solution was replaced with Tissue-Tek® (Sakura Finetec), and aggregates were frozen on a 21°C cutting block. Aggregates were cut into 16 µm slices with a cryostat-microtome (Microtome CM3050 S, Leica). Slices were transferred to SuperFrost® glass slides and subjected to immunofluorescence analysis.

2.5 Flow cytometry

Samples were incubated with monoclonal mouse IgM Tra1-60 (R&D Systems; 1:2000) antibody and appropriate goat anti mouse IgG Alexa 488 (Life Technologies; A11001; 1:1000) secondary antibody. Unspecific binding and autofluorescence were controlled by omitting the primary antibody. Analysis was performed on a FACSCalibur™ analytic flow cytometer (BD). Data were analysed and arranged using FlowJo Analysis Software (Tree Star Inc.).

2.6 Three-germ layer differentiation assay

HPSC aggregates were differentiated under adherent conditions in EB medium composed of Knockout-DMEM medium, 20% FCS, 0.5% non-essential amino acids 1% L-glutamine (all from Life Technologies) for mesodermal differentiation, in MEF medium composed of DMEM high glucose medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids and 1% L-glutamine (all from Life Technologies) for endodermal differentiation and N2-medium composed of DMEM-F12 medium supplemented with 1% N2-Supplement (all from Life Technologies), 0.08% insulin (Sigma) and 0.0017 g/mL glucose (Sigma) for ectodermal differentiation. Media changes were conducted every three to four days and cells were differentiated for four weeks.

2.7 Genome-Wide Human SNP Array

DNA isolation was performed from whole cell samples using the QIAamp DNA Mini Kit (51304, Qiagen). Isolation was performed according to the manufacturer's instructions. Karyotype stability was confirmed by genome wide human small nucleotide polymorphism (SNP) evaluation from isolated DNA samples using the Genome-Wide Human SNP Array 6.0 (Affimetrix).

2.8 TaqMan® hPSC Scorecard™ Panel

Aggregates were cultivated in the BioLevitator either in mTeSR or E8 medium. On the final day, medium was changed to EB medium composed of Knockout-DMEM medium containing 20% FCS, 0.5% non-essential amino acids and 1% L-glutamine (all from Life Technologies). Differentiating cells were cultivated for two weeks in the bioreactor, medium was changed every other day. Isolation of total RNA from two weeks differentiated hiPSC cells was performed according to the manufacturer's instructions using peqGOLD TriFast reagent (Peqlab). The RNA concentration was measured using the Nanodrop Calibrator (Thermo Fisher Scientific). Relative levels of gene expression were analyzed using the new TaqMan® hPSC Scorecard™ Panel (Life Technologies) and the ViiA7 real time PCR system (Life Technologies). The preparation and evaluation of the Scorecard was performed according to manufacturer's instructions.

2.9 Metabolic analysis

Glucose and lactate concentrations were measured using the YSI 2700 biochemistry analyzer (Kreienbaum).

2.10 Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test.

3 Results

3.1 Establishment of homogenous 3D hPSC cultures under dynamic conditions in mTeSR-medium

In order to establish a 3D culture system for hPSC, a parallel benchtop bioreactor system (BioLevitator) consisting of four independent vessels (LeviTubes) with a maximal working volume of 50 mL each was explored. For initial assessment and validation of inoculation densities, culture period and feeding strategy, one hiPSC and one hESC line were used. To support a robust production of cells, hiPSC were directly transferred from 2D mTeSR cultures as single cells to dynamic suspension culture in the bioreactor. All experiments were performed at 30 mL maximal culture volume. However, culture volumes of up to 50 mL were found to be practicable (data not shown). Aiming at high efficiencies, i.e. high cell yields from initial low inoculation densities, the expansion rate was calculated after four days of cultivation (Supporting information, Fig. S1A). A maximal fold increase of 4.9 ± 2.1 was achieved by inoculating 7.5×10^4 cells/mL. Flow cytometry analysis was used to determine the optimal culture period under the established feeding regime. As shown by flow cytometry stable and high Tra1-60 (>90%) expression could be achieved up to day 4 after inoculation in the bioreactor (Supporting information, Fig. S1B). According to these analyses, a culture period of four days after inoculation (day 1–4) yielded optimal results in terms of cell quality and quantity. Frequently, 80% media exchanges are performed on a daily basis in order to maintain hPSC in an undifferentiated and self-renewing state in bioreactor suspension culture [17, 21, 25]. With the intention to develop a more cost efficient and less laborious feeding strategy, we tested the addition of media as an alternative method. A comparative analysis showed that a daily addition of 5 mL mTeSR medium is sufficient to maintain hPSC self-renewing and highly Tra1-60 positive for up to 4 days after inoculation (Supporting information, Fig. S1B and S1C). The observed decrease of Tra1-60 expression from day 4 onwards corresponded to an increase in lactate production and glucose consumption (Supporting information, Fig. S1D). Based on these results, a culture period of 4 days (day 1–4) and a daily media addition (day 1–3) of 5 mL was chosen for low inoculation densities in the BioLevitator.

3.2 MTeSR-medium supports long-term expansion of hPSC in dynamic 3D cultures

A specific focus of our study was the development of a bioreactor-based process that could substitute conventional adherent 2D cultivation in order to save material costs, reduce hands-on time and to standardize the process of hPSC cultivation. Hence, serial passaging (10 passages) of hPSCs was performed in the bioreactor. Initially, hESC and hiPSC lines were expanded in mTeSR using Matrigel as matrix protein. To initiate suspension cultures, hPSC were harvested from cell culture plates by Accutase treatment, inoculated in the bioreactor at indicated cell densities at day 0, expanded for four days, passaged as a single cell suspension, and re-inoculated in the same conditions. We successfully produced size-specific aggregates through optimization of bioreactor hydrodynamic conditions by adapting the rotation speed and rotation period. HiPSC formed homogeneous round spheroids with an average diameter of 140 ± 36 µm at each investigated passage (Fig. 1A). In order to determine the growth capabilities of hPSCs, cumulative net cell numbers were determined at each passage during long-term cultivation in the bioreactor. HPSC maintained their typical exponential growth pattern, and average expansion rates (hESC: 5.42 ± 2.87; hiPSC: 5.09 ± 3.20) for both investigated cell lines turned out to be equivalent (Fig. 1B and 1C; Supporting information, Fig. S2B and S2C). The final cell yields per mL obtained under 2D and 3D conditions, were shown to be similar under similar inoculation densities, thus confirming the supportive growth condition provided by the suspension culture protocol (Fig. 1D; Supporting information, Fig. S2D). In accordance, flow cytometry and immunocytochemical analysis of sliced hPSC aggregates and aggregates that were switched to 2D conditions revealed homogenous expression of the pluripotency-associated markers Tra1-60, Oct-4 and Nanog in hESC and hiPSC cultures (Fig. 1E and 1F; Supporting information, Fig. S2A and S2F). In contrast, no cells were found to be positive for the germ layer markers AFP, SMA or Pax-6 (Fig. 1F; Supporting information, Fig. S2E and S2F). Finally, to validate the obtained cultures with respect to pluripotency and genomic integrity an EB differentiation assay as well as SNP analyses were performed after 10 consecutive passages in the bioreactor. Both, hESC and hiPSC cultures were shown to maintain three-germ-layer differentiation potential as well as genomic integrity (Fig. 1G and 1H; Supporting information, Fig. S2G and S2H).

The vast majority of protocols for differentiating hPSC towards specific somatic cell types are based on 2D culture conditions. Thus, for straightforward implementation of the presented suspension culture paradigm into established 2D differentiation workflows conversion from 3D to 2D conditions and vice versa would be desirable. As shown in Fig. 2, hPSCs cultured alternately under 2D and 3D bioreactor conditions maintained their typical colony-like morphology and high levels of Tra1-60 (>90%), Oct-4 and Nanog expression. Finally, we asked, whether the developed protocol supports expansion of hiPSC at higher inoculation densities. As shown in Fig. 3, hiPSC inoculated with 2.0×10^5 cells/mL yielded larger aggregates of 240 ± 60 µm at day 4 (Fig. 3A and 3B). However, the expansion rate for hiPSC was found to be similar to previ-

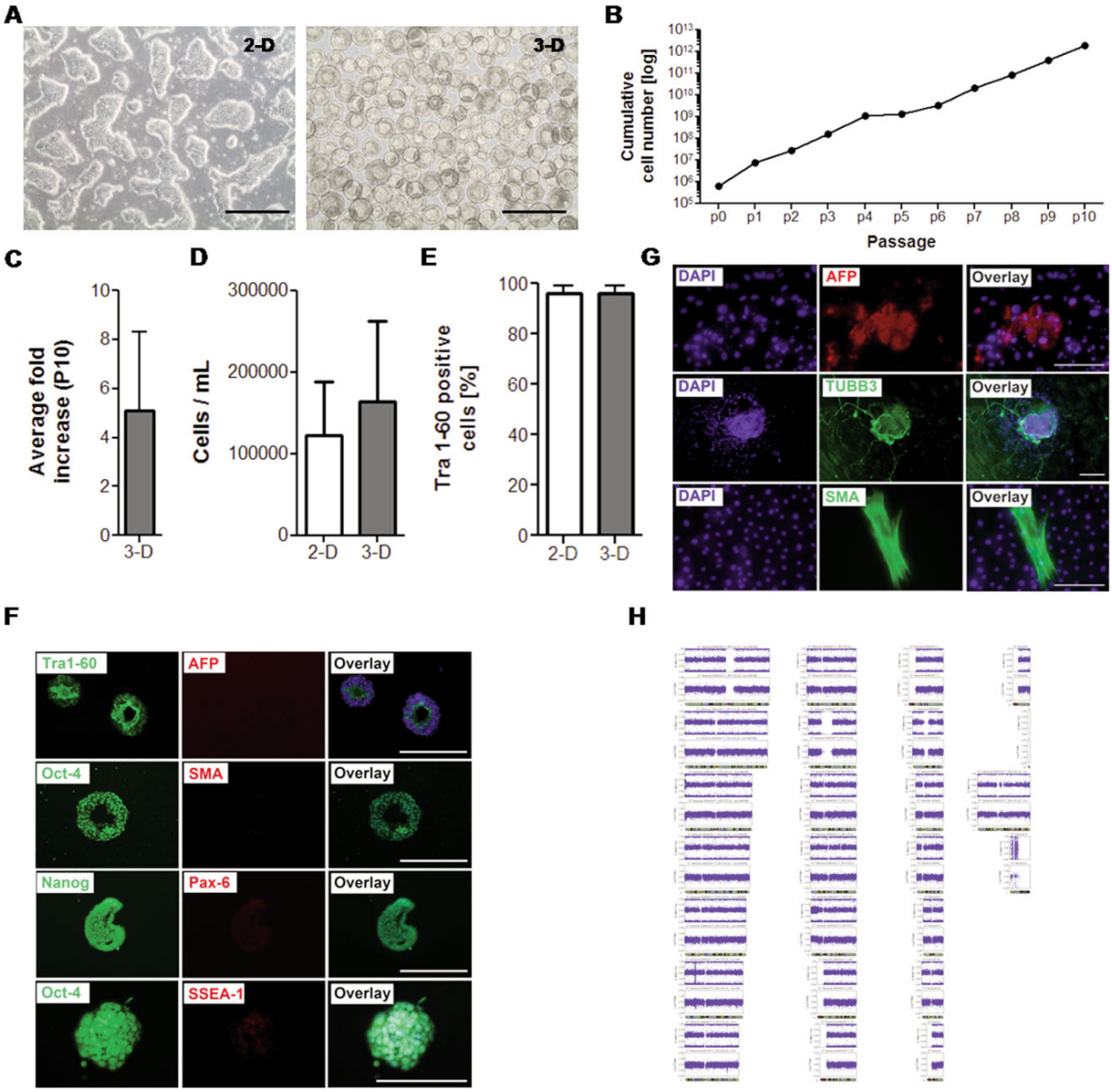

Figure 1. 3D long-term expansion of hiPSC in mTeSR-medium in the bioreactor. (**A**) Representative phase-contrast images of adherent hiPSC before inoculation (p0) and at day 4 of cultivation in the bioreactor (passage 1). (**B**) Mean cumulative cell numbers were calculated for 10 serial passages and blotted on a logarithmic scale (3D: $n = 2$). (**C**) The average fold increase was calculated as the mean fold increase of each passage ($n = 20$). (**D**) Mean cell yield under 2D and 3D (cells/mL) cell culture conditions normalized to the used media volumes (2D: $n = 4$, 3D: $n = 20$). (**E**) Flow cytometry analysis of the mean percentage of Tra1-60 positive cells during continuous 3D (p10) and 2D (p4) cultivation at each passage (2D: $n = 2$; 3D: $n = 2$). (**F**) At p10, aggregates were fixed and sectioned for immunofluorescence analysis of pluripotency-associated markers Tra1-60, Oct-4, and Nanog, the germ layer associated-markers AFP (endoderm), SMA (mesoderm), and Pax-6 (ectoderm) and the differentiation marker SSEA-1. Nuclei were counterstained with DAPI. (**G**). HiPSC from bioreactor cultures (p10) were subjected to differentiation under adherent conditions for 4 weeks. Subsequently, three-germ-layer differentiation potential was analyzed by immunofluorescence analysis with antibodies to AFP (endoderm), TUBB3 (ectoderm) and SMA (mesoderm). Nuclei were counterstained with DAPI. (**H**) Genetic integrity of hiPSC was confirmed by SNP analysis after p10 in the bioreactor. Scale bars: 200 µm. Abbreviations: p, Passage.

ous results from lower inoculation densities (4.3 ± 0.7) (Fig. 3C). Expression of the pluripotency-associated markers Tra1-60, Nanog and Oct-4 was maintained during bioreactor-based expansion as demonstrated by flow cytometry and immunocytochemical analysis (Fig. 3D and 3E).

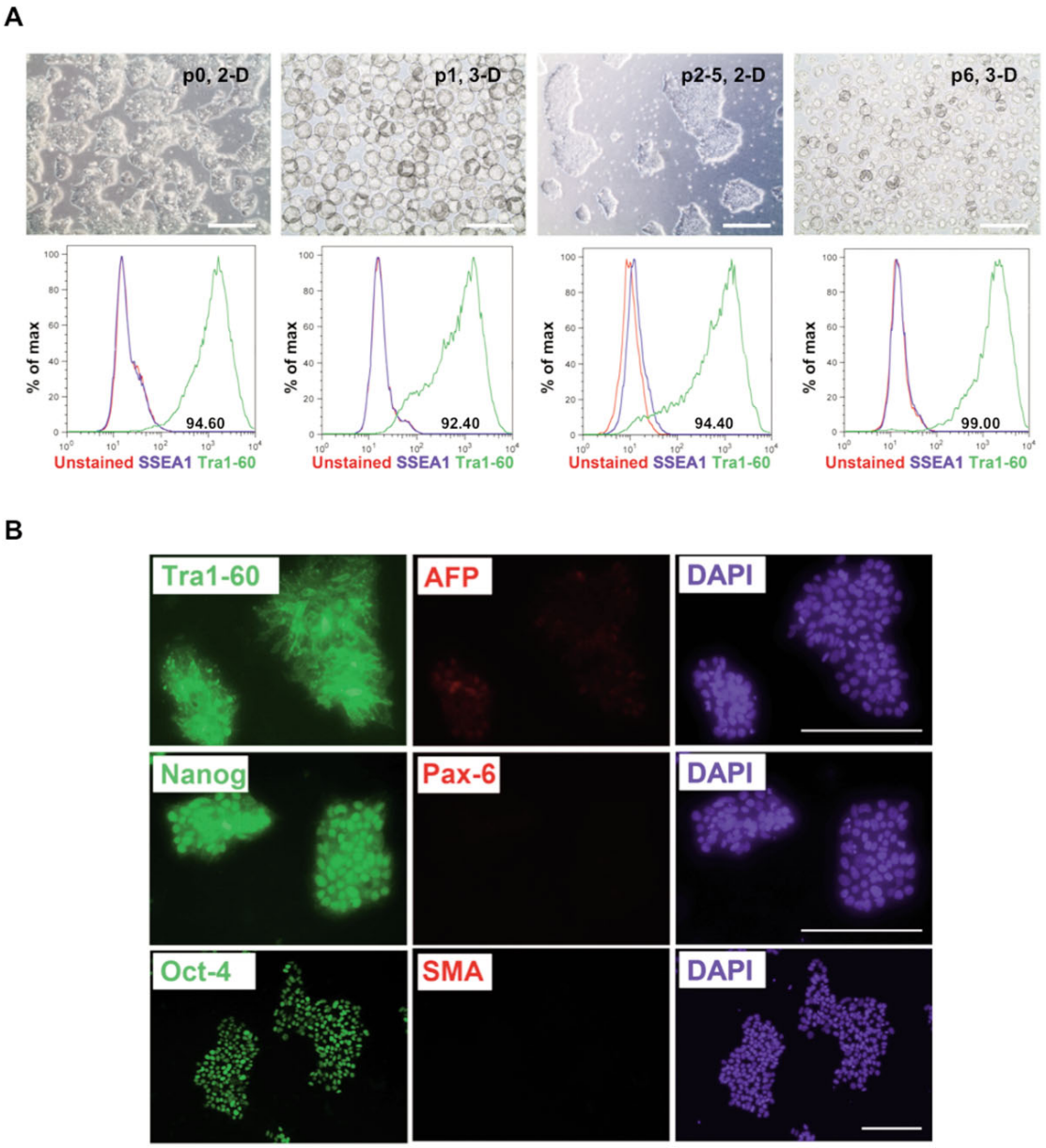

Figure 2. Seamless conversion between 2D and 3D cell culture conditions. HiPSC were harvested from adherent cultures (p0) and inoculated in the bioreactor (p1) as single cell suspension. After cultivation in the bioreactor, cells were harvested and replated as single cells into Matrigel coated cell culture dishes. After p5 under adherent conditions hiPSC cells were again inoculated in the bioreactor and cultured for additional two passages in suspension. (**A**) Representative phase contrast images of hiPSC under 2D and 3D culture conditions at indicated passages and corresponding flow cytometry analysis of Tra1-60 and SSEA1 positive cells. (**B**) HiPSC were replated after p1 in the bioreactor. Immunofluorescence analysis of the pluripotency-associated markers Tra1-60, Oct-4, and Nanog, the germ layer associated markers AFP, SMA, and Pax-6 three passages after replating. Nuclei were counterstained with DAPI (blue). Scale bars: 200 µm. Abbreviations: p, Passage.

Taken together, we established a protocol on the basis of mTeSR medium that allows parallel expansion and subcultivation of hPSC cultures in a bioreactor system. HPSC continuously expanded for more than one month in the bioreactor, remained pluripotent, karyotypically stable and highly proliferative. We could show that the developed protocol supports the undifferentiated growth of hiPSC for inoculation densities between 0.75 and 2.0×10^5 cells/mL. Most importantly, cultivation of hPSC can be easily switched between 3D and 2D conditions, thus facilitating implementation of this method into protocols including 2D differentiation steps.

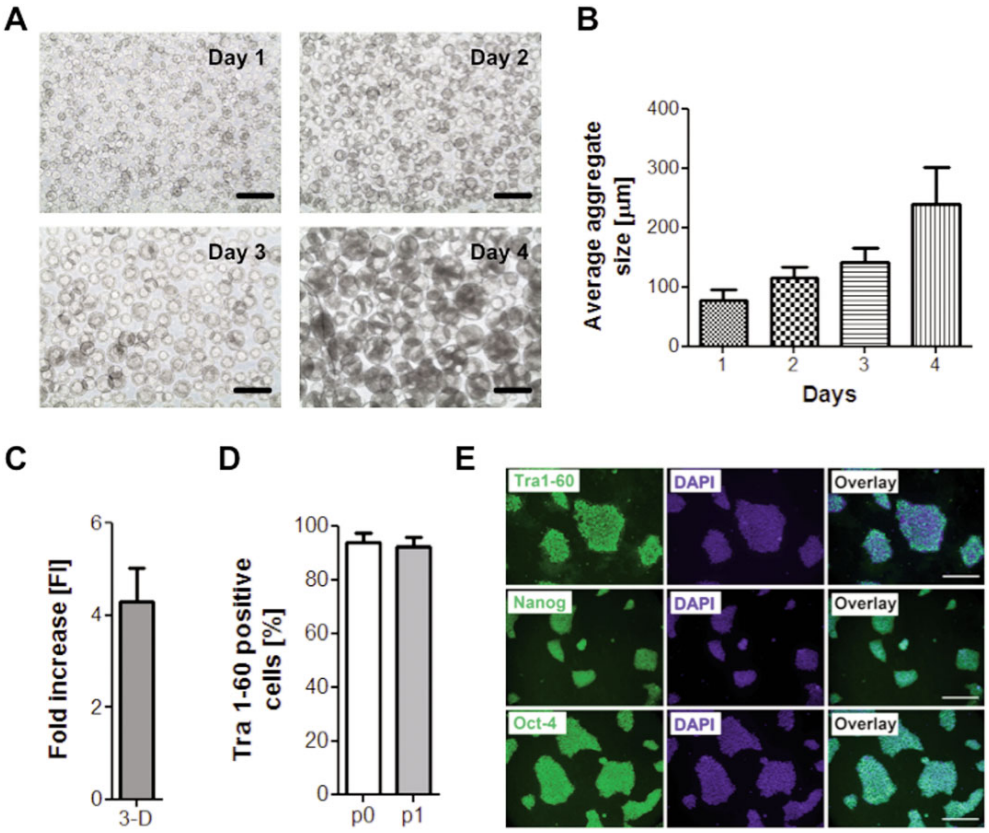

Figure 3. High density 3D expansion of hiPSC in mTeSR-medium in the bioreactor. (**A**) Representative phase-contrast images of bioreactor expanded hiPSC at indicated days of cultivation. (**B**) Graphical illustration of the average aggregate size measured at each day of cultivation. Images were analyzed using ImageJ software (National Institutes of Health, US) ($n = 3$). (**C**) The average fold increase was calculated after one passage ($n = 3$). (**D**) Analysis of the mean percentage of Tra1-60 positive cells during 3D cultivation performed by flow cytometry ($n = 3$). (**E**) HiPSC were replated after one passage in the bioreactor and analyzed by immunofluorescence with antibodies to Tra1-60, Oct-4, and Nanog. Nuclei were counterstained with DAPI. Scale bars: 200 µm. Abbreviations: p, Passage.

3.3 Protocols for mTeSR-based dynamic 3D hPSC cultures can be translated to chemically defined E8 medium

Recently the development of a significantly improved hiPSC culture medium, E8, which contains completely defined and xeno-free components, was described [12, 13]. Based on this novel hPSC medium, Wang and colleagues published a suspension culture system in a stirred bioreactor, which enables passaging and differentiation of hPSCs [21]. We performed a comparative analysis of mTeSR and E8 bioreactor-based suspension cultures under similar conditions.

In accordance with our previous results, similar expansion rates were obtained under both media conditions (Fig. 4A). In order to challenge the system, we additionally investigated inoculation densities of 0.33×10^5 cells/mL. Interestingly, in average an up to 20-fold increase was obtained for mTeSR cultures, and a more than 14-fold increase was observed under E8 conditions. In contrast to mTeSR suspension cultures, Tra1-60 expression decreased from 88.4 ± 1.7% at day 3 to 61.8 ± 29.3% at day 4 and further dropped to 36 ± 17% at day 5 in E8 medium (Fig. 4B). Metabolic analysis revealed different kinetics for glucose consumption and lactate production under E8 and mTeSR conditions (Fig. 4C). Glucose concentrations sharply dropped from day 4 (mTeSR) and day 3 (E8) onwards, while lactate concentrations increased accordingly. The observed metabolic activity correlated well with the decrease of Tra1-60 expression under both media conditions. Thus, translating the established mTeSR conditions to E8 medium is feasible but demands a reduced culture period.

Under both media conditions homogenous aggregates with similar morphological characteristics and of similar size 138 (E8: 138 ± 24 µm; mTeSR: 140 ± 36 µm; Fig. 4D and 4E) developed. Expression of the pluripotency-associated markers Tra1-60, Nanog and Oct-4 was maintained during bioreactor-based expansion as

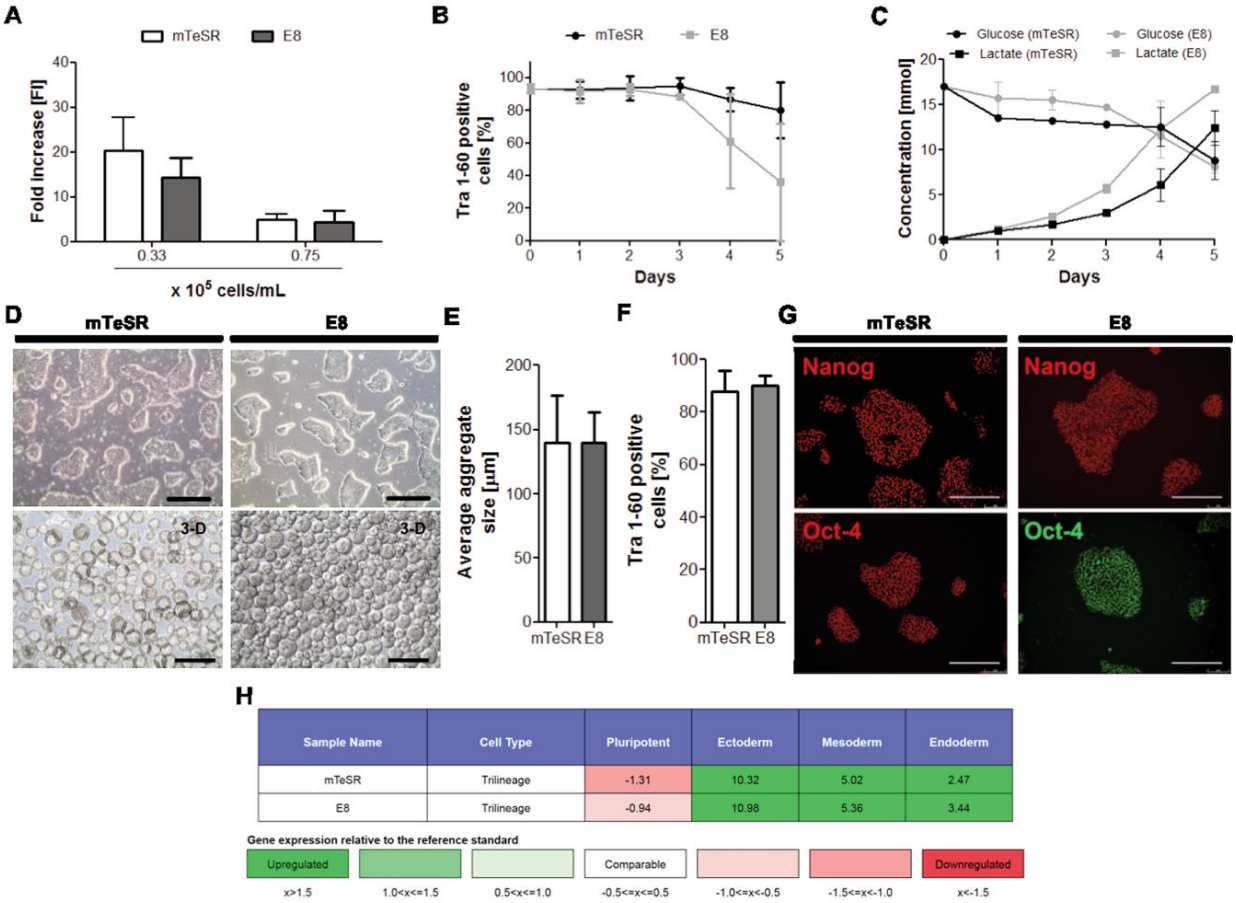

Figure 4. Comparative analysis of mTeSR and E8-based bioreactor cultures of hiPSC. Adherent mTeSR hiPSC cultures were harvested and inoculated at similar volumes and inoculation densities in either mTeSR or E8 medium as single cell suspension. (**A**) Average fold increase under 3D culture conditions was calculated for two different inoculation densities at the last day of cultivation ($n = 3$). (**B**) Daily flow cytometry analysis of Tra1-60 positive cells in E8 and mTeSR-based 3D bioreactor cultures ($n = 3$). (**C**) Metabolic activity of cells was monitored on daily basis by glucose consumption and lactate production ($n = 3$). (**D**) Representative phase contrast images of hiPSC aggregates cultivated either in mTeSR or E8 medium in the bioreactor. (**E**) Average aggregate size in mTeSR and E8-based bioreactor cultures was calculated by measuring 140 aggregates per condition with Image J ($n = 3$). (**F**) Mean percentage of Tra1-60-positive cells in mTeSR or E8-based hiPSC bioreactor cultures was determined by flow cytometry ($n = 3$). (**G**) Immunocytochemical analysis of the pluripotency-associated markers Oct-4 and Nanog after replating onto Matrigel in mTeSR or E8 medium. (**H**) TaqMan® hPSC Scorecard panel analysis of hiPSCs expanded either in mTeSR or E8 medium in the bioreactor. Following expansion in either of the two media, cells were differentiated in the bioreactor in EB medium. After 14 days total RNA was isolated to perform a TaqMan® hPSC Scorecard analysis according to the manufacturer's instructions. The manufacturer's proprietary algorithm predicts pluripotency and lineage bias based on a panel of 94 genes. Expression pattern blot of the specified pluripotency-associated and three-germ-layer (tri-lineage)-associated. Under both conditions pluripotency-associated gene expression was down-regulated and germ layer-associated gene expression was up-regulated in a similar manner. Scale bar, 200 µm.

demonstrated by flow cytometry and immunocytochemical analysis (Fig. 4F and 4G).

Next we evaluated the E8-based protocol for its ability to be translated to serial passaging in order to meet the requirements of scale-up processes and long-term cultivation in the bioreactor. To challenge our E8-based hPSC suspension culture system, we used a medium-quality batch of hESC maintained on feeder co-cultures in Knock-Out DMEM medium as a starting material (Supporting information, Fig. S3A). Such co-culture systems persist from the early days of hPSC derivation and represent the most widely used system for maintaining hPSC [15]. While feeder layers have proved to be a robust surface for long-term culture of hPSCs, there are undefined components secreted in the media causing batch-to-batch variability, which often leads to increased levels of spontaneous differentiation. Flow cytometry analysis of the hESC batch revealed a high level of spontaneous differentiation indicated by low numbers (≈48%) of Tra1-60 positive cells (Supporting information, Fig. S3B). HESC were enzymatically harvested from feeder cells by collagenase treatment (Supporting information, Fig. S3A).

Subsequently, the obtained hESC clumps were triturated to single cells by Accutase treatment, inoculated in the bioreactor, expanded for three to four days, passaged as a single cell suspension, and re-inoculated at the same conditions. The cells were maintained in the bioreactor for 37 days with 10 passages. Monitoring of Tra1-60 expression during long-term cultivation revealed a gradual improvement of the culture documented by an increase of Tra1-60 expression to 92% at passage 10. In order to determine the growth capabilities of hESC, cumulative net cell numbers were determined at each passage during long-term cultivation in the bioreactor (Supporting information, Fig. S3C). Growth analysis showed no significant differences for the obtained average fold expansion rates in mTeSR (5.42 ± 2.87) and E8 (7.1 ± 3.4) (Fig. 1C; Supporting information, Fig. S3D). Homogenous expression of the pluripotency-associated markers Oct-4, Nanog and Tra1-60 was observed throughout the aggregates as well as in suspension cultures that were switched to 2D conditions (Supporting information, Figure S3E and S3F). Furthermore, three-germ-layer differentiation potential after long-term bioreactor-mediated expansion was maintained as shown by in vitro differentiation of hESC (Supporting information, Fig. S3G).

To further assess pluripotency and three-germ-layer differentiation potential of hiPSCs propagated as suspension cultures in either E8 or mTeSR, the TaqMan® hPSC Scorecard Assay (Life Technologies) was performed. The assay quantitatively confirms trilineage differentiation potential on the basis of 94 predefined TaqMan® Gene Expression assays. Total RNA was isolated from 14 days differentiated hiPSC aggregates. Increased expression profiles of markers of all three-germ-layers could be detected in both conditions, with highest up-regulation of genes associated with the ectodermal lineage (Fig. 4H; Supporting information, Fig. S4). Heterogeneous differentiation of EBs is a significant challenge described previously and media conditions have been shown to bias the differentiation potential of hPSC [28, 29]. Scorecard values showed that the expression of pluripotency-associated markers was down-regulated confirming efficient differentiation of the hiPSC under both conditions. Most importantly the analysis demonstrated that trilineage differentiation potential was not fundamentally biased by the choice of media.

4 Discussion

Increasingly, researchers, biotech and pharma companies are implementing pluripotent stem cells as standard tools for basic research, drug development and toxicity screening [30–32]. Upon appropriate differentiation, hiPSC can be used to study both normal and pathologic human tissue development in vitro, to investigate disease mechanisms, and for the development of novel therapeutic agents or patient-specific cell replacement therapies. This increasing scientific and economic interest in hiPSC technology has also triggered a number of global large-scale hiPSC biobanking projects [33]. The intended exploitation of hiPSC technology for the development of new therapies for multiple diseases, including diseases with complex traits and with varying genetic and environmental contributions to disease manifestation presents unique logistical challenges. To provide a resource for such iPSC-based studies, large patient cohorts need to be reprogrammed and analyzed to enable clinically meaningful assays to be designed and interpreted. Thus biobanking, disease modeling and cell therapy have placed increasing demands on hiPSC technology. In particular, the high-throughput derivation of hiPSC lines from large patient cohorts and their subsequent expansion requires cell culture technologies that facilitate simple, robust, cost-efficient and parallel propagation of hiPSC. Here we developed a bioprocess for the semi-automated, parallel expansion of hPSCs as 3D aggregates in a bench top bioreactor system. We could show that hPSC can be expanded as pluripotent aggregates for extended periods of time either in mTeSR or E8 medium. High expansion rates were obtained for both investigated media conditions, even from very low inoculation densities. We showed seamless transition between 2D and 3D culture conditions, which enables the straightforward implementation of 2D and 3D differentiation protocols for downstream applications. In particular, 3D cell culture systems have recently experienced a rapid development and gained enormous scientific interest. A number of 3D differentiation protocols have been shown to produce cells with significantly increased functional maturation and more complex phenotypes when compared to conventional 2D systems [24, 34–37]. Such 3D models can be propagated as bioreactor cultures and are thus ideally suited for being implemented into the established suspension culture protocol [24, 35]. As a result of scientific advances since the discovery of hESC in 1998, hPSC have reached the point of utility for clinical applications [38, 39]. Such clinical applications require large cell numbers produced in a chemically defined condition by robust, reproducible and economic methods. Here we showed the translation of hiPSC suspension cultures to chemically-defined and xeno-free E8 culture conditions, thereby broadening the spectrum of potential applications of the protocol for clinical use.

In conclusion, we present a reproducible approach for the simple, parallel, economical, and scalable expansion of hPSC to meet the needs of hiPSC-based medical research.

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All co-authors of the manuscript have agreed to the submission of the manuscript. Oliver Brüstle is co-founder of and has stock in LIFE & BRAIN GmbH. All other authors declare no financial or commercial conflict of interest.

5 References

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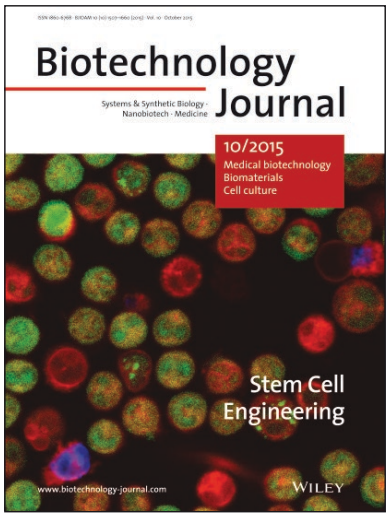

Cover illustration

Special Issue: Stem Cell Engineering. This special issue, edited by Joaquim Cabral and Sean Palecek, contains articles on culturing stem cells, their differentiation as well as on imaging techniques. The cover shows primary hematopoietic stem cells and lineage-committed cells from the bone marrow stained with Hoechst 33342 (blue), CMFDA (green) and CMTPX (red). Stem cells (stained blue) are in contact with neighboring niche cells (stained red and green) that regulate stem cell fate decisions. Image provided by Harley et al.

Biotechnology Journal – list of articles published in the October 2015 issue.

Research Article

Enzymatic passaging of human embryonic stem cells alters central carbon metabolism and glycan abundance

Mehmet G. Badur, Hui Zhang and Christian M. Metallo

http://dx.doi.org/10.1002/biot.201400749

Research Article

Spatial and temporal control of cell aggregation efficiently directs human pluripotent stem cells towards neural commitment

Cláudia C. Miranda, Tiago G. Fernandes, Jorge F. Pascoal, Simone Haupt, Oliver Brüstle, Joaquim M.S. Cabral and Maria Margarida Diogo

http://dx.doi.org/10.1002/biot.201400846

Research Article

Microarray profiling of preselected CHO host cell subclones identifies gene expression patterns associated with increased production capacity

Eva Harreither, Matthias Hackl, Johannes Pichler, Smriti Shridhar, Norbert Auer, Paweł P. Łabaj, Marcel Scheideler, Michael Karbiener, Johannes Grillari, David P. Kreil and Nicole Borth

http://dx.doi.org/10.1002/biot.201400857

Research Article

Compartment-specific metabolomics for CHO reveals that ATP pools in mitochondria are much lower than in cytosol

Jens-Christoph Matuszczyk, Attila Teleki, Jennifer Pfizenmaier and Ralf Takors

http://dx.doi.org/10.1002/biot.201500060

Research Articel

The stage-specific in vitro efficacy of a malaria antigen cocktail provides valuable insights into the development of effective multi-stage vaccines

Holger Spiegel, Alexander Boes, Robin Kastilan, Stephanie Kapelski, Güven Edgue, Veronique Beiss, Ivana Chubodova, Matthias Scheuermayer, Gabriele Pradel, Stefan Schillberg, Andreas Reimann and Rainer Fischer

http://dx.doi.org/10.1002/biot.201500055