Chapter 2
Human Embryonic Stem Cells

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Abstract Stem cells can be isolated from a variety of sources and they are typically classified based on their tissue of origin. Embryonic stem cells are, as the name indicates, derived from the inner cell mass of pre-implantation stage blastocysts at day 5–7 post fertilisation. These cells possess qualities such as pluripotency and a seemingly limitless capacity to proliferate *in vitro* in their undifferentiated state. Embryonic stem cells were first derived from mouse embryos in the early 1980s but have now been derived from a number of different species including rat, rabbit, sheep, pig, horse and human. This chapter focuses on human embryonic stem cells and describes techniques used for their derivation and culture. In addition, the basic properties of these cells are illustrated, including some examples of their capacity to differentiate to various precursors and functional cell types. Finally, some areas of applications for these cells are discussed with emphasis on their possible future use in regenerative medicine including current clinical trials.

Keywords Embryonic stem cells • Blastocysts • Surface markers • Transcription factors • Enzyme mediated passage • Pluripotency • Xeno-free culture

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2.1 Introduction

The developments in the human pluripotent stem cell field during the last 15 years are remarkable, and the scientific achievements made have substantially furthered our understanding of the opportunities that these cells provide for basic and applied research as well as for future regenerative medicine applications. There has been rapid progress in the development of improved derivation and culture technologies for human embryonic stem (hES) cells since the initial derivation of stable cell lines in 1998 (Thomson et al. 1998). One of the main driving forces behind this is the aim to generate high quality, clinically compliant, hES cell lines which can be used for cell therapy in humans. As such, the cell lines need to be manufactured according to good manufacturing practice (GMP) in order to comply with good clinical practice (GCP) which is a set of internationally recognised ethical and scientific quality requirements that must be observed for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects (2001/20/EC 2001; 2005/28/EC 2005). In addition, there are more immediate opportunities to use hES cells and their derivatives as *in vitro* tools to study, for example, human development and genetic diseases. Furthermore, the cells are also expected to contribute to improvements of the models currently used in drug discovery and by providing a source of a variety of human specialised cells which then can be manufactured under standardised conditions. However, in order to realise the opportunities that human pluripotent stem cells provide, there are a number of challenges that need to be addressed. Cost-efficient culture conditions which allow large scale production of the undifferentiated cells is one, efficient and robust protocols for the process of differentiation of the pluripotent cells to the desired end point is another. Characterisation and quality control also require specific attention and monitoring the phenotype and genomic stability of the cells during expansion and propagation is critical. For any kind of therapeutic applications, regulatory compliance needs to be factored in as well. Ideally culture systems would be based around fully defined components, using small molecules with a decreased dependency on biologics. Much effort has been spent on genetically modifying the cells, e.g. to make cells overexpress genes linked to desirable functionality such as metabolising enzymes and also to generate reporter lines. With the last years' advancements in the generation of induced pluripotent stem (iPS) cells, the technologies used for genetically modifying cells has certainly fuelled further research in this area of engineering stem cells. Further details on the iPS cells and the opportunities and challenges they provide are reviewed in a separate chapter of this book and will not be discussed further here. Below, we will cover basic aspects on the derivation process of hES cells and highlight different culture conditions for maintenance of the undifferentiated cells. We will also describe various ways to characterise hES cells in order to verify their unique properties. The pluripotency of the cells will be illustrated with some examples of differentiated cell types which can be generated from the hES cells, and applications in regenerative medicine will be discussed.

2.2 Derivation/Classification

2.2.1 Derivation

The different stages of human embryos which have been successfully used for derivation of new hES cell lines are blastocysts (Thomson et al. 1998), morulae (Strelchenko et al. 2004), late-arrested embryo (Feki et al. 2008; Gavrilov et al. 2009; Zhang et al. 2006) (Gavriolov 2009) or blastomere (Geens et al. 2009; Klimanskaya et al. 2006).

Initially, the method was adapted from the previously developed protocol for mouse ES cells (Evans and Kaufman 1981; Martin 1981). The substantial species differences and the lack of appropriate culture medium for human embryos were likely a large part of reason why it was not until the late 1990s that the first human embryonic stem cell line was isolated. Divergences in intracellular pathway signalling between mice and human have been demonstrated as one explanation to the differences in culture requirements (Brandenberger et al. 2004; Rho et al. 2006; Xu et al. 2002). In 1994, Bongso and co-workers managed to isolate and, to some extent, propagate inner cell masses (ICMs) from human blastocysts and these cells displayed stem-cell like properties (Bongso et al. 1994). This achievement in concert with the successful derivation of non-human primate ES cell lines in the mid-1990s (Thomson et al. 1995, 1996) paved the way for the subsequent derivation of stable hES cell lines in 1998 (Thomson et al. 1998). Since then, the generation of a large number of hES cell lines have been reported, numbering well over 1000 different lines (Loser et al. 2010) with more being added every year. Registries of currently available hESC lines are maintained by ISCF (http://www.stem-cell-forum.net/initiatives/isci/stem-cell-registry/), UK Stem Cell Bank (http://www.nibsc.org/), NIH (http://grants.nih.gov/stem_cells/registry/current.htm) and EU hESCreg (http://www.hescreg.eu/).

The majority of hES cell lines derived thus far have utilised donated surplus embryos from assisted conception laboratories, i.e. from the procedure of *in vitro* fertilisation (IVF). Both fresh and previously frozen material can be used for stem cell derivation. The embryos are cultured to the blastocyst stage before measures are taken to isolate the ICM cells. The first step is removal of the zona pellucida which can be achieved by the use of pronase (xeno-derived) or alternatively Tyrode's acid solution or mechanical dissection is a xeno-free method is preferred. Spontaneously hatched blastocysts can also be processed further without the need for zona pellucida removal (Heins et al. 2004). Subsequently, the blastocyst is treated with mouse antibodies directed against human trophoectoderm cells and guinea pig complement components. This process, called immunosurgery, lyses the cells by an antibody/complement reaction leaving the inner cell mass cell mostly intact, and these cells can then be sub-cultured further on a layer of mitotically inactivated mouse embryonic fibroblast feeder cells (mEF cells). The initial outgrowth from the inner cell mass cells is usually dissected mechanically under the microscope and transferred to new culture dishes after 1–2 weeks. An established hES

cell line is typically passaged every 5–10 days, depending on culture method and population doubling time. As soon as the hES cells are growing in a way allowing culture expansion, low passage samples should be cryopreserved as a seed bank, and also as larger well characterised Master Cell Banks (MCB). From these MCBs, Working Cell Banks (WCB) can be established, for further expansion and various endpoint applications. If the aim is to derive hES cells for downstream clinical use, i.e. to generate therapeutic cells, then the whole procedure needs to comply with regulatory demands, such as current Good Manufacturing Practice (cGMP), but also comply with ethical regulations (Crook et al. 2007; Murdoch et al. 2012).

The methodology for ICM isolation briefly described here is dependent on careful monitoring and execution for successful results since the viability of the resulting ICM isolate is fragile. A success rate in hES cell line generation of at least 5–10 % is reasonable to expect but greater than 30 % success rate has been reported (Chen et al. 2009; Sjogren et al. 2004). Negative results could be related to sub optimal culture conditions or other technical issues. Blastocyst quality is also an influencing factor for successful derivation of hES cell lines, and the use of higher quality embryos leads to a greater success rate (Lerou et al. 2008b). However hES cell lines have been established from blastocysts graded as of low quality (Dokras et al. 1993; Heins et al. 2004; Lerou et al. 2008a) and even from non-viable embryos, albeit with questionable quality (Feki et al. 2008). Assuming embryo culture is well conducted it appears that derivation from previously cryopreserved embryos or blastocysts should be as successful as derivation from fresh material (Sjogren et al. 2004). The proportion of cryopreserved embryos eligible and donated for research activities varies, however, there seems to be a positive correlation between the level of information and support from the IVF clinic staff and the willingness of patients to donate surplus material for research (Brett et al. 2009).

2.2.2 Classification

The essence of a hES cell is its ability to both self-renew and differentiate into other, more specialised cell types, such as muscle cells, neurons, connective tissue and epithelial cells, to mention a few. The hES cells are pluripotent, i.e. they can form cells representing the three germ layers endoderm, ectoderm, and mesoderm. To assess the quality of a recently derived hES cell line, the expression or selective absence of various surface markers, transcription factors, and other properties of the assumed pluripotent hES cells are monitored. Several of these markers have been found closely associated to the pluripotent state of hES cells and researchers normally apply a panel of several markers for the characterisation procedure (Heins et al. 2004; Thomson et al. 1998). Briefly, they include

- Cell membrane bound surface markers like the glycol lipids SSEA-1,-3, -4; and the keratin sulphate molecules TRA-1-60, -1-81.
- Transcription factors such as Oct-4, Sox2, and Nanog.

- Telomerase activity, as a measure of the hES cells ability to continuously go through mitosis.
- Alkaline phosphatase activity.
- *In vitro* pluripotency, assayed for by using markers for endo-, ecto-, and mesoderm on spontaneously differentiated cell material. Commonly used markers are the transcription factor forkhead box A2 (Foxa2) transcription factor for endoderm, β-III-tubulin for ectoderm and Arterial Smooth Muscle Actin (ASMA) as a marker for Mesoderm.
- *In vivo* pluripotency, assayed for by xenografting hES cells into an immune-deficient mouse and subsequently analysing the resulting teratoma for endo-, ecto-, and mesoderm derivatives. Typically, the cells are placed under the kidney capsule but other areas have also been used such as testis and skeletal muscle.

In Fig. 2.1a, the nuclei of a confluent layer of hES cells have been stained with an antibody for the transcription factor Nanog to illustrate its presence. Nanog was simultaneously discovered in 2003 as a pluripotency sustaining factor by Chambers and Mitsui (Chambers et al. 2003; Mitsui et al. 2003). Flow Cytometry (FC) allows a more exact quantification of markers than microscopic observations of immunostaining, and several markers could be analysed simultaneously, e.g. to asses co-expression of more than marker. In Fig. 2.1b, an FC plot illustrates a population of Oct-4 positive cells as an indication of pluripotency, with Fig. 2.1c illustrating the isotype negative control for the assay as a comparison. In addition to the above examples of properties linked to pluripotency, the genomic stability of the hES cells is normally assessed, employing standard G-banding karyotyping or more high resolution techniques such as high density array based SNP analysis. The absence of pathogens is also normally confirmed as well as the thawing recovery rate after cryo-preservation.

The International Stem Cell Forum launched a characterisation initiative a few years ago (The International Stem Cell Initiative or ISCI) as a global collaborative effort to reach consensus on basic methodology and criteria for the derivation,

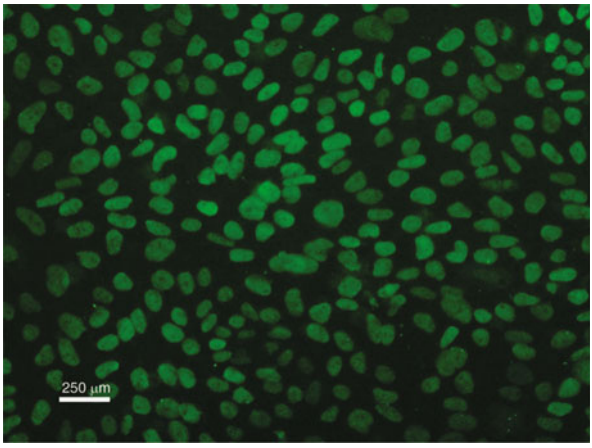

Fig. 2.1a Nanog nuclear staining of a confluent layer of hES cells. This methodology, known as immunocytochemistry, is commonly used to illustrate the presence of various factors and markers linked to certain properties of cells, e.g. pluripotency of hES cells

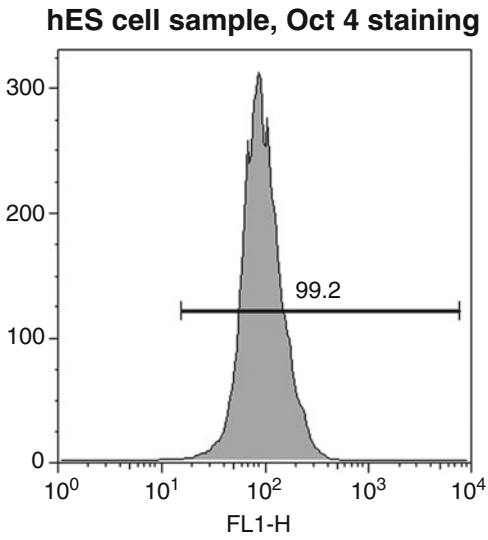

Fig. 2.1b Flow Cytometry (FC) diagram of hES cells positive to 9X, x % for Oct-4 transcription factor. By using quantitative methods like FC a more exact determination of the characteristics of stem cells is possible, compared to qualitative methods

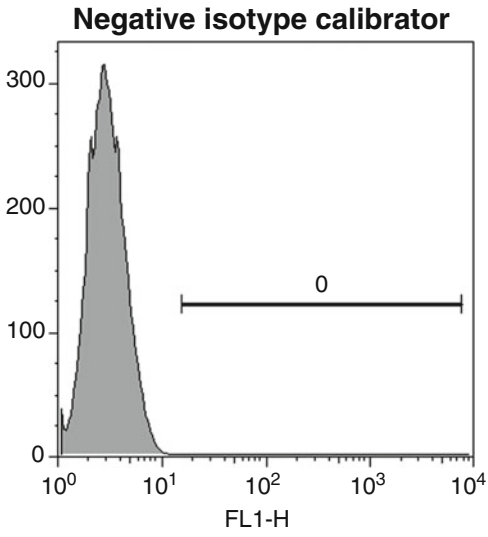

Fig. 2.1c FC isotype negative control. This illustrates how cells negative for the marker appear in the FC analysis

characterisation and maintenance of hES cells (Adewumi et al. 2007), also discussed by Stephenson et al. (Stephenson et al. 2007). The consensus of the scientific community in concert with the regulatory bodies for the major markets will be central to the eventual development and adoption of medical applications of hES cells.

2.3 Culture Conditions

2.3.1 General

It is anticipated that hES cells are more sensitive to sub optimal culture conditions than common somatic cell lines, hence demanding a more stable and controlled environment and a precise culture medium formulation to maintain the undifferentiated and pluripotent state during long term culture. A number of critical parameters should be accounted for when setting up a hES cell culture laboratory, including:

- Laboratory facilities – there should be a dedicated, fit for purpose, and secluded cell culture area with
  - Clean filtered air and stable temperature, preferably positive pressure.
  - A minimum of consumables storage in the direct cell culture area.
  - Good cleaning routines and easily cleaned equipment and surfaces.
  - High quality equipment that is regularly serviced and calibrated where appropriate, cell culture incubators maintaining a stable climate to avoid changes in pH, temperature and osmolality of the culture medium.
  - Heated stages fitted to the microscopes to avoid a decrease in culture temperature when outside of the incubator for inspection or manipulation.
- Aseptic handling – especially important if cultures are not supplemented with antibiotics. The staff should be trained and audited accordingly.
- Contact materials – all materials should preferably be tested for embryotoxicity or be of IVF grade. Surface cleaning detergents should be non-abrasive, non-volatile and non-toxic.
- High quality culture reagents, such as medium, growth factors and other reagents and solutions.
- Good cell culture laboratory routines in general, regular testing for mycoplasma, quarantine routines and preferable a quality system in place that regulates version control of protocols and deviations.

2.3.2 Culture Propagation Techniques – Manual Dissection

The classic method for culture of hES cells is in co-cultures with supporting feeder cells, typically mitotically inactivated mouse embryonic fibroblasts, that provide support in terms of conditioning of the culture medium, surface matrix component deposition and other direct cell-cell interactions (Ellerstrom et al. 2007; Heins et al. 2004; Thomson et al. 1998). Critical parameters for high quality hES cell colonies are the quality of the feeder cells, as well as their density and capacity to condition the culture medium. As further discussed below, other hES cell culture systems free of a feeder layer per se could still rely on medium being conditioned by feeder cells,

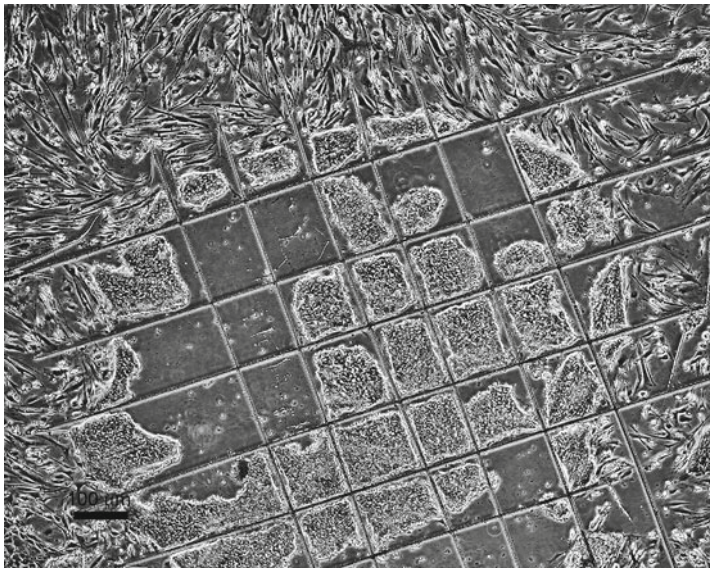

Fig. 2.2 Manually dissected hES cell colony, demonstrating the principles of this propagation method. The mature hES cell colony is sliced up in small fragments using a fine sharp object like a pulled-out glass capillary or a micro scalpel. The colony fragments are subsequently detached from the culture dish and transferred to a new dish with a fresh feeder cell layer. This procedure is typically repeated every 5–7 days, and culture medium is refreshed in between

thus the medium contain factors favourable for maintaining the hES cells in a pluripotent state (Prowse et al. 2005; Lim and Bodnar 2002), The hES cell culture medium could also be totally free of feeder cell influence and have more or less defined components as discussed below and reviewed in Amit and Itskovitz-Eldor 2006 and Amit 2007. The original method for propagation of hES cell cultures on feeder cells is by mechanical dissection. A fine sharp object, e.g. a capillary drawn out over a flame, micro scalpels or other bespoke stem cell cutting tools are used for slicing up a mature colony of hES cells into smaller pieces that is subsequently transferred to a new culture vessel with feeder cells. In Fig. 2.2, a hES cell colony has been sectioned and some pieces removed to illustrate the technique. hES cell colonies typically grow to rounded flat and homogenous colonies, a layer just a single, or a few cells thick. The colonies are clearly visible by eye and can be sizeable in diameter (mm scale). The culture schedules for individual hES cell line often have to be determined empirically, however, the colonies generally need to be passaged every 5–7 days. If allowed to overgrow, the hES cell colony will eventually start to show signs of differentiation, with random 3D structures appearing, migration of fibroblast-like cells and even formation of spontaneously beating cells and neural-like outgrowths. In addition, the feeder layer will deteriorate. Typically, the culture vessels with the feeder layer are prepared some time in advance, allowing conditioning of the culture medium. During the course of culture, culture medium is normally replaced, either fully or partially, at intervals. Centre well dishes like the

ones used for IVF are commonly used since they are designed to allow manipulation of their content, have a rim compartment that should contain culture medium or sterile buffered solution in order to minimise changes in osmolality of the centre well, and not the least, they are subjected to a rigorous quality control. Nevertheless, a number of different culture vessels can be used. When simultaneously maintaining more than one hES cell line in culture, routines should be established so the risk of cross contamination between lines is eliminated.

2.3.3 Enzyme Mediated Passage

The manual dissection method for hES cell passage described above is very labour intensive and requires staff skilled in micro dissection. It is also practically impossible to acquire enough cells for large scale experiments, not to mention sufficient number of cells for use in compound screening or future regenerative medicine applications. As a consequence, protocols for enzymatic digestion of hES cell colonies have been developed and reported by several groups (Ellerstrom et al. 2007; Richards and Bongso 2006; Sjogren-Jansson et al. 2005). There are several commercially available culture systems, with more or less defined components, allowing feeder cell independent stem cell culture in confluent monolayers with enzyme mediated passage. The important aspect of these culture systems is their ability to support hES cell growth on either a biologic or synthetic matrix without involvement of feeder cells and their inherent variability and undefined contribution. The advantages are apparent; it is less time consuming to passage the hES cells and a larger number of cells can be cultured by a single person. After establishment and initial mechanical passage, hES cells can be made progressively tolerant to enzymatic digestion, eventually allowing the cell colonies to be dissociated from old culture vessels and transferred into new ones with good viability. This requirement for adaptation may vary between lines. Colonies can be dissociated by enzymes, such as trypsin and collagenase IV, and also by treatment with EDTA. Needless to say, a system based on enzymatic digestion and large scale cultures need to be robust and validated to support long term pluripotency. Such a system is also ideal for automation of stem cell cultures. A certain level of control is lost when transferring from mechanical to enzymatic passaging methods, i.e. the selection of morphologically perfect specimens over differentiated, or partly differentiated cells. However many commercially available culture systems now maintain hESC with minimal differentiation which mitigates the impact of this change. Nonetheless, until there are more data and therefore more confidence in non-mechanical passaging methods it is likely that manual passaging will continue to be practised in many laboratories to generate and bank high quality starting material.

When hES cell colonies or confluent layers are enzymatically digested, two principles of passage can be applied. Either the cells can be dissociated to clusters of a few hundred cells or to a single cell suspension. Cell viability is largely preserved in the former procedure, however, the stress inflicted on the hES cells in the latter

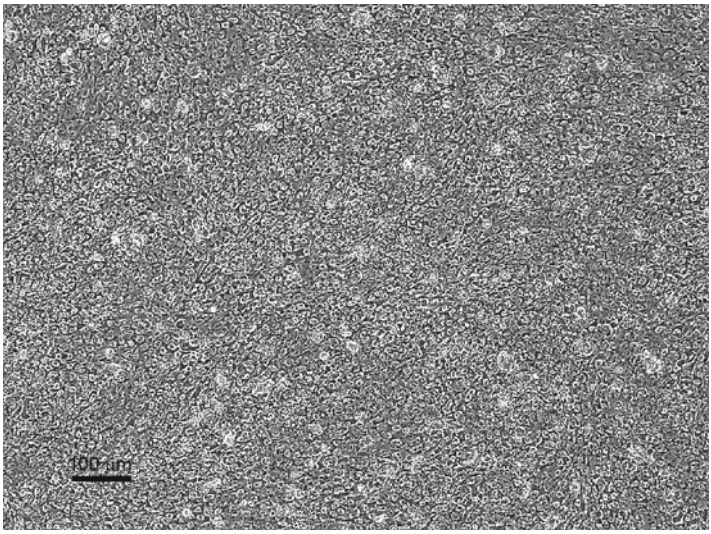

Fig. 2.3 A confluent monolayer of hES cells cultured without the presence of feeder cells. This technique allows scaled up, and also automated hES cell culture

will lead to apoptosis (Watanabe et al. 2007). Watanabe and co-workers made the significant finding that addition of a rho-kinase inhibitor to the medium that prevents apoptosis, thus allowing viable single cell suspensions of hES cells to be achieved. The molecule also improved cell recovery after cryopreservation (Li et al. 2009). Viable single cell passaging can also be achieved, in the absence of rho-kinase inhibitor, by the use of novel substrates such as laminin-521, these can also be used in conjunction with defined media formulations presenting an attractive proposition (Rodin et al. 2014). The ability to produce viable single cell suspension allows hES cells to be seeded in a reproducible and defined manner, i.e. to seed them into culture vessels at exact numbers, which is a prerequisite for e.g. compound screening campaigns or other applications that require absolute control over seeding densities. Single cell passaging also paves the way for easier automated cell culture according to specified protocols and seeding densities which result in more predictable cell growth. Figure 2.3 illustrates a confluent layer of hES cells in a feeder-free culture system.

Concerns were raised in 2004 by Draper and co-workers that prolonged exposure of hES cell cultures to enzymes would cause chromosomal aberrations (Draper et al. 2004), however other studies have later demonstrated that hES cells can be enzymatically propagated over longer periods of time with stable characteristics (Sjogren-Jansson et al. 2005; Suemori et al. 2006). As Catalina and colleagues suggest, the individual hES cell lines may have different pre-dispositions to chromosomal instability independent of culture conditions (Catalina et al. 2008). To address the concern of chromosomal changes, all hES cell cultures should either be regularly karyotyped or only cultured in a passage window that is unlikely to allow

chromosomal deviations to appear and propagate. hES cells not regularly subjected to enzymatic digestion have been shown to sustain a stable karyotype for up to almost 2 years in continuous culture but should still be regularly monitored (Caisander et al. 2006).

2.4 Scaling Up Cultures

For any clinical or industrial application of either undifferentiated hES cells or derivatives thereof, a scaled up, possibly even automated, production methodology is required. Protocols for directed differentiation of naïve hES cells to a specialised cell fate are often inefficient in terms of conversion from starting material, often due to extensive cell loss at early stages. It has been estimated that for a hES cell based repair of a heart damaged by ischemia, hES cells in the range of billions would be required (Passier and Mummery 2005). This is just one example and clearly the number of cells required at the start of a certain differentiation protocol will depend on the level of loss and number of differentiated cells required. At either end of this spectrum would be hESC derived RPE (retinal pigment epithelium) for ophthalmic diseases and hESC derived RBC (red blood cells) for transfusion. Efficacy is one challenge as well as efficiency, the cost of the process and purity of cell populations are two examples. To achieve billions of stem cells as a starting point is not practical with labour intensive protocols so the need for massive amounts of starting material has been a clear driver for the development of scalable culture systems, such as these discussed in the previous section. The scaled up culture process for hES cells needs to be as robust and straightforward as possible, to generate the necessary reproducibility and to be cost effective. Thus, the number of undefined components in the process should be kept to a minimum and ideally a hES cell culture system should be feeder independent and based on defined media and matrix, synthetic or recombinant. The culture procedure must also be subject to quality control, and preferably GLP procedures should apply. The application of MCB (master cell bank) and WCB (working cell bank) approaches will contribute to reproducibility and robustness in the culture system.

Recent progress has been reported where hES cells have been successfully cultured in amounts required for conducting compound screening campaigns in multiwell plate formats (Desbordes et al. 2008; Thomas et al. 2009) (Andrews et al. 2010) These achievements are essential for the use of hES cells in high throughput applications e.g. drug discovery and toxicology screening.

The definition and development of industrial cell production standards needs to be addressed, since scaled up hES cell culture is still a relatively new technology. The ability to manipulate hES cell cultures as single cell suspensions is a first critical step towards reproducible scaled up culture, and not the least, distribution of even cell numbers in multiwell plates for screening. The possibility of adding wholly or partly automated cell culture technology will further strengthen the capability to supply a consistent quality of cells in useful numbers (Thomas et al. 2009).

Future widespread use of hES cell derivatives in bio reactors will also demand cell numbers of an industrial scale.

2.5 Deriving Xeno Free hES Cells and hES Cells for Clinical Use

Routinely, all mammalian cell culture relies heavily on reagents sourced from animals, e.g. serum, amino acids, albumin, various matrices (e.g. collagen) and other factors. This is cost effective and gives enough consistency for the majority of applications. However, the undefined culture condition that is a consequence of the common use of bovine serum as a source of growth factors may be of concern for a number of reasons. One practical consideration is that of reproducibility due to batch-to-batch variation which impacts on the robustness of any culture system. Maybe of more concern in the context of the stem cell therapy area, is the risk of contamination by prions, viruses or other zoonoses of cells cultured in contact with animal derived material. Concerns were raised when it was observed that hES cells cultured in contact with animal material incorporated and expressed animal sialic acid on their cell surface, however, this has also been demonstrated to be reversible (Heiskanen et al. 2007; Martin et al. 2005; Nasonkin and Koliatsos 2006). To address this potential problem, derivation, propagation and banking of hES cell lines strictly without any contact with animal-sourced material, i.e. under "xeno-free" conditions, has been reported by several groups (Ellerstrom et al. 2006; Ludwig et al. 2006; Rajala et al. 2007; Richards et al. 2004) (Ilic et al. 2012). Desai and colleagues have produced a comprehensive review of the evolution of xeno-free hESC culture (Desai et al. 2015).

Xeno-free culture is still substantially more expensive than regular hES cell culture due to the high costs of the reagents, but also the need for dedicated plasticware and equipment as well as routines separated from the non-xeno free cell cultures. The International Stem Cell Banking Initiative is a group of stake holders striving to harmonise guidelines for stem cell banking worldwide, with the goal of delivering the best practice for clinical grade stem cell delivery (Crook et al. 2010).

In order to minimise the dependency on undefined contributions from feeder cells, serum and complex culture matrices like Matrigel (BD Biosciences), efforts have been made to elucidate the hES cell culture requirements for defined culture systems, resulting in commercially available defined culture systems for hES cells and iPS cells. For clinical use of hES cell or hES cell derived cells, cultures free of any animal components would desirably decrease the number of unknowns in the equation as would replacing biologics with small molecules. In addition to the biological and regulatory challenges, the industry and institutions need to generate enough cells suitable for therapeutic use and at the same time make it financially sound.

One of the great hopes of hES cells is to utilise them as raw materials for tissue engineering and replacement for damaged organs or tissues. A number of clinical situations have been identified as potential targets for this kind of approach. A therapy where insulin-dependent type-1 diabetics could receive functional beta cells that would integrate and normalise blood glucose levels would revolutionise the lives of thousands of those affected by this auto immune disease that targets and destroys the endogenous insulin producing cells. Replacing damaged neurons in patients suffering from spinal cord injuries could mean the difference between confinement to a wheel chair and normal mobility. The examples are plenty and great hope for future development is held by many patient groups and clinicians.

Since the above described scenarios include the transplantation of living cells into patients, the regulatory framework is comprehensive. For any future cell-based therapy, Good Clinical Practice (GCP) is a requirement for clinical trials, and this includes that the cells have been sourced and produced according to cGMP. This is a regulatory framework ensuring that an end product meets pre-set specifications. For a putative therapy based on hES cell derived functional cells, it includes the regulation of processes for

- Donation and procurement of the starting materials, e.g. blastocysts and feeder cells
- Testing and quality control
- Processing and manufacturing of the undifferentiated cells as well as their differentiated derivatives.
- Traceability of all reagents and materials as well as release criteria.

In 2007, Crook and co-workers published the first six hES cell lines derived and further processed in line with GMP regulations (Crook et al. 2007) and the most recent efforts in this area was published in 2012 (Ilic et al. 2012). These are important steps towards the clinic; however, there were and are still big challenges to overcome; to develop differentiated functional cells, verified and validated, of clinical value. Also, the six GMP compliant hES cell lines derived by Crook and co-workers were not derived in absence of animal material, i.e., they cannot be defined as xeno-free. However, from a GMP perspective, the use of reagents sourced from animals, like serum, is acceptable, although it has been argued that clinical grade hES cells, or hES cell derived cells need to be derived xeno-free (Unger et al. 2008). Nevertheless, in 2009, Geron Corporation was granted permission from the US Food and Drug Administration (FDA) to initiate the first phase 1 clinical trials for a spinal cord injury therapy, based on specialised cells derived from the hES cell line H1 (Alper 2009; Barde 2009). This hES cell line was originally cultured in contact with animal components and without all the intricate documentation and assurance that GMP compliance gives (Geron 2009; Thomson et al. 1998). Demonstration that some groups have overcome these constraints can be found in the completed, ongoing and approved clinical trials using hESC derived cells which are discussed briefly later in this chapter.

2.6 Differentiation Capacity

The ability of hES cells to differentiate into virtually any specialised cell type present in the adult body is one of the key features of these cells. Spontaneous differentiation occurs *in vitro* when the cells are cultured in conditions lacking the appropriate components that sustain pluripotency. In addition, several protocols for directed differentiation of hES cells into various specialised cell types have been reported in which the cells are guided along lineage restricted pathways to generate relatively pure populations of cells. A detailed review on this topic is beyond the scope of the present chapter and here we only exemplify the differentiation capacity of hES cells using two brief examples; cardiomyocytes and hepatocytes.

The differentiation of hES cells towards the cardiac lineage can be observed through the appearance of clusters of spontaneously contracting cells, as originally reported in 2000 (Itskovitz-Eldor et al. 2000). Different approaches have been developed to induce hES cells to differentiate to cardiomyocytes in culture. One is based on the formation of embryoid body-like structures under the influence of various cocktails of growth factors or small molecules in attempts to recapitulate heart development *in vivo* (Kehat et al. 2001; Yang et al. 2008). Another method is to utilise a co-culture system with hES cells and END-2 cells (a visceral endoderm mouse cell line), in which the pluripotent stem cells are directly exposed to cell-cell interactions as well as the secretome of the END-2 cells (Mummery et al. 2003). In recent years, derivation of cardiomyocytes from monolayers of hES and iPS cells has been demonstrated (Laflamme et al. 2007; Uosaki et al. 2011) and from hES cells cultured under defined conditions using a directed differentiation approach using small molecules (Parsons et al. 2011). Molecular, pharmacological, and electrophysiological studies have characterised hES cell-derived cardiomyocytes to various extents and these cells express many cardiac markers, including transcription factors, structural proteins, ion-channels, and different junction proteins (Beqqali et al. 2006; Synnergren et al. 2008). Despite the similarities with their adult counterparts, hES cell-derived cardiomyocytes still seem to mainly display a foetal cardiac phenotype, and future research is needed to develop protocols which can sustain *in vitro* maturation of the cells towards a phenotype more close to the adult human cardiomyocyte. For drug discovery applications it would be desirable to generate pure populations of e.g. ventricular cardiomyocytes, to study specific targets.

In 2003, the first report appeared which described the generation of hepatic-lineage cells from hES cells, with several publications following (Agarwal et al. 2008; Baharvand et al. 2008; Cai et al. 2007; Rambhatla et al. 2003) and more recently by Medine and co-workers (Medine et al. 2011). It is however a major challenge to generate truly metabolically competent cells, i.e., cells expressing relevant enzymatic activities. No studies to date have shown activity levels of different Cytochrome P450 enzymes that resembles those of freshly isolated human primary hepatocytes. Rather, the accumulated published work implicates that obtaining fully functional cells from hES cells is a major challenge (D'Amour et al. 2006),

(Guguen-Guillouzo et al. 2010). Progress has been made however, as demonstrated by recent publications on the derivation of insulin producing beta cells from hES cells (Bruin et al. 2014; Pagliuca et al. 2014).

2.7 Potential Applications for Therapies

Therapeutic applications based on hES cells add several dimensions to the challenges regarding hES cell culture, differentiation, and purification as discussed above. In addition, critical aspects such as safety and efficacy need to be clarified in detail before such cells can routinely progress to clinical trials, this has been achieved (for PhI trials) in some cases and there are reports of compassionate use of hESC derived cell products (Menasché et al. 2015). Indeed, much advancement has been made towards these ends and there are many diseases that are envisioned as suitable for targeting with stem cell therapies.

For example, despite important advances in pharmacological therapies and organ transplantation, heart failure represents an enormous clinical problem. The limitations of the current interventions have driven the search for stem cell-based techniques to repair and regenerate heart muscle. The opportunity to create the major cell types present in the human heart (i.e., cardiomyocytes, smooth muscle cells, and endothelial cells) from pluripotent stem cells and subsequently transplanting these into the site of injury is an appealing strategy. Pre- clinical studies have begun to investigate hES cell-based heart regeneration, and initial studies provided positive encouragement and demonstrated short term (4 weeks) functional improvement following transplantation of hES cell-derived cardiomyocytes to injured myocardium (Caspi et al. 2007; Laflamme et al. 2007; Leor et al. 2007). However, it was later demonstrated that the functional improvement was transient and no differences were observed between the cardiomyocyte transplanted group and the control animals at 12 weeks post-myocardial infarction (van Laake et al. 2007). Also, formation of fibrous tissue around the graft has been observed, hampering electrophysiological integration. However when hES cell derived cardiomyocytes were co-transplanted with hES cell derived endothelial cells and/or cardiac progenitor cells into mouse hearts, functional capillaries were formed and the cardiomyocytes were found to survive for up to 24 weeks. The formation of capillaries suggests the possibility of an increased blood supply to the graft area. Although the understanding of the mechanism of action is limited at present, it appears that transplantation of cells to the injured heart has some beneficial effects but there are other possibly prohibitive mechanisms that need to be elucidated further (van Laake et al. 2009, 2010). This hypothesis is being tested in a current clinical trial which is outlined below.

The possibility of generating relevant numbers and quality of hepatocytes for bio-artificial liver support technology and possibly also for *in vivo* liver regeneration is a thrilling prospect (Dalgetty et al. 2009), (Medine et al. 2011). In addition, achieving an unlimited access to competent liver cells would be of exceptional use

for studying drug targets, metabolism and toxicity (Jensen et al. 2009). On the other hand, *in vitro* differentiation of hES cells towards the hepatic lineage clearly is a challenging task (Snykers et al. 2009). A number of neurological disorders, such as Alzheimer and Parkinson's diseases, amyotrophic lateral sclerosis and multiple sclerosis (ALS and MS respectively) as well as spinal cord injury have been put forward as targets for hES cell derived therapies and are the subject of extensive research (Kim and Vellis 2009). Although many of the hESC derived therapies in development are still many years from the clinic great progress has been made more recently resulting in a number of clinical trials at various stages of approval and execution.

A comprehensive description of hESC derived cells in clinical trials is not possible here however it is useful to consider which types of therapy have already made it to clinical trial, although many are still to publish data or even recruit. Very preliminary data have been published from ACTs hESC derived RPE for Stargardt's macular dystrophy trial, these data are promising but preliminary and should not be extrapolated at this stage (Schwartz et al. 2015). A French academic led trial to assess the feasibility and safety of hESC derived cardiac-committed progenitor cells in patients with severe heart failure is currently recruiting with an estimated study completion date of June 2017 (clinicaltrials.gov Identifier NCT02057900). Viacyte have FDA approval for a trial of their device encapsulated hESC derived 'beta –like cells' in patients with TypeI diabetes (Borowiak and Melton 2009). The aim is to enrol 40 subjects in this trial and is estimated to collect the final data August 2017 (clinicaltrials.gov NCT02239354). Pfizer are sponsoring a trial to test a hESC derived RPE delivered on a patch that was developed at University College London; this trial is listed with an estimated enrolment of 10 subjects however is not yet open for recruitment (clinicaltrials.gov NCT01691261).

2.8 Conclusions and Future Development in Research

We have now had access to hES cells for over 15 years, and the progress of the scientific field has been spectacular. One could only speculate about what the next decade will generate in terms of greater understanding of human developmental biology, and all aspects of the generation of functional cells from hES cells for therapy as well as drug discovery applications. The field has started to transform from research and development phase into a state where therapeutic and industrial applications begin to be tangible and indeed have reached clinical trials despite the technical and ethical challenges. In terms of the therapeutic area, the regulatory bodies are fundamental in developing and harmonising the legal framework for the use of stem cells in the clinic, and also when it comes to drug safety assessment legislation.

The hES cell field has largely been directed by scientific drivers, however it becomes more obvious that as products mature financial drivers will become more significant. Any application based on hES cells or their derivatives needs to be

proven biologically effective, safe where applicable, but also economically justified, compared to the alternatives. Efficient ways of producing the required quality (GMP for therapeutic use) and quantity of cells need to be further developed and established with an emphasis on defined and/or xeno-free and automation where appropriate. In addition to potential therapeutic use, modified hES cells and/or derivatives thereof are likely to be important tools for further research and development, for example reporter gene containing hES cell lines, immortalised precursors and cells modified to overexpress genes of interest, e.g. ion channels or metabolising enzymes.

In terms of the generation of new hES cell lines, it has been argued that there is a continued need for this. As has been described, technology has moved apace since the early lines were derived and it would be prudent to use lines derived using these new, better defined, techniques as we progress with research in this field, especially in the therapeutic space, A number of the GMP lines currently available have been retrospectively qualified after having been derived as research grade and have been in contact with non-optimal reagents in their history. However there are now a number of true GMP lines which have been derived and maintained under GMP conditions from the outset. One can also argue that there might not be sufficient diversity among the existing hES cell lines to cover the needs for future cell-based therapies as well as for the development of novel drugs for treatment of disease (Civin and Rao 2006). However, studies of renal allograft donor-recipient relations give fuel for speculation that a relatively low number of hES cell lines, in the range of low hundreds or ever less, would cover the larger proportion of the population for future therapies (Nakajima et al. 2007; Taylor et al. 2005), as discussed by Daley and Scadden (Daley and Scadden 2008). Some compounds are known to be metabolised differently between individuals within various ethnic backgrounds as well as between ethnic groups, one example is the well known genetic diversity of the alcohol dehydrogenase. Tissue type matching will obviously be important for regenerative use but these metabolic differences will be of relevance in drug screening strategies. Some of this diversity can be achieved by the generation of new hESC lines whereas more specific modifications can be achieved through the use of the rapidly developing genome modification technologies such as zinc finger nucleases, CRISPR/Cas9, etc. (reviewed in Li et al. 2014).

The advent of hiPSC lines predictably led to the argument that establishing new hESC lines would be redundant. However, the variability in reprogramming methods and the impact these will have on the safety profile of these cells mean that there is still a case for using hESC cells for therapeutic applications. Uncertainty about the equivalence of iPSC to hESC also leaves the question as to whether or not hiPSC can be used as a simple replacement for hESC unanswered. This is discussed in a recent publication from Barad and colleagues (Barad et al. 2014). It the last edition of this book we said that only time, together with high quality peer/reviewed research and development, will contribute to the future directions of stem cells in general and hES cells in particular. Despite the many advances in technology and clinical applicability since then this statement still holds true.

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