Periodic Harvesting of Embryonic Stem Cells from a Hollow-Fiber Membrane Based Four-Compartment Bioreactor

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Different types of stem cells have been investigated for applications in drug screening and toxicity testing. In order to provide sufficient numbers of cells for such in vitro applications a scale-up of stem cell culture is necessary. Bioreactors for dynamic three-dimensional (3D) culture of growing cells offer the option for culturing large amounts of stem cells at high densities in a closed system. We describe a method for periodic harvesting of pluripotent stem cells (PSC) during expansion in a perfused 3D hollow-fiber membrane bioreactor, using mouse embryonic stem cells (mESC) as a model cell line. A number of 100×10^6 mESC were seeded in bioreactors in the presence of mouse embryonic fibroblasts (MEF) as feeder cells. Over a cultivation interval of nine days cells were harvested by trypsin perfusion and mechanical agitation every second to third culture day. A mean of 380×10^6 mESC could be removed with every harvest. Subsequent to harvesting, cells continued growing in the bioreactor, as determined by increasing glucose consumption and lactate production. Immunocytochemical staining and mRNA expression analysis of markers for pluripotency and the three germ layers showed a similar expression of most markers in the harvested cells and in mESC control cultures. In conclusion, successful expansion and harvesting of viable mESC from bioreactor cultures with preservation of sterility was shown. The present study is the first one showing the feasibility of periodic harvesting of adherent cells from a continuously perfused four-compartment bioreactor including further cultivation of remaining cells. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2015
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Introduction

Pluripotent stem cells (PSC) are a promising source for production of different cell types for applications not only in cell-based therapies but also in drug screening and toxicity testing.[1] In addition to embryonic stem cells (ESC), initially generated from mice[2,3] and later from humans,[4] human induced pluripotent stem cells (hiPSC) have been introduced as a promising alternative to ESC.[5,6] In order to provide adequate cell numbers at a well defined quality for *in vitro* or *in vivo* applications a scale-up of PSC-production under good manufacturing practice (GMP) conditions is necessary.

Current two-dimensional (2D) petri dish and flask techniques provide static open systems with discontinuous medium exchange; they are labor intensive and impractical for handling larger cell numbers. Bioreactor technologies offer the option to scale-up PSC production. For example, in stirred tank bioreactors PSC can be expanded in aggregates or grown on microcarriers. A major challenge of these culture formats is the prevention of shear stress caused by agitation.[7] Another problem, the time-consuming preadaptation passages of cell aggregates in static suspension cultures before transfer to dynamic suspension cultures,[8] was solved by Olmer et al.[9] by inoculating single cell suspensions supplemented with Rho-associated coiled-coil kinase (ROCK)-inhibitor. Furthermore, the accumulation of toxic metabolites can be prevented by applying continuous medium perfusion together with a cell retention device.[10–12]

An alternative approach to address these problems is the usage of hollow-fiber based bioreactor technologies that enable continuous perfusion and oxygenation of cultured cells

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without shear stress and allow for increased cell densities at physiological levels.[13,14] The culture platform used in the present study consists of two independent bundles of hollow-fiber membranes for counter-current medium perfusion interwoven with one bundle of oxygenation capillaries for gas exchange.[15] The integration of oxygenation fibers enables enhanced mass exchange and reduced gradient distances as compared to typical two-compartment bioreactors with external oxygenation.[16] The capillaries also provide a scaffold for the attachment of adherent cells like PSC. Culture experiments performed with different scale variants of the described bioreactor, obtained by multiplying the capillary units, provided comparable results in differently sized bioreactors.[15(p552)] The technology allows for expansion and follow-up differentiation[17] in a closed system with constant monitoring of culture parameters according to GMP requirements.

Successful short-term expansion of mouse embryonic stem cells (mESC) over three days in this bioreactor was shown.[18] However, prolonged culture resulted in spontaneous differentiation of mESC[19] or human embryonic stem cells (hESC)[20] into different cell types and tissues. An increase in colony size and cell density can cause spontaneous differentiation.[21] In addition, the diffusion of nutrients and oxygen into the center of clusters is impaired when the aggregates exceed a critical size, potentially leading to central cell necrosis.[18(p46)]

We hypothesize that enzymatic dissociation of PSC aggregates can be used for cell harvesting from the bioreactor system. In a further aspect, enzymatic treatment could prevent the cell differentiation by reducing the cell density and thus permit prolonged expansion of PSC in the bioreactor.

In this study, an enzymatic-mechanical method for long-term cultivation and sterile harvesting of adherently growing mESC from hollow-fiber membrane based four-compartment bioreactors was established, using mESC as a model cell line. For testing the possibility of periodic harvesting, cells were harvested every second to third culture day over a cultivation interval of nine days. Between the harvesting steps, the remaining cells were further cultured in the bioreactor to monitor their proliferation behavior. Harvested cells were characterized with respect to their growth potential and differentiation state.

Materials and Methods

Bioreactor technology

The four-compartment bioreactor consists of three independent but interwoven hollow-fiber capillary systems that serve for counter-current medium perfusion (two medium compartments) and decentralized oxygenation (gas compartment) of the cells which are located in the extracapillary space (cell compartment) (Figure 1a). A detailed description of the technology can be found elsewhere.[15(p550)] The laboratory-scale bioreactors used in this study have a cell compartment volume of 8 mL. Bioreactors are integrated into a perfusion device that is designed to provide controlled temperature, gas supply and medium perfusion conditions (Figure 1b). Bioreactors, tubing systems and perfusion devices were manufactured by Stem Cell Systems, Berlin, Germany.

Preparation of cells

Mouse ESC (strain 129/SVEV, CMTI-1, Millipore, Billerica, MA, USA) were cocultured with irradiated mouse embryonic fibroblasts (MEF) of the strain CD-1 (passage 5), provided by the Experimental Pharmacology and Oncology Berlin-Buch GmbH, Germany. Mouse ESC were seeded at a cell density of 3.5×10^4 cells/cm^2 into culture dishes coated with 0.1% gelatine (Millipore) and preseeded with inactivated MEF (gamma irradiation at 30 Gy) at a density of 3×10^4 cells/cm^2. Cells were cultured in T175 culture flasks (BD Falcon, San José, CA) or in lumox® multiwell plates suitable for immunofluorescence studies (Sarstedt, Nümbrecht-Rommelsdorf, Germany). Cells were cultured in mESC-medium based on DMEM (Biochrom, Berlin, Germany) containing 1,000 U/mL leukemia inhibitory factor (LIF; ESGRO, Millipore), 15% fetal calf serum (FCS; Millipore), 100 U/mL penicillin/100 μg/mL streptomycin (Biochrom), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), 7.14 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 0.1 mM nonessential amino acids and 0.1 mM nucleosides (both from Millipore). Every 2–3 days, cells were passaged enzymatically, using a 0.05% trypsin/0.02% EDTA solution (Biochrom).

Expansion of mESC in the bioreactor

Bioreactors were inoculated with 60×10^6 MEF on day 0 followed by the inoculation of 100×10^6 mESC (passage 37 or 39) on day 1. The bioreactors were maintained at 37°C over 9 days. The cell compartment was supplied with 40 mL/min of a gas mixture initially consisting of 95% air and 5% CO_2. Bioreactors were perfused at a constant recirculation rate of 30 mL/min. Fresh medium was supplied with an increasing feed rate from 6 to 12 mL/h according to the current glucose consumption of the cells. The pH value and oxygen partial pressure (pO_2) in the culture perfusate were measured daily with a clinical analyzer (ABL 700, Radiometer, Copenhagen, Denmark) and adjusted, if required, by variation of CO_2 supply rates to maintain a stable pH between 7.2 and 7.4.

Metabolic parameters

Samples from culture perfusates were taken on a daily basis for biochemical analyzes. Measurements of glucose and lactate concentrations were performed with a blood gas analyzer (ABL 700, Radiometer), while lactate dehydrogenase (LDH) activities, indicating potential cell damage, were determined with an automated clinical chemistry analyzer (Cobas® 8000, Roche Diagnostics GmbH, Germany). The metabolic rates in bioreactors were calculated on the basis of the system volume and feed rate as described elsewhere.[22]

Harvesting of mESC from the bioreactor

Every second to third culture day cells were harvested from the bioreactors. In the intervals between each harvesting step, cultures were continued for further expansion of the remaining cells. On culture day 9 bioreactors were opened immediately after harvesting to extract any remaining cells. For harvesting of cells from the bioreactors, two connecting lines were integrated into the tubing system between the medium inflow and outflow 1 and between the medium inflow and outflow 2, respectively (Figure 2a). In addition, a
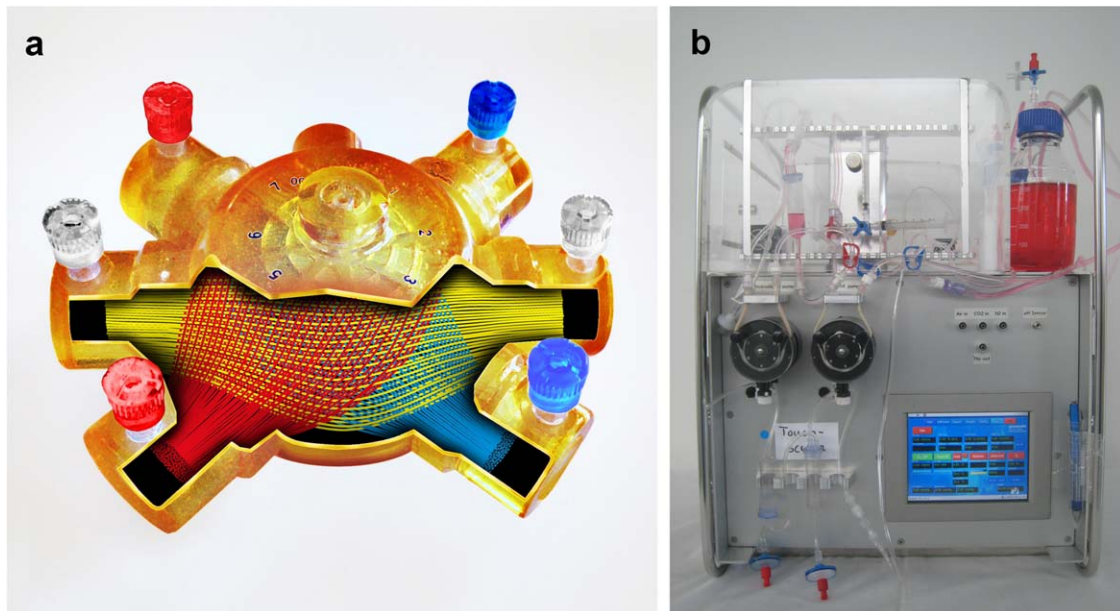
Figure 1. 3D bioreactor technology.

Schematic drawing of the bioreactor with a cell compartment volume of 8 mL showing the housing with tube connectors, partially opened to view the interwoven capillary system (a). The perfusion device is equipped with pumps for medium recirculation and medium feed, a gas mixing unit and a heating to maintain a constant temperature (b).

sterile glass vessel was connected to the cell compartment outlet of the bioreactor to collect cells during the harvesting procedure (Figure 2a). For harvesting the culture medium was first washed out by rinsing the bioreactor circuit with the threefold system volume of phosphate buffered saline (PBS; PAA, Dartmouth, MA) without calcium/magnesium to remove the FCS contained in the culture medium and flush nonadherent cells into the glass vessel. The PBS solution was then replaced by prewarmed 0.05% trypsin/0.02% EDTA solution. To enhance enzymatic incubation, 15 mL of trypsin/EDTA solution were added directly into the cell compartment, followed by continuous recirculating perfusion of the bioreactor with enzyme solution for a total of 16 min. Enzymatic digestion was stopped and cells were rinsed out from the cell compartment by flushing the circuit with DMEM culture medium with 15% FCS and 100 U/mL penicillin/100 μg/mL streptomycin. The detailed flushing procedure is shown in Figure 2. First, the cell compartment was directly perfused with a flow rate of 20 mL/min for 5 min collecting the cell suspension in the glass vessel (Figure 2b). Then the cell compartment was flushed alternately for 10 min with 20 mL/min via each medium in- and outlet (Figure 2c, d), and finally direct rinsing of the cell compartment was performed again for 5 min to rinse the detached cells into the glass vessel. During the enzymatic incubation and the different flushing modes, vigorous shaking was performed to enhance cell detachment. After completion of the harvesting procedure the system was filled again with mESC medium, and the perfusion mode was reinstalled for further expansion of remaining cells (Figure 2a).

The harvested cells were counted using trypan blue solution, seeded, and cultured on 2D culture plates for further three days in order to investigate the viability and sterility of the cells as well as for immunocytochemical analyzes.

To assess the number of remaining cells, bioreactors were opened on culture day 9 immediately after harvesting. Therefore, the excised capillaries were incubated in a 50 mL tube

for 15 min at 37°C with approximately 15 mL trypsin solution. After removal of capillaries, cells contained in the suspension were counted.

Immunocytochemistry

Feeder-dependent mESC maintained in 24-well plates (lumox®, Sarstedt) for three days were fixed with 4% formaldehyde solution (Herbeta Arzneimittel, Berlin, Germany) and permeabilized with ice-cold 80% (v/v) methanol (J.T. Baker, Deventer, The Netherlands). After rinsing with PBS, the cells were incubated in blocking buffer (PBS with 2% FCS [PAA] and 2.5% bovine serum albumin [BSA, Sigma-Aldrich]) for 60 min. Subsequently the cells were incubated for 60 min with 2 μg/mL of the primary antibody OCT3/4 (Santa Cruz, Santa Cruz, CA, USA) and for 150 min with 25 μg/mL SSEA-1 (R&D Systems, Minneapolis, MN, USA), followed by incubation with 2 μg/mL fluorochrome coupled secondary antibodies (Life Technologies) for 60 min, and staining of the nuclei with 0.17 μg/mL DAPI (Sigma-Aldrich) for 5 min. Subsequently the cells were mounted with Aqua Polymount solution (Polysciences, Warrington, PA, USA). Microphotographs were taken using a Zeiss Axiovert 200M microscope (Carl Zeiss, Göttingen, Germany) with a Retiga 2000R digital camera (QImaging, Surrey BC, Canada) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

RNA isolation and cDNA synthesis

RNA was isolated from control cultures and from harvested cells using the PureLink™ RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. Genomic DNA was digested on-column with RNase-free DNase-Set (Qiagen, Hilden, Germany). RNA concentrations were measured with a spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μg of RNA served as a template to synthesize complementary DNA with High Capacity cDNA Reverse Transcription
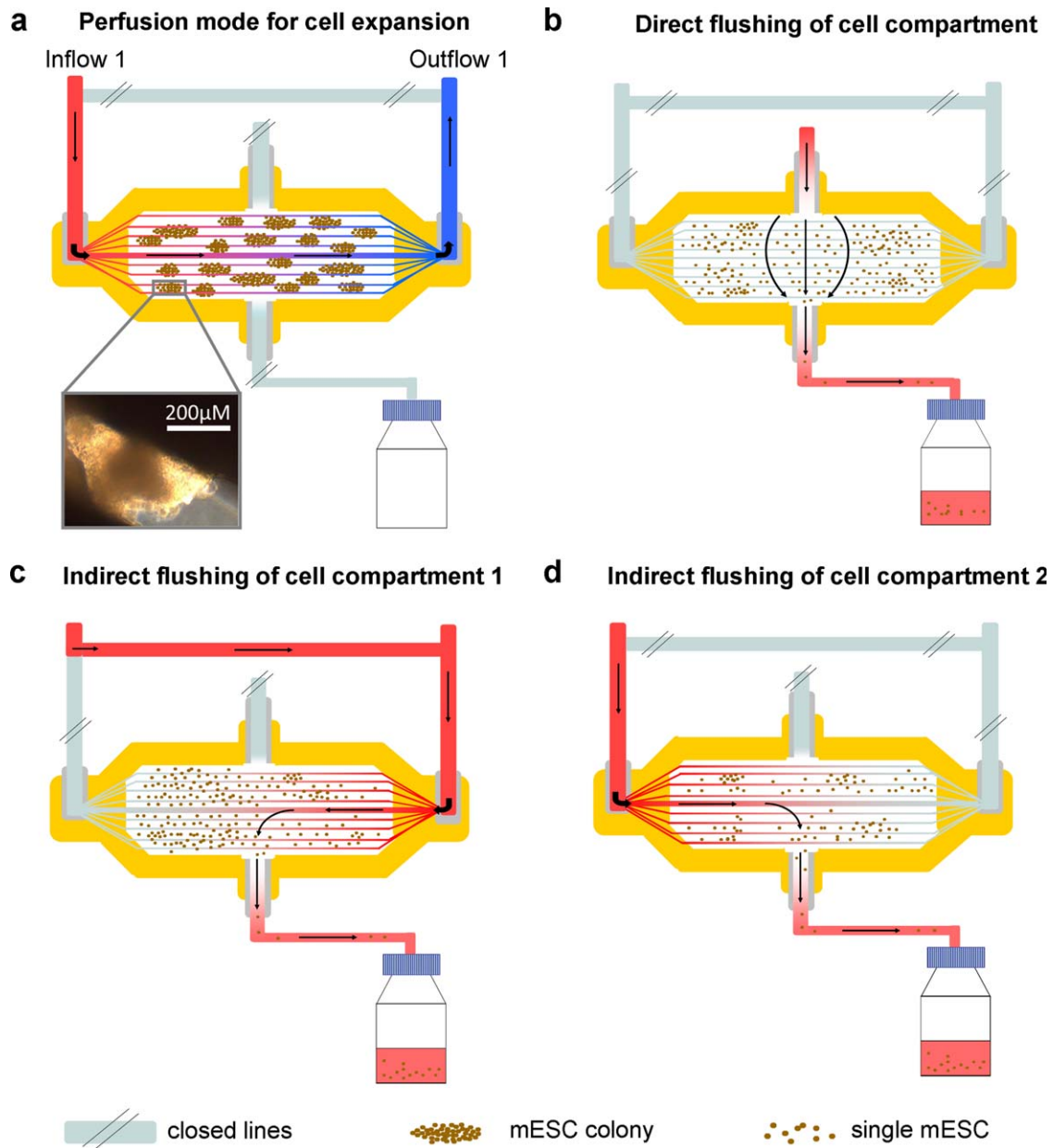
Figure 2. Different flushing modes during 3D bioreactor perfusion and cell harvesting.

The figure shows a schematic cross-section of the 8 mL bioreactor during cell expansion (a) and during cell harvesting (b–d). After incubation with trypsin, DMEM containing FCS is flushed directly through the cell compartment (b). Then the solution is flushed via each medium outlet into the cell compartment (c) followed by perfusion via each medium inlet (d). The harvested cell suspensions were collected and fractionated in a glass vessel. Insert shows a representative mESC aggregate (brightfield) obtained from a separate bioreactor not subjected to harvesting after three days of expansion (a).

Kit with RNase-Inhibitor and random hexamers (Applied Biosystems, Foster City, CA).

Real-time (TaqMan) PCR analysis

Expression analyzes were performed with specific primer/probes for mouse genes as indicated in Table 1 and assays were conducted as described elsewhere.[23] Expression of specific genes was normalized to that of the housekeeping gene (*Gapdh*) and fold changes of expression levels were calculated with the $\Delta\Delta$Ct method.

Statistical evaluation

Statistical analyzes were performed using GraphPad Prism 5.0 for Windows (GraphPad Software, SanDiego, CA).

Experiments were performed in triplicate and results are presented as means $\pm$ standard errors of the mean (SEM). The one-way ANOVA with Bonferroni's post hoc test was performed to analyze significance of differences in harvested cell numbers in the four fractions.

Results

Establishment of a cell harvesting method

Based on preliminary investigations with different rinsing modes and enzyme concentrations an optimized method based on enzymatic-mechanical detachment with subsequent rinsing was established (for detailed description see "Materials and Methods"). After 3 days of expansion (Figure 2a) cell

Table 1. Applied Biosystems TaqMan Gene Expression Assays®

Protein Symbol	Gene Symbol	Gene Name	Assay-ID (Applied Biosystems)
OCT-3/4	*Puo5f1*	POU domain, class 5, transcription factor 1	Mm00658129_gH
NANOG	*Nanog*	Nanog homeobox	Mm01617761_g1
AFP	*Afp*	alpha fetoprotein	Mm00431715_m1
GATA4	*Gata4*	GATA binding protein 4	Mm00484689_m1
NESTIN	*Nes*	Nestin	Mm00450205_m1
PAX6	*Pax6*	paired box gene 6	Mm00443081_m1
BMP4	*Bmp4*	bone morphogenetic protein 4	Mm00432087_m1
ASMA	*Acta2*	actin, alpha 2, smooth muscle, aorta	Mm01546133_m1
GAPDH	*Gapdh*	glyceraldehyde-3-phosphate dehydrogenase	4352932E

Table 2. Numbers of Cells Harvested from 3D Bioreactors by Fractionated Collection

Fraction	Flushing Mode	Time	1st Harvest*	2nd Harvest*	3rd Harvest*
0	PBS flushing	10 min	0.5 ± 0.2	0.3 ± 0.1	1.5 ± 1.3
1	Culture medium flushed directly through cell compartment	5 min	336.3 ± 130.8	244.7 ± 19.5	343.0 ± 278.5
2	Culture medium flushed indirectly through medium capillaries	10 min	33.5 ± 8.9	105.2 ± 94.4	39.8 ± 37.1
3	Culture medium flushed directly through the cell compartment	5 min	9.7 ± 4.4	21.7 ± 15.8	2.8 ± 1.8
Total of fractions 1–3	Combined flushing through the cell compartment and the medium capillaries	20 min	379.5 ± 118.0	371.5 ± 90.6	385.5 ± 317.5
Total	Total harvested cells from one bioreactor (fractions 1–3, resp.)			1136.6 ± 310.6	

*The cell numbers are given as means $\pm$ SEM ($\times 10^6$).

aggregates with a diameter of approximately 250 μm were obtained as shown for a separate bioreactor not subjected to harvesting (Figure 2a, insert). For initiation of the harvesting process, bioreactors were perfused with trypsin/EDTA solution in a recirculation mode. The obtained single cells were rinsed out in three steps (Figure 2), beginning with direct cell compartment perfusion (Figure 2b), followed by flushing of the cell compartment via medium in- and outlets (Figure 2c, d), and finally again direct rinsing of the cell compartment. Cell detachment was enhanced by mechanical agitation. The remaining cells after harvesting were further cultivated in the bioreactor to evaluate the possibility of long-term cell expansion in the system (Figure 2a).

Fractionated cell harvesting

During each harvesting procedure cells were collected in different fractions, including fraction 0 obtained by PBS flushing before trypsin incubation and those obtained by applying different flushing modes after trypsin incubation. In Table 2, the numbers of collected cells in the four fractions are shown, while Figure 3 shows the percentage of cells in individual cell fractions. The number of cells flushed out during PBS treatment prior to enzymatic perfusion (fraction 0) increased from 0.5×10^6 cells to 1.5×10^6 cells from the first to the third harvesting, still representing a minor proportion of cells compared to the other fractions. With the direct flushing mode applied immediately after trypsin perfusion (fraction 1) the majority of cells (75–92%, Figure 3) were removed from the bioreactor cell compartment at all three time points of harvesting, with cell numbers between 245×10^6 and 343×10^6 cells. Fraction 2 (6–20%), and fraction 3 (1–5%) contained only a minor proportion of cells. Total amounts of cells removed with every harvest averaged 380×10^6 cells (Table 2) and a cumulative number of $1.14 \pm 0.31 \times 10^9$ cells could be harvested from each bioreactor culture over 9 days (Table 2). The vitality of harvested cells in fraction 1–3 was assessed by trypan blue

exclusion method and was always 95% or higher (data not shown). After the third harvest on culture day 9 approximately 8×10^6 mESC (representing 8% of total cell amount, Figure 3) were still in the bioreactors, as determined by counting the remaining cells after opening the bioreactor. This indicates that the majority of mESC were removed with the enzymatic–mechanical based harvesting. From numbers of harvested cells for each harvesting time point and number of cells that were counted after opening the bioreactor an average doubling time of 31.9 ± 2.5 h was calculated.

Investigation of viability and expression of pluripotency markers

To evaluate the proliferation potential of the harvested mESC the cells were re-plated in 2D culture dishes and cultivated for further 3 days. For comparison, a standard mESC 2D culture was used as control (Figure 4). The colonies of mESC obtained after harvesting did not differ significantly in size from those in control cultures. All cultures were free of any microbiological contamination, which confirms the maintenance of sterility during harvesting procedure. Immunocytochemical staining of pluripotency markers in harvested cells that were replated in 2D culture dishes and cultivated for further three days showed that the pluripotency markers OCT3/4 and SSEA-1 were present in a similar intensity and distribution compared to the control (Figure 4). Counterstaining of nuclei with DAPI clearly confirmed the location of OCT3/4 within the nucleus, while SSEA-1 was expressed upon the surface of cells (Figure 4).

As a further parameter to assess the quality of harvested mESC the pluripotency markers *Nanog* and *Pou5f1* were analyzed on mRNA-level through qRT-PCR in samples taken directly after harvesting procedure. Figure 5a shows that there are no differences in expression levels of *Pou5f1* and *Nanog* in harvested mESC in relation to control cultures. In addition *Acta2* and *Bmp4* as markers for mesoderm were analyzed also showing no differences or even a decrease in
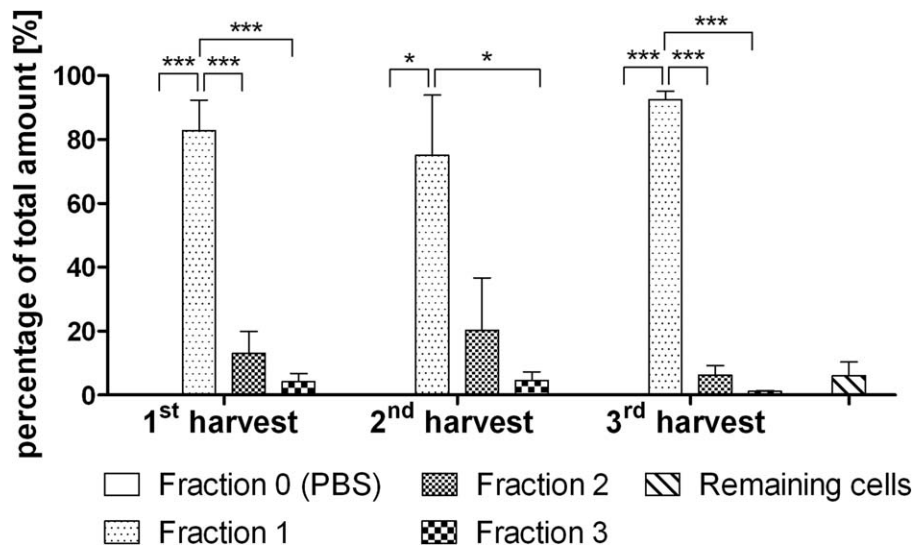
Figure 3. Percentage of mouse embryonic stem cells (mESC) harvested from 3D bioreactors in different fractions compared to the total amount of collected cells during periodic harvesting at three different time points.

Bioreactors were opened after harvesting on culture day 9 in order to count remaining cells ($n = 3$, mean $\pm$ SEM). *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (one-way ANOVA with Bonferroni's post hoc test).

gene expression compared to control cultures (Figure 5b). The same observations were made for the markers *Nes* and *Pax6*, typical for the ectodermal germ layer (Figure 5c). Only for the markers of the endodermal germ layer *Gata4* and *Afp* a 50- to 70-fold increase of gene expression was observed (Figure 5d).

Influence of periodic harvesting on metabolites and nutrients

For evaluation of cell growth within the closed bioreactor system glucose consumption and lactate production of cells were determined before and between every harvesting time point (Figure 6). The glucose consumption of mESC increased up to approximately 17 mg/h until the first harvest, which then resulted in a decrease by approximately 50% in glucose consumption (Figure 6a). Afterwards glucose uptake increased again until the second harvest to about 17 mg/h and again declined by nearly 50% after the harvest. Until the third harvest the glucose consumption reached a value of approximately 13.5 mg/h (Figure 6a). The lactate production was similar to glucose consumption, reaching maximum values of about 15 mg/h directly before harvesting and decreasing afterwards (Figure 6b). Lactate concentrations increased and reached values of 1.5 g/L or more before cell harvest and decreased to 0.5 g/L or less after harvest respectively (Figure 6c). The overall yield of lactate from glucose was around 0.8 g/g and no significant changes due to cell harvest were observed (Figure 6d). Although the lactate concentrations changed due to cell harvest the pH values remained stable between 7.1 and 7.4 by adjusting CO_2 supply rates (Figure 5e). Partial oxygen pressure (pO_2) increased slightly after harvesting procedure from around 130 to 150 mm Hg (Figure 6f).

The LDH release did not show changes due to cell harvest and did not exceed basal levels of 0.5 U/h (data not shown) indicating no significant cell injury.

Discussion

To enable the production of large cell amounts for potential future applications of PSC, including ESC or autologous hiPSC, a scale-up of culture technologies is desirable. Besides, after successful expansion and differentiation of PSC, the sterile recovery of vital cells is of interest for further analyzes and applications. Whereas nonadherent cells cultivated in 3D bioreactors can be harvested by using only mechanical agitation,[24] additional enzymatic reaction is needed for adherent cells like mESC, as already shown for microcarrier systems,[25] or fibrous bed bioreactors.[26]

In this study, an enzymatic-mechanical based method for repeated harvesting of PSC from a hollow-fiber bioreactor was established and used for prolonged expansion of the cells. A total of 1.14×10^9 mESC could be harvested from a single bioreactor culture with three harvesting procedures over nine days of culture, corresponding to an 11-fold expansion of cells, which is comparable to previously reported studies using aggregates or microcarriers.[26(p55),27] A doubling time of 32 h was calculated, which is in consistence with results from other groups reporting doubling times of 25 to 30 h for expansion of mESC in aggregates or on microcarriers.[28–30] Liu and colleagues[26(p55)] investigated the expansion of mESC in fibrous bed bioreactors over a period of 15 days and observed a doubling time of 60 h. The distinct longer doubling time as compared to the present study can be explained by a discontinuous medium exchange resulting in a limitation of nutrients and hence in a decreased growth potential. However, other groups reported shorter doubling-times of around 16 to 24 h[12(p85),31] which is only half of the doubling-time compared to the present study. The doubling-time and obtained cell numbers could be further optimized by adjusting medium feed rates in a way that lactate concentrations in the culture perfusates are below 1.5 g/L throughout the culture period, since lactate concentrations of 1.5 g/L or higher were shown to inhibit ESC growth.[32]

Since too high lactate concentrations may also induce differentiation of ESC[32(p450)] adjustment of medium feed rates could additionally prevent the observed increase in gene expression of endodermal markers *Afp* and *Gata4*. Another
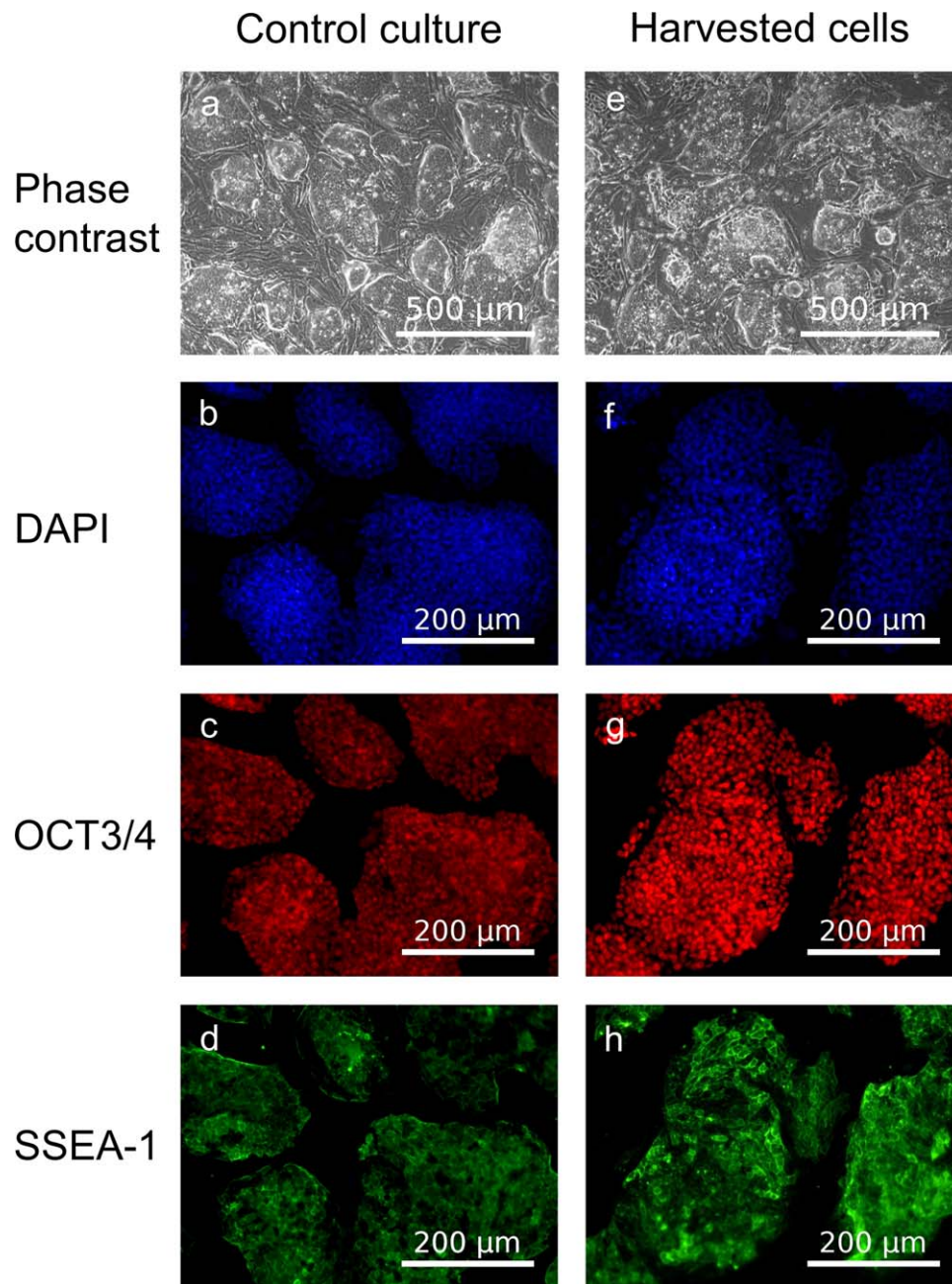
Control culture   Harvested cells

Figure 4.   Morphology and immunocytochemical analyzes of mouse embryonic stem cells (mESC) after harvesting from 3D bioreactors.

The figure shows mESC co-cultivated with feeder cells in 2D cultures for 3 days in control cultures and (a–d) cells from the second harvest from bioreactor representative for all experiments and harvesting time points (e–h).

potential reason for the observation of beginning differentiation of the cells could be the fact that during the first harvesting the pre-inoculated feeder cells were detached and flushed out from the bioreactor and were therefore not further available to support the undifferentiated state of mESC. As shown by Smith et al.[33] the factors provided by feeder cells, mainly LIF, are crucial for support of the self-renewal capacity of feeder-dependent mESC. Therefore, another possibility to prevent spontaneous differentiation would be to increase the LIF concentration in the bioreactor perfusate to compensate potential loss of feeder cells after the first harvest. Alternatively a feeder-independent cell line with chemically defined media could be used for future studies. Furthermore Gerlach et al.[18(p45)] reported that mESC started to differentiate after three days of bioreactor culture showing

a distinct decrease in expression of pluripotency markers and an increase in various differentiation markers. In contrast, in this study a stable expression of pluripotency markers *Pou5f1* and *Nanog* was observed and only endoderm specific markers increased while expression of other differentiation markers kept unchanged. Another explanation for these results could be lineage-specific priming. Several studies showed that mESC express heterogeneous levels of Nanog when they are cultured in medium containing serum and LIF[34,35] as in the present study. On the other hand, this effect was also observed in the presence of fully defined media (2i media) containing inhibitors of the fibroblast growth factor (FGF)/mitogen-activated protein kinase pathway and glycogen synthase kinase 3 (GSK3).[36] Moreover, Price and colleagues[37] showed that addition of Wnt3a to the
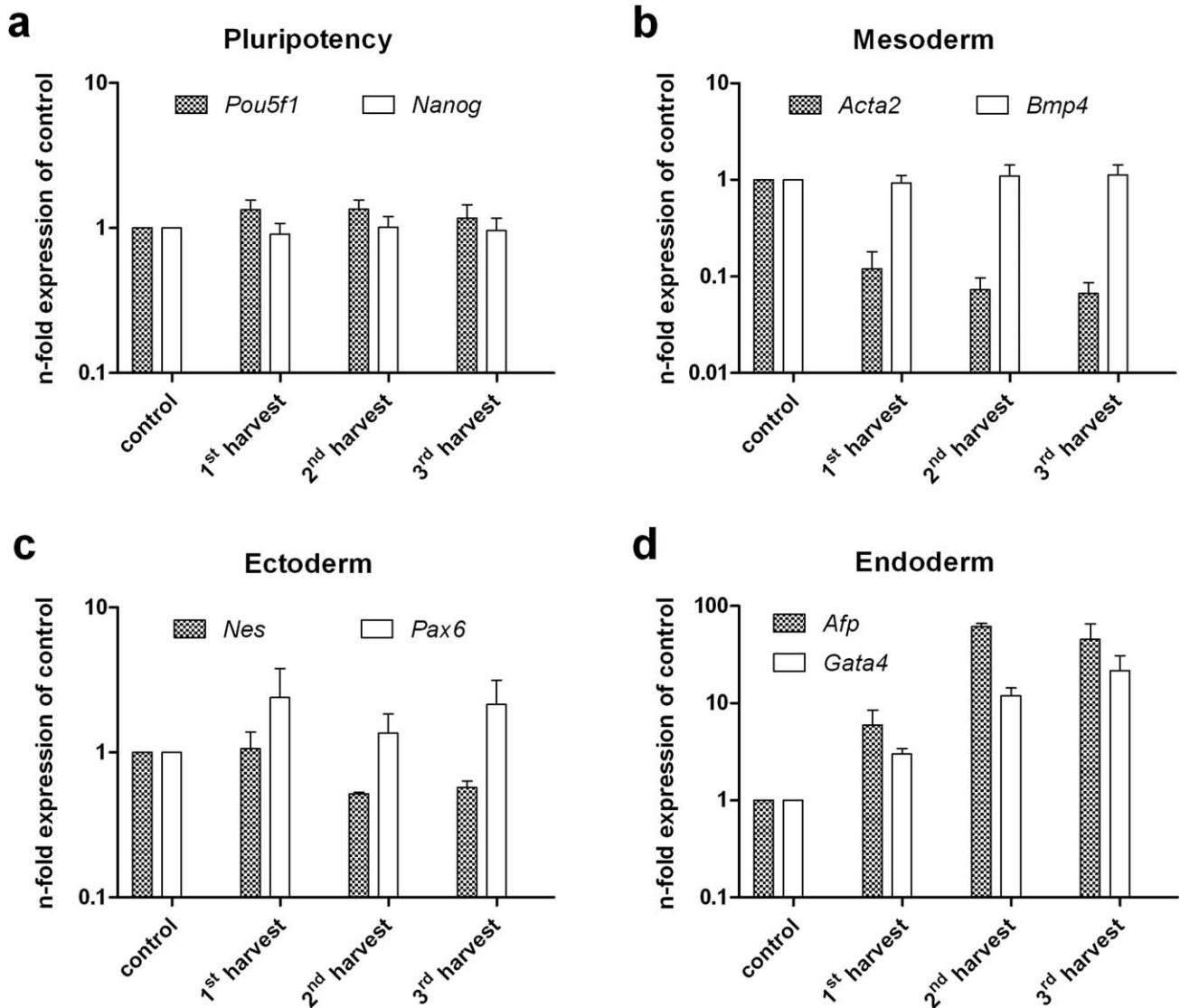
Figure 5. **Gene expression of pluripotency markers in mouse embryonic stem cells (mESC) harvested from 3D bioreactors in relation to control cultures.**

The figure shows the relative expression of pluripotency markers (a), mesodermal markers (b), ectodermal markers (c), and endodermal markers (d) of cells harvested from 3D bioreactors with three harvesting time points each over nine culture days ($n = 3$, mean $\pm$ SEM). Gene symbols: Pou5f1 = POU domain, class 5, transcription factor 1, Acta2 = alpha smooth muscle actin, Bmp4 = bone morphogenetic protein 4, Nes = Nestin, Pax6 = paired box gene 6, Afp = alpha-fetoprotein, Gata4 = GATA binding protein 4.

culture medium resulted in lineage-specific priming of mESC characterized by a low Nanog expression and upregulation of genes for primitive endoderm (Gata4, Sox17), while the expression of other pluripotency markers (Oct4, Sox2) remained unchanged. These findings indicate that various medium factors can influence the fate decision of PSC. In the bioreactor lineage-specific priming of mESC might also be caused by incomplete dissociation of some cell aggregates resulting in reduced oxygen and nutrient supply of the central areas of those aggregates. For example Lim et al.[38] showed an increased formation of primitive endodermal cells during embryoid body formation under hypoxic conditions. But the principle suitability of the established method for regular cell harvesting is reflected by the results from analysis of metabolic parameters before and between every harvest time point. The release of the intracellular enzyme LDH did not increase upon cell harvesting, indicating that the harvesting procedure did not cause significant cell injury. Time courses of glucose consumption and lactate production rates

indicate ongoing proliferation over the culture period of nine days. The course of cell growth was characterized by an increase within the first days, decreases due to cell harvest, which were followed by the continuation of increase in metabolic activity. However, as cell metabolism may change throughout the culture period further validation would be needed. In future studies several bioreactors could be run in parallel to have the possibility to open one bioreactor after each harvest and to count the remaining cells, which would allow determining the absolute cell number in the bioreactor. Counting of remaining cells, in addition to harvested cells and determination of the total cell amount in the bioreactor would also be needed for calculation of specific glucose and lactate production rates (mg/h/cell) to assess metabolic activity in response to process modifications. This could be combined with the application of an *in vivo* imaging system for the observation of the cell distribution within the bioreactor as it was already applied by Ratcliffe et al.[39] By using this imaging system during the harvesting procedure a potential
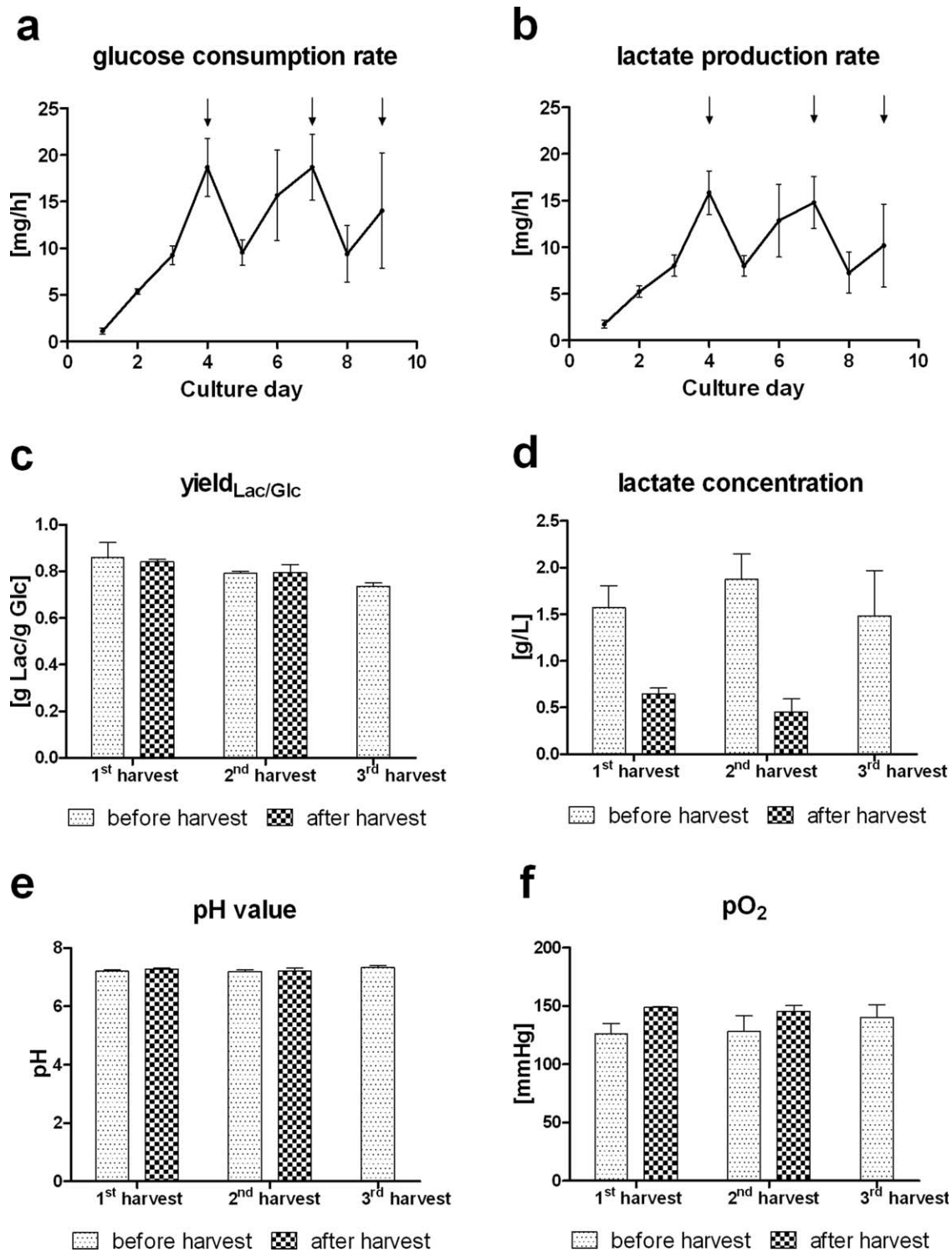
Figure 6. Influence of periodic cell harvesting on nutrients and metabolites during perfused 3D bioreactor culture of mouse embryonic stem cells (mESC).

Glucose consumption rates (a) and lactate production rates (b) were calculated daily. Arrows indicate harvesting time points. Resulting yield Lac/Glc (c) was calculated over 24 h before and 24 h after cell harvest. Lactate concentrations (d), pH values (e) and partial oxygen pressure (pO_2, f) were measured directly before and after harvest of mESC from 3D bioreactors. Bioreactors were opened after third harvesting on culture day 9 in order to count remaining cells resulting in only one dataset (before harvest) for the third harvest ($n = 3$, mean $\pm$ SEM).

inhomogeneous distribution of remaining cells could be revealed and allow counteracting via further targeted rinsing. This would also lead to decreased variances of harvested cell numbers and metabolic data after the first cell harvest.

To obtain even larger amounts of cells at one time point and not at time-displaced intervals of two to three days,

several bioreactors could be run in parallel as Abbasalizadeh et al.[8(p844)] demonstrated for expansion of PSC in stirred tank bioreactors. This would allow for production of clinical relevant numbers of 1–2×10^9 stem cells and their derivatives.[40] Alternatively a larger scale hollow-fiber membrane bioreactor could be used. In previous studies a good

comparability of cell functionality and metabolic activity was observed in differently scaled bioreactors with a volume of up to 800 mL.[15(p552)] Additionally the yield could be increased by various process modifications. For example, it was shown that in stirred-tank bioreactors the orientation of impeller,[41] and of agitator type and rate[7(p253)] play an important role for cell growth. Similarly, Gloeckner and Lemke[42] showed that an increase of the medium recirculation rate in a hollow-fiber membrane bioreactor resulted in increased cell numbers. Further attempts to enhance the cell activity aim to improve oxygen delivery to the cells, e.g., by adding sterile red blood cells to the culture medium.[43] Optimized bioreactor processes combined with an increase of harvesting cycles would enable regular cell delivery. To ensure sterility over prolonged periods, the technical set-up for harvesting procedure could be upgraded. For example, the usage of large medium bags with pre-connected transfer lines in combination with sterile quick connectors would reduce the contamination risk during transfer of liquids. If cell production in accordance to GMP guidelines is needed the bioreactor perfusion and cell harvesting can be performed using a laminar flow or under clean room conditions.

In order to transfer the method to repeated harvesting of human-relevant cells (e.g., hiPSC) in the bioreactor some adaptations of the established procedure may be necessary. On the one hand the usage of feeder-free culture conditions would be needed. In combination with the use of defined media this would enable standardized, xeno-free cultivation. Furthermore a potential harmful influence of feeder cells during drug testing studies could be excluded. On the other hand, a more detailed quality control including flow cytometry analysis and formation of embryoid bodies or teratoma studies should be applied. Additionally some process adjustments would be required due to a different behavior of the cells regarding cell growth and sensitivity to enzymatic treatment.[44] In other bioreactor systems based on microcarriers collagenase and TrypLE[TM] have been tested for cell passaging and harvesting of hESC[45] and for dissociation of hiPSC aggregates in suspension cultures Accutase[TM] and Accumax[TM] were successfully applied.[8(p836)]

Applications for post harvest cells can be seen not only in clinical applications but also in pharmacological studies. Therefore the harvested cells could be reseeded in conventional 2D cultures or in more complex culture systems such as miniaturized 3D bioreactors,[22(p9)] in 3D fibrous matrices[32(p453)] or in fibrous bed bioreactors.[26(p63)] The reseeded undifferentiated cells could then be used for studies on embryotoxicity or for differentiation into specific cell types and subsequent drug screening and toxicity testing.

Conclusion

The present study is the first one showing the feasibility of periodic cell harvesting from a continuously perfused hollow-fiber membrane bioreactor with subsequent further cultivation of ESC that remained in the bioreactor after harvest. The gained cells were shown to be viable and sterile, and could be further cultivated in 2D. The perfusion device allows for control of medium and gas flow rates. Additionally, the system provides the option for equipping the perfusion circuit with pH and pO_2 sensors for online measurement to ensure constant culture monitoring. Hence, the 3D bioreactor technology represents a promising tool for scaling up stem cell expansion in a highly controlled environment. The new opportunity of periodic harvesting of cells from the system would allow for the generation of high amounts of cells in a closed culture system for *in vitro* or clinical research.

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