Bioprocess Biosyst Eng (2005) 28: 95–107
DOI 10.1007/s00449-005-0003-6

MINI REVIEW

Stephan Michael Meier · Holger Huebner
Rainer Buchholz

Single-cell-bioreactors as end of miniaturization approaches in biotechnology: progresses with characterised bioreactors and a glance into the future

Received: 2 April 2005 / Accepted: 6 May 2005 / Published online: 11 August 2005

Abstract Incidents with single cells and their genesis have not been the major focus of science up to now. This fact is supported by the difficulties one faces when wanting to monitor and cultivate small populations of cells in a defined compartment under controlled conditions, in vitro. Several approaches of up- and down-scaling have often led to poorly understood results which might be better elucidated by understanding the cellular genesis as a function of its microenvironment. This review of the approaches of scale-up and scale-down processes illustrates technical possibilities and shows up their limitations with regard to obtainable data for the characterisation of cellular genesis and impact of the cellular microenvironment. For example, stem cell research advances underline the lack of information about the impact of the microenvironment on cellular development. Finally, a proposal of future research efforts is given on how to overcome this lack of data via a novel bioreactor setup.

Keywords Utem cells · Single cell bioreactor · Immobilisation · Scale down · Scale-up

Introduction

Up to now, biotechnical approaches on a laboratory scale often failed to be transferred into other scales of cultivation directly. Hence, extensive scaling studies have always been focused by scientists to facilitate economical profit from the newly obtained research data. Accordingly, groups of processes have been developed in several niches of biotechnology up to now, but general understanding of the systems is still mostly lacking. This primarily applies to mammalian cell culture in vitro, where an intrinsic understanding of the cellular genesis and microenvironment is still the subject of intensive research.

It is usual in engineering practice to derive a conclusion of a lack of understanding of ongoing processes if several scaling approaches fail consecutively. Admitting this fact, every scientist has to agree on first understanding infinitesimal incidents before returning to scaling processes. The smallest scale accessible in cell culture is the cell itself, but unfortunately lab-scale investigations still contain a certain scale-up technology. This is caused by the fact that cell populations are mostly focused on in vitro experiments, which come along with cell–cell interaction modifying or even inducing differentiation. Several techniques which facilitate population observations are accessible at present which govern flow cytometry like fluorescence activated cell sorting (FACS) and related techniques like fluorescence microscopy and confocal laser scanning microscopy (CLSM).

Finally, the lack of understanding of cellular genesis of mammalians is directly indicated by the growing number of scientist focusing on stem cell research.

S. M. Meier (✉) · H. Huebner · R. Buchholz
Institute of Bioprocess Engineering,
University of Erlangen-Nuremberg, Erlangen, Germany
E-mail: stephan.meier@bvt.cbi.uni-erlangen.de

Scale-up processes

As mentioned before, several scale-up experiments have already yielded a group of processes now being available at huge scales. A closer look at these processes uncovers the fact that the more genetically related to mammalians or humans the cultivated organisms are, the smaller the accessible scales.

Huge scale bioprocesses

Classical huge scale bioprocesses govern the regimes of waste water treatment—the absolute winners in scaling up—and procaryotic fermentation in stirred tank reactors, where ~100 m^3 [5; 22] of fermenter volume are feasible.

In eukaryotic systems, yeast fermentations have also been scaled up successfully into the same order of magnitude. These researches can mainly be attributed to the brewery and beverage industry as well as to large scale protein expression [16, 32, 51, 74, 88].

Some specialities among these processes even govern the scale-up of insect cell culture processes for advanced glycosilated protein expression [49, 55].

Scale up of processes involving marine organisms

Even processes involving marine organisms have been scaled up to hundreds of litres of culture volume [6, 8, 86]. Nevertheless, this is an excellent example of the difficulties arising from one additional parameter in scale modulation. Undemanding organisms like yeast and procaryotic bacteria are relatively easy to handle as the parameters of severe influence (nutrition, shear stress and oxygen tension) are easy to be changed and controlled. Marine organisms—being phototrophic or heterotrophic living entities—demand intensity and penetration of light as additional parameters to be closely looked at. Unfortunately, the impact of light is no longer scaleable with ease, as depth of penetration lays claim on time dependent regulation and is not linearly correlated with the fermenter volume.

Walter et al. [86] proposed a method of up-scaling these fermenters taking light penetration as an parameter of severe impact. From their calculations, the ''Medusa'' photo-bioreactor has been developed as an adaptation of standard aerated loop reactors to the demands of phototrophic microorganisms (Figs. 1, 2).

Scale up of mammalian cell cultures

Accordingly, mammalian cell cultures, which demand highly controlled conditions of several parameters, are at the pinnacle of scale up processes. Some genetically modified (and hence less demanding) cell cultures like genetically tailored CHO-cells have been successfully employed in larger production scales [5, 7], but none of them have ever scaled up primary cell-cultures to this extent.

Scaling up mammalian cell cultures is mostly associated with problems arising from the cellular microenvironment as well. The huger the scale of the setup the more difficult becomes the homogeneous adjustment of optimum cultivation parameters, which again might trigger the cells to undergo apoptosis or die via outer necrotic effects.

Effects like sufficient metabolite removal (e.g. CO_2) or maintenance with sustainable amounts of oxygen have attracted the attention of scientists and led to several approaches on how to achieve this [54, 57]. Nevertheless, the need for very sophisticated methods underlines the inherent problem of closely linked parameters, each one negatively influencing the other.

Taking aeration as an example, researchers [28] underline the significance of superficial gas velocity and bubble size distribution in mammalian cell culture systems with direct aeration. Direct aeration is assumed to be very effective due to the direct action of the oxygen supply [57], though it comes with associated problems of hydrodynamic shearing effects as well as foaming of the reactor [67].

Accordingly, membrane-based oxygen maintenance systems (i.e. indirect aeration) have been developed passing on the effectiveness of direct aeration [67]. Abandoning highly efficient transfer systems boost gradient problems, which can only be avoided via a more intense mixing of the culture broth using, for example, special roller bottle setups [3]. This again induces an alteration of the cellular microenvironment and might trigger unwanted side effects in the cultivated cells.

Up to now these limits persist especially due to the unknown cell death and apoptosis mechanisms of primary mammalian cells

Fig. 1 Loops of a 100 l ''Medusa''-photobioreactor under operation conditions, working via recirculation of synthetic sea water via continuous aeration of one tube of the loops. (Pictures kindly provided by Christian Walter, see [85])

Scale down progresses

Immobilisation research in general

Being aware of the need for having to generate special microenvironments to cultivate specific cells, scientist have focused on producing small compartments and funded a branch of research subsumable under the heading immobilisation and microencapsulation. The use of these techniques facilitates the creation of (micro-bioreactors), which mimic, to a certain extent the cell densities achieved in tissue culture in three dimensional arrangements [12, 61].

These micro-compartments can be established via creation of a micro-membrane using wide-spread encapsulation techniques based on alginate and related

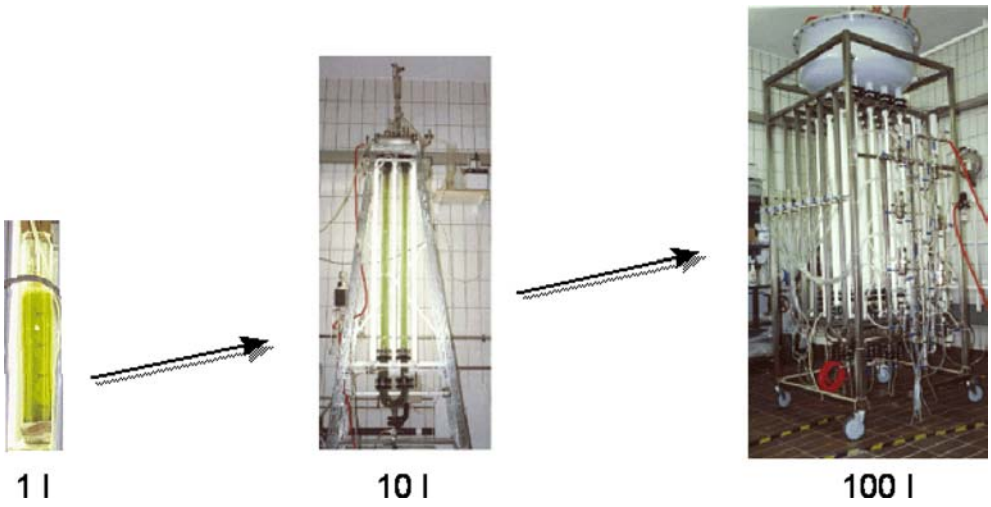

Fig. 2 Scale up of photobioreactors applicable for cultivation of phototrophic marine micro organisms, starting from the photobioreactor screening module (1 l) up-scaling to a ten litre pilot-plant phase (10 l) and ending up at maximum size of 100 l active volume. (Pictures kindly provided by Christian Walter, see [86])

matrices forming droplets with a co-acervate membrane [25, 59, 60]. Another way of establishing a microenvironment is a complete reactor re-design, incorporating smallest reactor compartments via hollow fibres or membranes in a flat arrangement [25].

Microencapsulation

With microencapsulation experiments, several different types of cells have already been cultured successfully [19, 47]. Cultivation governs several mammalian cells like parathyroid cells [63], hepatocytes [15, 80], neurons [69, 81], islets of langerhan's [12, 58] and T-cells, as well as transfected hybridoma [10] cells. As a speciality, even infected insect cells have been grown under these conditions [29].

Microencapsulation of infected insect cells

Cultivation of insect cells in microcapsules is an excellent example of elucidating the need for further investigations in diminution techniques. During the past few years Hübner [29] has found that infected Sf21 insect cells in microcapsules grown in standard spinner flasks of 1 l volume persist in vitality for more than a month, while "classical" suspension culture systems rapidly decrease in vitality after 1 week (Figs. 3, 4). These findings can only be associated with the changed culture system via encapsulation, as other parameters of cultivation were kept equal to those in the suspension culture. Hence, a change of the cellular microenvironment has led to a significant change in cellular behaviour. The reason for this change in behaviour urgently needs to be clarified. As suspension cultures of infected insect cells suffer greatly from the discharge of newly formed virus particles through the cellular membrane it is conjecturable that microcapsules are in some way able to physically stabilise the infected cells against the stress of virus release.

Microencapsulation of T-cells

In some of our recent experiments we have found a Jurkat T-cell-line to grow as fast in sodium-cellulose-sulphate capsules under disadvantageous conditions as their counterparts under standard suspension culture conditions. Cell proliferation has been tracked via CFDA-SE (Carboxy-Fluorescein-Di-Acetate-Succimidyl-Ester) dilution and flow cytometric analysis of the cells [47].

CFDA-SE dilution techniques are based on the permeation of the fluorochrome through the membrane of the cell where etsterases decarboxylate the CFDA, which thus becomes hydrophilic and trapped within the cell. Additionally, the succimidyl ester binds to amino groups of proteins and accordingly enhances the retention of the molecule in the cell. Division of the cells yields a halving of the fluorochromes per cell and hence can be detected in the flow cytometer.

Suspension culture has been compared to the immobilised cell culture. Both cultures have been grown under static culture conditions in RPMI 1640 media supplemented with 10% fetal bovine serum (FCS), where the diffusion drawbacks of the membrane of the microcapsules should have led to a diminished proliferation of the cells. Actually, no significant difference can be stated (Fig. 5), which leads to the assumption that microencapsulation has to inflict an additional positive influence upon the cells, compensating for the hindrance in diffusion. Further investigations are now done, eliminating diffusion barriers via diminution of the capsules as well as superposing convectional fluid patterns. These experiments are expected to result in faster growing cells in microcapsules compared to those in static suspension cultures.

Once again, these unusual findings urgently need to be clarified concerning their mode of action. It is conjecturable that the self-forming microenvironment of small cell populations has a major impact on their genesis in future development.

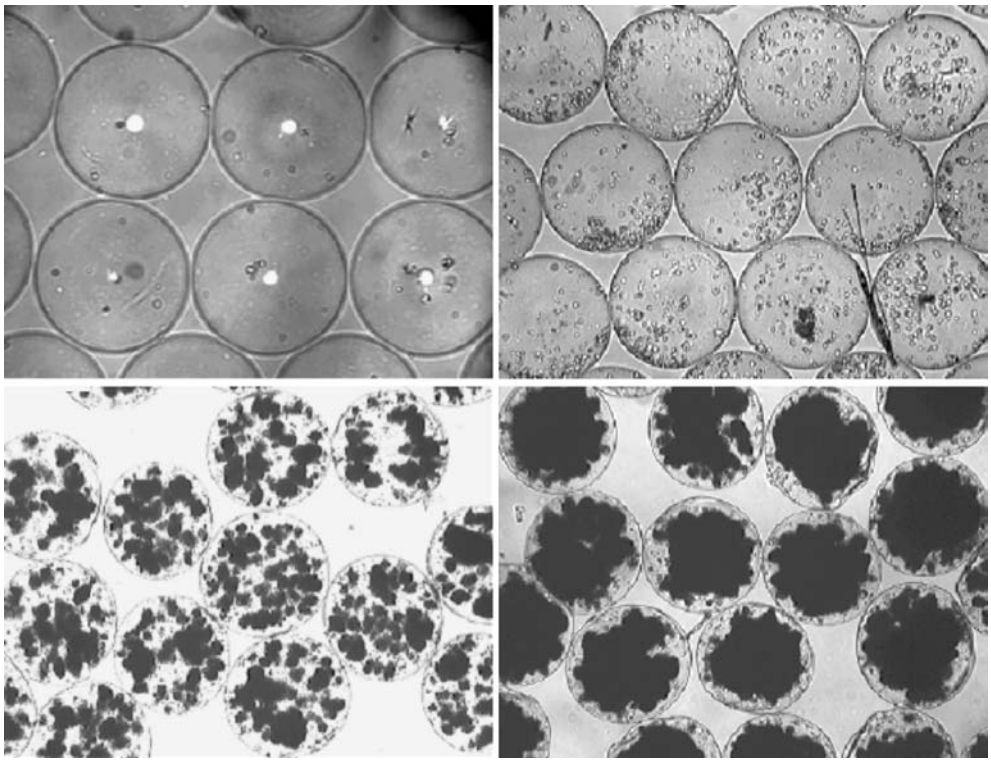

Fig. 3 Growth of Sf21 insect cells in micro-hollowspheres built up from Sodium cellulose sulphate and Poly(diallyldimethylammonium chloride) (PDADMAC) [29]

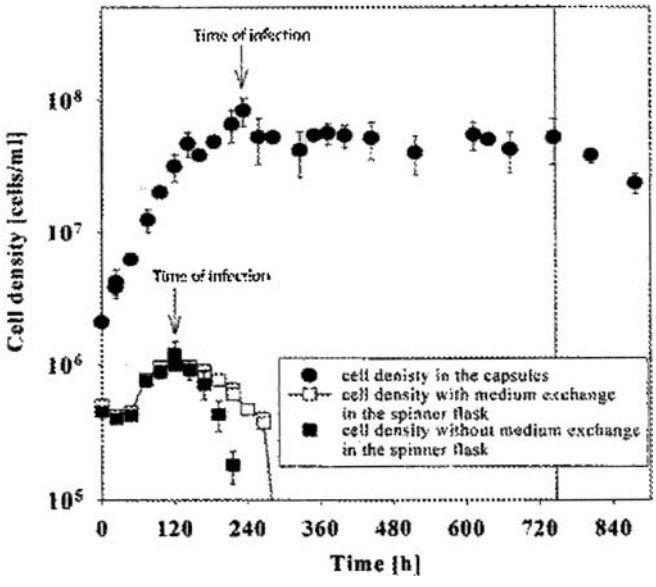

Fig. 4 Growth curve of an immobilised/suspension culture of Sf21 insect cells in cultivated in spinner flasks and infected with "Autographa californica Multiple nucleopolhedrovirus" (Ac-MNPV) [29]

Microencapsulation of chondrocytes

Microencapsulation additionally offers an opportunity to mimic an extra cellular matrix and hence has been successfully applied to a cultivation of cells from tissue found in complex matrices in vivo. Chondrocytes forming complex matrices on their own have been cultivated in microcapsules in recent years. A group of possible matrices has been especially proposed by different researchers governing "classical" alginate matrices [82, 68], as well as agarose matrices [30]. More sophisticated methods incorporate self-degradation of the matrices for later retransplantation of the in vitro expanded cells based upon a polyglycolic acid non-woven mesh with fibrin gel matrix assistance [1]. Photochemical matrix formation approaches have also been assessed for purposes of chondrocyte immobilisation, although significant cell losses have been observed using these techniques [17].

Other immobilisation/encapsulation examples

Microencapsulation in in vivo models

An immunological barrier has been intentionally established to facilitate delivery of smaller proteins or substances into the transplanted host's body, without facing allogenic rejection of the secreting cells [43, 79].

In this approach, sodium cellulose sulphate microcapsules have already successfully been transplanted into MRL/lpr (lupus responser) mice. Microcapsules have contained a murine hybridoma cell line secreting an anti–idiotypic murine antibody (mab) for treating rheumatoid diseases. After 11 weeks, untreated mice showed the normal development of their disease, while mice which received capsules intraperitoneally (i.p.) displayed no visible symptom [37].

Besides these findings several approaches facilitated continuous drug administration into the host body. This

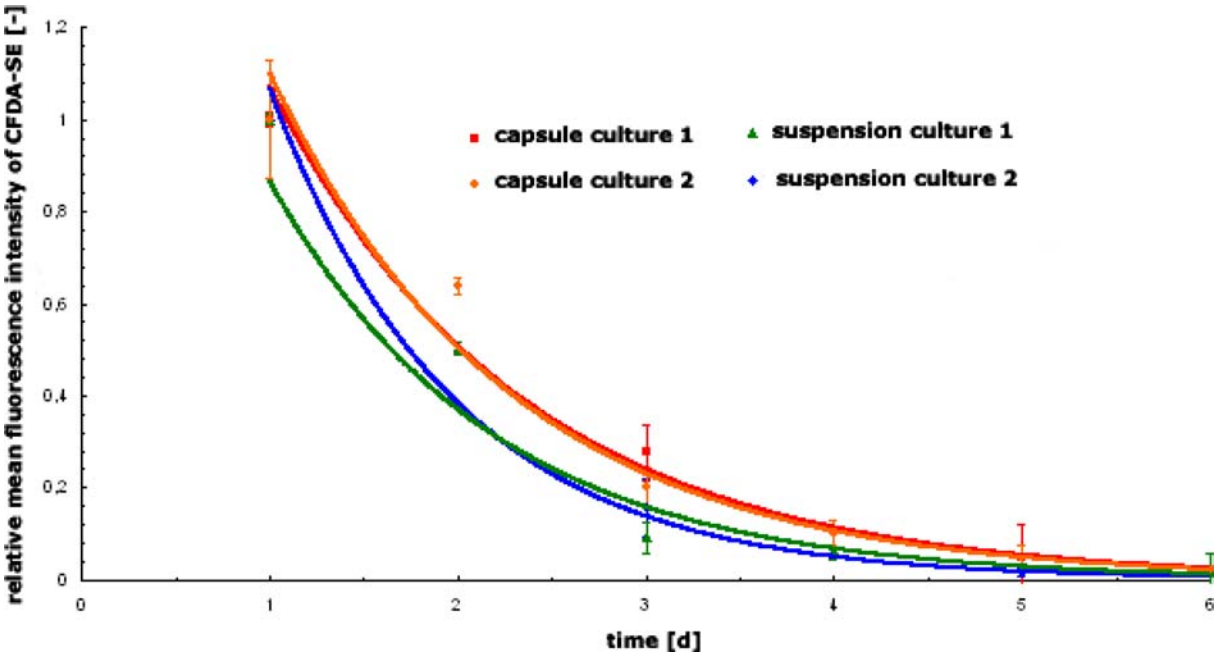

Fig. 5 Time dependent elution of the CFDA-SE signal of tagged Jurkat T-cells under different culture conditions (Data from the author, unpublished)

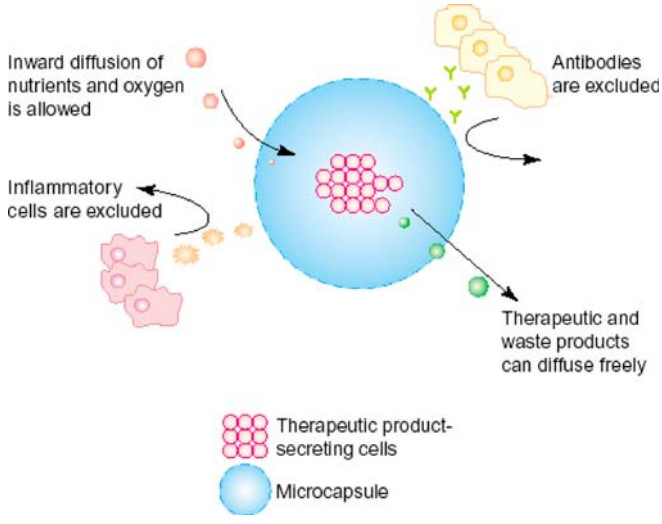

Fig. 6 Working principle of microcapsules in in vivo studies (59, 60)

has been undertaken with major focus on diabetes therapy, usually using insulin secreting islets of langerhan's cells [59, 60] (Fig. 6). In vivo models have already yielded several successes with xenogenic and allogenic insulin-secreting cells transplanted into immunosuppressed and immunocompetent mice [58], proving the ability of controlled release of microcapsules.

Different approaches of miniaturised immobilisation

Another well-established cultivation system employing smallest compartments is subsumable under the heading of 'hollow fibre reactor systems' or 'membrane reactors' [25]. These systems are an adoption of the spherical microcapsule arrangement to a linear hollow fibre or flat membrane arrangement with defined membrane properties. Hollow fibre reactor systems are already commercially available, yielding a wide variety of applications. Mainly those governing the production and in-situ extraction of proteins desirable for clinical applications [84].

Nevertheless, some scientists have found potential in these systems for an artificial liver bridging system [36, 73, 77]. Unfortunately, final success does not seem to be close at hand, as those systems are still under clinical studies from 1996 [73]. The main advantage of the hollow fibre arrangement is the easily definable exclusion properties of the membranes as well as the scale-up possibilities via coupling several hollow fibre modules in parallel [84].

Starting from the well-known setup of microtiterplate cultivation some successful approaches have already been realised in the field of analysis in these compartments [31, 33]. Additionally, microbial growth has been studied in very small microtiterplate equivalent fermenter compartments (~5 ml) yielding similar growth kinetics compared to standard laboratory cultivation approaches of 3 l volume [66].

Cellular development in humans and stem cell research

Research progresses up to now

In recent years, it has been discovered that different types of stem cells persist in the human body. All of them share distinctive functions and patterns which mark them as a member of the stem cell sub-population. Specific prop-

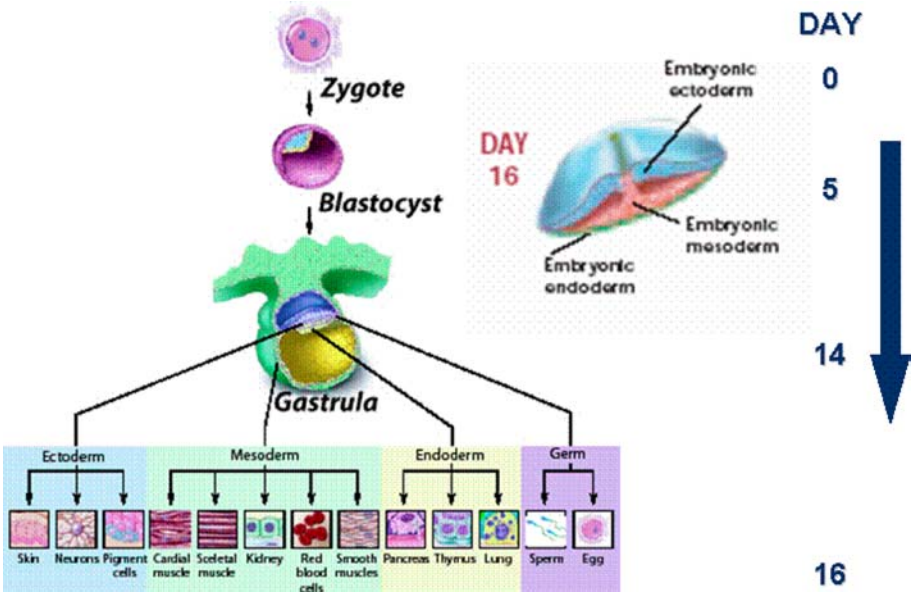

Fig. 7 Human development during the first 16 days of maturation from the fertilized egg (modified from Kirschstein et al. 35)

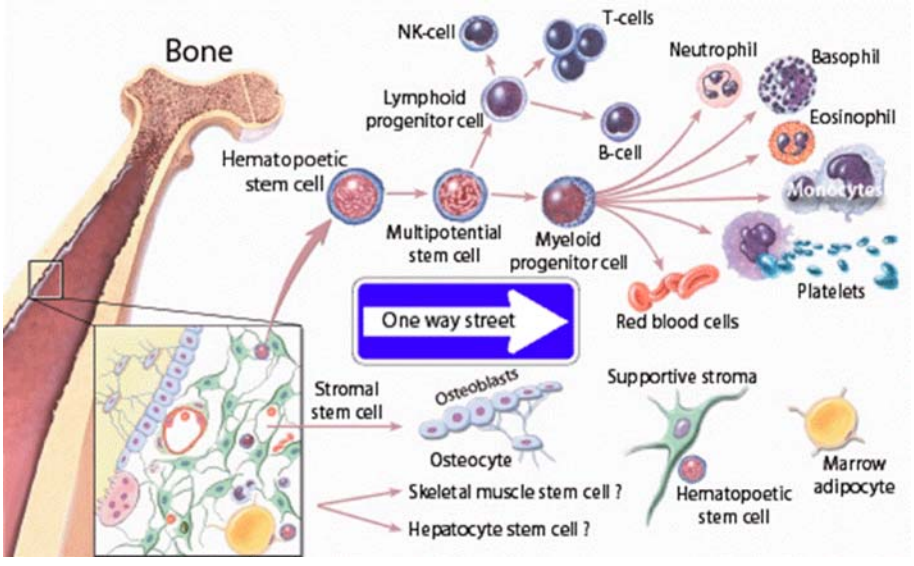

Fig. 8 The postulated one way street of stem cell development following the haematopoietic and (partially) mesencheymal pathway (modified from Kirschstein et al. 35)

erties of stem cells govern the ability of nearly indefinite self-renewal and capability of differentiation into other types of specialised cells like skin cells and nerve cells [35].

Within the population of stem cells several types need to be distinguished concerning these abilities. Top down, one can define three subpopulations, the totipotent stem cell, the pluripotent stem cell and the unipotent stem cell. The fertilised egg or zygote is the only known totipotent stem cell. Embryonic stem cells defined by their origin—that is from one of the earliest stages of the development of the embryo, called the blastocyst—are called pluripotent stem cells (Fig. 7). Additionally, there is a population of adult stem cells restricted to the differentiation into cell types of the tissue it originated from. Accordingly, adult stem cells derived from bone marrow are restricted to differentiate into any of the mesoderm cell subpopulations and are defined as unipotent [35, 56].

Several in vitro experiments where stem cells were cultured under altered conditions underlined these facts. Nevertheless, only few niches of differentiation primers are known up to now. Those results are often outcomes of intense trial and error assays. This again underlines the need of an assay system that allows fast and easy analysis of the impact of certain substances on the cellular genesis. Generally, the reason and mechanisms of stem cell differentiation are still subject to intense research and are not widely known.

Culture systems up to now

Progress in miniaturisation of cell culture techniques mentioned previously have already made an impact on other fields of biotechnology. Even stem cell research now employs similar approaches to artificially generate miniaturised compartments. Comparability is evident considering the wide spread ''hanging drop'' setup for embryonic body (EB) formation, for example. These techniques facilitate spatially controlled cultivation of embryonic stem cells (ES) and have led to several new results [14, 45].

Additionally, the successful cultivation of EBs has led to a further expansion of this culture approach by making it accessible to further commercialisation of products derived from expanded stem cells. It emerges that some smooth mixing and perfusion systems are able to generate larger samples of EBs for later investigation. Nevertheless, these systems still do have to fall back on the utilisation of feeder layer cultures, at least at a preliminary stage [24], indicating again the lack of knowledge about the microenvironment composition.

Generally, stem cell research is mostly restricted to in vivo mouse models or employs feeder layers, both of them generating an undefined microenvironment. Only few niches of differentiation of different types of stem cells have been found in vitro without employing these techniques. A general differentiation pathway from an embryonic stem cell to a fully differentiated cell is still lacking. Nevertheless, pancreatic islet-like structures [46], neuronal cells [2, 34] and endothelial cells [83] have been grown from embryonic cells in different models.

Examples of known differentiation pathways in vitro

The best known differentiation pathway up to now is the pathway of haematopoiesis from a haematopoietic progenitor cell to a fully developed, functional immunocompetent cell. This is due to the fact that techniques for isolation and characterisation of haematopoietic cells have already been available for a longer time. (Fig. 8)

An excellent example for the better understanding of this specific system is the work of Spangrude et al. [76] and Morrison et al. [53, 52] that reveals a broad pattern of influencing substances on haematopoietic growth and differentiation. They elucidate the key role of cytokines like IL-3, IL-6, granulocyte-colony-stimulating factor (GCSF), erythropoietin and thrombopoietin via the generation of platelet, red and white blood cells in vitro from haematopoietic stem cells.

Starting from murine embryonic stem cells Potocnik et al. [65] had been able to establish lymphocyte differentiation by just modulating the partial pressure of oxygen down to 5% in an in vitro system. Even specific macrophage cells have been differentiated in vitro via IL-3 and macrophage colony stimulating factor (M-CSF) addition by Lieschke and Dunn [44] in an standard microtiterplate reactor setup. These results underline the major impact of even slight differences in the cellular environment on the later cellular genesis.

Also partially uncovered concerning its differentiation pattern as a function of microenvironmental effects is the mesodermal cell lineage. Scientists have uncovered the effects of dexamethasone, vitamine D3 and bone-morphogenic-protein-2 (BMP-2) on mesenchymal stem cells and have found adipocytes, chondrocytes, osteoblasts and tenocytes to develop from them [38, 62, 64]. Additionally, astrocytes and neurons [72] as well as skeletal muscles, have been derived from these stem cells via induction with 5-azacytidine [85].

Besides the differentiation outcomes induced by a chemically modified microenvironment, novel cultivation approaches which include mechanical stresses have been correctly identified to influence cellular behaviour as well as even a cell-line model [39].

Starting with embryonic stem cells, scientists [26] have managed to postulate a pathway for neuronal development, finding that retinoic acid is the key player concerning the microenvironment influences.

Other cellular origins of stem cells are, by comparison, white spots on the map of cell differentiation in vitro.

The in vivo models and their confusing outcomes

Moving away from the in vitro model facilitates linkages between cells, which, at a first glance, have nothing in common. For example, researchers [27] have injected haematopoietic stem cells and 'muscle-derived-stem-cells' into dystrophic mice and have found that both populations were able to restore normal health status of the mice partially (Fig. 9). On the other hand, neuronal stem cells have been transplanted into irradiated mice [4] and have later been found to have developed into blood cells. Stem cells can accordingly form myeloid cells as well [71].

Finally, some mouse model experiments give rise to the hope that, in future, even complex organ failures like acute liver failure might be treated via a cellular therapy, as pancreatic cells as well as haematopoietic stem cells have already been transferred into hepatocytes in vivo [40, 87]. This also elucidates that a 'non-stem-cell' can de-differentiate and re-differentiate to form a completely different type of cell under correct conditions.

How to lay a spot on cellular development as a function of the microenvironment

Genetic analysis does not hold the answer

All the facts mentioned previously cannot simply be answered by genetic analysis, as genetics would prohibit a cell to completely change its expression pattern. There

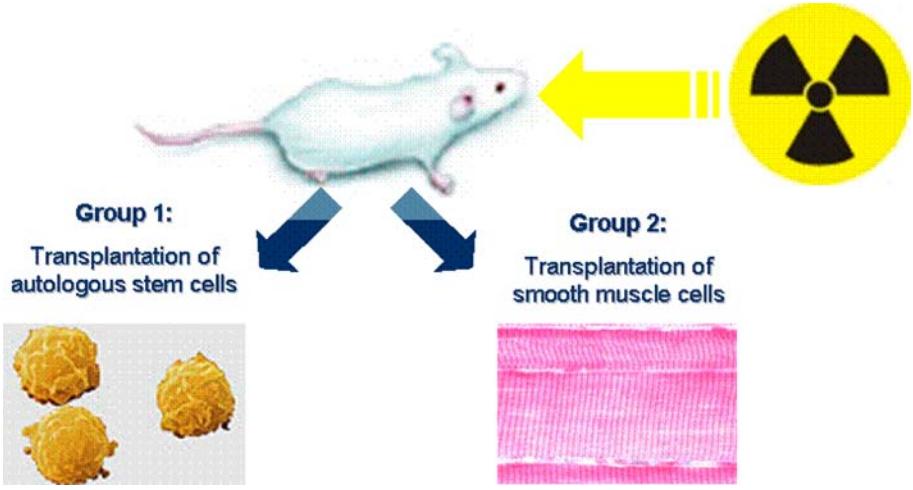

Fig. 9 Experimental setup from Gussoni et al. [27] yielding successfully restoring health in lethally irradiated mice via stem cell and smooth muscle cell retransplantation. (modified from Kirschstein et al. [35])

is no gene decoded up to now which enables cells to dedifferentiate and re-differentiate in a completely altered manner.

In recent years 3 billion $ [11, 13] have been spent to characterise the human genome. These investigations have been done in the hope of understanding human development and differentiation of cells in a more accurate way [18]. Despite of the great achievement of fully decoding the human genome [20], it turns out that all results are not able to explain the development of a human from a single cell [23].

Accordingly, other parameters had to have a major impact on this fact. It is conjecturable that changes in the microenvironment of living cells play a key role in these developments, but a technique to evaluate these impacts on a single cell level is still lacked.

Modern culture assessment systems

Flow cytomeric analysis

Flow cytometer techniques assess cell populations via an offline method employing a nozzle hydro dynamically focussing and decollating the cells to allow excitation of fluorochromes coupled on the cells [75].

Unfortunately these techniques are, up to now, not able to monitor on-line the cellular genesis of single cells. Only shifts in population distributions can be assessed, which come along with probing an existing culture. These techniques are reliant on the analysis of a distinct population governing at least several hundred cells to allow statistical analysis. Single cells would yield only one event being analysable yielding no statistical analysis and hence no reliable result. Additionally, current flow cytometers with sorting option interfere severely with the microenvironment of the cell, due to the inherent problem of having to hydro dynamically focus the cells in a liquid stream. Accordingly, FACS analysis can never correctly display a genesis of distinct cells.

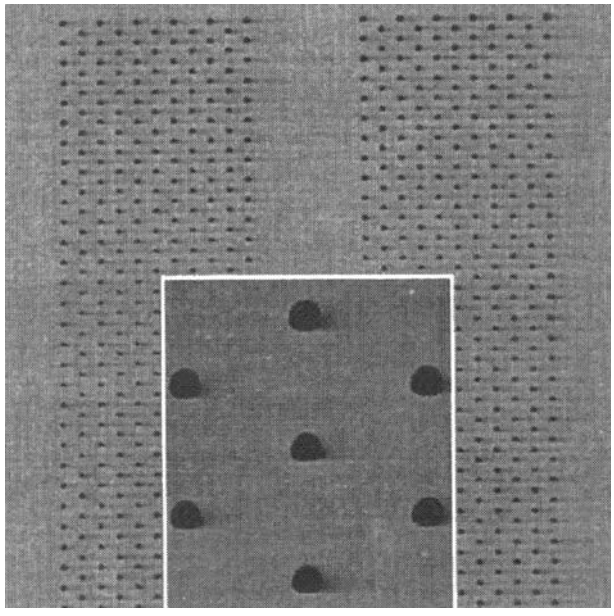

Fig. 10 REM picture of a membrane used by Büscher for single cell retention made by laser modification of a polymer membrane (diameter of one "hole" 5–5.5 μm) [9]

Fluorescence microscopy and immunohistochemistry

Based on classical phase contrast microscopy techniques, fluorescence microscopy lacks the advantage of reliable statistical analysis due to the small, stagnant sample volume. Still it holds the basic advantage of spatial resolution of the junction site of the fluorochrome at least in an 2-D arrangement [52]. Accordingly, scientists employ these techniques if spatial resolution is crucial for a conclusion from the experimental setup and if 2-D resolution is sufficient.

A limited 3-D resolution is accessible if immunohistochemistry is applied on the standard fluorescence microscopy experimental setup, as slights of tissue can be assessed stepwise and results can be correlated to the 3-D spatial arrangement [50]. Novel methods developed

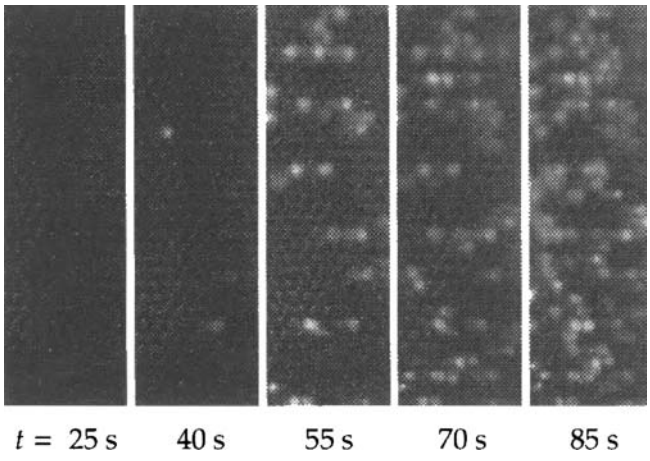

Fig. 11 Time resolved inoculation of a membrane layer (see Fig. 10) with decollated cells and retention of cells at discrete places. Accordingly an inoculation with single cells is feasible (see $t = 40$ s) [9]

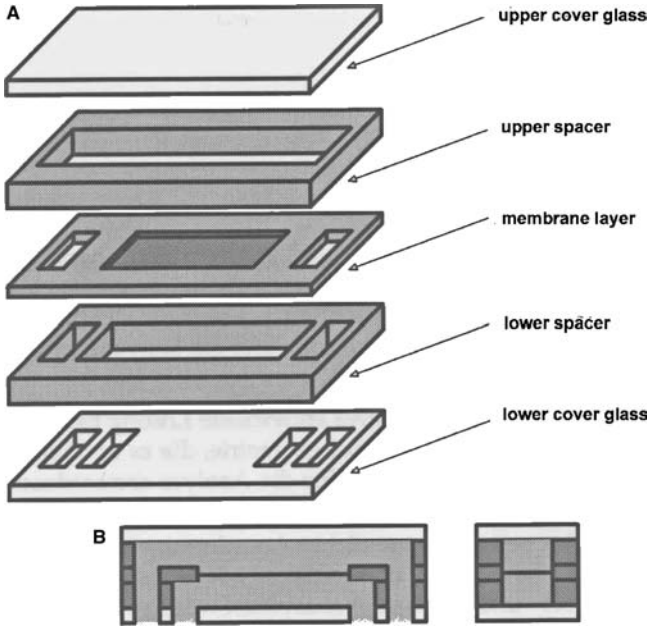

Fig. 12 Exploded view of the single cell reactor and assembly. [9]

during the maturation of in situ hybridisation also apply fluorescent probes to resolve even DNA and mRNA in an fluorescence microscopy arrangement [42].

Confocal laser scanning microscopy (CLSM)

In contrast to classical confocal fluorescence microscopy not a broad band excitation source (UV-Hg-Lamp) is used and restricted to certain band-widths via filter application, but a laser is used to ensure narrowest band width distribution of exciting light. Additionally CLSM no longer enlightens the whole sample, but a grid is overlaid and each grid-knot is excited separately. This yields that CLSM can automatically focus through the sample. Doing this another lens system is applied to focus on the "pinhole" to exclude light emitted from other slights of the sample.

Finally all gathered information can be rearranged to build up a truly three dimensional picture of the analysed sample [70].

The spatial and temporal resolution of CLSM systems have reached theoretical values of up to now 0.2×0.2×0.6 μm in x, y and z direction at a sampling sequences of 0.1–30s, although 25 Hz are feasible [21].

Isolated cell reactor setup

Assuming the above stated lack of knowledge as a target for novel bioreactor design an isolated cell fermenter is urged to be developed, which allows priming isolated cells with specific substances in vitro, which are supposed to have a major impact on the cellular differentiation genesis.

To allow a crosstalk of cells via signalling substances a multi well setup would be favourable. This would come along with the desired effect of decoupling cell–cell contact effects from signalling effects via soluble factors. Even though feeder layer cultures are a common experimental setup to induce cellular differentiation in vitro [41], it is still unknown whether the feeder layer acts via contact signalling or via secreted soluble factors. It is conjecturable that a combination of both prevails, but a decoupling of both parameters would at least be helpful for a sensitivity analysis of these parameters.

Some approaches already successfully decouple the influences of soluble factors from contact signalling of cells, employing commercially available cell culture inserts with different micro porous membranes. Nevertheless cell populations are employed and hence a contact driven cell–cell signalling in between cells of the same population is *not* excluded [78].

Additionally, this experimental setup has to fulfil several aspects required for mammalian cell culture as well as measuring technique demands. These requirements govern sustainable delivery of essential media components in a highly defined way regardless of spatial arrangement of the cells, the possibility of priming the isolated cells with defined amounts of differentiation inducing factors and a possibility of online monitoring the cellular genesis.

At a preliminary stage a reactor system needs to be developed fulfilling upper demands via design of a multi well-plate being translucent for optical analysis of the cells. This setup would utilize a CCD-camera to simultaneously monitor different fluorescence signals by appropriate filter application. CCD-cameras allow continuous monitoring of "entrapped" cells, which are tagged with specific antibodies indicating the differentiation status of the cell.

Antibody application is already a widespread technique in the field of haematopoiesis due to extensive flow

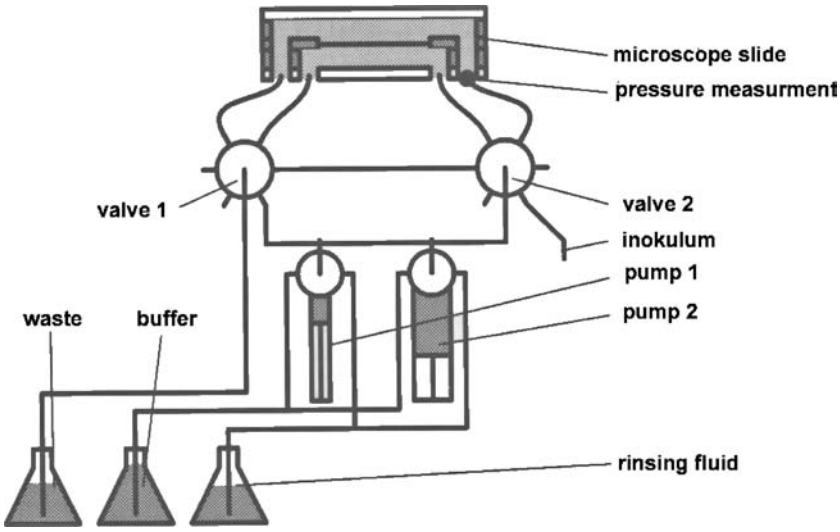

Fig. 13 Complete reactor setup, using regular maintenance of the cells via "pump 2"/"buffer", and possibility of priming immobilised cells via "pump 1" and rinse containment. (modified from 9)

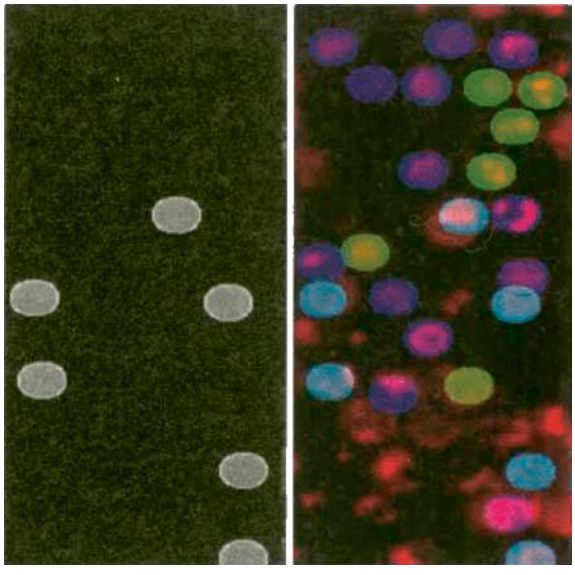

Fig. 14 CCD-view of the membrane layer after fluorescence excitation and application of a "blue-filter" for later cellular expression profiling (modified from 9)

cytometry [75] and MACS development advances in recent years, and is known to resolve even expression-densities on cells via binding of antibodies on specific epitopes on a cellular surface or protein.

An applicable reactor setup fulfilling most of the upper requirements has already been developed by Büscher [9]. This setup is already characterised in respect to its optical resolution and has already been run under sterile conditions with peripheral blood mononuclear cells (PBMC's)

Büscher's setup utilises several membranes with highly defined incorporated "holes" being used for cell entrapment, ranging from 1 μm to 5.5 μm in diameter (Fig. 10), which can retain singularised cells. Even timely resolved inoculation densities seem to be feasible (Fig. 11) for later single cell assessment.

The entire setup consists of a translucent observation chamber which even can be fed with defined substances via usage of pump 1. (Figs. 12, 13)

Application of suitable filters additionally allows a resolution of coupled fluorochromes (Figs. 12, 15) and hence can be applied to later analyse differentiation patterns of cells being immobilised on the membrane as a function of superposed gradients of soluble factors.

Future targets

Taking blood cells as a first target of research an uncovering of way of action of proteins influencing haematopoiesis might be feasible on a single cell level in vitro. Using the single cell assessment approach of Büscher this might be feasible at a preliminary approach. Timely resolved influences of several cytokines on singularised cells can then be assessed decoupled from the influences of cellular crosstalk being observed in standard cultivation approaches done in well-based culture conditions.

Coupling with information from genetic research of these experiments should be able to bridge the gap between genetics and proteomics.

A far more demanding task would be taking stem cells from the blastocyst rather than haematopoietic cells and triggering them specifically to form a distinct differentiated cell type in vitro without the employment of feeder layers or other uncharacterised supplements like FCS.

Starting from the postulate implicit in Fig. 8, the culture setup mentioned previously might even elucidate the falseness of the "one-way-street" assumption, or result in a better understanding of the factors influencing the pathway defined in Fig. 7. Finally, it might come to an understanding of the "confusing outcomes" from the experiments from Gussoni et al. which are already contradictory to the postulate in Fig. 8.

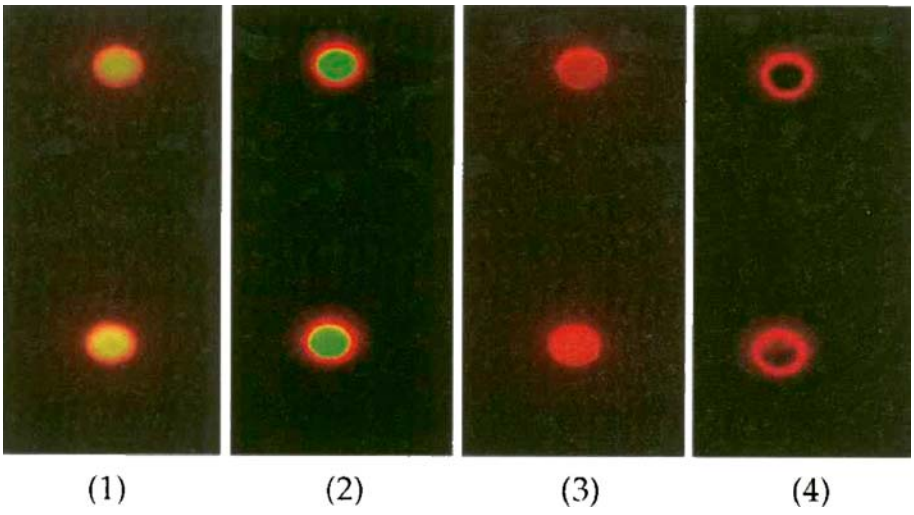

Fig. 15 Green and red fluorescent particles: *1* unprocessed data, *2* after compensation of overlayed fluorescent signals, *3* only red fluorescence of the unprocessed data, *4* only fluorescent data of the already compensated data. [9]

It might additionally be thought of several approaches utilising cell populations from other,"more differentiated" stem cells e.g. from the mesenchymal lineage or other bone marrow derived adult stem cells.

References

1. Ameer GA, Mahmood TA, Langer R (2002) A biodegradable composite scaffold for cell transplantation. J Orthop Res 20:16–19
2. Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI (1995) Embryonic stem cells express neuronal properties in vivo. Dev Biol 168:342–357
3. Berson RE, Pieczynski WJ, Svihla CK, Hanley TR (2002) Enhanced mixing and mass transfer in a recirculation loop results in high cell densities in a roller bottle reactor. Biotechnol Prog 18:72–77
4. Bjornson CR, Rietze RL (1999) Turning brain into blood: a hematopietic fate adopted by adult neural stem cells in vivo. Science 283:534–537
5. Blüml G, Reiter M, Zach N, Gaida T, Schmatz C, Assadian A, Strutzenberger K, Katinger H (1994) In: Spier RE, Griffith JB, Berthold W (eds) Animal cell technology. Butterworth Heinemann, Oxford, pp 267–269
6. Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechno l70:313–321
7. Boudreault P, Tremblay JP, Pe'pin MF, Garnier A (2001) Scale-up of myoblast culture process. J Biotechnol 91:63–74
8. Burja AM, Abou-Mansour E, Banaigs B, Payri C, Burgess JG, Wright PC (2002) Culture of the marine cyanobacterium, *Lyngbya majuscula* (Oscillatoracae), for bioprocess intensified production of cyclic and linear lipopeptides. J Microbiol Methods 48:207–219
9. Büscher M (2003) Entwicklung eines automatisierten Verfahrens zur quantitativen und qualitativen Zellanalyse. Tenea Verlag, Berlin, ISBN 3-86504-029-2
10. Cadic-Amadeuf Ch, Margerin Ch, Baquey Ch, Dupuy B (1993) Modified antibody secretions by embedding hybidoma cell lines in agarose gels, colloids and surfaces. Biointerfaces 1:91–96
11. Carroll SB (2003) Genetics and the making of homo sapiens. Nature 422:849–857
12. Chae SY, Lee M, Kim SW, Bae YH (2004) Protection of insulin secreting cells from nitric oxide induced cellular damage by crosslinked haemoglobin. Biomaterials 25:843–850
13. Collins FS, Morgan M, Patrinos A (2003) The human genome project lessons from large-scale biology. Science 300:286–290
14. Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW (2002) Efficiency of embryotic body formation and hematopoietic development from embryotic stem cells in different culture systems. Biotechnol Bioeng 78(4):442–453
15. Dautzenberg H, Stange J, Mitzner S, Lukanoff B (1996) Encapsulation by polyelectrolyte complex formation—a way to make hepatocyte cultures safe, efficient and online available. Progress Biotechnol 11:181–188
16. Dowd C, Bekes F (2002) Large-scale expression and purification of high-molecular-weight glutenin subunits. Protein Expr Purif 25:97–104
17. Elisseeff J, McIntosh W, Anseth K, Riley S, Ragan P, Langer R (2000) Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. J Biomed Material Res 51:164–171
18. Fahrer AM, Bazan JF, Papathanasiou P, Nelms KA, Goodnow CC (2001) A genomic view of immunology. Nature 409:836–838
19. Fiirster M, Mansfeld J, Dautzenberg H, Schellenberger A (1996) Immobilization in polyelectrolyte complex capsules, encapsulation of a gluconate-oxidizing Serratia marcescens strain. Enzyme Microbial Technol 19:572–577
20. Frazier ME, Johnson GM, Thomassen DG, Oliver CE, Patrinos A (2003) Realizing the potential of the genome revolution. The genomes to life program. Science 300:290–293
21. Fricker MD, Meyer AJ (2001) Confocal imaging of metabolism in vivo: pitfalls and possibilities. J Exp Bot 52:631–640
22. Fuchs C, Köster D, Wiebusch S, Mahr K, Eisbrenner G, Märkl H (2002) Scale-up of dialysis fermentation for high cell density cultivation of *Escherichia Coli*. J Biotechnol 93:243–251
23. Furey TS, Diekhans M, Lu Y, Graves TA, Oddy L, Randall-Maher J, Hillier LW, Wilson RK, Haussler D (2004) Analysis of human mRNAs with the reference genome sequence reveals potential errors, polymorphisms, and RNA editing. Genome Res 14:2034–2040
24. Gerecht-Nir S, Cohen S, Itskovitz-Eldor J (2004) Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation. Biotech Bioeng 86:493–502
25. Glockner H, Lemke H-D (2001) New miniaturized hollow fibre bioreactor for in vivo like cell culture, cell expansion and production of cell derived products. Biotechnol Prog 17:828–831
26. Gottlieb DI, Huettner JE (1999) An in vitro pathway from embryonic stem cells to neurons and glia. Cells Tissue Organs 165:165–172
27. Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394

28. Handa A, Emery AN, Spier RE (1987) On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble column bioreactors. Dev Biol Stand 66:241–253
29. Hübner H (1999) Microencapsulation. Encyclopedia of fermentation, biocatalysis and bioseparation. Wiley, New York, ISBN-0471-13822-3
30. Idowu BD, Knight MM, Bader DL, Lee DA (2000) Confocal analysis of cytoskeletal organisation within isolated chondrocyte sub-populations cultured in agarose. Histochem J 32:165–174
31. John GT, Klimant I, Wittmann C, Heinzele E (2002) Integrated optical sensing of dissolved oxygen in microtiter plates: a novel tool for microbial cultivation. Biotech Bioeng 81:529–536
32. Johnson SK, Zhang W, Smith LA, Hywood-Potter KJ, Swanson ST, Schlegel VL, Meagher MM (2003) Scale-up of the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in *Pichia pastoris*. Protein Expr Purif 32:1–9
33. Kensy F, Zimmermann HF, Knabben I, Anderlei T, Trauthwein H, Dingerdissen U, Büchs J (2004) Oxygen transfer phenomena in 48 well MTPs: determination by optical monitoring of sulfite oxidation and verification by real-time measurement during microbial growth. Biotech Bioeng 89:698–708
34. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee S-H, Nouven J, Sanchez-Pernaute R, Bankiewicz K, McKay R (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50–56
35. Kirschstein R, Skirboll LR (2001) Stem cells: scientific progress and future research directions. National Institutes of Health Department of Health and Human Services
36. Klammt S, Stange J, Mitzner SR, Peszynski P, Peters P, Liebe S (2002) Extracorporal liver support by recirculating albumin dialysis analysing the effect of the first clinically used generation of the MARS system. Liver 22:30–34
37. Kloth C, Riese C, Cho M, Buchholz R, Marx U (1995) Mab production and treatment of rheumatoid disease using encapsulated anti-idiotypic hybridoma cells. In: Beuvery EC, Griffiths JB, Zeijlemarker WP (eds) Animal cell technology: developments towards the 21st century. Kluwer, Dordrecht, pp 765–769
38. Kramer J, Hegert C, Guan K, Wobus AM, Müller PK, Rohwedel J (2000) Embryonic stem cell derived chondrogenic differentiation in vitro. activation by BMP-2 and BMP-4. Mech Dev 92:193–205
39. Kumar A, Murphy R, Robinson P, Wei L, Boriek AM (2004) Cyclic mechanical strain inhibits skeletal myogenesis through activation of focal adhesion kinase, Rac-1 GTPase, and NF-κB transcription factor. FASEB J 18:1524–1535
40. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234
41. Lee JB, Song JM, Lee JE, Park JH, Kim SJ, Kang SM, Kwon JN, Kim MK, Roh SI, Yoon HS (2004) Available human feeder cells for maintenance of human embryotic stem cells. Reproduction 128:727–735
42. Levsky JM, Singer RH (2003) Fluorescence in situ hybridization: past, present and future. J Cell Sci 116:2833–2838
43. Li RH (1998) Materials for immunoisolated cell transplantation. Adv Drug Deliv Rev 33:87–109
44. Lieschke GJ, Dunn AR (1995) Development of functional macrophages from embryonal stem cells in vitro. Exp Hematol 23:328–334
45. Liu SR, Liu HQ, Pan YQ, Tang SP, Xiong J, Hui N, Wang SL, Qi ZT, Li LS (2004) Human embryonic germ cells isolation from early stages of post implantation embryos. Cell Tissue Res 318:525–531
46. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R (2001) Differentiation of embryonic stem cells to insulin secreting structures similar to pancreatic islets. Science 292:1389–1394
47. Lyons AB (2000) Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods 243:147–154
48. Mansfeld J, Fiirster M, Hoffmann T, Schellenberger A (1995) Coimmobilization of Yarrowia lipolytica cells and invertase in polyelectrolyte complex capsules. Enzyme Microbial Technol 17:11–17
49. Maranga L, Cunha A, Clemente J, Cruz P, Carrondo MJT (2004) Scale-up of virus-like particles production effects of sparging, agitation and bioreactor scale on cell growth, infection kinetics and productivity. J Biotechnol 107:55–64
50. Mitsiades C, Sourla A, Doillon C, Lembessis P, Koutsilieris M (2000) Three-dimensional type I collagen co-culture systems for the study of cell-cell interactions and treatment response in bone metastases. J Musculoskelet Neuronal Interact 1:153–155
51. Montes FJ, Catalan J, Galan MA (1999) Prediction of k_La in yeast broths. Process Biochem 34:549–555
52. Morrison IEG, Karakikes I, Barber RE, Fernandez N, Cherry RJ (2003) Detecting and quantifying colocalization of cell surface molecules by single particle fluorescence imaging. Biophys J 85:4110–4121
53. Morrison SJ, Hemmati HD, Wandycz AM, Weissmann IL (1995) The purification and characterisation of fetal liver heamtopoietic stem cells. Proc Natl Acad Sci USA 92:10302–10306
54. Mostafa SS, Gu X (2003) Strategies for Improved dCO2 Removal in Large-Scale Fed-Batch cultures. Biotechnol Prog 19:45–51
55. Murphy CI, McIntire JR, Davis DR, Hodgdon H, Seals JR, Young E (1993) Enhanced expression, secretion and large-scale purification of recombinant HIV-1 gp120 in insect cells using the baculovirus egt and p67 signal peptides. Protein Expr Purif 4:349–357
56. Niemeyer P, Seckinger A, Simank HG, Kasten P, Südkamp N, Krause U (2004)Allogene Transplantationsmöglichkeiten humaner mesenchymaler Stammzellen. Orthopädie 33:1346–1353
57. Nehring D, Czermak P, Vorlop J, Luebben H (2004) Experimental study of a ceramic microsparging aeration system in a pilot-scale animal cell culture. Biotechnol Prog 2:1710–1717
58. Omer A, Duvivier-Kali VF, Trivedi N, Wilmot K, Bonner-Weir S, Weir GC (2003) Survival and maturation of microencapsulated porcine neonatal pancreatic cell clusters transplanted into immunocompetent diabetic mice. Diabetes 52:69–75
59. Orive G, Hernandez RM, Gascon AR, Igartua M, Pedraz JL (2003a) Cell microencapsulation technology for biomedical purposes novel insights and challenges. Trends Biotechnol 24:207–210
60. Orive G, Hernandez RM, Gascon AR, Igartua M, Pedraz JL (2003b) Survival of different cell lines in alginate-agarose microcapsules. Eur J Pharm Sci 18:23–30
61. Orive G, Ponce S, Hernandez RM, Gascon AR, Igartua M, Pedraz JL (2002) Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginate. Biomaterials 23:3825–3831
62. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. Proc Natl Acad Sci USA 92:4857–4861
63. Picariello L, Benvenuti S, Recenti R, Formigli L, Falchetti A, Morelli A, Masi L, Tonelli F, Cicchi P, Brandi ML (2001) Microencapsulation of human parathyroid cells: an "in vivo" study. J Surg Res 96:81–89
64. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
65. Potocnik AJ, Nielsen PJ, Eichmann K (1994) In vitro generation of lymphoid precursors from embryonic stem cells. EMBO J 13:5274–5283

66. Puskeiler R, Kaufmann K, Weuster-Botz D (2005) Development, parallelization and automation of a gasinducing ml scale bioreactor for high throughput bioprocess design for high-throughput bioprocess design (HTBD). Biotech Bioeng 89:512–523
67. Qi HN, Chetan TG, Michaels JD, Henzler H-J, Jovanovic GN, Konstantinov KB (2003) Experimental and theoretical analysis of tubular membrane aeration for mammalain cell bioreactors. Biotechnol Prog 19:1183–1189
68. Ramdi H, Tahri Jouti MA, Lievremont M (1993) Immobilized articular chondrocytes: in vitro production of extracellular matrix compounds. Biomater Artif Cells Immobilization Biotechnol 21:335–341
69. Roberts T, De Boni U, Sefton MV (1996) Dopamine secretion by pc12 cells microencapsulated in a hydroxyethyl-methacrylate-methyl methacrylate copolymer. Biomaterials 17:267–275
70. Roderfeld M, Matern S, Roeb E (2003) Konfokale Laserscanning-Mikroskopie: Der Blick in die Zelle. Dtsch Med Wochenschr 128:2539–2542
71. Rohwedel J, Maltsev V, Bober E, Arnold HH, Heschieler J, Wobus AM (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Dev Biol 164:87–101
72. Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256
73. Sauer LM, Kardassis D, Zeillinger K, Pascher A, Gruenwald A, Pless G, Irgang M, Kraemer M, Puhl G, Frank J, Müller AR, Steinmüller Th, Denner J, Neuhaus P, Gerlach JC (2003) Clinical extracorporal hybrid liver support–phase 1 study with primary porcine liver cells. Xenotransplantation 10:460–469
74. Selber K, Tjerneld F, Collén A, Hyytiä T, Nakari-Setälä T, Bailey M, Fagerström R, Kan J, van der Laan J, Penttilä M, Kula M-R (2004) Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. Process Biochem 39:889–896
75. Sengbusch G, Hugemann B (1974) Fluorescence microscope attachment for flow-through cytofluorometry. Exp Cell Res 86:53–58
76. Spangrude GJ, Smith L, Uchida N, Ikuta K, Heimfeld S, Friedman J, Weissmann IL (1991) Mouse hematopoietic stem cells. Blood 78:1395–1402
77. Stange J, Mitzner SR, Risler T, Erley CM, Lauchart W, Goehl H, Klammt S, Peszynski P, Freytag J, Hickstein H, Löhr M, Liebe S, Schareck W, Hopt UT, Schmidt R (1999) Molecular adsorbent recycling system (MARS), clinical results of a new membrane-based blood purification system for bioartificial liver support. Artif Organs 23:319–330
78. Taichman RS, Reilly MJ, Verma RS, Emerson SG (1997) Augmented production of interleukin-6 by normal human osteoblasts in response to CD34 hematopoietic bone marrow cells in vitro. Blood 89:1165–1172
79. Uludag H, De Vos P, Tresco PA (2000) Technology of mammalian cell encapsulation. Adv Drug Deliv Rev 42:29–64
80. Uludag H, Hwang JR, Sefton MV (1995) Microencapsulated human hepatoma (HepG2) cells capsule to capsule variations in protein secretion and permeability. J Control Release 33:273–283
81. Vallbacka JJ, Nobrega JN, Sefton MV (2001) Tissue engineering as a platform for controlled release of therapeutic agents: implantation of microencapsulated dopamine producing cells in the brains of rats. J Control Release 72:93–100
82. van Susante JL, Buma P, van Osch GJ, Versleyen D, van der Kraan PM, van der Berg WB, Homminga GN (1995) Culture of chondrocytes in alginate and collagen carrier gels. Acta Orthop Scand 66:549–556
83. Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E (1996) Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. Blood 88:3424–3431
84. Voisard D, Meuwly F, Ruffieux P-A, Baer G, Kadouri A (2003) Potential of cell retention techniques for large scale high density perfusion culture of suspended mammalian cells. Biotech Bioeng 82:751–765
85. Wakitani S, Saito T, Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426
86. Walter C, Steinau T, Gerbsch N, Buchholz R (2003) Monoseptic cultivation of phototrophic microorganisms-development and scale-up of photbioreactor system with thermal sterilization. Biomol Eng 20:261–271
87. Wang X, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M (2001) Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. Am J Pathol 158:571–579
88. Znad H, Bales V, Kawase Y (2004) Modeling and scale up of airlift bioreactor. Comput Chem Eng 28:2765–2777