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Challenges and possible clinical applications of human embryonic stem cell research

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Abstract

Human embryonic stem cells (hESC) are harvested from the inner cell mass of the pre-implantation embryo and possess several unique characteristics. First, they are self-renewing, meaning they can grow indefinitely in an appropriate culture environment and secondly, they are pluripotent, which means they have the potential to become nearly every cell of the human body. Consequently, hESC offer a unique insight into basic human development *in vitro*, allow better understanding of the genetic and molecular controls of these processes, and are of pharmaceutical interest to test or develop new drugs. The most exciting and high-profile potential application of hESC research is the possibility that such cells can be used for regenerative medicine. Still, several obstacles have to be overcome before clinical applications can be considered: (i) xeno-free derivation and culture of hESC is necessary; (ii) hESC should be safe after transplantation and (iii) their identity and behaviour should be well-known.

Keywords: *embryo, stem cells, regenerative medicine*

Abbreviations

2i	2 inhibitors
ALS	Amyotrophic Lateral Sclerosis
bFGF	Basic fibroblast growth factor
BMPs	Bone Morphogenetic Proteins
BIO	6-bromoindirubin-3-oxime
CGH	Comparative genomic hybridization
EC	Embryo carcinoma cells
ESC	Embryonic stem cells
hESC	Human embryonic stem cells
mESC	Mouse emmrbyonic stem cells
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
EpiSC	Epiblast stem cells
ERK1/2	Extracellular signal-regulated protein kinases 1/2
FGF	Fibroblast Growth Factor
G-banding	Giemsa-banding
GDFs	Growth Differentiation Factors
HLA	Human Leukocyte Antigen
ICM	Inner cell mass
Id	Inhibitor of differentiation
IVF	In vitro fertilization
KO-DMEM	Knockout Dulbecco's Modified Eagle Medium
LIF	Leukaemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
NK cells	Natural Killer cells
PD98059	Parke Davis 98059
PICMI	Post inner cell mass intermediate
(q)RT-PCR	(Quantitative) Real Time Polymerase Chain Reaction
SC-1	Pluripotin
SNP	Single nucleotide polymorphism
TE	Trophectoderm
TFs	Transcription factors
TGF-β	Transforming Growth Factor-β
XaXa	Two active X chromosomes
XiXa	One X-chromosome inactivated

INTRODUCTION

In 1998, Thomson and colleagues established the first human embryonic stem cells (hESC) from the inner cell mass of a human blastocyst stage embryo (1). The unique ability of hESC to proliferate indefinitely in culture and to differentiate into all cell types from the three germ layers (ecto-, endo- and mesoderm) created an explosion of interest and the emergence of a rapidly advancing field. Hence, hESC have become an indispensible tool across a wide range of scientific disciplines and hold great promise for treating many human diseases. From a basic science perspective, the study of how hESC differentiate into more specialized cells and tissues provides novel insight into human developmental biology. The use of hESC derived from embryos with genetic disorders provides *in vitro* models to study the pathology of diseases in clinically relevant cell types. Both normal and disease-model hESC have also been utilized in pharmaceutical research, using targeted cell types for *in vitro* drug and toxicology testing and drug development (2, 3). The most intriguing and potentially beneficial applications of hESC, however, are situated in the field of regenerative medicine (3). A large proportion of human diseases are the result of cellular dysfunction, degeneration, or damage. It is hypothesized that differentiated hESC can be used to effectively replace or repair damaged cells and restore normal function for a whole series of diseases (see figure 1). From infertility perspective, a long-term objective is to create *de novo* patient-

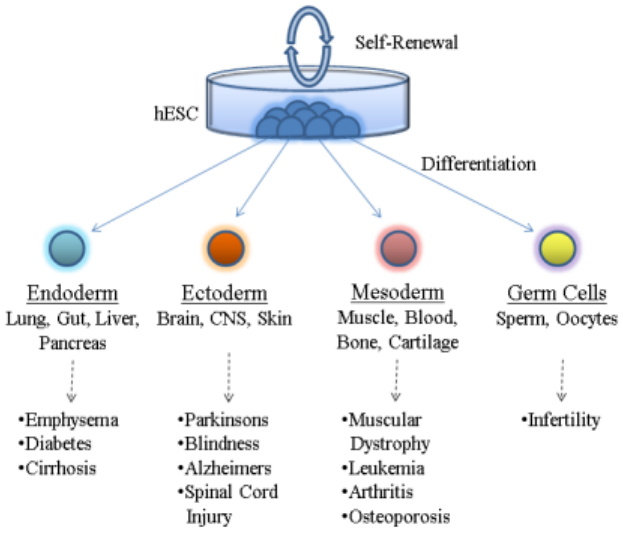

Figure 1. The differentiation potential of human embryonic stem cells and examples of tissue specific diseases that are being investigated.

specific gametes through the technology of therapeutic cloning for sterile patients. This would imply the creation of human somatic cell nuclear transfer embryos using a somatic cell of a patient (4); the derivation of hESC from these cloned embryos (5); and finally the *in vitro* differentiation of these hESC into oocytes or sperm. These gametes would contain the same genetic content as the individual from who the somatic cell originated to reconstruct the cloned embryo.

Although advancing rapidly, the field of hESC research still must overcome substantial challenges both fundamentally and clinically. Understanding the signalling mechanisms that underlie both the undifferentiated and differentiated stem cell state is a fist hurdle. Large scale gene expression profiles failed so far to reveal unique factors that control self-renewal in hESC (6, 7). Comparative analysis has further provided evidence for similarities and differences between hESC lines in self-renewal, and spontaneous and directed differentiation. These differences may be associated with inherited variation in the sex, stage, quality and genetic background of embryos used for hESC line derivation, and/or changes acquired during passaging in culture and the used culture conditions (5, 8). There is a general consensus that gene expression in hESC lines is strongly affected by the used culture methodology (8). One major challenge of hESC research thus remains to identify factors that will enable researchers to propagate and differentiate pure populations of embryonic stem cells under defined conditions *in vitro* (9).

A whole plethora of culture conditions is currently being used for the derivation and culture of hESC which can influence the genetic, epigenetic and transcription profiles when comparing lines cultured or derived in different conditions. Hence, a uniform chemical defined culture environment is highly warranted. The increasing possibility of using hESC-derived cells for future clinical applications requires their derivation and maintenance to be clinically-grade and safe for the patient. In this context, xeno- and feeder-free derivation and culture of hESC has become a major topic of research (10). Small molecules targeting differentiation pathways are nowadays more and more used to replace animal-derived components to maintain the undifferentiated state of embryonic stem cells (11).

For clinical applications, it is a prerequisite that the transplanted cells are chromosomally normal and do not provoke tumour formation. Prolonged *in vitro* culture of hESC has been shown to generate genetic instability in hESC, typical for the process of 'culture-adaptation' (12). Also epigenetic changes have been demonstrated, although more haphazardly and independently from the *in vitro*-culture time (13). Research is being conducted to avoid immune-rejection or tumorigenicity following hESC transplantation and to effectively differentiate hESC into functional cell types (14, 15). A combination of HLA matching of the hESC lines and immunosuppressive medication is most probably the most efficient way. When undifferentiated hESC are transplanted *in*

vivo, they generate large tumours called teratomas which consist of benign masses of cells of differentiated tissue, but they can also give rise to teratocarcinomas containing malignant cells. Strategies are being investigated to eliminate this risk. These collaborative efforts are now beginning to pay off, with the first clinical trials using hESC to treat spinal cord injury and macular degeneration ongoing (16).

Another major challenge is revealing the true nature of existing hESC. Recently published studies have provided evidence that inner cell mass (ICM) cells undergo significant changes during the outgrowth phase of mouse ESC derivation (17). A single-cell gene expression analysis, performed using cells from whole-mouse embryos plated in conventional embryonic stem cell culture conditions, has shown that the molecular profile of a subgroup of cells changes dramatically as they progress from ICM to ESC status (18). These data are limited, and due to the lack of data on gene expression during crucial phases of immediate post-implantation development in humans, it remains unclear precisely which embryonic cell is the *in vivo* counterpart of hESC. This will also help us to better understand the origin of hESC, because recent findings suggest that all existing hESC lines might be of epiblast origin and thus not from ICM (19). The different states of pluripotency in mouse that have been found in mouse will help to elucidate this (20, 21).

PRODUCTION OF HUMAN EMBRYONIC STEM CELLS

Originally, stem cells were classified according to their origin or their *in vitro* or *in vivo* differentiation potential. Based on their origin, we can distinct embryonic, foetal and adult stem cells. Next to these, scientists have discovered in 2006 a method in which somatic cells can be reprogrammed to an embryonic-like state (22). These so-called induced pluripotent stem cells (iPSC) are of particular interest as they can provide autologous cells for therapeutic applications (23). Although this breakthrough may eventually provide an alternative to the use of hESC, comparisons between iPSC and hESC are revealing differences that could impede their clinical use (23). Of particular concern, iPSC may exhibit gene expression profiles associated with cancer and increased levels of cancer-associated microRNAs (24). There are also questions regarding the biological equivalent of iPSC compared to hESC regarding their epigenetic state and developmental potential (25).

Based on their ability to differentiate into other cell types, stem cells are classified as being (i) totipotent: able to differentiate into all embryonic and extra-embryonic cells, (ii) pluripotent: able to differentiate into all cells derived from three germ layers, (iii) multipotent: able to differentiate into a limited number of cell fates, mostly those of a related family of cells and (iv) unipotent: produce only one cell type or tissue. In this review, only

human embryonic stem cells will be further discussed which are considered pluripotent.

Three fundamental steps are involved in the process of hESC derivation – 1) embryo and inner cell mass (ICM) culture, 2) initial hESC outgrowth and identification and 3) hESC maintenance, characterisation and propagation. Donated embryos originally created for infertility treatment are primarily used for hESC derivation. There are two main categories of donated embryos from which hESC lines arise (26). Patients can donate their cryopreserved embryos if they no longer wish to use them for fulfilling their child-wish. These embryos are cryopreserved at any stage of development and are of good quality as they were originally intended for clinical treatment. The second major source of embryos used for hESC derivation comprises fresh spare embryos containing poor quality traits. These inferior quality embryos do not meet clinical criteria of embryo transfer or cryopreservation and can be donated for research rather than being discarded. For efficient hESC derivation, embryos are mostly cultured until the blastocyst stage, although hESC lines have been derived from earlier stages onwards including isolated blastomeres (27-31). Still the highest efficiency can be obtained from the blastocyst stage (32), which contains two distinct cell types: (i) the trophectoderm (TE) giving rise to extra-embryonic tissues such as the placenta and chorion; and (ii) the inner cell mass (ICM) ultimately giving rise to the foetus. The ICM cells are also the source of the pluripotent embryonic stem cells. Immunosurgery is frequently used to isolate the ICM, but also partial or whole blastocysts can be plated for stem cell derivation. For the latter, the blastocyst typically attaches to the substrate, flattens, and the TE cells grow and spread radially during the first several days of culture. Areas of ICM organization can subsequently be excised from the surrounding TE and cultured on a new substrate following whole blastocyst plating (5). Several substrates can be used to support hESC derivation, the most popular being inactivated mouse embryonic fibroblasts (MEFs) as a feeder layer (see further). The time interval between ICM plating and first stem cell outgrowth is rarely reported on (33). In nine published reports, the time of initial outgrowth from the ICM ranged from 4 to 19 days, with the majority of hESC-like cells emerging between 7 and 11 days (34-39). Morphologically, individual hESC can be identified by their high nucleus to cytoplasm ratio and prominent nucleoli. The initial dense colony arising from the plated ICM should be mechanically dissected into uniform clumps with a sharp needle and further cultured on fresh MEFs (40). For maintenance, rapidly self-renewing hESC colonies require a specific culture environment (see further) and frequent passaging into smaller colonies of around 50 to 1000 cells to allow for unhindered growth (41) by mechanical or enzymatic means such as collagenase IV, dispase, or trypsin (42). Aggressive enzymatic passaging, however, has been linked to hESC lines acquiring genetic abnormalities (43, 44).

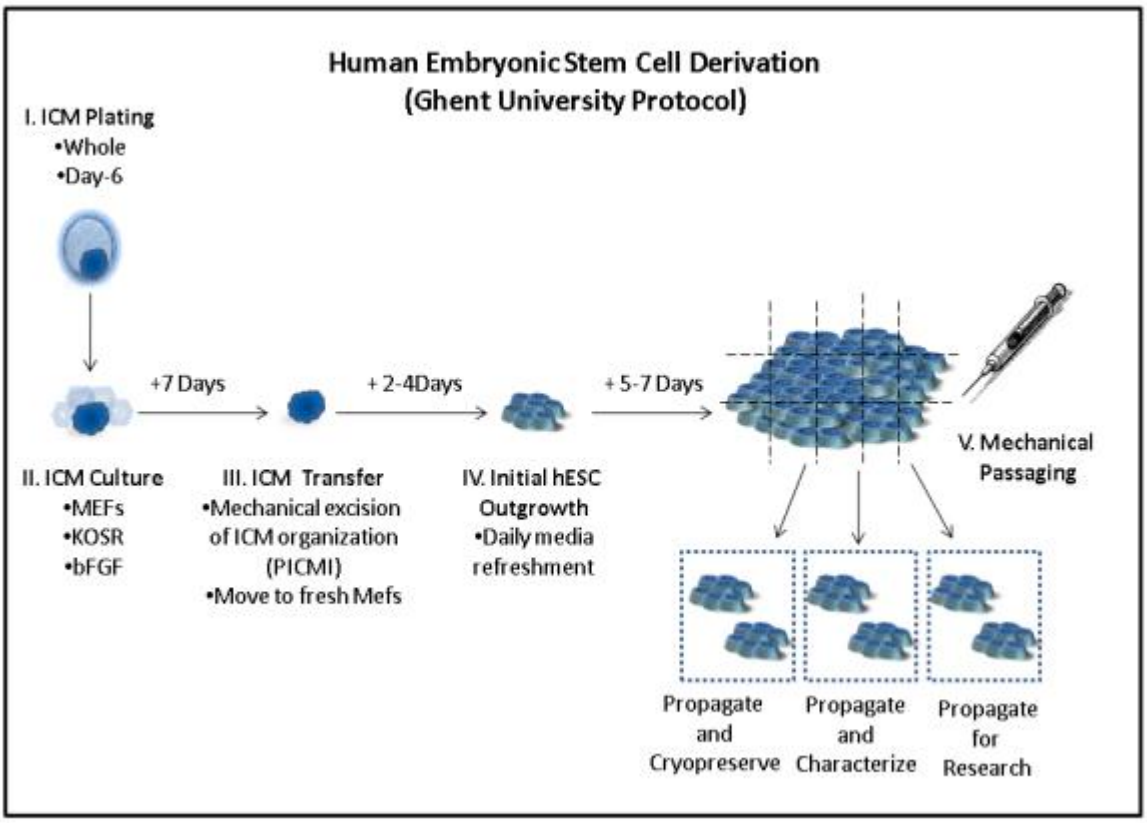

Figure 2. A summary of the steps involved in hESC derivation process at Department for Reproductive Medicine, Ghent University Hospital.

It has to be noted that hESC generally do not survive well following complete dissociation into single cells and this may allow for the selection of chromosomally abnormal cells (41). The standard culture medium for hESC consist of KO-DMEM supplemented with a variety of factors, basic fibroblast growth factor (bFGF) being the most important one and refreshment of culture media is advised on alternating days (5). Once novel hESC colonies have been established, the new line can be propagated indefinitely and cryopreserved. It is essential that thorough characterisation of the new hESC lines is carried out (see next section).

CHARACTERISATION OF HUMAN EMBRYONIC STEM CELLS

Established hESC lines are evaluated by a set of surface and transcription markers to prove their undifferentiated self-renewal state and by tests to evaluate their differentiation potential. Commonly used reliable markers that are typical for the undifferentiated status, include the glycolipid antigens

SSEA3 and SSEA4, TRA-1-60, TRA-1-81, protein antigens CD9, Thy1, and the class 1 HLA antigens (45). Alternatively, cell surface markers can also detect the loss of self-renewal to assess the culture health such as SSEA-1, A2B5, CD56, GD2, and GD3 which should not be expressed in undifferentiated hESC (46). Next to these surface markers, gene expression profiling aids in the characterisation of the undifferentiated pluripotent state of hESC. Previous research showed that a core set of transcription factors (TFs), such as Oct4, Nanog, Sox2 and Klf4 are essential for the maintenance of self-renewal and pluripotency in embryonic stem cells in both mice and human (9). These factors function together in a regulatory loop to maintain pluripotency and self-renewal by upregulation of genes involved in key signalling pathways and downregulating genes involved in other developmental processes (47) and thus are considered as a 'stemness fingerprint'. In addition to the core transcription factors mentioned, genes that are regulated by NANOG can also be used as consistent hESC markers. These genes include GDF3, GABRB3, EBAF, PODXL, NODAL, ZFP42, LIN28, EOMES, and SFRP2 (48). Not surprisingly, there are many genes commonly expressed by hESC and human embryonic carcinoma cells including Oct4, DNMT3B, Sox2 and FoxD3 (49). Gene expression detection by (q)RT-PCR and commercially available microarray kits are becoming common techniques used for detailed hESC characterization.

It is highly important to determine the normal euploid karyotype of all newly derived hESC lines and to reassess their genetic stability during prolonged in vitro culture, which is subject to chromosomal instability (50). Especially changes in chromosomes 12 and 17 are detected, which is a hallmark of many tumours (51). By these aberrant chromosomal constitutions, hESC can easily propagate towards cancer-like or embryonic carcinoma cells (52). Karyotyping is typically performed by classical G-banding or spectral karyotyping to assess whole chromosomal complement. Brimble et al. (53) demonstrated that enzymatically passaged hESC (more disintegrated in single cells) acquired chromosomal abnormalities more frequently than mechanically passaged hESC. If hESC lines are to be used therapeutically, more sensitive techniques such as array comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) array may be necessary to detect genomic stability at a higher resolution (54). Apart from this genetic stability, evaluation of the epigenetic stability of hESC lines has gained increased interest. The 5'-promoter regions of many transcriptional genes clusters of the dinucleotide CpG, which are methylated at transcriptionally silent genes and demethylated upon transcriptional activation. In differentiated cells, the *Oct4*, *Nanog* and *Sox2* promoter regions are silent and methylated, whereas in hESC these promoters are active and unmethylated.

Finally, the differentiation potential of newly established hESC lines needs to prove their most defining feature, pluripotency. Chimera formation and

tetraploid-embryo complementation are the most stringent assays for testing developmental potential of mESC but are evidently not possible with hESC. Therefore *in vitro* embryoid formation with coincident histological analysis and teratoma formation after injection into immuno-deficient mice are currently the only two methods available to test the pluripotency of hESC (45). Following teratoma and embryoid body formation, immunocytochemical analysis and gene expression profiling indicative for cells of the three germ layers is carried out. It has to be noted that embryonic stem cells from non-human primates have failed to contribute to chimeric offspring thus far after blastocyst injection (55). So the full differentiation capacity of hESC has not been determined yet. Whether this is due to the different pluripotent states that have recently been proposed will be further discussed.

MOLECULAR PATHWAYS INVOLVED IN EMBRYONIC STEM CELL SELF-RENEWAL

Some signalling pathways have been shown essential to keep hESC in their undifferentiated state, which are different from mESC, and mostly agonizing and antagonizing each other. For hESC, the TGF-β /Activin/Nodal, Wnt and FGF pathways are the core signalling pathways to maintain self-renewal (56). The TGF-β superfamily contains the TGF-β proteins, activin and nodal, growth differentiation factors (GDFs) and BMPs, all of which are involved in maintaining the stem-cell state (9). Several studies have reported that activin or nodal can synergize with several other signalling proteins, more specifically FGF2 or WNTs, to promote stem-cell maintenance. One study even showed that activin supplementation alone elicits FGF2 production which directly supports self-renewal (57). However, the bulk of the evidence suggests that both activin and FGF2-mediated signalling pathways need to be activated for hESC maintenance. They activate the transcription factors SMAD2 and/or SMAD3, leading to downstream expression of the key pluripotency transcription factor Nanog in human ESC (9). Basic FGF was the first factor found to be crucial for hESC maintenance and is nowadays always incorporated in the derivation and culture medium. HESC express receptors for FGFs, thereby producing FGF2 and activating signalling through ERK1 and ERK2 in these cells (58). The precise interaction of these FGF2-mediated and MEK-ERK mediated signalling and the network of pluripotency transcription factors is still unknown (9). Similarly, the exact role of the Wnt pathway remains uncertain as it could have only a proliferative function (59, 60).

In contrast, the first key element for maintaining self-renewal in mESC was the leukaemia inhibitory factor (LIF). LIF is activating the pro-self-renewal pathway JAK/STAT3 (signal transducer and activator of transcription 3) pathway, which is functioning through regulation of c-*Myc*, *Klf4, Klf5, Oct4, Sox2* and *Nanog*; and the pro-differentiation MAPK pathway (61). The action of LIF signalling requires

the presence of serum, which can be replaced by BMPs which -induces the expression of Id (inhibitor of differentiation) proteins and attenuates the activity of the pro-differentiation MAPK-responsive transcription factors (62). Importantly, mouse ESC can also be maintained in a combination of small molecule inhibitors that block the MAP-kinase signalling pathway which normally leads to differentiation (63, see further). Thus, the combined actions of LIF and BMPs selectively promote mESC maintenance in their undifferentiated status. Intriguingly, small molecules that inhibit or activate all these signalling pathways have become a useful tool to manipulate cell fate (see next sections).

CHALLENGES IN HESC RESEARCH

EFFICIENCY OF HESC DERIVATION

The International Stem Cell Registry now has documented more than thousand novel hESC lines derived in laboratories worldwide. Unfortunately, discrepancies in the reporting of hESC derivation methodology make it difficult to assess its efficiency. It is almost impossible to ascertain from the literature a true efficiency for deriving a hESC line from embryos. The number of used embryos is not always revealed or presented in an inconclusive way. Furthermore, the quality of the used embryos or blastocysts is rarely described. It has to be noted that the larger the experience of a group in embryology and/or cell culture, the higher the success rates of hESC derivation will be (64; own experience). Still, the chances of success of producing human ESC lines largely depend on whether blastocyst formation can first be achieved and the quality of used blastocysts (32). The reported efficiency of hESC derivation from fresh poor quality embryos that develop into blastocysts ranges from 4.1 to 25% (5). In comparison, using frozen-thawed good quality embryos, the hESC derivation efficiency ranges from 13 to 50 % between identical replicates (65). Given the lack of pre-implantation embryo scoring details, it long was uncertain whether the quality of used embryos would influence hESC derivation success rates. Recording of the number of embryos allocated to research and developing to blastocysts and subsequent stem cell derivation would also give important feedback to the IVF lab regarding their culture system and success rates (33). Ström et al. (66) concluded that there was no influence of embryo morphology when fresh embryos were classified as good versus poor quality embryos on the establishment of hESC lines. Still, the individual traits or scores of embryos were not correlated directly to hESC derivation efficiency. More information on the latter was recently reported by O'Leary et al. (5). They showed that although embryos with different poor quality traits (fragmentation level, multinucleation, delayed development, abnormally fertilised embryos) were all able to make blastocysts with good-quality ICMs, the ICMs were unequal in their ability to derive hESC. Good-quality ICMs from embryos with two or more poor quality traits were unable

to generate hESC lines, in contrast to good-quality ICMs originating from embryos with a single poor-quality trait (5). These results suggest that when experiments aiming at hESC derivation are designed, a proper randomisation of the embryos allocated to different experimental groups is warranted. Other publications suggested the possible influence of the cohort of embryos within a patient on derivation success (65, 67, 68). In one study, the efficiency of hESC derivation in five patients was 66.6% (18 hESC lines from 27 blastocysts) in contrast to the study's overall efficiency of 32.1% (65). Apart from embryo cohort influence, this implies also that patient characteristics might influence hESC derivation success. It is well known that patient-specific parameters such as maternal age and responsiveness to ovarian stimulation can also impact embryo development and competency in IVF (69, 70). Indeed it was shown recently that embryos originating from older patients or from cycles that did not result in pregnancy had significantly diminished blastocyst development and ICM quality (71). Interestingly, embryos originating from women older than 37 years were not able to support successful hESC derivation, which corresponds to the poor prognosis of pregnancy success with increasing age in assisted reproductive technology. HESC generating cohorts had the highest blastocyst formation rates, which contained the highest percentage of good-quality ICM and had the highest pregnancy rate, indicative for an overall increase in cohort health (71). So, from these studies it can be concluded that embryo traits, cohort and patient parameters of the embryos used for hESC derivation attempts will affect the success rate and thus can influence outcome parameters.

To date, no studies have been reported to increase the derivation efficiency of hESC except for one. Fan et al. (72) showed that a modified embryo culture medium supplemented with both recombinant human LIF and bFGF significantly increased blastocyst formation rates which resulted in a seven-fold increase in derivation efficiency. Given the control group was performed one year prior to the experimental group and improper randomisation of the embryos was performed, results from that study could be biased. The original protocol by which the first mESC were successfully established was only efficient for a very limited number of mouse strains (especially the 129 mouse strain) and was not widely transferable to other species. In this "permissive" mouse strain, LIF/STAT3 signalling was sufficient to obtain pluripotent stem cells (73, 74). These findings proved that distinct pluripotent states can be specified by culture conditions but also that the genetic background determines the requirements for exogenous factors to obtain the pluripotent stem cells. In 2008, the signalling pathways that regulate the naive state of mouse ESC pluripotency became clearer (63). Hence, it was determined that small molecule inhibition of GSK3 signalling by Chir99021 and FGF-MAPK signalling by PD0325901 in the presence of LIF could maintain mESC in defined,

feeder-free conditions (63). This new culture system, known as '2i+LIF', also allowed the efficient derivation of naïve ESC from any non-permissive mouse strains tested as well as from the rat (75). Subsequently, a combination of three factors (LIF and 2 small molecules: PD98059 and BIO) was shown to yield a fivefold higher mouse ES cell derivation rate compared with that of LIF alone (76). More recently, the combination of the Rho kinase inhibitor Y-27632 preventing apoptosis and accutase significantly increased the derivation rate in the C57BL/6×129/Sv mouse strain (77). So it seems that supplementing derivation conditions with appropriate external signalling can significantly increase derivation efficiency. This has not been proven yet in hESC derivation attempts.

CHEMICAL DEFINED CONDITIONS FOR HESC CULTURE

Although the derivation and culture of hESC has progressed enormously since the first publication over a decade ago (1), currently used procedures are usually still ill-defined, differ substantially from each other and mostly contain animal derived products rendering them unsuitable for possible future regenerative cell therapies (78). Clinical applications of hESC based cell-therapies require their derivation and maintenance to be clinically-grade and safe for the patient, as defined by both the European Medicines Agency and the Food and Drug Administration. All sources of animal- or human-derived contamination should be eliminated. Stable long-term maintenance of self-renewing and pluripotent hESC traditionally involved the use of feeder cells, mostly mouse embryonic fibroblasts (MEFs). The culture medium necessary for the cultivation of hESC contains various animal proteins and additional exogenous factors. These undefined culture conditions present many problems. In particular, the use of serum products (with batch-to-batch variation) can compromise the consistency of the hESC culture, and complicate comparative biological studies between different hESC lines.

Several groups have attempted to exclude individual animal components by using feeder-free matrices (79, 80) feeder cells of human origin (35, 81-83), or defined xeno-free media (80). Crook et al. claimed to have created the first six hESC lines suitable for therapeutic use but animal derived components such as serum replacement were still used and the established lines were not maintained for a long period (84). One year earlier, Ludwig et al. already reported the derivation of hESC lines in 'defined conditions' but these lines were shown to be karyotypically unstable after prolonged passaging (80). Recently, Ilic et al. reported the derivation and culture of five hESC lines under clinical-grade conditions, one of which was presented to the UK Stem Cell Bank for further evaluation with the goal to make the cell line available to other researchers (85).

Another more fundamental approach aiming towards more chemically defined conditions has focused on the signalling pathways involved in self-

renewal and pluripotency. Small molecules, targeting specific signalling pathways and/or mechanisms, have been shown to be useful chemical tools in manipulating cell fate, state and function, and are playing increasingly important roles in elucidating the fundamental biology of stem cells (11). For example, after screening of 50 000 small molecules in the absence of feeder layers, serum and LIF, pluripotin (SC-1) was identified as a potent and specific small molecule supporting mESC expansion in the undifferentiated state (86). Burton et al. (87) succeeded in the maintenance of hESC in the absence of both feeders and cytokines by using the compound erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). Recently, Tsutsui et al. (88) reported a "golden combination" of small molecules (a MEK inhibitor, GSK3 inhibitor and ROCK inhibitor) enabling maintenance of hESC on a fibronectin-coated surface by single cell passaging. It has to be taken into account that all these studies have started from existing hESC already exposed to animal-derived components during derivation and initial culture. All these combined efforts should soon lead to the derivation of hESC in chemically defined conditions which would be more suitable for future clinical cell-based therapies using cells derived from hESC.

(EPI-)GENETIC STABILITY OF HESC AND TUMOUR FORMATION

Maintaining a hESC line by weekly mechanical passaging is time, money and effort consuming. But it has been proven to be the safest and most reliable method for the long term propagation of hESC, as the development of genetic abnormalities during culture has not been reported (89). Bulk culture of hESC normally involves enzymatically passaging of hESC which leads to a dramatic expansion of hESC. However, it coincides with an increase in genetic instability, methylation changes and mitochondrial mutations, similarly observed in cancer and Embryo Carcinoma (EC) cells (43, 53). EC cells are the transformed unstable counterparts of hESC, which display very similar cytogenetic abnormalities to hESC with acquired genetic abnormalities such as trisomy 12, 17 and X (43, 53). Even subkaryotypic changes and point mutations in coding regions might arise in stem cell cultures (90) which demand more accurate and expensive methods for the evaluation of the genomic integrity, such as array comparative genomic hybridization and single-nucleotide polymorphism array (SNP array). Recently, SNP arrays showed that structural variants occur sporadically in a large study including 125 hESC lines (12). Next to genetic errors that may occur during *in vitro* culture, it has been shown that hESC undergo a range of epigenetic modifications upon prolonged culture in serum free culture conditions, including dramatic changes in methylation. Holm et al. found that loss of methylation in ESC is directly related to tumorigenesis (91). Loss of imprinting leads to a higher growth rate, a shortened cell cycle time, cellular immortality, resistance to TGFβ, and foci formation on a confluent monolayer. Evaluation of some imprinted genes

revealed some abnormal epigenetic states and expression of for example IGF2 and H19 in some hESC lines (92). It is clear that a deeper understanding of the molecular mechanisms that control the genetic stability of hESC is required and culture methods that prevent genetic instability will need to be optimized, since large amounts of undifferentiated stem cells of high quality are needed for future regenerative medicine approaches.

Apart from the genetic stability within the hESC culture, a major concern with future hESC-based cell therapy is that of tumorigenic potential. Therefore, it is imperative to assess the heterogeneity of a culture, as the engraftment of undifferentiated or incorrectly differentiated cells may present a substantial tumorigenic risk to the recipient (93). In this respect, a particular difficulty is the ability to monitor cell distribution after transplantation to distinct them from host cells. This is especially relevant when the cells are administered intravenously, rather than locally, since broad dissemination is likely to occur. It is not yet possible to quantify the tumour risk associated with the introduction of hESC-derived cells *in vivo*. This risk will be ameliorated by developing appropriate purification protocols and the means for monitoring contamination. Transgenic methods are being investigated to modify hESC lines in such way that only differentiated cells survive after engraftment as has recently been proposed in iPSC-based therapies (94), although such genetic modification could also alter the cell characteristics.

DIFFERENT STATES OF STEM CELL PLURIPOTENCY

Despite their same ICM origin, hESC are clearly dissimilar from their mESC counterparts. Since all stages of embryogenesis in mice are experimentally accessible, different types of stem cells with distinct characteristics have been derived which suggested that the differences between mouse and human probably correspond to different stages of embryonic development from which they originate (20). Pluripotent stem cells were derived from the epiblast of the post-implantation mouse embryo in growth conditions similar to hESC, giving rise to the so called epiblast stem cells (EpiSC) (95, 96). As such, pluripotent cell types were classified into two fundamentally distinct states of pluripotency (21, 97): (i) ‘the naive state’ = the inner cell mass-like pluripotent state, which is typical for mESC derived from the pre-implantation ICM and (ii) ‘the primed state’ = epiblast-like state, characteristic for mouse EpiSC isolated from the post-implantation embryos. Naive ESC can be cloned with high efficiency as packed dome colonies, efficiently contribute to chimeras, maintain both X chromosomes in an active state (XaXa), and are relatively refractory in their potential for primordial germ cell differentiation (9). In contrast, primed stem cells are characterized by a flattened morphology, show intolerance to single cell passaging, are highly inefficient in chimera contribution, have undergone X-chromosome inactivation (XiXa), and are poised for differentiation into primordial

germ cell precursors. Importantly, the signalling pathways/external signals for unlimited self renewal are also different and mostly antagonizing each other (9, 21): the growth of primed stem cells depends on signalling by Activin, FGF2, ERK1/2, and TGF-ß while BMP4 and LIF/STAT3 signalling stabilizes naive ESC.

Given the shared morphology, culture requirements and signalling pathways, it has been suggested that existing hESC are more similar to mouse EpiSC (9). Little is known about the temporal changes that occur as the ICM progresses to the stem cell outgrowth state or the importance of timing for these events. A single-cell gene expression analysis in mice had shown that the molecular profile of a subgroup of cells changes dramatically as they progress from ICM to ESC status (18). The cellular organization and molecular changes that occur during the transition from human ICM to hESC were recently demonstrated (19). It was shown for the first time that the human ICM in culture developed first to an epiblast-like structure before the generation of pluripotent hESC. This structure, which was termed the post inner cell mass intermediate (PICMI), was found to be an essential intermediate in all of the hESC derivations (19). The PICMI possessed a unique molecular signature and combined characteristics of early and late mouse epiblast and even showed some primordial germ cell markers. The PICMI was shown to be a closer progenitor of hESC than the ICM and its existence, together with the observed dynamics of hESC derivation, had further elucidated the origin of hESC (19). This was the first proof that all existing human ESC contained a primed state of pluripotency (98). Importantly, primed mouse EpiSC cells do not contribute (or very poorly) to chimera formation which is one of the most stringent tests of differentiation capacity. In contrast, naive mESC contribute very efficiently to chimeras (9). As the existing hESC are thought to be primed, it is very likely that the differentiation potential is also limited which has consequences for regenerative purposes (21). Interestingly, in response to external signals, the two different pluripotent stem cell types show high plasticity and can be converted to each other.

CLINICAL APPLICATIONS USING HESC

Despite much progress and accrued knowledge at the basic science research level, the clinical use of hESC is still in its infancy. This lag in application of hESC from its isolation over 10 years ago is largely due to concerns of tumour formation (see above), as well as ethical and related legal issues. Still, the lack of detailed characterisation of most established hESC lines causes a major concern for manufacturers and end-users since an enormous investment is necessary when taking a cell line through to a medicinal product. A minimum characterisation is required to deposit a clinical grade line which should include passage number before submission, DNA fingerprinting, karyotype, viral and sterility testing, viability and functional

differentiation assays and expression of pluripotent markers (33). The application of hESC based cell therapies has been proposed for a whole series of diseases, mostly aging-associated diseases (99). The latter has become a primary focus in the biomedical field since the number of individuals aged 65 years and older is expected to dramatically increase within the near future due to the baby boom generation between 1945 and 1964.

Current hESC research is very much focussed on the optimisation of *in vitro* differentiation protocols to enrich pure, homogeneous populations of specific functional cell types belonging to the three germ layers that can be used for transplantation. The differentiation of hESC towards mesoderm derivates such as cardiomyocytes has attracted great interest (100). The high prevalence of heart disease, along with the scarcity of hearts and heart tissues available for transplantation and the associated clinical and autoimmune problems of transplantation, make this line of research imperative. HESC are known to differentiate to myocytes morphologically similar to cardiomyocytes which display normal cardiomyocyte function and electrophysiological properties by culturing in a specific condition medium or treatment with bone morphogenetic protein-4 (BMP-4) and activin A (100). Until now, clinical trials of hESC have not been started for cardiac diseases, but the successful application in repairing heart failure in the mouse system has encouraged scientists to develop such therapy for human patients.

Endodermal derivatives from hESC include cells that populate the lung, liver, pancreas, urinary bladder, pharynx, thyroid, parathyroid, and digestive system (99). Type 1 diabetes for example is a disease in which a specific type of cell, insulin-producing pancreatic beta cells, is damaged or destroyed by the patient's own immune system. Although the strategy of treating diabetes with beta cell transplantation is clinically possible, it is also very much limited by the shortage of donors. Therefore, the generation of insulin-secreting cells from hESC has gained much interest. Unfortunately, similar to hepatocytes differentiation, *in vitro* beta cell differentiation from hESC is very hard and inefficient. By using a step-wise *in vitro* differentiation protocol, some progress has been achieved recently (101). In a similar manner, a highly robust population of functional hepatocytes was proven possible (102). Still, differentiation of endodermal cell types from established hESC remains inefficient. The successful derivation of a new state of naive pluripotent hESC might alleviate this problem.

Interestingly, the dominant differentiation pathway in hESC cultures leads to the formation of ectoderm derivates including cells of the nervous system and the epidermis (99). One of the most exciting and most advanced possible therapeutic applications of hESC is for patients who have been paralyzed by catastrophic spinal cord damage. A USA Biotech company Geron began Phase I safety trials of its technique for converting stem cells into a type of neuronal cell,

known as oligodendrocyte progenitor cells, intended for injection into the patient's spinal cord at the site of injury. The intention, which has been repeatedly demonstrated in animal tests, was that the newly-injected oligodendrocytes might repair the damaged insulation around the severed nerve cells of the spinal cord, and thereby enable those cells to once again send signals to the patient's limbs and organs. Due to internal re-organisation within the company, this clinical trial has unfortunately recently been halted (103). Therapies using hESC-derived cells on neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS) are also under investigation by the generation of functional neurons. For example, hESC-differentiated dopaminergic (DA) neurons that can secrete dopamine were produced for the treatment of Parkinson's disease and found to have therapeutic effect in animal models (99). Retinal pigment epithelium cells are another specific cell type derived from neuroectoderm that are of interest for hESC based cell therapy. Two clinical trials testing retinal cells derived from hESC (for age-related macular degeneration and Stargardt's macular dystrophy) have recently reported positive preliminary results as there were visual improvements in the patients (103). No signs of tumor or other abnormal growths, retinal detachments, or immune rejection of the hESC-derived cells were noticed, but the final results are only expected in 2013. It has to be noted that these preclinical and clinical hESC based trial phases has largely been funded by private corporations without government support.

HESC have also been implicated as potential cell-based therapy for cancer treatment. Recent studies have demonstrated that hESC can differentiate into NK cells which produced cytokines and performed antibody-mediated cytotoxicity on targeted cells (104). In perspective of infertility treatment, hESC have been differentiated towards sperm and oocyte precursor cells, but functional gametes were not obtained yet. In mice, functional sperm was successfully obtained from naive mESC (105) which reinforces the need for creation of naive hESC which differentiation potential towards gametes could be more efficient.

CONCLUSIONS

For possible clinical applications of hESC derived cells, there are still many scientific challenges that must be addressed. The field of regenerative medicine is relatively young and it would be wrong to overpromise on the speed and scope of such research to patients and their families. Firstly, we need to define the real pluripotent state of existing hESC given the significant overlap with mouse EpiSC. Is it possible to derive naive hESC or does this state of pluripotency not exists in human? What are the differentiation capacities of naive hESC compared to their primed counterparts? These are fundamental questions that should be first answered before any clinical application should be considered. We also need to make sure that the hESC derived cells

behave in predictable ways and do not produce tumours. Thorough characterisation of established hESC lines using state-of-the-art molecular techniques is a very important first step in this. Finally, we need to figure out how to get human embryonic stem cells to differentiate down specific pathways in a well-controlled process using defined conditions.

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