Assessment of stem cell pluripotency using an *in vitro* 3D perfusion-based culture model

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“Science never sleeps”

anonymous

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Abbreviations

AB-PAS	Alcian blue-Periodic acid
AFP	Alpha-1-Fetoprotein
ALS	Amyotrophic lateral sclerosis
AP	Alkaline phosphatase
ßFGF	Fibroblast growth factor ß/2
ßtub3	Beta-3-tubulin
CD31	Cluster of Differentiation 31
CDX2	Caudal type homeobox 2
CFD	Computational fluid dynamic
CGH	Comparative genomic hybridization
CHES1	Swiss (CH) Embryonic stem cell line 1
CHES6	Swiss (CH) Embryonic stem cell line 6
DNA	Deoxyribonucleic acid
EBs	Embryoid bodies
ECC	Embryonic carcinoma cell
ECM	Extra cellular matrix
EpiSCs	Epiblast stem cells
ESCs	Embryonic stem cells
FBS	Fetal serum
GFAP	Glial fibrillary acidic protein
HAND1	heart and neural crest derivatives 1
HD	Hanging drop
hESCs	Human embryonic stem cells
hPSCs	Human pluripotent stem cells
HTS	High-throughput screening
ICM	Inner cell mass
IMDM	Iscove's Modified Dulbecco's Medium
iPSCs	Induced pluripotent stem cells
KLF4	Krüppel-like factor 4
KO-DMEM	Knockout-DMEM
KSR	Knockout-serum replacement
LIF	Leukemia inhibitory factor
LU5	Pan Cytokeratin
MAPK	Mitogen-activated protein kinase
mESCs	Mouse embryonic stem cells
mPSCs	Mouse pluripotent stem cells
Oct4	Octamer-binding transcription factor 4

PE	Primitive endoderm
PGD	pre-implantation genetic diagnosis
PGS	pre-implantation genetic screening
RI	Rock inhibitor
SCNT	Somatic cell nuclear transfer
SMA	Smooth muscle actin
SNP	Single-nucleotide polymorphism
SOX17	Sex determining region Y-Box 17
SOX2	Sex determining region Y-box 2
SSEA3	Stage specific embryonic antigen 3
SSEA4	stage specific embryonic antigen 4
TF	Transcription factor

Summary

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have the capacity to self-renew while maintaining the ability to differentiate into cell types derived from all three germ layers (endoderm, mesoderm, ectoderm). They have a high potential as a cell source in the field of regenerative medicine, drug development, disease modelling and early embryonic development. Multiple criteria have been proposed to evaluate hPSCs such as morphology, karyotype, cell surface phenotype, gene expression profile, *in vitro* differentiation as well as *in vivo* differentiation. However, there are many inconsistencies in the way of generating and reporting the results, most particularly concerning the formation of teratoma *in vivo*, which is the gold standard for the demonstration of pluripotency. The place of implantation, the number of injected cells as well as the duration of the assay introduce high level of variability with various consequences, including absence of detectable teratoma formation.

In the first part of the thesis I report the generation of four hESC lines under the same conditions. They were characterized for their pluripotency status, their gene expression profile and their differentiation behaviour *in vitro*. We showed that although derived and cultured in identical conditions, the 4 hESC lines exhibit differences in their gene expression pattern and their propensity to spontaneously commit during their maintenance. We also demonstrated that these differences have further consequence for their directed differentiation into neuronal tissue.

In the second part of the thesis I present the establishment of a new perfusion-based 3D *in vitro* culture system, which allows the formation of teratoma-like structures derived from all three germ layers. We show that the perfused culture allows spontaneous differentiation of hPSCs into the three embryonic lineages in a more efficient, balanced and reliable way than previously used *in vitro* systems. This leads to the formation of teratoma-like tissue structures similar to those observed *in vivo*. Finally, we defined a quantitative system to grade teratomas, which indicates that our *in vitro* culture is as efficient but more controlled and reproducible than the *in vivo* assay. That grading method could also potentially be used to compare hPSC lines properties in our system.

Taken all results together, my thesis illustrates the complexity of hPSCs and the extensive need of standardized processes to derive, to maintain and characterize their pluripotency status.

General Introduction

A. Stem cells

1. Overview

Stem cells are biological cells found in almost every multicellular organism. They are the source of every cell types during development. In adults, they allow normal regeneration of tissues (like skin or blood) but are also required to repair damaged tissues. They are able to self-renew, but as well to differentiate into various cell types. During periods of growth and regeneration, stem cells are able to divide symmetrically to generate two identical copies thereby expanding stochastically their cell numbers (1). One way to accomplish both self-renewal and differentiation is the asymmetric cell division whereby each stem cell divides to one daughter cell, that maintains stem cell identity and to one that further differentiates into another type of cell with a more specialized function (2). This capacity is crucial to maintain the number of stem cells, while still allowing the production of new more differentiated cells (1).

2. Different types of stem cells have different biological functions and differentiation potency

Stem cells are present both in embryonic and adult organisms. However, the so called embryonic stem cells (ESCs) and somatic, or adult, stem cells exert different biological role and therefore exhibit different potency to differentiate into other types of cells. Stem cells are thus often categorized based on their differentiation potency (Figure 1) (3).

2.1 Embryonic stem cells (ESCs)

ESCs are formed in pre-implanted embryos, after division of the zygote until the blastocyst stage (Figure 1). At early stages, from the fertilized egg up to the 4-cell stage embryo, the blastomeres have the greatest differentiation potential and are considered to be totipotent. These cells are capable of developing into a complete organism as they can give rise to every type of cell of the embryonic body as well as to extra-embryonic cell types (chorion, placenta) (4). At the 16-cell stage (morula), the cells start to differentiate and to develop in an inner cell mass (ICM) or into cells of the outer trophoblast. During this development, the cells belonging to the ICM lose their totipotency and become pluripotent. They are still able to give rise to every cell types that derive from all three embryonic germ layers (endoderm, mesoderm and ectoderm) but are no more capable to give rise to extra-embryonic tissues. Those two types of stem cells are the only one

that can give rise to zygotes, through generation of germ cells, which are reprogrammed during fertilization to generate new totipotent stem cells. Because of their unlimited division capacity, they are virtually immortal (5, 6).

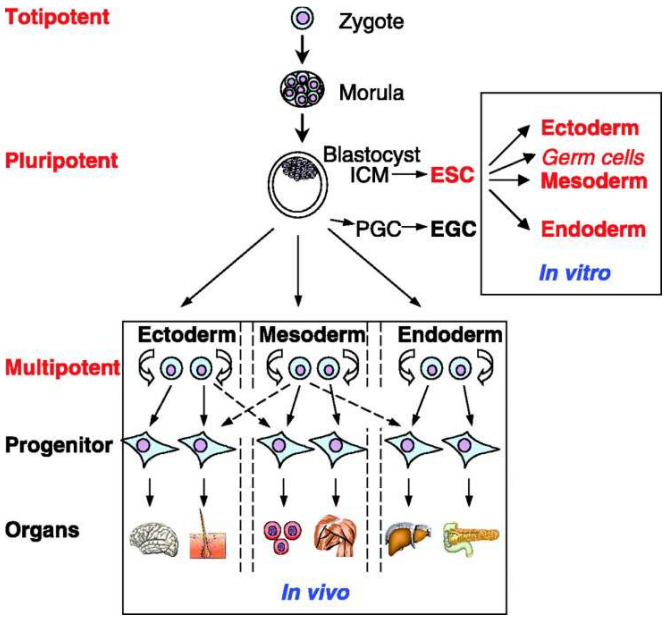

Figure 1: Stem cell hierarchy. The zygote until the morula stage is defined as totipotent, since a whole organism can be built. The cells of the ICM of the blastocyst are able to differentiate into the three germ layers and to the primordial germ cells. In adult tissue, multipotent stem cells are able to give rise to cells within the germ layer but also to cells of other lineages. Progenitor cells are restricted to differentiate into cells within one tissue (adapted from (3))

2.2 Somatic stem cells

Somatic stem cells are found in a variety of tissues in the body. They are more specialized cells and thus more restricted in their differentiation potential. They originate in one germ layer and can usually only form different derivates of the same germ layer (like mesenchymal stem cells and endothelial stem cells). They are at best multipotent (lineage restricted) and are committed to give rise to several distinct but closely related cell types (e.g. fat-to-muscle within the mesodermal lineage). They can also be oligopotent (like bone marrow stem cells) and then able to differentiate into a few different cell types within one tissue. Somatic stem cells can even be *unipotent* only being able to produce one cell type but still self-renewing which distinguish them from progenitor cells. None of those cells are immortal but they exhibit high level of division (7, 8).

3. Biological and experimental recovery of stemness

3.1. De-differentiation and transdifferentiation

While pre-existing stem cells are used during the natural process of tissue renewal, newly generated stem cells through de-differentiation of somatic cells can also be involved, particularly in the case of tissue regeneration after injury (Figure 2). Thereby a fully differentiated cell retrieves back to a less-differentiated stage within its own lineage. This way, the cells proliferate before they re-differentiate and subsequent replace those cells that have been lost. In some cases, de-differentiated cells may re-differentiate into a different cell type of the same lineage (e.g. fat-to-muscle within the mesodermal lineage) but can also transdifferentiate to cells of other germ layers (e.g. bone marrow-to-neurons between mesodermal and ectodermal lineages) (9, 10). Two models actually exist to describe the process of transdifferentiation. As explain above, one proposes that the cell must first dedifferentiate to a precursor or a stem cell stage before it can start to differentiate into a cell type of another lineage. The other proposes that transdifferentation can occur without going via the intermediate precursor step. However, this phenomenon was not observed during a natural process, but rather happens during experimental induction of transdifferentiation (10, 11).

3.2. Recovery of pluripotency

While somatic cells may sometime give rise to adult stem cells through de-differentiation, they do not seem to be able to naturally de-differentiate back to a pluripotent state. The only process where cells are fully reprogrammed naturally is during fertilization, in which totipotent stem cells are produced to differentiate into any type of cells in the body. Nevertheless, somatic cells can to be reprogrammed artificially by introducing specific transcription factors to reach the pluripotent state again (reviewed in (11, 13))

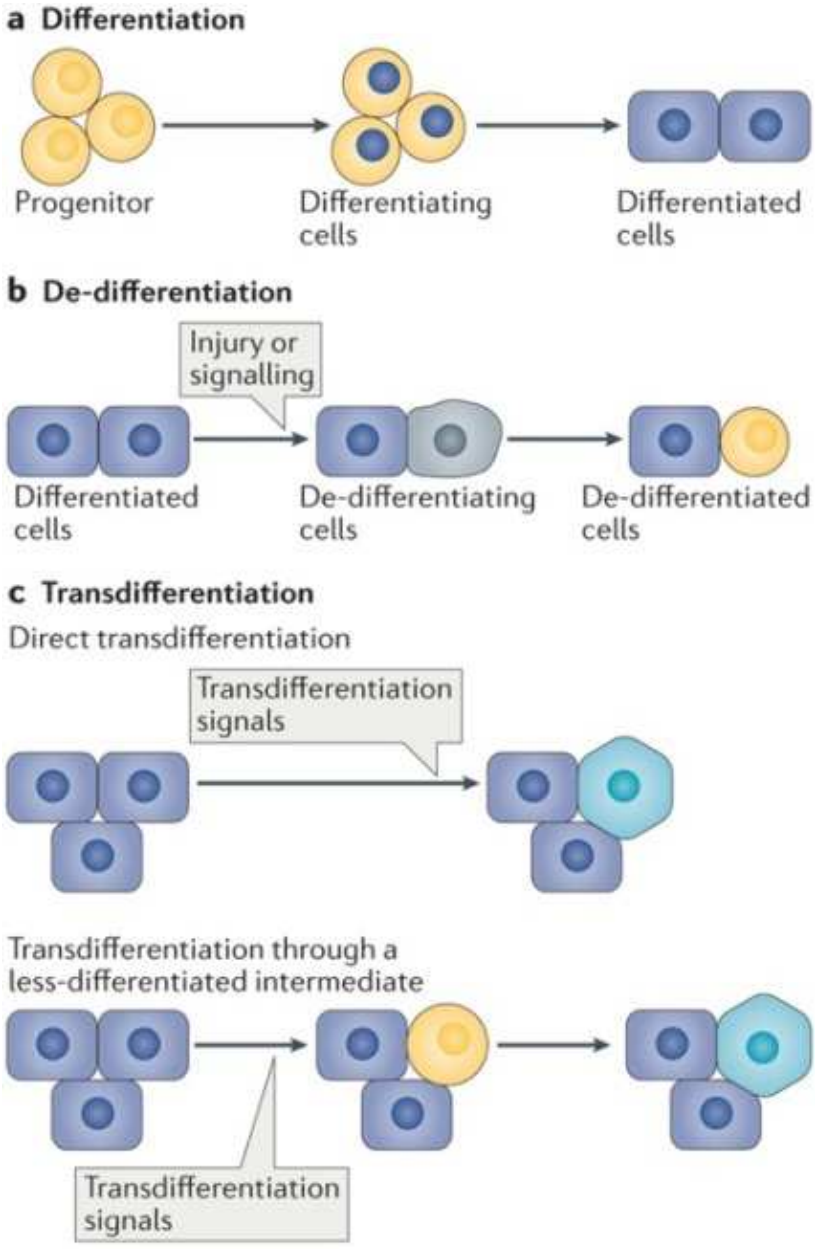

Figure 2: Different model of differentiation. (a) During development and regeneration stem cell or progenitor cells differentiate to a specific cell type. (b) In case of injury, differentiated cells may de-differentiate to an early fate to become a progenitor or a stem cell again. (c) In some cases, differentiated cells may differentiate into another mature cell type (also of another lineage), which is called transdifferentiation. It can occur either directly or through the de-differentiation step before they re-differentiate to the new mature cell type (adapted from (12)).

B. Derivation and maintenance of natural and induced pluripotent stem cells

As already mentioned the essence of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and experimentally induced pluripotent stem cells (iPSCs) is the ability both to intrinsically self-renew and to differentiate into other cell type that derive from all three germ layers, such as muscle, intestine, neurons or skin cells (5, 6). Due to their characteristics, they are promising in different fields of research and medicine and a lot of effort by many laboratories during the last decades has now allowed the isolation, maintenance and amplification of many hPSC lines in culture (14).

1. hESCs

1.1 A brief historic overview

The derivation of the first human embryonic stem cell line was reported in 1998 based on the derivation of mouse embryonic stem cells (mESCs) in 1981 (5, 15, 16). The development of ESCs evolved out of the pioneering work on mouse teratocarcinoma, which is the malignant form of the teratoma, comprised of several undifferentiated tissues besides the differentiated tissues (17). They isolated PSCs (embryonic carcinoma cells or ECCs) out of the teratocarcinoma and demonstrated that they could differentiate into various different cell types. The expanded research on ECCs also demonstrated that it was possible to generate chimera by injecting ECCs to a murine blastocyst. Furthermore, those ECC were used as an *in vitro* model for early mammalian ESCs and to investigate different processes occurring during mammalian development (17).

Nevertheless, these cells had several limitations, they had often chromosomal abnormalities and the capacity to differentiate into different cell types remained limited. Since it was possible to induce a teratocarcinoma by injecting a blastocyst ectopically, Martin and Evans realized independently from each other that it might be possible to derive pluripotent stem cells directly from blastocysts. These findings led then finally to the derivation of mPSCs directly from the mouse blastocyst (15, 16). Next, primate ESCs from rhesus monkeys and later on from marmoset were isolated in 1995 (18, 19). In 1998, the same laboratory was able to derive hESCs out of excessed blastocysts, which were donated by infertile couples undergoing assisted reproduction. The technique to isolate hESCs was not very different from the one to generate mESCs. They isolated the ICM of single blastocysts and plated the cells on mouse embryonic fibroblasts. During establishment, they realized that the properties of the hESCs are different from those of

mESCs and that they require different culture conditions. By contrast, the primate ESCs resemble much more hESCs in terms of morphology, genes expression and lack of response to the growth factor LIF (20).

1.2 Derivation of hESCs

The establishment rate of generating a hESC line from a donated embryo is around 3–10% and is highly dependent on the technique and the developmental quality of the embryo (21). Most hESCs are derived from human embryos that have been originated during assisted reproduction and being in excess for clinical needs. In some countries, the law allows the generation of embryos specifically for research purposes (such as UK and Belgium) but for ethical reasons most of the generated hESC lines are derived anyway from surplus embryos (22). Swiss law allows the generation of hESC lines from surplus embryos donated for stem cell research exclusively (Stammzellenforschungsgesetz (StFG), in vigour since 2003). However, most of the established hESC lines, including those obtained in our laboratory (23) were derived from blastocyst-stage embryos (5-7 days after fertilization) by isolating the ICM. Some hESC lines have been generated from earlier-staged embryos or even from single blastomers (24, 25). This procedure has been performed to avoid the destruction of human embryos that are in a later stage of development but the likelihood for producing a hESC line is lower (22).

1.3. Maintenance of hESCs

When the first hESC lines were established, very similar culture medium and conditions as those for the culture of mESCs were used. However, since novel involved signaling pathways was detected, new reagents to maintain hESCs culture were developed and the culture conditions evolved considerably since then (26).

Several components required for the growth and the maintenance were identified, such as (i) basic medium, (ii) protein source, (iii) factors used to stimulate pluripotency (e.g. FGF-2), (iiii) cell support (e.g. feeders, matrigel). A huge variety of different compounds exist to maintain the culture conditions of hPSC *in vitro* (22).

The most commonly used basic culture medium and protein source to culture hPSCs is the knockout-DMEM (KO-DMEM) supplemented with knockout-serum replacement (KSR). The medium has a reduced osmolality, which favors growth of hPSCs. KSR, a mixture of required

factors, replaces regular fetal serum (FBS) and thus enables growth and differentiation while avoiding the presence of multiple undefined components (22).

The hESCs were originally cultured on mitotically inactivated mouse embryonic fibroblasts as feeder cells. To avoid the number of xeno-components, they were progressively replaced by mitotically inactivated human fetal fibroblasts. Through the secretion of essential growth factors, feeders support the self-renewal of hESCs. New culture conditions have been established where hESC could be cultured on a feeder-free basis requiring a matrigel or laminin layer (27, 28).

Furthermore, hPSC culture requires extrinsic growth factors to support pluripotency. The leukemia inhibitory factor (LIF) supports the undifferentiated state for mESCs. In some conditions, it is also used in hESC culture. However, the most commonly used growth factor for hESC culture is FGF-2 (or ßFGF). It promotes self-renewal in two different ways: it directly activates the mitogen-activated protein kinase (MAPK) pathway in the cell, and indirectly it has an influence on the feeder cells to modify TGFβ1 and activin A signalling pathway. These three pathways play a central role in the maintenance of pluripotency and self-renewal (29).

hESC have a poor survival rate after their dissociation of the cells into single cells, which is regularly done for passaging other cell types. hESCs are more sensitive to treatments and are vulnerable to cell death. Thus, the cells need to be passaged as aggregates either mechanically using a scalpel or by enzymatic dissociation. However, in some cases single cell dissociation needs to be performed (e.g. for embryoid body formation). The latter requires Rho-associated protein kinase (ROCK) inhibitor (RI) to maintain cell survival by preventing dissociation-induced apoptosis (30).

1.4 The pluripotency state in human as compared to mouse ESCs

Despite similar origins there are several differences between hESCs and mESCs: (i) the colony morphology, whereby the hESC have a flattened appearance as compared to the mESC being “dome” shaped (ii) some pluripotency markers differ, e.g. hESCs express SSEA-3 and SSEA-4 instead of SSEA-1 as in mESCs, (iii) hESCs are sensitive to single cell dissociation and therefore need to be passaged as cell aggregates, in contrast to the mESC, (iiii) different signaling pathways are involved in the maintenance of pluripotency. mESCs depend on the LIF/BMP4 pathway whereas hESC use the FGF/TGFß signaling pathway. These differences were always associated to the fact that different developmental programs exist between human and mice.

However, this assumption was questioned when it was discovered that different types of PSCs can be isolated from mice (31) (Figure 3).

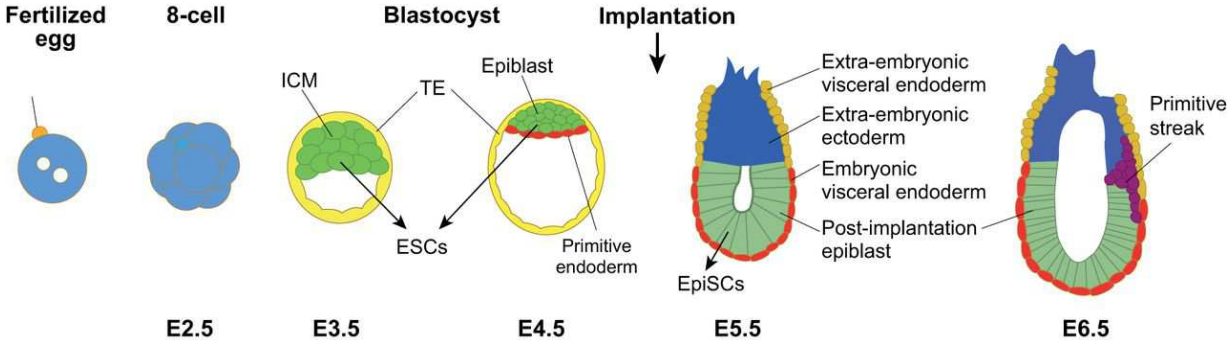

Figure 3: The pluripotent lineage in the mouse embryo. Until the embryonic day (E) 2.5, cells are totipotent. At E3.5 the ICM of the blastocyst contains cells expressing pluripotency and extra-embryonic endoderm. At E4.5 the epiblast and the primitive endoderm lineages segregate. At this stage mESCs are retrieved from the epiblast and are called naïve. Shortly after this step, the embryo implants in the womb. The embryo further develops and the EpiSCs derive which are in a primed pluripotent state (adapted from (32)).

ESCs are isolated from the ICM of the pre-implanted mouse or human blastocysts. Epiblast stem cells (EpiSCs) are isolated from mouse post-implantation embryonic epiblast stage embryos (Figure 3). Due to ethical aspects, the derivation of EpiSCs in human was not attempted. ESCs are often termed as "naïve" pluripotent stem cells. They are unprepared to make lineage decisions and have to pass a maturation process. In contrast, the EpiSCs are defined as "primed" pluripotent stem cells. They are partially specified and prone to a specific cell fate. The EpiSCs express the main pluripotency markers (Oct4, Sox2 and Nanog), are able to differentiate *in vitro* and can build a teratoma. However, the mouse naïve and primed ESCs differ in the way that naïve ESCs are able to form chimeras upon blastocyst injection whereas primed ESCs are incapable. It has been proposed that the EpiSCs correspond to a more advanced developmental stage. Furthermore, they differ from the ESCs in the expression of several different transcription factors (33, 34). The hESCs show an expression pattern more similar to mouse EpiSCs than to the mouse ESCs and may represent a later step of differentiation than the mouse ESCs. However, their naïve or primed nature cannot be evaluated since human chimeras cannot be built due for obvious ethical reasons (32, 35). Different attempts were done to generate human naïve cells,

which relied on transgene expression using specific inhibitors to target different signaling pathways (31).

1.5 Variation of the hESCs

Although all hESC lines share the same properties, differences between lines often exist in terms of genetic stability during long-term culture and differentiation potential into specific cell types (36, 37). The mentioned variability in generating and maintaining hESCs makes the interpretation and conclusions of inter-line differences difficult. No conclusion can be drawn if the difference is due to inherent genetic variation or due to environmental influences like the culture conditions. Furthermore, it has to be considered that the maintenance of the pluripotency state in hESCs is artificial as compared to the human development. This can be a further explanation for the different behavior of derived hESCs (38).

2. Induced pluripotent stem cells (iPSCs)

Although natural dedifferentiation of adult cells sometime occurs in vertebrates, it apparently never achieves a pluripotent state (11). Yamanaka et al. succeeded in reprogramming a somatic cell to generate PSCs by utilizing retroviral transduction of Oct4, Sox2, Klf4 and c-myc , first in mice and later also in human (6, 39). The successful possibility of the generation of iPSCs was based on three important findings in research over the last decades. First, the technique of somatic cell nuclear transfer (SCNT) has shown that different somatic cells keep the same genetic information as early embryonic cell. Gurdon successfully cloned a frog by using intact nuclei from a somatic cell and fused it with an enucleated egg. With the same technique, the sheep Dolly was developed having the same genetic background as the mammary cell donor (40). Those cloning results demonstrate that the genome of a fully differentiated cell has the capacity to support the development of an entire organism. Nevertheless, this technique is challenging and not ideal for genetic studies. Second, the technique of deriving and culturing ESCs arose and improved. Third, a whole network of transcription factors involved in the maintenance of cellular identity during development was discovered. They act by switching on specific genes, while lineage-inappropriate genes are suppressed (reviewed in (41)).

Initially Yamanaka et al. screened 24 transcription factors that might have an influence in the maintenance of pluripotency. They systematically narrowed the necessary factors down until they landed on four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4. These were sufficient to

induce the development of stem cell-like colonies (39). This approach has later been successfully adapted to human cells (6). In the past years, different reprogramming methods have been developed to derive iPSCs with an increased efficiency in reprogramming cells. First retro-or lentiviral vectors were used to deliver the mentioned transcription factors, but the risk exists that their transcriptional activity remains in the pluripotent cells. Thus, several other techniques were established to generate iPSCs while avoiding the integration of any viral vector into the genome of the treated cells, such as nonintegrating viral vectors (e.g. sendai-virus) (42).

IPSCs cells are mitotically active and have the capacity of self-renewal. Moreover, they can differentiate *in vitro* into derivates of all three germ layers and they can build teratoma *in vivo*. They have the same morphology than the hESCs and can be cultured in the same conditions. Their pluripotency status is usually characterized by performing the same tests than for hESCs (41, 43, 44).

3. iPSCs versus hESCs

There are two main advantages existing of the iPSCs technology towards the hESCs. First, no ethical issues are existing in the generation of hESCs, where an embryo needs to be destroyed in order to isolate cells. Second, in the field of cell replacement therapy, the problem of the immune incompatibility between the donor cell and the recipient, which could lead to the rejection of the transplanted cells, can be eliminated by using patient-derived iPSCs (45). However, the debate still continues to what extent iPSCs recapitulate the characteristics of hESCs. By comparing the transcription profile of iPSCs and hESCs, some differences were encountered. Some show that the profiles are nearly identical with only a small group of genes being expressed differentially (46). Other comparisons show that some differences in the expression profile of iPSCs and ESC exist, but are not consistently observed in all iPSCs lines. By investigating more into detail the individual reprogramming experiments, significant difference between iPSCs and hESC can be observed that can be referred to the reprogramming step where (i) iPSCs not efficiently silenced all the genes from the somatic cell from which they derived and (ii) iPSCs failed to induce some ESC specific pluripotency genes (47). This leads to the assumption that iPSCs may have an epigenetic memory. Two studies analyzed the epigenetic memory, referred to DNA methylation and histones modifications of mouse iPSCs and they could show that iPSCs, which were differentiated back to the cell of origin showed an advantage over another differentiated cell from another lineage (48)(49). This phenomenon could only be observed during early passages (P4-P6) of iPSCs indicating that the reprogramming process needs a longer time to be accomplished as

expected. Moreover, the step of reprogramming using specific transcription factors may lead to spontaneous aberrations in the genome since most of these genes are associated with neoplastic development (50). Finally, iPSCs and hESC show also variations in their capability to differentiate, the former exhibiting in some cases lower performances. Also in this context, it is difficult to compare the potential of iPSCs to differentiate into specific cell type due to different culture conditions and various differentiation protocols. However, by a direct comparison of differentiation potential between iPS and hESC, the outcome of differentiation can be evaluated in different ways; by scoring the efficiency of differentiation by quantifying the obtained cells using a specific marker or by assessing the quality of the differentiated cells. IPSCs and hESCs show variations, either the show the same or in some cases as well lower performance of differentiation. The possible inferior performance of iPSCs could be explained by a higher tendency for aberrations, which subsequently might influence cell perception of external differentiation signals. All these differences may mainly arise due to incorrect reprogramming behavior although the efficiency of reprogramming may still be optimized (47).

C. Uses and promises of the hPSC technology

A main challenging aspect in biology is the understanding of the development of the human body. Furthermore, disease associated prevention and treatment of diseases are of a major interest. Much knowledge about human development is being extrapolated from model organisms which gave us an insight into the general principles of development and signaling pathways, such as the drosophila (e.g. discovery of the hox genes in controlling the body plan) (51).

The mouse is the primary model organism for the study organ development in humans. Both the mouse and the human genomes share a high percentage of similarity. However, they differ in several species-specific genes and in some organ functions. Moreover, important events of embryonic development are also different, in particular during gastrulation. Subsequently, these dissimilarities lead partly to the disability of recapitulating some human diseases in the mouse model. The same genotype may be lethal in one species whereas in the other it may be viable. For that reason, it is essential to conduct some biomedical research directly in human models, which, in the case of embryonic development, is mostly limited to *in vitro* systems. HPSC have two major advantages to be suitable for human development studies and disease modelling. Since they are primary cells, they have the potential to self-renewal and they have the capacity to differentiate into every adult cell type. These features enable the study of the relationship between genotype and phenotype and to obtain a high number of cells for further purposes like cell therapy (51, 52).

1. Model for normal human development

A model for the early embryogenesis is the embryoid body formation (see D.3.). Briefly, hPSCs are cultured as aggregates in suspension and are able to differentiate spontaneously into cells of the three germ layers. The EB formation is mostly used as the initial step for the differentiation into any specific cell type (53). Expression patterns in human EBs demonstrate that several genes are activated which are involved in the early embryogenesis, the gastrulation and the formation of the germ layers. Therefore, the EBs are a useful model to understand the mechanisms that drive the human embryogenesis. As an example, the study with EBs illustrated that NODAL and LEFTY, which belong to the transforming growth factor (TGFß) superfamily, are involved in the gastrulation process. Inhibition of NODAL/LEFTY lead to the disruption of mesodermal differentiation (51).

HPSCs are also used to mimic cell fate specification via direct differentiation. To mimic the tissue specific development during embryonic development, first the hESCs must be differentiated into the three germ layers and then further to any specific cell type through the addition of recombinant growth factors and/or small molecules. For example, it is known that hESCs are exposed to activin A to differentiate into endoderm, to BMP4 and activin A for mesodermal differentiation and to inhibitors of BMP4 and WNT signaling to be able to differentiate into the ectoderm. In those tissues, in which no specific knowledge is available, the *in vivo* environment can be recapitulated using cells isolated from an environment in which the desired cell type is present. For example, a study illustrated the following; they wanted to understand why in the inner ear lost hair cells could not be regenerated. First, they differentiated mESC into the ectoderm and by the exposure to ear-related factors, otic progenitor cells were built. They further plated these progenitors on stromal cells of the ear to induce the formation of hair cells (54).

HPSCs also offer opportunities to understand developmental mechanisms. Studies have been carried out to investigate the role of the signaling of TGFß during pancreatic development. By using hPSCs, they could show that TGFß inhibits the differentiation of pancreatic progenitors into the lineage that gives rise to the ß-cells of the pancreas (reviewed in (51)).

2. Disease modelling and drug discovery

Apart from studies of the normal development, hPSCs are also utilized to study abnormal development and specific diseases. In a first step, a cell line need to be established carrying the defect of interest, which can be used to investigate the phenotype of a particular disease. Different models exist to generate genetic disorders (Figure 4).

HPSCs carrying genetic disorders can be generated from healthy or aberrant cells. Isolated hESCs from healthy donors may be genetically modified at a specific locus so that a disease phenotype can be further characterized. Additionally, hESCs can develop spontaneously chromosomal aberrations during culture. These cells can be isolated and further investigated, such as cells with the karyotype causing the turner syndrome, displaying monosomy X. HESCs may also be isolated from human embryos carrying specific mutations or aberrations, which were identified by pre-implantation genetic diagnosis (PGD) or pre-implantation genetic screening (PGS). These embryos, which otherwise would be discarded due to the diagnosed abnormality, may be used as *in vitro* models for particular disorders. Since only a reduced number of disorders can potentially be identified by PGD or PGS the ability to reprogram somatic cells yield to new

possibilities in the field of disease modelling. The technique of somatic cell nuclear transfer (SCNT), whereby a nucleus of a somatic cell can be reprogrammed and subsequently implanted into an enucleated oocyte allows the derivation of hESCs from patient cells.

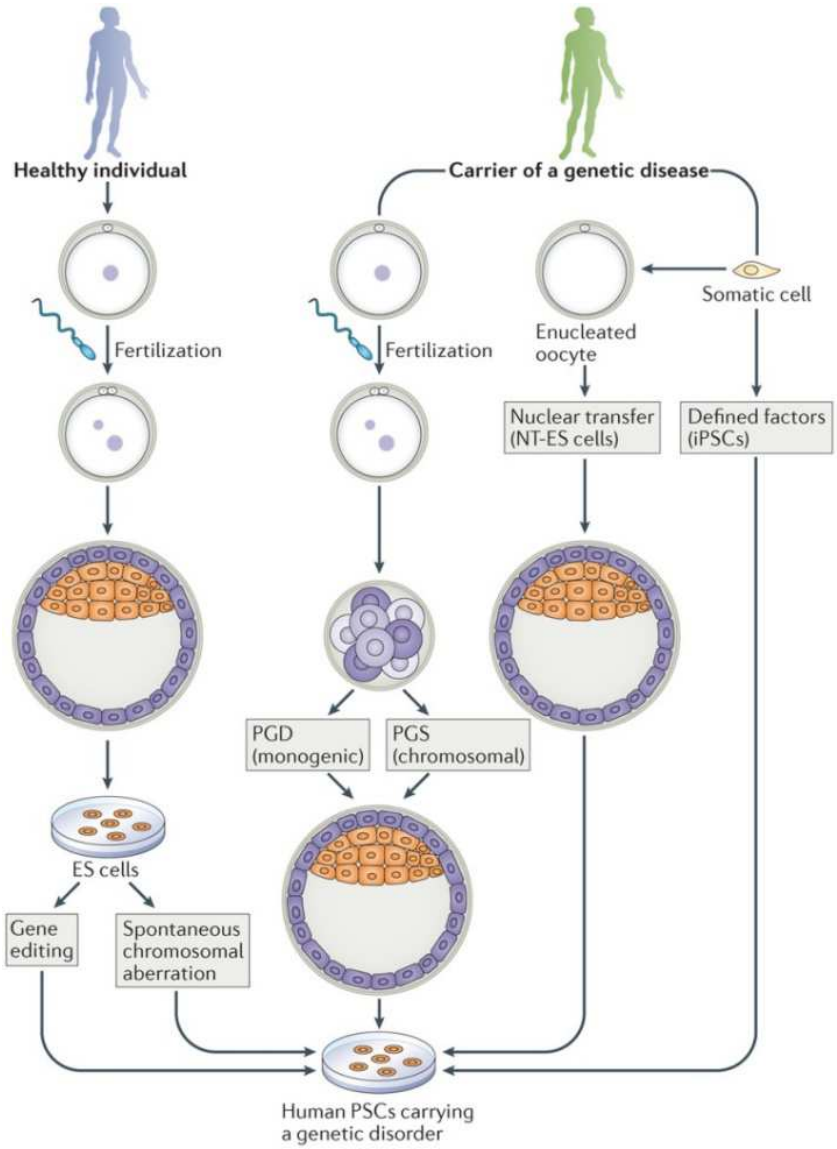

Figure 4: HPSC carrying a genetic disorder can be generated by using healthy or aberrant cells. Either the isolated hESC acquire spontaneous aberration during culture or the hESC can be genetically edited. The cells can be also used from a carrier of a genetic disease. Either disease-specific hESC cells are identified during PGD or PGS before *in vitro* fertilization is carried out. Another possibility is be reprogramming of a somatic cell of a patient by SCNT of hESC or by generating iPSCsC (adapted from (52)).

The alternative process is the reprogramming of a somatic cell into iPSCs. The reprogramming of patient cells has the advantage that the iPSCs from multiple patients can be easily generated and enables the analysis of similar mutations but with different genetic backgrounds. Several laboratories generated successfully different disease-related iPSCs from fibroblasts of patients carrying a specific mutation. The presence of the mutation was confirmed in the newly generated iPSCs line. Phenotypes of neurological disorders were the first diseases that could have been recapitulated *in vitro* due to the extended knowledge and demonstration of normal neuronal development. Amyotrophic lateral sclerosis (ALS) was modelled by generating iPSCs of dermal fibroblasts taken from two patients, which are both heterozygous for the L144F mutation in the superoxide dismutase gene. iPSCs were differentiated into motor neurons and investigated for different factors (reviewed in (52)).

As reported, the disease modelling helps first to understand the cellular and molecular mechanisms related to it. Second, it enables to approach particular drug screening strategies using hPSCs. When a specific phenotype is identified to be treated, two main approaches are used to identify potential beneficial drugs. Briefly, an iPSC, which carry a disease-specific genetic aberration, can be further differentiate to the disease-affected cell type (e.g. neurons for neurodegenerative diseases). The candidate drug approach contains a small number of compounds that are investigated on affected cells. This strategy is used when already knowledge about a disease exists, such as one specific pathway, which is known to be responsible for the induction of a specific disease. Thus, a defined set of drugs is tested to identify the most potent therapy. The effect of existing drugs is also validated and confirmed in the iPSCs models. The high-throughput screening (HTS) approach is based on investigating the potential effects of more than 1 million compounds. However, with this method, it needs to be guaranteed to re-evaluate the phenotype again specifically, like for example when a disease has an electrophysiological defect. A large number of cells are needed to test a wide range of compounds. Drug discovery in neurological disorders is more progressed than in other medical fields. So far, 25 neurological diseases, for which iPSCs-derived neural cells have been develop to model the disease and were used to screen potential drug components. However, most of these diseases are linked to monogenic or chromosomal aberrations rather than to complex genetic disorders. Moreover, it needs to be considered that the approach of using iPSCs for drug development is still at the very beginning and the first step of a long phase until the identified drug can be used on the market for

treating patients. The future aim of hPSC-based therapies is to model every genetic disease, even complex ones. These current existing approaches have already confirmed the high value of hPSCs in modelling and treating various diseases (reviewed in (52)).

3. Regenerative medicine

One promise of iPSCs cells is the replacement of diseased or injured cells within specific organs. The main challenge of this approach is the ability to provide iPSCs cells or iPSCs-derived cells to the target tissue while maintaining the organ viable and functional. There are two ways how the cells can be delivered into the affected organs; (a) via intravenous injection with the expectation that they will return to the concerned organ and can be engrafted successfully. Thereby cells are mostly injected naked in a buffer with the disadvantage of a high rates of cell death (45), or (b) via local delivery of the cells during open surgery into the organ. In this situation, injectable or implantable scaffolds are used to support engraftment and cell survival (tissue engineering approach). There are several applications being used, in which pluripotent stem cell derivates (such as cardiomyocites and A9 dopamineric neurons) are already in phase 1 or 2 clinical trials. A phase 1 clinical trial is the first step towards the development of a new treatment in humans. In this phase, the maximal amount of treatment that can be given to a patient is evaluated. In the phase 2, more persons are recruited to determine the effect of the tested drug. As an example, ES cell-derived cardiomyocytes were used to repair cardiac muscle damage after myocardial infarction. A patient, also receiving a coronary bypass operation was grafted in the infarcted area with the derived cardiomyocyte progenitors. The cells were embedded in fibrin to enable the integration of the grafted cells into the heart tissue. The symptoms of the disease significantly improved after 3 months and contractibility was observed in the previously kinetic myocardial region. However, it is not yet clear if it is due to the bypass operation or the engraftment (55). To reach the step of the clinical study, it is indispensable that the safety of PSC-derived cells is properly tested (e.g. for chromosomal stability and mutations in oncogenes) in the preclinical phase to avoid any adverse events in the patients (56).

4. Prospect of human organ growth inside animals

One future goal of stem cell technology is to grow functional and transplantable tissue or full organs *in vitro*. An important first step towards this approach was established by the formation of a human-animal chimera. First, a mouse-rat chimera was built using the CRISPR-CAS9 genome editing tool to delete specific genes which are responsible to build heart, pancreas or eye in the

mouse blastocysts. Subsequently, they introduced rat PSCs into the embryo and the resulting mouse offspring could develop the organs from rat stem cells. Surprisingly the rat stem cells were able to build a gall bladder in the mouse, even though in the rat this organ is not developed anymore over the last million years. This shows that the gall bladder is not developed anymore because the potential remains hidden by a rat-specific developmental program. Next, they induced hiPSCs into a pig blastocyst. They chose this animal since the organ size of pigs resembles that of humans. Several types of hiPSCs were tested to be implanted in the blastocyst of the pig, like naïve, intermediate and primed hiPSCs. The intermediate hiPSCs turned out to be the most suitable ones since the embryos at a particular stage managed to survive the experimental procedure. They could successfully build a human-pig chimera embryo, which was subsequently implanted into a sow and developed for 28 days. With the help of fluorescence tagging they could detect within the pig embryo about 1 living human cell in 100’000 pig cells. Next, they aim to improve the efficiency and push the human cells into the formation of particular organs using the CRISPR-CAS9 method, as applied for the mouse-rat chimera (reviewed in (57)).

D. Characterisation of hPSCs

Multiple criteria have been proposed to evaluate the pluripotency status of newly generated hPSCs. In general, PSCs are verified by their status of undifferentiation investigating the morphology, cell surface phenotype and gene expression profile and by their capacity to differentiate into various cell types assaying the differentiation potential *in vitro* and *in vivo*. Furthermore, euploid karyotype, epigenetic status and extended proliferative capacity are essential criteria to be characterized. Nevertheless, these assays vary in specificity with which they analyze the state of pluripotency (58, 59).

1. Assessment of hPSC morphology

hPSCs acquire a typical morphological appearance when they are grown in culture. The cells typically have a round shape with a large nucleus, large nucleoli and a scant cytoplasm. They are arranged in compact colonies with distinct boarders and sharp-edges. The morphology of the newly derived hPSCs can already be used as a first screen during the colony selection (60).

2. Markers of pluripotency

A widely-tested panel of markers of the undifferentiated state of hPSCs exist. The international stem cell initiative has been conducted a study in which they characterized 59 hESCs lines from 17 different laboratories worldwide. Even though there is a huge diversity in the techniques to generate and maintain hESC lines they could identify similar expression patterns for a number of pluripotency markers (61).

A number of transcriptions factors (TF) playing a crucial role in regulating the maintenance of self- renewal were identified, such as octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (SOX2) and Nanog. These TFs are known to be the key regulatory genes to maintain pluripotency and self-renewal and act cooperatively with each other in a complex way (62). This network has the ability to positively regulate genes needed for maintenance of pluripotency state while repressing genes encoding lineage-specific regulators. When any of these master transcription factors is no longer expressed to keep pluripotency the differentiation program will be initiated (63). Oct4 is a member of the POU-homeodomain family and is a key player to sustain totipotency or pluripotency and is highly expressed in the ICM of the blastocyst (64, 65). A balanced Oct4 level is required to maintain the pluripotency state of cells cultured *in vitro* (66). In addition, Oct4 plays a role in the regulation of gene expression

networks. Depending on its expression during development it has an influence on the lineage commitment to early endoderm and mesoderm by overexpression whereas a repression of Oct4 leads to a loss of pluripotency and de-differentiation to the trophectoderm (67). SOX2 belongs to the SOX transcription factors and is also regulating the pluripotent state as well as the differentiation to different cell types during development (68). SOX2 controls the differentiation to specific cell types, such as the nervous system and anterior foregut endoderm during early development (69, 70). It further regulates the progenitor cells in adult tissue of the brain and trachea (71, 72). Nanog is also known to be essential for pluripotent cell development and maintenance. Furthermore, it controls the epiblast versus the primitive endoderm decision in the blastocyst (66). Since these TFs play a crucial role in the guarantee of the undifferentiated and self-renewal status, they became important markers for the identification of the pluripotency status when a newly derived hPSC line was generated.

Furthermore, glycolipids and glycoproteins that were originally identified on embryonic carcinoma cells became later biomarkers for pluripotent stem cells, such as SSEA-3, SSEA-4 TRA-1-60 and TRA-1-81 (73). Interestingly, after the induction of differentiation into cell of the three germ layers each marker show a different kinetic of disappearance. For example, OCT4 and TRA-1-60 were the first markers that are down-regulated whereas Nanog is detectable for a longer time period after the initiation of differentiation (74).

The alkaline phosphatase (AP) is another key marker to identify pluripotency. AP is an enzyme, which is highly positive in the ICM. As soon as the differentiation has started the AP expression it is downregulated. Thus, high AP activity is related to a high number of PSCs (75)

These markers are the most commonly used in stem cell research and are tested both on RNA and protein level by qPCR and immunohistochemistry respectively. However, several arrays exist with specific genes that are involved in the maintenance of pluripotency and self-renewal and are often used to have a deeper insight in the expression pattern of the new hPSC lines (76).

3. *In vitro* differentiation- embryoid body (EB) formation

When specific factors that maintain stemness of the hPSCs are removed during culture (e.g. ßFGF), the cells spontaneously differentiate into cells of the different germ layers. By dissociating hPSCs from the colonies and after their transfer in suspension, the cells are able to aggregate and build spherical three-dimensional embryoid bodies (EBs) (77). Due to cell to cell

interactions the cells within an EB are able to differentiate spontaneously into derivates of all three germ layers. With this possibility to differentiate, the EBs recapitulate the early embryonic development. The EB formation is the most commonly used *in vitro* assay to prove the capacity of the hPSCs to differentiate (53).

The first indication that an EB starts to differentiate is the spontaneous formation the primitive endoderm (PE) on the surface of the EBs. The cells of the PE further differentiate into visceral and parietal endoderm. These generated cells build a membrane that separates the PE cell layer on the surface from the remaining undifferentiated cells. Cells that are not directly in contact with this membrane undergo apoptosis leading to the formation of central cystic EBs. During further differentiation within the EB, different phenotypes of all three germ layers arise (77).

However, the major challenge of this assay is the heterogeneous differentiation capacity of the EBs. The differentiation outcome is highly dependent upon the quality of the EBs that is mainly determined by their individual size (78, 79). The size of an EB is primarily depends on the number of hPCS within each single EB and subsequently depends on the cell-cell interaction (80). While too small EBs do not survive the differentiation process, too big EBs undergo central necrosis and become cystic due to reduced access to mass transport (81).

There are two principles to assess the differentiation potential of hPSCs. EBs are either used to prove the capability to differentiate spontaneously into the three germ layers (so called stochastic EBs) or they are used as intermediate for the direct differentiation towards specific cell types of the three germ layers such as neural and cardiac cells. Depending on the approach that is implemented, the uniformity of the size of the EBs is crucial for the reproducibility of the differentiation process. By contrast, for the illustration of diverse tissues derived from the three germ layers the ability to build EBs of different sizes poses advantage (82).

Numerous approaches have been developed to induce EB formation (83). To induce stochastic EBs, cells are put in suspension culture in dishes with hydrophobicity. Thereby the seeded hPSCs don’t attach to the surface and naturally stick to each other and build aggregates. The composition of the culture medium that is used has an influence in the viability and the differentiation process of the EBs. It was reported that EBs cultured in a lower glucose concentration than normally in combination with the growth factor ßFGF increased the variability of different tissue-like structure within an EB (84).

However, in order to generate specific tissue types through the formation of EBs, a different culture system must be applied. The homogenous size of the EBs has been demonstrated to simplify the differentiation process. Different methods have been developed to form EBs with defined sizes. The hanging drop (HD) method provides the hPSCs a good environment to build EBs. The number of cells that aggregate in a hanging drop is more controlled by an exact number of cells of the initial population to be hung as a drop from the lid of a petri dish. One EB per drop will be formed from a predetermined number of hPSCs in a defined volume of liquid leading to a more homogenous distribution of the size of the produced EBs.

After 2 days, the EBs are placed in suspension for further differentiation. This method has the disadvantage of limited preparation of EBs due to the limited volume of the hanging drop. EBs of homogenous size are built in a 96 well-plate. Similar to the HD culture, one EB is composed of a predetermined number of hPSCs per well but by contrast it enables medium exchange and the management of a large volume of cell suspensions. However, the number of cells to build an EB affects the differentiation of the resulting EB. Another alternative technique to produce EBs is the stirred flask method, in which the cells are cultured in a special flask with a magnetic stirring to ensure a continuous rotation of the culture medium. Compared to the static culture, the aggregation of the cells is easier and the cells are exposed to a better gas exchange as compared to the static culture. It has also the advantage to scale up the EB production (80, 82, 84, 85).

To verify the germ layer formation of EBs, the expression of germ layer specific genes and the demonstration of tissue-like structures must be studied. Upon EB differentiation, gene expression studies allow the identification of the germ-layer specific markers and the lack of pluripotency genes. Histology later allows a more detailed assessment of differentiation into various tissues (82).

4. In vivo differentiation- teratoma formation

4.1 Definition of a teratoma

The name “teratoma” has its origin in the Greek word “teras” which means “monster” (86). By definition, a teratoma contain tissues derived from all three embryonic germ layers, the endoderm mesoderm and ectoderm, like hair, teeth and bone (87). This type of tumor belongs to the non-seminomatous germ cell tumors (N.S.G.C.T). All the tumors of this category are the result of abnormal development of pluripotent cells (Germ cells and embryonic stem cells). Teratoma

which derive from germ cells are mostly developed in the gonads (testis and ovary). In contrast teratoma derived from embryonic stem cells occur mostly in the sequestered midline of the human body (88).

4.2 Clinical implications of teratoma

Mature teratomas consist of various differentiated somatic tissue that are foreign of the particular tumor site. Immature teratoma are composed of immature structures or incompletely differentiated tissues, like primitive neuroectodermal structures. Furthermore, the degree of immaturity correlates with the proliferation rate (89, 90).

Regardless of location of the body, the grade of maturity of the teratoma is assessed according to a cancer staging system, which is important to grade the severity of the tumor, which gives indication for surgery or radiotherapy. The teratoma is classified using the grading system outlined by Gonzales-Crussi in 1982 (91):

Grade 0: Mature (benign)
Grade 1: immature, probably benign
Grade 2: immature, possibly malignat (cancerous)
Grade 3: Frankly malignant. If the teratoma is frankly malignant, the tumor is a cancer for which additional cancer staging may be performed.

Furthermore, a teratoma can be classified by its content; one distinguishes between *solid teratoma*, containing only tissues from *cystic teratoma*, contain only pockets of fluid or semi-fluid such as sebum, or fat; and *mixed teratoma* containing solid and cystic parts. (88, 92))

4.3 Experimental teratoma

The most stringent proof of pluripotency is the generation of chimera via germ line transmission as it is applied for testing pluripotency of mPSCs. Thereby, mPSCs are injected into a blastocyst of a wild-type mouse and the first resulting breed is called chimera. It is defined as an organism with genetically different cell populations derived from more than one fertilized egg (93). However, this test of forming a human chimera is not applicable for hPSCs due to ethical concerns (61, 94). That is why the spontaneous differentiation of hPSCs *in vivo* is assessed by teratoma formation in immundeficient mice, which is defined as the gold standard for the proof of pluripotency (95, 96). Essentially hPSCs are injected into different transplantation sites (such

as testis, subcutaneously, capsula of the kidney) in immune-deficient mice until a potential tumour is developed.

Up to now, the histogenesis of the teratoma is poorly understood but the cells mimic the post-implantation early embryonic development. The tumor consists of different somatic tissues with various degrees of maturation. Some teratoma show organized structures of adult tissue but most contain structures resembling early stages of development, such as neural rosettes (97). HPSCs are considered as pluripotent when the derived tumor is identified as a teratoma by displaying various different types of tissues deriving from all three germ layers (endoderm, mesoderm and ectoderm) (98).

4.4 In vivo teratoma assay- state of the art

The method of inducing a teratoma differ widely among different research groups in several aspects, such as the preparation of cells, the number of transplanted cells, the choice of the transplantation site and the incubation time (99, 100). One study that was performed in 2009 to investigating the susceptibility to form hESC-derived teratomas according to the transplantation site (kidney capsula, muscle, subcutaneous space, peritoneal cavity, testis, liver and epididymal fat pad) in SCID mice. The effect of matrigel was assessed as well. Interestingly, in all generated tumors within 8 weeks a pronounced liquid cyst, interfering with histological analysis were detected. They found out that the most experimentally convenient and reproducible way to build a teratoma was the intramuscular injection of hESCs without matrigel. Another research group published an *in vivo* teratoma assay which showed the most reproducible method is based on the subcutaneous injection of 100`000 cells together with inactivated feeder cells and Matrigel into an immune-deficient mouse. The assay was highly reproducible and 100% efficient. As we can see from these two examples, the techniques to ensure a teratoma vary a lot. It has also been shown that the numbers of cells to induce a teratoma is varying among different manuscripts; for example, a range between 3000 to 5 million of hPSCs exists for testicular injection. In case the cells grow on feeder layers, no single cells can be generated. As a result, the number of injected cells are vaguely described as being “50 clumps of 300 cells” Since these great number of variables exist in generating a teratoma *in vivo*, one cannot be sure if the failure to induce a teratoma with the newly derived hPSCs is due to inappropriate conditions of the assay or due to an abnormality of the generated hPSC line (reviewed in (101)).

Even if the *in vivo* assay is presented in the manuscript, mostly the information reported for generated a teratoma remain utterly vague. The number of animals used for an experiment is rarely reported and the failure to produce teratoma is almost never explained (101).

In most cases the generated teratoma is assessed by histochemical analysis using the conventional Hematoxylin and Eosin staining. The generated structures that are derived from all three germ layer are identified mostly by a pathologist who further classifies them as derivates from endoderm, mesoderm and ectoderm. Most of the studies report at least one tissue per germ layer, such as gastrointestinal glands for endoderm, cartilage for mesoderm and neural tubes for the ectoderm (102-105). In some cases, specific markers are tested by immunohistochemistry to identify differentiation into the three germ layers, such as smooth muscle showing smooth muscle cells derived from the mesoderm, ßtub3 detecting young neurons from the neuroectoderm and different types of cytokeratin to demonstrate the presence of epithelial cells (106).

This summary of the inconsistencies in the methodology used and in the poor reporting of the results show that there is a need of a standardization of the *in vivo* teratoma assay, which is supposed to be the gold standard for demonstrating pluripotency of hPSCs (99).

5. Karyotype

A diploid karyotype is another condition to be fulfilled for a newly generated hPSC line. Once a new hPSC line is established, it is expected to stay chromosomally stable. HPSCs are often karyotyped using standard GTG banding metaphase spreads, comparative genomic hybridization (CGH) but also more precise techniques like single-nucleotide polymorphism (SNP) arrays (107, 108).Chromosomal changes of hPSCs in long-term culture have been reported several times by various laboratories (109-111). In hESCs the most reported aberrations are the gain of chromosomes 12 and 17, duplication of 1q11q32 and 20q11.21 region or the aneuploidy of chromosome (109, 112). These mutations confer often to proliferation advantages, higher culture adaption and resistance to apoptosis. It has been reported that the quality of the blastocyst has an impact on the chromosomal status of any newly derived hPSC line. In some cases, aneuploidy has been observed already at the stage of the blastocyst (113). For iPSCs two different aberrations were described such as the gain of trisomy of chromosome 8 and 12 but also deletion of chromosome 8 and 17. Furthermore the amplification of portions of chromosome 1, 17, or 20 is frequently observed. It is not yet fully clear whether these chromosomal variations in iPSCs

originate at an early stage or later during culture or whether they are caused by the reprogramming process (108, 114). Chromosomal aberrations in iPSCs can also occur during the culture of the somatic cells, which underwent several cell divisions before they were transformed to iPSCs (115).

The method of passaging the hPSC has been shown to have an effect on chromosomal changes. The mechanical technique by cutting the colonies into small pieces with a blade has turned out to be less aggressive than enzymatic passaging, therefore the euploid population may be better preserved (116). It has also been shown that hPSCs with a higher passage are more vulnerable to karyotypical changes than hPSCs with a lower passage. However, some generated hPSCs are more prone to chromosomal changes already at early passage than others (117). Due to these facts, it is important that a karyotype is performed more regularly during long-term culture to ensure that the hPSC line remains chromosomally stable. This is important to test since the existence of chromosomal abnormalities in hPSCs is often associated with carcinogenesis and impaired *in vitro* and *in vivo* cellular behaviour (115).

E. The tumorigenicity of hPSCs

HPSCs share many characteristics of tumor and cancer cell lines (106, 118). Both have a fast proliferation rate, a tendency of genomic instability, a high telomerase activity and similar gene expression patterns (50).

The acquisition of tumorigenic potential of hPSC has to be considered especially in regenerative medicine (115). Since hPSCs have cancer-cell characteristics it is challenging that these cells stay normal in their behaviour. They are able to transform their genetic background during long-term culture and may turn to aneuploidic hPSCs (see D.5). It has been shown that the abnormal cells tend to give rise to a teratocarcinoma *in vivo* and not as it should, to a teratoma. A teratocarcinoma is classified as a germ cell tumor that is composed of tissue elements from all three germ layers, but it also contains undifferentiated malignant cells, known as embryonal carcinoma cells (119).

Not only aneuploidic hPSCs can result in a teratocarcinoma. It has been reported that mESCs with a normal karyotype build a teratocarcinoma in immune-incompetent mice. IPSCs are generated by reprogramming a somatic cell by the use of four transcription factors OCT4, SOX2, c-Myc and KLF4. These factors are also known to be highly expressed in different types of cancer (120, 121). Several approaches have been performed by generating hiPSCs without c-Myc or with combining the reprogramming with chemical inhibitors. In comparison to hESCs they show a higher tendency of acquiring chromosomal abnormalities (122, 123).

In consideration of the future clinical use of these cells, the tumorigenic potential of a generated hPSC line needs to be investigated more deeply. Newly derived hPSCs should be tested for the presence of specific undifferentiated markers after differentiation. By assessing the possibility of a teratoma formation one has to investigate more into detail the characteristics of the generated tumor, more specifically whether it may turn to malignancy. Additionally, karyotype analyses should be conducted not only after the derivation of an hPSC line but also during culture before it is used for patient treatment (76).

F. Perfusion-based bioreactor culture system

A bioreactor is defined as an engineered device that supports biological processes under defined and controlled conditions. It is a closed environment with a stable condition such as pH, temperature, nutrient supply and waste removal. Bioreactors offer the possibility to scale-up, which improves the reproducibility and cost-effectiveness (124, 125). To generate a 3D tissue-like structure *in vitro* several requirements are necessary; cells need to be incorporated with cell-to-cell interactions on a 3D scaffold, which act as a template for tissue formation by providing mechanical stability and regulating cell function. The cell-scaffold construct needs to be under suitable conditions so that necessary nutrients can support the tissue differentiation and maturation (126, 127).

An ideal scaffold should act as the natural *in vivo* extra cellular matrix (ECM) imitating its biochemical and biomechanical property. In particular, natural scaffolds are composed of similar macromolecules property as the ECM and are based on different types of proteins such as collagen and fibrin among the most common. Collagen I is the most abundant protein in the mammalian body and therefore it is the most widely used natural scaffold (128).

Cell seeding on scaffolds is the first essential step to ensure tissue formation. The cells need to be seeded with the highest possible efficiency. It has been reported that manual cell seeding mostly leads to irregular cell distribution and poor cell integration into the scaffold. With perfusion-based bioreactors, the cell seeding efficiency can be more ensured.

The perfusion provides a higher mass transfer to the cells to support larger viable and uniform tissue and to avoid necrosis in the inner part of the scaffold (129, 130). Thereby a homogenous cell distribution within the scaffold followed by tissue formation and growth within the scaffold can be guaranteed for prolonged times (127, 131, 132).

Different types of perfusion-based bioreactors have been demonstrated to ensure an efficient cell seeding and reduction of external mass-transfer limitation (Figure 5) (125). The spinner-flask bioreactor ensures an appropriate cell seeding via convection (Fig.5a) while the rotating-wall vessel bioreactors guarantees a high mass transfer with low shear stress (Fig. 5b). In the hollow-fiber bioreactor, the medium perfuses either through or around the semi-permeable fibers which successfully maintain highly metabolic cells (Fig. 5c). The direct perfusion bioreactor allows uniform cell distribution by medium flowing directly through the scaffold (Fig. 5d).

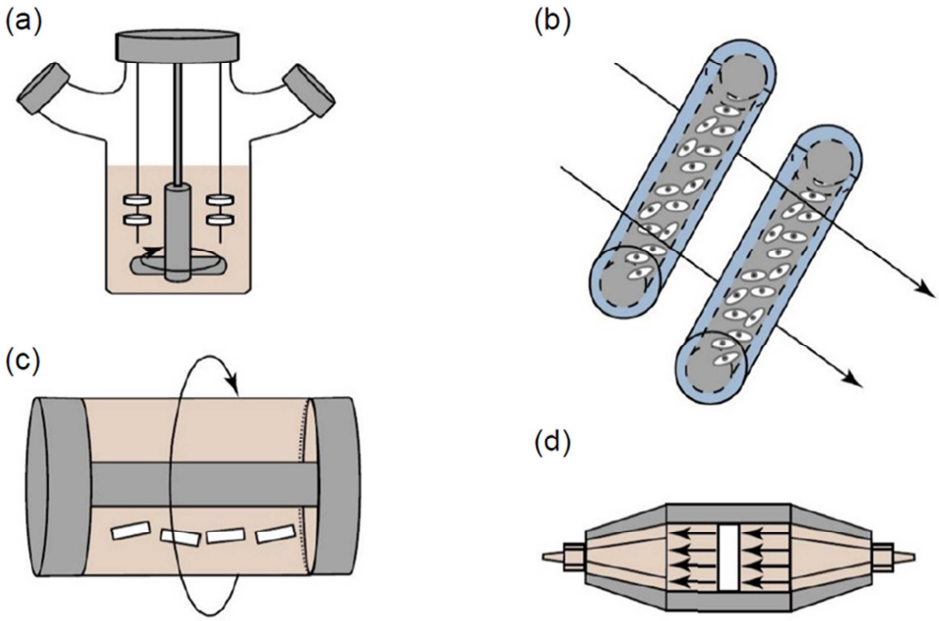

Figure 5: Perfusion-based bioreactors for tissue engineering. (a) Spinner-flask bioreactors were used for cell seeding into 3D scaffolds and for subsequent culture of the construct. (b) Rotating-wall vessels offer a dynamic culture environment to the construct, with high mass-transfer and low shear stress. (c) Hollow-fiber bioreactors are used to enhance mass transfer during cell culture of highly metabolic and sensitive cells. (d) Direct perfusion bioreactors in which medium flows directly through the scaffold, often used for seeding and culturing of constructs (adapted from (125)).

The quality of the generated tissues under perfusion also depends on the applied flow rate. Different magnitudes of flow-mediated shear stress exposed to the cells have an impact to the generated tissue. For example, higher magnitudes of shear have been correlated with increased mineralized-matrix production by bone-marrow of cells. Moreover, a scaffold with a homogeneous distribution of pores has been shown to control more precisely the shear stresses over time. In this context, it is evident that the scaffold used for the approach of tissue generation should not be limited to biocompatibility but also in the evaluation of pore structures, which must be adapted to particular flow conditions (125, 133).

The mass transport of oxygen in tissue cultures is crucial for the maintenance of cell viability and function. Moreover, it could be demonstrated that the differentiation stage of various cells can be changed by applying different oxygen levels during culture (e.g. 5% O_2 versus 20% O_2 to chondrocytes). Thus, it is evident to quantify the level of oxygen within the perfused tissue to make sure that a specific flow rate supply sufficient oxygen (133).

Computational fluid dynamic (CFD) softwares have been developed in the past years which are powerful tools to calculate the appropriate flow rates, shear stresses and mass transport within a construct (125).

Bioreactors not only offer the possibility to generate tissues but also acts as a 3D culture model to mimic *in vivo* environment (125). It is also a platform to regulate the stem cell microenvironment, the so-called "niche" which affects the stem cell fate (134). Moreover, this system is promising in the field of disease modelling and drug discovery, as for example in cancer research by mimicking the tumorigenic development to further test specific anticancer compounds (129, 135) . Moreover, it has to be considered that the bioreactor approach is a promising new model system, which in combination with hPSC cells can lead to a further understanding of human developmental aspects. All these different approaches using the bioreactor leads as well to a tremendous reduction of animal use, since the *in vivo* tool has been used so far to model human diseases with the aim to understand the mechanisms and subsequently the effect of different drug compounds (51).

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Aim of the thesis

The generation and use of human pluripotent stem cell (hPSC) lines are expected to have a substantial impact on medical and biological science. Their capacity of unlimited self-renewal on one hand and of broad differentiation capacity into any cells types on the other hand make them powerful and versatile tools to investigate early human development and diseases. Moreover, with the increasing ability to direct their differentiation *in vitro* into selected cell types and to modify them by molecular engineering, clinicians hope to use hPSC-derived products for therapeutic purposes. However, different techniques to generate new lines are being used and the way of analyzing their pluripotent character varies widely between the lines. Furthermore, and maybe consequently, functional, phenotypical and molecular differences between lines are observed. Thus, extensive and standardized characterization of hPSCs lines might be an important prerequisite to allow stem cells and stem cell technology to fulfill their expectations.

In **chapter I** I describe the characterization of four hESC lines that we generated and maintained using the same protocol. We compared their stemness and differentiation properties and defined their differences, despite their maintenance under identical conditions.

In **chapter II** I propose the establishment of a perfusion-based culture system to generate a teratoma-like tissue *in vitro*. Teratoma formation *in vivo* after hPSCs grafting, i.e. the formation of nontumorigenic advanced tissues and structures derived from the three embryonic layers, is nowadays the standard to prove pluripotency. However, there are many inconsistencies in the protocols used to induce a teratoma *in vivo* and in the way the results are analyzed and reported. For these reasons, we have set up and characterized an animal-sparing, more standardized and controlled method to ensure a proper assessment of pluripotency, which could be used as an alternative or complementary to the current *in vivo* teratoma formation assay.

Chapter I:

„Gene Expression Profiles of Similarly Derived Human Embryonic Stem Cell Lines Correlated with Their Distinct Propensity to Exit Stemness and Their Different Differentiation Behavior in Culture“

Enclosed is the paper published in Cellular Reprogramming, 2014 Jun, 16(3):185-95
In this paper I contributed to the characterization of the newly derived hESC lines

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Gene Expression Profiles of Similarly Derived Human Embryonic Stem Cell Lines Correlate with Their Distinct Propensity to Exit Stemness and Their Different Differentiation Behavior in Culture

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Abstract

Four normal-karyotype human embryonic stem cell (hESC) lines were generated using the same protocol and maintained under identical conditions. Despite these precautions, gene expression patterns were found to be dissimilar among the four lines. The observed differences were typical of each cell line, correlated with their distinct propensity to exit stemness, created heterogeneity among the cells during cell line maintenance, and correlated with their altered capacity as a source of differentiated cells. The capacity of some cell lines to give rise to more, and more mature, neurons within comparable time frames of directed differentiation reflected the distinct proportions of cells already predifferentiated at the onset. These findings demonstrate that the subsequent stages of neural differentiation were altered both in a quantitative and timely fashion. As a consequence, cell lines with apparent better and quicker ability to produce neurons were actually the less capable of reproducing proper differentiation. Previous data suggested that cell lines able to generate more neurons faster would be more suitable to clinical application. Our analysis of the differentiation process strongly suggests the opposite. The spontaneous tendency to predifferentiate of any particular hESC line should be known because it clearly impacts further experimental results.

Introduction

THE FIRST HUMAN EMBRYONIC stem cells (hESCs) were derived from the inner cell mass (ICM) of embryos donated for research (Thomson et al., 1998). Since then, hESCs have been recognized as a potent tool in basic research and they are considered to hold great promise for regenerative medicine (Siniscalco et al., 2008; Srivastava et al., 2008; Sterthaus et al., 2009). However, it has also been reported that despite the expression of a common pluripotent phenotype, hESC lines derived from single-stage embryos display important variations in their gene expression patterns (Abeyta et al., 2004; Guenther et al., 2010; Laurent et al., 2010; Skottman et al., 2005), possibly affecting their behavior during differentiation (Lappalainen et al., 2010; Osafune et al., 2008; Tavakoli et al., 2009).

The causes of the variability of gene expression patterns among hESC lines are unknown. The different genetic background of the human embryos used to derive the lines or differences in the culture conditions used during derivation and/or expansion of the cell lines rank among possible causes (Skottman et al., 2006; Tavakoli et al., 2009). These differences are likely to impact on any experimental outcome, while the practical consequences still need to be identified. The capacity of some hESC lines to give rise to more neurons after an identical period of directed differentiation has been

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documented (Lappalainen et al., 2010; Tavakoli et al., 2009), but the degree of different hESC lines all derived under very similar conditions to enter the neural differentiation process in a synchronized way, a prerequisite to obtain a high rate of defined neuron-subtypes, has never been analyzed in detail.

Four new hESC lines were generated in our institution using the same protocol. This gave us the opportunity to address the question of inherent versus derivation-dependent or culture-dependent differences in gene expression and differentiation behavior of hESC lines. Although the derivation and maintenance of the hESC lines were carried out under very similar conditions, inherent differences in their gene expression patterns became soon evident. These differences correlated with their distinct tendency to predifferentiate spontaneously during stem cell maintenance. We describe several aspects of their differentiating behavior, found to be altered as a result of an initial greater propensity of some cells to predifferentiate. We discuss those observations in terms of care in the choice of cell lines adapted to specific diagnostic or therapeutic purposes.

Materials and Methods

Regulatory aspects and laboratory environment

All procedures related to the donation of surplus embryos deriving from assisted reproduction technology (ART) and their use for research purposes were in accordance with the Swiss law on ART and the Swiss law on human ESC research. This project was approved both by the local ethics committee (EKBB) and by the Swiss Federal Office of Public Health (BAG) (see Supplementary Data available at www.liebertpub.com/cell/). All experiments were carried out in a laboratory equipped with grade A, particle–free technology. This laboratory is separated from, but in close proximity to, the ART unit. All procedures and working conditions in the embryology laboratory of the ART unit were modified to xeno-free conditions, including full traceability of the components of all processes.

hESC derivation and culture

After fertilization, embryos were cultured at 37°C in a humidified gas mixture containing 6% CO_2 and 6% O_2 for 3 days in K-SICO (Cook Medical, www.cookmedical.com), without medium changes in 25-μL droplets under oil (K-SIMO; Cook Medical). On day 3, the medium was changed to K-SIBO (Cook Medical). On day 6 or 7, the donated blastocysts were mechanically disrupted into two pieces. The pieces with the ICM were further cultured in hESC medium on a human feeder layer [35 Gy γ-irradiated foreskin fibroblasts; American Type Culture Collection (ATCC CRL2429)]. After 6–8 days of culture, the outgrowth was mechanically split into six to 10 pieces and again placed onto a new feeder layer. After further 6–8 days, the first hESC colonies started to appear. For stem cell culture, knockout DMEM-KO culture medium (Invitrogen, catalog no. 10829-018) was supplemented with 20% KSR (Invitrogen, catalog no.10828-028), 2 mM L-GlutaMax (Invitrogen, catalog no. 35050-038), 0.05 mM β-mercaptoethanol (Invitrogen, catalog no. 31350-010), 0.1 mM nonessential amino acid (Invitrogen, catalog no. 11140-035), and 8 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, catalog no. 234-FSE-025/CF) in the presence of penicillin (50 U/mL) and streptomycin (50 μg/mL; Invitrogen catalog no. 15140-122) and is denominated henceforth hESC-M.

Characterization of hESC lines with the alkaline phosphatase assay and with immunohistochemistry

Alkaline phosphatase (AP) staining and immunofluorescence were performed according to the recommendations provided by the manufacturer (Millipore, catalog no. SCR001). For AP-staining, ESCs were cultured for 4–5 days, fixed, and incubated in the staining solution Fast Red Violet for 15 min. For immunofluorescence staining, cells were incubated with primary antibodies against the stemness markers Oct-4, SSEA-4, TRA-1-60, and TRA-1-81 at 1:200 (Millipore, catalog no. AB3209). Secondary antibodies fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin M (IgM)/IgG (Sigma, catalog no. NF-1010) or tetramethylrhodamine (TRITC)-labeled donkey anti-rabbit IgG (Sigma T-6778) were used at 1:200 for 30–60 min at room temperature.

In vivo *differentiation analysis*

Eight- to 10-week-old immune-incompetent nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River Laboratories, Germany) were used to demonstrate the *in vivo* differentiation potential of the newly derived hESC lines. Housing and experimental protocols were approved by the Basler Cantonal Veterinary Department and were in accordance with Swiss Animal Protection Law. About 100–200 cells from 60 colonies of the CHES2, CHES3, or CHES5 line were mixed with Matrigel (BD Biosciences, catalog no. 354277) and injected subcutaneously into two NOD/SCID mice. Two months later, the mice were sacrificed, the tumors removed for Hematoxylin & Eosin (H&E) staining and histological evaluation with a conventional light microscope (Dialux 20; Leitz, Zeiss Axio Imager A.1). Immunohistochemistry was performed on a Ventana Immunostainer using Cytokeratin (CK22, Biomeda,1:500), neuron-specific enolase (NSE), desmin (DES), vimentin (VIM; Ventana/ Roche), and smooth muscle actin (SMA; DAKO), antibodies prediluted by the manufacturer.

Differentiation in vitro

For random differentiation into the three germ layers, hESC colonies were mechanically scraped off the feeder layer. To form embryoid bodies (EBs), two to three pieces, each consisting of around 50–100 cells, were placed into 50-μL droplets of hESC medium without bFGF. Some EBs were analyzed after 4 days by quantitative PCR (qPCR). For further differentiation, the EBs were plated onto a gelatinized dish and cultured for 8–14 days in the hESC medium in which FCS was substituted for KSR.

For directed neural differentiation, the colonies of each hESC line were removed from the feeders, dissociated into small clumps, and expanded in suspension in low-attachment-well plates (HydroCell™, Nunc, catalog no. 2014-05) (Steiner et al., 2010). The cells were amplified in hESC-PRO medium corresponding to neurobasal medium (Invitrogen, catalog no. 21103049) supplemented with 14% knockout DMEM-KO culture medium (Invitrogen, catalog no.10829-018), 1× nutridoma-CS (Roche, catalog no. 11363743001), 2 mM L-GlutaMax (Invitrogen, catalog no. 35050-038),

penicillin (50 U/mL), and streptomycin (50 μg/mL; Invitrogen, catalog no. 15140-122), 0.1 mM nonessential amino acids (Invitrogen, catalog no. 11140-035), 25 ng/mL recombinant human Activin A (Prospec CYT-569), 0.5 μg/mL human laminin (Sigma, catalog no. L4544), and 20 ng/mL recombinant human bFGF (R&D Systems, catalog no. 234-FSE-025/CF). Clumps were partially dissociated once a week by gentle shaking and cultured in the presence of 10 μM ROCK inhibitor (Sigma, catalog no. Y0503) for 1 day. For one experiment, hESC-PRO was substituted with TeSR1 (StemCell Technologies, catalog no. 05850). To start neuroectodermal induction, the clusters were gently dissociated and plated on 5 μg/mlL (collagen IV, Sigma, catalog no. C5533) at a density of 90,000 cells/cm^2 and cultured in neural differentiation medium consisting of Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen, catalog no. 31331-028), 1×N-2 Supplement (Invitrogen, catalog no. 17502-048), 0.1 mM nonessential amino acids, penicillin (50 U/mL), and streptomycin (50 μg/mL; Invitrogen, catalog no. 15140-122), and 600 ng/mlL recombinant human noggin (Prospec CYT-475) for 7 days. The cells were then further cultured for an additional 2 weeks in the same medium but in the absence of noggin. Immunofluorescence was performed using mouse anti-βIII tubulin (1/200; Sigma, catalog no. T5076) or rabbit anti-βIII tubulin (1/200; Covance, catalog no. PRB-435P), rabbit anti-MAP2 (1/50; Sigma, catalog no. HPA 012828), and mouse anti-Nestin (1/50; R&D Systems, catalog no. MAB1259). Secondary antibodies Alexa Fluor 488 or 546 conjugated with either anti-mouse or anti-rabbit IgGs were used at 1/1000. The nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, catalog no. 32670).

Quantitative PCR

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, catalog no. 740955) according to the manufacturer's instructions. For each sample, 300 ng of total RNA was reverse-transcribed into single-stranded cDNA by the SuperScript®II Reverse Transcriptase (Invitrogen, catalog no. 18064.014) in presence of 0.5 μM of deoxynucleotide triphosphates (dNTPs; Invitrogen, catalog no. 10297-018) and a mix of 5 ng/μL random hexamer primers (Invitrogen, catalog no. 48190-011).

One-tenth of each cDNA was used for each PCR reaction, performed following the guidelines given by the manufacturer (SABiosciences; Embryonic Stem Cells PCR Array, catalog no. PAHS-081) by using Power SYBR® Green PCR Master Mix (Applied Biosystems, catalog no. 4367659) in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). For each sample, two qPCR measurements were performed, and the average was used to calculate the change fold. The qPCR primers used were synthesized by Microsynth (Balgach, Switzerland) and are described with their used annealing temperature in Figure S4 (Supplementary Data are available at www.liebertpub.com/cell/).

Results

Establishment of four new hESC cell lines

From August, 2008, to August, 2010, a total of ~800 infertile couples were treated with ART. During this time interval, 15 blastocysts were donated for hESC derivation, but only seven were used with our standard derivation protocol (see Materials and Methods). All donated embryos arose from pronucleate oocytes that were cultured after intracytoplasmic sperm injection and developed up to the blastocyst stage. Four hESC lines were generated from those seven embryos following a fixed and established protocol (Inzunza et al., 2005).

Between days 6 and 8 after fertilization, the hatched blastocysts were cultured on a human foreskin fibroblast feeder layer (CRL2429). Three seeded blastocysts produced no outgrowth (Table 1). The growing cell mass of the remaining four blastocysts was cut mechanically into six to 12 pieces and further cultivated on a feeder layer for another 6 days. When they developed ESC-like colonies, they were separated and grown over prolonged time periods. All four lines, CHES2, CHES3, CHES5, and CHES6, have been fully characterized and registered, and their characteristics have been published in the European Human Embryonic Stem Cell Registry (hESCreg).

Cells were karyotyped using standard GTG banding metaphase spreads, and normal chromosome complements were found in all four hESC lines (Fig. 1A–D). For CHES2, a comparative genomic hybridization was performed at passage 22 to detect rearrangements or multicopy amplifications, but no aberrations were detected. All cell lines (CHES2 at passage 70, CHES3 at passage 40, CHES5 at passage 40, and CHES6 at passage 48) were characterized with immunohistochemistry, and the expression of a number of typical stemness markers, such as AP, TRA60, TRA81,

Table 1. Processing and Grading of Donated Blastocysts Used for hESC Derivation

Blastocyst grading,[a], *day of grading and plating*	*Outgrowth*[b]	*Treatment of embryo or outgrowth*	*Name of hESC line*
6/a/b, day 6	Yes	Plated after self-hatching	ChES2
5/b/b, day 6	Yes	Cut off of hatched side (ICM outside)	ChES3
5/c/c, day 6 but plated on day 7 after collapse	No	Blastocyst degraded did not survived day 7	—
3–5/b/b, day 6	Yes	Started to hatch, mechanical squeezed and cut in half but only one half attached	—
6/a/a, day 7	Yes	Plated after self hatching and cut in half	ChES5
5/a/b, day 6	Yes	Cut off from hatched side (ICM outside)	ChES6
5/b/b, day 7 but collapsed on day 8	No	ZP opened by knife and plated	—

[a]Grading of blastocyst according to Gardner et al. (2000).
[b]Cell cluster forming after embryo plating on feeder.
hESC, human embryonic stem cell; ICM, inner cell mass; ZP, zona pellucida.

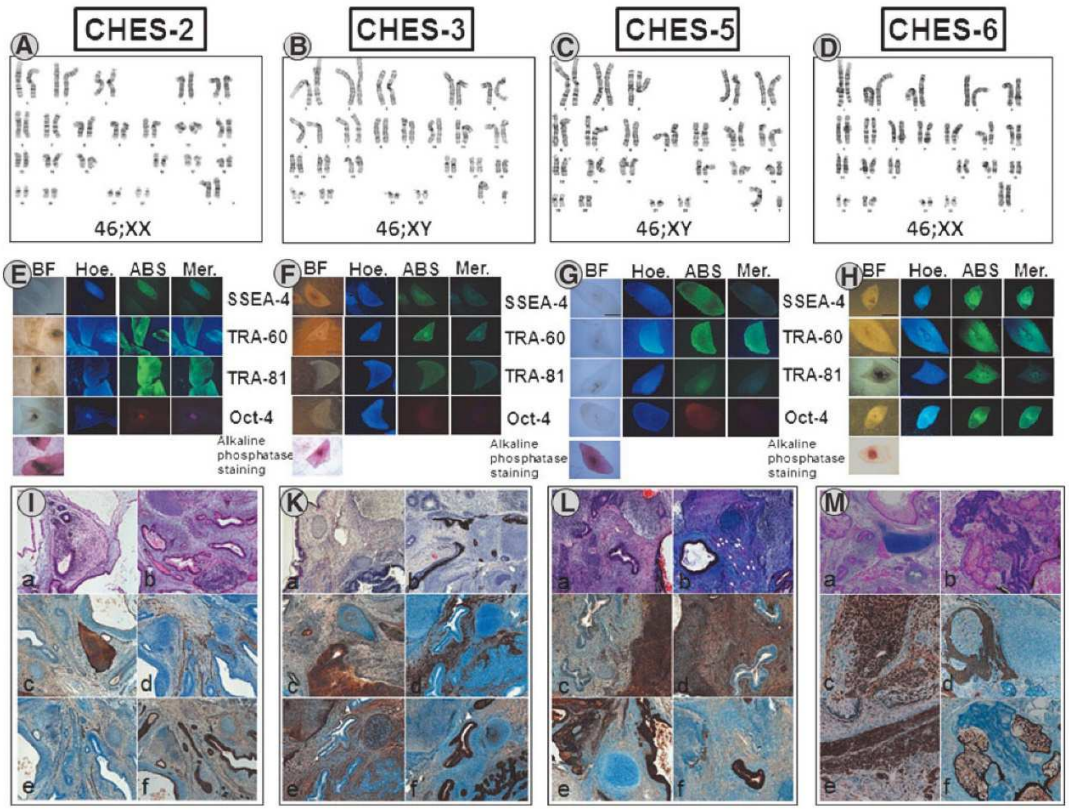

FIG. 1. Characterization of new established lines CHES2, CHES3, and CHES5 by karyotyping, immunohistochemistry of the hESCs and histological teratoma analysis (**A–D**) Karyotype analysis of CHES 2, CHES 3, and CHES 5. (**A**) CHES2 analyzed in passage 10, 46XX. (**B**) CHES3 analyzed in passage 8, 46XY. (**C**) CHES5 analyzed in passage 6, 46XY. (**D**) CHES6 analyzed in passage 23, 46XX. (**E–H**) Immunohistochemistry of CHES2, CHES3, and CHES5. BF, bright field; Hoe, Hoechst staining; ABS, antibody staining; Mer, merge. Magnification, 50×; bar, 1 mm. (**E**) CHES2. (**F**) CHES3. (**G**) CHES5. (**H**) CHES6. (**I–M**) Histology showed a tumor composed of tissue components derived from all three germ layers (**I**) CHES2: (**a**) Immature neuro-ectodermal tissue with neuropil and rosette formation is predominant in the center of this image. Conventional histology, H&E, magnification 100×. (**b**) Tumor area with mesodermal spindle cell stroma and endodermal glandular tissue components. H&E, magnification 100×. (**c**) Immunohistochemical staining for neuron specific enolase highlighted neural elements in brown. NSE, magnification 100×. (**d**) Mesodermal smooth muscle differentiation was depicted by smooth muscle actin immunohistochemistry. SMA, magnification 100×. (**e**) Mesodermal skeletal muscle differentiation was revealed by immunohistochemistry for desmin. DES, magnification 100×. (**f**) Epithelial differentiation of endodermal and ectodermal structures was shown by immunohistochemistry for panepithelial pancytokeratin immunohistochemistry for cytokeratin 22. CK22, magnification 100×). (**K**) CHES3: (**a**) Tumor area with endodermal cysts and mesodermal stroma including cartilage islands. H&E, magnification 100×. (**b**) Neurectodermal rosettes adjacent to pigment epithelium as abortive retina anlage. H&E, magnification 100×. (**c**) Neurectodermal rosettes and neuropil highlighted in immunohistochemical reaction for NSE. NSE, magnification 100×. (**d**) Mesodermal spindle cell stroma with smooth muscle differentiation as shown in SMA immunohistochemistry. SMA, magnification 100×. (**e**) Mesodermal spindle cell stroma and hyaline cartilage with diffuse expression of vimentin. VIM, magnification 100×). (**f**) Epithelial endodermal structures with cytokeratin expression. CK22, magnification 100×. (**L**) CHES5: (**a**) Solid tumor area with neurectodermal components with formation of rosettes and neuropil. H&E, magnification 100×. (**b**) Formation of myxoid interstitial matrix, particularly in mesodermal hyaline cartilage island and adjacent mesodermal loose connective tissue. Alcian Blue PAS, magnification 100×. (**c**) Expression of NSE in neurectodermal tumor area. NSE, magnification 100×. (**d**) Diffuse expression of Vimentin in mesodermal spindle cell stroma. VIM, magnification 100×. (**e, f**) Epithelial endodermal tumor components with cytokeratin expression, in contrast to absence of cytokeratins in hyaline cartilage. CK22, magnification 100×. (**M**) CHES6: (**a**) Immature neurectodermal tissue with neuropil and rosette formation is predominant in the center of this image. Conventional histology, H&E, magnification 100×. (**b**) Solid tumor area with neurectodermal components with formation of rosettes and neuropil. H&E, magnification 100×. (**c**) Immunohistochemical staining for neuron-specific enolase highlighted neural elements in brown. NSE, magnification 200×. (**d**) Mesodermal smooth muscle differentiation was depicted by smooth muscle actin immunohistochemistry. SMA, magnification 100×. (**e**) Mesodermal skeletal muscle differentiation was revealed by immunohistochemistry for desmin. DES, magnification 200×. (**f**) Epithelial differentiation of endodermal and ectodermal structures was shown by immunohistochemistry for panepithelial pancytokeratin immunohistochemistry for cytokeratin 22. CK22, magnification 100×).

TABLE 2. qPCR DATA GENERATED WITH PCR ARRAY COMPARING NEW DERIVED hESC LINES WITH CONTROL LINE HS401

Regulation (compared to HS401)			
Genes[a]	*CHES2*	*CHES3*	*CHES5*
CDX2	2.96[b]	2.34	**3.33**
GAL	−2.90	−1.96	**−3.85**
GBX2	**5.94**	1.70	**3.43**
LEFTY1	−1.59	−2.77	**−5.91**
NEUROD1	**3.93**	−2.08	1.01
NODAL	−1.94	−1.53	**−4.94**
NOG	2.98	1.05	**3.46**
PAX6	**13.98**	1.25	**4.65**
SERPINA1	**4.12**	1.94	1.56
SYCP3	−1.01	−1.82	**−3.61**

[a]Only genes with three fold regulation are listed.
[b]Fold regulation of new hESC line compared to HS401.

SSEA-4, and OCT-4, was confirmed (Fig. 1E–H). These hESC lines were capable of undergoing *in vitro* differentiation to EBs containing cells belonging to the three germ layers (see Fig. S1). These EBs can then be further differentiated into cardiomyocytes and neurons (data not shown). For full characterization of these cell lines, teratomas were formed *in vivo* showing derivative tissues of all three germ layers as well (Fig. 1I–M).

Comparison of the gene expression patterns of the newly derived hESC lines

Three hESC lines were then characterized by qPCR array, including 44 ESC-specific genes and 40 selected ESC differentiation genes (see Fig. S2). CHES3 exhibited a gene expression profile similar to the commonly used reference line HS401 (Table 2) (Martin-Ibanez et al., 2008). For none of the genes examined in both cell lines was a more than three-fold difference in the expression levels detected. In contrast, when compared to HS401, four genes were differentially expressed in CHES2 and eight in CHES5. Some are stem cell–specific genes and were differentially expressed at either a higher or lower level: GBX2 (+5.9) in CHES2, GAL (−3.9), GBX2 (+3.4), LEFTY1 (−5.9), NODAL (−4.9), and NOG (+3.5) in CHES5. Furthermore, those two cell lines expressed higher levels of some differentiation genes—neuro-ectodermal markers NEUROD1 (+3.9) and PAX6 (+13.9) as well as the endodermal marker SERPINA1A (+4.1) were overrepresented in CHES2, whereas PAX6 (+4.7) and the trophoblastic marker CDX2 (+3.3) were more expressed in CHES5. In addition, the germ cell marker SYCP3 (−3.6) appeared underexpressed in the latter.

The expression levels of these genes in the hESC lines were also compared with those of neonatal foreskin fibroblasts to account for any potential contamination by the feeder cells (see Fig. S3). When compared to fibroblasts, a number of pluripotency markers were highly overexpressed in all hESC lines (GAL 67–260 times, LEFTY1 110–652 times, NODAL 38–189 times, GBX2 4.2–25 times), whereas Nog was overexpressed more than three times only in CHES2 and CHES5. However, other stem cell markers did not appear to be overrepresented because they were already expressed at similar (LIFR, TERT, NANOG) or even higher levels (Il6ST, IFITM2, FGF5, and COMMD3) in the foreskin fibroblasts (see Fig. S4c and S4d, respectively).

Some genes classified as differentiation markers (FN1, LAMA1, LAMB1, LAMC1, and DES) were expressed at similar levels in both stem cells and fibroblasts. For that panel of genes, the relative expression profile was not significantly different among the various hESC lines (see Fig. S4b). However, other differentiation markers were clearly overexpressed in hESCs when compared to fibroblasts. The neural marker PAX6 (7.9–110 times), the trophoblast marker CDX2 (3.7–5.3 times), and the visceral endoderm marker SERPINA1 (3.0–8.0 times), as previously found, but also the visceral endodermal marker α-fetoprotein (AFP; 14.9–28.9 times), the

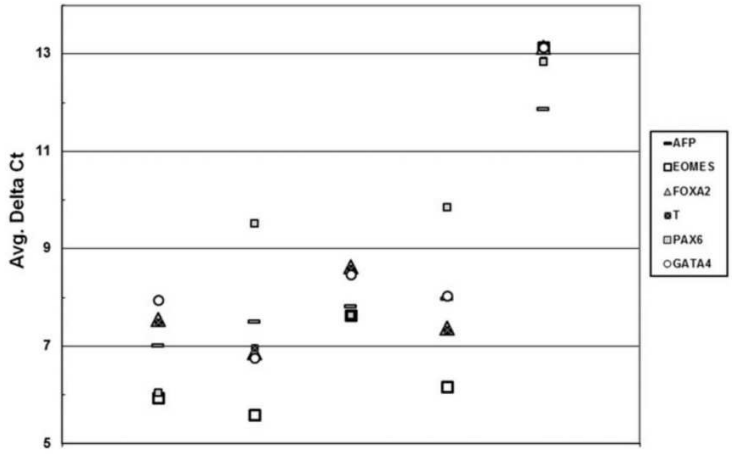

FIG. 2. qPCR data generated from qPCR array. Average delta Ct values of CHES2, CHES3, CHES5, HS401, and CRL2429 fibroblasts are shown. Differentiation markers overexpressed in the hESC lines are compared to CRL2429.

trophoblast marker EOMES (45.7–186.8 times), the extra-embryonic endoderm FOXA2 (22.8–78.0 times), and the mesoderm marker T-Brachyury (Bra; 19.4–59.4 times) were overexpressed in hESC lines. Those correspond mostly to genes not expressed in fibroblasts (Fig. 2). In the panel of genes used here, each cell line exhibited a different expression profile, which was not influenced by the amount of feeder cell transcripts in the samples (see Fig. S3). Furthermore, the similarities of the expression profiles of HS401 and CHES3 and those of CHES2 and CHES5 were striking.

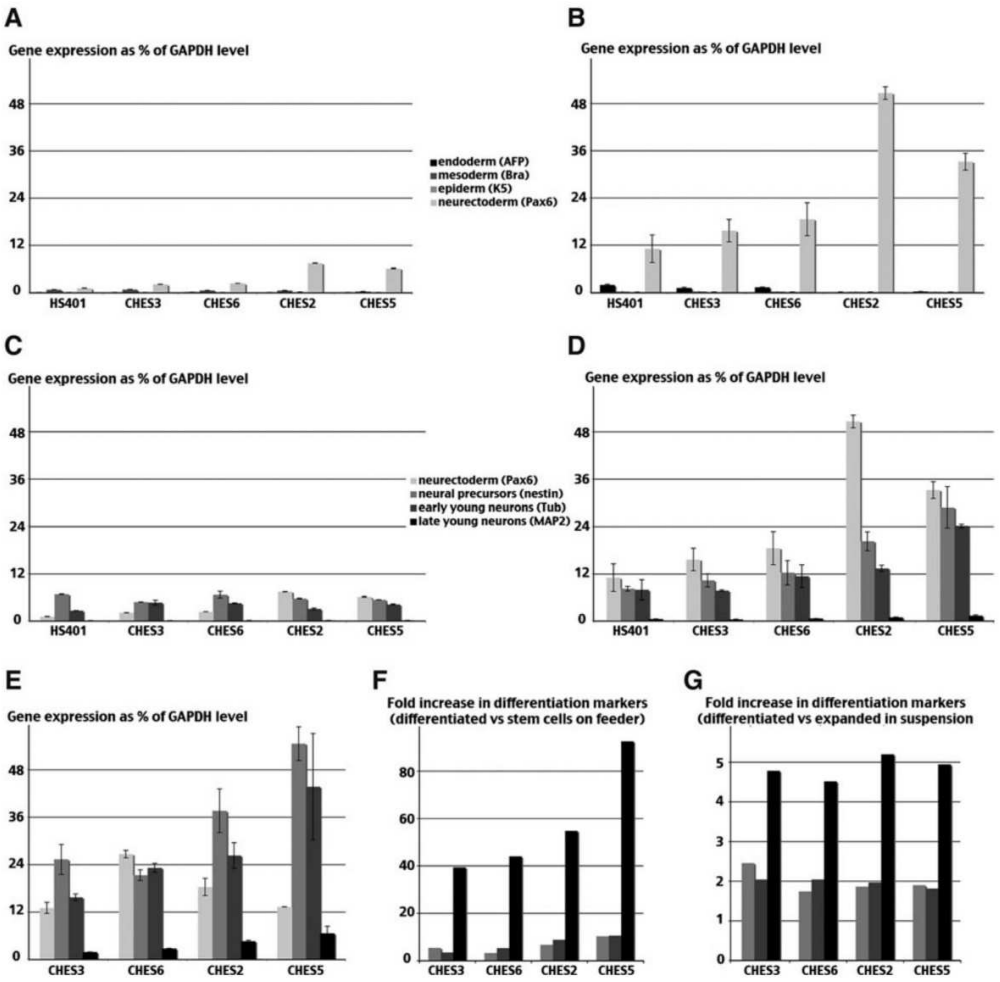

FIG. 3. Comparison of spontaneous and directed neural differentiation of CHES2, CHES3, CHES5, and CHES6 cell lines. Expression of various differentiation markers was quantified by semiquantitative RT-PCR in ESCs maintained on feeders (**A** and **C**), expanded in suspension culture (**B** and **D**), and after 3 weeks of directed differentiation into the neural lineage (**E**). For each gene, the relative expression level is expressed as a percentage of the housekeeping gene *GAPDH*. Spontaneous commitment into the three embryonic germ layers was confirmed by the endodermal marker α-fetoprotein (AFP), the mesodermal marker Brachyury, and the two ectodermal markers K5 and Pax6 (for epidermis and neurectoderm, respectively) in cells cultured on fibroblast feeders (**A**) or amplified in suspension culture (**B**). Spontaneous differentiation into the neural lineage was compared by using the neurectodermal marker Pax6, the neural precursor marker Nestin, the early marker of young neuron β-tubulin III (Tub), and the marker for more differentiated neurons MAP2 both in cells cultured on fibroblast feeder (**C**) or amplified in suspension culture (**D**). The level of differentiation achieved for cells first amplified in suspension, then plated on collagen IV, and cultured for 3 weeks in neural differentiation medium was determined by quantifying the relative expression level of the same markers (**E**) and compared to their expression in undifferentiated cells cultured on feeders (**F**). The differentiation rate of each cell line was estimated by comparing the expression level of those genes measured after 3 weeks of allowed neural differentiation to their level in suspension culture immediately before plating (**G**).

Spontaneous differentiation propensity and directed neural differentiation capabilities of the four hESC lines

To test the biological significance of the observed differences in gene expression among the various newly derived hESC lines, we compared their capacity for neural differentiation under identical conditions. As shown in Figure 3A and in accordance with the qPCR array, their different ability to maintain stemness when cultured on fibroblast feeders gave rise essentially to the expression of neuro-ectodermal markers (*i.e.*, Pax6 in the experiment), whereas markers of other layers were detectable by qRT-PCR at a much lower level (*i.e.*, AFP as a marker for endoderm, T-Brachyury for mesoderm, and keratin-5 for nonneural ectoderm).

Furthermore, large-scale expansion in a defined microcarrier-free suspension culture system (Steiner et al., 2010) appeared even more permissive because spontaneous neuroectodermal commitment increased while retaining the line-specific differences already observed (Fig. 3B). CHES3 and CHES6 appeared more prone to remain undifferentiated, whereas the CHES2 and CHES5 cell lines had a greater tendency toward spontaneous commitment. In addition, low levels of neural differentiation were also observed in both culture conditions as the marker of neural precursor nestin, and the marker of early neuronal development βIII-tubulin was detected as well (Fig. 3C and D). That predifferentiation tendency appeared similarly constrained for both cell lines when cultured on feeders (Fig. 3C), whereas in the more permissive suspension culture, predifferentiation was more pronounced, especially in CHES2 and CHES5 cell lines, which expressed low but, when compared with the other two lines, significantly higher levels of MAP2, a marker of more advanced neurons (Fig. 3D).

When plated on collagen and cultured in neural differentiation medium, all cell lines displayed a similar pattern of morphological changes typical of neuronal differentiation (not shown). To quantify neuronal differentiation, we measured the relative levels of differentiation markers after 3 weeks in culture. As shown in Figure 3E, markers of both neural precursors and young neurons increased in all cell lines. When compared to the essentially undifferentiated initial cell populations present in cultures on feeder, the expression level of the neural precursor Nestin was five to 10 times higher, and the expression level of the neuronal marker MAP2 was increased 40–90 times. However, CHES5 achieved a level of differentiation twice that of CHES3 and CHES6, whereas CHES2 exhibited an intermediate profile (Fig. 3F). In contrast, when the expression levels of the various differentiation

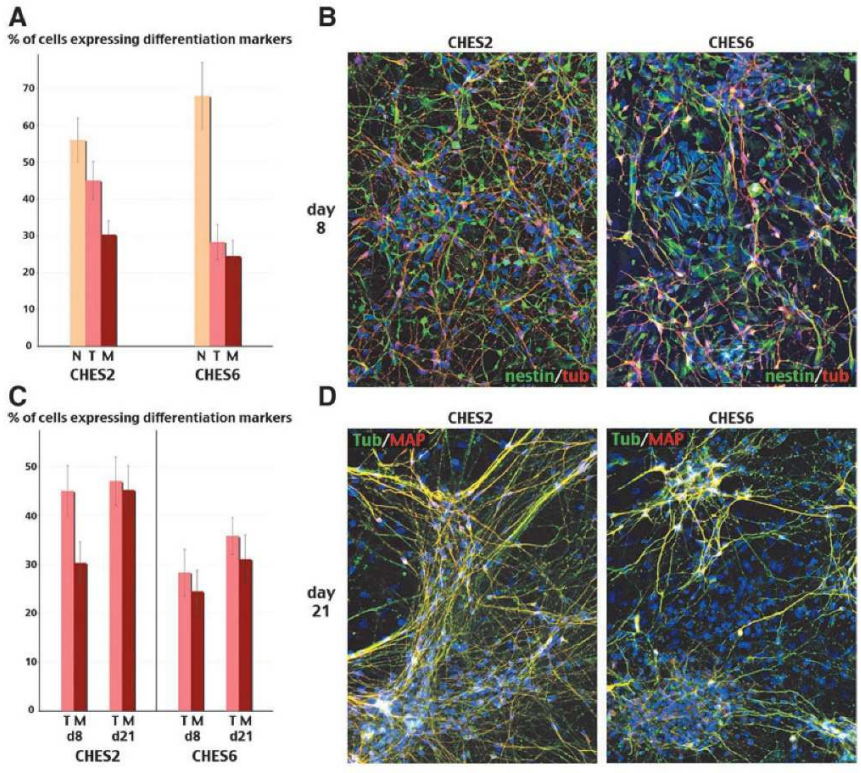

FIG. 4. The number of cells expressing neural differentiation markers was determined after 8 (**A**) and 21 days of neuronal differentiation (**C**). Immunostaining was performed after 8 (**B**) and 21 days (**D**) of neuronal differentiation. Cells were counted after immunofluorescence staining against nestin (N), β-tubulin III (T), and MAP2 (M) and expressed as percentage of the total cell number ($DAPI^+$ cells).

markers at the end of the experiment were compared with their levels immediately before plating, the rate of increase in differentiation markers expression was indistinguishable among the four cell lines (Fig. 3G).

We confirmed by immunocytochemistry that the differences in global expression of differentiation markers indeed reflect distinct capacities of different hESC lines to give rise to neurons during a given time period of directed differentiation. For this purpose we analyzed the percentage of cells expressing those markers in two cell lines (CHES2 and CHES6) with distinct gene expression profiles (Fig. 4). After 8 days in differentiating culture conditions, almost 100% of cells in both lines were actually engaged in the neural lineage, either as neural precursors expressing nestin or as young neurons expressing βIII-tubulin (Fig. 4A). However, CHES6 cells were mostly typical bipolar nestin-positive neural precursors, only around a quarter of the culture being composed of young neurons (Fig. 4, A and B). In contrast, nearly twice as many of the CHES2 cells differentiated to neurons (Fig. 4A), and cells expressing nestin exhibited a more advanced morphology resulting in cells displaying an ambiguous morphological status, halfway between still true precursors and very young neurons but not yet expressing βIII-tubulin (Fig. 4B). Although the number of neurons increased slightly in both cultures during the two subsequent weeks of differentiation, the difference between the two cell lines was only marginally reduced (Fig. 4C). As neurons maturated and progressively co-expressed βIII-tubulin and MAP2, differences in maturation remained clearly visible between both cell lines. After 3 weeks of differentiation, most CHES2 neurons were expressing MAP2, whereas a significant number of CHES6 neurons were still only expressing βIII-tubulin (Fig. 4C and D). These differences in the advancement of neural differentiation among both cell lines are also visible in their morphology: βIII-tubulin and MAP2 coexpressing neurons of CHES2 exhibit more developed extruding processes, which even started to form networks (Fig. 4D). Thus, the higher level of differentiation exhibited by hESC lines with a higher propensity to predifferentiate spontaneously implied both the generation of more neurons and the formation of more mature neurons during the same time interval.

Consequences of spontaneous predifferentiation for further behavior of neural cell populations during the differentiation process

This property of some hESC lines to predifferentiate was also responsible for an asynchrony in the differentiating cell populations, which rendered more difficult the distinction between the successive phases of the neuronal differentiation process. As clearly visible for CHES6 in Figure 5, in which predifferentiation of the starting population was moderate, those phases could be visualized by following the stepwise evolution of expression of selected markers during differentiation. A transient upregulation of Pax6 expression

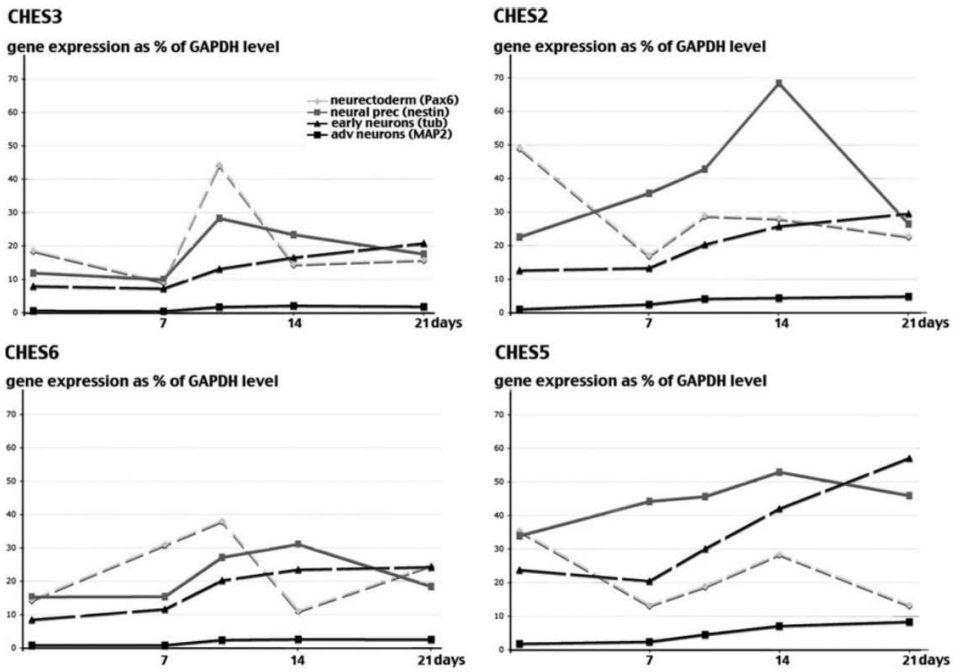

FIG. 5. Expression time-course of neural differentiation markers in differentiating CHES2, CHES3, CHES5, and CHES6 cell lines. Expression of neural genes Pax6, nestin, β-tubulin III (Tub), and MAP2 in differentiating CHES2, CHES3, CHES5, and CHES6 lines was quantified 7, 10, 14, and 21 days after initiation of directed neural differentiation (as in Fig. 4).

corresponding to the commitment of stem cells into neurectoderm was followed by a wave of Nestin expression as neuroectodermal cells transformed into neural precursors. Those precursors then gave rise to neurons expressing first βIII-tubulin, then MAP2. In the absence of mitogenic factors needed to sustain the proliferation of precursor cells, such as bFGF, the level of Nestin expression decreased proportionately with the increase in the expression of neuronal markers, indicating the progressive replacement of proliferating progenitors by maturating neurons. In contrast, in cell lines such as CHES5, which was characterized by a more heterogeneous cell population at the onset, the expression profile of each marker was a mix of several overlapping waves (Fig. 5). Nestin expression increased first in already committed Pax6-positive cells and only secondarily in cells newly committed from stem cells. It then correlated first with a decrease in Pax6 expression and persisted while the secondary wave of neurectoderm formation became visible. This gave rise to the paradoxical impression that the order of the various differentiation waves was inverted in CHES5 as compared to CHES6.

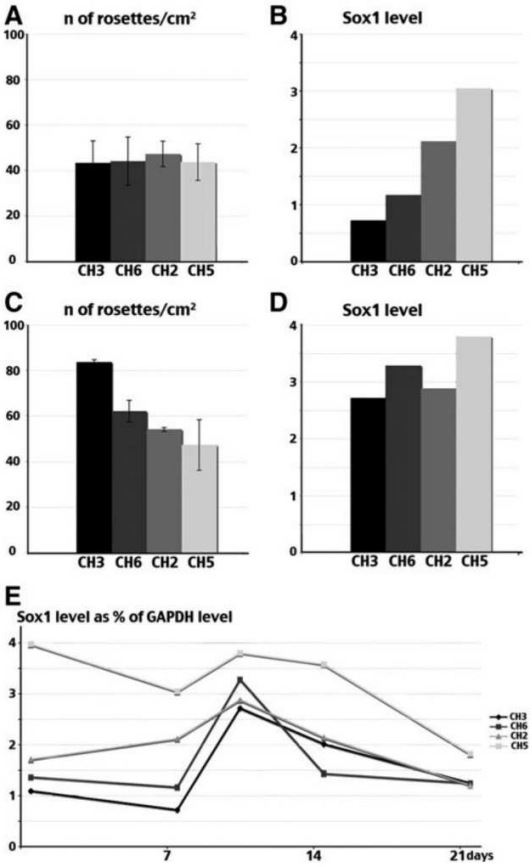

FIG. 6. Formation of neuroepithelial tube-like structures (rosettes) during differentiation of CHES2, CHES3, CHES5, and CHES6 cell lines. The number of rosettes formed in differentiating cultures was counted 7 days (**A**) and 10 days (**C**) after the initiation of directed neural differentiation. The level of maturity of the rosettes was evaluated by quantifying the expression of Sox1 by semiquantitative RT-PCR in the corresponding cultures (**B, D**). Time course of Sox1 expression during a 3-week period of directed differentiation (performed as in Fig. 5) is shown for each cell line (**E**).

Variable levels of predifferentiation among different hESC lines were also associated with distinct abilities to form rosettes, the characteristic structures that form *in vitro* as neural precursors, which acquire the contractile epithelial properties needed to form the neural tube. After 1 week of differentiation, the number of rosettes was similar in all cell lines (Fig. 6A). However, as expected for more advanced cultures, rosette-forming cells in CHES2 and CHES5 expressed higher levels of Sox1, a marker of late mature rosettes in the human (Fig. 6B). After 10 days, the situation had changed radically (Fig. 6C). While the number of rosettes had increased in CHES3 and CHES6, CHES2 and CHES5 had barely progressed to form new rosettes. At that time, they were mostly mature rosettes expressing Sox1 in culture of all cell lines (Fig. 6D). Interestingly, the ability to increase the number of rosettes during that 3-day interval correlated with the ability of differentiating CHES3 and CHES6 cell populations to produce a sharp peak of Sox1 expression, reflecting synchronous differentiation, whereas stagnation of the number of rosettes in CHES2 and CHES5 was associated with rather flat kinetics of Sox1 expression (Fig. 6E).

Discussion

Four new hESC lines were derived under very similar, if not identical, conditions. No isolation of the ICM was performed, and there was almost no need for manipulation of the embryo, thereby reducing the mechanical stress. The characterization of the hESC lines by comparative genomic hybridization (CGH) array and immunohistochemistry of both *in vitro* and *in vivo* differentiated hESCs did not indicate any abnormalities in the genomic integrity of the cells nor in their stemness properties. Notwithstanding these measures, our data also clearly demonstrated a number of differences in the gene expression patterns among all four hESC lines and the important consequences for their behavior during directed differentiation.

The origin of the variability among hESC lines remains unclear. As with lines derived in different laboratories, these differences could be explained by the distinct embryo culture conditions (day 0–6) used in ART laboratories, similar to what is known in the mouse (Fernandez-Gonzalez et al., 2004; Rinaudo and Schultz, 2004). In addition, the variability could be also acquired during the derivation of new lines, because different media are used in different settings until a stable line is established. However, in our setting all of these uncertainties were avoided, because all four lines were derived under identical conditions.

One other potential cause of variability could be caused by the timing of blastocyst processing. The human blastocyst normally arises around day 5 after fertilization, but the optimal timing for processing depends on the actual expansion status of the embryo. Thus, blastocysts are processed between days 5 and 8. During this critical time interval, the preimplantation blastocyst gradually shifts toward a postimplanting blastocyst, which changes the medium requirements as well

as the expression profile of the embryo. In our hands, the best day for processing was day 6. Three of the cell lines (CHES-2, CHES-3, and CHES6) were processed at that time and only CHES-5 at day 7 (Table 1). An influence of the timing of blastocyst processing is not sufficiently plausible to explain the variability among CHES-2, CHES-3, and CHES-6, which were derived from blastocysts achieving a similar developmental stage after an identical culture period. We could not exclude such an influence in the differences between CHES5 versus the three other lines. However, authors of a recent study used embryos at different stages of development to derive ESC lines without causing a higher level of variability in gene expression profile among the lines (Giritharan et al., 2011). Thus, even in the case of CHES-5, it may still be possible that a different timing in blastocyst processing did not play a major role in the differences observed.

Our results confirm another recent study dedicated to compare cell lines generated under standardized conditions in which authors similarly observed a significant range of variability between cell lines (Lappalainen et al., 2010). One possible explanation could be that different cell lines are genetically determined to have different requirements or sensitivities to external factors used for maintenance of stemness *in vitro*. In accordance, we observed that medium composition greatly influenced the propensity of cells of a given line to remain pluripotent or to predifferentiate, but also changed transiently the extent of variation among different cell lines (A.C. Feutz, unpublished results). This suggests that slightly different compositions of the culture medium may be required to maintain stemness in each individual cell line.

Our hESC lines express differentiation markers at low levels characteristic of the three embryonic lineages, indicating spontaneous commitment during stem cells maintenance. Although the expression profiles of those differentiation markers are unique for each cell line, lines with the higher level of spontaneous commitment (CHES-2 and CHES-5) show striking similarities in expression profiles. That suggests that a common weakness may underlie their impaired ability to stay undifferentiated. The differences in gene expression profiles among different hESC lines were particularly pronounced in genes determining neuro-ectodermal differentiation, such as *NeuroD1* and *Pax6*. This bias likely reflects the default status of neuro-ectodermal differentiation, which, in contrast to differentiation toward other lineages, does not require the presence of any specific inducers or cell interactions. As previously described (Lappalainen et al., 2010; Tavakoli et al., 2009), we observed that an increased tendency to spontaneously commit during stem cell maintenance correlates with an apparent increased efficiency to generate neurons when differentiation is allowed. However, while previous data have crudely described the divergent capacity of hESC lines to differentiate to neural cells, we found that our four hESC lines differentiate at similar rates and that the generation of more, or more mature, neurons after an identical time period simply reflects their distinct status already present at the onset of the differentiation process.

Furthermore, while previous data suggested that cell lines able to generate more neurons faster would be more suitable for clinical applications, our analysis of the differentiation process suggests that it may not necessarily be the case. Indeed, as shown in the most permissive situation, different levels of initial neural commitment were associated with clear abnormalities in the neural differentiation process. Following the timely evolution of expression of various markers during differentiation, we observed discordant behavior resulting from heterogeneity in the starting cell populations. For cell lines with the greater tendency to commit spontaneously, the progressing waves of ongoing differentiation of the already committed cells overlapped with those of newly committed cells. Such discordance is responsible for the observed lower ability to form rosettes in these populations, because rosette formation implies simultaneous acquisition of neuro-epithelial properties in neighboring cells. It may also explain the previous description of the different response to neuronal patterning factors of two similarly derived hESC lines, interpreted at that time as difference in differentiation potential (Wu et al., 2007). Indeed, the selective generation of a neuronal subtype *in vitro*, a prerequisite for a number of expected uses of hESCs, including in regenerative medicine, requires addition of regionalizing factors during a precise and narrow window of neuronal development (Gaspard and Vanderhaeghen, 2010; Okada et al., 2008). Treatment of heterogeneous cultures, with an increased proportion of cells at different stages of their development, would thus necessarily lower the ratio of the desired neuronal types or even bias the differentiation toward the generation of unwanted ones. Additional experiments are clearly needed to confirm that, at least in some cases, a higher level of neuron production correlates with increased difficulties to produce homogeneously specific neuron types.

Conclusions

The use of hESCs for drug discovery and toxicology requires cell lines with different genotypes but standardized differentiation behavior. The best strategy to achieve that goal may imply either selection of cell lines with closely related properties or control of the cells' tendency to predifferentiate spontaneously. In any case, the gene expression profile and the tendency to predifferentiate for each newly created hESC line should be well known and tested in a variety of different culture systems. The selection of a panel of cell lines with similar properties is clearly possible and straightforward. However, this strategy finds its limitation when a given cell line is not exchangeable, *i.e.*, when a precise or compatible genotype is required, as in transplantation of tissue-engineered cells. That problem is even more pronounced when induced pluripotent stem cells (iPSC) lines are concerned. This indicates that a variety of different culture conditions may be required to maintain the stemness of stem cells derived from various sources.

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Author Disclosure Statement

All authors declare no conflict of interest.

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Chapter II:

„Assessment of stem cell pluripotency using an *in vitro* 3D perfusion-based culture model“

This chapter contains my main project. The corresponding manuscript is ready for submission in a peer-reviewed journal.

Assessment of stem cell pluripotency using an *in vitro* 3D perfusion-based culture model

1. Abstract

To demonstrate pluripotency status of newly derived human pluripotent stem cells (hPSC), the degree of undifferentiation and the capacity to differentiate into the three germ layers both need to be verified. The *in vivo* teratoma assay ranks as the most universally accepted methods to ascertain pluripotency. The conventional *in vitro* method to verify spontaneous differentiation is the embryoid body (EB) formation. However, both methods lack standardization and are burdened by a high level of variability in both methodology and readout. In addition, the *in vivo* teratoma assay is animal consuming because it requires the use of immune-incompetent mice for transplantation of hPSCs. To replace that test, we now evaluated a three-dimensional (3D) perfusion-based *in vitro* culture system (perfused culture). Human embryonic stem cells (CHES6) and iPS cells were cultured on a collagen scaffold under perfusion flow. EBs cultured in suspension and hPSCs transplanted into immune-incompetent mice were used as controls. Results show that perfusion of the scaffold is essential for tissue viability with a high potential of structure formation. Comparison of perfused culture with the EBs revealed similar expression pattern of differentiation markers of the three germ layers but results were more consistent in the perfused culture. The perfused culture system distinguished normal stem cell lines from an abnormal cell line (CHES1) by its ability to generate teratoma-like tissue within 3 weeks. Based on an established grading system to identify the degree of differentiation of one structure per germ layer (intestinal epithelium, smooth muscle and neural tubes) the perfused culture reached the same degree of differentiation as the *in vivo* teratoma at 3 and at 8 weeks of culture in contrast to the EB culture. The development of teratoma-like tissue of hPSCs was 90% in the perfused culture but only 33% *in vivo*. Our findings present an animal-free applicable system that can be used as a possible pluripotency assay for screening newly derived lines before they will be further investigated. This system would bear relevance towards definition of more practical and better standardized regulatory guidelines for assessment of hPSCs.Furthermore, it can also be used as a model to investigate early human development and further used for drug discovery.

2. Introduction

Human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are characterized by their capacity of self-renewal in an undifferentiated state and their ability to differentiate into cells of all three germ layers: endoderm, ectoderm and mesoderm (1, 2). Due to their unique capacity and with the advent technology for the derivation of hPSCs, these cells are promising for widespread application like regenerative medicine, disease modelling and drug development (3). Consequently, it is crucial that the pluripotency status of newly derived hPSC lines is properly characterized.

Multiple criteria have been proposed to evaluate the pluripotency status of newly generated hPSCs. In a first stage, the undifferentiated status is assessed by morphological analysis and through the expression of distinct markers typical of undifferentiated hPSCs such as Nanog, Oct4, Sox2, SSEA4 and TRA-1-81 (4).

Furthermore, the differentiation capacity is assessed by *in vitro* and *in vivo* differentiation of the cells into the three germ layers. The gold standard to prove pluripotency is the formation of transplanted hPSCs into teratoma in immunodeficient mice (5). A teratoma is defined as a tumor consisting of tissues generated spontaneously from all three germ layers (6). Despite the obvious eminence of this assay, it is burdened by lack of standardisation. There are no uniform protocols for the site of implantation (7), the number of injected cells (8) and the duration of observation until teratoma formation (9). Moreover, this assay raises ethical concerns since hPSCs are grafted in mice, which induces pain and suffering of the animals used in the assay (10).

Up to now, the conventional assay for *in vitro* differentiation, the embryoid body formation (EBs), is used to mimic the spontaneous differentiation of the hPSCs *in vitro* (11). EBs are three-dimensional aggregates of hPSCs cultured statically in suspension, that are able to differentiate into cells of all three germ layers in the absence of any specific growth factor (12). However, the quality of the EBs is highly dependent on the culture condition, the number of cells and the size of the EBs at onset of culture (13, 14). Due to the high variability of differentiation of each single EB cultured in suspension, this method gives rise to heterogeneous populations in terms of cell and tissue morphology (15). For that reason the differentiation potential of hPSCs *in vitro* is often assessed by direct differentiation of EBs into particular cells of the three germ layers, such as neurons and cardiomyocytes (16).

3D perfusion-based bioreactors have previously been demonstrated to allow the formation of tissues in a collagen-scaffold over prolonged times periods, as they guarantee the maintenance of cells in a controlled environment (17-22). The perfusion provides access of nutrients even towards cells residing in the centre of the scaffold. The bioreactors enable efficient cell growth while maintaining optimal surviving rates (23). Currently, the perfusion-based bioreactor has been used to produce several different tissue types in tissue engineering (17, 21, 22). A feeder-based bioreactor has been established previously for the formation of teratoma-like tissue out of undifferentiated stem cells (24). Although structures of all three germ layers were generated successfully, this bioreactor system has not found widespread application due to the complexity of the device.

For this reason, we focus on a user-friendly 3D perfusion-based bioreactor system (perfused culture) for the the establishment of an *in vitro* system, that allows the formation of teratoma-like tissue. The novel method should be animal-sparing and well standardized, simple to handle and allow to obtain results faster than with the conventional *in vivo* assay. The differentiation behavior of hPSC cultured in the perfused system was examined by the analysis of differentiation parameters in the culture medium, at RNA level and by histology. These results were compared with the EB culture and the *in vivo* teratoma assay in immunodeficient mice.

3. Material and Methods

3.1 Cultivation of human embryonic stem cells (hESCs)

3.1.1 hESC lines

The hESC line CHES-6 was derived from the inner cell mass (ICM) of a hatched blastocyst and its pluripotency has been characterized as already published in our previous study (25). The abnormal hESC line (CHES1) was provided by Prof. A. Feki, Geneva, Switzerland (26).

3.1.2 Cultivation and irradiation of human foreskin fibroblasts

Human foreskin fibroblasts (American Type Culture Collection (ATCC CRL2429)) were expanded on Petri dishes under standard conditions using IMDM (Gibco, catalog no. 12440053), 10% FBS (GIBCO, catalog no.16000-044) and 50 U/ml Penicilin, 50 mg/ml Streptomycin (Invitrogen, catalog no. 15140-122). The fibroblast cultures were mitotically inactivated using gamma-irradiation (35 Gy). The irradiated fibroblasts were plated in a concentration of 0.1 $x10^6$ cells/ml and kept in culture for 5 days before they were used as feeders for sustaining the growth of hESC. The feeder cell culture medium corresponds to the fibroblast medium.

3.1.3 Maintenance culture of hPSCs cells

hPSCs (CHES6, CHES1 and iPS) were grown on a feeder layer of irradiated human foreskin fibroblasts in culture medium consisting Knockout DMEM (Invitrogen, catalog no.10829-018), 20% of Knockout Serum Replacement (Gibco, catalog no.10828028), 2 mM L-Glutamax (Invitrogen, catalog no. 35050-038), 1% of nonessential amino acids (Invitrogen, catalog no. 11140-035), 0.05 mM mercaptoethanol (Invitrogen, catalog no. 11140-035), 50 U/ml Penicilin, 50 mg/ml Streptomycin (Invitrogen, catalog no. 15140-122) and 8 ng/ml human recombinant basic fibroblast growth factor (βFGF; R&D Systems, catalog no. 234-FSE-025/CF). The hPSCs were cultured under standard conditions (37 °C and 5% CO_2 in humidified air) and the media were changed on a daily basis. The hESC colonies were passaged by mechanically scraping off the feeder layer into 4-16 pieces, depending on the size of the colony. They were transferred with a pipette to new feeder layer, which was washed twice with PBS and subsequently hPSC medium supplemented with βFGF was added. The cells were passaged once per week.

3.2 3D culture conditions

3.2.1 Embryoid body formation and cultivation

hESC cells were scratched from the feeder layer after treatment with Accutase (Gibco, catalog no. A1110501) for 15 minutes at 37 ºC. The generated dissociated cells were further cultured in suspension at a concentration of 0.3 x10^6cells/ml for 24 hours in hPSC culture medium without the growth factor βFGF but supplemented with 10 µM ROCK inhibitor (RI) (Sigma, catalog no. Y0503) in non-tissue culture-treated dishes (Costar 24 well plate, Corning, catalog nr. 3473). After 24 hours, the generated aggregates were then further cultivated in suspension using standard hESC medium without any supplements (no ßFGF, no RI) for up to 8 weeks.

3.2.2 Culture conditions for static and perfused culture systems

For the 3D non-perfused (static) culture conditions, 12-well-plates were coated with 2% agarose to avoid adherence. The EBs were re-suspended in 10µl and seeded on a collagen type I scaffold sponge (Ultrafoam®, Avitene) with a diameter of 4mm. After letting the cells adhere to the scaffold for 30 minutes at 37 °C, 2ml of basic hPSC medium without any growth factor or inhibitor (see maintenance culture of hPSCs) was added to the well and were subsequently cultured for 8 weeks. Media change was performed every other day and collected and stored at -80°C for further analysis.

For the 3D perfused system, a commercially available bioreactor was used (Cellec Biotek AG). Collagen type I scaffold sponge (Ultrafoam®, Avitene) with a diameter of 8mm were hydrated in culture medium and then placed in the bioreactor between two silicon rings plus an additional reducer ring to a final available area for direct perfusion with a diameter of 4mm. Once in the bioreactor, the aggregates, generated out of 0.3 x10^6 dissociated cells, were seeded on a collagen sponge positioned in the bioreactor. Subsequently 10ml of basic hPSC medium without any supplement was added. The perfusion flow rate was set at 0.1 ml/min to guarantee a superficial velocity of 100µm/sec. Media change was performed every other day and collected and stored at -80°C for further analysis.

3.3 Generation of hPSCs-derived teratoma

The *in vivo* teratoma formation of the different hPSC lines was generated in immune-incompetent non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice (Charles River Laboratories). All animal experiments were performed in accordance with the Swiss Animal Protection Law with the approval of the Basler Cantonal Veterinary Department. Around 50 colonies with about 200 of undifferentiated hESCs were mixed with matrigel (BD, catalog no. 356237) and injected subcutaneously into the flank of an 8-to 10- week-old NOD/SCID mouse. The resulting tumors were excised 3 and 8 weeks later.

3.4 Measurement of AFP in the culture medium

The releasing protein AFP (alpha-fetoprotein) was measured every other day in the circulating medium of the bioreactor and in the supernatant of the static conditions. The presence of AFP (10 µl) was determined with an electrochemiluminescence assay (ECLA) according to standard procedures. These measurements were performed on the automatic Coba 800 system (Roche Diagnostics) in the central laboratory facilities of the University Hospital of Basel.

3.5 DNA measurement

We examined the cell growth and survival in the static and perfused culture by measuring the quantity of DNA. Briefly, samples of both culture conditions were incubated with 0.15% of collagenase IV at 37 °C (Gibco, catalog no. 17104-019) until the collagen scaffold was completely digested (30-60 min). The amount of DNA was measured with the CyQuant Cell Proliferation Assay (Invitrogen, catalog no. C7026) according to manufacturer's instructions. The fluorescence was measured with Synergy H1 Hyprid multi-mode microplate reader (BioTek Instruments). The amount of DNA was calculated using a regression curve.

3.6 Real-time quantitative PCR

Total cellular RNA was extracted by using NucleoSpin RNA II kit (Macherey–Nagel, catalog no. 740955) as described in the manufacturer`s instructions. 300 ng of total RNA was used for first strand cDNA synthesis by SusperScript II Reverse Transcriptase (Invitrogen, catalog no. 18064.014) with 0.5 µM of deoxynucleotide triphosphates (dNTPs; Invitrogen, catalog no. 10297-0189) and 5 ng/ µL random hexamer primers (Invitrogen, catalog no. 48190-011). The cDNA of each sample was used to detect expression of genes listed in table 1. The primers were

synthesized by Microsynth (Balgach, Switzerland). One-tenth of each cDNA was used per qPCR reaction by using Power SYBR-Green PCR Master Mix (Applied Biosystems, catalog no. 4367659. The qPCR reaction was done in an ABI 7500 Fast Real- Time PCR System (Applied Biosystems). The gene expression was normalized using GAPDH as reference gene.

Table 1: Primer sequences for qPCR

Primer	**Sequences**
AFP	F: 5'-GGC AGC CAC AGC AGC CAC TT-3' R: 5'-GCA CTG GCC AAC ACC AGG GT-3'
Brachyury	F: 5'-AAG GCC CAG GTC CCG AAA GAT G-3' R: 5'-CCG TGT GCT CCT CCA CTG CTT TG-3'
CDX2	F: 5'-CAG AGC CCT TGA GTC CGG TG-3' R: 5'-GGC TCA GCC TGG AAT TGC TCT G-3'
GAPDH	F: 5'-AGC CAC ATC GCT CAG ACA CC-3' R: 5'-GTA CTC AGC GGC CAG CAT CG-3'
GFAP	F: 5'-TCA TCG CTC AGG AGG TCC TT-3' R: 5'-CTG TTG CCA GAG ATG GAG GTT-3'
HAND1	F: 5'-GCC GTG AGA GCA AGC GGA AAA-3' R: 5'-GGG CCT CGG CTC ACT GGT TT-3'
Nanog	F: 5'-TGG TGT GAC GCA GAA GGC CTC AGC A-3' R: 5'-CCC AGT CGG GTT CAC CAG GCA TCC C-3'
OCT4	F: 5'-ATG GCG TAC TGT GGG CCC CAG GTT-3' R: 5'-TCC GGG GAG GCC CCA TCG GAT TGT-3'
PAX6	F: 5'-CGT GGA ATC CCG CGG CCC CC-3' R: 5'- GAG TCC GGC AGT GGC CGC CC-3'
α-SMA	F: 5'-AAG GCC GGA GCT AGG AGT CCA-3' R: 5'-GAG GCA AAG GGC TGG TCC CTG-3'
SOX17	F: 5'-GGC GCA GCA GAA TCC AGA-3' R: 5'-CCA CGA CTT GCC CAG CAT-3'
βtub3	F: 5'-GAG ATC CTG CAC ATC CAG GCC GG-3' R: 5'-TCC GCT CCA GCT GCA AGT CCG-3'

3.7 Immunohistochemistry (IHC)

Tissue samples were retrieved after 3 or 8 weeks of culture in each of the various conditions and fixed in 4% paraformaldehyde, then embedded in paraffin (TPC15 Medite) and subsequently cut serially in 5 µm sections. Hematoxylin and eosin (H&E) staining were carried out following the standard protocol. IHC was performed using the standard streptavidin-biotin-peroxidase procedure. Tissue sections obtained from the different study groups were treated with 10% normal goat serum for 30 min and incubated with the primary antibodies listed in table 2 overnight at 4 °C. Blockage of endogenous peroxide activity was performed after 30 min of incubation with 3% H_2O_2 (Sigma, cat.no. H1009). The antigen-antibody reaction was visualized using the avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, PK-6101) and diaminobenzidine (DAB) as the chromogen (Impact DAB EqV, Vector Laboratories, SK-4103). Sections were counterstained with hematoxylin. For every antibody-negative control was performed by omitting the primary antibody. The histological and IHC sections were analysed with an Olympus BX63 Apollo microscope.

Table 2: Antibodies for IHC

Antibodies	**Dilution**	**Supplier**
Anti- AFP	1:200	Cell Marque (EP209)
Anti- Brachyury	1:100	Abcam (ab20680)
Anti- Calretinin	RTU*	Leica Biosystems Bond (PA0346)
Anti- CDX2	RTU	Abcam (ab765419)
Anti- CD31	RTU	Leica Biosystems Bond (PA0250)
Anti- Desmin	RTU	Leica Biosystems Bond (PA 0032)
Anti- Ki67	RTU	Biosystems (275R-18)
Anti- LU5	1:1000	BMA Biomedicals (T-1302)
Anti- Pax6	1:100	BioLegend (Poly19013)
Anti- p63	RTU	Leica Biosystems Bond (PA0103)
Anti- α-SMA	RTU	Leica Biosystems Bond (PA0943)
Anti- Synaptophysin	RTU	Leica Biosystems Bond (PA0299)
Anti- βtub3	1:2000	Abcam (ab18207)

* RTU= Ready to use

3.8. Coloration of sections

Alcian-blue and periodic acid Schiff (AB-PAS) staining were used to stain structures containing glycogens, often found in mucus. The combination of the AB- and the PAS-techniques are commonly used to differentiate between neutral and acetic mucosubstances (2450077). The stainings were performed according to the manufacturer's instructions using 1% Alcian blue (Sigma, cataog no. A5268), 0.3 % periodic acid and the Schiffs reagent (Morphisto, catalog no. 11686**).** *Masson fontana* staining was used for the visualisation of argentaffin cells and melanin in the tissues. The staining was performed according to standard protocol using ammonical silver solution (25% of ammoniac and 10% of silver nitrate) and 0.1% of nuclear fast red solution (Merck, catalog no. 1.00121.0500). Melanin reduces the ammoniacal silver nitrate to metallic silver and this leads to the black appearance.

3.9 Immunofluorescence

The viability staining was performed with the live/dead fixable dead cell stain kit (Molecular probes L23101). The scaffold-based generated tissues were retrieved from the bioreactor and from the static culture followed, were firstly washed with PBS. The staining of the tissue was performed in 1 ml PBS by adding 2 µM calcein (stains living cells in green) and 4 µM ethidium homodimer (stains dead cells are stained red) for 30 min at room temperature and protected from light. Subsequently, the tissues were washed with PBS and analysed in a chamber slide. 3D images were acquired by Nikon confocal A1 microscopy with a Plan Apo 10x NA 0.75 objective using 0.25 µm z-stack step size. Three images per construct have been performed. The images were constructed and analysed with the Software Imaris X 64 7.6.5.

3.10 Grading system

Every sample of the three experimental groups (EBs, perfused culture and *in vivo*) was serially sectioned and stained for each of the selected markers (CDX2, α-SMA and βtub3). Subsequently, every section of each condition was analysed and for each marker expression the highest visible grade was given. The grading system distinguishes four grades of structural integrity:

- Grade 0: negatively stained sections for these particular markers.
- Grade 1 : poorly differentiated structures (disorganized).
- Grade 2 : moderately differentiated structure showing some degree of organization.
- Grade 3 : well differentiated and recognizable structures.

3.11 Statistical analysis

All experiments were performed at least three times independently. Graphs and statistical analysis (unpaired one-way and two-way ANOVA) were implemented in Prism. P-values of < 0.05 was considered statistically significant and are highlighted with *, p-values of <0.01 are highlighted with **, p-values of <0.001 are highlighted with ***. Error bars represent the standard error of the mean (SEM).

4. Results

4.1 Set up of a 3D perfusion-based bioreactor system to culture differentiating hESCs (CHES6).

In a first phase, two seeding procedures were tested to ensure the optimal seeding and culture condition of CHES6 in the perfused system. Dissociated CHES6 were either cultured statically in suspension in presence of rock inhibitor (RI) for 24 hours or injected directly into the bioreactor system supplemented with RI (Figure 1A). Both procedures were expected to allow aggregation of the cells. In the first method, small aggregates of CHES6 were further seeded manually on the scaffold of the perfused system and subsequently cultured in the presence of standard hPSC medium in the absence of any growth factor and RI. In the second method, CHES6 were injected directly into the perfused system, in which aggregates are formed under constant perfusion with medium containing the RI allowing the cells to integrate onto the scaffold. In both methods, medium was changed after 24 hours to the standard hPSC medium without the RI or growth factors.

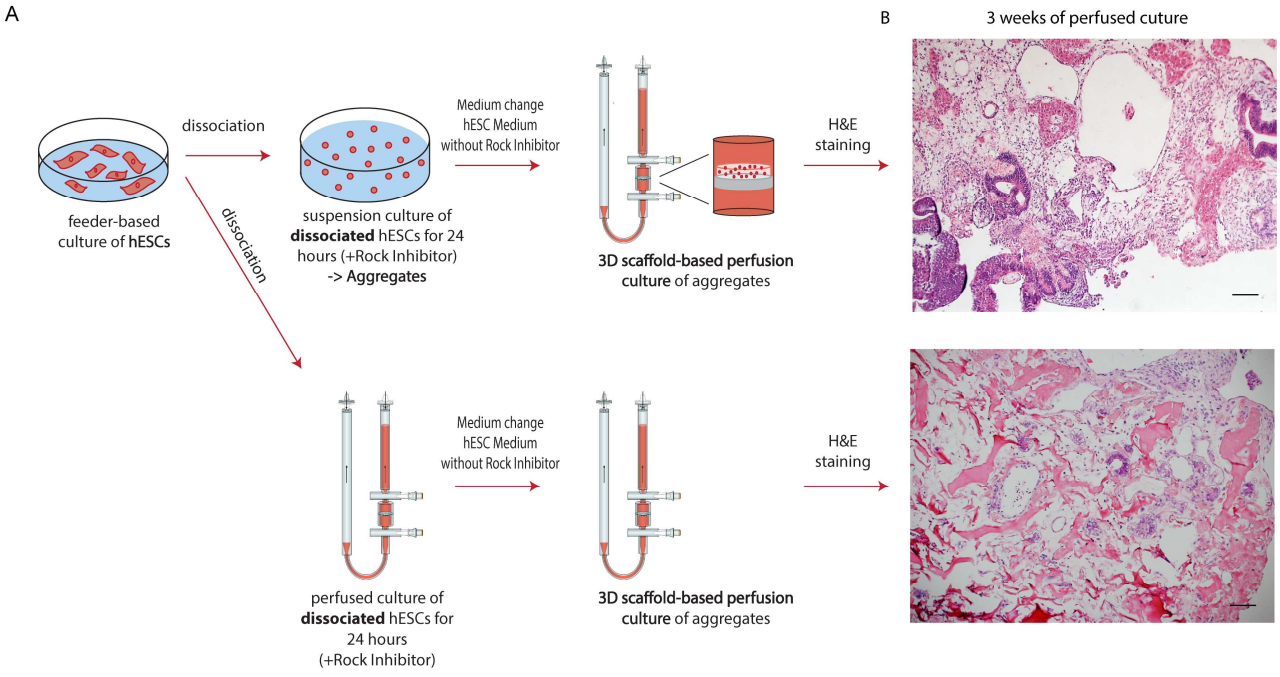


Figure 1: Assessment of different seeding procedure in the perfused culture. (A) Experimental scheme for the two seeding methods for culturing a teratoma-like tissue. (B) H&E staining of representative sections from the dissociated cell culture (lower panel) and the aggregates culture (upper panel) after 3 weeks of perfused culture (scale bar, 100 µm).

Cells were then cultured in differentiation-permissive medium for 3 weeks followed by histological analysis of the tissues samples obtained (Figure 1B). The first method using pre-formed aggregates in suspension gave rise to various distinct structures. In contrast to the second method where the cells are aggregated within the scaffold, cells were mostly spread over the whole scaffold and formed only few small structures (Figure 1B). Therefore, we concluded that a first aggregation of cells in static suspension was needed to secure efficient structure formation in the perfused culture.

Since we load our system with aggregates formed in suspension, the exact number of cells used to load the perfused system could not be evaluated. We tested if the concentration of dissociated cells placed in suspension build always the same amount of aggregates. Therefore, we measured the number of cells to be seeded on the scaffold as the number of dissociated cells taken from the feeder-based CHES6 culture and placed in suspension. We started culturing 0.3 $x10^6$ of dissociated cells in suspension and measured the amount of DNA of the aggregates formed after 24 h of culture in suspension (Figure 2A). The aggregates formed out of 0.3 $x10^6$ of dissociated cells cultured in suspension contained 160 ng/ml of DNA (Figure 2B). Hence, the concentration needed to form a teratoma-like tissue in the perfused culture is 0.3 $x10^6$ of dissociated cells.

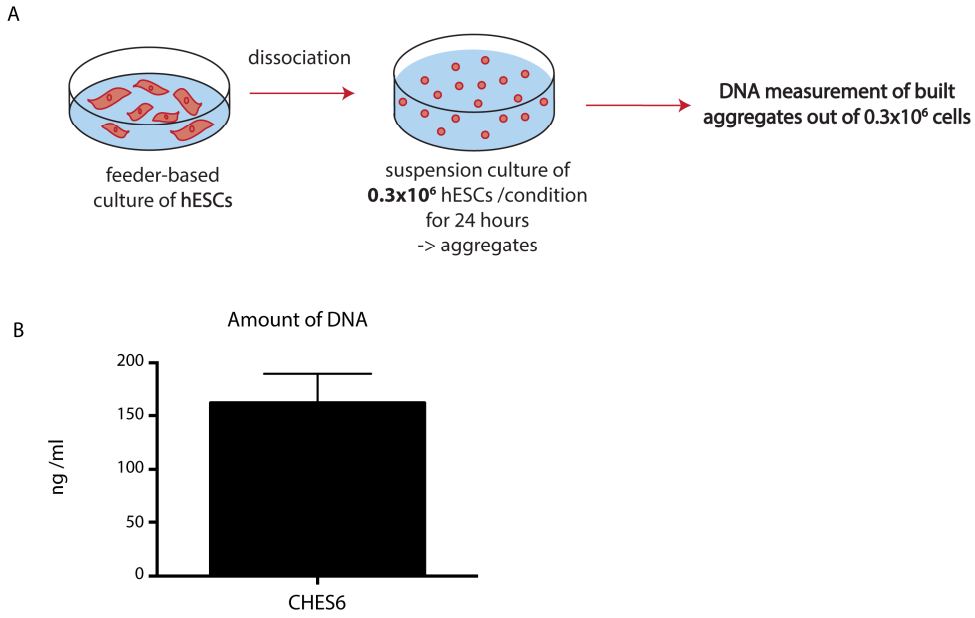


Figure 2: Standardization of the seeding procedure in the 3D-perfused culture. (A) Experimental scheme for the measurement of the amount of DNA of the generated aggregates out of 0.3 x 10^6 dissociated stem cells. (B) Amount of DNA measured in the generated aggregates of CHES6 after 24 hours of static suspension culture (n=5).

We then examined whether the 3D-scaffold system indeed required perfusion with circulating culture medium to achieve better results. To that purpose several determinants of cell growth and differentiation were compared between the perfusion-based culture and a non-perfused scaffold condition (static culture) (Supplementary Figure S1). Briefly, aggregates derived from CHES6 were embedded into a collagen scaffold and cultured dynamically under perfusion or statically in a 12-well plate during the same time interval. We first compared the viability of the hPSCs in static and perfused cultures, using calcein AM and ethidium homodimer-1 dyes, staining living cells and dead cells respectively (Figure 3A). After dissociation and seeding, and before the cells adapted to the new culture conditions, we initially observed a significant amount of cell death. After one week of culture around 80% of cells survived in both conditions, whereby in the static culture the SEM was higher within the triplicates (83% ± 2 SEM in perfused culture and 75% ± 10.3 SEM in static culture). Moreover, we observed that the inner part of the scaffold was mainly composed of dead cells with a rim of living cells.

Between 1 and 3 weeks of culture, the number of living cells increased under perfusion. As a consequence of culture stabilization, the percentage of living cells increased, reaching almost 100% in the perfused system (96% ± 0.9 SEM). Instead, in the static culture the cell survival was lower (77% ± 5.0 SEM). After 8 weeks of culture, the proliferation of cells stopped, as the differentiation process was initiating and more advanced structures progressively formed. In both culture conditions cell viability decreased. This likely reflected the lack of various survival factors required *in vitro* by the numerous types of differentiated cells produced in such a heterogeneous differentiation system, with cells loss not being anymore balanced by the generation of new cells.

These results were confirmed by measuring the DNA content for each culture condition over time (Figure 3B). After 1 week of culture, the amount of DNA was lower in the static culture. In the presence of equivalent cell viability in both culture conditions (Figure 3A), the lower amount of DNA in the static cultures indicates a lower rate of cell recovery and proliferation. Compared to 1 week of culture, after 3 weeks the amount of DNA increased 4x with the static culture and more than 6x with the perfused culture (Figure 3B). After 8 weeks a decrease of the DNA content was observed in both conditions, which was significantly lower under perfusion (Figure 3B).

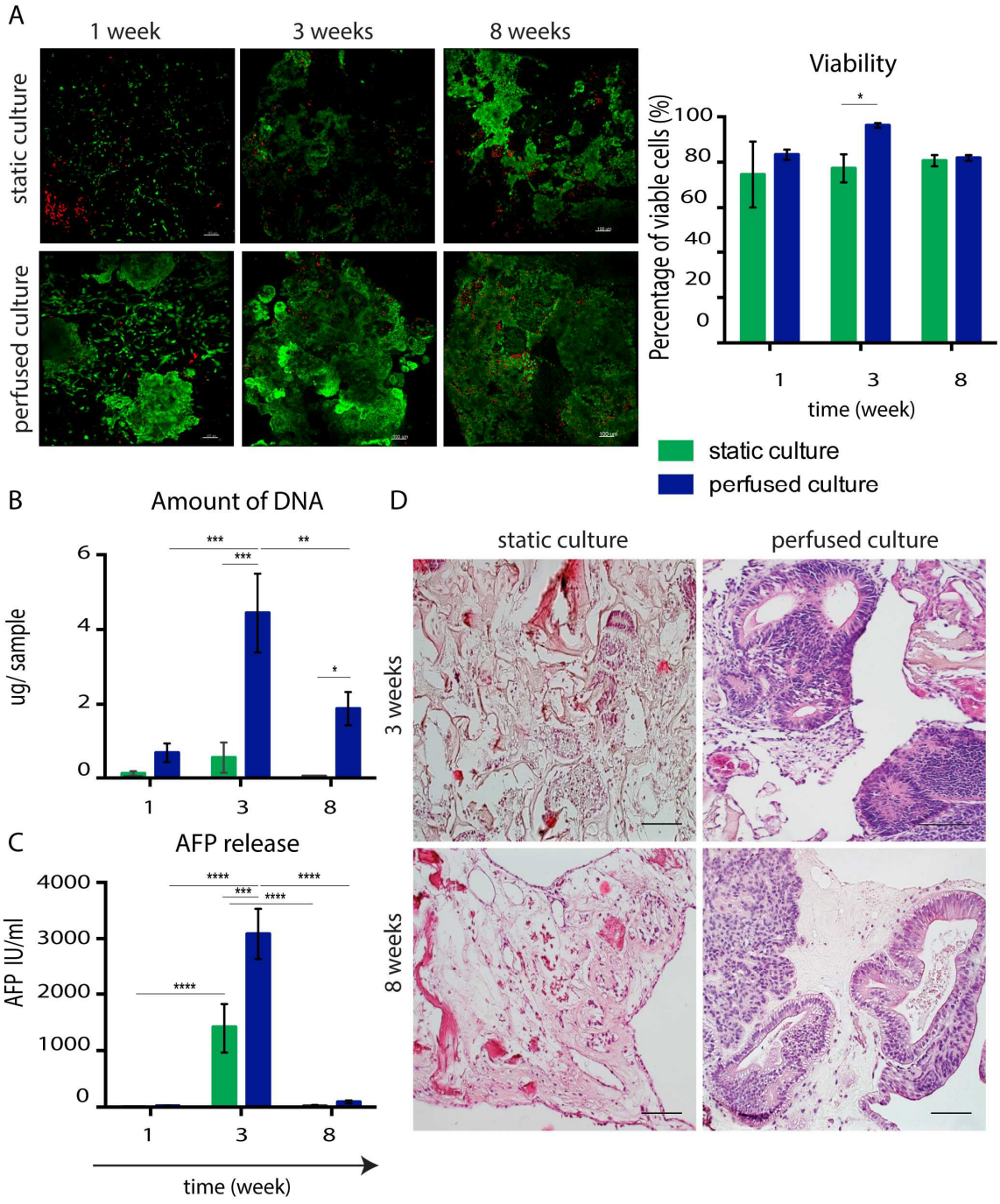


Figure 3: Cell and tissue characteristics of the perfused and static cultures. (A) Cell viability staining (green= living cells, red= dead cells). Fluorescent microscopic images of representative samples of tissue grown after static or perfused culture, after 1, 3 and 8 weeks. Quantitative analysis of the percentage of living cells (green) cultured in both conditions (n=3). (B) Measurement of the amount of DNA of cells grown in either static or perfused cultures over time (n=3). (C) AFP-release into the surrounding culture medium measured over time during two different culture conditions (n=6). (D) H&E-stained representative sections of samples retrieved after 3 and 8 weeks of static or perfused cultures (scale bar, 100 µm). Error bars indicate ±SEM. *$P < 0.05$, ***$P < 0.01$.

We then analysed the effect of perfusion on the ability of the cells to differentiate and to become organized into specialized structures. We determined the initiation of differentiation by measuring the release of AFP, an early marker of endoderm, into the culture medium (27) (Figure 3C). After 1 week of culture almost no AFP-release was detected, while after 3 weeks the AFP-levels strongly increased in both conditions giving an evidence of cell commitment. The release was significantly higher, almost 3-fold, in the perfused culture system as compared to the static culture. This difference suggests that the lower cell growth observed in static culture condition (Figure 3B) was not due to a premature entry into the differentiation process. After 8 weeks of culture AFP-release decreased in both conditions, illustrating the transient expression of AFP during differentiation. These results demonstrate that the commitment of differentiation to the endoderm was more efficient in the perfusion system than in the static counterpart.

We then examined the formation of differentiated structure with Haematoxylin & Eosin staining (H&E) after 3 and 8 weeks of culture in both conditions. After 3 weeks, we observed that cells cultured under static conditions were spread over the whole scaffold, but were almost unable to form recognizable organized structures. In contrast, under the perfused culture conditions cells were able to produce organized structures (Figure 3D). After 8 weeks of culture, cystic structures were identified in both culture conditions, but better organized structures were formed under perfused culture.

Collectively, the perfusion-based culture system provides an environment which promotes higher cell viability, better differentiation, and supports the generation of tissue-like structures. Therefore, we decided to further validate the perfused system as a valuable culture tool for the formation of teratoma-like tissue *in vitro.*

4.2 Spontaneous differentiation of CHES6 into the three germ layers during perfused culture

We examined the time course of the differentiation process during perfusion of the 3D-scaffold by determining the expression levels of early and late markers of the three germ layers. We compared these results with those of the conventional *in vitro* EB culture model described above, often used as an initial method for initiating spontaneous differentiation towards the three germ layers (28). In both culture conditions, after one week of culture, Oct4 and Nanog, common stemness markers for self-renewal, were not expressed anymore (Figure 4).

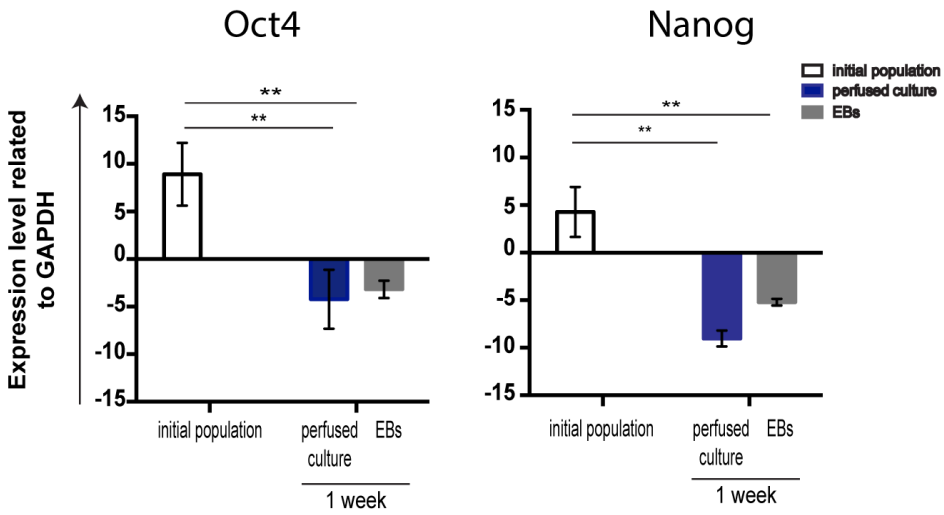


Figure 4: Pluripotency status of hPSCs cultured in two different conditions (A-B) Assessment of the expression level of two pluripotency markers, Oct4 and Nanog, after 1 week of perfused and EB culture in comparison to the initial undifferentiated population. Error bars indicate ±SEM. $^{**}P < 0.01$.

Concomitantly, we investigate the degree of commitment assessing the expression level of the specific germ layer markers AFP, brachyury, and PAX6 respectively for endoderm, mesoderm, and neuroectoderm. In both culture conditions, after 1 week, the degree of commitment increased with the expression of all three markers three- to five-fold more than in the initial cell population (Figure 5).

In the perfused culture, the expression of AFP and PAX6 expression continued to increase during the two following weeks (two-fold and three-fold, respectively), while the expression levels of brachyury remained stable (Figure 5A). As expected, as these genes are expressed only transiently during the specification of the three germ layers, their expression dropped after 8

weeks of culture suggesting that the process of differentiation was ongoing and that the committed cells were progressing into more specialized cells (29, 30).

In the EB culture, the situation was much more erratic. While the three genes became expressed after 1 week of culture, only AFP continued to be expressed over the entire observation period, corresponding to normal and undisturbed development. The expression of brachyury prematurely decreased after 3 weeks, while the expression of PAX6 persisted longer than expected (Figure 5A), indicating that the EB culture system is less favourable to the differentiation process than the perfused culture. By IHC, we confirmed the presence of cells for all three markers in both culture conditions (Figure 5B), but the defined structures were found only in the perfused culture system (right panel).

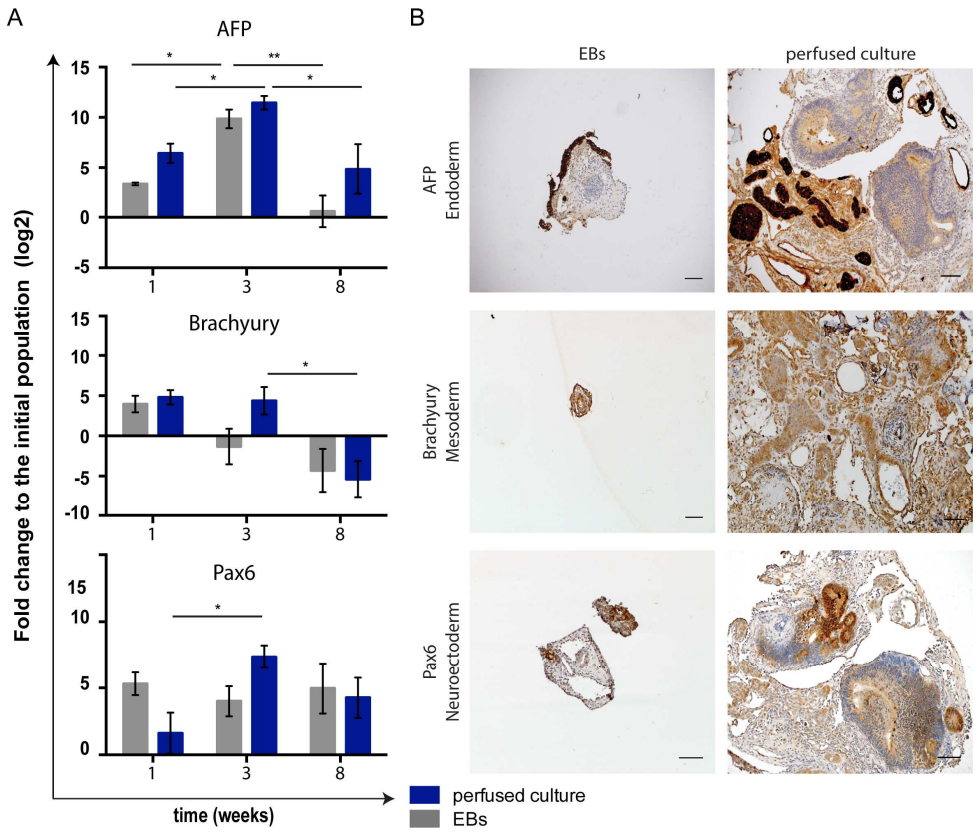


Figure 5: Assessment of the commitment potential of hPSCs under perfused culture. (A) Comparative RT-qPCR analysis of the early markers AFP, brachyury and PAX6, representing each of the three germ layers after 8 weeks (n=3). (B) IHC of AFP, brachyury and PAX6 in the generated teratoma-like tissue (right panel) and in the EBs (left panel) after 3 weeks of culture (scale bar, 100 µm). Error bars indicate ±SEM. *$P < 0.05$, **$P < 0.01$.

To examine whether committed cells were able to differentiate further into more specialized tissue types during prolonged culture, the expression of more advanced differentiation markers was compared between the two *in vitro* cultures by RT-qPCR (Figure 6). For the endoderm, we focused on SRY-box 17 (SOX17), which is implicated in endoderm determination and maintenance (31) and CDX2, which is a major regulator of genes expressed in the intestinal epithelium (32) (Figure 6A). For the mesoderm, the transcription factor heart and neural crest derivatives 1 (HAND1), which is required for the differentiation into heart tissue (33) and alpha smooth muscle-actin (α-SMA) which is a key mediator of the differentiation of heart and muscle tissue (34) were studied (Figure 6B). Regarding the neural lineage, ß-III-tubulin (ßtub3), a marker for young neurons (35) and glial fibrillary acidic protein (GFAP), a marker for astrocytes (36), were examined (Figure 6C). Expression of all these markers was detected after 3 weeks in both culture conditions, confirming that the two systems allowed further differentiation after cell commitment.

In the perfused culture, the evolution of expression level of those differentiation markers between 3 and 8 weeks was coherent with their order of appearance during normal *in vivo* development. In endodermal lineage, SOX17, which marks also early endodermal cells, was highly expressed after 3 weeks, while the expression of CDX2, which labels differentiated intestinal cells, rose only after 3 to 8 weeks (Figure 6A). In the mesodermal lineage, expression of markers of different types of muscle cells were already detectable at 3 weeks, but their level increased as differentiation proceed (Figure 6B). Finally, in neural lineage, the neuronal marker ßtub3 was already highly expressed after 3 weeks, while the astrocytic marker GFAP was poorly detectable at that time, but became predominant after 8 weeks (Figure 6C). That ranking is reminiscent of the sequential generation of neural subtypes during embryonic development; first neurons, then glial cells.

In the EB culture, expression of all markers was detected at levels similar to that observed in the perfused culture, indicating that differentiation was globally similarly efficient in the two systems. However, the time-course of differentiation seems to follow much more poorly what happens during *in vivo* development. On one hand, SOX17 in endoderm or α-SMA in mesoderm behaved similarly to what was observed in the perfused culture. On the other hand, CDX2 in endoderm, HAND1 in mesoderm and GFAP in neurectoderm showed faster upregulation. For CDX2 and HAND1, expression level was unexpectedly even higher at 3 weeks than after 8

weeks of differentiation. In neurectoderm, the disturbance in differentiation time-course led apparently to simultaneous rather than sequential generation of neuronal and glial cells. Thus, the perfused culture seems to better reflect the organization of tissues, which allows the interaction of cells required to reproduce normal developmental events.

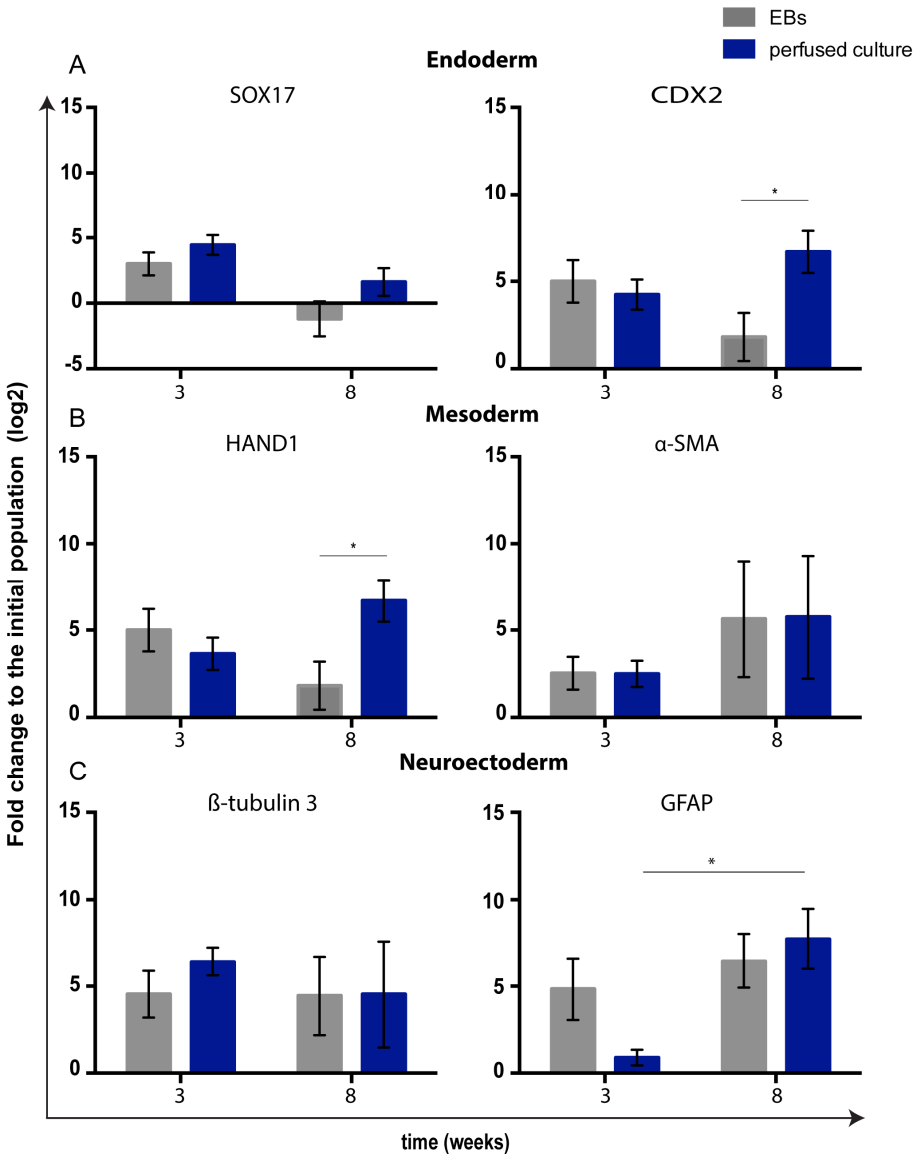


Figure 6: Comparative analysis of the late differentiation potential of hPSCs over time with RT-qPCR. (A) The endodermal markers SOX17 and CDX2 were examined in the perfused culture and the EB culture after 3 and 8 weeks in culture. (B) The expression of the mesodermal markers HAND1 and α-SMA were tested in both conditions after 3 and 8 weeks in culture. (C) The expression of the neuroectodermal marker ßtub3 and of the astrocytic marker GFAP were determined in the perfused culture and in the cultured EBs after 3 and 8 weeks. Error bars indicate ±SEM. *$P < 0.05$.

4.3 Structure formation along the three germ layers

Since we are particularly interested in the feasibility of cells cultured into 3D-perfused system to build various structures derived from the three germ layers, we assessed different specific markers linked to particular structures in the generated teratoma-like tissue. Indeed, a broad spectrum of different markers was expressed at 3 and/or 8 weeks of perfused culture (Figure 7)

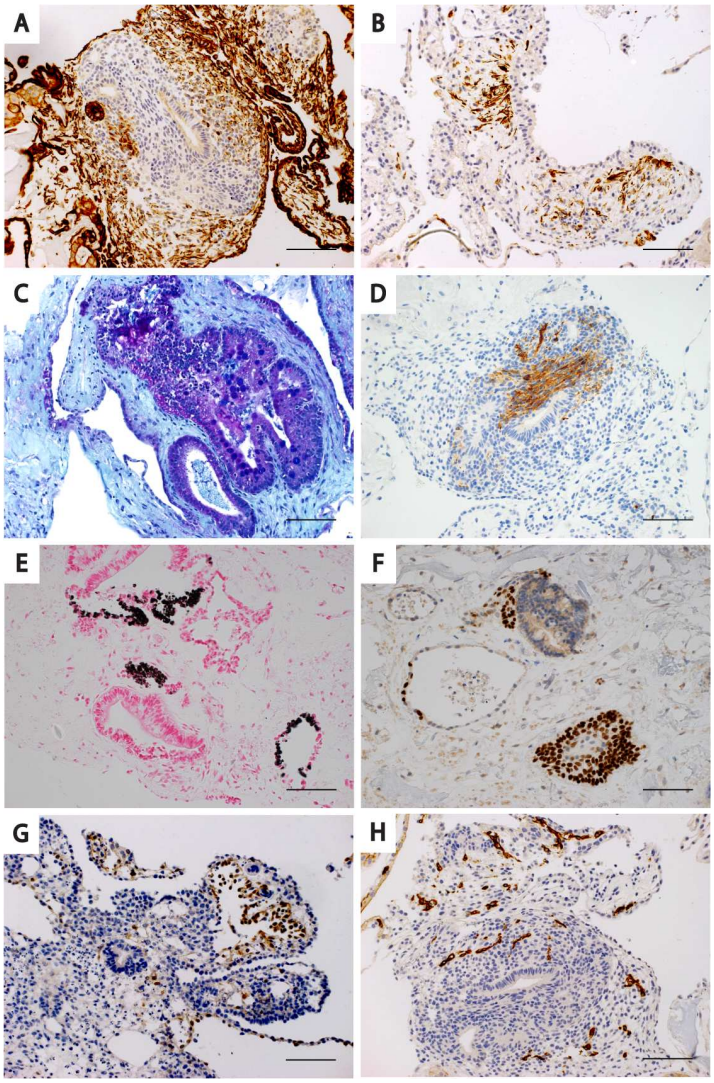


Figure 7: Histological overview of teratoma-like tissue generated in the perfused culture. (A) Pancytokeratin (Lu5) staining decorating epithelial cells. (B) Desmin positivity in myogenic cells. (C) Positive AB-PAS staining showing glands. (D) Synaptophysin expressed by neural and neuroendocrine cells. (E) Singh-staining showing melanin containing cells. (F) P63 expression displaying squamoid and basaloid cells (G) Calretinin immunostaining exhibiting mesothelial cells. (H) CD31 positivity in endothelial cells of capillary vessels. (scale bar= 100 µm).

Epithelial cells were identified by a positive staining of pancytokeratin (LU5), while a negative staining of this marker was used to detect nervous tissue (like neuronal tubes) (7A). Staining for desmin was used to identify differentiation to muscular cells (7B). With Alcian-Blue and the Periodic Acid-Schiff reaction (AB-PAS) glands with or without goblet cells were detected (7C). Cells of neural structures and neuroendocrine tissue were identified by a staining for synaptophysin (7D). By melanin staining (Singh) cells of the retinal epithelium were identified (7E). Cells with squamoid and/or basaloid differentiation were highlighted with the marker p63 (7F). Mesothelium was confirmed by the expression of calretinin (7G) and CD31 expression displayed endothelial cells of capillary vessels (7H).

4.4 Reliability of the system

As all experiments described above were carried out with the hESC line CHES6, the performance of the perfusion-based bioreactor was examined with two other stem cell lines. One stem cell line was a hiPSC line (iPS) newly generated in our laboratory, while the other is a teratocarcinoma-like hESC line (CHES1). This latter cell line has an abnormal karyotype (61 XX and presence of iso-chromosomes) and is unable to differentiate into normal teratoma tissue. When grafted *in vivo*, it forms malignant tumors still containing many undifferentiated cells (26, 37). After three weeks of culture in the perfused culture, different parameters were compared in the generated tissue of the three cell lines CHES6, iPS and CHES1 (Figure 8). As previously demonstrated with the reference CHES6 line, after 3 weeks of culture proliferation of cells still continued, but the differentiation process was already progressing. Therefore, we selected that time point to compare the performance of the three cell lines in the perfused culture.

Staining with the proliferation marker Ki67 (Figure 8A) and visualisation of structure formation by histology after H&E staining (Figure 8B) demonstrated that the iPS switch from proliferation to differentiation followed a time course very similar to CHES6. In the teratoma-like tissue formed with iPS, the ratio of still proliferating cells was roughly equivalent to what was observed with CHES6 (Figure 8A) and various structures of different origins could be recognized (Figure 8B). The pluripotent stem cell characteristics of iPSC line were confirmed by its ability to form teratoma *in vivo* after transplantation into immunodeficient mice (Supplementary Figure S2). With CHES1, in contrast, a strikingly high number of proliferating cells was detected (Figure 8A) and no distinct structure of any of the three germ layers was recognized (Figure 8B).

We have previously shown that AFP release can be used as a non-destructive indirect indicator to assess stem cell commitment to the endoderm and that the highest release of AFP into the circulating culture medium can be detected after 3 weeks of culture. We therefore examined AFP-release into the culture media of the three cell lines. As with CHES6, high levels of AFP were measured in the circulating culture medium during the differentiation of the iPSC line (around half the level found in CHES6) (Figure 8C). In contrast, no AFP-release could be detected in the circulating medium during the differentiation of CHES1 (Figure 8C). Absence of commitment after three weeks of culture of CHES1 was confirmed at the RNA level, as no significant expression of AFP, nor of brachyury and PAX6 was measured (data not shown).

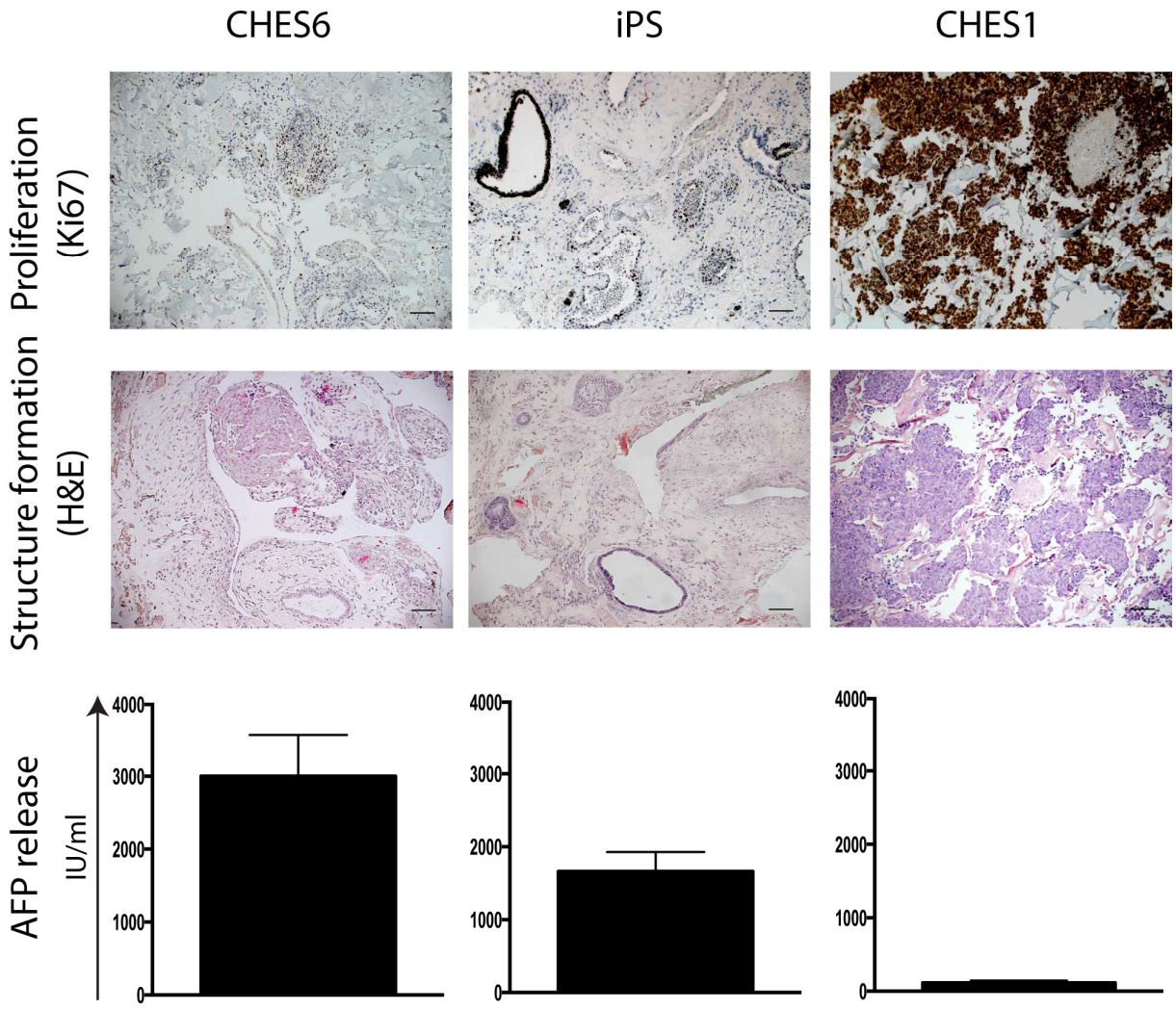


Figure 8: Assessment of the performance of the perfused system with 3 different hPSC lines after 3 weeks in culture. (A) Comparative analysis of the proliferation of three different hPSC lines by a Ki67 staining. (B) H&E-stained representative sections of samples generated by 3 different hPSC lines after 3 weeks of culture (C) AFP-release in the perfused culture using 3 different hPSC lines (n=4) (scale bar, 100 µm). Error bars indicate ±SEM.

In conclusion, these results clearly demonstrate that the 3D perfused bioreactor allows the distinction of the pluripotency between a normal hPSC line from that of an abnormal hPSC line by the attempt of generating a teratoma-like tissue after three weeks of culture.

4.5 Grading of structures from the three germ layers

We performed a semi-quantitative analysis to evaluate the differentiation quality of the structures formed either *in vivo* after transplantation into immunodeficient mice or *in vitro* in the perfused culture or from cultured EBs. We established a grading system based on the differentiation degree of particular structures derived from the three germ layers. It was adapted from a tumor grading system, previously defined by the WHO (38). The structure of intestinal epithelium, muscle fibers and early neural tubes are commonly used structures to identify teratoma *in vivo*, mostly after staining with H&E (9). We selected one marker per germ layer to properly identify each of these structures representing the three germ layers: CDX2 for endoderm as expressed in intestinal epithelium, α-SMA for mesoderm expressed in muscular fibres and ßtub3 as expressed in young neurons originate in neural tubes representing neuroectoderm. The grading system distinguishes four grades of structural integrity (Figure 9). The detailed procedure of scoring the stained sections is explained in *Material and Methods.*

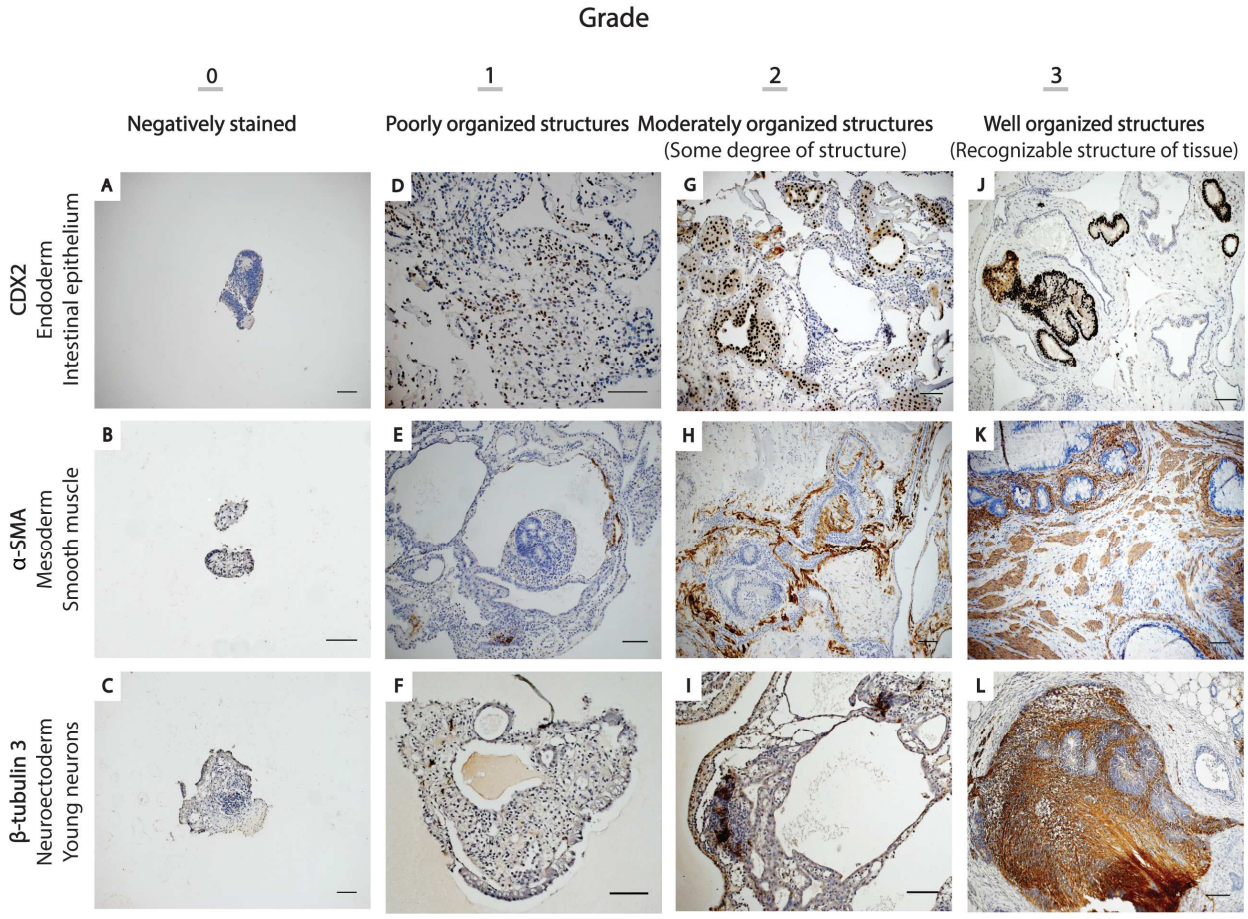


Figure 9: Scoring system with different grading categories. (A-C) Negatively stained sections for CDX2, α-SMA and ßtub3. (D-F) Single cells are stained with the particular marker. (G) Abortive intestinal epithelium. (H) Subepithelial linear cell groups (I). Abortive neural tube formation (J) Intestinal epithelium (K) Muscle fibers (L) Neural tube formation + organized cell groups (scale bar, 100 µm).

After 3 weeks of culture (earliest possible time point with highest expression of commitment *in vitro*) and after 8 weeks (time needed to build a teratoma *in vivo*) we compared the scoring results achieved with the three different culture conditions. By comparing the two *in vitro* cultures both after 3 and 8 weeks for all markers the grade level of the structures produced in the perfused system was significantly higher than the level reached with the EB culture (Figure 10A). The grade level of the structures built in the perfused system could reach the same level as the *in vivo* conditions even after 3 weeks of culture. In contrary, the grade level achieved with the *in vitro* growth of EBs never reached the same grade level as with the *in vivo* system at both time points. In addition, the quality of the structures built in each EB was very diverse. Some EBs differentiated into organized structures whereas others acquired a cystic appearance. In most differentiated structures the cells expressed the required markers, but the observed structures usually remained disorganised and did not form a recognizable appearance.

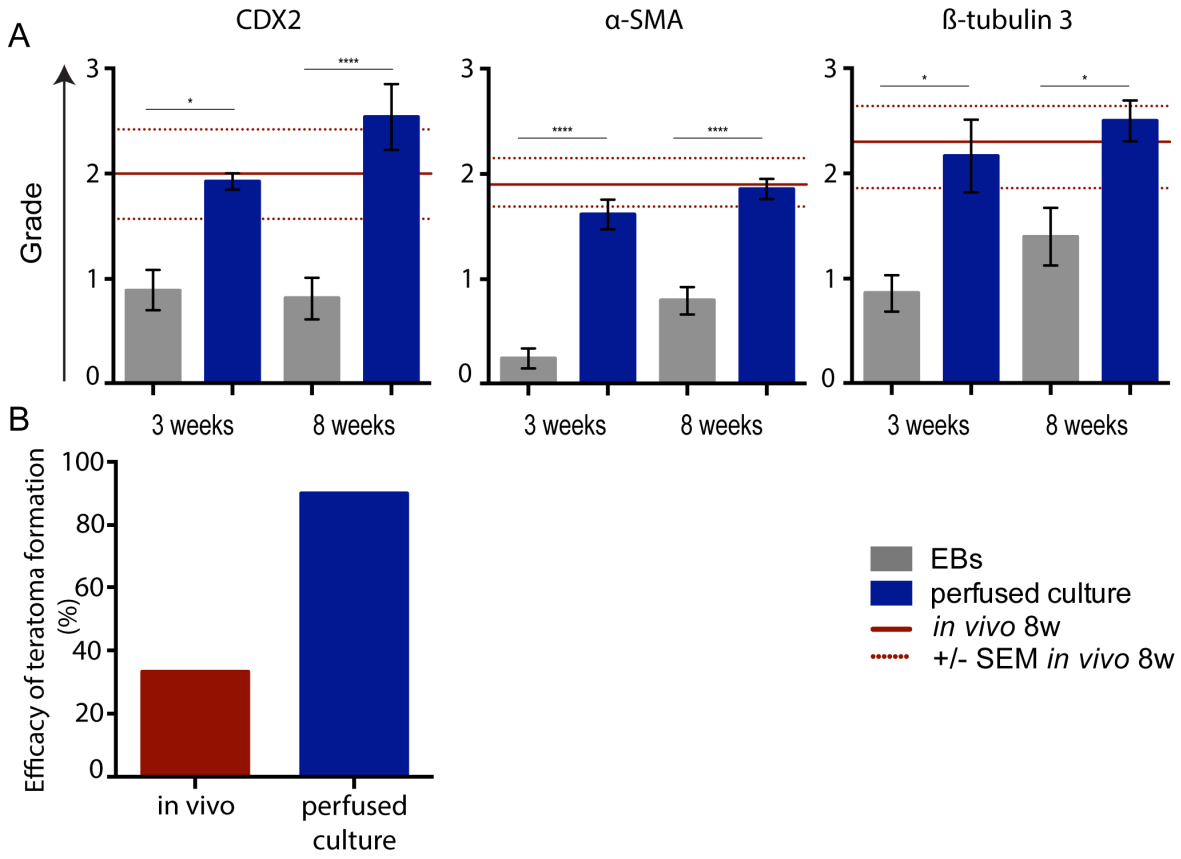


Figure 10: Comparison of the differentiation degree of particular structures among the three different culture conditions. (A) Comparative analysis of the grade-level of the markers CDX2, α-SMA and ßtub3 in the two *in vitro* systems and the *in vivo* system. (B) Comparative analysis of teratoma formation efficacy between the *in vivo* system and the perfused culture. Error bars indicate ±SEM. *$P < 0.05$, ***$P < 0.001$.

Finally, we compared the degree of efficacy rate of the conventional *in vivo* teratoma assay with the perfused system (Figure 10B). Out of 9 *in vivo* experiments, a real teratoma was detected in only 3 cases, while one mainly consisted of a big liquid cyst. In contrast, the perfusion-based culture produced teratoma-like tissues in 90 % of all experiments (9 out of 10 cases). The perfused culture system is more reproducible than the conventional *in vivo* assay.

In summary, the grading of the EB *in vitro* culture is clearly inferior to that reached with the perfused system. The grading of the tissue quality achieved with the perfused bioreactor system is similar to the grading of the tissue quality in *vivo*, but was in our hands much more reproducible. In addition, teratoma-like tissue can already be obtained after three weeks of perfused culture, whereas *in vivo* growth to mature teratoma tissue mostly required 8 weeks after transplantation.

5. Discussion

In this study we present a well-controlled and reproducible culture system for the demonstration of pluripotency of newly derived hPSC lines. Our experiments clearly illustarted that hESCs (CHES6) and iPSCs are able to produce teratoma-like tissue in the 3D perfusion-based bioreactor system.

We demonstrated that the perfused culture of both hESCs and hiPSCs supports cell growth and differentiation resulting in the rapid generation of viable tissues of the three germ layers. Our findings are in line with similar observations with other cell types cultured in this system in which was demonstrated that the dynamic culture environment of the perfused bioreactor ensures a continuous support of nutrients and oxygen, which leads to a maintenance of viable tissue and to the feasibility of the formation of larger tissue-like structures over prolonged time periods (19, 21-23).

The differentiation potential of hPSC *in vitro* is typically examined through the analysis of the expression of genes characteristic of the three germ layers after EB formation (28). Therefore, we investigated if our perfused system is able to let CHES6 commit and differentiate spontaneously into the three germ layers similar as with the EBs. In both the perfused culture system and in the culture of EBs Oct4 and Nanog, which are key transcription factors for the maintenance of pluripotency, are expressed in the undifferentiated starting population of CHES6 (39, 40). As soon as the cells start to differentiate, the expression of the pluripotency markers gradually disappears, whereas the expression level of differentiation genes starts to rise (41). The assessment at molecular level of commitment and late differentiation into to the three germ layers over time has clearly shown that the perfused system recapitulates better the *in vivo* developmental events than the EB culture. During early differentiation until 3 weeks, the cells highly express the commitment markers AFP, brachyury and PAX6. At 8 weeks, the expression levels of two markers (AFP and PAX6) already decrease, while the expression of brachyury is still stable. This is in accordance with the known transient expression of these markers during embryonic development (27, 42), while, *in vivo*, PAX6 is re-expressed later to regulate adult neurogenesis (43, 44). By investigating the expression of later markers of the three germ layers between 3 and 8 weeks of culture, we observed that after the commitment, the differentiation

process is ongoing and that upregulation of differentiation markers belonging to each germ layer roughly follows their order of appearance during embryonic development (45-47).

However, after 8 weeks of culture, there was a remarkable variability in the expression of the various differentiation markers among samples. This can be attributed to the prolonged culture of CHES6 in the basic hPSC medium in the absence of any supplements supporting specific differentiation of certain tissue types. A defect in such specific requirements is also likely to be involved in the observed increase of cell death after 8 weeks in a culture system, which exhibits a remarkable capacity to sustain cell viability for several weeks. The markers of commitment and late differentiation are indeed expressed in the EBs but much more inconsistently.

By the histological evaluation, which is the more stringent assessment of spontaneous differentiation (28), we could detect a clear difference in tissue differentiation between the two *in vitro* assays. The culture of EBs led to more heterogeneous results characterized by diverse qualities of structures. The expression of differentiation markers indicated that expected differentiated cells were indeed generated in the EB but they mostly failed to produce recognizable structures. With the newly established grading system, we could realize that the structure formation never reached the same grade level as the *in vivo* assay, regardless of the duration of the culture. Others have published similar observations, demonstrating expression of markers of the three germ layers in the absence of any organized structures in 35-day-old EBs (48). Another study demonstrated the generation of a feeder-based perfusion system to generate teratoma-like tissue *in vitro*. Also there the culture of EBs resulted in a much lower degree of differentiation and tissue formation as the perfused culture (24). The variability of marker expression and structure formation among the EBs can be attributed to the stochastic nature of differentiation in such culture. Furthermore, it is known, that the differentiation potential of the EBs depends on their initial condition, given by their cell number and their size (13, 49). Depending on the size, the EBs may either develop organized structures or become cystic due to reduced mass transfer (15). The resulting heterogeneity of their terminal differentiation lead to a lower grading of the differentiated structures.

In the perfused system, we could detect various structures derived from the three germ layers at 3 and 8 weeks of culture. To define the differentiation capacity of hPSCs into a teratoma *in vivo* at least one structure per germ layer needs to be demonstrated. The identification of the tissue is

usually done by H&E-staining of the sectioned tumor (9). The following structures, morphologically easy to detect, are usually reported as a proof of spontaneous differentiation *in vivo*: intestinal glands of the endoderm, cartilage of the mesoderm and neural tubes derived from the neuroectoderm (50-53). These structures were also detected in the *in vivo* grown teratoma produced by CHES6 and iPS. In the teratoma-like tissue generated in the perfused culture we detected intestinal glands and neural tubes, but not cartilage-like tissue. One explanation for the disability to the formation of cartilage-like structure is the basic culture medium, which is not supplemented with additional growth factors, such as TGFß, which is known to be an inducer of chondrogenic differentiation (54). Another possible reason could be that the system doesn't have an appropriate niche for its generation as compared to the *in vivo*. In the *in vivo* assay matrigel is co-inoculated with hPSC as it is known to enhance the formation of teratoma *in vivo* (55). Matrigel is a gelatinous extracellular matrix (ECM) that contains various ECM proteins and growth factors such as ßFGF and TGFß (56). Thereby the cell survival and growth of the tumor are enhanced. Moreover, it promotes differentiation into various types of tissue (55, 57). The use of matrigel might have an influence in the chondrogenic differentiation *in vivo* due to these growth factors, but it is not clear to which extend and how exactly since the concrete composition of the matrigel is poorly understood (58). Furthermore, the mouse model itself may have another impact on the growth and differentiation pattern of the teratoma (59). Thus, instead of cartilage, we selected muscle fibers to represent the mesoderm. With the grading system, we could show that the perfused culture was able to mimic teratoma-like tissues from all three germ layers reaching similar grading levels as the teratoma tissues *in vivo* at 3 and 8 weeks of culture. These results demonstrate that the dynamic cell mass exchange in the bioreactor may mimic the cell mass exchange occurring *in vivo.*

In comparison with the *in vivo* growth of teratoma in immunodeficient mice, the present bioreactor system produces teratoma-like tissue faster, as it was formed already after 3 weeks of culture with an efficiency of 90%. In contrast in only 33% of the cases a teratoma *in vivo* could have been built, which underlines the inconsistency of this assay (9, 60). Several approaches have been carried out to standardize the *in vivo* assay by defining distinct parameters. The best engraftment site, the minimum cell number and the best cell preparation have been defined by several laboratories to guarantee the best condition for teratoma formation *in vivo* (8, 55). Our

controlled and reproducible bioreactor system eliminates several of the possible variables existing in the *in vivo* assay.

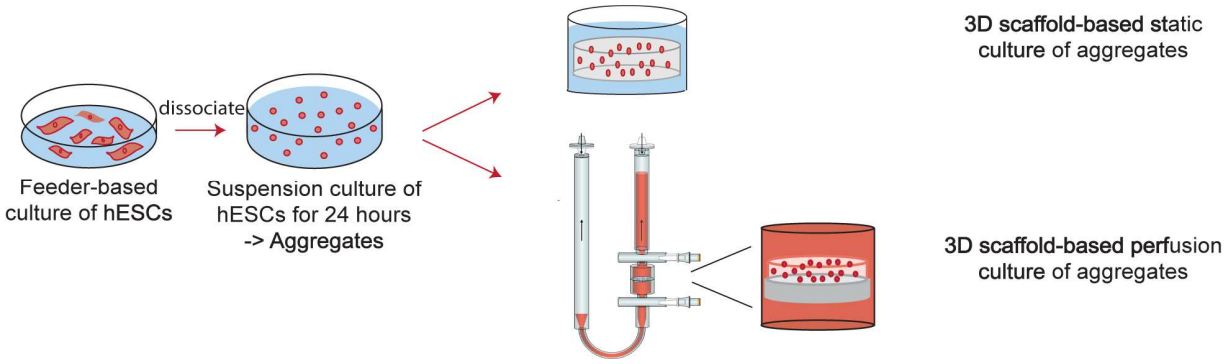
Another disadvantage of the in vivo assay is its duration, which can last up to 15 weeks (9). With the perfused bioreactor system, we could already detect at 3 weeks of culture if the newly derived hPSCs have a normal or abnormal behavior, even by a non-invasive way measuring the AFP-release into the culture medium. This has been clearly demonstrated by culturing the teratocarcinoma-like line CHES1. Already after 3 weeks of culture, no structure formation was detected in the generated tissue. This was confirmed by the absence of AFP-release, into the surrounding culture medium. This result was in accordance to published knowledge concerning this abnormal hESC line (26, 37).

An important aspect of the use of this new perfusion-based *in vitro* assay is the reduction of animal experimentation. The generation of a tumor in an immunodeficient mice may cause pain and harm (10). In consideration of the widespread use of hPSCs and with the technique of generating iPSCs, the number of animals to proof pluripotency by teratoma formation would thereby increase drastically .

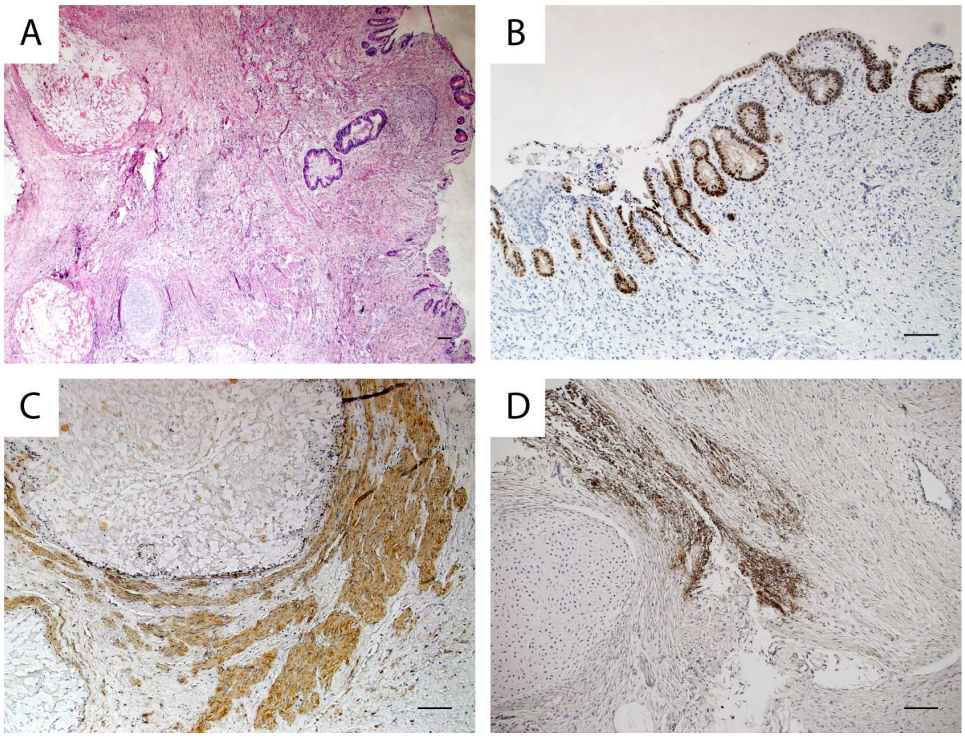
When compared to one other existing feeder-based bioreactor for the production of teratoma-like tissue, we could generate similar results revealing that the perfused culture is able to generate teratoma-like tissue similar to the *in vivo* culture (24). However, our bioreactor system is more user-friendly and it has been validated to an applicable tool using histological analyis.

Based on these results we propose our novel bioreactor system to be used as a screening tool for the demonstartion of pluripotency, with which new hPSC lines can be tested within 3 weeks for their normal or abnormal differentiation behaviour. This model may be further used to compare different hPSC lines in their differentiation capacity using the established grading system. Since hiPSCs are promising in the field of regenerative medicine and disease modelling, the demand for the generation of newly derived hPSC lines is constantly increasing (61). Thus, this system can be further optimized for the use of disease modelling or for clinical application. Thereby the patien-derived iPS lines may be differentiated in a favoured tissue under perfused conditions. Moreover, it can become a valid system in the field of reprotoxicity, since the teratoma formation simulates the early embryonic development.(10).

6. Supplementary Data



Supplementary Figure 1: Experimental scheme for the generation of a teratoma-like tissue under perfused and static conditions.



Supplementary Figure 2: Histological overview of a generated teratoma *in vivo* with iPS after 8 weeks of incubation. (A) H&E-staining of the teratoma tissue (B) CDX2 expressed by cells of early intestinal tissue (C) α-SMA expression displays smooth-muscle like structures (D) Staining of btub3 showing young neurons (scale bar= 100 µm).

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Summary and conclusion

PSCs are powerful tools in basic research and promising for treating injuries, for disease modelling and drug discovery. Technological advances have made it progressively easy to generate iPSCs. Although the various generated lines are considered to be very similar in terms of stemness and their ability to differentiate, differences in their characteristics among lines also exist based on the origin, derivation and culture conditions.

In **chapter I** we assessed the difference in gene expression and differentiation behaviour in 4 generated hESC lines. We demonstrated that inherent differences were evident although the hESCs were derived and cultured in the same way. Spontaneous commitment during stem cell maintenance could be verified. However, the origin of variability among the hESC lines remains unclear. Several explanations exist, such as that the cells are genetically determined to be differently sensitive to external factors used to maintain stemness *in vitro*. It has been shown by various studies that the culture medium has a remarkable influence in the property of the cell to remain pluripotent or to start to pre-differentiate. Thus, it is important that the degree of stemness and the pre-differentiation potential of every newly created hESC line is well characterized. Our results clearly document the difficulty in the handling and characterization of newly created hPSC lines since the derivation- and culture- dependent variability together with the inherent differences clearly have an impact on the property of hPSC lines.

A high variability not only exist in the derivation and maintenance of the hPSC lines but also in the techniques used for the characterization of these lines. The appropriate analysis and the preciseness of any particular test is determined by the investigator. This was also observed in the *in vivo* teratoma assay, which is the gold standard to demonstrate the pluripotency of stem cells. Despite its eminence, this test has never been standardized in terms of graft site, number of cells implanted and the cell preparation. These factors are all known to influence the development of teratoma *in vivo*. In addition, the way to analyze and report the result is considered as variable and inconsistent. This assay raises ethical concerns since hPSCs are inoculated into animals and when a tumor is formed, the animal suffers pain. Thus, in **chapter II** we demonstrated the establishment of a 3D perfusion-based culture system to generate a teratoma-like tissue, which is more standardized and reproducible as compared to the conventional *in vitro* and *in vivo* assay.

The technique of 3D perfusion-based *in vitro* cultures has shown to be essential to ensure the generation of a teratoma-like tissue.

Overall this work implies the complexity and the subsequent challenge of hPSCs in their proper definition and evaluation of pluripotency. HPSCs have the unique characteristic of their potential to differentiate into multi-lineages and their ability to self-renewal, which permits them to proliferate in their undifferentiated status. Consequently, these cells are promising for widespread application like understanding early development, cell therapy and disease modelling due to their unique capacity (1). However, the pluripotent state remains dynamic leading to genetic and epigenetic variation between the lines despite being functional pluripotent. It appears that the definition of the pluripotent state turns out to be more complex as expected and illustrates a weakness in the field of characterization of these cells. Some years ago, specific assays for the characterization of hPSCs have been established but they lack standardization (2). With the establishment of our *in vitro* system to build a teratoma-like tissue as described in chapter II, we could successfully go one step towards a standardized and applicable assay. However, to become a valid alternative or supplement of the existing assays for proving the differentiation capacity of the cells, additional validation steps will be required.

In general, further work has to be addressed for the validation of pluripotency of novel hPSC lines. The so far existing assays are the main base for the evaluation of the pluripotency status but further refinements of these tests should be investigated. Moreover, for the purpose of using hPSCs for cell therapy and translation in clinical practice, quality and safety issues must be solved in order to generate hESCs and iPSCs (3). Depending on the future use of these cells, different levels of stringency in terms of pluripotency may be required. In case of the cell therapy application, it may not be essential that the line has the correct molecular pluripotency but rather the functional pluripotency being able to differentiate into cells that derive from all three germ layers (4).

Additionally, the acquisition of genomic alterations during long-term culture must be prevented or, when they occur, detected. Therefore, it is essential that the pluripotency status of existing hPSC lines is assessed regularly especially before the cells will be further used. Culture conditions and derivation processes (especially for iPSCs) need to be constantly optimized to maintain the characteristics of hPSCs thereby avoiding genetic aberrations. The new existing

molecular tools (e.g. next generation sequencing) are promising in understanding the pluripotent state at a molecular level (5). The next generation sequencing is more often used but more work should be investigated in establishing a user-friendly application of the analysis of this data set.

Together, further research efforts will have to address refined standards to assess the pluripotency status depending on the further applications of the cells. In this context, new technologies have to be officially involved in the evaluation process of hPSC lines. Ultimately, reaching this goal, a continuous and strong collaboration in the stem cell community is required to develop these novel standards. In this way, by an appropriate combination of the different assays, the use of hPSCs can hold its promise of bringing basic research and translation one step forward.

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