Hepatic Differentiation of human embryonic stem cells in a 3D bioreactor environment

vorgelegt von

Diplom-Ingenieur

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Von der Fakultät III - Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften
Dr. Ing.

genehmigte Dissertation

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Tag der wissenschaftlichen Aussprache: 07. März 2014

Berlin 2014
D83

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Abbreviations

ALF	acute liver failure
BAL	bioartificial liver device
BMP	bone morphogenic protein
BRY	brachyury
CLF	chronic liver failure
DE	definite endoderm
DMSO	dimethyl sulfoxide
ELAD	extracorporeal liver assist device
ELISA	enzyme-linked immunosobent Assay
ES	embryonic stem
FGF	fibroblast growth factor
hESC	human embryonic stem cells
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
ICM	inner cell mass
IHC	immune-histochemistry
IMDM	Iscove´s modified Dublecco´s medium
IPS	induced pluripotent stem cells
i.s. Injection	intrasplenic injection
IVF	in vitro fertilization
LDH	lactate dehydrogenase
LT	liver transplantation
MEF	mouse embryonic fibroblasts

NOD	non-obese diabetic
NSG	NOD SCID gamma
Oct-4	Octamer-binding transcription factor 4
OSM	Oncostatin M
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PS	primitive streak
RT-PCR	reverse transcription polymerase chain reaction
s.c. Injection	subcutaneous injection
SCED	single cell enzymatic dissociation
SCID	severe combined immunodeficiency
SOX	sex determining region Y
VEGF	vascular endothelial growth factor
Wnt3a	Wingless-type MMTV integration site family, member 3A

1. Introduction

In the past years great progress was made in the field of regenerative medicine research. Although cell based therapies utilizing pluripotent cells are still far away from clinical practice, it is clear that differentiated cells from IPS or hESC could play an important role in the treatment of degenerative and congenital diseases in the future. Besides the understanding of the generation of safe stem cell derivatives the reliable and cost effective supply of relatively large amounts of cells is an elemental requirement.

Embryonic stem cells owe the ability to differentiate into cell derivatives of all three germ layers. In combination with their theoretically unlimited proliferative capacity hESC could play a central role in developmental biology research as well as regenerative medicine utilizing differentiated derivatives not only for direct cell transplantation, but also in temporary supportive therapies. In first approaches undifferentiated ESC were directly transplanted into various organs and tissues. The cells did not restore organ function, but formed teratoma [1 - 3].

Approaches investigating the supportive effect of transplanted differentiated embryonic stem cells have been conducted with promising results [4]. But also the formation of teratoma originating from transplanted endodermal hESC derivatives was reported [5] in a mouse model. In general neural stem cells (NSC) of fetal and embryonic origin share the majority of characteristics [6], and NSC of fetal origin have been demonstrated to exhibit no tendency to form tumors in an animal model [7] in contrast to NSC differentiated from hESC [8]. But after the application of fetal neural stem cells the formation of a multi-focal donor-derived brain tumor was reported in a human patient [9]. Although progress on the safety of a potential medical application of differentiated stem cells has been made by the elimination of tumorigenic stem cells from differentiated hESC populations [10], the application of cell derivatives in humans still remains dicey with uncertain clinical outcome. Only recently a phase I clinical trial utilizing differentiated oligodendrocyte progenitor cells (OPC) in patients suffering from spinal cord injuries was discontinued due to unimpaired function after cell application [11].

Interestingly, early results of a phase I/II open label study treating patients suffering from dry age-related macular degeneration (n=1) or Stargardt´s disease (n=1) with hESC derived retinal pigment epithelium cells show no adverse effects like teratoma formation, rejection or inflammation. Further on a visual improvement was seen in both patients [12].

A more secure potential clinical application of differentiated hESC might be the extracorporeal organ support. Reports have been published for bioartificial liver devices utilizing porcine or human hepatocytes [13]. These cells could be replaced by differentiated hESC and thus avoid the risks and problems related to xenobiotic materials or scarcity of human hepatocytes. In addition the design of the bioreactor device excludes the possibility of direct cell transfer from the bioreactor device into the patient and thus eliminates the risk of tumor formation due to unintended transplantation of differentiated hESC.

1.1 In vitro culture of human embryonic stem cells (hESC)

The development of human embryo begins after the conjunction of the parental gametes. The zygote starts to divide and the first molecular differences in the arising cells become apparent in the 4 to 8 cell stadium [14]. This is followed by the formation of a morula (16 cell stadium). After further cell divisions (70 to 100 cells) and cavitation the blastocyst is formed (5 days after fertilization). It is comprised of a blastocoel cavity that is filled with fluid, the inner cell mass (ICM) and the surrounding trophoectodermal cells (trophoblast) [15].

When in 1998 Thomson et al. first reported the successful isolation and cultivation of embryonic stem cells from the inner cell mass (ICM) of a human blastocyst, a useful tool for developmental biology and drug discovery as well as hope for a revolution in transplantation medicine arose.

Human embryonic stem cells possess the unique ability of self-renewal for long periods in culture and form derivatives of all three embryonic germ layers [16]. The differentiation of hESC has been extensively studied in the past and protocols for the

differentiation in neuronal [17], cardiac [18], cartilage [19], pancreatic [20], hepatic [21, 22] and other cell types were established. In parallel the understanding of molecular mechanisms responsible for the unique characteristics of hESC grew over time, and the culture conditions for the proliferation of undifferentiated hESC were successively more standardized and further developed from animal or human feeder-dependent coculture [23, 24] over xeno-culture conditions [25] to defined xeno-free conditions [26].

1.2 Hepatic differentiation of human embryonic stem cells

The endodermal and subsequent hepatic differentiation has extensively been studied in developmental models such as zebra-fish, worm and mouse. In these studies the existence of a progenitor cell population (mesendodermal cells) capable of further differentiating into cells of the mesodermal and endodermal germ layer [27] was described. Interestingly several signals involved in the differentiation into the primitive streak, mesendoderm and further into mesoderm and endoderm seem to be conserved [28].
Due to this, approaches for the endodermal differentiation of human embryonic stem cells were adapted from protocols for mouse ESC, in which four main pathways (Activin/Nodal, bone morphogenic proteins [BMP], FGF and Wnt) had been reported to play a crucial role in lineage commitment.

1.2.1 Cell therapies for clinical treatment of liver failure

Every year about 2 million people die of liver disease while waiting for a suitable organ. Acute and chronic liver failure (ALF) & (CLF), which can be due to a plurality of physical, chemical, or infectious reasons is a devastating disease and associated with significant morbidity and a relatively high mortality (60-90%) [29]. Currently the only effective therapy for ALF is liver transplantation (LT). It was first performed in 1983 and its clinical outcome improved with growing experience and enhanced operative management. Today LT is a surgical procedure performed in large centers

with a 1 year survival rate of more than 90% [30]. Due to the scarcity of donor organs the LT is not an option for all patients. In fact less than 30% of the patients on the waiting list receive a transplant [31].

However, since the liver is capable of extensive self-regeneration, for example after partial hepatectomy [32], or other kinds of injury, there is an alternative for a certain amount of patients waiting for a transplant. Liver assist devices have been developed and tested since the 1950s. In general two different approaches are used for liver support. The first kind of devices is based on the idea that patients with ALF or CLF are mostly affected by small dialyzable molecules such as ammonia and phenols that accumulate to toxic concentrations. However, evidence has been provided that the substances, which cause hepatic encephalopathy, multi-organ failure and further insults in case of ALF or CLF, are not just these small toxins but also mediators of systemic inflammation like various cytokines, chemokines, anaphylatoxins, etc [33]. On the other side also anti-inflammatory mediators can be found at elevated levels which have been shown to inhibit the proliferation of hepatocytes and mediate pro-fibrotic and pro-cancerogenic signals [34].

This led to the development of a second kind of devices for the support of ALF and end-stage CLF patients.

These bioartificial liver (BAL) devices use mammalian hepatic cells as central element to metabolize not only toxic components but also the mediators (also in combination with detoxification devices). It has been shown that bioartificial liver support can help to bridge patients with ALF by detoxification [35] until a suitable organ is available or the regeneration of the patient´s liver is advanced to a point where no liver transplantation is needed. The insufficient availability of human liver cells for hepatocyte based bioartificial liver devices led to the use of porcine hepatocytes [36] or human hepatic cell lines [37].

However, the concerns of potential immunological problems in case of using xenogeneic cells and the risk of tumor cell transfer into patients in case of using tumor-derived cell lines [38] avert clinical application. Furthermore it has been shown that the C3A cell line, a derivate of the hepatic tumor cell line HepG2, which is used in the Extracorporeal Liver Assist Device (ELAD), caused no significant beneficial effect on the survival rate of patients in a pilot-controlled clinical trial [39]. These problems could be overcome by the use of differentiated hESC, capable of unlimited

proliferation and differentiation into hepatocytes. Mature or premature hepatic cells derived from hESC could also be used for cell transplantation therapy in patients suffering from ALF or CLF, if the presence of remaining undifferentiated hESC with the risk of tumor formation can be excluded in the preparation [40]. Until now no clinical application of hESC in liver therapies has been reported, based on ethical issues and safety risks due to the high oncogenic potential of the cells.

1.2.2 Use of hepatocytes for pharmacological studies

Pharmacological companies invest enormous amounts of money into the development of new drugs. Before the application in human phase I studies any new drugs are tested in animal trials to reduce the possibility of human side-effects. However, the metabolism of various animal species used for pharmacological studies features significant differences compared to the metabolism in humans [41, 42]. To overcome the most obvious inter-species differences, efforts have been taken to generate trans-gene animals with a more human like metabolism [43]. However, several differences still remain in such models and thus limit the informative value of animal experiments. It has recently been reported that hepatocytes cultured in a miniaturized bioreactor model can be cultured under maintenance of cytochrome P450 activity even under serum free conditions [44]. But although relatively small cell numbers are needed for one bioreactor run, the general shortage in human primary hepatocytes remains a challenge in large-scale pharmaceutical studies. Differentiated hESC could replace primary hepatocytes and thus enable large-scale pharmaceutical studies with cells exhibiting a set of hepatocyte specific enzymes closer to physiological conditions compared to the use of cells of non-human origin.

1.2.3 Bioreactor system for dynamic 3D culture of hepatic cells

An elegant alternative to animal experimental studies of drug biotransformation kinetics and in addition a valuable additional tool for the prediction of adverse events in humans might be the use of 3D bioreactor systems inoculated with hepatic cells.

In addition to the gain of informative value by the use of bioreactors inoculated with human cells, the culture system switch from conventional 2D culture in static plastic dishes to 3D bioreactor systems offers a number of advantages. Bioreactor systems provide a more homogeneous environment with the possibility to constantly monitor and adjust culture parameters. Limitations of conventional 2D culture in the control of culture environment parameters like the accumulation of metabolites, the fluctuation in nutrient concentration, the adjustment of oxygen partial pressure as well as pH can be addressed in 3D bioreactor systems.

The bioreactor system used in this study was designed and used for extracorporeal liver support [45, 46, 13]. The bioreactor consists of interwoven hollow fiber membranes that form four independent compartments. Two of these components are utilized for the media perfusion of the cell compartment. The fourth compartment is used for the oxygenation. The combination of interwoven fiber membranes for media and gas supply in repetitive units enables a decentralized mass exchange, a reduction of distance dependent concentration gradients and a scalability of the bioreactor system itself. The cell compartment is nerved by these interwoven capillaries that allow for formation of tissue like structures in between.

The use of different 3D bioreactor systems has already been addressed in various studies with human hepatocellular carcinoma cell lines [47] as well as with primary human and animal hepatocytes [48 - 50]. Although the use of human hepatocytes, isolated from tumor free tissue after partial hepatectomy, in such 3D bioreactor systems is at the moment the most promising approach, the use of human primary cell is associated naturally with a number of disadvantages. It is known that primary hepatic cells have a high inter-individual variation in cytochrome P450 expression induced not only through different genetic background and sex, but also by age, smoking habits, abuse of drugs and many more factors [51]. In addition the amount of primary cells needed for pre-clinical trials would exceed the amount of available

healthy tissue suitable for cell isolation and bioreactor inoculation. Alternative approaches using hepatic carcinoma cell lines were disappointing due to the limited expression of hepatic enzymes in these transformed cells compared to primary hepatocytes [52]. Interestingly, it was shown that the culture of the human hepatoma cell line HepaRG in bioreactors results in a higher hepatocyte specific cytochrome P450 activity compared to the cells cultured in conventional 2D cultures [53]. A positive effect on hepatocyte specific functions in 3D bioreactor systems has also been reported for primary hepatocytes [54]. Therefore a promising alternative to the use of primary hepatocytes might be the use of differentiated human embryonic stem cells in a 3D bioreactor system.

1.3 3D bioreactor systems for the expansion of hESC

Today stem cells are cultured mainly in conventional 2D cultures. This method bears several advantages like relatively easy morphological evaluation of the cells and the possibility to manipulate colonies directly, but it is also very labour intensive, and makes large scale expansion in an extend needed for regenerative medicine therapies practically impossible. Recently an approach for the automation of cell expansion and labour reduction has been published [55]. However, other disadvantages of conventional 2D cultures, especially the discontinuous medium supply, leading to an accumulation of metabolic products and the scarcity of nutritive substances, and also the rather unphysiological 2D configuration of the cell layer still remains.

The culture and expansion of stem cells in bioreactors could provide an attractive alternative. The advantage of these culture systems lies not only in the reduction of effort, it also offers the possibility of continuous monitoring and adjustment of nutrient supply, waste elimination, pH - and oxygen concentration. It has been reported that the growth kinetics of hESC in a stirred-tank bioreactor in combination with micro-carriers were superior in comparison to conventional 2D cultures under maintenance of the undifferentiated state [56].

1.4 Hepatic differentiation of hESC in 3D bioreactor systems

Not only the undifferentiated expansion, but also the differentiation of stem cells in 3D bioreactor systems bears numerous advantages compared to 2D approaches. It has been reported that the formation of embryoid bodies in a stirring bioreactor with porous alginate scaffolds resulted in a superior vascularization compared to embryoid bodies gained after conventional approaches in 2D [57]. A higher proliferation rate, probably as a result of the more constant and optimized culture conditions, could also be reported within the same publication.

The directed differentiation of hESC in 3D bioreactors still holds a number of unanswered questions. The scientific progress made in the field of developmental biology gives us more and more insight into the molecular pathways that are involved in proliferation and differentiation of hESC into various lineages. The experiments made on the direction of differentiation into defined cell derivatives were mostly made in conventional 2D cultures. One of the essential findings from the diverse differentiation approaches was that not only the precise sequence of signal molecules, but also its concentration, the cell density, cell-cell contacts, the extra-cellular matrix, the nutrient supply, oxygen partial pressure and many other factors play a central role in signal conduction and thus cell differentiation. The culture environment that can be provided in a 3D bioreactor is fundamentally different from that in a culture dish. The potential of that culture system was demonstrated in a study investigating the teratoma formation from hESC in a 3D bioreactor in comparison with *in vivo* mouse teratoma formation [58]. Therefore the established protocols for the differentiation have to be adapted when applied to another environment. Initial studies on hepatic differentiation in the bioreactor system have been conducted [59] and showed a hepatic commitment of the cells.

1.5 Current limitations of hepatic differentiation of hESC

The directed hepatic differentiation of stem cells in conventional 2D culture has been reported to rely on the precise sequence of signal molecules. In contrast the nature of 2D culture systems brings fluctuation of nutrients, metabolic products, pH and signal molecules by the medium exchange.

The culture in a bioreactor system with continuous feed offers a fundamentally different environment. Culture parameters like nutrient content, metabolic waste concentration, and pH can be monitored and can be influenced by adjustment of the feed rate and gas mixture.

Two different applications are imaginable for bioreactors containing hepatic differentiated cells. The use in pharmaceutical research, in which a robust expression of the hepatic enzyme machinery is desired, and the use in acute or chronic liver failure as a therapeutic tool to support the liver function of patients. For the application in a clinical environment as extracorporeal liver assist device the use of materials of animal origin should be avoided.

When addressing the use of hepatic cells in pharmaceutical research the expression of enzymes of the cytochrome P450 family is of central interest. In a recent study cryopreserved human primary hepatocytes were cultured for 4 hours in conventional 2D culture and compared with commercially available hepatic differentiated hESC regarding their drug metabolizing capacity [60]. The hESC-derived differentiated cells showed only a fractional amount of activity of the investigated cytochrome P450 isoforms. Interestingly, it was shown that when the primary hepatocytes were cultured for 48 hours in conventional 2D culture instead, the drug metabolizing capacity decreases dramatically to a level comparable to hepatic differentiated hESC. It has been shown that the 3D bioreactor culture of primary human hepatocytes has a positive effect on the long-term maintenance of cytochrome P450 isoform activity [54] in comparison to conventional 2D culture. It therefore appears reasonable to culture hepatic differentiated hESC under 3D bioreactor conditions to support the drug metabolism enzyme expression in pharmaceutical research.

When addressing the potential clinical use of differentiated hESC three major

problems arise, the realization of the production of sufficient cell numbers, the functionality of the cells and the safety of medical application. In a phase I clinical study with a bioartificial liver assist device utilizing primary porcine liver cells in medical treatment of eight patients suffering from acute liver failure about 1.8E10 to 4.4E10 cells were used per application / patient [35]. Reports describing the cell numbers recovered after hepatic differentiation are rare, but it is known that the definitive endodermal commitment of hESC is a highly selective step associated with a high number of cell loss due to apoptosis.

Reports about the efficiency of differentiation of hESC into hepatocyte like cells vary from about 70% [61] of the cells expressing human Albumin to about 94% [62]. Although the purity of cell preparations obtained by these approaches is remarkably high, the identity of the non-hepatic cells still remains of large clinical interest since teratoma formation has been reported after transplantation of hESC committed to the endodermal lineage [5].

Until today the transplantation of mature hepatocytes is an attractive option in the treatment of metabolic disorders such as urea cycle defects or Crigler-Najjar disease. Although the transplanted cells only work transiently, hepatocyte transplantation can stabilize the medical condition and bridge the patient until classical liver transplantation [63].

A more promising alternative for cell-based therapies in regenerative medicine is the transplantation of fetal hepatic stem cells. The supportive effect of these cells can last for several years instead of several months as reported for the transplantation of mature hepatocytes [64, 65]. But ethical concerns make the use of fetal tissue difficult.

But whatever hepatic cell source is used, relatively high cell numbers are needed to treat a patient. In case of immature (fetal) cells reports range from 15 million fetal stem cells for a 2 year old patient to about 100 to 150 million cells in adults [64, 65]. Even more cells are needed if using adult hepatocytes since the cell engraftment generally accounts to not more than 3% of the host organ cells in a normal liver environment [66].

Taken into consideration that differentiated hESC feature only partly the metabolic activity of primary hepatocytes [60] the therapeutic value of differentiated cells is at the moment limited.

Thus, further development of differentiation methods is needed to improve the

functional performance of hESC-derived (progenitor) cells, to ensure the clinical safety of cell preparations and to optimize the practicability of cultivation processes for clinical use.

1.6 Aim of this study

The aim of this study was to investigate the effects of a 3D bioreactor environment on the hepatic differentiation of human embryonic stem cells.

Two different strategies were investigated for the differentiation of hESC along the hepatic lineage in a 3D bioreactor environment.

The first strategy aims the reduction of xenobiotic material by establishing feeder free culture conditions for hESC. Further on published protocols for the hepatic differentiation were evaluated in regard to the degree of hepatic differentiation via real-time PCR as well as cell yield. In the following suitable protocols were adapted for hESC differentiation in 3D bioreactor culture systems and the cell differentiation was monitored by the determination of the metabolic activity (glucose and lactate concentrations), the measurement of factors indicating processes of differentiation (beta HCG) and endodermal commitment (AFP) as well as the expression of hepatic and non-hepatic transcripts via real-time PCR.

In the second strategy the hepatic differentiation in a 3D bioreactor system was conducted in cooperation with Cellartis according to a protocol for the hepatic differentiation in conventional 2D culture published by Brolén et al., 2010 [67]. Cells were cultured using the Single Cell Enzymatic Dissociation (SCED) technique. The 3D bioreactor experiments were conducted with or without proteins and cells of animal origin. In a two-step differentiation approach cells were first committed to definitive endodermal lineage and then further proliferated and maturated into hepatic cells. The differentiation was monitored by determination of the metabolic activity, the measurement of factors indicating ongoing differentiation (beta-hCG) and definitive endodermal commitment (AFP) as well as immune histochemistry to study the formation of hepatic structures at the end of differentiation.

An overview of the strategies followed within this study in given in figure 1.

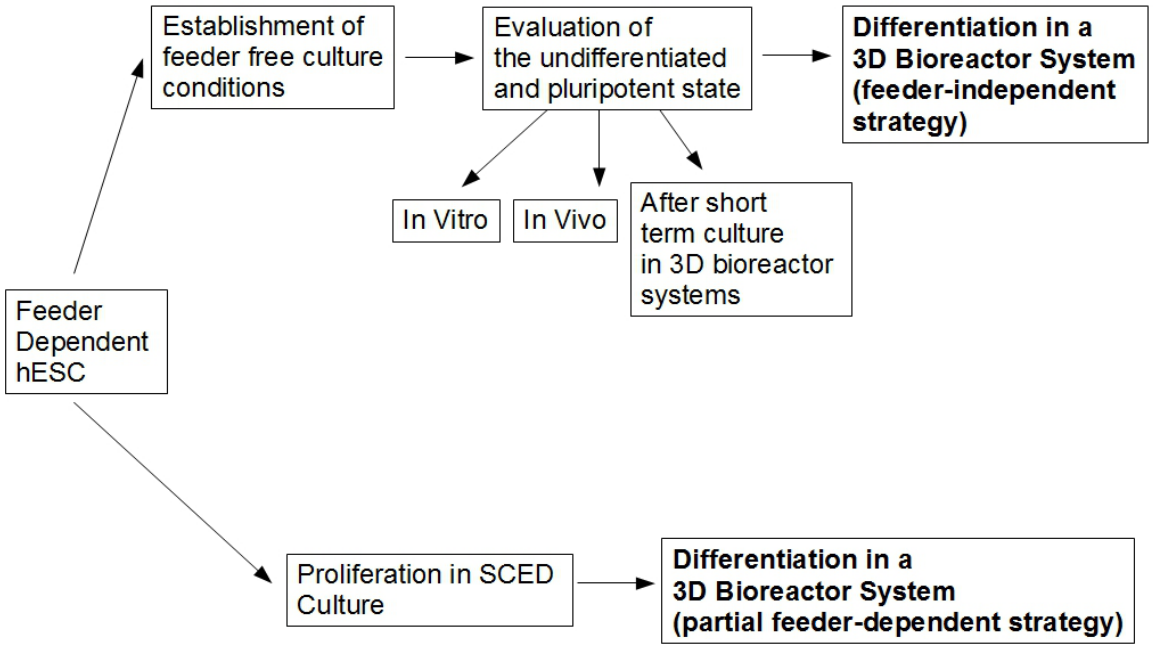


Figure 1: Strategies for the hepatic differentiation of hESC in 3D bioreactor systems. SCED = Single Cell Enzymatic Dissociation

2. Material and Methods

2.1 Materials

2.1.1 Equipment and Software

Table 1: Equipment

Equipment	Manufacturer
Bench drill; OPTI B23 Pro	Optimum-Maschinen Germany GmbH, Hallstadt
Bioreactor perfusion device	Stem Cell Systems, Berlin
Blood gas analyzer; ABL 700 Series	Radiometer, Brønshøj, Denmark
Centrifuge; Varifuge 3.OR	Heraeus Instruments GmbH, Hanau
Clinical analyzer; modular PPP analyzer	Roche Diagnostics, Heidelberg
Device for gas valves	Vögtlin Instruments, Aesch, Switzerland
Dako pen wax crayon	Dako, Hamburg
Fluorescence microscope; Axiovert 200M	Carl Zeiss, Göttingen
Fluorescence microscope camera; Retiga 2000R	QImaging, Surrey, BC, Canada
Fluorescence microscope lamp; AttoArc, HBO 100 W	Carl Zeiss, Göttingen
Horizontal air flow	Telstar, Terrassa, Spain
Light microscope; Axiovert 40 CFL	Carl Zeiss, Jena
Light microscope camera; MicroPublisher 3.3 RTV	QImaging, Surrey, BC, Canada
Incubators; Cytoperm CO_2 /O_2	Heraeus Instruments GmbH, Hanau
Laminar air flow; HB2472	Heraeus Instruments GmbH, Hanau
Microcentrifuge; 5417R	Eppendorf, Hamburg
Microtome; Microm HM355s	Microm-International-GmbH, Walldorf
Paraffin embedding center AP 250	Microm-International-GmbH, Walldorf
Perfusor; Secura FT	B.Braun, Melsungen

Plate-reading fluorometer; FumostarOptima	BMG Labtech GmbH, Ortenberg
Radiator; OB29/4 (^{137}Cs γ source)	STS, Braunschweig
Realtime cycler; Mastercycler ep Realplex 2	Eppendorf, Hamburg
Stereo microscope; Nikon SMZ1000	Nikon Instruments Europe BV, Amsterdam, Netherlands
Spectrophotometer; Nanodrop	Thermo Fisher Scientific, Waltham, MA, USA
Thermal-Cycler; Veriti 96 Well	Applied Biosystems, Foster City, USA

Table 2: Software

Software	Manufacturer
Get red-y 5	Vögtlin Instruments, Aesch, Switzerland
GraphPad Prism 5.0	GraphPad Software, San Diego, CA, USA
Image Pro Plus	Media Cybernetics, Silver Spring, USA
QCapture Pro 5.1	QImaging, Surrey, BC, Canada
OpenOffice	Apache OpenOffice Foundation, Los Angeles, CA, USA
Mendeley Desktop	Mendeley Ltd., New York, USA

2.1.2 Disposables

Table 3: Cell culture disposals

Component	Manufacturer
6-, 12- and 24-well plates	Falcon, BD Biosciences, San Jose, CA, USA
6-well plates, Nunclon^{TM} surface	Nunc, Roskilde, Denmark
24-well plates, lumox®	Sarstedt, Nümbrecht-Rommelsdorf
96-well plate; Fluoronunc™	Nunc, Roskilde, Denmark
"*In Vitro* Fertilization" (IVF) dishes	Falcon, BD Biosciences, San Jose, CA, USA
Cell culture flasks (25 cm^2 - 175 cm^2)	Falcon, BD Biosciences, San Jose, CA, USA

Stem Cell Cutting Tool™	Vitrolife AB, Göteborg, Sweden
Falcon tubes (15mL/50mL)	BD Biosciences, San Jose, CA, USA
Glass slides; super frost plus	R. Langenbrinck, Emmendingen
Whatman™ paper	Schleicher & Schuell GmbH, Dassel
Cover slides	Carl Roth, Karlsruhe
Thermo-Fast 96-Well-PCR-Platten	Thermo Fisher Scientific, Waltham, MA, USA

Table 4: Bioreactor systems and tube system components

Component	Company
Lab Scale Bioreactor (8 ml BR)	Stem Cell Systems, Berlin
Analytical Scale Bioreactor (2 ml BR)	Stem Cell Systems, Berlin
Miniaturized Bioreactor (0,2 ml BR)	Stem Cell Systems, Berlin
Miniaturized Bioreactor (0,4 ml BR)	Stem Cell Systems, Berlin
Tubing System for bioreactor perfusion	Stem Cell Systems, Berlin
Combi-stopper luer lock	Fresenius Kabi, Bad Homburg vor der Höhe
Combidyn® adapter	B.Braun, Melsungen
Disposable cannula	B.Braun, Melsungen
Gas filter	Sartorius, Göttingen
Glas vessel (250 mL/500 mL)	Schott, Mainz
Heidelberger extension tubing	B.Braun, Melsungen
Perfusion line	B.Braun, Melsungen
Syringes (1-50 mL)	B.Braun, Melsungen
Three-way valves	B.Braun, Melsungen
Vessel lid with integrated luer lock	Stem Cell Systems, Berlin

2.1.3 Chemicals and solutions

2.1.3.1 Chemicals and inorganic solutions

Table 5: Chemicals and inorganic solutions

Component	Company
4',6-diamidino-2-phenylindole dihydrochloride (DAPI)	Molecular Probes, Eugene, USA
4% paraformaldehyde solution	Herbeta Arzneimittel, Berlin
Aqua Polymount	Polysciences Inc., Warrington, PA, USA
Eosin a	Carl Roth, Karlsruhe
Ethanol	Herbeta Arztneimittel, Berlin
Harris' hematoxylin solution	Sigma-Aldrich, St. Louis, MO, USA
Methanol	J.T. Baker, Deventer, Netherlands
ParaClear Intermedium; ProTaqs Clear	Quartett Immundiagnostika Biotechnologie GmbH, Berlin
Paraffin	Merck, Darmstadt
RNase ZAP®	Life Technologies, Carlsbad, CA, USA
TRIZOL® reagent	Life Technologies, Carlsbad, CA, USA
Trypan blue, 0.5% w/v	Biochrom, Berlin
UltraPure™ Dnase/RNase-free Distilled Water	Life Technologies, Carlsbad, CA, USA
Vitro-Clud®	R. Langenbrinck, Emmendingen
Teleosteangelatin	Sigma-Aldrich, St. Louis, MO, USA

2.1.3.2 Cell culture media solutions, additives and cytokines

All solutions and additives were stored according to the manufacturer´s instructions.

Table 6: Cell culture media solutions and additives

Solution / Additive	Company
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA
Fetal Calf Serum (FCS)	PAA, Dartmouth, MA, USA
1x Phosphate buffered saline (PBS) w/o Ca^{2+} Mg^{2+}	PAA, Dartmouth, MA, USA
Dulbecco´s Modified Eagle´s Medium	Biochrom, Berlin

(DMEM)	
DMEM / F12 medium	Life Technologies, Carlsbad, CA, USA
L-alanyl-L-glutamine “stable glutamine”	Biochrom, Berlin
Gentamycin	Biochrom, Berlin
Beta-mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Sodium-butyrate	Sigma-Aldrich, St. Louis, MO, USA
Very Low Endotoxin (VLE)-RPMI 1640	Biochrom, Berlin
Knockout Serum Replacement	Life Technologies, Carlsbad, CA, USA
0.05%/0.02% (w/v) Trypsin/EDTA solution	Biochrom, Berlin
Dispase	Life Technologies, Carlsbad, CA, USA
Knockout DMEM	Life Technologies, Carlsbad, CA, USA
B-27® Supplement Minus Insulin	Life Technologies, Carlsbad, CA, USA
VitroCol® Human Collagen Product	Inamed, Fremont, CA, USA
Iscove´s MEM	Biochrom, Berlin
Retinoic acid	Sigma-Aldrich, St. Louis, MO, USA
Insulin	Roche Diagnostics, Heidelberg
Transferrin	Sigma-Aldrich, St. Louis, MO, USA
Ly294002	Merck, Darmstadt
Monothioglycerol	Sigma-Aldrich, St. Louis, MO, USA
mTeSR™1 medium	Stemcell Technologies, Vancouver, BC, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Non essential amino acids (NEAA)	Biochrom, Berlin
Glutamax®, 200 mM	Life Technologies, Carlsbad, CA, USA
Hepatocyte basal medium (HBM)	Lonza, Walkersville, USA
HCM Single Quot Kit	Lonza, Walkersville, USA
MatrigelTM (growth factor reduced)	BD Biosciences, San Jose, CA, USA
0.1% gelantin solution	Sigma-Aldrich, St. Louis, MO, USA
SB431542	Merck, Darmstadt
Iscove´s Modified Dulbecco´s Medium (IMDM)	Life Technologies, Carlsbad, CA, USA
TrypLE®	Life Technologies, Carlsbad, CA, USA
mFreSR™	StemCell Technologies

Cytokines were resuspended according to the manufacturer´s instructions and aliquots were prepared for long-term storage.

Table 7: Cytokines

Cytokines	Company
Hepatocyte Growth Factor (HGF)	PeproTech, London, UK
Oncostatin M (OSM)	PeproTech, London, UK
Basic Fibroblast Growth factor (bFGF)	PeproTech, London, UK
Activin A	PeproTech, London, UK
Wingless-type MMTV integration site family, member 3a (Wnt3a)	R&D Systems, Minneapolis, USA
Bone morphogenic protein4 (BMP4)	PeproTech, London, UK
FGF4	PeproTech, London, UK
FGF10	PeproTech, London, UK

2.1.3.3 Kits and assays

Table 8: Kits and assays

Component	Company
High Capacity RNA-to-cDNA Kit with Rnase-Inhibitor	Applied Biosystems, Foster City, USA
PureLink® RNA Mini Kit	Life Technologies, Carlsbad, CA, USA
RNase-free DNase Set	Quiagen, Hilden
TaqMan® Fast Universal Master Mix	Applied Biosystems, Foster City, USA
TaqMan Gene Expression Assays	Applied Biosystems, Foster City, USA
AFP, HS00173490_m1, Assay	Applied Biosystems, Foster City, USA
GAPDH, Hs03929097_g1, Assay	Applied Biosystems, Foster City, USA
Nanog, Hs02387400_g1, Assay	Applied Biosystems, Foster City, USA
POU5F1 (Oct ¾), Hs00999632_g1, Assay	Applied Biosystems, Foster City, USA
Neurofilament, Hs00196245_m1, Assay	Applied Biosystems, Foster City, USA
GATA II, Hs00231119_m1, Assay	Applied Biosystems, Foster City, USA
Cyp3A4, Hs00604506_m1, Assay	Applied Biosystems, Foster City, USA
Albumin, Hs00910225_m1, Assay	Applied Biosystems, Foster City, USA
SOX7, Hs00846731_s1, Assay	Applied Biosystems, Foster City, USA
SOX17, Hs00751752_s1, Assay	Applied Biosystems, Foster City, USA
Cyp2D6, Hs02576168_g1, Assay	Applied Biosystems, Foster City, USA
Cyp2B6, Hs03044634_m1, Assay	Applied Biosystems, Foster City, USA
Cyp2C9, Hs00426397_m1, Assay	Applied Biosystems, Foster City, USA
Cyp1A2, Hs00167927_m1, Assay	Applied Biosystems, Foster City, USA
CXCR4, Hs00607978_s1, Assay	Applied Biosystems, Foster City, USA
Human Albumin ELISA Quantitation Set	Bethyl Laboratories, Montgomery, TX, USA

2.1.3.4 Solutions for immune histochemistry and antibodies

Table 9: Blocking solution

Component	Concentration
BSA	2.5 % (w/v)
FCS	2.00% (v/v)
Teleosteangelatin	0.20% (v/v)
PBS	95.3% (v/v)

Table 10: Citrate buffer

Component	Concentration	Company
Citric acid	1.8 mM	Sigma-Aldrich, St. Louis, MO, USA
Sodium citrate	8.2 mM	Merck, Darmstadt

Antibodies were diluted in blocking buffer at defined concentrations (see table 11).

Table 11: Antibodies

Antigen	Host species	Isotype	Immunogen	Manufacturer	Dilution
SSEA-4	mouse	monoclonal IgG	human	R&D-Systems, Minneapolis, USA	1:30
Oct-4	rabbit	polyclonal IgG	human	Santa Cruz, Dallas, USA	1:100
AFP	mouse	monoclonal IgG1	human	Santa Cruz, Dallas, USA	1:100
CK 18	mouse	monoclonal IgG1	human	Santa Cruz, Dallas, USA	1:100
CK 19	mouse	monoclonal IgG1	human	Santa Cruz, Dallas, USA	1:100
SOX 17	mouse	monoclonal IgG3	human	R&D-Systems, Minneapolis, USA	1:100
Nestin	rabbit	polyclonal IgG	human	Santa Cruz, Dallas, USA	1:100
Desmin	mouse	monoclonal IgG1	human	Sigma-Aldrich, St. Louis, USA	1:100

Vimentin	mouse	monoclonal IgG1	human	Sigma-Aldrich, St. Louis, USA	1:40
SOX 7	rabbit	polyclonal IgG	human	Santa Cruz, Dallas, USA	1:50
Albumin	mouse	monoclonal IgG2a	human	Abcam, Cambridge, UK	1:100
Cyp 1A2	rabbit	polyclonal	human	Novus Biologicals, Littleton, USA	1:100
Cyp 3A4	rabbit	polyclonal	human	Novus Biologicals, Littleton, USA	1:100
Cyp 2C9	rabbit	polyclonal	human	Novus Biologicals, Littleton, USA	1:100
Cyp 7A1	rabbit	polyclonal	human	Santa Cruz, Dallas, USA	1:100
Cyp 2B6	rabbit	polyclonal	human	Santa Cruz, Dallas, USA	1:100
Cyp 2D6	rabbit	polyclonal	human	Bioss, Boston, USA	1:100
Secondary Antibodies					
Alexa Fluor 488	goat		mouse	Life technologies, Carlsbad, USA	1:1000
Alexa Fluor 594	goat		rabbit	Life technologies, Carlsbad, USA	1:1000

2.1.3.5 Cell lines and culture media

The research on human embryonic stem cells was performed with the approval by the Robert Koch Institute, Berlin, Germany, according to the German stem cell law.

Table 12: Cell lines

Cell Line	Provider
Mouse embryonal fibroblasts (MEF)	provided by Dr. I. Fichtner, MDC, Berlin-Buch
hESC line H1 feeder-dependent	WiCell Research Institute, Madison, WI, USA
hESC line H1 feeder-independent	WiCell Research Institute, Madison, WI, USA
hESC line SA002	Cellartis, Göteborg, Sweden
hESC line SA121	Cellartis, Göteborg, Sweden
Human foreskin fibroblasts	American Type Culture Collection, Manassas, VA, USA

Culture media were used within two weeks after supplementation with additives and were stored at 4°C.

Table 13: Composition of MEF Medium

Component	Final Concentration
DMEM	87% (v/v)
FCS	10% (v/v)
L-Glutamine (c = 200 mM), 100x	1% (v/v) (c = 2 mM)
NEAA (c = 20 mM), 100x	1% (v/v) (c = 0.2 mM)
Penicillin/Streptomycin (c = 10000 U/ml/10000µg/ml), 100x	1% (v/v) (c = 100 U/ml / 100 µg/ml)

Table 14: Composition of HFF Medium

Component	Final Concentration
IMDM	89% (v/v)
FCS	10% (v/v)
Penicillin/Streptomycin (c = 10000 U/ml/10000µg/ml), 100x	1% (v/v) (c = 100 U/ml / 100 µg/ml

Table 15: Composition of mouse-feeder-dependent hESC Medium

Component	Final Concentration
DMEM	77.5% (v/v)
Knockout Serum Replacement	20% (v/v)
NEAA (c = 20 mM), 100x	1% (v/v) (c = 0.1 mM)
Glutamax (c = 200 mM), 100x	1% (v/v) (c = 2 mM)
Beta-Mercaptoethanol	1% (v/v) (c = 0.1 mM)
bFGF	4 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 16: Composition of medium for SCED culture

Component	Final Concentration
DMEM	77.5% (v/v)
Knockout Serum Replacement	20% (v/v)
NEAA (c = 20 mM), 100x	1% (v/v) (c = 0.1 mM)
Glutamax (c = 200 mM), 100x	1% (v/v) (c = 2 mM)
Beta-Mercaptoethanol	0.1 mM
bFGF	10 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 17: Composition of feeder-independent hESC Medium

Component	Final Concentration
mTeSR™1 Basal Medium	79.5% (v/v)
mTeSR™1 5x Supplement	20%
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

The following media were used for hepatic differentiation of hESC:

Table 18: Medium one of Differentiation Approach one (M1.1)

Component	Final Concentration
Knockout DMEM	77.5% (v/v)
Serum Replacement	20% (v/v)
L-Glutamine (c = 200 mM), 100x	1% (v/v) (c = 2 mM)
Beta-Mercaptoethanol	0.1 mM
NEAA (c = 20 mM), 100x	1% (v/v) (c = 0.2 mM)
BFGF	4 ng/ml
DMSO	1% (v/v)
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 19: Medium two of Approach one (M1.2)

Component	Final Concentration
HBM	97% (v/v)
HCM SingleQuot Kit	2.5% (v/v)
HGF	10 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 20: Medium three of Approach one (M1.3)

Component	Final Concentration
HBM	97% (v/v)
HCM SingleQuot Kit	2.5% (v/v)
HGF	10 ng/ml
Oncostatin M	10 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 21: Medium one of Approach two (M2.1)

Component	Final Concentration
RPMI 1640	96.5% (v/v)
Glutamax (c = 200 mM)	1% (v/v) (c = 2 mM)
B27	2% (v/v)
Sodium Butyrate	1 µM
Activin A	100 ng/ml
Wnt3a	50 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 22: Medium two of Approach two (M2.2)

Component	Final Concentration
HBM	97% (v/v)
HCM SingleQuot Kit	2.5% (v/v)
HGF	10 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 23: Medium three of Approach two (M2.3)

Component	Final Concentration
HBM	97% (v/v)
HCM SingleQuot Kit	2.5% (v/v)
HGF	10 ng/ml
Oncostatin M	10 ng/ml
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 24: Medium one of Approach three (M3.1)

Component	Final Concentration
RPMI 1640	97.5% (v/v)
FBS	0.5% (v/v)
Activin A	100 ng/ml
Glutamax (c = 200 mM)	1% (v/v) (c = 2 mM)
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 25: Medium one of Approach four (M4.1)

Component	Final Concentration
RPMI 1640	97.5% (v/v)
B27	2% (v/v)
Activin A	100 ng/ml
Glutamax (c = 200 mM)	1% (v/v) (c = 2 mM)
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 26: Medium one of Approach four (M4.1)

Component	Final Concentration
RPMI 1640	97.5% (v/v)
B27	2% (v/v)
BMP4	20 ng/ml
bFGF	10 ng/ml
Glutamax (c = 200 mM)	1% (v/v) (c = 2 mM)
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 27: Medium one of Approach five (M5.1)

Component	Final Concentration
RPMI 1640	97.5% (v/v)
B27	2% (v/v)
bFGF	20 ng/ml
BMP4	10 ng/ml
Ly294002	1µM
Activin A	100 ng/ml
Glutamax (c = 200 mM)	1% (v/v) (c = 2 mM)
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

Table 28: Medium for embryoid body formation (EB-Medium)

Component	Final Concentration
DMEM/F12	78.5% (v/v)
Serum Replacement	20% (v/v)
L-Glutamine (c = 200 mM)	1% (v/v) (c = 2 mM)
Beta-Mercaptoethanol	0,1 mM
Gentamycin (c = 10 mg/ml), 200x	0.5% (v/v) (c = 50 µg/ml)

2.1.3.6 Polymerase chain reaction (PCR) primers and temperature profile

Table 29: PCR Primers

Germinal sheet	Primer	Orientation	Sequence	TM [°C]	Product Size [bp]
Pluripotent	**Oct-4**	forward	CCCTGGTGCCGTGAAGCTGG	65.85	180
		reverse	ACCTTCCCAAATAGAACCCCCAGGG	*65.90*	
	Nanog	forward	AGGAAGACAAGGTCCCGGTCAA	*63.29*	*256*
		reverse	GGTGCTGAGGCCTTCTGCGT	*65,20*	
Ectoderm	**Neurofilament**	forward	ACGAGGTGTCCGAGAGCCGT	*65.49*	*282*
		reverse	CCTCGCCTTCCAAGAGTTTCCTGT	*64.65*	
	Beta-III Tubulin	forward	GCGGGGCCGCGGCTATAA	*65.31*	*172*
		reverse	GACTTCCCAGAACTTGGCCCCGA	*66.48*	
Mesoderm	**GATA2**	forward	ACGCCCATCCACCCCTCCTC	*66.01*	*285*
		reverse	CCTGGGCAGCAGTCAGGTGC	*65.83*	

Endoderm	**AFP**	forward	GCCCACTCCAGC ATCGATCCC	*65.21*	*235*
		reverse	TGTTGCTGCCTT TGTTTGGAAGCA	*64.46*	

Table 30: Temperature Profile [°C]

Temperature [°C]	Time [seconds]	Number of cycles
94	180	1
94	30	*35*
58	45	
72	45	
72	600	*1*
4	Forever	-

2.2 Methods

2.2.1. Preparation, culture and inactivation of mouse embryonic fibroblasts

2.2.1.1 Culture of MEF

Mouse embryonic fibroblasts (MEF) from CF-1 mice at day 13 to 14 of pregnancy were provided by the group of Dr. Fichtner / Dr. Eckert, MDC, Berlin-Buch. The MEF were cultured in an incubator at 37°C in a 5% CO_2 atmosphere. MEF - Medium was changed every two to three days. Once cultures reached about 90 to 95% confluence, the medium was aspirated, the cells were washed once with PBS and then incubated for 5 minutes with a pre-warmed trypsin-EDTA solution at 37°C. Then the digestion was stopped by addition of MEF – medium and the non-adherent cells were spun down for 5 minutes at 300 g. After that the cells were resuspended in MEF Medium, counted and inoculated in T175 culture flasks at a splitting ratio of 1:4.

2.2.1.2 Culture of human foreskin fibroblasts

Human foreskin fibroblasts (HFF) were cultured in an incubator at 37°C in a 5% CO_2 atmosphere. The medium was changed every 48 to 72 hours. Once cultures reached about 100% confluence and the cells showed a spindle like shape, the culture was once washed with PBS and then incubated for about 5 minutes with pre-warmed trypsin-EDTA solution at 37°C. The digestion was stopped by addition of HFF medium and the cells were spun down for 5 minutes at 300g. After that the cells were resuspended, counted and inoculated in culture flasks at a split ratio of 1:4 to 1:6.

2.2.1.3 Inactivation of MEF and HFF

Mouse embryonic fibroblasts were cultured and proliferated until the 4th passage. HFF were used until the 40th passage. Then cells were treated with trypsin-EDTA to detach them from the culture flasks and re-suspended in MEF medium. The cells were counted using a hemocytometer and inactivated by exposure to 30 Gray by a γ – radiation source. After inactivation cells were either used immediately for the coculture with human embryonic stem cells, or they were cryopreserved for later use.

2.2.2 Culture of human embryonic stem cells in co-culture with MEF or HFF

2.2.2.1 Preparation of IVF-dishes for the culture of human embryonic stem cells

The coculture of hESC and MEF was carried out in *in vitro* fertilization (IVF) dishes. At least 24 hours prior to hESC transfer, IVF dishes were pre-coated with a 0.1% gelatine solution for 30 minutes at 37°C. After this, the gelatine solution was aspirated and the plates were rinsed once with PBS. Then 1 ml of MEF - medium was added to the center well and 1 ml of the resuspended MEF cells at a concentration of 7.6x10^4 cells per ml was added per well. This resulted in a feeder cell density of 4x10^4 per cm^2. Additionally hESC medium was added to the peripheral ring of the dish. The feeder cell containing IVF dishes could be used for the coculture with hESC for about 7 to 10 days. For hESC medium composition see table 15.

2.2.2.2 Culture and passage of human embryonic stem cells in co-culture with mouse embryonic fibroblasts

One hour prior to manual passage of hESC, the MEF medium of the prepared IVF dishes was removed and the plates were washed once with PBS. Then 2 ml of prewarmed hESC medium were added. The hESC cultures to be passaged were checked for visible hallmarks of differentiation and undifferentiated hESC colonies of appropriate size were marked. To passage the hESC, preselected colonies were dissected into rectangles with an edge length of about 300 µm by using a Stem Cell Cutting Tool under a stereo-microscope. The microscope table was equipped with a heating unit to ensure a constant temperature environment. After colonies had been divided by cutting as described, the pieces were slightly pushed to loosen up and get into solution. After that about 10 pieces were aspirated using the cutting tool, and firmly distributed in an IVF dish.

2.2.2.3 Preparation of culture flasks for single cell enzymatic dissociation (SCED) culture

About 30 minutes prior to HFF inoculation the culture flasks were pre-coated with a 0.1% gelatine solution at 37°C. The solution was aspirated and the plates were rinsed once with PBS. The HFF were inoculated at a density of $5x10^4$ cells per cm^2 in SCED medium.

2.2.2.4 Culture and passage of hESC in SCED culture

The hESC were washed with PBS and incubated for 5 minutes with pre-warmed TrypLE® solution. The digestion was stopped by addition of medium for SCED cultures. The cells were spun down at 300g, the supernatant was aspirated, the cells were resuspended in Medium for SCED culture and split at a ratio of 1:4 to 1:10. Medium was changed every 48 to 72 hours.

2.2.3 Feeder-free culture of human embryonic stem cells

The hESC cell line H1 adapted for feeder-free culture was maintained in mTeSR1 medium and routinely passaged according to the manufacturer´s recommendations.

Briefly cells were cultured in Matrigel coated 6 well dishes, and passaged using dispase solution every five to six days at a split ratio of about 1:4. A complete medium exchange was performed daily.

2.2.3.1 Evaluation of the undifferentiated state of human embryonic stem cells

To evaluate the undifferentiated state of cultured hESC, standard procedures including immune histochemistry and polymerase chain reaction (PCR) for the detection of markers for undifferentiated hESC as well as differentiated cells were performed.

PCR primer sequences and annealing temperatures are given in table 29, the PCR temperature profile is given in table 30. Additionally IHC stainings were performed. Antibodies, host species, manufactures and used dilutions are given in table 11.

2.2.4 Evaluation of the pluripotent potential of human embryonic stem cells

2.2.4.1 Evaluation of the pluripotent potential in conventional 2D culture

To evaluate the potential of cultured hESC to differentiate into cells of the three germ layers, undifferentiated cells were cultured under conditions that support spontaneous differentiation for 17 days. Medium was changed every 48h to 72h and RNA was isolated after 5, 10 or 17 days. The supernatant was analyzed for differentiation markers (bHCG and AFP) as well as for glucose, lactate and LDH concentration.

2.2.5 Hepatic differentiation of human embryonic stem cells in conventional 2D cultures

2.2.5.1 Differentiation of stem cells in 2D cultures

In order to establish a suitable protocol for hepatic differentiation of human embryonic stem cells, five different approaches recently published were compared and further investigated. Strategies and references for definitive endodermal (DE) commitment and hepatic maturation of committed cells are given in table 31.

Table 31: Approaches for the hepatic differentiation of hESC

Approach number	Reference	Strategy for definitive endodermal (DE) commitment	Strategy for the proliferation / maturation of DE committed cells
1	Hay et al. 2007 [22]	8 days in medium containing • DMSO • bFGF	10 days in medium containing • Insulin, Transferrin, Epidermal Growth Factor (EGF), Ascorbic Acid, Hydrocortisone, Bovine serum Albumin (= **HCM®**) • HGF 4 days in medium containing • HCM® • HGF • OSM
2	Hay et al. 2008 [68]	3 days in medium containing • Activin A • Wnt3a • Sodium Butyrate • B27	10 days in medium containing • HCM® • HGF 4 days in medium containing • HCM® • HGF • OSM
3	Agarwal et al. 2008 [69]	3 days in medium containing • Activin A • FBS	6 days in medium containing • FGF 4 • Knockout Serum Replacement

		2 days in medium containing • Activin A • Knockout Serum Replacement	9 days in medium containing • HCM® • Dexamethasone • FGF 4 • HGF • OSM
4	Si Tayeb et al. 2010 [70]	5 days in medium containing • Activin A • B27	5 days in medium containing at 4% O_2 • B27 • BMP4 • bFGF 5 days in medium containing • HCM® • OSM
5	Touboul et al. 2010 [71]	3 days in medium containing • bFGF • BMP4 • Ly294002 • Activin A	3 days in medium containing • FGF 10 • B27 2 days in medium containing • FGF 10 • SB431542 • B27 • Retinoic Acid 10 days in medium containing • B27 • Insulin • Transferrin • Monothioglycerol • BSA • FGF4 • HGF • EGF

The differentiation approaches were conducted as shown in table 32. A schematic overview of the strategies is given in table 31. The medium compositions used in hepatic differentiation experiments are given in table 18 to table 27. All approaches were based on a two-step procedure, including differentiation of the hESC into definitive endoderm (DE) in the first step, followed by maturation to functional hepatocytes in the second step.

Table 32: Overview of hepatic differentiation experiments

Day	Approach 1	Approach 2	Approach 3	Approach 4	Approach 5
-1	100 % medium change (MteSR™1)	100 % medium change (MteSR™1)	100 % medium change (MteSR™1)	100 % medium change (MteSR™1)	100 % medium change (MteSR™1)
0	wash plates 3x with PBS, change to Medium1.1	wash plates 3x with PBS, change to Medium2.1	wash plates 3x with PBS, change to Medium3.1	wash plates 3x with PBS, change to Medium4.1	Precoated dishes (FCS), add medium 5.1
1	100 % medium change (Medium1.1)	100 % medium change (Medium2.1)	-	100 % medium change (Medium4.1)	100 % medium change (Medium5.1)
2	100 % medium change (Medium1.1)	100 % medium change (Medium2.1)	100 % medium change (Medium3.1)	100 % medium change (Medium4.1)	100 % medium change (Medium5.1)
3	100 % medium change (Medium1.1)	100 % medium change (HCM2.1)	100 % medium change (Medium3.2)	100 % medium change (Medium4.1)	100 % medium change (Medium5.2)
4	100 % medium change (Medium1.1)	100 % medium change (HCM2.1)	-	100 % medium change (Medium4.1)	100 % medium change (Medium5.2)
5	100 % medium change (Medium1.1)	100 % medium change (HCM2.1)	Remove Medium3.2, passage cells with Trypsin, inoculate cells on collagen 1 coated dishes, culture in medium 3.3	Remove Medium 4.1, add medium4.2; incubate at 4% O2	100 % medium change (Medium5.2)
6	100 % medium change (Medium1.1)	100 % medium change (HCM2.1)	-	-	100 % medium change (Medium5.3)
7	100 % medium change (HCM1.1)	-	100 % medium change (Medium3.3)	100 % medium change (Medium4.2)	100 % medium change (Medium5.3)
8	100 % medium change (HCM1.1)	100 % medium change (HCM2.1)	-	-	100 % medium change (Medium5.4)
9	100 % medium change (HCM1.1)	-	100 % medium change (Medium3.3)	100 % medium change (Medium4.2)	-
10	100 % medium change (HCM1.1)	100 % medium change (HCM2.1)	-	100 % medium change (HCM4.1)	100 % medium change

					(Medium5.4)
11	100 % medium change (HCM1.1)	-	100 % medium change (HCM3.1)	-	-
12	-	100 % medium change (HCM2.2)	-	100 % medium change (HCM4.1)	100 % medium change (Medium5.4)
13	100 % medium change (HCM1.1)	-	100 % medium change (HCM3.1)	-	-
14	-	100 % medium change (HCM2.2)	-	100 % medium change (HCM4.1)	100 % medium change (Medium5.4)
15	100 % medium change (HCM1.1)	-	100 % medium change (HCM3.1)	**End of experiment**	-
16	-	**End of experiment**	-		100 % medium change (Medium5.4)
17	100 % medium change (HCM1.2)		100 % medium change (HCM3.1)		-
18	-		-		**End of experiment**
19	100 % medium change (HCM1.2)		100 % medium change (HCM3.1)		
20	-		**End of experiment**		
21	**End of experiment**				

	End of DE differentiation
	End of experiment

Glucose and lactate measurements in samples from the supernatant were performed with a blood gas analyser. Lactate dehydrogenase (LDH), Alpha fetoprotein (AFP) and Human Chorionic Gonadotropin (beta HCG) were determined with an automated clinical chemistry analyzer. At the end of both differentiation steps, RNA was isolated for analysis of the differentiation outcome. Immune histochemistry was performed on cells after DE commitment and at the end of differentiation. Additionally cell counts using a Neubauer hematocytometer were performed.

2.2.5.2 Evaluation of the stability of lineage commitment

To evaluate the stability of the lineage commitment after differentiation, cells were cultured under conditions that support either the maintenance of hepatic functions or spontaneous differentiation. RNA samples were collected after 0, 3, 7 and 14 days. The supernatants were analyzed for differentiation markers (bHCG and AFP), glucose, lactate and LDH concentrations.

2.2.6 Bioreactors variants used for the hepatic differentiation of hESC in 3D culture

2.2.6.1 Bioreactor models

For hepatic differentiation of hESC under 3D culture conditions a multi-compartment hollow-fiber bioreactor technology for high-density perfusion culture of cells [72, 73] was used. The bioreactor is composed of three independent hollow-fiber capillary membrane systems, which are interwoven thus offering decentralized gas and medium exchange. The cells are cultured within the cell compartment, which is nerved by oxygen and medium capillaries. Two of the capillary systems are made of hydrophilic microporous polyethersulfone (mPES, Membrana, Wuppertal) with a molecular weight cut-off of approximately MW 500,000. They serve for counter-current medium perfusion. The third capillary system is made of hydrophobic multilaminate hollow fiber membrane oxygenation capillaries (MHF, Mitsubishi, Tokyo, Japan). By this way the supply with nutrients and oxygen of the cells within the cell compartment, as well as the removal of potentially toxic metabolites and carbon dioxide are ensured. The mass transfer into the cell compartment can by carried out via diffusion or laminar flow depending on the perfusion mode selected. In counter-current medium perfusion mode the in- and outlet of each medium capillary system are open. Thus medium exchange is carried out via diffusion. During cross-flow perfusion mode the capillary outlet of the one, and the medium inlet of the other

capillary system are mechanically blocked, thus mass exchange is mainly carried out via laminar flow.

This complex interwoven membrane system is potted in a two-component polyurethane housing. Direct access to the cell compartment (for cell inoculation, cell sample collection or direct substance administration) is enabled by open ending silicon capillaries.

Three differently sized variants of the bioreactor technology were used in this study.

The capillary system of the Analytical Scale bioreactor (4 ml cell compartment volume) and the Lab Scale bioreactor (17.2 ml cell compartment volume) are made of multiple capillary layers each consisting of one type of capillaries (either medium or oxygenation capillaries). Medium and gas capillary layers are arranged in an alternating order.

The miniaturized bioreactor disposes of only four (miniaturized bioreactor with 0.4 ml cell compartment volume) respectively two layers (miniaturized bioreactor with 0.2 ml cell compartment volume).

An overview of the bioreactor models used for the hepatic differentiation of hESC is given in table 33 and figure 2.

Table 33: Bioreactor variants used in differentiation experiments

	Miniaturized Bioreactor	**Analytical Scale Bioreactor**	**Lab Scale Bioreactor**
Volume cell compartment [ml]	0.2 – 0.4	4.0	17.2
capillary surface area [cm^2]	28 -56	293	986
Volume bioreactor system [ml]	12	20	40

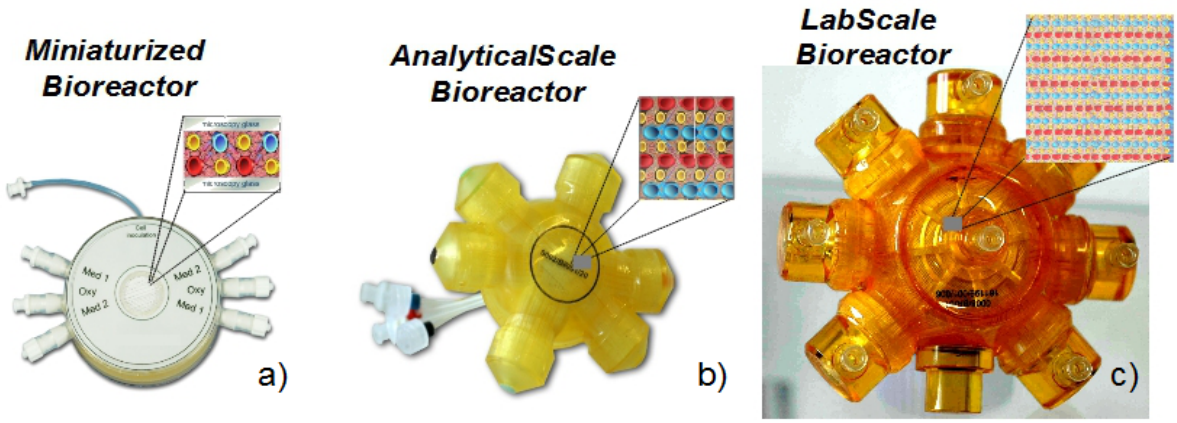


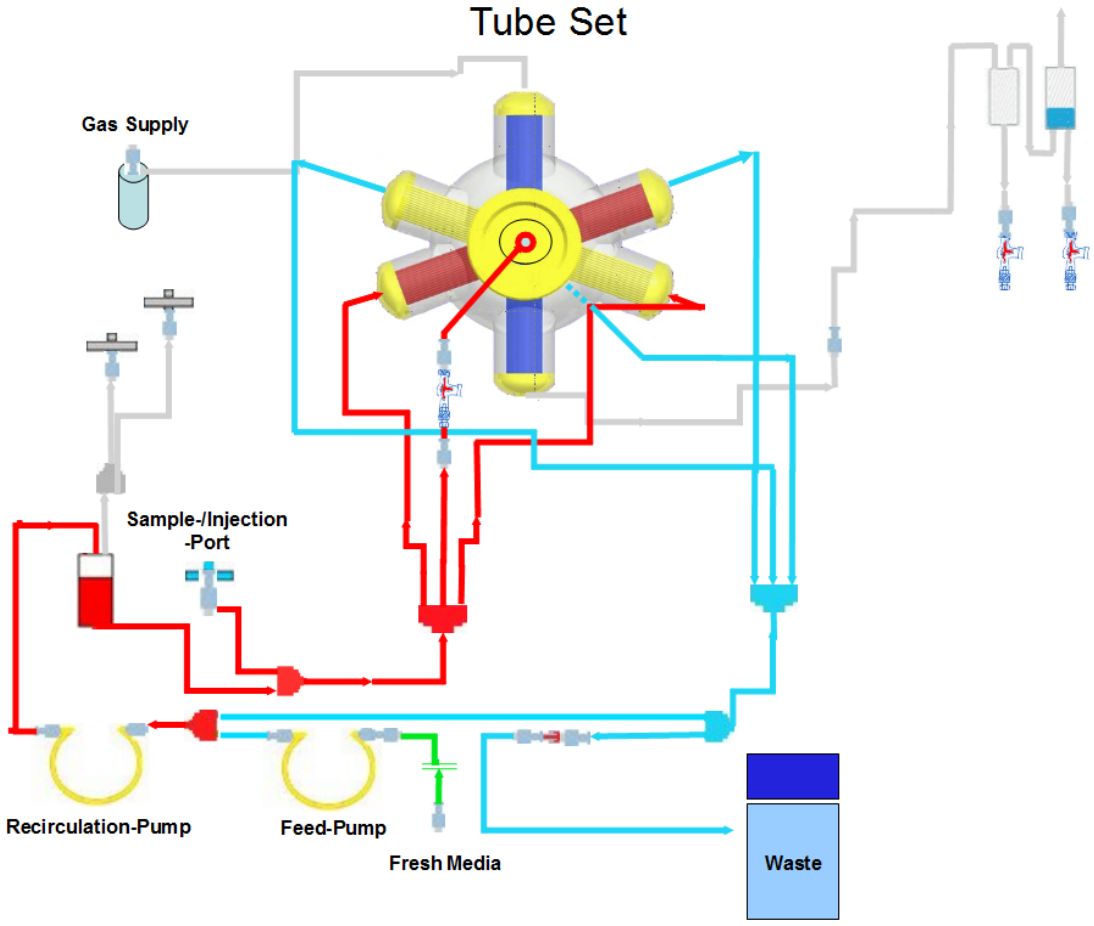
Figure 2: Bioreactor variants used in this study. a) Miniaturized bioreactor, the medium capillaries (red and blue) are arranged in an alternating way with the oxygenation capillaries (yellow). The capillary layers in Analytical Scale bioreactors (b) and Lab Scale bioreactors are either dedicated for medium supply (red and blue) or for oxygenation (yellow) as illustrated in the inserts.

2.2.6.2 Bioreactor tubing system

The bioreactor tubing system (Stem Cell Systems, Berlin) includes a bubble trap to avoid air insertion and a connection for sample removal as well as substance addition. A schematic overview of the bioreactor tubing system is given in figure 3.

The bioreactor and the tubing system were sterilized by formaldehyde treatment at least 7 days before use.

Figure 3: Tubing System. Schematic illustration of the tubing system for bioreactor systems.



In addition gas supply, gas outlet tubes and sampling / injection ports are shown.

2.2.6.3 Bioreactor perfusion system

The bioreactors connected to their tubing system were operated in perfusion systems (Stem Cell Systems, Berlin) designed and built for the long term maintenance of bioreactor cultures. The device includes a processor controlled heating unit, a gas mixing unit and pressure controlled pumps for medium recirculation and feed (figure 3). During the experiment perfusion devices were connected via an integrated USB port to a PC and the perfusion parameters (pressure, temperature, pump speeds) were continuously recorded and graphically monitored using a stand-alone measurement program created with LabVIEW (National Instruments, Munich, Germany). The program also provides the opportunity for remote monitoring via the Internet.

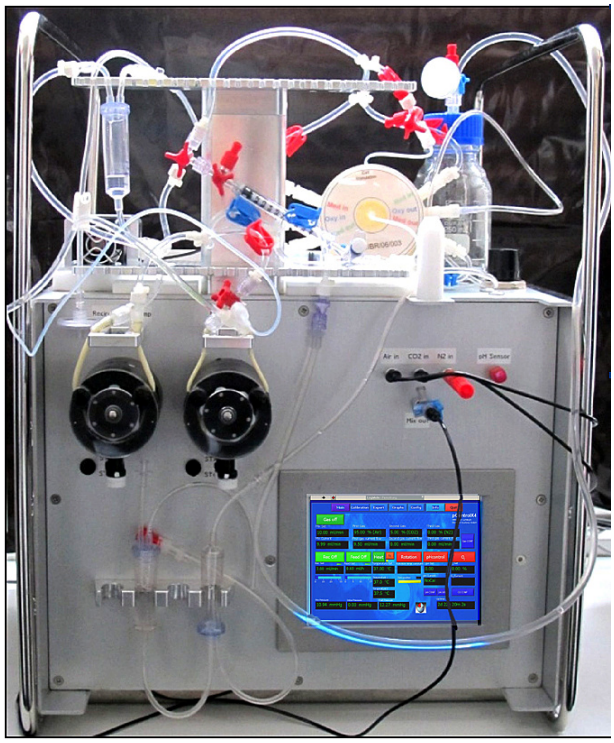


Figure 4: Perfusion device, equipped with a recirculation and fresh medium feed pump, a gas mixing unit, and a heating device. A bioreactor (in this picture a miniaturized bioreactor) is placed on top of the perfusion device. After attachment of the cover temperature, gas mixture supply, recirculation and feed pump rates are controlled by an integrated personal computer with touchscreen interface.

2.2.7 Evaluation of the pluripotent potential of hESC in 3D bioreactor systems

To evaluate the pluripotent potential of hESC under 3D culture conditions, undifferentiated feeder independent cells were cultured under conditions that support spontaneous differentiation for 17 days. Differentiation markers (bHCG and AFP) as well as glucose, lactate and LDH were measured. After bioreactor shutdown RNA samples were isolated and further processed for Real-Time PCR analysis.

2.2.8 Hepatic differentiation of human embryonic stem cells in 3D bioreactor systems

Feeder independent and feeder dependent human embryonic stem cells were differentiated according to protocols established for the differentiation of hESC in conventional 2D culture. The protocols were adapted regarding the inoculated cell number and medium exchange to the requirements of different bioreactor models. An overview of the conducted bioreactor experiments is given in table 34.

Table 34: Hepatic differentiation of hESC in bioreactors

	Approaches with feeder independent cells		**Approaches with partial feeder dependent cells**		
	Approach 1	**Approach 2**	**Cellartis Approach 1**	**Cellartis Approach 2**	**Cellartis Approach 3**
Reference	Hay et al., 2007 [22]	Hay et al., 2008 [68]	Brolén et al., 2010 [67]	Brolén et al., 2010[67]	Brolén et al., 2010 [67]
hESC Cell line	H1	H1	SA121	SA121	SA002
Bioreactor variants	Miniaturized Bioreactor (0.4ml)	Miniaturized Bioreactor (0.4ml)	Miniaturized Bioreactor (0.2 ml)	Analytical Scale Bioreactor	Lab Scale Bioreactor
Number of bioreactor runs	2	2	2	1	2
Number (vital) of inoculated cells (passage)	25E6	25E6	10E6 (p53)	50E6 (p34)	8.5E7 (p74) - hESC 4.75E7 (p4) - MEF
Experiment duration	21 + 3 days	17 + 3 days	25 days + 7 days + 5 days	30 + 3 days	46 + 3 days

In all five approaches the cells were first committed to the definitive endodermal lineage, then proliferated and finally maturated.

An overview of the factors used in each step is given in table 35.

Table 35: Overview of factors used in various differentiation approaches

	Medium for proliferation of undifferenti-ated hESC	Factors for DE commitment	Factors for proliferation of DE committed cells	Factors for the maturation of differentiated cells
Approach 1 (feeder independent)	mTeSR™1 Medium	• DMSO 1% (v/v) • bFGF 4 ng/ml for 8 days	• Insulin, Transferrin, Epidermal Growth Factor (EGF), Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • HGF 10 ng/ml for 10 days	• Insulin, Transferrin, Epidermal Growth Factor (EGF), Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • HGF 10 ng/ml • Oncostatin M 10 ng/ml for 4 days
Approach 2 (feeder independent)	mTeSR™1 Medium	• Activin A 100 ng/ml • Wnt3a 50 ng/ml • Sodium Butyrate 1 µM • B27 for 3 days	• Insulin, Transferrin, Epidermal Growth Factor (EGF), Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • HGF 10 ng/ml for 10 days	• Insulin, Transferrin, Epidermal Growth Factor (EGF), Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • HGF 10 ng/ml • Oncostatin M 10 ng/ml for 4 days
Cellartis Approach 1 (partial feeder dependent)	Standard Medium	• Activin A 100 ng/ml • Sodium Butyrate 1 µM for 24 hours • Activin A	• aFGF 50 ng/ml • bFGF 5 ng/ml • BMP2 25 ng/ml • BMP4 100 ng/ml for 7 days • DMSO 1% (v/v)	• Insulin, Transferrin, EGF, Ascorbic Acid, Hydrocortisone, Bovin serum Albumin • Dexametasone

		100 ng/ml • Sodium Butyrate 0,5 µM for 6 days	for 48 hours	0.1 µM • bFGF 2 ng/ml • HGF 2 ng/ml • Oncostatin M 10 ng/ml for 16 days
Cellartis Approach 2 (partial feeder dependent)	Standard Medium	• Activin A 100 ng/ml • Sodium Butyrate 1 µM for 24 hours • Activin A 100 ng/ml • Sodium Butyrate 0,5 µM for 4 days	• aFGF 50 ng/ml • bFGF 5 ng/ml • BMP2 25 ng/ml • BMP4 100 ng/ml for 8 days • Insulin, Transferrin, EGF, Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • bFGF 2 ng/ml • HGF 20 ng/ml for 9 days	• Insulin, Transferrin, EGF, Ascorbic Acid, Hydrocortisone, Bovin serum Albumin • Dexametasone 0.1 µM • bFGF 2 ng/ml • HGF 2 ng/ml • Oncostatin M 10 ng/ml for 8 days
Cellartis Approach 3 (partial feeder dependent)	Standard Medium	• Activin A 100 ng/ml • bFGF 4 ng/ml for 48 hours • Activin A 100 ng/ml • bFGF 4 ng/ml • FCS 0.2 % (v/v) for 3 days	• aFGF 100 ng/ml • bFGF 5 ng/ml • BMP2 50 ng/ml • BMP4 200 ng/ml • FCS 0.2 % (v/v) for 8 days • Insulin, Transferrin, EGF, Ascorbic Acid, Hydrocortisone, Bovine serum Albumin • bFGF 2 ng/ml • HGF 20 ng/ml for 9 days	• Insulin, Transferrin, EGF, Ascorbic Acid, Hydrocortisone, Bovin serum Albumin • Dexametasone 0.1 µM • bFGF 2 ng/ml • HGF 2 ng/ml • Oncostatin M 10 ng/ml for 24 days

2.2.9 Determination of hepatocyte-specific functions

2.2.9.1 Cytochrome P450 (CYP) activity assay

The determination of CYP activity was performed using a mixture of three different drugs that are transformed by different CYP450 isoenzymes. The specific isoenzyme for each drug and the final concentration in the medium are given in table 36.

Table 36: Substrates for testing hepatocyte-specific functions

Drug	Converted by isoenzyme	Final concentration in medium	Product
Phenacetin	CYP1A1/1A2	26 µmol/l	Acetaminophen
Midazolam	CYP3A4	3 µmol/l	1-OH-midazolam
Diclofenac	CYP2C9	9 µmol/l	4-OH-diclofenac

Immediately before starting a CYP experiment, the filter at the sample port was removed to avoid sticking of the lipophilic drugs to the membrane, and 1 ml of culture medium was drawn from the circuit, which served as blank value for the analysis. During the incubation with the drugs the feed pump of the bioreactor circuit was switched off to provide a constant reaction volume. Samples (200 µl) for analysis of substrates and their metabolites were taken 24 hours after drug administration. After each experiment the bioreactor circuit was rinsed with 15 to 50 ml of culture medium (depending on the bioreactor circuit volume) and was then reset to the standard operation mode.
The analysis of substrate and metabolite concentrations was performed by LC-MS analysis by Pharmacelsus GmbH, Saarbrücken.

2.2.9.2 Albumin ELISA

An Albumin ELISA was performed using the Human Albumin ELISA Quantitation Set according to the manufacturer´s instructions. The optimal horseradish peroxidase (HRP) detection antibody dilution for this kid was tested before and set to 1:50,000.

2.2.10 Immunohistofluorescence

2.2.10.1 Embedding of bioreactor samples in paraffin blocks

At the beginning of the preparation process samples from the bioreactor cell compartment were transferred into a 4% paraformaldehyde solution for at least 10 minutes. This was followed by sequential application of differently concentrated ethanol solutions (50%, 70%, 85%, 96%, 100%) in ascending order for 30 minutes respectively.
Afterwards the samples were incubated in Paraclear for at least 1 hour. Then the probes were transferred into preheated (60°C) liquid paraffin. After 12 hours the samples were transferred into embedding forms and the liquid paraffin was cured by cooling.
The paraffin embedded bioreactor samples were cooled down in a -20°C freezer for at least 12 hours to ensure proper rigidity of the paraffin.
Paraffin blocks were sliced into 2.5 µm thick slides using a Microm HM355S microtome and attached to glass slides. To ensure proper adherence and dehydration glass slides were dried for at least 24 hours at room temperature or for 1 hour at 37°C in a dry incubator. Slides were stored at room temperature until further processing.

2.2.10.2 Preparation of slides for IHC

To de-paraffinize the samples, the slides were transferred into Paraclear for at least 2 hours, followed by sequential application of ethanol solutions with various concentrations (100%, 96%, 80%, 70%) in descending order over 5 minutes, respectively.
Afterwards, the slides were treated in a pressure cooker for 15 minutes (effective cooking time) in citrate buffer (the buffer composition is given in table 6).
After the buffer solution containing the slides had been cooled down in an ice bath, samples were transferred into a dark chamber with high humidity and blocking buffer. The blocking buffer composition is given in table 5.

2.2.10.3 Immune histochemical staining

After 1 hour the blocking buffer was removed, and fresh blocking buffer containing primary antibodies was applied. The primary and secondary antibodies used are shown in table 7.
After 1 hour incubation the buffer was removed and the slides / wells were washed with PBS. Afterwards blocking buffer containing the secondary antibodies was added and incubated for 1 hour. This was followed by a washing step again using PBS. In the next step a DAPI solution was added and incubated for 10 minutes. After extensive washing the stained cells on the slides / wells were mounted using Aqua Polymount and covered using glass slides.

2.2.10.4 Hematoxylin & Eosin (H&E) Staining

Prior to the H&E staining the slides were treated in Paraclear for about 2 hours. This was followed by sequential application of ethanol solutions with various concentrations (100%, 96%, 80%, 70%, 50%) in descending order for 4 minutes (100%) or 2 minutes (all other concentrations).

After the ethanol treatment the slides were transferred into distilled water for a couple of seconds and afterwards transported into a hematoxylin solution. After 10 minutes the slides were transferred into cold tap water and incubated for 10 minutes. This was followed by a 2 minutes eosin treatment. The eosin stain was washed out by a 5 second bath in distilled water. After that the slides were again transferred into a 70% (v/v) ethanol bath for about 5 seconds, followed by a 96 % (v/v) ethanol bath for about 30 seconds and finally a 100% (v/v) ethanol bath for about 2 minutes. The slides were then incubated in paraclear for another 5 minutes. The tissue containing parts of the slides were then coated with Vitro-Clud and covered using glass cover slides.

2.2.11 RNA Isolation, processing and analysis

Cells were lysed by addition of TRIZOL® agent. The solution was either further processed or stored at -20°C. The isolation was performed using the PureLink® RNA Mini Kit according to manufacturer´s instructions for TRIZOL® lysed samples. After accomplishment of the isolation protocol the RNA content was determined via NANODROP® spectrometry. Afterwards RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit with RNAse-Inhibitor according to the manufacturer´s instructions. The cDNA was stored at -80°C until it was analyzed via Real Time PCR.

2.2.12 *In vivo* experiments

2.2.12.1 Evaluation of the tumorigenic potential

The tumorigenic potential of feeder independent undifferentiated, definitive endodermal and fully hepatic differentiated hESC (differentiated according to the protocol published by Hay et al. 2008 [68]) was evaluated by *in vivo* transplantation experiments performed in cooperation with Dr. Klaus Eckert and Maria Stecklum (EPO GmbH, Berlin-Buch). The cells were gently detached and suspended in a 100

µl mixture of Matrigel and cells in case of subcutaneous injection and a total volume of 40 µl in case of intrasplenic injection. Various cell numbers were injected subcutaneously into NOD/SCID or NOD scid gamma (NSG) mice. After 120 days the tumor tissue was removed and the tumor size and weight were determined. Subsequently samples were further processed for RNA analysis and histological analysis. Histological analysis was performed by S. Brunk (Institut für Tierpathologie, Berlin). An overview of the performed *in vivo* experiments is given in table 37.

Table 37: Overview of performed *in vivo* experiments for the determination of the tumorigenic potential of undifferentiated, definitive endodermal committed and hepatic differentiated hESC.

	NOD/SCID			**NSG**		
	Number of cells per animal	**Number of animals**	**Side of injection**	**Number of cells per animal**	**Number of animals**	**Side of injection**
un-differentiated hESC	1E5	9	s.c.	1E5	5	s.c.
	1E6	21	s.c.	1E6	14	s.c.
	1E7	9	s.c.	1E7	14	s.c.
	5E5	9	i.s.	5E5	5	i.s.
	1E6	3	i.s.	5E6	5	i.s.
	1E7	3	i.s.	-	-	-
Definitive endodermal committed cells	1E5	3	s.c.	1E5	5	s.c.
	1E6	3	s.c.	1E6	5	s.c.
	-	-	-	5E5	5	i.s.
Hepatic differentiated cells	1E5	9	s.c.	1E5	2	s.c.
	1E6	9	s.c.	1E6	2	s.c.
	5E5	9	i.s.	5E5	2	i.s.

2.2.13 Statistical evaluation

The hepatic and spontaneous differentiation in conventional 2D culture has been performed three times (n=3). The hepatic differentiation in 3D bioreactor cultures according to approach two and Cellartis approach one have been conducted twice (n=2), the remaining bioreactor experiments were performed once (n=1). The results are presented as means +/- standard deviation (SD) of data from the experiments.

3. Results

3.1 Establishment of hESC culture, reduction of components of animal origin

Three different human embryonic stem cells lines were used in this study. Culture conditions were continuously improved in regard of the reduction of animal components, and improvement of labor sensitivity and reproducibility.

3.1.1 Feeder dependent culture of hESC

The mouse embryonic fibroblast dependent human embryonic stem cell line SA002 and H1 were cultured according to previously established protocols. The quality of cells and their grade of differentiation were evaluated by marker expression (immunohistochemistry), gene expression (real-time PCR) and morphology. Undifferentiated hESC exhibit a typical and unique morphology. The cells grow in flat and homogenous colonies. The cells exhibit a high nucleus to cytoplasm ratio and lay tightly packed with prominent nucleoli and close cell-cell contacts (figure 5a).

Any deviation from these characteristics can be interpreted as sign of differentiation (figure 5b).

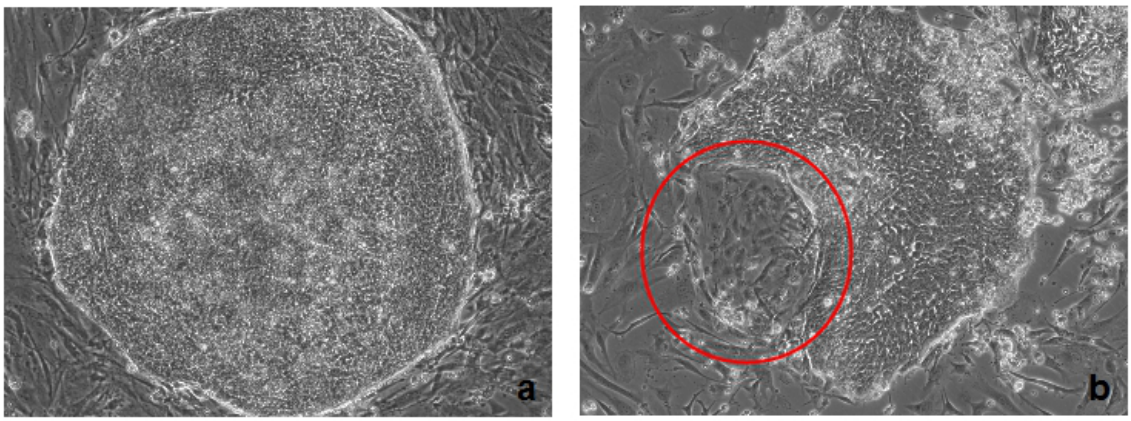


Figure 5: MEF dependent hESC; a) undifferentiated colony of hESC, b) colony of hESC with signs of differentiation (red circle), 100x total magnification

3.1.1.1 Standard culture

Half of the medium was exchanged by fresh medium every 24 hours. Feeder dependent hESC cultures that exhibited no signs of differentiation were cut into squares using a stem cell knife. The resulting pieces were gently detached from the culture dish and transferred into a new in vitro fertilization dish.

The hESC were cultured for more than 20 passages under the described culture conditions. The expression of markers indicating the pluripotent state were confirmed via immune cytochemistry and RT-PCR. The manual dissection of undifferentiated hESC colonies resulted in about 5 to 10 squares depending on size and quality of the cells. In average about 20 to 30 squares were harvested per plate. Differentiated areas were spared, resulting in a reduction of differentiated cells in the following passage. About 10 squares were transferred into a new in vitro fertilization dish.

3.1.1.2 Single Cell Enzymatic Dissociation (SCED) Culture

In order to obtain larger cell numbers for bioreactor experiments the cells were detached enzymatically and transferred in gelatin coated standard culture flasks that had been inoculated with human foreskin fibroblasts 24 hours before. Cells were passaged every 6 to 8 days. In comparison to the co-culture with MEF, HFF induced a change in the growth patterns of hESC. The cells grew not as flat colonies on top of the (MEF) feeder layer, but within the HFF layer. Microscopic evaluation of the cell quality was therefore not possible. The SCED cultures were expanded for up to 5 passages showing no signs of differentiation as verified by immune cytochemistry and RT-PCR analysis.

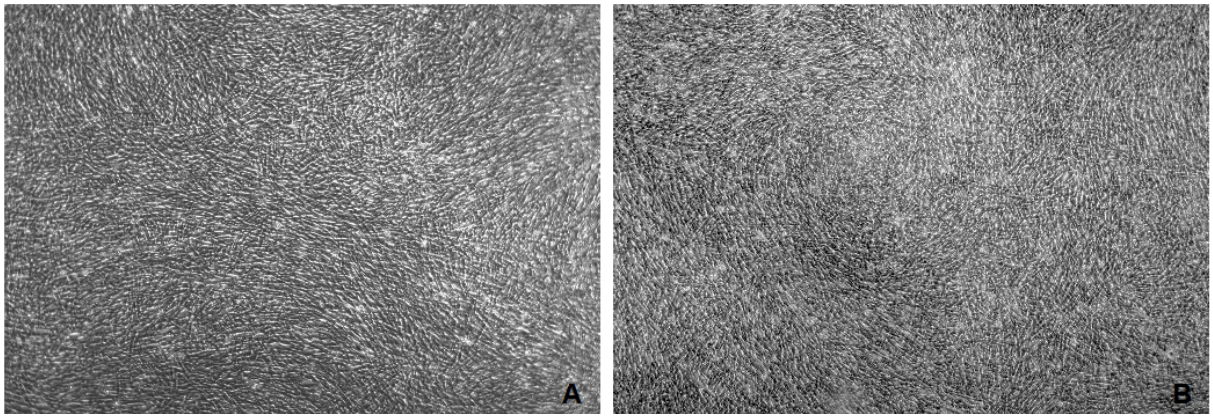


Figure 6: SCED culture, (A) culture one day after enzymatic passage, (B) SCED culture right before passage; 50x total magnification

3.1.2 Feeder independent culture of hESC

In order to simplify the culture of hESC, to avoid (MEF) batch variation and to minimize components of animal origin in culture conditions the feeder independent culture of hESC was established. The culture of the feeder independent cell line H1 (WiCell Research Institute, Madison, WI, USA) was established according to the supplier´s recommendations.

3.1.2.1 Standard culture

Feeder independent hESC grown in mTeSR™1 exhibited morphological characteristics similar to feeder dependent hESC. They possessed a prominent cell nucleus and a high nuclear to cytoplasm ratio. The cells within the colonies were tightly packed. The quality of hESC cultures was regularly evaluated by microscopic analysis of cell morphology, by real-time PCR and by immune cytochemistry. In figure 7 a representative colony stained for Oct-4 (7b), Nanog (7c) and SSEA4 (7f) is shown.

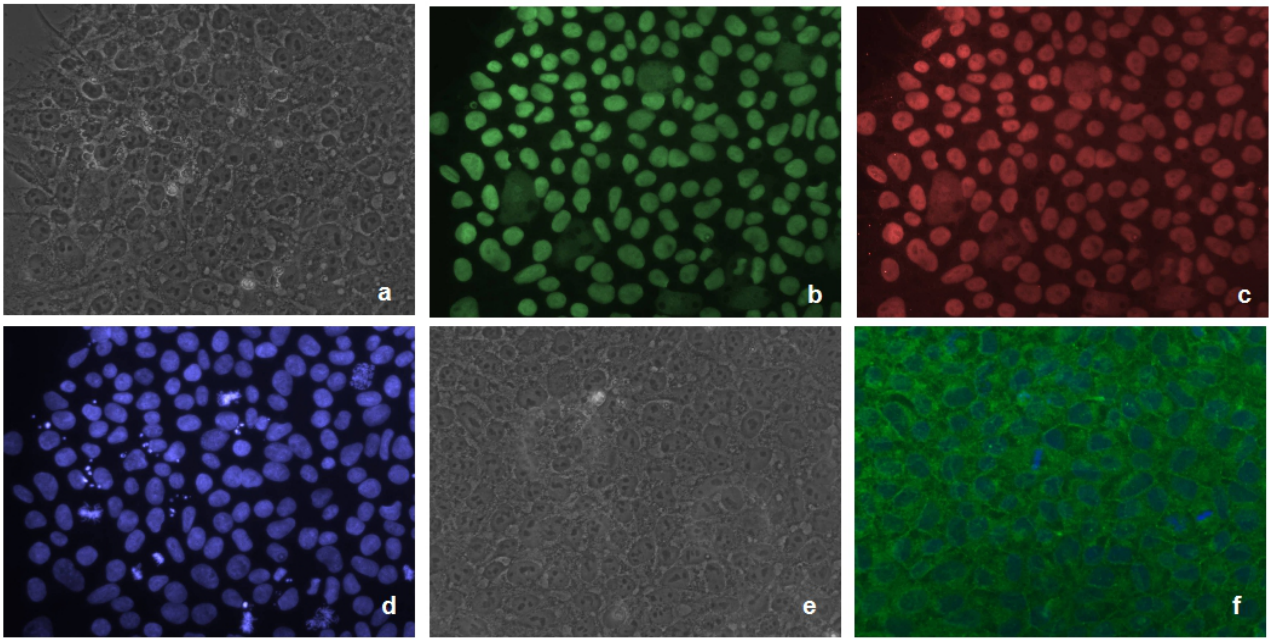


Figure 7: Immune cytochemical staining of undifferentiated hESC in conventional 2D culture; a) – d): hESC colony stained for Oct-4(b), Nanog(c), DAPI(d) and phase contrast picture (a); e) phase contrast picture of a hESC colony, f) same colony stained for SSEA4 (green) and DAPI (blue), 400x total magnification in all pictures.

A high expression of pluripotency markers Oct-4 and Nanog besides a weak expression of lineage specific markers (Neurofilament, AFP) was found in the reverse transcription (RT) PCR analysis (see table 38). No expression of GATAII was found in the samples analyzed.

Table 38: Expression of pluripotency markers and lineage specific markers in undifferentiated feeder independent hESC (H1), “++” = strong expression, “(+)“ weak expression, “-” = absent expression

PCR Product	Expression
Oct-4	++
Nanog	++
GATAII	-
Neurofilament	(+)
AFP	(+)

3.1.2.2 Spontaneous *in vitro* differentiation in conventional 2D cultures

In order to determine the potential of cultured stem cells to form cells of the three germ layers feeder independent hESC (H1) were differentiated in conventional 2D cultures for 17 days using EB-Medium. Medium was changed every 48 to 72 hours and factors indicating differentiation (bHCG, AFP) were measured as well as glucose, lactate and lactate dehydrogenase (LDH). Additionally RNA samples were taken after 5, 9 and 17 days of differentiation and analyzed via real-time PCR.

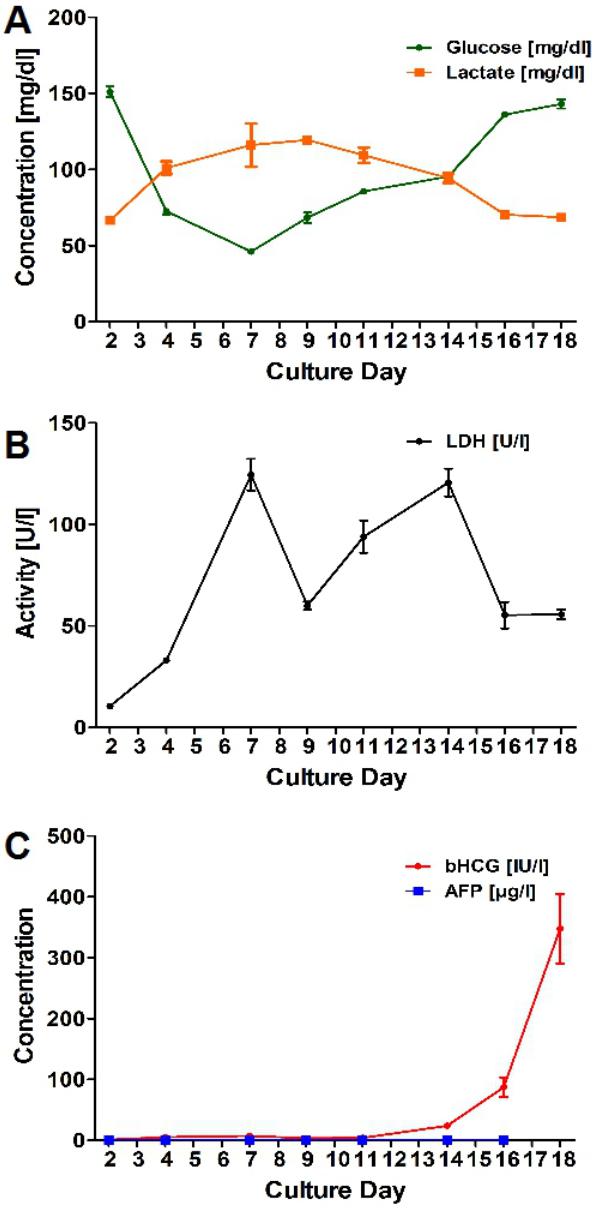


Figure 8: Time-course of glucose, lactate (A), LDH (B), bHCG and AFP (C) during spontaneous differentiation of hESC in 2D culture (n=3).

The time-course of metabolic activities, enzyme release and differentiation factor release are shown in figure 8. The glucose consumption and lactate production increased until day 7, indicating cell proliferation. This was followed by a decrease of metabolic activity to a level comparable to activities measured at day 2.

The LDH concentration measured in the supernatant also increased until day 7. The

concentration remained at high levels.

The bHCG concentration remained at very low concentrations during the whole experiment. A strong increase in AFP concentration was seen from day 14 until the end of the experiment.

The RNA samples taken on day 5, 9 and 17 were reverse transcribed and the expression of genes for the three germ layers (AFP, Neurofilament and GATA II) as well as the expression of genes characterizing the undifferentiated state of hESC was analyzed. The expression was normalized to GAPDH. In figure 9 the fold expression change compared to undifferentiated hESC is shown.

The expression of markers indicating the pluripotent state of hESC (Oct-4 and Nanog) was already reduced after 5 days and remained stable over the duration of the experiment. The expression of the mesodermal marker GATA II was already strong on day 5, increased to day 9 and slightly decreased until day 17.

The endodermal marker AFP was not induced after 5 days in spontaneous differentiation. The expression level increased continuously till day 17. A weak induction of the ectodermal marker neurofilament was found only at the end of the experiment.

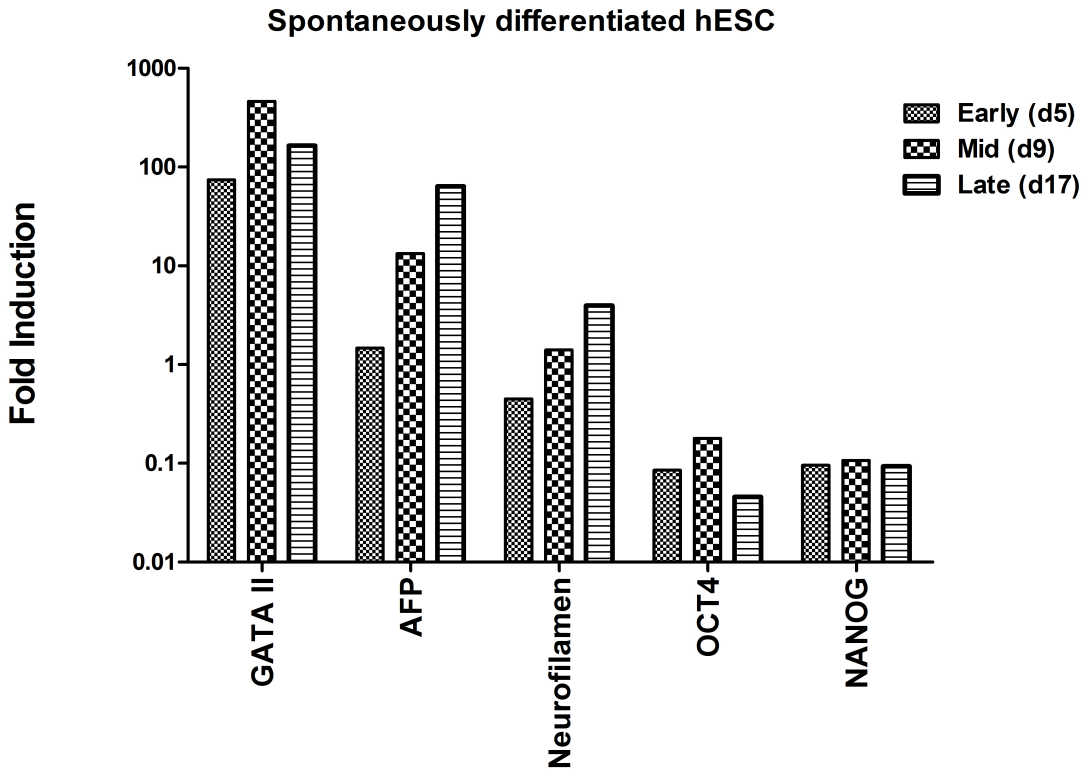


Figure 9: Fold expression of GATA II, AFP, Neurofilament, Oct-4 and Nanog in spontaneously differentiated hESC normalized to undifferentiated hESC (=1) after 5 (Early), 9 (Mid) and 17 (Late) days in conventional 2D culture.

A change in morphological appearance was seen during the spontaneous differentiation (Figure 10). The experiment was started when the cultures were about 70% confluent. In the first phase of the experiment the cells formed a confluent cell layer, a dramatic change in cell morphology was not seen. Once the culture reached 100% confluence (at day 5), the cells tended to form colonies made of multiple layers of inhomogeneous cells. In addition larger cell aggregates formed that grew in size over time. Due to the high cell density morphological changes of the cells could not be detected after day 5.

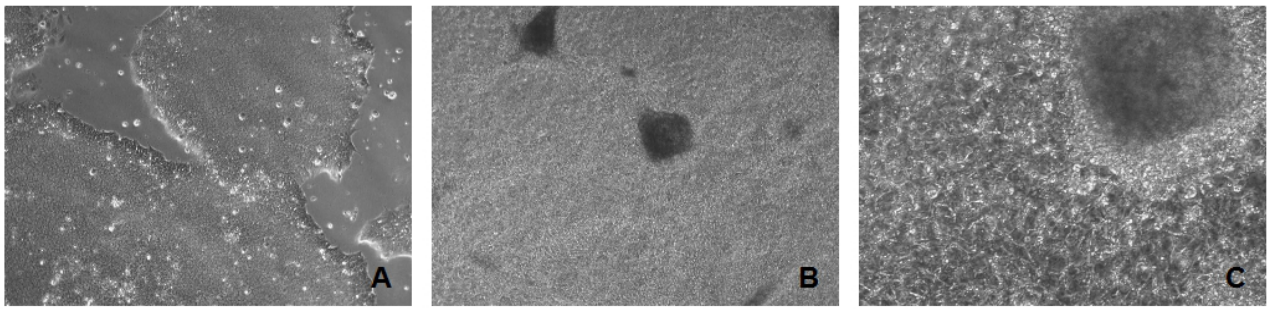


Figure 10: hESC, undifferentiated at d 0 (A) and after 17 days of spontaneous differentiation in conventional 2D culture, A and B: 100x total magnification, C: 320x total magnification.

3.1.2.3 Spontaneous *in vitro* differentiation in 3D bioreactors

In order to determine the potential of spontaneously differentiating hESC in 3D bioreactors, 25E6 undifferentiated feeder independent hESC (H1) were inoculated in a miniaturized bioreactor model (0.4 ml cell compartment volume). The cells were differentiated for 17 days, daily factors indicating differentiation (bHCG, AFP) as well as glucose, lactate and lactate dehydrogenase (LDH) were measured (Figure 11). The metabolic activity indicated by glucose consumption and lactate production was low during the experiment. A high concentration of LDH was seen during the first days, with a maximum concentration found on day 4. During the whole experimental duration only low concentrations of HCG were measured, whereas the concentration of AFP was rising from day 11 on. At bioreactor shutdown RNA was isolated and further processed for Real-time PCR analysis.

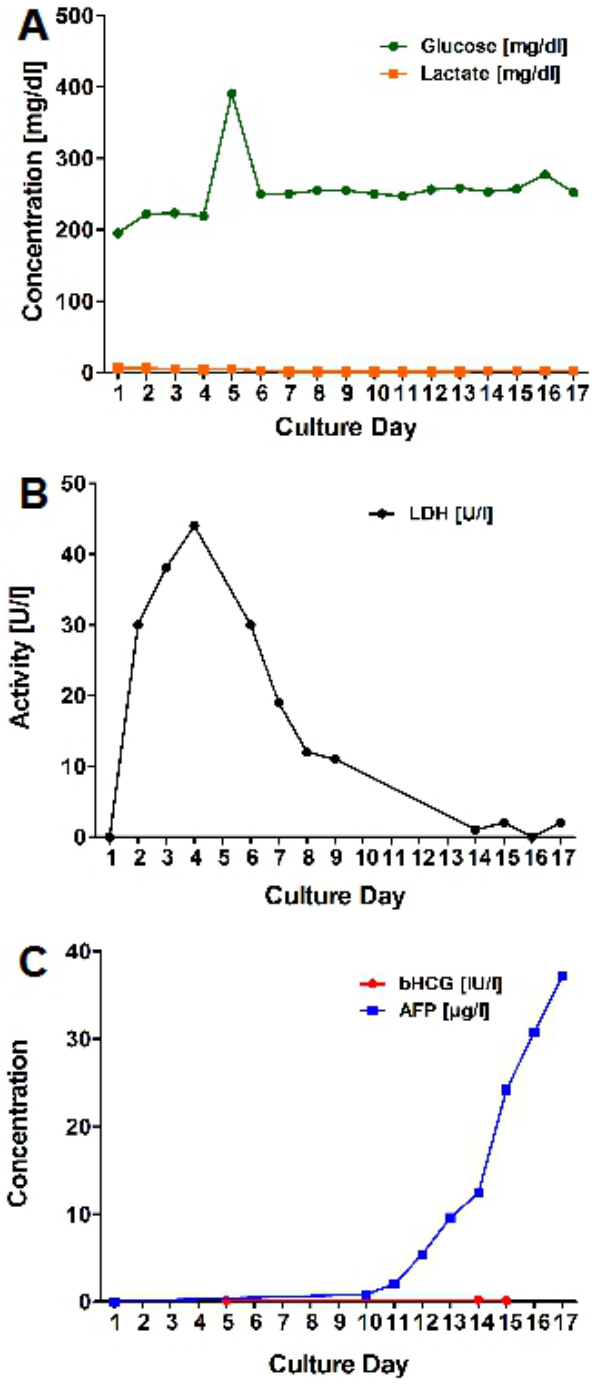


Figure 11: Time-course of glucose and lactate (A), LDH (B), bHCG and AFP (C) during spontaneous differentiation of hESC in a 3D bioreactor.

The RNA isolated after bioreactor shutdown were reverse transcribed and the expression of genes for the three germ layers (AFP, Neurofilament and GATA II), and

of those characterizing the undifferentiated state of hESC (Oct-4, Nanog) and of endodermal / hepatocyte-specific genes (SOX7, SOX17, Albumin, Cyp 2D6, Cyp 2B6, Cyp 3A4, Cyp 2C9) was analyzed. The expression was normalized to GAPDH. In figure 12 the fold expression change compared to undifferentiated hESC is shown. A strong induction of AFP expression was found after 17 days of bioreactor culture, while only a moderate induction of GATA II, neurofilament and hepatocyte specific transcripts was found. In contrast the expression of markers indicating the undifferentiated state of hESC (Oct-4 and Nanog) was found to be down-regulated.

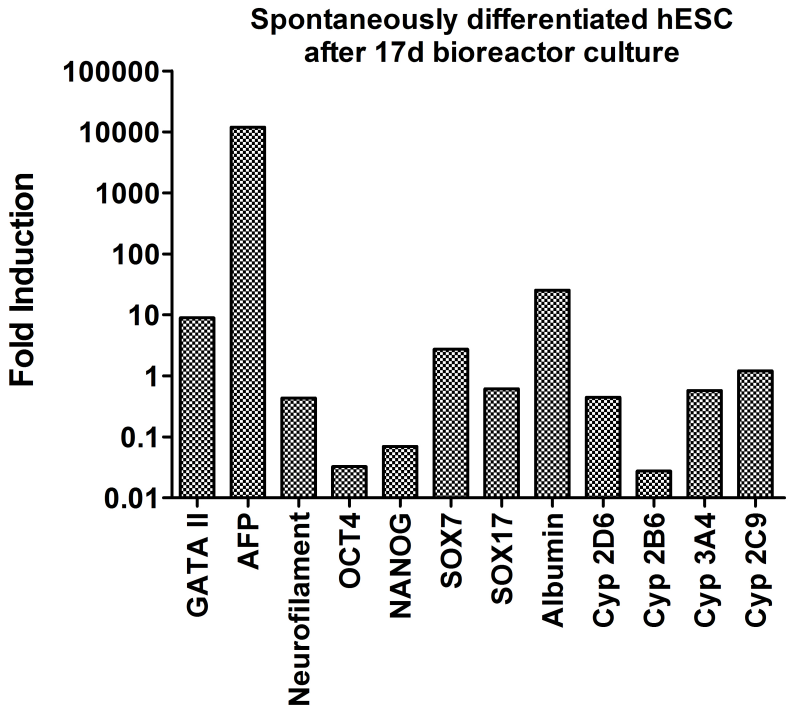


Figure 12: Fold induction of GATA II, AFP, Neurofilament, Oct-4, Nanog, SOX7, SOX17, Albumin, Cyp 2D6, Cyp 2B6, Cyp 3A4 and Cyp 2C9, normalized to undifferentiated hESC (n=1).

3.1.2.4 The *in vivo* tumorigenic potential of feeder independent hESC (H1)

Undifferentiated hESC (H1) were injected subcutaneously into NOD/SCID mice or NSG mice as described under methods. After 120 days 3 solid tumors could be isolated out of NOD/SCID mice that received $1x10^7$ (3 mice) cells each and 2 tumors out of NSG mice that received $1x10^6$ (1 mouse) or $1x10^7$ (1 mouse) cells each. Three of the harvested tumors were processed and HE staining was performed. In two of these tumors cell derivatives of all three germ layers were identified (Figure 13). In one tumor cells of the ectodermal germ layer were not identified clearly.

In order to determine the tumorigenic potential of differentiated cells, feeder independent hESC (H1) were differentiated to the definitive endodermal and hepatocyte state according to Hay et al. 2008. No teratoma was found in NOD/SCID (n=33) or NSC (n=21) mice after the subcutaneous or intrasplenic application of definitive endodermal or hepatic differentiated cells ($1x10^5$ to $1x10^6$ cells per injection).

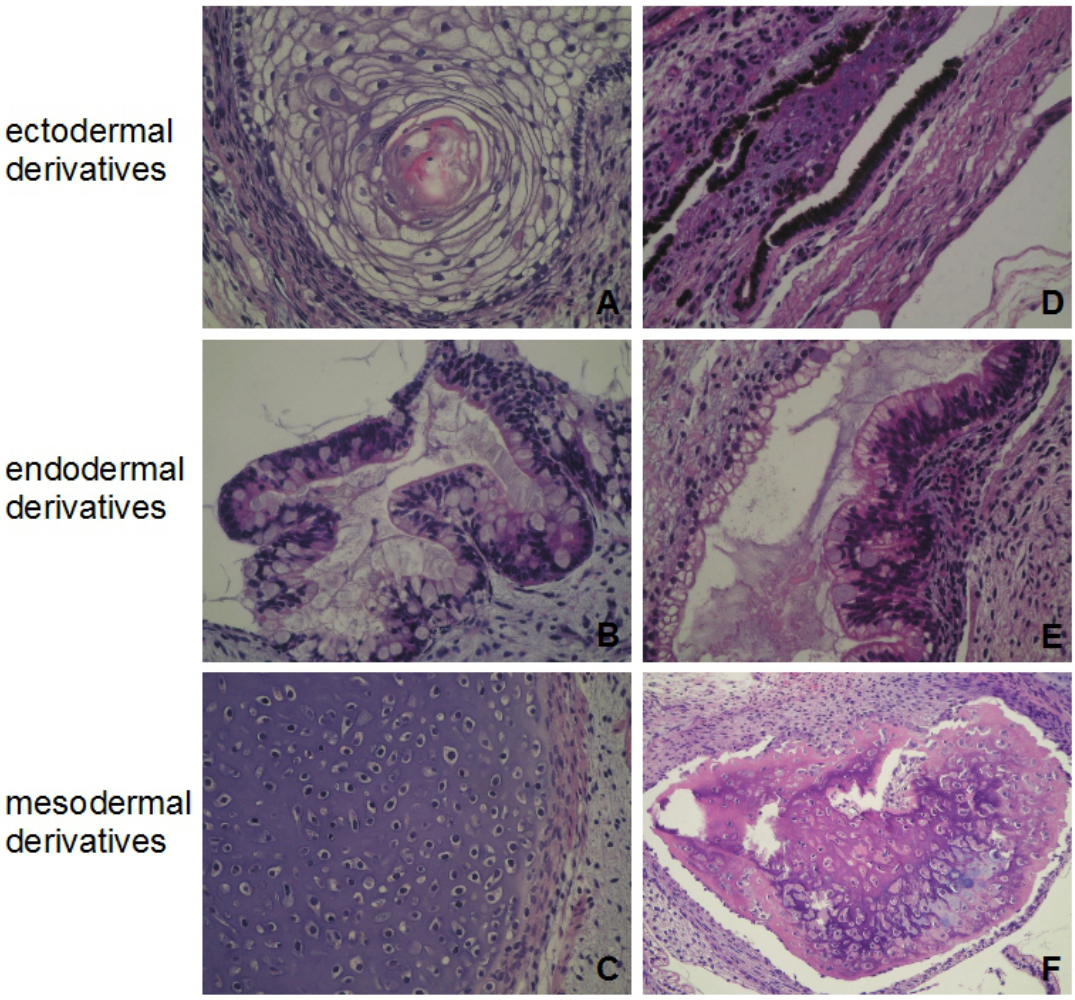


Figure 13: HE-staining of samples from teratoma: A) keratin rich epithelium; B) goblet cells and ciliated epithelium; C) cartilage; D) pigmented epithelium and neuroectodermal tubular structures; E) goblet cells and ciliated epithelium; F) cartilage and skeletal muscles, HE-stainings and histological evaluation was performed by S.Brunk, Institut für Tierpathologie, Berlin.

3.2 Hepatic differentiation

3.2.1 Hepatic differentiation in 2D

Five different protocols (Table 31) were investigated in order to establish a method for the hepatic differentiation of hESC under conventional 2D conditions. In all approaches undifferentiated cells first undergo commitment to the definitive endodermal (DE) lineage. Subsequently after completion, cells were treated with cytokines that stimulate cell proliferation. The hepatic differentiation was accomplished by a combination of cytokines that support maturation of hepatic cells (see table 31). During the differentiation process the concentration of factors that indicate metabolic activity (glucose and lactate), differentiation (AFP and bHCG) and cell integrity (LDH) were measured in the supernatant. RNA samples and cells for IHC were collected before differentiation, after accomplishment of DE-commitment and at the end of the hepatic differentiation. In addition samples from hESC that received mTeSR1™ medium during the process of differentiation were collected as control. The cell number of vital cells was determined at the beginning, after DE-commitment and after accomplishment of hepatic differentiation.

3.2.1.1 Metabolic parameters during hepatic differentiation

The hESC that underwent hepatic differentiation according to the first approach showed the highest metabolic activity among all protocols investigated (Figure 14). A significant drop in glucose consumption a well as lactate production was seen around day 15, which is likely a result of the altered medium change cycle beginning at day 12.

In the second approach the metabolic activity dropped significantly after the start of differentiation. The metabolic rate increased again after day 6, which can be interpreted as result of the pro-mitotic effect of the cytokines given after day four.

The third approach showed a metabolic rate that lied between the rates of approach one and two. No metabolic conversion of glucose could be observed after transferring the cells. The collection of samples was stopped for this approach at day nine.

The cells in approach 4 and 5 perished within the first days of DE differentiation. The course of metabolic rates in approaches four and five was characterized by a dramatic drop. After day three no activity could be observed. The collection of samples was discontinued at day five.

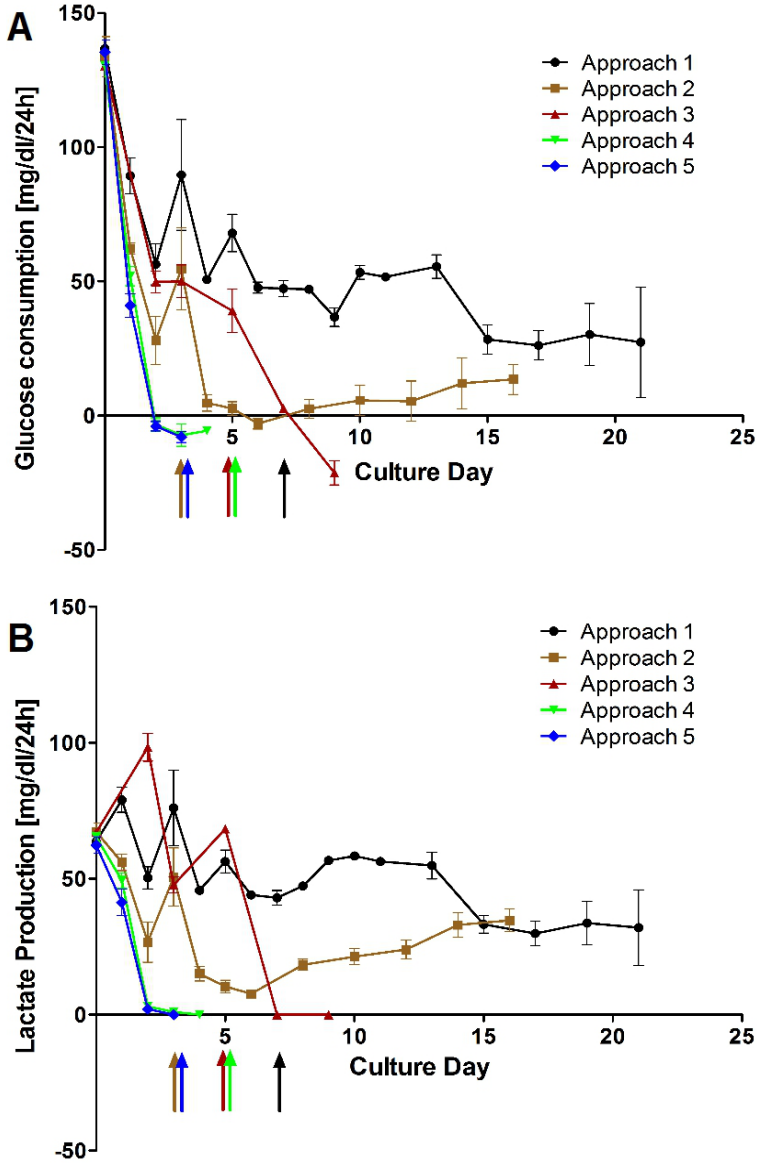


Figure 14: Time course of (A) glucose consumption and (B) lactate production of the 5 different approaches investigated in conventional 2D culture. Arrows indicate the end of endodermal commitment of the cells in colors corresponding to the approaches (n=3).

3.2.1.2 Cell integrity and cell numbers during hepatic differentiation

The induction of DE commitment of the hESC seemed to have a vast impact on cell integrity. In all approaches the LDH concentration reached high levels in the supernatant (Figure 15). In Approach one the first step of differentiation had the lowest impact on cell integrity. This assumption is supported by the cell numbers counted after DE commitment (Figure 16). During the second step of the hepatic differentiation no significant increase in LDH concentrations was measured in any of the approaches.

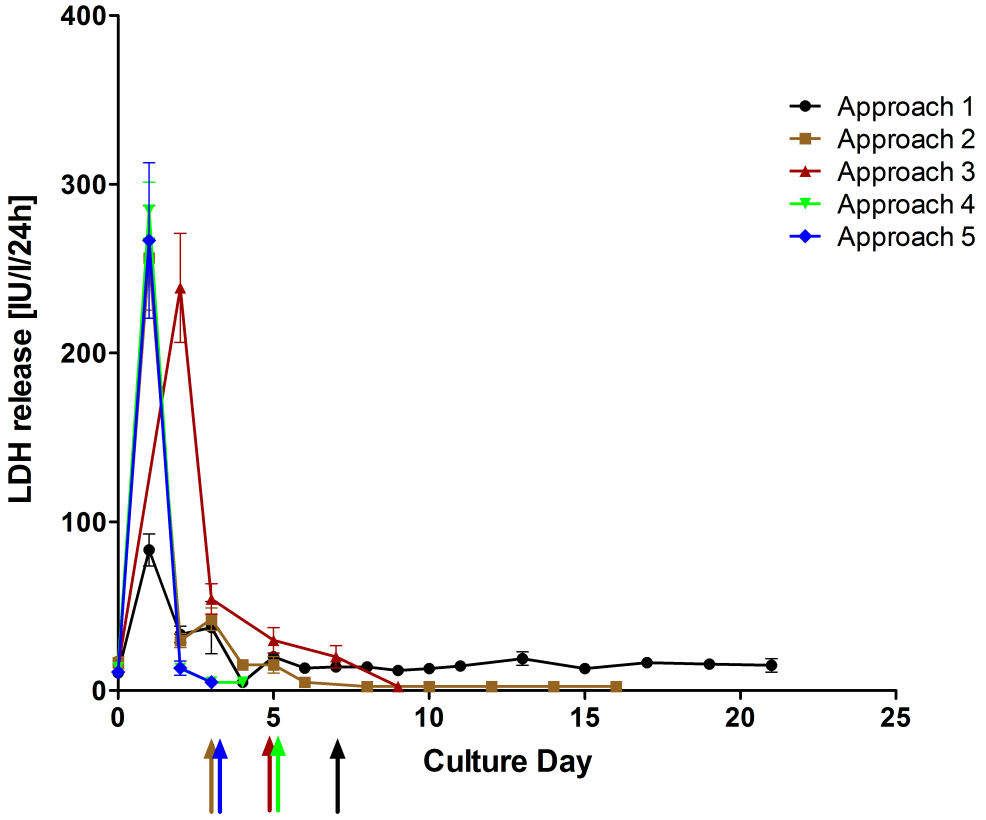


Figure 15: LDH release of the 5 different approaches measured during hepatic differentiation. Arrows indicate the end of endodermal commitment of the cells in colors corresponding to the approaches (n=3).

The high LDH concentrations during definitive endodermal differentiation are consistent with the number of cells found after DE and at the end of differentiation compared to the cell number before starting the differentiation process (Figure 16 A

& B). The amount of cells was significantly higher for both time points in approach 1 compared to approach 2.

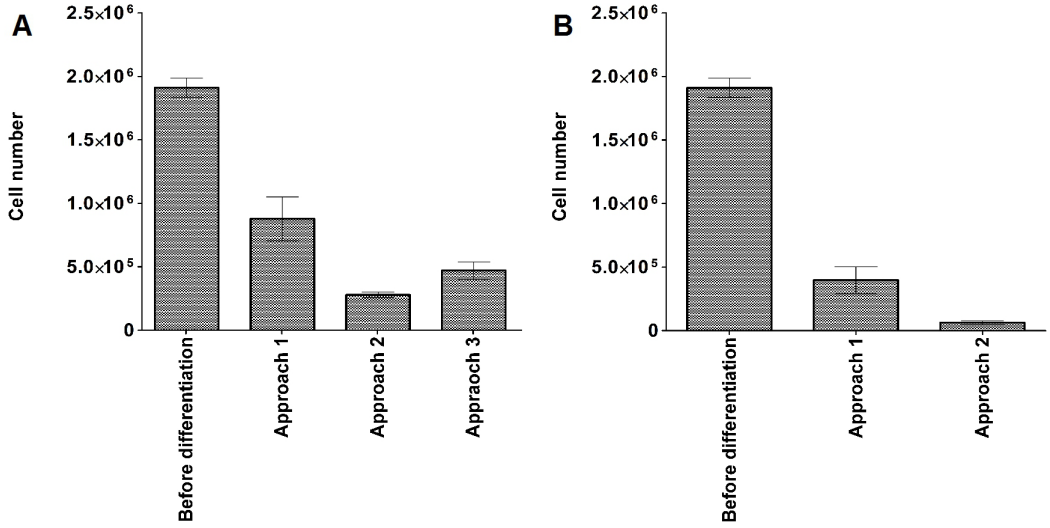


Figure 16: Cell numbers of differentiating hESC after accomplishment of DE differentiation (A) and at the end of differentiation (B) (n=3).

During the experiment markers indicating differentiation first accrued in elevated levels at around day 8. The time courses of AFP and bHCG concentrations in approach 1 and 2 are given in figure 17. In approach 1 a significant increase of bHCG was seen from day 8 on. The highest concentration was measured around day 10. Afterwards the concentration decreased and reached basal levels at around day 15. The concentration of AFP in cells differentiating according to approach 1 was constantly low over the time of the experiment.

In approach 2 constantly low levels of bHCG were measured during the differentiation. The levels of AFP increased constantly from day 8 on.

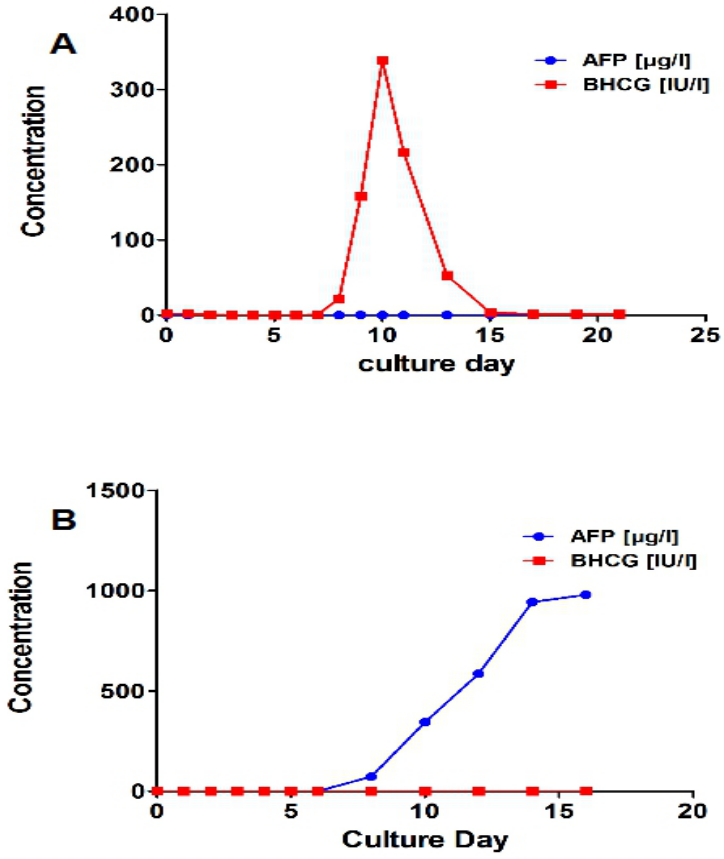


Figure 17: Concentration of bHCG and AFP during differentiation of hESC according to approach 1 (A) and approach 2 (B).

The cells underwent specific morphological changes during differentiation, as compared to undifferentiated cells (Figure 18, F).

DE differentiated cells in the first approach showed an inhomogeneous morphology (Figure 18, A and B). Round cells that were tightly packed could be seen next to spindle shaped cells. At the end of differentiation the cells were less heterogeneous.

In the second approach the cells acquired a “cobblestone” like morphology (Figure 18, C and D). During further progress in differentiation, the cells grew in size and the cell nucleus became apparent.

In the third approach the cells appeared less “organized” compared to the second approach, but still homogenous (Figure 18, E). Subsequently after accomplishment of the DE step the cells were transferred into new culture flasks. The cells were not

able to adhere, and perished right after passage.

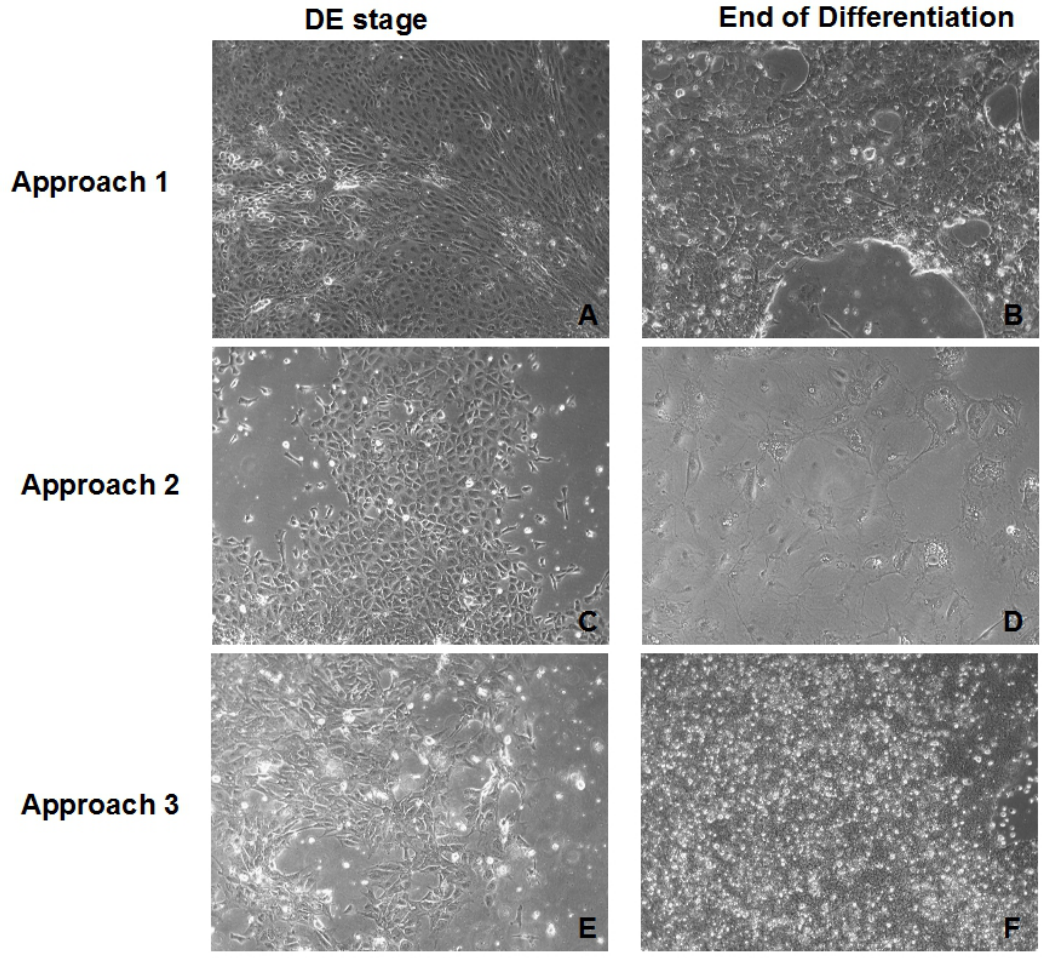


Figure 18: hESC after accomplishment of DE commitment (A, C, E) and after maturation (B, D) in conventional 2D culture; phase contrast microscopy; (F) undifferentiated hESC on d0; 100x total magnification.

The results from real time PCR analysis of the RNA isolated after DE commitment and at the end of differentiation are shown in figure 19.

An induction of GATA II expression after DE commitment was observed in approach 1. At the end of differentiation the expression of GATA II was still strongly induced.

In the second approach a strong induction of GATA II was also seen after DE commitment, but in contrast to the first approach the expression of SOX17 was induced as well. At the end of differentiation the cells in the second approach showed a strong induction of AFP, Albumin, SOX17 and Cytochrome P450 2C9 expression.

Definitive endodermal cells of the third approach showed already high expression of

AFP and SOX17.

After the first step a robust expression of markers for undifferentiated stem cells (Oct-4 & Nanog) could be seen in all approaches. The expression of these markers was significantly reduced after accomplishment of the differentiation.

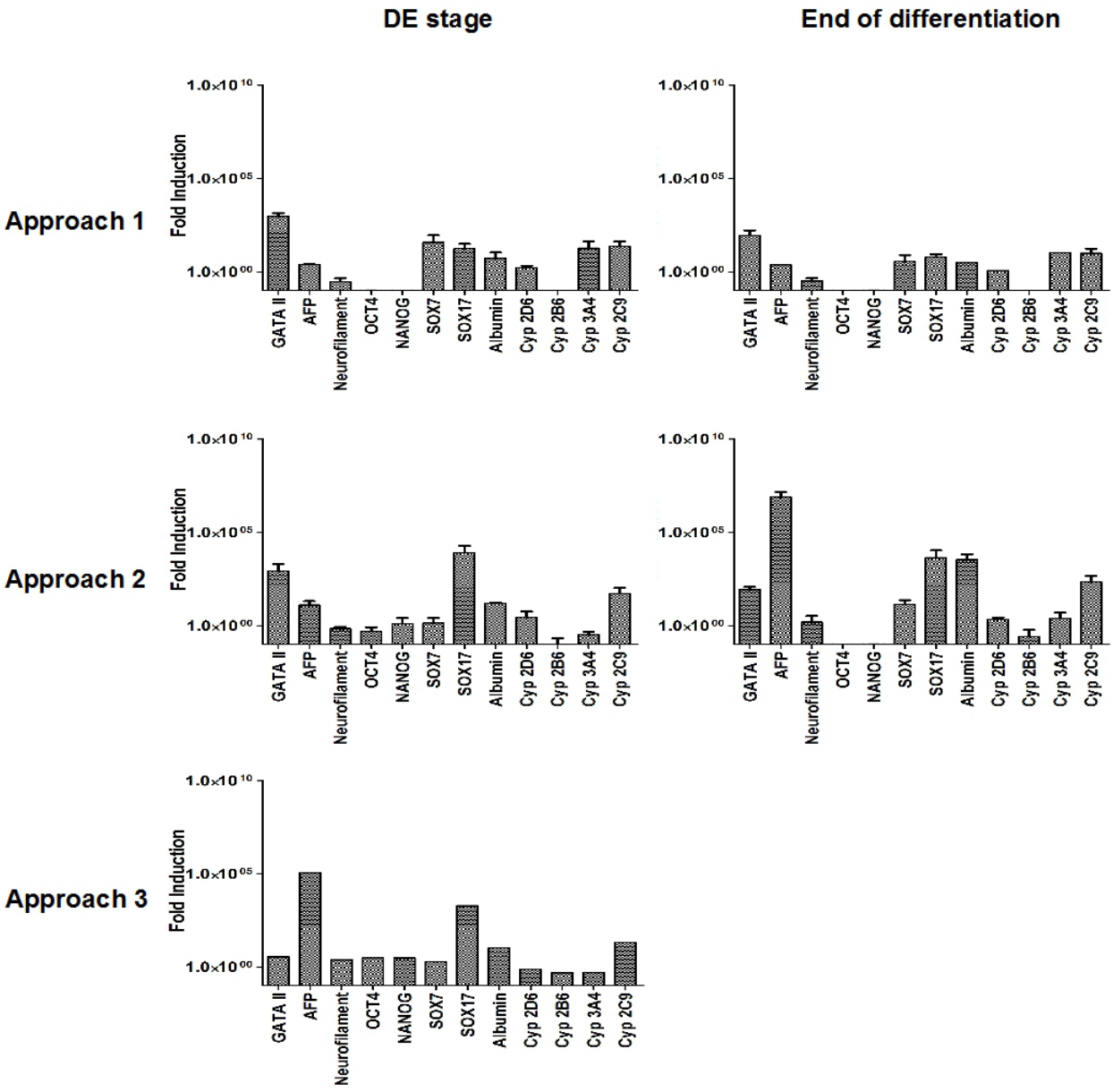


Figure 19: Expression of genes characteristic for undifferentiated cells (Oct-4, Nanog) and for cells differentiated into endodermal (AFP), mesodermal (GATA II), ectodermal (Neurofilament) and hepatic (SOX17, Albumin, Cyp2D6, Cyp2B6, Cyp3A4, Cyp2C9) cells in hESC after DE commitment and at the end of differentiation in conventional 2D culture, normalized to undifferentiated cells (n=3).

The cells differentiated according to the protocol of the first approach exhibited a CK18/CK19 double positive phenotype. They were not positive for vimentin. Representative pictures are shown in figure 20.

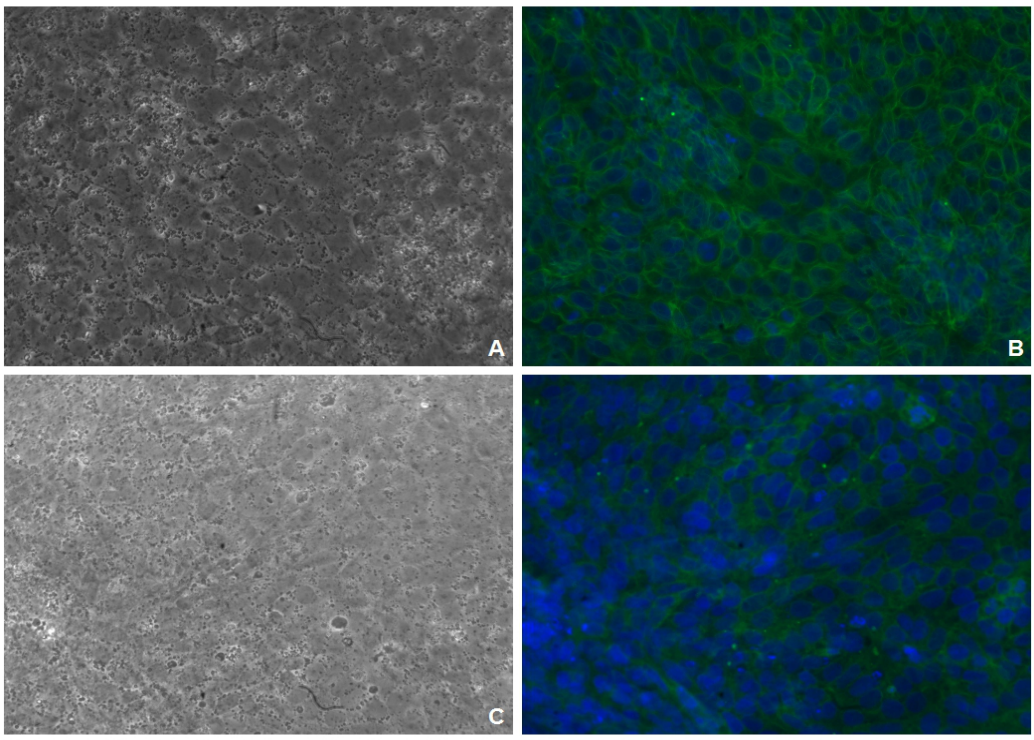


Figure 20: Immune cytochemistry staining of hESC in conventional 2D culture after conducted differentiation according to approach 1; (B) CK18 (green) & DAPI (blue); (D) CK19 (green) & DAPI (blue); (A) & (C) phase contrast microscopy picture; 400x total magnification.

The cells differentiated according to approach 2 were positive for CK18 and CK19 (Figure 21, B and D). In addition a non-uniform positive immunoreaction was seen also for the mesodermal marker vimentin (Figure 21, F).

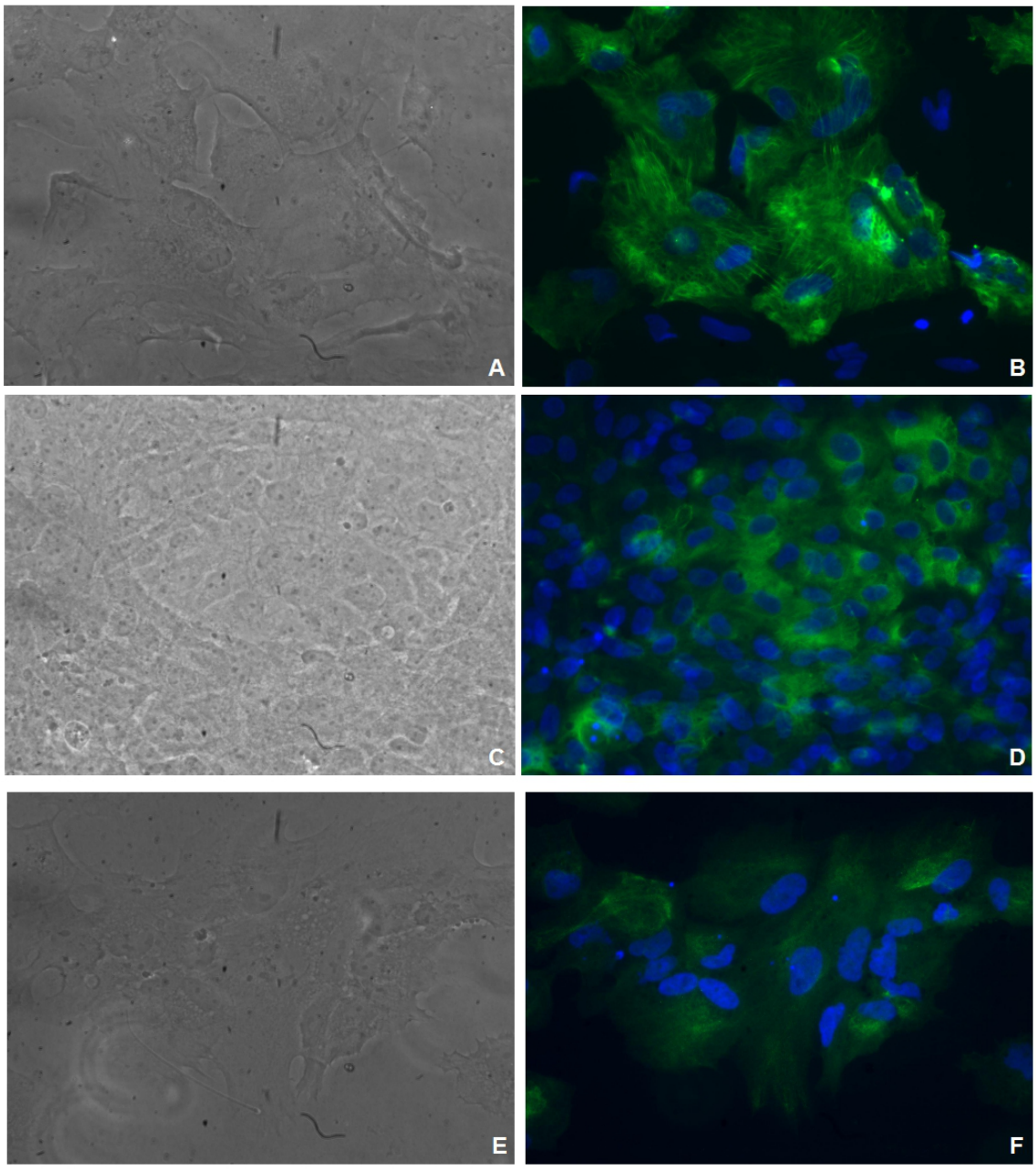


Figure 21: Immune cytochemical staining of hESC after conducted differentiation according to approach 2; (B) CK18 (green) & DAPI (blue); (D) CK19 (green) & DAPI (blue); (F) vimentin (green) & DAPI (blue); 400x total magnification in all pictures.

3.2.1.3 Stability of hepatically differentiated cells

In order to determine the stability of differentiation hESC were differentiated according to approach 2 and either cultivated in medium that supports hepatic maturation (HCM2.2) or medium that supports spontaneous differentiation (EBMedium) for 14 days. During the experiment duration the concentration of factors that indicate metabolic activity (glucose and lactate), differentiation (AFP and bHCG) and cell integrity (LDH) were measured in the supernatant. RNA samples were collected at the end of differentiation, after 3 days in HCM2.2 or EB medium, and after 14 days.

The metabolic activity of the cells in both culture media was comparable (figure 22 A and B). The higher glucose concentration in EBMedium is a result of the higher concentration in the basal medium (450 mg/dl for the EBMedium, 185 mg/dl for the HCM2.2)

The levels for LDH (figure 22 C) were also similar in both approaches indicating a comparable cell decline.

AFP levels proceeded comparably until day 26 of culture (figure 22 D). From that day the levels were higher in the culture that received HCM2.2 medium.

Concentrations for bHCG were elevated in HCM2.2 medium from day 20 to day 24 before they reached levels similar to those in EBMedium again (figure 22 E).

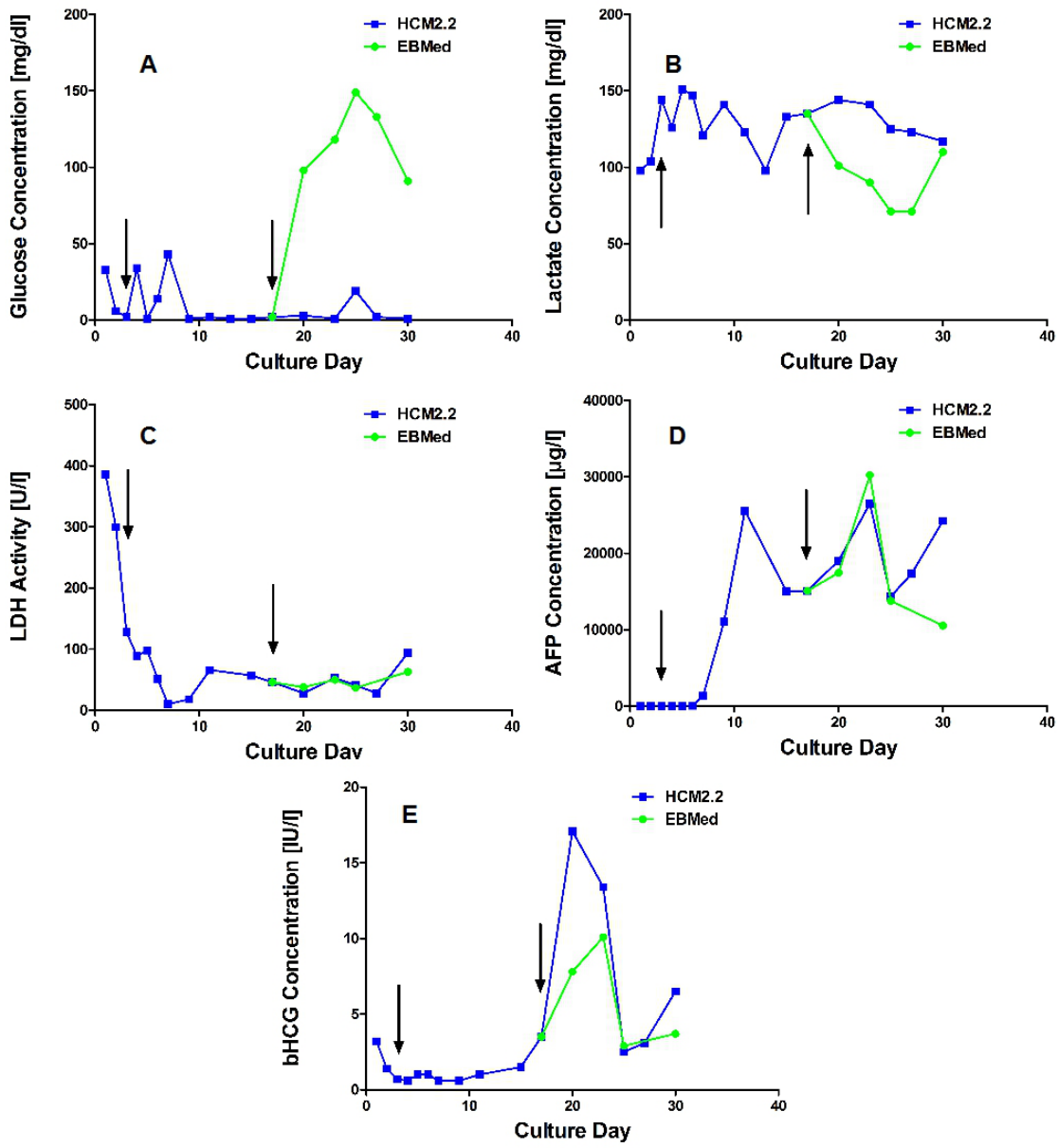


Figure 22: Graphs for glucose (A), lactate (B), LDH activity (C), AFP (D) and bHCG (E) concentration during hepatic differentiation (until d17) and subsequent evaluation of stability of differentiation. The arrows indicate the end of the definitive endodermal commitment (left arrow) and the end of differentiation (right arrow).

The real time PCR analysis of RNA samples collected at day 0, day 3 and day 14 after the end of hepatic differentiation showed only minor changes in expression of the investigated genes (see figure 23).

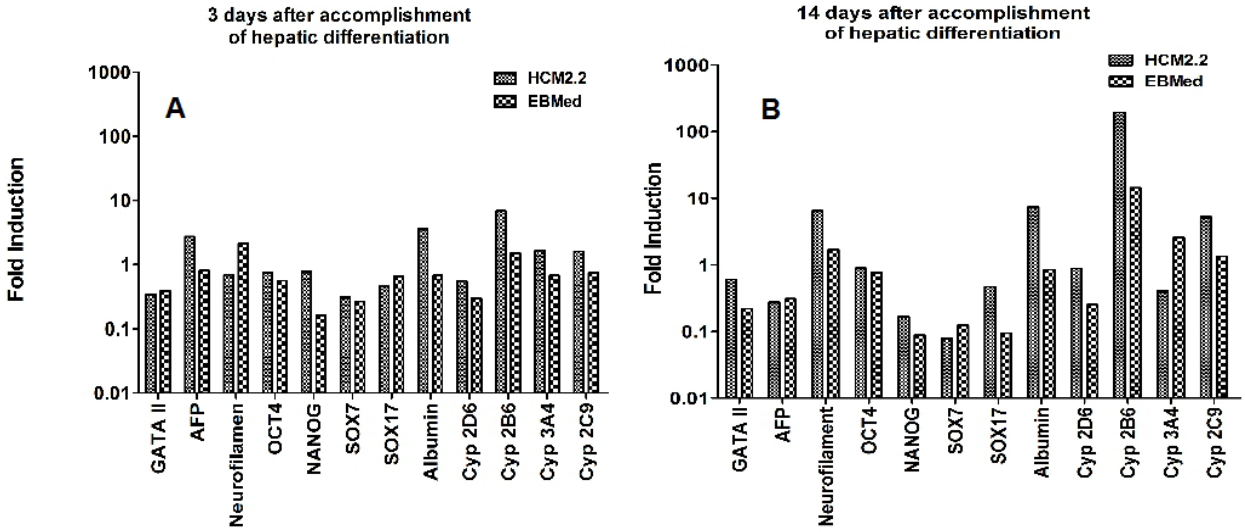


Figure 23: Gene expression profile of differentiated hESC, after 3 days (A) or 14 days (B) in EBMedium or HCM2.2 medium showing markers for undifferentiated cells (Oct-4, Nanog) and for cells differentiated into endodermal (AFP), mesodermal (GATA II), ectodermal (Neurofilament) and hepatic (SOX17, Albumin, Cyp2D6, Cyp2B6, Cyp3A4, Cyp2C9) cells, Expression has been normalized to hepatic differentiated hESC (d0) (n=1).

The expression of markers for non-endodermal germ layers (GATA II and Neurofilament) was not significantly changed in HCM2.2 nor in EB-Medium. The RNA expression of Albumin and Cytochrome P450 2B6 was induced in HCM2.2 medium, a moderate induction of Cytochrome P450 3A4 was found in EBMedium.

Genes indicating an undifferentiated state (Oct-4, Nanog) of hESC were expressed at levels similar to those measured at day 0 or below.

The differentiated cells underwent further morphological changes depending on the culture medium (Figure 24). The cells cultured in HCM2.2 medium did not proliferate, they formed colonies with sharp edges. The cells were less homogeneous in total as compared to cells cultured in EBMedium and the cell size was reduced.

The cells that were further cultured in EBMedium appeared more homogeneous, and formed flat colonies consisting of cells with prominent nucleoli.

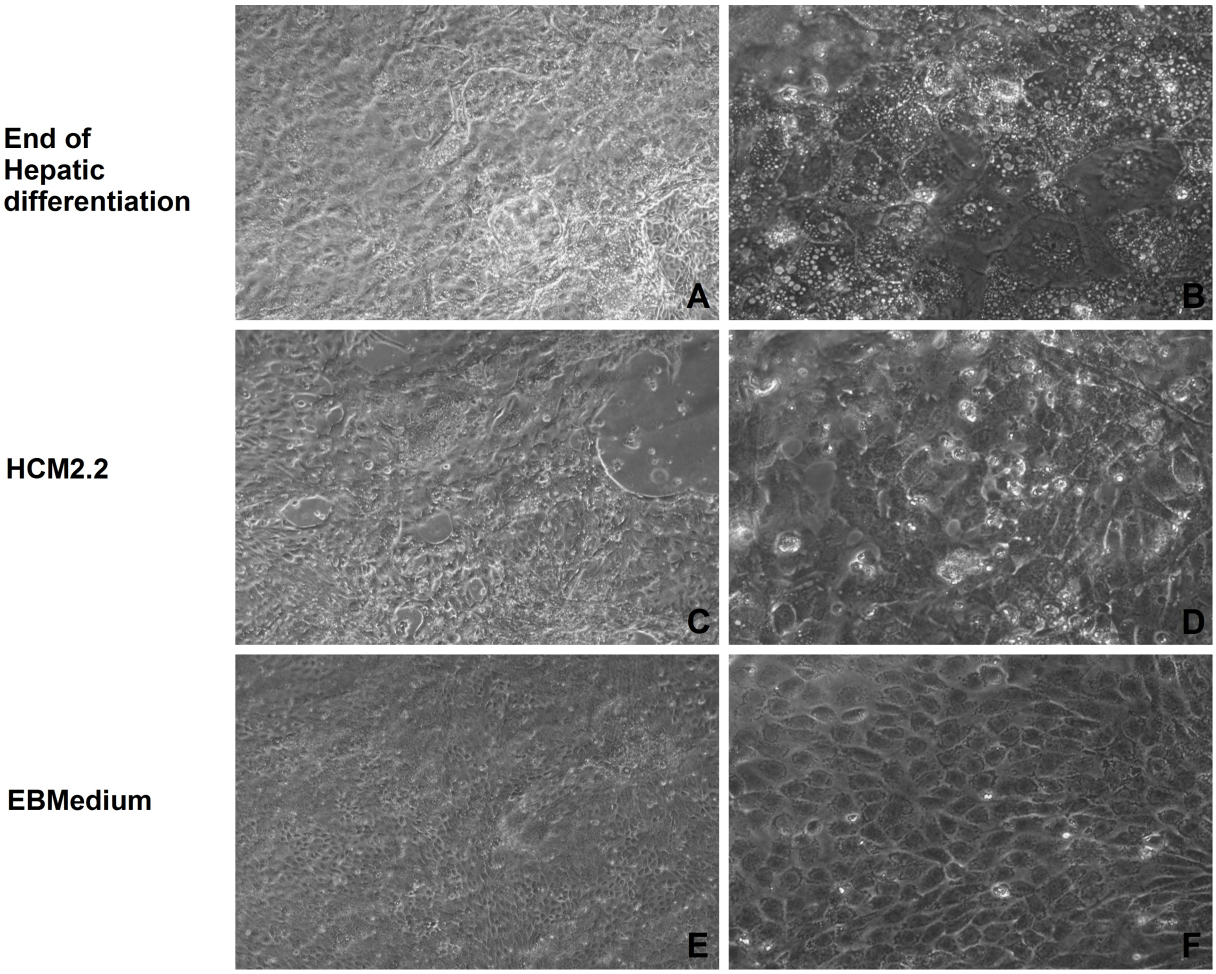


Figure 24: hESC after accomplishment of hepatic differentiation, (A) 100x total magnification, (B) 320x total magnification; hepatic differentiated hESC after 14 days in HCM2.2 (C, D) or EBMedium (E, F) after accomplishment of differentiation; (C, E) 100 x total magnification, (D, F) 320 x total magnification.

3.2.2 Culture of hESC in 3D bioreactor systems

The culture of hESC was carried out in various bioreactor models. Before starting the differentiation experiments a 48 to 72 hour recovery period in mTEsR medium was granted. In order to determine the level of differentiation that hESC acquire during that period, a miniaturized bioreactor was inoculated with 60 million undifferentiated cells followed by a culture period of 48 hours. Daily metabolic parameters (glucose, lactate), parameters that indicate cell integrity (LDH) and differentiation (AFP and bHCG) were measured. A decrease in glucose concentration and corresponding to that an increase in lactate was seen after inoculation of the cells (figure 25 A). The LDH activity increased continuously over the time of the experiment (figure 25 B). An increase of bHCG or AFP concentrations above the detection limit was not detectable in the samples drawn from the bioreactor recirculation (data not shown). At bioreactor shutdown RNA samples were taken and further processed for real time PCR analysis.

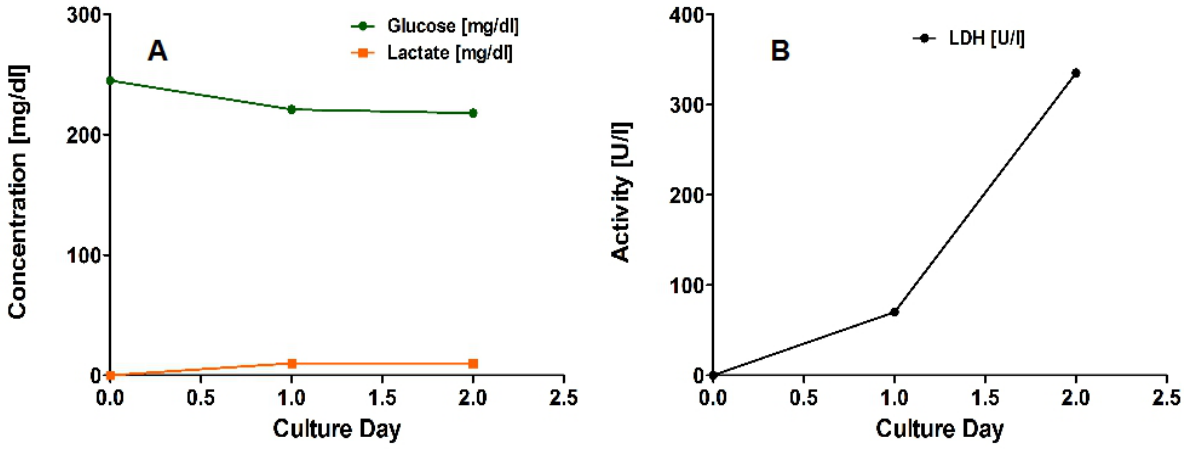


Figure 25: Glucose, lactate concentration (A) and LDH activity (B) during 48 hours culture of undifferentiated hESC under conditions that support the proliferation of undifferentiated cells (n=1)

The expression of the majority of investigated genes did not change dramatically, a stable expression of genes indicating the undifferentiated state (Oct-4 and Nanog) was found in the cells after 48 hours of bioreactor culture. A strong induction of

expression was found for SOX17 (Figure 26).

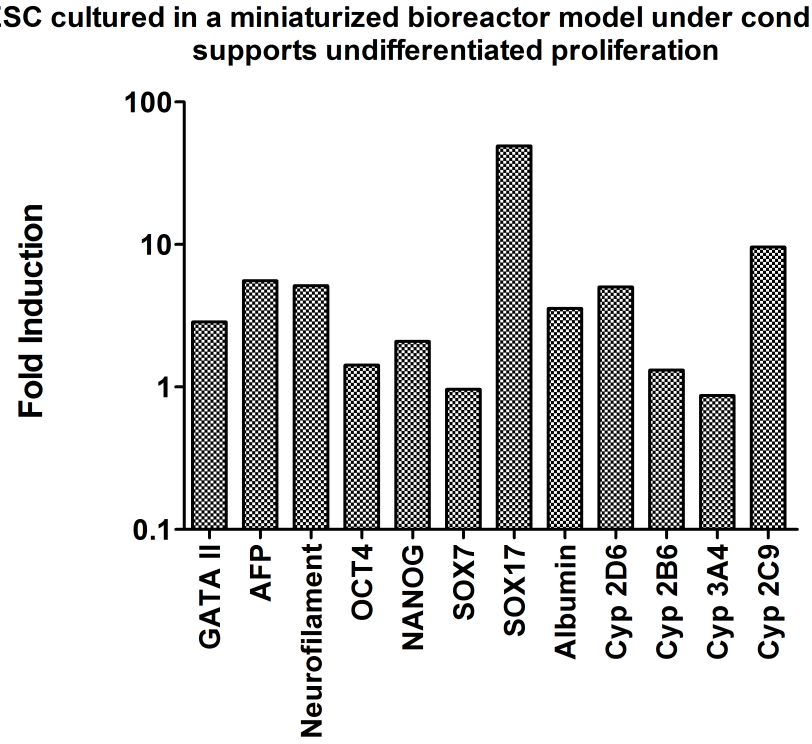


Figure 26: Fold induction of genes characteristic for undifferentiated cells (Oct-4, Nanog) and for cells differentiated into endodermal (AFP), mesodermal (GATA II), ectodermal (Neurofilament) and hepatic (SOX17, Albumin, Cyp2D6, Cyp2B6, Cyp3A4, Cyp2C9) cells hESC after 48 hour bioreactor culture in mTesr1™ medium. The expression of genes was normalized to undifferentiated hESC before inoculation.

3.2.3 Hepatic differentiation in 3D bioreactor systems

3.2.3.1 Hepatic differentiation of feeder independent hESC in 3D bioreactor systems or conventional 2D culture according to approach 1

In this approach 2.5E7 feeder independent hESC (hESC cell line H1) were inoculated into a miniaturized bioreactor (0.4 ml cell compartment volume). The cells were differentiated according to the protocol published by Hay et al. (2007) [22]

adapted for 3D bioreactor cultures. After inoculation into the bioreactors, a 48 hour period in mTeSR1™ medium for adaptation and recovery of the cells from enzymatic passaging was applied. In parallel conventional 2D cultures were performed. For the hepatic differentiation in conventional 2D cultures hESC cultures with about 70% to 80% confluence (about 2*10E6 cells per well of a 6-well plate) were differentiated according to approach 1.

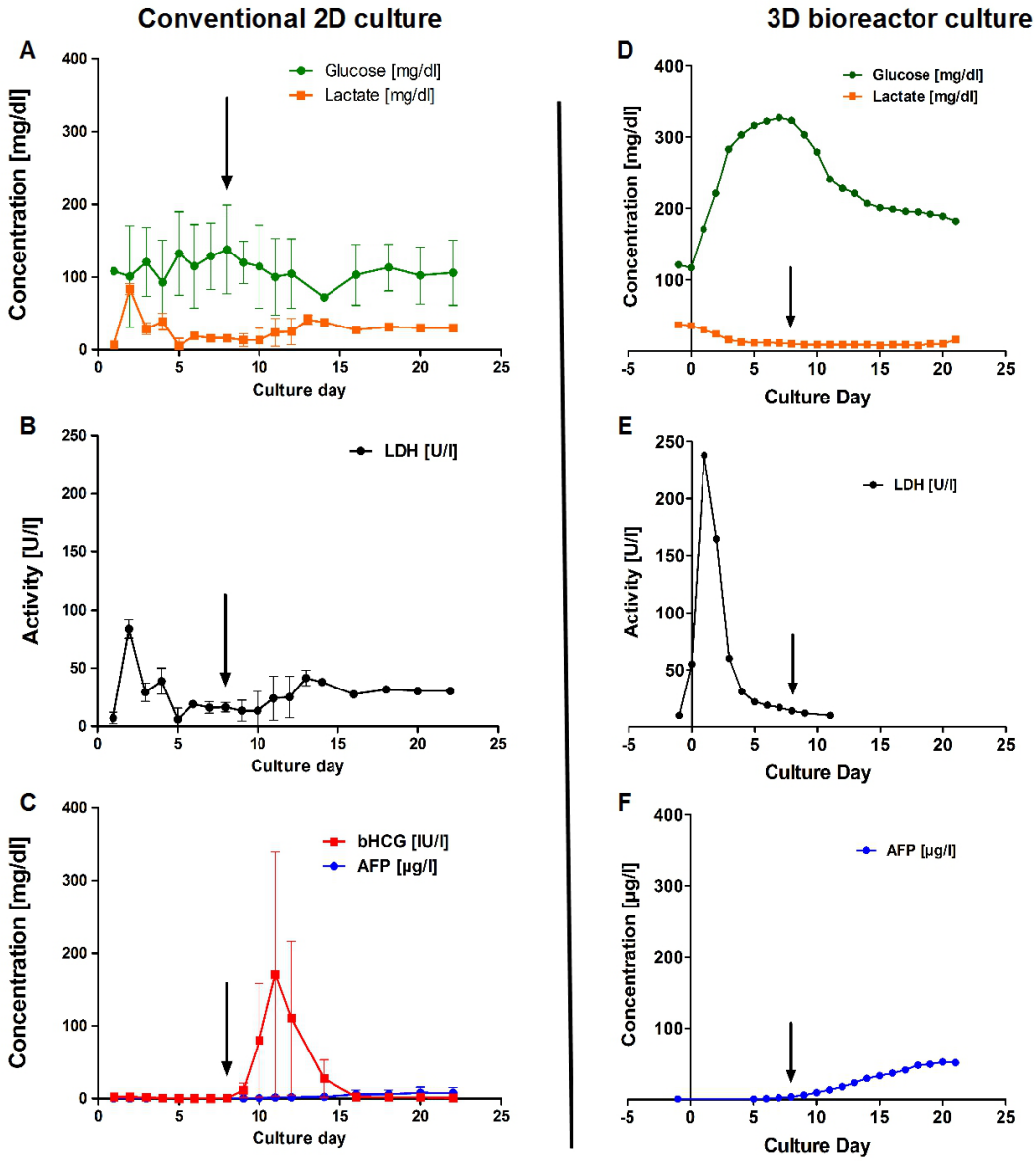


Figure 27: Time course of glucose and lactate (A), LDH activity (B), AFP and bHCG (C) concentrations in a bioreactor culture (right hand side, n=1) or conventional 2D culture (left hand side, n=4) differentiated according to approach 1; arrows indicate the end of endodermal commitment of the cells.

The glucose metabolism in 2D cultures was robust and higher than the activity seen in the 3D bioreactor culture (Figure 27). LDH activities indicating cell stress and apoptosis were found to be higher in bioreactor cultures compared to 2D cultures. This is likely to be more a result of bioreactor inoculation induced cell stress than LDH release due to apoptosis during DE commitment. HCG was only detectable in 2D cultures and not in 3D cultures. It was found to have the highest concentration around day 11. AFP was detected at only low levels in 2D cultures compared to bioreactor culture. A constant increase in AFP concentration was seen from day 5 on.

At bioreactor shutdown cell were harvested and RNA was isolated. After reverse transcription the expression was normalized to undifferentiated hESC after 48 hours bioreactor culture. At the end of hepatic differentiation in conventional 2D cultures RNA was isolated and processed in a similar fashion. The expression of hepatic differentiated cells under 2D culture was normalized to undifferentiated hESC at d0.

Results of gene expression analysis are shown in figure 28. A robust induction of AFP and Albumin expression in the 3D bioreactor culture higher than the induction found in 2D cultures was detected. An induction of the mesodermal marker GATA II could be found in 2D cultures and at a minor level in the 3D bioreactor culture as well. The expression of markers indicating the pluripotent state of hESC (Oct-4, Nanog) was down regulated in both 2D and 3D cultures, but could still be found at relevant levels in 3D bioreactor cultures.

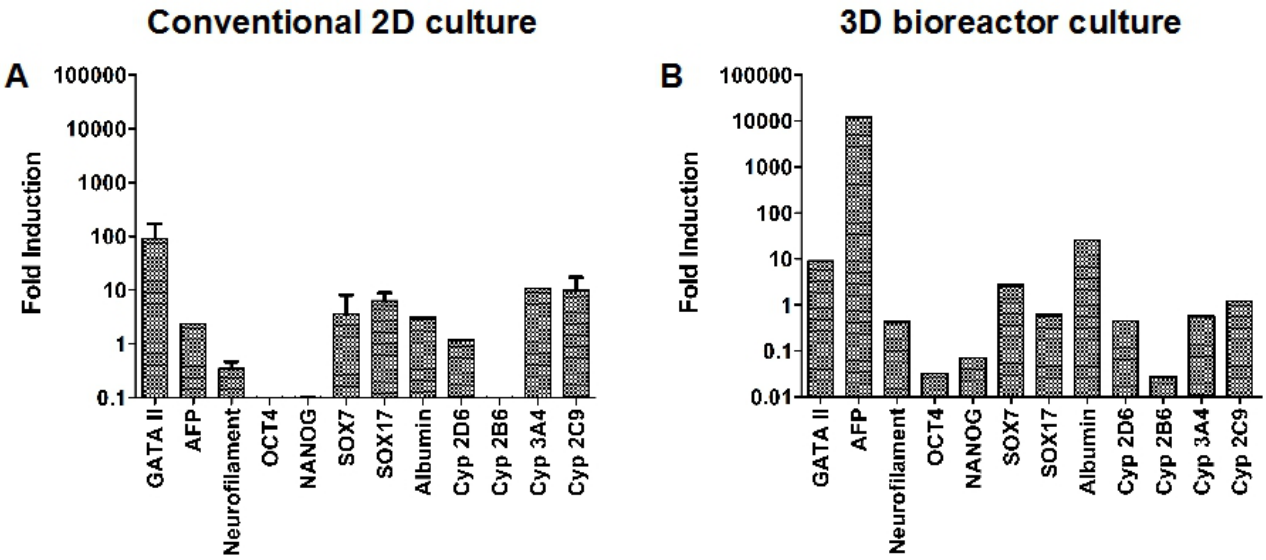


Figure 28: (A) Fold induction of genes characteristic for undifferentiated cells (Oct-4, Nanog) and for cells differentiated into endodermal (AFP), mesodermal (GATA II), ectodermal (Neurofilament) and hepatic (SOX17, Albumin, Cyp2D6, Cyp2B6, Cyp3A4, Cyp2C9) cells in hESC after 21 days differentiation according to approach 1 in conventional 2D culture (A) or in 3D bioreactor (B). The expression was normalized to undifferentiated hESC (A) respectively undifferentiated hESC after 48 hours of 3D bioreactor culture (B) (n=2).

3.2.3.2 Hepatic differentiation of feeder independent hESC in 3D bioreactor systems and conventional 2D cultures according to approach 2

In this approach $2.5x10^7$ feeder independent hESC (cell line H1) were inoculated into miniaturized bioreactors (n=2) (0.4 ml cell compartment volume). The cells were differentiated according to the protocol published by Hay et al. (2008) [68] adapted for bioreactor cultures. The cells were prepared and seeded as described for approach one.

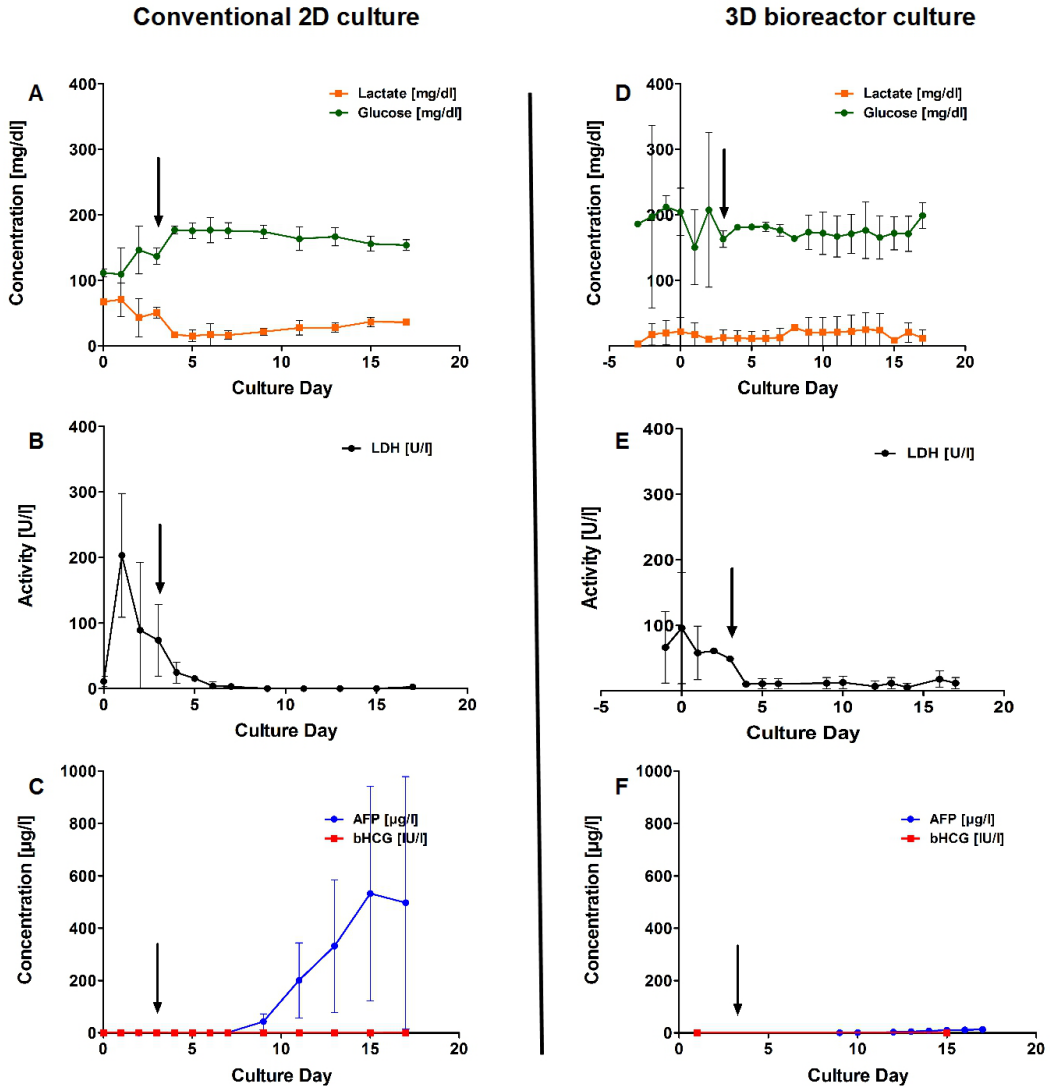


Figure 29: Time course of glucose & lactate (A), LDH activity (B), AFP & bHCG (C) concentration of a bioreactor culture (right hand side) (n=2) or conventional 2D culture (left hand side) (n=4) differentiated according to approach 2**;** the arrows indicate the end of definitive endodermal commitment of the cells.

The cells inoculated in 3D bioreactor systems had a robust metabolic activity (figure 29 D). The LDH release after cell inoculation was moderate, and the LDH release under definitive endodermal differentiation was surprisingly low (figure 29 E). Factors marking differentiation (AFP, bHCG) of hESC were barely detectable (figure 29 F).

The metabolic activity of cells during hepatic differentiation in conventional 2D culture was stable (figure 29 A), but a strong LDH release was measured during the first stage of differentiation (figure 29 B). A high concentration of AFP was measured,

while bHCG concentrations were below the detection limit (figure 29 C).

At the end of 2D or 3D culture experiments cells were harvested and RNA was isolated. Analysis of marker gene expression was performed as described for approach 1.

A strong induction of AFP was found in both culture systems (figure 30 A and B), the expression of the ectodermal marker neurofilament was significantly stronger induced in 3D bioreactors. SOX17 and Albumin were stronger induced in conventional 2D cultures compared to 3D bioreactor systems.

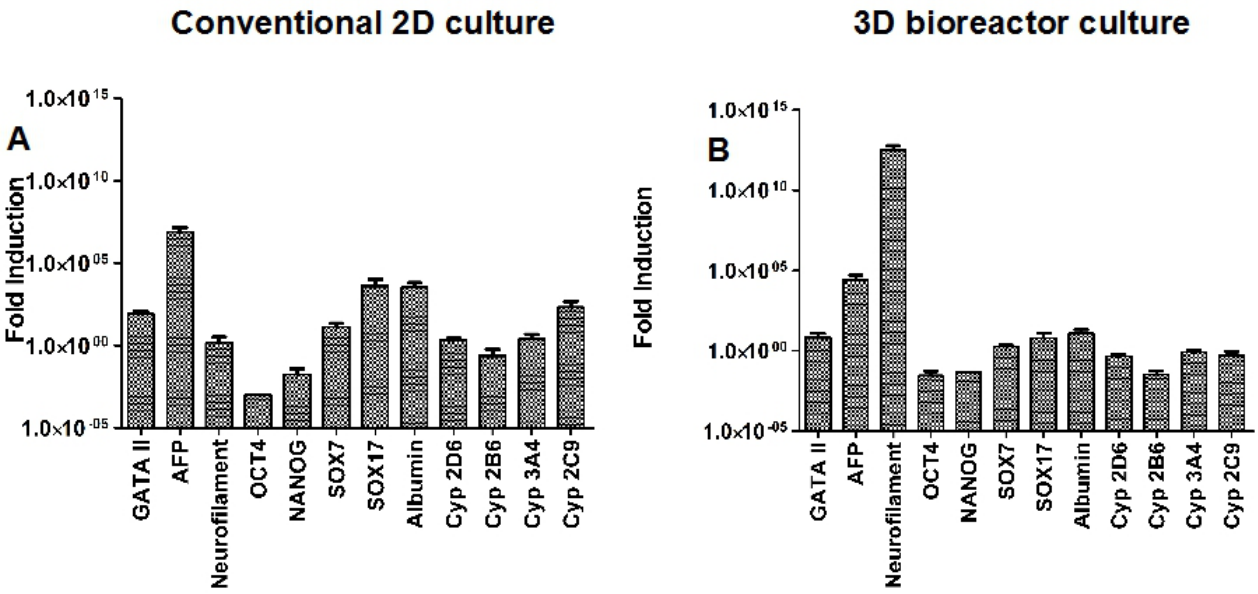


Figure 30: Fold induction of genes characteristic for undifferentiated cells (Oct-4, Nanog) and for cells differentiated into endodermal (AFP), mesodermal (GATA II), ectodermal (Neurofilament) and hepatic (SOX17, Albumin, Cyp2D6, Cyp2B6, Cyp3A4, Cyp2C9) cells in hESCs harvested at bioreactor shutdown after differentiation according to approach 2 (n=2) or after differentiation in conventional 2D culture (n=4).

3.2.3.3 Hepatic differentiation of partial feeder dependent hESC in 3D bioreactor systems according to Cellartis approach 1

In order to investigate the hypothesis that cell injury occurring during the DE commitment might influence the differentiation and maturation of cells in 3D bioreactors, feeder independent hESC (cell line: SA121) were initially cultured and differentiated in conventional 2D cultures (1E6 per bioreactor). After accomplishment of the definitive endodermal commitment (d8) the cells were enzymatically detached and inoculated into miniaturized bioreactors (0.2 ml cell compartment volume). The cells were further proliferated, differentiated and maturated for 25 days. The time course of glucose consumption, lactate production, LDH, AFP and bHCG release is shown in figure 31.

The metabolic activity of the cells strongly decreased after the cells were inoculated into the bioreactors (on day 8). A high LDH release was seen during definitive endodermal commitment but not after bioreactor inoculation. Beta-HCG was measured at low levels until about day 19, AFP was measured at low levels from day 15 on.

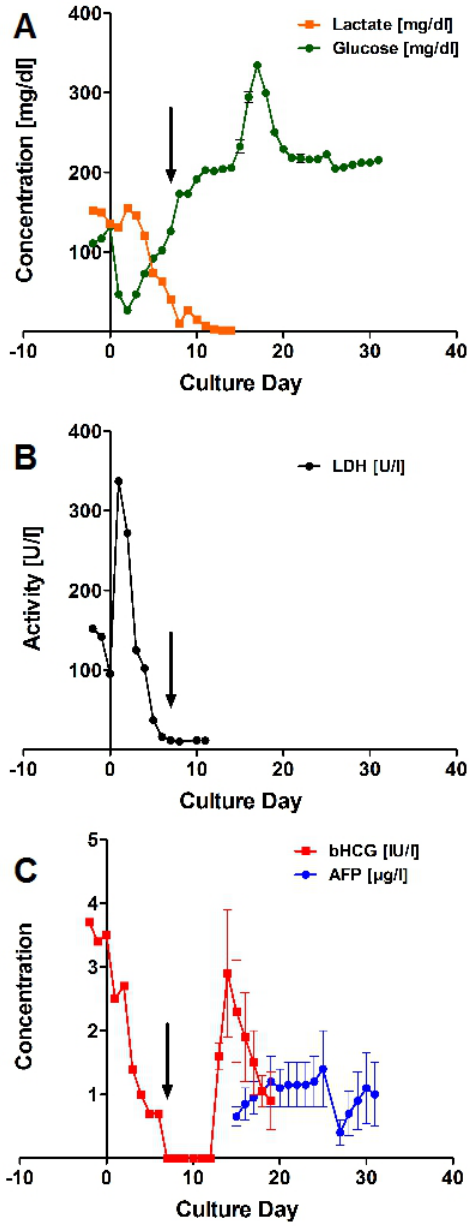


Figure 31: Time course of glucose & lactate (A), LDH activity (B), AFP & bHCG (C) concentration taken from 3D bioreactor cultures differentiated according to Cellartis approach 1. Arrows indicate the end of DE commitment and the transfer of cells into the 3D bioreactor systems (n=2).

3.2.3.4 Hepatic differentiation of partial feeder dependent hESC in 3D bioreactor systems according to Cellartis approach 2

In this approach feeder independent hESC (cell line: SA121) were inoculated into an Analytical scale bioreactor. In total 5E7 viable hESC were differentiated for 30 days after a 3 days adaptation period. The time course of glucose consumption, lactate production, LDH, AFP and bHCG release is shown in figure 32.

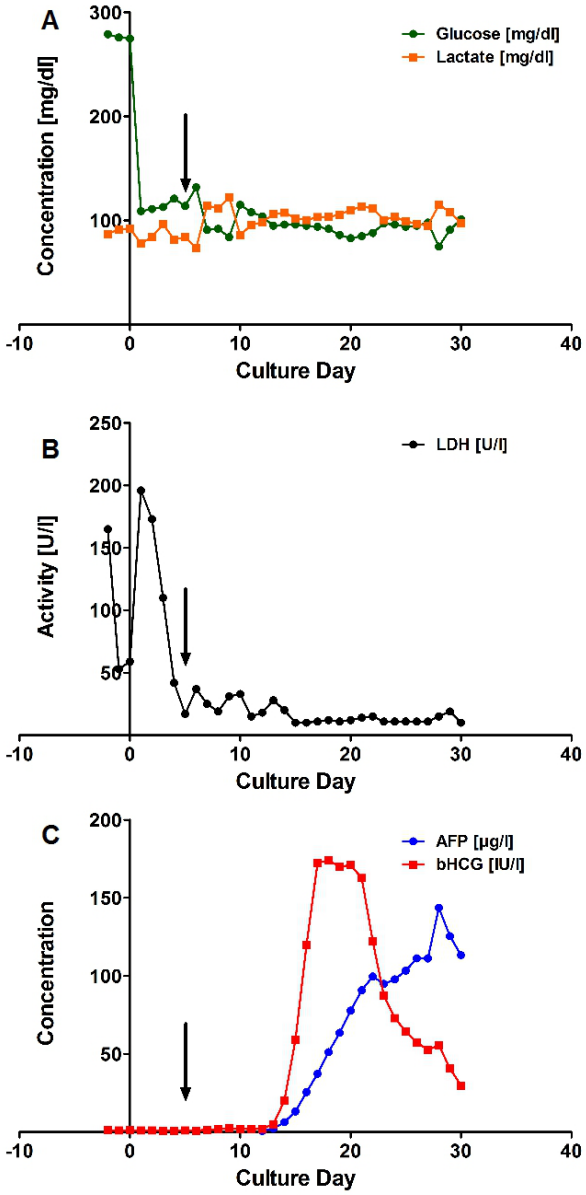


Figure 32: Time course of glucose & lactate (A), LDH activity (B), AFP & bHCG (c) concentration of a bioreactor culture differentiated according to approach Cellartis 2.

The metabolic activity of the inoculated cells was stable over the duration of the experiment. Two peaks in the time course of LDH concentration were obvious. The first increase occurred subsequent to cell inoculation and the second during endodermal commitment. After endodermal commitment the LDH activity levels decreased and stabilized.

The concentration of beta-hCG increased from day 13 to day 16, stayed at this high concentration until day 21, and afterwards declined. The AFP concentration increased from day 13 on and reached its maximum around day 28.

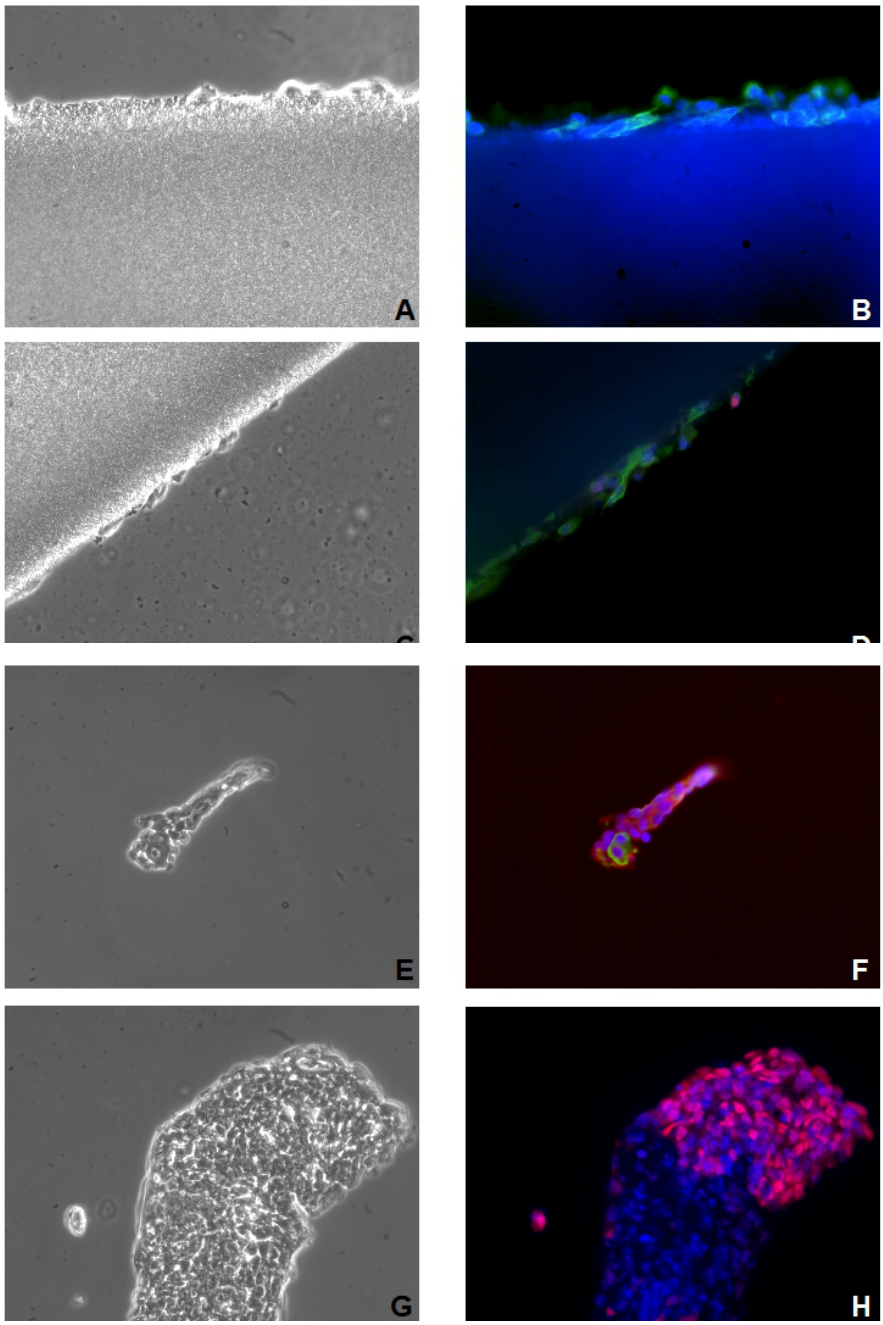


Figure 33: Immune histochemistry of cells harvested after bioreactor culture following differentiation according to approach Cellartis 2 (B) CK19 (green) & DAPI (blue); (D) CK18 (green), Ki67 (red) and DAPI (blue); (F) CK19 (green), AFP (red), DAPI (blue); (H) Oct-4 (red), DAPI (blue); (A), (C), (E), (G) phase contrast microscopy picture; 400x total magnification in all pictures.

The samples taken after bioreactor shutdown were stained for various hepatic and non-hepatic markers (figure 33). Interestingly the cells showed a CK18 / CK19 positive phenotype. A relatively high number of these stained positive for Ki67, indicating mitotic activity. The majority of the CK18/CK19 positive cells also stained positive for AFP. The co-expression of vimentin was seen as well in these cells.

Interestingly cells expressing the pluripotency marker Oct-4 could also be identified. They were mostly found in larger aggregates.

3.2.3.5 Hepatic differentiation of partial feeder dependent hESC in 3D bioreactor systems according to Cellartis approach 3

In this approach feeder dependent hESC (cell line SA002) were inoculated in lab scale bioreactors after SCED culture propagation. The cells were differentiated for 46 days after a three-day recovery period after cell inoculation. In total two bioreactor runs were conducted following that protocol. In the first run 8.5E7 viable hESC were inoculated. The second bioreactor was inoculated with the same amount of hESC and additionally with 5E7 MEF. The time course of glucose consumption, lactate production, LDH activity, AFP and bHCG release is shown in figure 34.

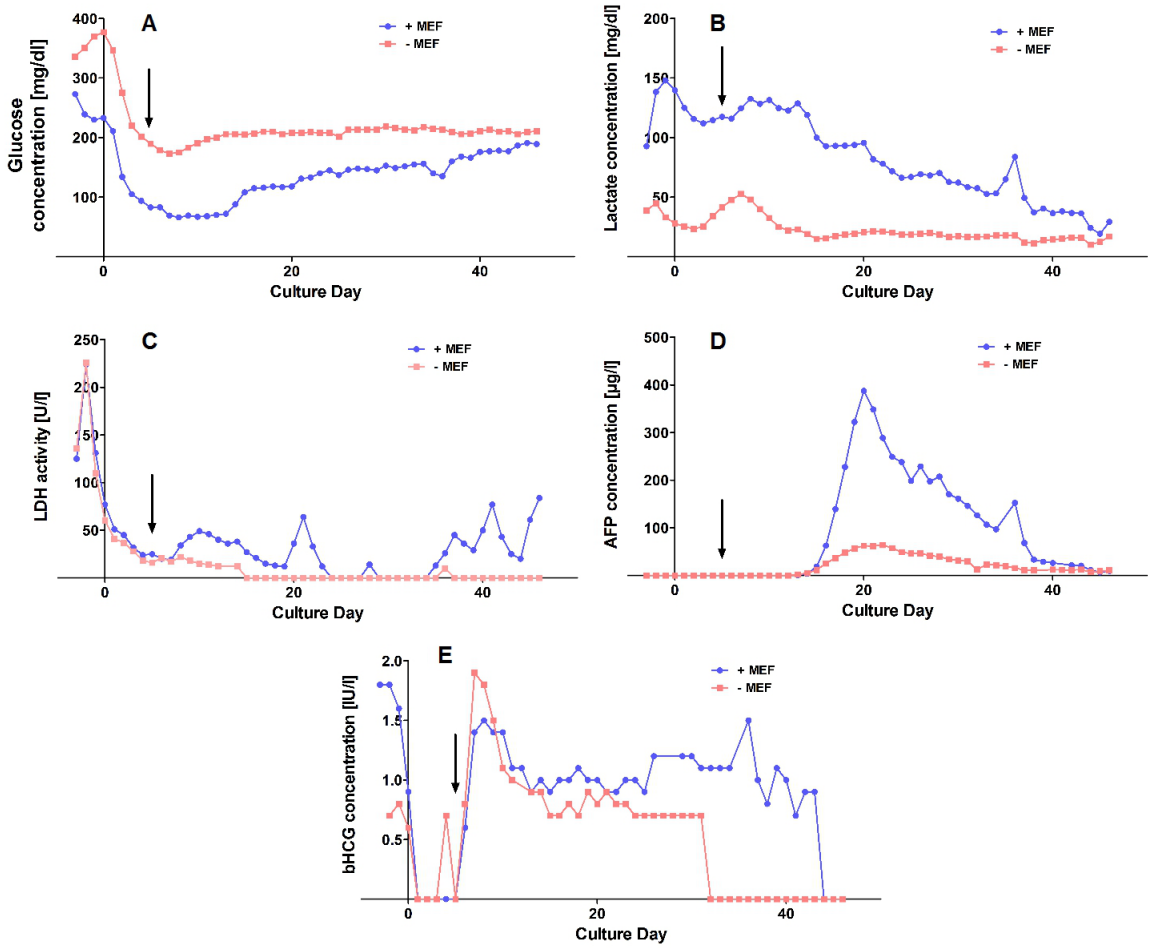


Figure 34: Time course of glucose (A), lactate (B), LDH activity (C) , AFP (D) and bHCG (E) concentrations of two bioreactor cultures differentiated according to approach Cellartis 3 (n=2). The arrows indicate the end of the endodermal commitment of the cells.

The metabolic activity of the two bioreactors was stable over the duration of the experiment, with some differences between the bioreactors (Figure 34). The consumption of glucose of cells inoculated with MEF was higher compared to the activity seen in the bioreactor without additional feeders.

In both bioreactors a high concentration of LDH can be seen right after cell inoculation. This is probably a result of cell stress during inoculation/adaptation to 3D conditions.

The curves for AFP and bHCG look similar in both bioreactor runs, although concentrations are about 7 times higher in the bioreactor culture with additional MEF.

After bioreactor shutdown samples for immune histochemistry were taken. The

sample collection for RNA and immune histochemistry analysis in the bioreactor without additional feeder cells failed probably due to the low number of viable cells after accomplishment of the differentiation.

In the microscopic evaluation of the samples two different cell types imposed. The first cells type stained positive for CK18 without (in the majority of cells) a positive coexpression of AFP. HNF4a and Albumin were expressed as well in those cells. The second cell type was not found to express this set of markers, but vimentin and in some cases Ki67 (Figure 35).

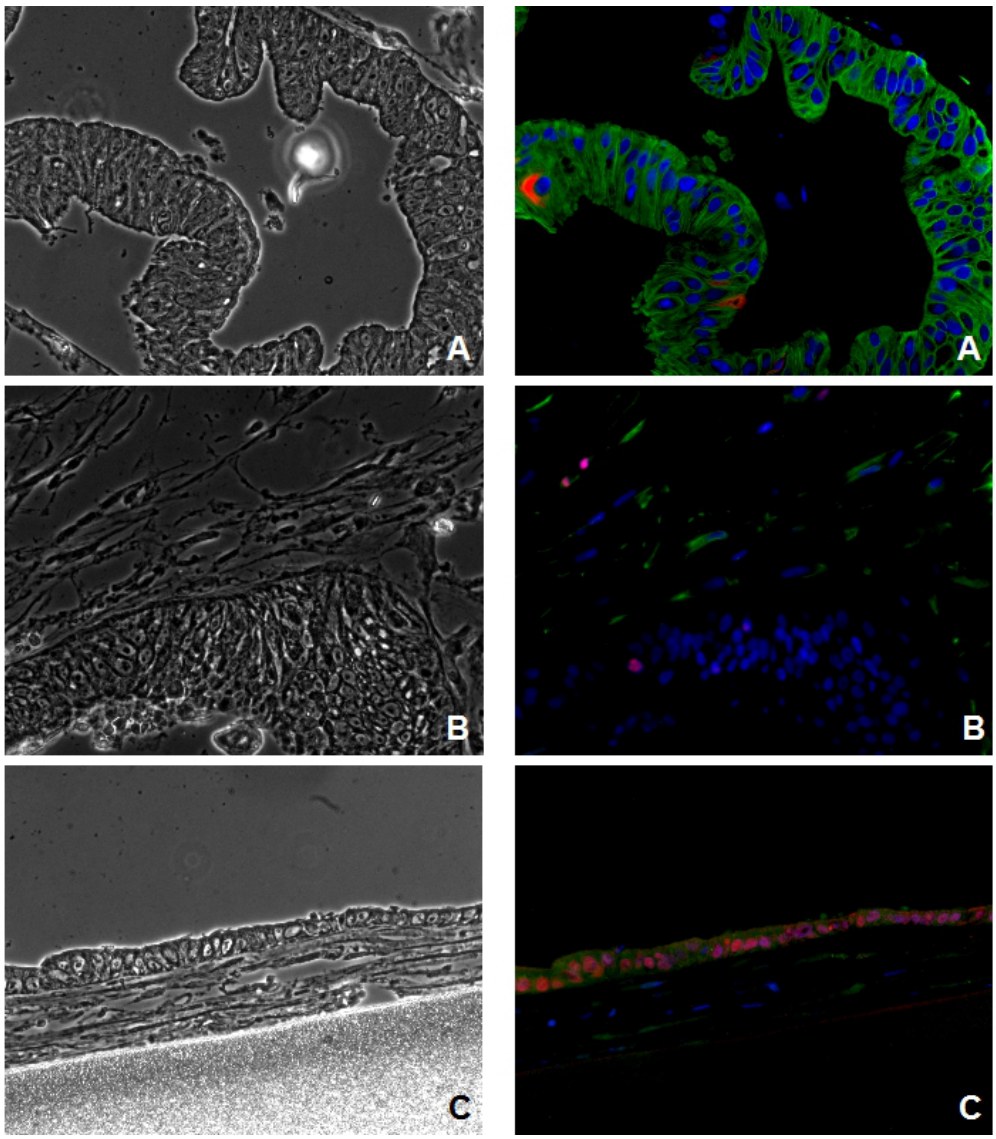


Figure 35: Immune histochemistry of cells harvested after bioreactor culture following differentiation according to approach Cellartis 3, CK18 (green) and AFP (red) (A); Vimentin (green) and Ki67 (red) (B); HNF4a (red) and Albumin (green) (C); 400x total magnification.

4. Discussion

In this study two different strategies for hepatic differentiation of hESC were followed in parallel: The first strategy focused on differentiation of hESC in a 3D bioreactor environment using a minimum of xenobiotic material. First the hESC were adapted to feeder independent conditions. After evaluation of this culture method in terms of maintaining the undifferentiated state of the cells, the hESC were differentiated according to various protocols. Two of these protocols were successfully established and employed in a 3D differentiation approach.

The second differentiation strategy performed in cooperation with Cellartis laid a stronger focus on experiments in 3D bioreactor systems. Initial point was a protocol for the hepatic differentiation in conventional 2D culture (Brolén et al., 2010) [67]. This method was modified and adapted to 3D bioreactor culture conditions.

4.1 The establishment and improvement of culture conditions of hESC

The first experimental data of this study were collected using the feeder dependent hESC line SA002. The standard maintenance culture utilized mouse embryonic fibroblasts (CF-1) and included the manual dissection of colonies every 5 to 7 days into small pieces. The fragments were then transferred into prepared culture dishes. Besides the fact that this method is very labor intensive and requires highly trained personal, the dependence on feeder cells of animal origin holds a risk for pathogenic agent transfer over species barriers, and a high feeder batch variation [74]. However, this method also holds a number of advantages compared to other protocols including the enzymatic dissociation of cultures during passage. It has been shown that the enzymatic treatment might induce genomic alterations [75] and it is possible to pre-select colonies that show hallmarks of differentiation during manual passage. Thus, manual passaging provides the chance of gaining a culture richer in undifferentiated cells after passage.
In order to prepare larger cell numbers for 3D bioreactor experiments the cells were

cultured in single cell enzymatic dissociation (SCED) culture conditions [76]. Here the concentration of bFGF was increased and inactivated human foreskin fibroblasts (HFF) served as feeder cells instead of MEF. Interestingly the grow pattern changed completely under these conditions. The hESC showed an integrative grow pattern. Microscopic evaluation of the cell morphology was practically impossible in contrast to standard culture conditions in co-culture with MEF.
In order to further standardize and simplify the long-term maintenance of hESC, culture conditions for feeder independent hESC were established. The cultures only sparely differentiated under conditions promoting the undifferentiated proliferation. The expression of markers indicating the pluripotent state of hESC, such as Oct-4 and Nanog [77] were routinely evaluated via PCR, Real-Time PCR and immune histochemistry. The hESC were adapted to feeder independent culture in mTeSR1™ medium. It is known that this medium contains human TGF beta and bFGF. Several reports have shown the necessity of TGF beta superfamily member Activin / Nodal, FGF and Wnt signaling to sustain the undifferentiated state and pluripotency [78, 79] of hESC by the activation of a core network that involves the transcription factors Oct-4, SOX2 and Nanog [80].

Oct-4 is a POU-family homeobox transcription factor that is exclusively expressed in pluripotent cells of the inner cell mass of a blastocyst [81] and early germ cells. It has been shown that the expression of Oct-4 is essential for the pluripotency of the cells from the inner cell mass of a blastocyst and in Oct-4 deficient embryos the ICM cells are restricted to the extraembryonal trophoblast lineage [82]. Interestingly alterations in Oct-4 expression levels result either in endodermal differentiation (in case of up regulation of expression) or mesodermal and endodermal differentiation (in case of down-regulation of expression) [83].

The SOX (SYR-related HMG box) family member SOX2 is not exclusively expressed in embryonic stem cells, but also in several differentiated cells [84]. It has been reported that the over-expression of SOX2 leads to trophectoderm differentiation whereas the reduction of SOX2 expression leads to trophectodermal and partial endodermal differentiation [85]. The necessity of SOX2 in the maintenance of the undifferentiated state of hESC has been questioned since in some cell lines it has been shown to be absent [86] and its absence is possibly compensated by other SOX

family members.

The homeobox transcription factor Nanog is in humans exclusively expressed in the cells of the ICM, early germ cells and hESC [87]. The down-regulation of Nanog has been shown to induce the differentiation of human embryonic stem cells to extraembryonal lineages [88]. The over-expression of Nanog in hESC has been demonstrated to contribute to the maintenance of the undifferentiated state in the absence of feeders or conditioned media for a period of time [89]. Interestingly the expression of Nanog has also been found in several neoplasia [90, 91] and was reported to improve proliferation and life span together with Oct-4 in human mesenchymal stem cells over-expressing these two transcription factors[92].

It has been reported that Nanog, Oct-4 and SOX2 play an essential role in the regulation of a global transcriptional network responsible for the maintenance of the undifferentiated state of stem cells [93]. Interestingly Oct-4 and SOX2 seem to regulate the expression of genes like Nanog and Utf1 and others, but also their own expression [94]. It was also reported that the expression of Nanog seems to be independent from that of Oct-4 and SOX2. In order to determine the undifferentiated state of cultured hESC the expression of Oct-4 and Nanog was determined routinely.

In order to determine the *in vitro* pluripotent potential hESC were cultured under conditions that support the spontaneous differentiation [95] for 17 days in conventional 2D culture. The data suggest a stable metabolic activity, the LDH activity was constantly high, suggesting apoptotic and / or necrotic processes during culture. During the last third of the experimental duration considerable amounts of beta-hCG were measured in the supernatant. This hormone is physiologically expressed during pregnancy by the syncytiotrophoblast and has recently been found to act pro-mitotically and support neuroectodermal development [96]. RNA was isolated after 5, 9 and 17 days and analyzed via real time PCR. Already after 5 days the expression of markers indicating the pluripotent state of hESC dropped dramatically. Surprisingly no further reduction was seen after 9 or 17 days, indicating a possible remain of pluripotent cells. The expression of the endodermal marker AFP increased over time and the mesodermal marker GATA II was expressed strongly and constantly in all three time points measured. A weak expression of neurofilament was found at day 5

and day 9, but it was increased at day 17 indicating a progressive commitment to the ectodermal lineage. Comparative kinetics for the expression of lineage specific genes have been described recently [97]. In summary the capability of cultured hESC to differentiate in conventional 2D culture into all germ layers was demonstrated.

In order to investigate the effects of 3D bioreactor culture on the potential of hESC to spontaneously differentiate and to form cells of the three germ layers, hESC were cultured for 17 days under conditions that support embryoid body formation. The metabolic activity was at a low level and stable, LDH concentrations in daily samples increased until day 4, and dropped afterwards constantly. The concentration of AFP increased from day 10 on, indicating a strong commitment to the endodermal lineage. At bioreactor shutdown RNA was isolated, the subsequent real-time analysis revealed a decreased expression of the pluripotency markers Oct-4 and Nanog to levels comparable to levels seen after 17 days of spontaneous differentiation in 2D. Surprisingly the expression of markers for the mesodermal (GATA II) and ectodermal (neurofilament) lineage were only slightly induced under the given conditions, whereas the expression of AFP was strongly induced. This can be interpreted as a result of the given culture conditions that either support the proliferation or survival of cells that underwent endodermal lineage commitment or aggrieves cells on non-endodermal lineages. Although the induction of markers for the three germ layers was seen during this experiment and in previous studies [58] the proportions and kinetics seem to vary depending on the culture system chosen. In summary the capability of the 3D bioreactor system to support spontaneous differentiation into the three germ layers was demonstrated.

In addition to the *in vitro* experiments performed for the demonstration of the pluripotent state of hESC adapted to feeder free conditions, *in vivo* experiments were conducted as well. In total 97 mice were treated with different amounts of undifferentiated cells (table 37). In three of these animals the formation of a solid tumor was seen after 120 days. In two of the three animals dissected a teratoma composed of derivatives of all three germ layers was identified by HE staining and histopathological interpretation. Only in one animal no ectodermal structures could be identified properly. In general the tumorigenic potential of feeder free cultured

hESC seems to be rather low. Further investigation is needed to understand the reduced tumorigenic potential of the hESC under feeder free culture conditions.

4.2 Hepatic differentiation in conventional 2D culture

In order to establish a protocol for the hepatic differentiation in 2D culture five different approaches were tested. In four of the five approaches Activin A was used for the definitive endodermal commitment (Table 32). After the definitive endodermal commitment of the cells, the cell number was determined and RNA was isolated. During the DE differentiation a combination of one or more of the signal molecules Activin a, Wnt3a, BMP4, bFGF, Ly294002, sodium butyrate and DMSO were used.

The Nodal/Activin A pathway does not only play a central role in the maintenance of the undifferentiated state in hESC, it also induces the formation of mesendoderm and subsequently definitive endoderm and mesoderm in a concentration dependent manner [21]. Interestingly the extent of DE differentiation has been reported to be modulated by FGF and Wnt pathway signaling, but in the absence of Activin/Nodal signaling they can neither maintain hESC pluripotency nor commit cells to the mesendodermal lineage in chemically defined medium [98, 79]. In conclusion, Activin A seems to act upstream of the FGF and Wnt pathway, and it has been shown that the inhibition of Activin/Nodal signaling results in the promotion of neuroectodermal differentiation [99].

The bone morphogenic protein member BMP4 is known to play a crucial role in the development of various tissues like lung, bone and tooth [100 - 102]. The stimulation with BMP4 together with FGF2 has been shown to induce mesendodermal differentiation [103]. Further it has been reported that the combination of BMP4 and Activin A generates endodermal cells expressing FOXA2 and SOX17 [104]. In addition it has been reported that the combined successive stimulation of hESC with Activin A, BMP4, FGF2 and VEGF (vascular endothelial growth factor) promotes the generation of a multipotent mesoderm progenitor population that can give rise to various mesodermal cells [105]. In conclusion BMP4 signaling seems to modulate

mesendodermal and mesodermal specification.

The transcription of Wnt3a has been reported to experience activation during the induction of primitive streak PS and mesendoderm formation by Activin A [78]. In consistence with these findings, Wnt signaling has been shown to induce brachyury (BRU)-positive PS / posterior mesodermal formation in serum free conditions, and when combined with an antagonist of BMP a differentiation into PS / definitive endodermal and anterior mesodermal cells can be induced [106]. These findings led to the development of protocols using Activin A and Wnt3a for efficient differentiation of hESC to definitive endodermal cells. Despite minor differences between these protocols, in each of these approaches a short-term (24 – 48 hours) stimulation with Activin A and Wnt3a is followed by a 48 hour long stimulation with Activin A in presence of low serum concentrations.
The fibroblast growth factor pathway has been shown to play a crucial role in embryonic development and the maintenance of the pluripotent state in hESC [107]. Interestingly, when the downstream effector of FGF signaling MAPK is blocked, hESC lose pluripotency markers and differentiate towards the primitive endoderm and trophoectoderm lineage [108]. FGF has been shown to act in a synergistic way in combination with Activin A in the commitment of hESC to the definitive endodermal lineage [98]. In addition it has recently been shown that sodium butyrate can influence the cell fate of definitive endodermal cells in a dose dependent manner [109].

Sodium butyrate is a small molecule that has various effects on cell cultures including the inhibition of proliferation, induction of differentiation, induction or repression of gene expression and histone hyperacetylation by the inhibition of histone deacetylase [110]. It has been reported to support the definitive endodermal commitment in human ESC [111] as well as the hepatic differentiation of DE committed cells in mice [112].
Another small molecule used for the hepatic differentiation is dimethylsulfoxide (DMSO). It was reported to have a dose dependent effect on the expression of a large number of genes [113]. In the past DMSO has been widely used for the cryopreservation of cells, but has recently been found to also affect the undifferentiated state of hESC [114]. In protocols for hepatic differentiation of hESC it

has been used for the endodermal commitment [22] and in later stages of differentiation [115].
The phosphoinositide 3-kinase (PI3K) and mTOR pathway inhibitor LY294002 is a pharmaceutical drug that is currently under clinical evaluation in the treatment for various cancer types [116]. Further on this drug was reported to inhibit insulin signaling during definitive endodermal commitment of human IPSC [117] and hESC [118].

After the endodermal commitment of the hESC, cells were found to be viable in differentiation approaches one, two and three. The cells of the fourth and fifth approach perished shortly after the beginning of the differentiation. The determination of cell numbers after endodermal differentiation showed that the highest cell number was achieved in approach one, followed by approach three and two. In general, the DE commitment of the cells is a process that is associated with a large scale decline of cells. This is clearly illustrated by a dramatic reduction in cell number compared to the amount of cells before differentiation and the high LDH activity.
Real time PCR analysis revealed a successful commitment to the endodermal lineage in approach 2 and 3 by the strong expression of SOX17 [119].
Interestingly the expression of the mesodermal marker GATA2 was significantly higher in approach 1 and 2 compared to the third approach. Together with the highest expression of the endodermal marker AFP in the third approach the definitive endodermal commitment seemed to be more robust in the cells differentiated according to that protocol compared to the other two approaches. But despite the superior endodermal lineage commitment in the third approach, the cells vanished after the transfer into a new culture dish. Due to the fact that the LDH activity measured in the supernatant of the third approach was not elevated in the first medium exchange after the passage of the cells it is likely that the cells already declined during passage. Considering the fact that the DE commitment alone has already been demonstrated to induce apoptosis it seems to add an additional challenge to passage cells directly after this sensible step.
In the most promising approaches (one and two) the differentiation was continued to attain fully differentiated hepatic cells.

After definitive endodermal commitment of the hESC the cells were stimulated by a cocktail composed of insulin, hydrocortisone, EGF, HGF, ascorbic acid, transferrin and oncostatin M [68].

The effect of glucocorticoids like hydrocortisone on fetal liver cells has been studied intensively during the last decades. It has been shown that hydrocortisone administration prolongs the expression of AFP and delays the expression of Albumin in fetal mouse liver hepatocytes [120]. In adult liver cell lines it has been demonstrated that the administration of glucocorticoids prolongs the expression of Albumin and transferrin [121] and keeps primary cells ex vivo longer in their differentiated and functional state. It addition it has been demonstrated that dexamethasone induces the expression of HNF4 and C/EBP-alpha [122].

The addition of the TGF-superfamily members HGF and EGF has been demonstrated to induce the expression of AFP, HNF4 as well as genes of the biliary lineage [122]. Further reports showed a positive effect of HGF on the proliferation and differentiation of DE committed hESC towards the hepatic lineage [123]. This is consistent with reports showing the necessity of HFG and EGF signaling for biliary cell formation [124].

The fibroblast growth factor pathway has been found to be activated during embryoid liver development [125]. Interestingly in an *in vitro* culture approach with endodermal differentiated human embryonic stem cells an inhibition of hepatocyte differentiation under increased FGF concentrations was shown [126].

The Interleukin 6 related cytokine Oncostatin M (OSM) has been investigated intensively and has been reported to induce the up-regulation of hepatocyte specific functions, including lipid synthesis, glycogen synthesis, detoxification and ammonia clearance [127] as well as morphological changes and enhanced inter-cell adhesion [128]. Interestingly, the lack of OSM signaling in high-density cultures does not lead to abolished liver differentiation, indicating an unknown and OSM independent differentiation / maturation pathway. Further on it has been shown that not only a proper cytokine stimulation by Oncostatin M and high cell density seems to be necessary for the final maturation of hepatocytes but also a certain composition of the extra cellular matrix [129].

The number of cells in the cultures was determined after accomplishment of hepatic differentiation. A further reduction in total number was found in both approaches, but

not in an extent seen after DE commitment. The cultures were treated with the same cytokine cocktails as shown in table 32. Interestingly considerable bHCG concentrations were only measured in approach one, whereas AFP was only detected in approach two. It was first measured at detectable concentrations around day six and increased constantly from there on. This finding is consistent with the real time RNA analysis of the cultures. The extent of hepatic differentiation was very limited in approach one indicated by a weak expression of SOX17 and Albumin. In the second approach all these markers were significantly stronger expressed, although the RNA expression of several cytochrome P450 isoforms was low compared to primary hepatocytes (data not shown). This finding is consistent with the fact that the expression of AFP was still quite strong at the end of differentiation, indicating an immature state of the cells [130], since AFP has been reported to be expressed during fetal development in the liver, and in adult livers only during excessive tissue regeneration or in association with malignant growth.
The immune histochemical analysis of the cells revealed a weak cytokeratin18 and cytokeratin19 double positive immune reaction in the first approach. This again indicates a rather immature state of differentiation. A comparable combination of cytokeratin expression was described for human fetal liver cells [131]. It was obvious that the cell population was not homogenous and areas with cells that showed more and less intense immune reaction altered.
The immune histochemical analysis of the cells of the second approach revealed a double positive reaction for cytokeratin18 and cytokeratin19 as well. But in comparison with approach one the cells were larger in size and the reaction was more intense. In addition, the cell population also stained positive for vimentin in some areas. Interestingly this combination of markers has been described previously for adult human liver progenitors isolated from ischemic liver tissue [132].

In order to investigate the stability of the hepatic differentiation performed according to approach two, cell were cultured after accomplishment of differentiation for additional 14 days in medium that either supports further maturation by a stimulation with HGF, EGF, hydrocortisone and oncostatin M (HCM2.2) or in medium that supports spontaneous differentiation (EBMed).
Over the 14 days of additional culture the cells exhibited a stable and comparable

metabolic activity. The LDH activity in the supernatant was constantly low. The AFP release remained at a constantly high level with a significant drop in cultures maintained in EBMed at the end of the experiment. Surprisingly the bHCG levels increased slightly after accomplishment of differentiation in both media and reached their maximum two days for the HCM2.2 media and respectively four days for the EBMed. Thereafter the levels decreased to basal levels.

The real time RNA analysis revealed a relatively stable expression profile after three days in media supporting either the maturation or the spontaneous differentiation. The expression of Albumin and cytochrome P450 2B6 was slightly increased in the culture maintained in HCM2.2 compared to the culture in EBMed after accomplishment of differentiation. The expression of markers indicating an undifferentiated state in hESC or a commitment to a non-hepatic lineage (GATA II or neurofilament) was stable.

A further induction of Albumin and cytochrome P450 2B6 expression was seen in HCM2.2 media after 14 days. The expression of AFP was found to be only slightly decreased. Markers indicating the undifferentiated state of hESC or the commitment to non-hepatic lineages were still stable after 14 days. In conclusion the state of differentiation gained after accomplishment seemed to be stable. Despite the differences in media composition the expression levels of the investigated markers did not alter dramatically. A further maturation of the cells that would be associated with a decreased expression of AFP and an increased expression of Albumin and cytochrome P450 isoforms was not seen despite constant stimulation with various cytokines. It has been shown that not only the precise cytokine stimulation and a certain extracellular matrix but also cell-cell interactions found at sufficient levels only in high density cultures are required for the maintenance of liver specific functions in human hepatocytes [54] as well as in hepatic tumor cell lines [53]. Thus, it appears rational to assume that the degree of maturation can be further increased by the modification of the cell density.

4.3 The culture of hESC in 3D bioreactor systems

The dissociation of hESC has been reported to be highly associated with cell decay [133]. Although progress has been made on this issue by the introduction of ROCK inhibitors, the cells appear still fragile after passage (own observations, data not shown). To increase cell survival in bioreactor differentiation experiments, an additional adaptation period between 24 and 72 hours was applied after cell inoculation before the start of differentiation. To determine the effect of the adaption period on the undifferentiated state of the inoculated hESC a 48h bioreactor run was performed.

The metabolic activity of the inoculated cells was low but stable, and LDH activity was high after 48h indicating a certain degree of cell decay but also viable cells and thus a successful inoculation (Figure 25). The concentrations of AFP and bHCG were below detection limit.

The real time RNA analysis revealed no significant drop in the expression of markers indicating the pluripotency of hESC (Oct-4, Nanog). The expression of markers for lineage commitment was only slightly increased (GATAII, AFP, neurofilament).

4.4 Hepatic differentiation in 3D bioreactor systems

In order to investigate the effect of a 3D culture system on the hepatic differentiation, hESC were inoculated and differentiated according to approach one or two. One of the main goals of this strategy was the hepatic differentiation under conditions that allow potential clinical application. Both approaches were conducted utilizing feeder independent hESC and protocols with chemically defined media.

4.4.1 Feeder independent Approach 1

In the first approach cells were inoculated in miniaturized bioreactors (cell compartment volume 0.4 ml) and a 48h additional adaptation period after inoculation in medium supporting the undifferentiated growth was applied before the start of differentiation.

The cells showed a stable but lower metabolic activity compared to the same approach in conventional 2D cultures. A high LDH activity was measured during the first days of bioreactor culture with its maximum around day one. This was seen in various bioreactor experiments and can be interpreted as a result of inoculation associated cell decay. Interestingly, cells differentiated according to this approach in conventional 2D cultures tended to release bHCG into the medium with maximum concentrations measured around day 10 to 12. HCG is known to be released next to various other signal molecules by the syncytiotrophoblast during pregnancy. It has further been reported that trophoblast (a precursor of the syncytiotrophoblast) differentiation of hESC can be achieved by the addition of exogenous BMP4 or by spontaneous differentiation [134]. Under 3D bioreactor conditions though, the levels of bHCG concentrations remained below detection limit and instead considerable amounts of AFP were measured indicating endodermal commitment of the cells. AFP is known to be not only expressed in the developing liver, but also in cells of the yolk sac that develops from extra-embryonic endoderm. It has been reported that extraembryonal cells can be identified by a high expression of SOX7 and SOX17 [135]. Interestingly, the induction of SOX7 expression appears to be robust in 3D bioreactor cultures and exceeds the level of SOX17 induction. However, taking into account that the induction was normalized to gene expression after 48 hours of 3D bioreactor culture (Figure 26) and a strong induction of SOX17 was found already at that time-point, the induction of SOX17 exceeds SOX7 about 10 times. A strong expression of SOX17 and FOXA2 has been described for cells committed to the definitive endodermal lineage [118]. In addition, an induction of Albumin expression was found, both in cells after 48 hours bioreactor culture and in the cells after hepatic differentiation in a 3D bioreactor system according to approach one. Therefore it appears that the change of the culture system did not only change the general lineage commitment, but also supports the hepatic differentiation under the given

cytokine stimuli.
Notably, although the expression of markers indicating the undifferentiated state of hESC (Oct-4 and Nanog) seems to be dramatically diminished in conventional 2D culture, a basic expression was still present in the sample taken from the 3D bioreactor culture.

4.4.2 Feeder independent Approach 2

In order to investigate the potential of the hepatic differentiation of hESC in 3D bioreactor systems according to approach two, cells were inoculated in miniaturized bioreactors (cell compartment volume 0.4 ml). An additional adaptation period of 48 hours was conducted compared to conventional 2D cultures. Here again, a basic but reduced metabolic activity compared to the 2D culture was seen in the bioreactor cultures. A high LDH activity after cell inoculation with a subsequent LDH release due to the endodermal commitment of the cells was seen in the 3D culture. In both culture systems the LDH activity decreased after DE commitment. HCG was measured at moderate concentration in 2D and 3D cultures. Interestingly from day nine on, constantly increasing concentrations of AFP were measured in 2D cultures, while in 3D culture these were only barely over detection limit. This might be due to the potentially low survival rate of the inoculated cells, and the unfavorable medium volume to cell number rate, but analysis of the expression revealed only a subordinate commitment to the hepatic fate in the 3D bioreactor system. Instead a strong induction of the ectodermal marker was found. Interestingly, several reports have been published that describe the neural differentiation using a chemical inhibitor of TGF-beta superfamily receptors, including the receptor for Activin and BMP [136].
A possible explanation for these results might be based on the fact that after cell inoculation in 3D bioreactor systems larger cell aggregates accumulate, and that in central areas of these only decreased cytokine concentrations occur. A further explanation of the obviously decreased Activin A cytokine response might be found in the hydrophobic / hydrophilic character of the membranes used for medium / gas supply in the bioreactor cell compartment. The possible permanent or temporary

adsorption of signal molecules might have a crucial impact on the lineage commitment of the cells.
The main goal of the following experiments conducted in collaboration with Cellartis was the establishment of a protocol for the hepatic differentiation in 3D bioreactors with a more mature phenotype. The experiments were conducted using feeder-dependent hESC and inactivated MEF.

4.4.3 Partial feeder dependent Approach Cellartis 1

In order to investigate the possibility that cell injury due to cell decay during DE commitment in 3D bioreactors might negatively influence the hepatic differentiation, hESC were differentiated for 7 days and after accomplishment of DE commitment cells were detached and inoculated into miniaturized 3D bioreactors (cell compartment volume 0.2 ml).
The metabolic activity measured during the following 25 days was very low, with LDH concentrations below the detection limit. A high LDH activity was seen during DE commitment, but not after bioreactor inoculation, indicating that cell decay occurred already during detachment, and not after inoculation of the cells into the 3D bioreactor system. Interestingly, despite the very low metabolic activity, detectable concentrations of bHCG and AFP were found, indicating a certain amount of differentiation towards syncytiotrophoblastic and hepatic cell fate. In conclusion the inoculation of cells that have previously been committed to the DE lineage does not result in an improved hepatic differentiation. The cells appeared fragile (as seen before in Approach 3 of the 2D experiments) after DE commitment, and thus cell passaging results in a reduced survival rate.

4.4.4 Partial feeder dependent Approach Cellartis 2

In this complex approach cells were differentiated for a total of 30 days in an analytical scale bioreactor (cell compartment volume 4.0 ml). The metabolic activity was increased compared to the previous bioreactor experiments utilizing smaller bioreactor variants (Figure 32). After a three day adaption period the cells were committed to the DE lineage. This is consistent with the high LDH activity found during the first five days. Interestingly, bHCG and AFP were again detectable at considerable levels during differentiation. Elevated bHCG levels became detectable at around day 13 and maximum concentrations were measured during day 17 and 21. AFP was also detected at elevated levels at around day 14. The concentration constantly increased until the end of the experiment indicating an ongoing hepatic differentiation.

This is consistent with the immune histological stainings that revealed a CK18, CK19 and AFP triple positive reaction of the cells, as it has been described for immature hepatic progenitor cells [132]. In addition, some of these immature cells stained also positive for Ki-67, indicating ongoing proliferation.

Besides cell aggregates that stained positive for markers of the hepatic cell fate, also areas with cells expressing the pluripotency marker Oct-4 imposed. The co-expression of Nanog was not investigated, but is likely [137]. Notably, Oct-4 cells were only found in relatively large aggregates, indicating the formation of niche like structures that might have deprived the cells from exogenous signals promoting the differentiation of the cells. In conclusion cells were identified that had differentiated towards the hepatic cell fate, but still carry characteristics of immature cells. In addition cells were found that express Oct-4 and thus can be considered to be at a very early state of differentiation.

4.4.5 Partial feeder dependent Approach Cellartis 3

In order to enhance the degree of maturation of hepatic differentiated hESC lab scale bioreactors with a cell compartment volume of 17.2 ml were inoculated either with or without additional inactivated MEF and cultured for an extended period of time (46 days) compared to approach Cellartis 2 (30 days).

The metabolic activity of the bioreactor that was additionally inoculated with MEF was significantly higher than that of the bioreactor without feeder cells (Figure 33). Interestingly, although the total number of cells inoculated was higher in the MEF containing culture the LDH activity after cell inoculation and DE commitment was comparable, indicating an improved cell survival not only after inoculation, but also after DE differentiation. Since inactivated MEF exhibit a limited live span the increased LDH activity measured in hESC co-cultured with MEF can be explained by the continuous decay of the MEF. Notably the concentrations of AFP found in samples drawn from MEF containing cultures were significantly higher compared to feeder-free cultures. But since the concentrations of bHCG measured were nearly identical until about day 30, this fact can not only be explained by improved cell survival during early stages of development, but possibly also by improved commitment to the hepatic lineage. Due to technical issues at culture day 30 the sample collection of the feeder-free culture was not successful.

The immune histological stainings (Figure 35) of cell material from the bioreactor run with addition of MEF revealed two distinct cell types. The first one showed a positive result for the CK18 staining. A CK19 positive reaction was not found. The co-expression of CK18 and AFP was only sparely found, indicating remaining immature cells. In addition, the CK18 positive cell type also stained positive for HNF4a (a transcription factor responsible for the expression of various cytochrome P450 isoforms) and Albumin, as markers of mature hepatocytes [138].

4.5 Conclusion

The aim of the first strategy was the reduction of xenobiotic materials in the differentiation of hESC under 3D culture conditions. The culture of hESC was successfully adapted from feeder dependent to feeder independent conditions under maintenance of their pluripotent potential *in vivo* and in *vitro*. A protocol was established for the hepatic differentiation of hESC in conventional 2D culture, and the resulting cell population was characterized via real time PCR and immune histological staining. The expression of hepatocyte specific genes in the first approach was significantly lower than in the second approach when conducted in conventional 2D culture. Although the cells showed hallmarks of hepatocytes, the level of enzyme expression found in hESC-derived hepatocytes was not comparable to primary human hepatocytes (data not shown). Instead a robust expression of a gene indicating immature hepatocytes (AFP) was found. The prolonged culture of differentiated hESC was not sufficient to further maturate the cells.
The transfer of the established protocols from 2D to 3D culture conditions revealed unexpected effects regarding the amount and the direction of the differentiation compared to 2D cultures. Possible reasons for the altered differentiation kinetics may lie in the adsorption of signal molecules to the bioreactor capillaries, the formation of large cell aggregates associated with the formation of a concentration gradient of signal molecules and nutrients, and also the interference of cell debris or enhanced endogenous signaling due to higher cell densities. Further investigations are needed to address these questions. Surprisingly the expression of hepatocyte specific genes was higher in the first approach compared to the second approach when conducted in a 3D bioreactor system.

The main goal of the second strategy was the adaption of a protocol for the differentiation of hESC into hepatic cells published for conventional 2D cultures. The interference of cell injury occurring during DE commitment was investigated and the inoculation of DE committed cells subsequent to endodermal differentiation in 2D cultures was found to be not practicable. It was demonstrated that hESC that underwent definitive endodermal commitment feature a fragile state, and therefore the stress due to cell passaging and inoculation into the bioreactor system can

probably not be compensated. The complete hepatic differentiation in 3D bioreactors was then investigated and revealed an immature cell type after 30 days of culture with remaining potential pluripotent cells. Finally the hepatic differentiation over an extended period of time was investigated and revealed a more mature phenotype identified by the expression of adult hepatic markers. In addition the absence of cells expressing markers of pluripotency was shown. Further a beneficial effect of co-inoculated inactivated MEF on hESC cell survival and commitment to a hepatic lineage was revealed. Despite the expression of the adult liver markers the metabolic capacity of the bioreactor cultures needs to be further evaluated.

In general, the results of the 3D experiments demonstrated the possibility of hepatic differentiation of hESC in this culture system. However, the experiments were only conducted in laboratory-scale bioreactors with a cell compartment volume ranging from 0.2 to 17.2 ml. The therapeutic effect of these bioreactor models in extracorporeal liver support therapy would not be sufficient to have significant impact on a patient with acute or chronic liver failure. The capability of a small scale up has already been demonstrated during the experiments. The cultures demonstrated a stable and comparable performance in larger bioreactor models. A larger version of the bioreactor that has already been used in clinical application [35] would meet the requirement to house sufficient numbers of cells for extracorporeal liver support utilizing differentiated hESC.

4.6 Outlook

With a growing understanding of the molecular pathways involved in the endodermal commitment and the further differentiation and maturation of the hESC a further optimization of protocols for the differentiation in 3D bioreactor systems also in regards to cost efficiency should be addressed. For example, it has recently been published that the combination of small molecules like rapamycin in combination with reduced amounts of cytokines results in an enhanced commitment to the definitive endodermal lineage [118].

The various approaches utilizing the bioreactor system performed during this study

illustrated the fragile nature of hESC that underwent cell passage. The high LDH activities detected after cell inoculation in combination with a lower metabolic activity compared to hESC in conventional 2D culture allows the assumption that a portion of the inoculated cells did not adapt to the 3D culture conditions of a bioreactor and underwent apoptosis. A lot of work has been invested into the identification and characterization of small molecules and their mechanism of action. Thiazovivin, pyrintegrin [139] and the ROCK inhibitor Y-27632 [140] are only three of these small molecules that might improve stem cell survival after bioreactor inoculation.

A further possibility to improve stem cell attachment, survival and differentiation after bioreactor inoculation might be achieved by the incorporation of various surface coatings. A study has recently been published that describes the benefits on survival and proliferation of undifferentiated hESC cultured in 3D culture systems coated with laminin and vitronectin [141]. In addition, it has been shown that the expression of hepatocyte specific cytochrome P450 enzymes is dependent also on the extracellular matrix [142].

5. Abstract

Human embryonic stem cells (hESC) are pluripotent cells capable of unlimited proliferation and of differentiation into cell derivatives of all germ layers. With a growing understanding of mechanisms that control cell differentiation, stem cell based therapies could play an important role in the future treatment of diseases. Further on stem cell derived differentiated cells could also be used in pharmaceutical research for *in vitro* tests of drug metabolism and toxicity.

The goal of this study was to investigate the hepatic differentiation of hESC in 3D bioreactor systems. Two different approaches were pursued.

The first strategy focuses on the reduction of xenobiotic materials. Two protocols were adapted for hepatic differentiation in a 3D bioreactor system.

In 2D cultures, the commitment to a hepatic lineage in protocol two was found to be superior compared to approach one. The differentiation kinetics was altered when the differentiation was conducted in 3D bioreactor systems. The hepatic commitment was now found to be increased in protocol one compared to protocol two.

The second approach was conducted in cooperation with Cellartis based on a protocol for hepatic differentiation of hESC in conventional 2D culture.

The results showed no improvement of differentiation in case of the inoculation of DE committed cells. The differentiation over a 30 day period resulted in an immature phenotype, while in a 46 day bioreactor experiment cells were found expressing adult liver markers.

In conclusion the results of this study suggest that the 3D bioreactor technology could be useful for the hepatic differentiation of hESC. In addition, the study provides evidence for a possible up-scaling by the use of larger bioreactor models, the use in pharmaceutical research and clinical applications.

6. Zusammenfassung

Humane embryonale Stammzellen (hESC) sind pluripotente Zellen, die zur unbegrenzten Teilung und zur Differenzierung in Zellen aller drei Keimblätter in der Lage sind. Mit einem wachsenden Verständnis der an der Zelldifferenzierung beteiligten Mechanismen könnten stammzellbasierte Therapien eine wichtige Rolle in der zukünftigen Behandlung von Erkrankungen spielen. Darüber hinaus könnten aus pluripotenten Stammzellen hergestellte differenzierte Zellen in der pharmazeutischen Forschung für *in vitro* Tests zum Metabolismus und zur Toxizität von Arzneimitteln genutzt werden. Ziel dieser Arbeit war die Untersuchung der leberspezifischen Differenzierung von hESC in 3D Bioreaktorsystemen. Es wurden dafür zwei verschiedene Ansätze verfolgt.

Der erste Ansatz zielte auf die Minimierung des Einsatzes von Materialien tierischen Ursprungs ab. Insgesamt wurden hierbei zwei Protokolle für die hepatische Differenzierung in 3D Bioreaktorsystemen adaptiert.

In 2D-Kultur war der Grad der hepatischen Differenzierung im zweiten Protokoll dem im ersten Protokoll überlegen. Im 3D-Kultursystem änderte sich das Differenzierungsmuster. Der Grad der hepatischen Differenzierung im ersten Protokoll stellte sich nun höher als im zweiten Protokoll dar. Der zweite Ansatz wurde in Kooperation mit Cellartis durchgeführt und basiert auf einem Protokoll für die hepatische Differenzierung in 2D Kultur. Die Ergebnisse des zweiten Ansatzes zeigten keine Verbesserung der Differenzierung bei der Inokulation von bereits endodermal differenzierten Zellen. Die Differenzierung über 30 Tage resultierte hier ebenfalls in einem unreifen Phänotyp. Hingegen konnten in einem Experiment mit einer 46-tägigen Differenzierung Zellen identifiziert werden, die adulte Lebermarker exprimierten.

Die Resultate dieser Arbeit zeigen, dass die verwendete Bioreaktortechnologie für die hepatische Differenzierung geeignet sein könnte. Weiterhin stellen sie den möglichen Nutzen des Systems für pharmazeutische Studien, klinische Anwendungen sowie die Möglichkeit eines „Scale-Up“ durch die Verwendung größerer Bioreaktormodelle dar.

7.1 List of own Publications

Stachelscheid H, Wulf-Goldenberg A, Eckert K, Jensen J, Edsbagge J, Björnquist P, Rivero M, Strehl R, Jozefczuk J, Prigione A, Adjaye J, **Urbaniak T**, Bussmann P, Zeilinger K, Gerlach JC. “Teratoma formation of human embryonic stem cells in three-dimensional perfusion culture bioreactors.” *J Tissue Eng Regen Med*. 2013; 7(9): 729-741

Darnell M, Schreiter T, Zeilinger K, **Urbaniak T**, Söderdahl T, Rossberg I, Dillner B, Berg AL, Gerlach JC, Anderson TB. “Cytochrome P450-dependent metabolism in HepaRG cells cultured in a dynamic three-dimensional bioreactor.” *Drug Metab Dispos.* 2011; 39(7): 1131-1138

Cochrane SW, Zhao Y, Perry SS, **Urbaniak T**, Sun XH. “Id1 has a physiological role in regulating early B lymphopoiesis.” *Cell Mol Immunol.* 2011; 8(1): 41-49

Gerlach JC, Lübberstedt M, Edsbagge J, Ring A, Hout M, Baun M, Rossberg I, Knöspel F, Peters G, Eckert K, Wulf-Goldenberg A, Björquist P, Stachelscheid H, **Urbaniak T**, Schatten G, Miki T, Schmelzer E, Zeilinger K. “Interwoven four-compartment capillary membrane technology for three-dimensional perfusion with decentralized mass exchange to scale up embryonic stem cell culture.” *Cell Tissue Organs* 2010; 192(1): 39-49

Stachelscheid H, **Urbaniak T**, Ring A, Spengler B, Gerlach JC, Zeilinger K. “Isolation and characterization of adult human liver progenitors from ischemic liver tissue derived from therapeutic hepatectomies.” *Tissue Eng Part A*. 2009; 15(7): 1633-1643

Dong J, Mandenius CF, Lübberstedt M, **Urbaniak T**, Nüssler AK, Knobeloch D, Gerlach JC, Zeilinger K. “Evaluation and optimization of hepatocyte culture media

factors by design of experiments (DoE) methodology.” *Cytotechnology* 2008; 57(3): 251-261

7.2 List of Poster Presentations

Urbaniak T, Freyer N, Knöspel F, Zeilinger K. “Sucessful hepatic differentiation of human embryonic stem cells in 3D bioreactor cultures”, 48th Annual Meeting of the European Association for the Study of the Liver (EASL), April 24 – 28, 2013, Amsterdam, The Netherlands

Urbaniak T, Jensen J, Knöspel F, Lübberstedt M, Björquist P, Gerlach JC, Zeilinger K. “Hepatic differentiation of human embryonic stem cells in a 3D bioreactor environment.”, 27th Annual Meeting of the German Association for the Study of the Liver (GASL), January 28 – 29, 2011, Regensburg, Germany

Urbaniak T, Stachelscheid H, Knöspel F, Gerlach J, Zeilinger K. “Enhanced Proliferation of human adult liver progenitor cells under culture conditions supporting human embryonic stem cell propagation.”, 2nd International Congress on Stem Cells and Tissue Formation, July 6 – 9, 2008, Dresden, Germany

Urbaniak T, Stachelscheid H, Gerlach JC, Zeilinger K. “Proliferation of hepatic progenitors isolated from adult human liver in coculture with mouse embryonic fibroblasts.” 2nd Biennial Leopoldina Conference and Final Meeting of the Stem Cell Priority Program 1109 of the German Research Foundation (DFG), September 24 - 27, 2006, Dresden, Germany

Stachelscheid H, **Urbaniak T**, Gerlach JC, Zeilinger K. “Characterization of two distinct proliferating liver epithelial cells isolated from liver tissue after ischemic treatment.” 3rd International Meeting of the Stem Cell Network North Rhine Westphalia, May 15 - 16, 2006, Münster, Germany

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9. Danksagung

An dieser Stelle möchte ich all jenen danken ohne die die Anfertigung dieser Arbeit in dieser Form nicht möglich gewesen wäre.
Ein großes Danke an Frau Dr. Katrin Zeilinger und Herrn Prof. Dr. Jörg Gerlach für die Möglichkeit der Anfertigung dieser Arbeit, für die konstruktive Kritik, die vielfältige Unterstützung und die Freiheit eigene Ideen umzusetzen. Des weiteren möchte ich mich bei allen aktuellen und ehemaligen Mitgliedern der Bioreactor Group (BCRT, Charite) bedanken, ein ganz besonderes Danke an Frau Dr. Ing. Fanny Knöspel, Herrn Marc Lübberstedt und Frau Nora Freyer für die exzellente Zusammenarbeit. Für die Durchführung der Tierexperimente möchte ich ganz besonders Frau Maria Stecklum und Herrn Dr. Klaus Eckert von der EPO (experimental pharmacology and oncology) Berlin Buch danken.
Einen großen Dank auch an Frau PhD Janne Jensen und Herrn PhD Petter Björquist die eine große Unterstützung bei der Planung der ersten Reaktorenexperimente waren. Einen besonderen Dank auch an Herrn Prof. Dr. Roland Lauster, nicht nur für die exzellente Lehre während des Studiums, sondern vor allem auch für die Zeit danach.
Des weiteren möchte ich allen Mitarbeitern von Stem Cell Systems danken. Ohne deren kontinuierliches Bestreben nach Verbesserung in der Fertigung wäre diese Arbeit nicht möglich gewesen. Am Ende möchte ich im Besonderen meiner Familie Danken, für deren Liebe, Geduld und Vertrauen. Für all die, die vor mir waren, die mit mir sind, und die nach mir kommen.
Die Arbeiten zu der Dissertation wurden im Rahmen des von der Investitionsbank Berlin (IBB) aus Mitteln des Europäischen Fonds für regionale Entwicklung (EFRE) geförderten Vorhabens „Glykanbasierte Biomarker für die Qualitätssicherung in der Stammzelltherapie“ (FKZ: 10147245) und des von der Europäischen Kommission geförderten Vorhabens „Vitrocellomics“ (LSHB-CT-2006-018940) durchgeführt.

10. Eigenständigkeitserklärung

Ich erkläre an Eides Statt, dass ich die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir wie oben angegeben vorgenommen worden.

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Weiter erkläre ich, dass mir die geltende Promotionsordnung für die Technische Universität Berlin vom 23. Oktober 2006 bekannt ist.

Thomas Urbaniak Schwedt, 23. Januar 2014