Article

Derivation of clinical-grade human embryonic stem cells

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Abstract

Embryonic stem cells proliferate in vitro while maintaining an undifferentiated state, and are capable of differentiating into most cell types under appropriate conditions. These properties imply great potential in the treatment of various diseases and disabilities. In fact, the first clinical trials with hESC for treating spinal cord injuries will begin next year. However, therapeutic application of human embryonic stem cell derivatives is compromised by the exposure of existing lines to animal and human components, with the subsequent risk of contamination with retroviruses and other pathogens, which can be transmitted to patients. The scientific community is striving to avoid the use of xenogeneic or allogeneic components in the process of derivation new hESC lines. This review summarizes attempts that have been made to avoid these contaminants and the breakthroughs achieved in the derivation of clinical-grade hESC that could be used for therapeutic purposes.

Keywords: clinical use, contaminants, human embryonic stem cells, inner cell mass

Introduction

The first studies with embryonic stem cells were carried out by Cole and collaborators in the early 1960s using rabbit blastocysts (Cole et al., 1964, 1966). Although the achievements in rabbits were groundbreaking, mice are more commonly used as research animals today, and it is in mouse that the most recent advances in embryonic stem cell biology have paved the way for similar strategies in the use of human embryonic stem cells (hESC). Mouse embryonic stem (mES) cells are isolated from inner cell masses of murine preimplantation embryos (Evans et al., 1981; Martin, 1981). mES cells are capable of proliferating indefinitely in an undifferentiated state and are pluripotent, making them a perfect vehicle for genetic manipulations in mice. The properties of these cells were an initial signpost for the scientific and therapeutic potential of embryonic stem cells. Since the establishment of the first human embryonic stem cell line in 1998, the hope has existed that such lines could constitute an unlimited cell source for replacement therapy in the treatment of various diseases and disabilities (Thomson et al., 1998).

Embryonic stem cells reproduce extensively in vitro while maintaining an undifferentiated state, and are capable of differentiating into most cell types under the correct conditions. Furthermore, they can be genetically manipulated (Zwaka et al., 2003). These characteristics make embryonic stem cells an ideal source for cell replacement therapy (Bjorklund and Lindvall, 2000). They could also provide a unique model for the study of human development and congenital anomalies, as well as in promising fields such as tissue engineering and in-vitro gene therapy models. The potential of transplanted hESC in cases of severe degenerative diseases is clear (Fishel et al., 1984; Keller et al., 1999; Edwards, 2002). Many human diseases result from defects in a single cell type. If defective cells could be replaced with the appropriate stem cells, progenitor cells, or cells differentiated in vitro, it might be possible to clinically treat disease and injury at a cellular level (Thomson et al., 1998).

In fact, several studies have shown that neuronal cells (Reubinoff et al., 2001; Schuldiner et al., 2001; Zhang et al., 2001), cardiomyocytes (Kehat et al., 2001), pancreatic β cells (Assady et al., 2001), haematopoietic cells (Chadwick et al., 2003), leukocytes (Zhan et al., 2004), endothelial cells...
(Levenberg et al., 2002), vascular cells (Gerecht-Nir et al., 2004), trophoblasts (Xu et al., 2002) and germ cells (Clark et al., 2004) can be induced from hESC. These results point to the clinical application of hESC in the treatment of conditions such as Parkinson’s, heart disease and diabetes, among others. Very recently, Hwang and colleagues have generated hESC using somatic cells from patients with diseases or injuries (Hwang et al., 2005). Patient-specific, immune-matched hESC are hoped to be of great biomedical importance for studies of disease and development, and for advanced clinical applications of stem cell transplantation. In addition, the derivation of hESC carrying genetic alterations associated with human disease would be a significant tool for the study of the aetiology and pathology of those diseases (Pickering et al., 2005; Verlimsky et al., 2005).

Embryonic stem cells can also be used in the study of a range of basic developmental biological processes, such as identification of the factors involved in the regulation of developmental processes, differentiation into certain cells or tissues, and screening for drugs or toxins (Smith, 2001).

Geron, the company that funded the first derivation of hESC (Thomson et al., 1998), has announced it will start clinical trials with hESC to treat spinal cord injuries in the summer of 2006 (Vogel, 2005). Keirstead and collaborators (Keirstead et al., 2005) have differentiated hESC into oligodendrocyte precursors. Oligodendrocytes produce the protective myelin sheath that allows neurons to propagate signals along their axons. This sheath is often lost during spinal cord injuries. Oligodendrocyte precursors, when injected into the spinal cord, promote recovery in rats that have suffered spinal cord injury (Keirstead et al., 2005). These studies involved the injection of mouse cells destined to form oligodendrocytes into injured or diseased animals, and the subsequent partial restoration of myelination (Brustle et al., 1999; Lu et al., 2002). Keirstead and collaborators demonstrated that hESC exert similar effects (Keirstead et al., 2005). In rats that received oligodendrocyte precursors 7 days after injury, the injected cells survived and helped repair the spinal cord myelin. However, the same cells, when injected into mice with 10-month-old injuries, did not produce a therapeutic effect, suggesting that oligodendrocyte precursor cells from hESC are a promising therapeutic modality for recent injuries.

The extreme flexibility and capacity for growth of hESC makes them ideal for producing large quantities of differentiated cells that can be used in therapeutic applications. However, hESC therapies are not without risks. There are many parameters that need to be controlled before the cells are introduced into patients.

**Derivation of hESC**

Human embryonic cells are derived from preimplantation stage embryos, a process which involves culturing embryos to the morula or blastocyst stage. In most cases, these are excess or poor quality embryos that have been donated for research and would otherwise be discarded. Initial derivations of hESC were performed in the Thomson laboratory at the University of Wisconsin, using the inner cell masses (ICM) of human blastocysts and placing them on inactivated murine feeder cells (MEF) (Thomson et al., 1998). The resulting cell population was then maintained as a cell line through continuous subculture.

Several laboratories have since published the derivation of additional lines using similar protocols (Reubinoff et al., 2000; Lanzendorf et al., 2001; Amit and Itskovitz-Eldor, 2002; Richards et al., 2002). Many laboratories are currently deriving additional hESC lines, but the overall success rate for derivation is as yet unclear. Indeed, the percentage of lines successfully derived from ICM ranges from 5 to 100% (Hoffman and Carpenter, 2005). An exact comparison of the success rate between different groups is difficult, since some groups report the number of donated embryos employed while others report the number of blastocysts used. Many groups have failed to generate any hESC lines despite employing multiple embryos. In addition, some laboratories have not published the results of successful derivations of cell lines. What is clear is that the number and quality of the embryos used for derivation, plus the techniques employed, are crucial factors to be considered with respect to this process. For example, legislation in some countries allows derivation only from embryos that have been frozen for at least 5 years. This legal requirement imposes a limitation on the derivation process since, 5 years ago, the technology available for freezing embryos was not as advanced or as efficient as it is now, and therefore the quality of embryos from that time is comparatively poor (Simón et al., 2005). Moreover, derivation efficacy seems higher in fresh versus frozen embryos in spite of the fact that there are examples of the opposite results (Sjögren et al., 2004).

Although some groups report the number of ICM used in their derivation processes, a surprisingly limited amount of information has been published concerning the number of embryos used to generate ICM and the resulting cell lines. Very few reports provide information on the quality or grade of the embryos used to derive the cell lines (Mitalipova et al., 2003; Cowan et al., 2004). Although hESC lines have been derived from embryos of poor quality (Mitalipova et al., 2003; Zeng et al., 2004), recent results suggest that hESC are more efficiently derived from high-quality embryos (Oh et al., 2005; Simón et al., 2005). Cell lines have also been isolated from morula-stage embryos (Strelchenko et al., 2004) and even from later stage blastocysts (7–8 days) (Stojkovic et al., 2004). Thus, existing hESC lines have been derived from embryos with varying characteristics. The methods used for isolation of hESC, immunosurgery, zona removal, spontaneous and mechanical hatching introduce another parameter, such as the timing of hESC derivation from the blastocysts. These differences may have profound effects on the quality or characteristics of the resultant cell lines. Since the derivation process has been optimised, it may be possible to assess the number and quality of embryos required to derive hESC lines. In the future, it is likely that fewer embryos will be required to obtain a given number of hESC than in earlier attempts.

Despite the mentioned differences in the derivation processes, all hESC lines derived to date exhibit similar expression patterns of standard stem cell markers (Table 1).

**Potential problems in hES cell therapy**

Initially, hESC are cultured on a feeder layer of mouse embryonic fibroblasts, after the feeder cells have been irradiated or treated
with mitomycin to prevent them from dividing (Thomson et al., 1998). Feeder cells provide an ideal environment for the growth and maintenance of hESC, because the feeder cells detoxify culture medium, secrete many unique proteins that facilitate cell growth, and form and remodel the extracellular matrix (Lim and Bodnar, 2002).

**Animal contamination**

The majority of the hESC derived in early experiments, including all National Institutes of Health-approved lines, were isolated and maintained (as they still are) using fetal calf serum (xenoproteins) and mouse embryonic fibroblasts as feeder layers (xenosupports) or as a source of conditioned medium for propagation of the cells in an undifferentiated state (Xu et al., 2001). A conditioned medium from feeders is harvested from a culture of feeder cells after 24 h growth and production of growth factors. Zoonosis, the transmission of pathogens from animals to humans, is currently a serious concern within the field.

The exposure of human cell lines to animal components presents a serious risk of contamination with unidentified retroviruses and other pathogens that could be transmitted to the patient and the wider population through the therapeutic application of hESC lines (FDA recommendations) (Cooper et al., 2000).

Recently, Amit and colleagues published interesting results after testing five hESC cell lines derived using mouse feeder cells in order to observe possible signs of murine retroviruses, which exist in the genome of all mouse cells. They identified receptors for the mouse leukaemia virus. However, they found no evidence that the virus had infected any of the human cells, even after being grown on mouse feeders for years (Amit et al., 2005). Yet, in spite of these results, the same authors acknowledge that the use of animal products may still be a risk.

More recently, researchers identified another potential drawback to using mouse feeder cells or mouse fibroblast-conditioned media. A foreign sugar molecule was detected on the surface of hESC grown with mouse feeders. HESC can incorporate the non-human sialic acid Neu5Gc from the mouse feeder layer and/or the medium, leading to an immune response to Neu5Gc mediated by natural antibodies that are present in most humans (Martin et al., 2005). Therefore, any therapy utilizing hESC lines derived from or grown on mouse embryonic fibroblasts or conditioned medium is unlikely to be successful. In addition, there are potential implications for the incorporation of Neu5Gc with regard to general hESC biology. Many of the characteristic markers of hESC (stage-specific embryonic antigens SSEA-3 and SSEA-4 and tumour rejection antigens TRA-1–60 and TRA-1–81) are glycolipids or glycoproteins, many of which can carry sialic acids (Thomson et al., 1998). As sialic acids are involved in self-recognition events, the presence of Neu5Gc instead of Neu5Ac (the primary sialic acid in humans) could lead to unexpected impairment of cell function and tissue development (Varki, 2001).

There are several methods for eliminating Neu5Gc (or any other unknown animal antigen or pathogen) from existing cultures, but none guarantees the complete eradication of the sugar. The only truly safe option would appear to be the derivation of hESC without exposure to any animal components.

**Human contamination**

Progress has been made towards elimination of xenogenic components in hESC derivation and culture. Richards and

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**Table 1.** Some representative human embryonic stem cell (hESC) lines derived using different feeder fibroblasts and serum sources. See Hoffman and Carpenter. (2005) for more extensive list. MEF, mouse embryonic fibroblasts; HFM, human fetal muscle; HFSK, human foreskin; HUEC, human uterine endometrium; H-Placenta, human placenta; EDF-H/SH, hESC-derived fibroblast feeders. FBS, fetal bovine serum; HS, human serum; NS, non-specified; FCS, fetal calf serum; SR, serum replacement.

<table>
<thead>
<tr>
<th>Feeder source</th>
<th>hESC line derived</th>
<th>Serum source</th>
<th>Latest hESC passage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF²</td>
<td>H1, H7, H9, H13, H14</td>
<td>FBS</td>
<td>50+</td>
<td>Thomson et al., 1998</td>
</tr>
<tr>
<td>MEF²</td>
<td>HES-1, HES-2</td>
<td>FBS</td>
<td>50+</td>
<td>Reubinott et al., 2000</td>
</tr>
<tr>
<td>MEF²</td>
<td>ES-76, ES-78-1, ES-78-2</td>
<td>FBS</td>
<td>50+</td>
<td>Lanzendorf et al., 2001</td>
</tr>
<tr>
<td>H/FM</td>
<td>Several</td>
<td>HS</td>
<td>20+</td>
<td>Richards et al., 2002</td>
</tr>
<tr>
<td>MEF²</td>
<td>3-1-3-1-4, 1-6</td>
<td>NS</td>
<td>50+</td>
<td>Amit and Itskovitz-Eldor, 2002</td>
</tr>
<tr>
<td>H/SK</td>
<td>BG01, BG02, BG03, BG04</td>
<td>FBS</td>
<td>50+</td>
<td>Mitaliupova et al., 2003</td>
</tr>
<tr>
<td>H/FSK</td>
<td>HS181, HS20/</td>
<td>FCS</td>
<td>30+, 10+</td>
<td>Hovatta et al., 2003</td>
</tr>
<tr>
<td>HUEC</td>
<td>HS293, HS306</td>
<td>SR</td>
<td>56+, 41+</td>
<td>Inzunza et al., 2005</td>
</tr>
<tr>
<td>H-Placenta</td>
<td>Miz-hES-9, Miz-Hes-14,</td>
<td>SR</td>
<td>55+, 40+,</td>
<td>Lee et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Miz-hES-15b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-Placenta</td>
<td>UCSF1, UCSF2</td>
<td>SR</td>
<td>17+, 7+</td>
<td>Genbeche et al., 2005</td>
</tr>
<tr>
<td>Lysed MEFs</td>
<td>VAL-1, VAL-2</td>
<td>SR</td>
<td>44+, 24+</td>
<td>Simon et al., 2005</td>
</tr>
<tr>
<td>EDF-H1, EDF-H2</td>
<td>SH7</td>
<td>SR</td>
<td>30+</td>
<td>Kimamskaya et al., 2005</td>
</tr>
<tr>
<td>SH1, EDF-SH2</td>
<td></td>
<td></td>
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<td>Wang et al., 2005b</td>
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</table>
colleagues successfully grew and maintained undifferentiated hESC, which had initially been derived on mouse embryonic fibroblasts, using human feeder layers obtained from human fetal muscle and skin and adult Fallopian tube epithelial feeder layers (Richards et al., 2002). In fact, this group was responsible for the first derivation of hESC using human feeder layers (fetal muscle) and human serum (Richards et al., 2002). However, growth of the hESC lines on fetal muscle, fetal skin and adult Fallopian tube was slower than that on mouse embryonic fibroblast cells. In addition, the limited availability of fetal cells from human abortuses and the ethical concerns surrounding their use, constituted a handicap. The same authors were able to grow hESC using feeder layers composed of fibroblasts from adult biopsies (Richards et al., 2003). Other human feeder cells, such as human foreskin fibroblasts, have also supported the growth of hESC previously derived using mouse embryonic fibroblasts (Amit et al., 2003). These approaches require the screening of donor sources for potential pathogens. Experience with organ and tissue allotransplantation has shown that human immunodeficiency virus (HIV)-1, HIV-2, Creutzfeldt–Jakob disease, hepatitis B or C viruses and other infectious agents can be transmitted from human donor cells to the recipient. Hovatta and colleagues derived hESC lines using commercially available human foreskin fibroblasts, but in the presence of fetal calf serum (Hovatta et al., 2003). More recently, the same group has successfully obtained hESC using the same human feeders but in the presence of serum replacement, thereby avoiding the use of animal serum (Inzunza et al., 2005). Interestingly, studies undertaken by Koivisto and collaborators have shown that the proliferation of hESC is higher in the presence of serum replacement than in human serum or fetal calf serum (Koivisto et al., 2004).

Other groups continue to derive hESC using different human feeders in the absence of animal components. Genbacev and collaborators established cellular lines from human placental fibroblasts, which have been used to derive four new hESC lines (UCSF-1, UCSF-2, Genbacev et al., 2005; VAL-1, VAL-2, Simón et al., 2005) (Figure 1). In both cases, extensive pathogen testing as well as exhaustive studies of the lines’ abilities to support growth of hESC lines were performed validating the human placental fibroblast lines for use in future therapies. During development in vivo, undifferentiated hESC are surrounded by the placenta, and fibroblasts are a principal component of the placenta. Thus, the use of human placental fibroblasts as support feeders during derivation and subsequent expansion in an undifferentiated state offers a close approximation to the in-vivo stem cell milieu.

The derivation process was designed to minimize contact with animal cells/products/proteins (Simón et al., 2005). For example, the zona pellucida was removed using acid Tyrode’s solution rather than pronase, and the lines were formed without performing immunosurgery, thereby eliminating exposure to animal antibodies and complement factors. The derivation and culturing processes took place in the absence of fetal calf serum using serum replacement instead. In customary culture systems, the enzyme solution often used for passaging the cells is a source of animal factors. Since the cells were passaged via manual dissection, they did not come into contact with these animal enzymes. As the placental fibroblasts were screened for human and animal pathogens and no serum was used during the derivation process, the risk of zoonosis was greatly reduced. However, it is important to note that animal components were used in the establishment and propagation of the human placental fibroblast feeders. Further experiments are in progress to derive both the feeders and the hESC in the absence of serum and animal proteins.

Alternatives to xenogeneic and allogeneic cultures

The use of feeder cells for the prolonged culture of undifferentiated hESCs limit the medical application of the latter, as xenogeneic and allogeneic feeders can transmit pathogens and other unidentified risk factors (Richards et al., 2002, 2003; Hovatta et al., 2003). The use of conditioned media of xenogeneic or allogeneic origin raises concerns about the inter- and intra-species transfer of viruses (Gearhart, 2004).

In an innovative approach, Stojkovic and colleagues have avoided the use of any animal or human fibroblast feeders by obtaining fibroblast-like cells from the spontaneous differentiation of hESC, and using the differentiated cells

Figure 1. Morphological features of VAL-1 (a) and VAL-2 (b) after 100 passages on placental fibroblasts.
as a feeder layer to support the undifferentiated growth of hESC colonies (Stojkovic et al., 2005b), although it is acknowledged that this is not a complete solution. Recently, Xu and collaborators demonstrated that conditioned medium of fibroblast-like cells differentiated from hESC was capable of supporting hESC growth under feeder-free conditions (Xu et al., 2004). Another step forward is the use of these human embryonic autogenic feeders in the derivation of a new hESC line that displays typical hESC morphology, as well as appropriate immunohistochemical and molecular properties, even after prolonged culture (over 10 months) (Wang et al., 2005b). Human embryonic autogenic feeder layers are promising because feeders differentiated from the first clinical-grade hESC line could be used as monolayers for growing isolated inner cellular masses (ICM), thereby eliminating the transfer of pathogens. As previously mentioned, the genome of a hESC can be altered (Pfetzer et al., 2002; Zwaka et al., 2003). Another potential advantage of this autogenic system is the possible introduction of genes for production of antibiotic-resistant or growth-promoting factors to facilitate the selection of hESC with special traits after gene modification or to grow hESC without the addition of exogenous growth factors (Wang et al., 2005b). However, it must be noted that differentiation into fibroblasts may not be definitive. Also, in the routine passaging of hESC, there is a certain percentage of spontaneous differentiation, which could create a mixture of colonies from different origins. DNA fingerprinting would provide crucial information for cell identification if mixing between different hESC lines were to occur.

The necessity of feeder cells for the derivation and propagation of undifferentiated hESC limits the large scale culture demands for both clinical applications and genetic manipulation of hESC.

The identification of conditioned media components essential for hESC self-renewal is a crucial aspect in the eventual application of hESC in the treatment of human disease. Significant studies carried out by two groups have described the possibility of maintaining the undifferentiated growth of pluripotent hESC in the absence of feeder layers and conditioned medium (Wang et al., 2005a; Xu et al., 2005). They have demonstrated that the bone morphogenetic protein antagonist noggin is critical in preventing differentiation of hESC in culture. In fact, the combination of noggin and basic fibroblast growth factor (bFGF) appears to maintain the prolonged growth of hES cells while retaining all hESC features. The use of low levels of bFGF has been found to activate ectodermal and mesodermal markers in certain hESC (Schuldiner et al., 2000). This supplemented medium may prove a critical step forward in developing a completely defined culture system compatible with future therapeutic applications of hESC. This medium did support the growth of at least three different hESC lines tested by two different groups; however, the ultimate test will be whether it supports the hESC derivation process.

Feeder-free derivation of embryonic stem cells from human blastocysts was not a reality until Klimanskaya and colleagues successfully derived pluripotent human embryonic stem cells (Klimanskaya et al., 2005). They have obtained a hESC line in the absence of cellular support or culture medium containing animal derivatives. Their system eliminates the risk of transmitting animal pathogens to hESC, a risk that would invalidate the use of these cells in therapy. The use of an extracellular matrix minimizes the possibility of pathogen transmission; however, in this case the matrix was prepared from mouse embryonic fibroblasts, and so the problem of xenocomponent immunogenicity remains.

A significant step forward in the field of the extracellular matrix has been made by Stojkovic and colleagues. They have demonstrated the growth and maintenance of hESC in human serum as a matrix in the presence of conditioned media from fibroblasts derived from hESC using serum replacement supplemented with human bFGF (Stojkovic et al., 2005a). These findings constitute a breakthrough, which avoids the presence of xenocomponents in the propagation medium, thereby decreasing the chance of immune response against foreign antigens.

Serum replacement is now used by most of the groups that culture human embryonic stem cells, but these serum replacement media also contain animal proteins, when ideally they should contain only human proteins. The company that distributes serum replacement is currently working on the formulation of a serum replacement that is made up of solely human derivatives.

According to new EU directives (2003/94/EC and 2004/24/EC), human embryonic stem cells for transplantation must be cultured using conditions of good manufacturing practice (GMP) in order to guarantee the safety and quality of the cells (Richards et al., 2003; Cowan et al., 2004). Incorporating GMP implies impeccable record keeping, qualified personnel, high sanitary standards, equipment verification, validation of processes and complaint management. The task is demanding but understandable, as the safety of cell transplantation recipients must be a priority. Identifying all culture constituents meeting GMP is now an important challenge for those wishing to derive clinical-grade human embryonic stem cell lines. GMP-grade embryos should also be used to derive GMP-grade hESC lines, since prolonged culture of hESC lines using GMP conditions does not assure that these lines are clean if those lines were previously derived and maintained under non-GMP conditions.

**Conclusion**

There is an urgent need for the generation and characterization of more hESC lines, as each cell line may have its own specific applications (Findikli et al., 2005). The availability of more hESC lines for comparison would aid in defining criteria for the most robust methods to grow hESC. From further observations (manuscript in preparation) and reports of other groups (Lee et al., 2000; Chung et al., 2002; Kim et al., 2002; Ward et al., 2004), it is clear that each hESC line varies in terms of differentiation potential, though they do appear to share characteristics when in an undifferentiated state. For example, despite the fact that VAL-1 and VAL-2 are cell lines derived from embryos originating from the same donor, VAL-1 cells easily differentiate into mesoderm tissues, while the VAL-2 line has a better ability to differentiate into endoderm. The derivation and study of many different hESC will provide a greater insight into their clinical application. In fact, an International Stem Cell Initiative has established a set of standards for the
characterization of 75 existing hESC lines. VAL-1 and VAL-2, among others, are being evaluated using these criteria. The goal is to understand the similarities and differences between different hESC lines so that results from different laboratories can be compared in a standardized manner (Andrews et al., 2005).

It will be necessary to achieve more extensive knowledge of the characteristics of hESC, in order to control or modify them prior to their use at a clinical level. For example, controlling immune responses, preventing both genetic and epigenetic chromosomal changes after extended passages, and modulating the process of differentiation are crucial aims to fulfil. However, the achievement of clinical grade hESC is growing closer. Progress in this area has been amazingly rapid, with significant results emerging almost daily, describing important steps towards the clinical application of hESC.

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