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Derivation, growth and applications of human embryonic stem cells

Miodrag Stojkovic, Majlinda Lako, Tom Strachan and Alison Murdoch[1]

Institute of Human Genetics, University of Newcastle, Newcastle upon Tyne, NE1 3BZ, UK and [1]Newcastle Fertility Centre at Life, NHS, Newcastle upon Tyne, NE1 4EP, UK

Correspondence should be addressed to M Stojkovic; Email: miodrag.stojkovic@ncl.ac.uk

Abstract

Human embryonic stem (hES) cells are pluripotent cells derived from the inner cell mass cells of blastocysts with the potential to maintain an undifferentiated state indefinitely. Fully characterised hES cell lines express typical stem cell markers, possess high levels of telomerase activity, show normal karyotype and have the potential to differentiate into numerous cell types under *in vitro* and *in vivo* conditions. Therefore, hES cells are potentially valuable for the development of cell transplantation therapies for the treatment of various human diseases. However, there are a number of factors which may limit the medical application of hES cells: (a) continuous culture of hES cells in an undifferentiated state requires the presence of feeder layers and animal-based ingredients which incurs a risk of cross-transfer of pathogens; (b) hES cells demonstrate high genomic instability and non-predictable differentiation after long-term growth; and (c) differentiated hES cells express molecules which could cause immune rejection. In this review we summarise recent progress in the derivation and growth of undifferentiated hES cells and their differentiated progeny, and the problems associated with these techniques. We also examine the potential use of the therapeutic cloning technique to derive isogenic hES cells.
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Background

Embryonic stem (ES) cells are derived from the pluripotent inner cell mass (ICM) cells of the preimplantation blastocysts (reviewed in Smith 2001). The derivation process involves plating of the blastocysts on mouse embryonic fibroblasts (MEF) and expansion of the outgrowth into established ES cell lines. ES cells are capable of unlimited self-renewal by symmetric division. They are also able to divide asymmetrically giving rise to one daughter cell resembling its mother, and another daughter cell giving rise to multiple types of differentiated cells representing all primitive embryonic germ layers. The latter can be tested by injecting ES cells into immunocompromised mice to produce teratomas that contain differentiated derivatives of all three germ layers.

The first successful derivation of human ES (hES) cells was reported by Thomson *et al.* (1998) in which the authors isolated ICM cells plated onto mitotically inactivated MEF cells. Two years latter Reubinoff *et al.* (2000) confirmed that hES cells could be efficiently derived from surplus embryos demonstrating at the same time the differentiation potential of hES cells under *in vitro*

conditions. Since then, rapid progress has been achieved and numerous studies have described the derivation of new hES cell lines including methods of growing both undifferentiated hES cells and their differentiated progeny. These milestones relied heavily on the gradual progress made in the previous 20 years when murine ES (mES) cells, primate ES cells and embryonic carcinoma (EC) cells derived from testicular teratocarcinomas were extensively characterised (Draper *et al.* 2002, Draper & Fox 2003). The last 6 years have witnessed an exponential increase in experiments aimed at improving culture conditions (Xu *et al.* 2001, Amit *et al.* 2003), genetic manipulation (Zwaka & Thomson 2003) and differentiation regimes to produce human cells for transplantation and drug testing (Assady *et al.* 2001, Kaufman *et al.* 2001, Zhang *et al.* 2001, Kehat *et al.* 2002, Schulz *et al.* 2003). However the challenge remains to produce mature, functional and pure derivatives of cell types that can be utilized for transplantation purposes. To facilitate these developments, a fuller understanding of the biology of undifferentiated cell types and genes that are important in

the maintenance of the pluripotency of hES cells is necessesary.

Human ES cells express some of the classical markers of pluripotent cells such as OCT4, alkaline phosphatase and show high levels of telomerase activity (Thomson *et al.* 1998, Reubinoff *et al.* 2000). In spite of this, they show several morphological and behavioural differences from mES cells: they grow more slowly and tend to form flat rather than spherical colonies (Odorico *et al.* 2001, Amit & Itskovitz-Eldor, 2002). While leukemia inhibitory factor (LIF) modulated through gp130 and JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathways is sufficient to prevent undifferentiated mES cells from differentiating, it cannot prevent the spontaneous differentiation of hES cells (Reubinoff *et al.* 2000, Amit & Itskovitz-Eldor, 2002). In contrast to mES cells, which are recognised by monoclonal antibodies raised to stage-specific embryonic antigen 1 (SSEA-1), hES cells and ICM cells are recognised by monoclonal antibodies raised to SSEA-3 and SSEA-4 (Henderson *et al.* 2002). A set of antibodies raised to hEC cell antigens, TRA-1-60, TRA-1-81, GCTM-2, TG-30 and TG-343, that are associated with a pericellular matrix keratin sulphate/chondroitin sulphate also recognise hES and hEC cells but not mES cells (Laslett *et al.* 2003). In contrast to mES cells, hES cells also express CD9, Thy1 and major histocompatibility complex class 1 (Henderson *et al.* 2002). These differences between mES and hES cells suggest that although some of the pluripotentiality genes are expressed in both mES and hES cells their function and downstream signalling pathways may differ. This highlights the importance of studying stem cell biology in humans given the relevance of stem cell applications in theraupetic medicine.

The capacity of hES cells to differentiate into almost all of the cell types of the human body highlights their potentially promising role in cell replacement therapies for the treatment of human diseases. Unfortunately, there are several hurdles to be overcome before such potential may be realised. For instance, the processes of ICM isolation, and *in vitro* culture of hES cells requires that human embryos and cells derived from them are in contact with animal ingredients or feeder cells. Large-scale culture of hES cells is time consuming and labour intensive, the presence of feeder cells may interfere with attempts to genetically modify the hES cells and prolonged growth *in vitro* may cause chromosomal aberrations.

In this review we summarise recent progress in hES cell research and we also highlight several problems associated with the derivation and growth of hES cells.

What is important for the derivation of hES cells?

Sufficient numbers of donated embryos and an *in vitro* culture system that allows development of early embryos into blastocysts with well-formed ICMs are crucial factors in the successful derivation of hES cells. To date, the majority of described hES cell lines were derived from day 5 to day 8 blastocysts produced for clinical purposes after *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection. Meanwhile, one hES cell line has been derived from blastocysts developed from embryos reconstructed using a somatic cell nuclear transfer (SCNT) technique (Hwang *et al.* 2004).

The very first study that describes successful separation of human ICM cells and their continued culture for at least two passages *in vitro* was published by Bongso and co-workers (1994). The authors demonstrated that separated ICM cells either differentiate or produce cells with typical hES-cell-like morphology positive for alkaline phosphatase staining and with normal karyotype. This mechanical isolation of ICM has been substituted by the removal of trophectoderm (TE) cells by immunosurgery using specific antibodies raised either against BeWo cells (Thomson *et al.* 1998, Lanzendorf *et al.* 2001), or whole human serum (Reubinoff *et al.* 2000, Park *et al.* 2003, Pickering *et al.* 2003, Hwang *et al.* 2004, Stojkovic *et al.* 2004) or red blood cells (Cowan *et al.* 2004). Nearly all described hES cell lines have been efficiently derived using the immunosurgery procedure, however mechanical isolation is advantageous since there is no contact of blastocysts with animal antibodies. On the other hand, there is a risk that not all of the TE cells may be removed during mechanical isolation and these may subsequently overgrow and inhibit the growth of ICM cells (Pickering *et al.* 2003).

Published data suggest that the success rate in deriving hES cell lines is highly dependent on the quality of recovered blastocysts, isolation conditions and experience of the group (Pera *et al.* 2000, Mitalipova *et al.* 2003): Thomson *et al.* (1998) used 14 blastocysts to derive 5 hES cell lines; Reubinoff *et al.* (2000) derived 2 hES cell lines from 4 blastocysts; Lanzendorf *et al.* (2001) derived 3 hES cell lines from 40 blastocysts; Park *et al.* (2003) derived 3 hES cell lines from 13 isolated ICMs; Pickering *et al.* (2003) derived 3 hES cell lines from 58 embryos; Mitalipova *et al.* (2003) derived 4 hES cell lines from 19 embryos; we (Stojkovic *et al.* 2004) derived 1 hES cell line from 7 blastocysts; and previously Cowan *et al.* (2004) described the derivation of 17 hES cell lines from 97 isolated ICMs (in total 286 early embryos and 58 blastocysts). These data are very encouraging since human IVF embryos have revealed an unexpectedly high incidence of postzygotic chromosomal abnormalities which reduce the quality of recovered embryos and contribute to early pregnancy loss after embryo transfer (Delhanty & Handyside 1995, Trounson & Bongso 1996, Handyside & Delhanty 1997, Munne 2002). Surprisingly, it has been demonstrated that even aneuploid zygotes can be used as a source for derivation of hES cells (Suss-Toby *et al.* 2004) that could be used for research purposes.

An exact comparison of the success rate between different groups is difficult to make since some of the groups report the numbers of donated embryos and other groups

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report the numbers of blastocysts used. In addition some of the groups have used a small number of donated embryos/blastocysts from which it is difficult to extract complete data. There is also a problem of publication bias, where many groups have either used many embryos but failed to generate any hES cell lines or after successful derivation their data were not deemed sufficiently innovative and were therefore not published. However, a common factor in nearly all studies which describe successful derivation of hES cells is that the blastocysts used for immunosurgery are recovered in sequential two-step culture system using G1 and G2 media; this system is designed not only to allow for changes in nutrient requirements and metabolism as development of early human embryos proceeds but also to facilitate the development of highly viable blastocysts (Gardner & Lane 2003, Gardner *et al.* 2004).

We successfully derived and fully characterised one hES cell line (hES-NCL1) from day 8 blastocysts recovered in a more complex three-step *in vitro* culture system (Stojkovic *et al.* 2004). Based on the sequential culture of human embryos (Gardner & Lane 2003), this includes culture of early stages until day 3 in G1 medium, until day 6 in G2.3 medium and then culture of recovered day 6 blastocysts in medium conditioned by buffalo rat liver cells (BRL-medium). Using this protocol, all day 6 blastocysts expanded or hatched on Day 8 demonstrating that the addition of the third step is effective in supporting the hatching and growth of the blastocysts. In addition, day 8 blastocysts possess significantly more ICM cells (~ 50) than their day 6 counterparts (~ 30 ICM cells; Fig. 1) which is probably due to the presence of hyaluronic acid (HA) in the second step and different growth factors such as LIF, insulin-like growth factor-II (IGF-II), and transforming growth factor β (TGFβ) in BRL-medium (Stojkovic
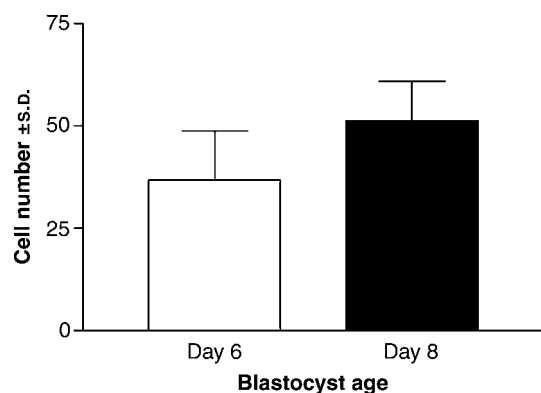

Figure 1 Numbers of ICM cells in day 6 (IVF = day 0) and day 8 human blastocysts. Day 6 blastocysts were recovered after two-step *in vitro* culture and day 8 after three-step *in vitro* culture: until day 3 early embryos were cultured in G1 medium, until day 6 in G2.3 medium and then recovered day 6 blastocysts were cultured for an additional 2 days in medium conditioned by buffalo rat liver cells. The cell numbers of 11 ICMs isolated from day 6 blastocysts were compared with the cell numbers of 13 ICMs isolated from day 8 blastocysts. $P < 0.05$ (Wilcoxon rank-sum test).

et al. 1995). HA plays a very important role in early embryogenesis (Stojkovic *et al.* 2003) and, when added to culture medium, HA increases the cell number of mouse (Gardner *et al.* 1999, Figueiredo *et al.* 2002) and bovine blastocysts (Stojkovic *et al.* 2002). Added to serum-free medium, LIF increases the blastocyst rate, hatching rate and cell number of mammalian preimplantation embryos (Fry 1992, Dunglison *et al.* 1996) but whether greater numbers of ICM cells in day 8 blastocysts allow more-efficient derivation of hES cells is difficult to say. When plated on feeder cells, hES clumps with 50–100 hES cells have more chances to attach and proliferate than smaller cell clumps (Reubinoff *et al.* 2000), and for successful derivation of hES cells the ICM has to be placed on feeders as a cell clump not as single cells since ICM cells are held together tightly with numerous junctional complexes (Fong *et al.* 2001). Therefore, we believe that the third step could be beneficial in the derivation of hES cells since it allows further proliferation of ICM cells within the blastocyst and allows the ICM cells a further 2 days to adapt to the culture conditions they will be subject to after isolation.

Growth of undifferentiated hES cells

Continuous culture of isolated ICM cells and hES cells in an undifferentiated state still requires the presence of feeder layers. Previously described feeder or feeder-free systems including MEF, STO, foetal muscle, skin and foreskin cells, adult Fallopian tube epithelial cells (Richards *et al.* 2002, 2003, Amit *et al.* 2003, Hovatta *et al.* 2003, Park *et al.* 2003), adult marrow cells (Cheng *et al.* 2003), or dishes coated with animal-based ingredients with the addition of MEF-cell-conditioned medium (Xu *et al.* 2001, Rosler *et al.* 2004). Such systems are not optimal for the derivation and growth of clinical-grade hES cell lines since they bear a risk of cross-transfer of different infectious agents. In addition, there are reports that describe high differentiation rates of hES cells and genomic instability of hES cells after prolonged *in vitro* growth (Amit *et al.* 2003, Carpenter *et al.* 2004). For instance, some hES cell lines display a certain level of aneuploidy (Carpenter *et al.* 2004) including the gain of chromosome 17q (Draper *et al.* 2004), trisomy 20 (Rosler *et al.* 2004) or aberrant X chromosome (Inzunza *et al.* 2004). Statistically significant differences in SSEA-4 expression, some differences in telomere length, downregulation of *collagen*, *STAT4*, a *lectin* and two genes involved in TGFβ signalling has been described in different hES cell lines derived in the same laboratory and cultured under feeder-free conditions (Carpenter *et al.* 2004, Rosler *et al.* 2004). This suggests that applied feeders and feeder-free systems for growth of undifferentiated hES cell lines are suboptimal since hES cell lines show subtle differences in gene expression upon long-term culture (Rosler *et al.* 2004).

Characterisation of hES cell lines

Following successful attachment of the ICM to the feeder cell to form a primary hES colony (Fig. 2), mechanical or enzymatic disaggregation may be performed to produce further hES colonies. This first step is followed by time-consuming and labour-intensive propagation, cryopreservation and characterisation of the new hES line. Fully characterised hES cell lines are those that show typical stem cell markers (Fig. 3), express high levels of telomerase activity, show normal karyotype and differentiation potential under *in vitro* and *in vivo* conditions and preferably have been shown to maintain these characteristics over extended periods of culture. Many new hES lines are
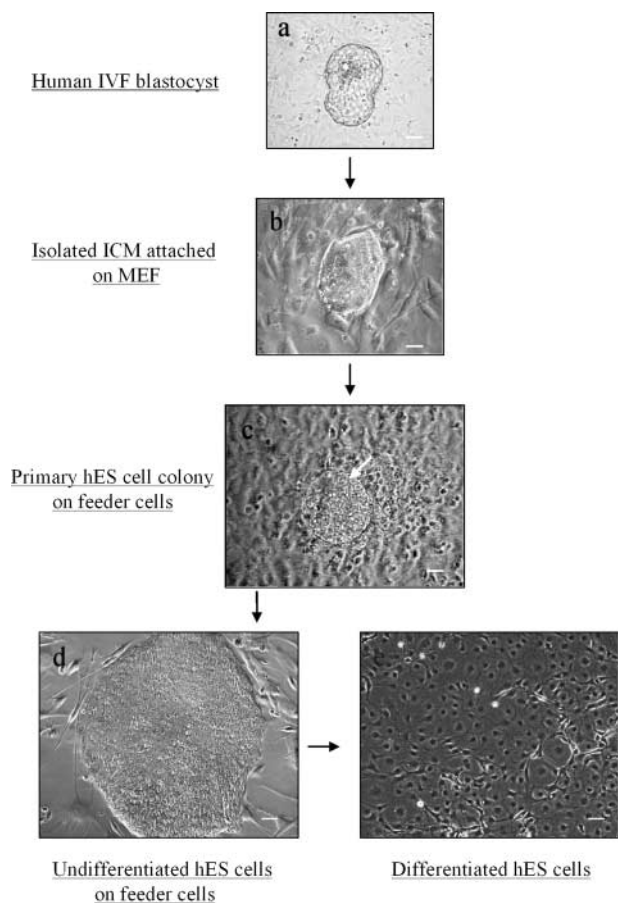

Figure 2 Derivation of hES cells. (a) Day 8 (IVF = day 0) hatching blastocyst derived after *in vitro* fertilisation. Alternatively, blastocysts could be received after intracytoplasmic sperm injection or nuclear transfer procedure. Note the presence of well-formed ICM (asterisk). (b) ICM isolated by immunosurgery and attached to the mouse feeder cells (MEF). Mechanical isolation of ICM is another possibility in order to remove trophectoderm cells. (c) Thirteen-day-old primary hES cell colony grown on MEF. Alternatively, feeder cells could also be of human origin. Note the presence of cells with typical hES-cell-like morphology (white arrow). (d) Undifferentiated hES-NCL1 colony grown on human feeder. (e) Spontaneous differentiation of hES-NCL1 cells with neuronal-precursors morphology. Induced differentiation of pluripotent hES cells could be achieved after addition of specific factor(s). Scale bars: 200 μm (a, c, d, e) and 100 μm (b).

described without complete characterisation, for example, formation of teratoma after injection into severe combined immunodeficient (SCID) mice and demonstration of karyotypic stability after long-term growth are often omitted. Some characterisation work with limited numbers of markers has been performed on 26 of the 60 or so reported hES cell lines (Carpenter *et al.* 2004) but to date it is not known how many hES cell lines exist in laboratories throughout the world (Gearhart 2004). To our knowledge there are probably more than 120 hES cell lines worldwide, 78 of which have been included in the National Institutes of Health (NIH) registry but not all of them are fully characterised and available to public institutions (for more information see www.stemcells.nih.gov/registry). Unfortunately, numerous newly derived hES cell lines are stored at early passage and are not fully characterised due to lack of resources.

It is important to note that not even fully characterised hES cell lines demonstrate comparable expression of cell surface (SSEA3, SSEA4, TRA1-60, TRA1-81, GTCM2, TGT343) and intracellular markers (*Nanog, OCT4, Rex1*), and not all hES cell lines have the same genetic profile even when cultured under the same conditions (Rosler *et al.* 2004). Comparing three different hES cell lines it was found that 52% of genes examined were expressed in all three independently derived hES cell lines and the expression of 48% genes was limited to just one or two hES cell lines (Abeyta *et al.* 2004). In addition, not all derived hES cell lines maintain their pluripotency under the same conditions, their potential for large-scale culture and growth under feeder-free protocols, or their ability to form teratomas after injection into SCID mice. Moreover, their capacity to differentiate spontaneously into different cell types under *in vitro* conditions (Richards *et al.* 2002) is variable. To our knowledge there is no study which describes the epigenetic status and stability of different hES cell lines or even one hES cell line after long-term culture. This is very important since early development and specific tissue characteristics are epigenetically regulated (Dean *et al.* 2001), every hES cell line possesses a unique expression signature, and it is of concern that application of genetically and epigenetically unstable hES cells in transplantation therapies could be detrimental.

Taken together, these data make it very difficult to evaluate the real number of derived hES cell lines and their authentic profiles. Therefore, complete characterisation of the hES cell lines – including their molecular status ('stemness') – and continuous genetic, epigenetic and chromosomal analysis of hES cell lines during prolonged *in vitro* growth are important steps. One possible solution to this cumulative problem is the establishment of national or international hES cell banks which would allow equal and detailed characterisation of deposited hES cell lines and provide scientists with all necessary information to choose the most suitable hES cell line for their own research.
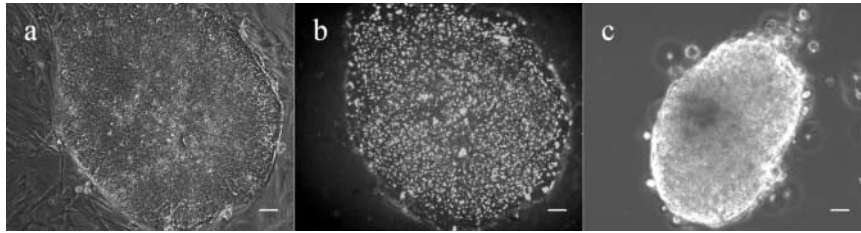
Figure 3 Human ES cells: (a) grown on mouse feeder cells; (b) stained with specific cell-surface marker (TRA1-60). When hES cells are transferred to non-adherent culture plates, they spontaneously form differentiated structures termed embryoid bodies (c). Embryoid bodies possess cells of all three germ layers and are frequently used to isolate differentiated cells under *in vitro* conditions. *In vivo*, differentiated cells could be isolated from teratomas formed after injection of hES cells into severe combined immunodeficient mice. Scale bars: 200 μm.

Improvement of conditions for growth of hES cells and their induced differentiation

It is not only the use of animal feeders and/or ingredients for growth of hES cells that limits the large-scale culture and medical applicability of hES cells. An additional obstacle is the slow population-doubling time of hES cells (∼36 h). In comparison, mES cells have ∼12 h population-doubling time (Amit *et al.* 2000) and the growth of mES cells under feeder-free conditions is straightforward in the presence of LIF or LIF-related cytokines (reviewed in Ginis *et al.* 2004). Again, hES cells do not appear to require LIF for their propagation and for maintenance of pluripotency (Thomson *et al.* 1998), thus identification of growth factors and components of extracellular matrix (ECM) which allow undifferentiated proliferation of hES cells under feeder-free conditions would be very helpful in the establishment of defined and xeno-free culture conditions. At the same time, identification of the molecular signature of undifferentiated hES cells and their differentiated progeny including ligand/receptor pairs and secreted inhibitors of different signalling pathways such as fibroblast growth factor (FGF) and/or TGFβ/bone morphogenetic protein (BMP) pathways (Sato *et al.* 2003) are necessary to improve conditions not only to prevent differentiation but also to induce differentiation of hES cells. This will be extremely difficult since different growth factors have unique effects on the growth of undifferentiated hES cells and their differentiation (Schuldiner *et al.* 2001). For instance, one of the most routinely used mitogenic and growth factor for propagation of undifferentiated hES cells is basic FGF (bFGF) which has, however, activating potential on ectodermal and mesodermal markers (Schuldiner *et al.* 2000) and acts as a differentiation factor for immortalised hypothalamic neurons (Park *et al.* 2004).

Another possibility to identify growth factors is to analyse feeder-cell-conditioned medium. Lim & Bodnar (2002) identified 136 unique protein species such as IGF-binding protein 4, pigment epithelium-derived factor, SPARC (secreted protein, acidic and rich in cysteine), nidogen-2, galectin 1 and peroxiredoxins from analyses of conditioned medium from MEF cells, and the STO cell line. Some of the identified proteins are known to participate in cell growth and differentiation, ECM formation and remodelling. This is one step further but more studies are necessary to investigate the proteome of hES cells and different feeders since STO cells do not produce sufficient amounts of soluble factors for self-renewal of hES cells growing without direct contact with STO cells (Park *et al.* 2003).

Another possibility to keep the hES cell undifferentiated or to direct hES cells along a specified lineage is genetic manipulation, i.e. transfection of hES cells or use of small interfering RNAs (siRNAs). It has been reported that the transfection efficiency of primate ES cells is lower than that of mES cells (Furuya *et al.* 2003). However, hypoxanthine phosphoribosyltransferase-1 and OCT4 genes were successfully targeted by homologous recombination (Zwaka & Thomson 2003) and the siRNAs technique was effective at knocking down gene expression (Vallier *et al.* 2004) in hES cells. Using homologous recombination in the cultured hES cells, specific gene defects could be repaired and, following differentiation into the appropriate tissue, hES cells would then be transplanted back to the patient (Rhind *et al.* 2003). Together with therapeutic cloning this offers exciting possibilities for deriving new modified and patient-friendly hES cell lines.

Therapeutic cloning

Undifferentiated hES cells express only low levels of molecules which activate an immune response. During differentiation, one of these molecules (major histocompatibility complex 1) is up-regulated (for review see Drukker & Benvenisty 2004). Therefore, the application of hES cell derivatives in regenerative medicine could be harmful due to the non-self proteins expressed on the grafted cells which may result in immune rejection of the graft in the absence of immunosuppressive therapy. A potential way of avoiding this disadvantage is therapeutic cloning (TC). The objective of this technique is to produce pluripotent hES cells that carry the nuclear genome of the patient and then induce them to differentiate into cells which may be transplanted back into the patient (Rhind *et al.* 2003). The SCNT technique requires the introduction of a nucleus

from an adult donor cell into an enucleated oocyte to generate a nuclear transfer (NT) embryo (Fig. 4). In culture, NT blastocysts can give rise to NT hES cells that have the potential to become almost any type of cell present in the adult body. This strategy could be used for derivation of isogenic or 'tailor-made' hES cells since all nuclear genes would be recognised as from the same origin (Hochedlinger & Jaenisch 2003). In animals, transplantation of cells derived using the TC technique has been successfully applied in parkinsonian mice (Barberi *et al.* 2003) and in humans TC might substantially improve the treatment of many incurable diseases (Alzheimer's, Parkinson's,
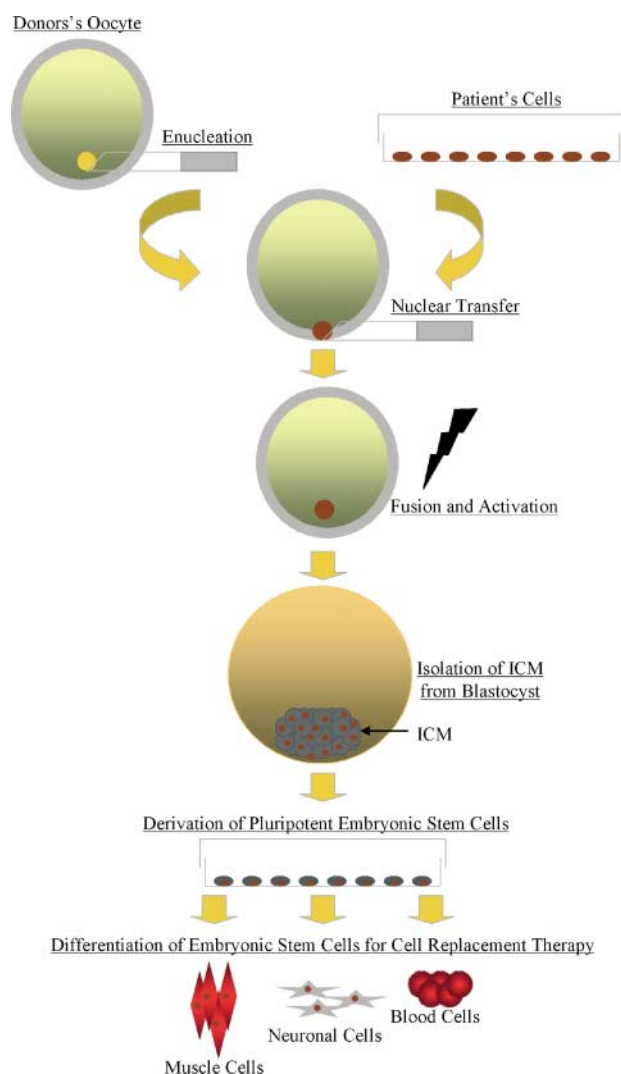

Figure 4 Steps of the therapeutic cloning technique. The nucleus of a donor's unfertilised oocyte is removed (enucleation) and replaced with the nucleus of a patient's own cells (nuclear transfer). After activation, the oocyte starts to cleave and derived NT blastocysts could be used for derivation of hES cells that have the potential to become almost any type of cell present in the adult body. This strategy offers an end to the use of immunosuppressive therapy in cell transplantation since all nuclear genes of differentiated hES cells would be recognised as having the same origin.

diabetes) since therapy for these diseases is currently limited by the availability or immunocompatibility of tissue transplants. In addition, NT hES cell lines could be useful in the development of *in vitro* human disease models for basic research, drug discovery and toxicology. Drug researchers use stem cells as a new resource for investigating the mechanism of action of new target compounds and assessing their safety (Street *et al.* 2003). Furthermore, derived NT embryos and NT hES cells offer an excellent opportunity to study: the effect of oocyte-derived mitochondrial proteins in somatic cells obtained by NT; the role of mitochondrial DNA; and cell reprogramming on the genetic and epigenetic status of derived NT hES cells.

Some scientists argued that primate embryos could never undergo successful SCNT, since meiotic spindle removal from recipient oocytes, which occurs during oocyte enucleation, appears to prevent embryo reconstruction (Simerly *et al.* 2003). However, the previous success of Korean scientists who were able to derive the first NT hES cell line (Hwang *et al.* 2004) demonstrates that the major obstacles in the derivation of NT-derived hES cells are indeed of a technical and logistic nature, i.e. low numbers of high-quality donated oocytes (Birmingham 2003).

Differentiation ability of hES cells

Human ES cells have the potential to differentiate into nearly all cell types of the human body. *In vitro* they are able to generate embryoid bodies (structures with three germ layers formed by pluripotent hES cells grown in three-dimensional culture, Fig. 3) which express marker genes of all three germ layers and for different cell types (Schuldiner *et al.* 2000). Human ES cells have the ability to differentiate into neuronal, skin, adrenal and keratinocyte (Reubinoff *et al.* 2000, Zhang *et al.* 2001, Green *et al.* 2003), blood, endothelial, kidney, bone, mucle, heart (Kaufman *et al.* 2001, Kehat *et al.* 2002, 2003, Levenberg *et al.* 2002, Xu *et al.* 2002, He *et al.* 2003), pancreas and liver cells (Schuldiner *et al.* 2000, Assady *et al.* 2001; reviewed in Eiges & Benvenisty, 2002). Induced differentiation of hES cells into cardiomyocytes which are electrophysiologically comparable with normal human cardiomyocytes and foetal ventricular myocytes has been reported by Mummery *et al.* (2003) and induced neuronal cell differentiation of hES cells has been achieved using retinoic acid and nerve growth factor (Schuldiner *et al.* 2001).

Studies in animal models show that transplantation of ES-derived cells can successfully treat a variety of chronic diseases including cardiovascular diseases, diabetes or traumatic spinal cord injury (reviewed in Odorico *et al.* 2001, Gerecht-Nir & Itskovitz-Eldor 2004, Shamblott & Clark 2004) which again underlines the promising role of hES cells in tissue regeneration and modern medicine.

Conclusions

Derivation of hES cell lines is time-consuming and labour-intensive work. However, derivation of a new generation of clinical-grade hES cell lines is more than necessary; it requires not only *in vitro* production of blastocysts cultured in defined systems, isolation of ICM cells using human antibodies or mechanical methods, growth of hES cells in culture systems which contain non-compromising and patient-friendly feeder or recombinant-based media ingredients, but also unique legislation. In the European Union this will lead to the necessary upgrading of IVF and stem cell units since the European Parliament adopted a directive (2004/23/EC) setting high quality and safety standards for human tissues and cells used in therapy. The directive sets out quality requirements related to the donation, procurement and testing of tissues and cells including oocytes, sperm and hES cells (for more information see http://europa.eu.int/eur-lex/en/). After derivation, new hES cell lines should be investigated frequently for possible chromosomal aberrations, genetic/epigenetic profile after prolonged growth and the ability to differentiate.

Certainly, application of hES cells for therapeutic purposes is still premature; however, the results achieved in hES cell research during the last few years, including derivation of new hES cell lines using SCNT, demonstrate rapid progress and the increased importance of hES cells in both basic research and the long-term future of modern medicine.

Acknowledgements

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