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Review

PGD-derived human embryonic stem cell lines as a powerful tool for the study of human genetic disorders

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Abstract

Human embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocyst embryos. They are established from spare embryos that have been obtained by in vitro fertilization (IVF) and donated for research purposes. The ICM-derived cell lines have two unique properties, they can be propagated indefinitely in culture and have the potential to develop into practically any cell type in vitro and in vivo.

Human embryonic stem (hES) cells carrying specific mutations can be used as a valuable tool for studying genetic disorders in human. One favorable approach to obtain such mutant ES cell lines is their derivation from affected preimplantation genetic diagnosed (PGD) embryos. This review focuses on the importance of deriving human ES cell lines from genetically abnormal embryos, especially in cases where no good cellular and/or animal models exist.

Keywords: Human embryonic stem cells; Preimplantation genetic diagnosis (PGD); Genetic disorders

Contents

1. Introduction

Human embryonic stem (hES) cells carrying specific mutations can be used as a powerful cell system in modeling human genetic disorder for which no good research models currently exist. Such in vitro systems may have great importance in the study of human genetics, especially for the exploration of new therapeutic protocols, including gene therapy-based treatments and disease-oriented drug screening and discovery. In addition, they can be used to gain new insights on developmentally regulated events that occur during human embryo development and that are responsible for the manifestation of genetically inherited disorders.

2. hES cells as a model for early human embryogenesis

Pluripotent stem cells are undifferentiated cells that are capable of forming practically any cell type in the body. They are transiently found during embryo development, in preimplantation embryos and in fetal gonads, but they can also be maintained as established cell lines (Eiges and Benvenisty, 2002). hES cells

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represent a special type of pluripotent cell lines. They are derived from the inner cell mass (ICM) cells of blastocyst stage embryos and are considered to have the greatest developmental potential, differentiating into the widest range of cell types (Thomson et al., 1998; Reubinoff et al., 2000). Furthermore, they can be triggered to differentiate both in vivo and in vitro. In vivo, they can differentiate spontaneously by forming benign tumors known as teratomas. Teratomas are induced by injecting undifferentiated hES cells into immunodeficient mice and are composed of various cell types which are derivatives of endoderm, mesoderm and ectoderm germ layers (Thomson et al., 1998; Reubinoff et al., 2000; Amit et al., 2000). In vitro, hES cells can be induced to undergo spontaneous differentiation by growing in suspension culture (Itskovitz-Eldor et al., 2000). Under these conditions hES cells tend to form cell aggregates, which grow and proliferate extensively, resulting in the formation of large cell structures termed embryoid bodies (EBs). The formation of EBs is a gradual process and is accompanied by morphological changes. It begins with the formation of densely packed cell aggregates, which continue to grow and undergo cavitation by accumulating fluid. These morphological changes coincide with cell differentiation so that they become cystic and fully mature by day 30 in suspension culture. At that stage, they are composed of many different cell types that are derived from all three germ layers. Spontaneous differentiation of hES cells by the formation of EBs has been illustrated by the detection of gene expression of several lineage-specific markers, such as alpha-fetoprotein, cardiac-actin and neurofilament, which are considered as being typical markers for terminally differentiated cells of endoderm, mesoderm and ectoderm origin, respectively (Schuldiner et al., 2000).

It has been proposed that differentiating hEBs mimic, to some extent, early human embryonic development (Dvash et al., 2006). This concept has been originally practiced in the mouse, where growing EBs have been used as an in vitro model system for studying developmentally regulated biological phenomena. Good examples of biological developmentally regulated events which have been studied using this system are X inactivation in females (reviewed by Heard, 2004) and globin gene switching (Lindenbaum and Grosveld, 1990), which are both tightly linked with embryo development and cell differentiation. Using DNA microarrays, which allow determining the gene expression profile of a given cell population, it was possible to follow the differentiation program of hEBs as they grow in culture (Dvash et al., 2004). Gene expression patterns of hEBs at different time points during their maturation were determined and compared, making it possible to cluster temporally expressed genes into several subgroups which represent different stages in embryo development, from the blastocyst stage to late organogenesis, through primitive ectoderm, gastrulation and early organogenesis. Furthermore, it is possible to recover some of the molecular pathways that are successively activated during embryo development. For example, by examining the expression of temporally expressed genes during hEBs formation, it was possible to follow the gene cascade of the nodal signaling pathway, which has been shown to play a major role in the determination of embryonic axes during gastrulation in the mouse (Hamada et al., 2002). These findings support the impression that hEBs can recapitulate, at least at the cellular level, early human embryonic development and may be used as a model system for studying certain developmental stages, which are otherwise inaccessible for research. Moreover, it may allow studying molecular pathways which operate in succession and are developmentally regulated, as they occur in vivo.

3. Currently available models for the study of genetically inherited disorders and their limitations

Modeling human genetic diseases can be achieved using primary cell cultures biopsied from patients. This approach is, however, restricted by the limited range of tissues from which cells can be obtained. For example, in syndromes such as fragile X or ML4, which are manifested mainly in nerve cells, biopsy and culture of specific cell types is impossible. Another limitation of cell cultures as a model for studying human genetics is their short life span unless they are transformed, and transformed cells usually carry chromosomal abnormalities and are cancerous in nature. Moreover, these cellular models allow studying only a restricted time point during a continuous, developmentally regulated pathway.

The above-mentioned limitations of cell culture systems have led to the use of genetically engineered animals to generate better research models. The mouse, which has been the most popular model for mammals in general and for humans in particular, is usually used for stable introduction of genetic modifications into its genome, including site-directed mutagenesis. Site-directed mutagenesis involves the replacement of a specific sequence in the genome of the cell with a mutated copy by homologous recombination. By targeting both alleles, it is possible to create "loss-of-function" phenotypes that can be applied for functional studies of specific genes in culture as well as in intact animals. Many of these models, however, do not faithfully represent the abnormal phenotype as manifested in human (Dvash and Benvenisty, 2004) and often diverge considerably from that of the human phenotype due to intra-specific variations (evidenced by anatomical, developmental and even some biochemical differences). Moreover, there are several naturally occurring mutations in humans which have no counterparts in rodents. For example, the unstable triplet repeat expansion disorders which have no complement in mice (i.e., Fragile X, myotonic dystrophy, androgen receptor, spinobulbar muscular atrophy and Huntington disease). Finally, fundamental differences in DNA and gene expression levels limit the power of knock-out (KO) mice in accurately mimicking human disorders, such as in the case of Lysch Nyhan syndrome, which is caused by the absence of the enzyme HPRT. The biochemical defect of the disease involves the accumulation of uric acid which is due to an inability to convert hypoxanthine to inosine-5′-monophosphate. In rodents, uric acid does not accumulate due to the presence of another enzyme (urate oxydase) which converts uric acid to allantoin. In addition, no clear neuronal behavior defects were observed in KO mice. Thus, in many respects, the available KO mice cannot serve as a good research model for the disease (Bedell et al., 1997; Elsea and Lucas, 2002).

4. Generation of genetically abnormal hES cell lines

The limitations of the currently available models, as described above, have emphasized the need for an alternative system that will better mimic certain genetic abnormalities. The creation of hES cell lines might allow studying such early processes that are otherwise inaccessible for research. Moreover, in the context of human genetics, the availability of mutant hES cells, which harbor specific mutations at discrete sites, should be most valuable for the study of some pathologies. There are two strategies for obtaining mutant cell lines (Fig. 1). One is to artificially introduce specific mutations in a pre-existing cell line by homologous recombination. Using this approach, a cellular model system for Lesch Nyhan syndrome was generated by targeting the HPRT gene in XY wild-type hES cells (Zwaka and Thomson, 2003, Urbach et al., 2004). The HPRT-deficient hES cells display the major biochemical defect that characterizes Lesch Nyhan patients, which involves the accumulation of uric acid, and cannot be mimicked by the mouse model due to inter-species genomic differences (Bedell et al., 1997; Elsea and Lucas, 2002).

The other approach is to establish a hES cell line directly from an affected embryo so that the resulting cell line will be carrying the naturally inherited mutation (Pickering et al., 2005; Verlinsky et al., 2005; Mateizel et al., 2006). hES cells derived from genetically affected embryos would be advantageous over genetic intervention, since there would be no need to genetically manipulate the cells in order to target a gene. In addition, it allows the generation of cell lines that harbor genetic modifications that are otherwise inaccessible, such as very small deletions, few base pair substitutions and even triplet repeat expansions. Furthermore, this approach could obtain hES cell lines which have numerical or structural chromosomal abnormalities. Such cell lines may be used as a model system for the study of specific chromosomal loss and rearrangements. The derivation of mutant hES cells depends on the availability of genetically affected embryos. Such embryos are obtained from carriers of genetic disorders who are undergoing preimplantation genetic diagnosis (PGD). PGD requires in vitro fertilization (IVF) and allows the selective transfer of disease-free embryos into the uterus to ensure a pregnancy of a healthy embryo.

Another approach that had been previously shown to be feasible in the mouse is to derive hES cells with naturally occurring mutations from nuclear transfer derived blastocysts (Munsie et al., 2000). In this method, a nucleus from a somatic cell of an affected individual is introduced into an enucleated oocyte, resulting in an affected cloned embryo. This embryo can now be used for the derivation of a mutant ES cell line. The advantage of this strategy over earlier approaches would be its use in cases of multifactorial diseases and other disorders with yet

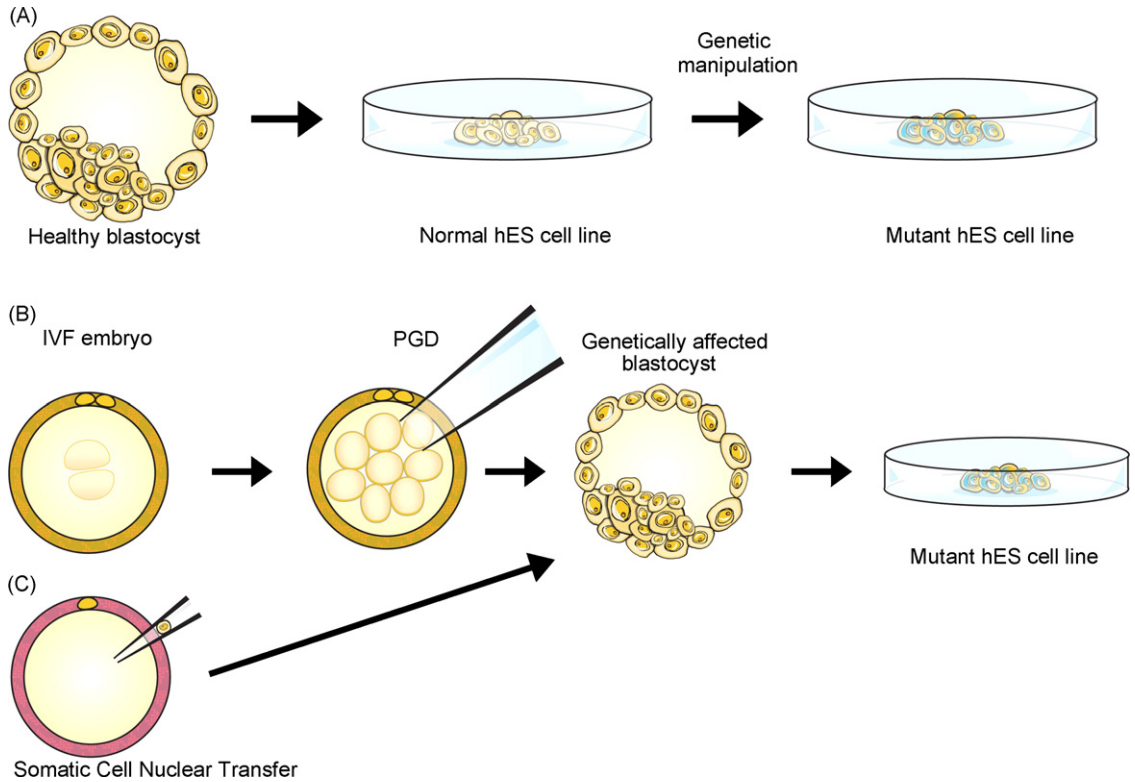

Fig. 1. Different approaches to establish mutated human embryonic stem (hES) cell models for the study of human disorders. (A) Models for human disorders can be created by the use of human ES cells through genetic manipulation. Targeted mutations can be obtained by homologous recombination in wild-type human ES cells that have been derived from genetically normal pre-embryos. (B) Models for human disorders can be also created by the use of ES cells that have naturally occurring mutations. Such cells can be established from genetically abnormal pre-embryos, which are obtained through preimplantation genetic diagnosis (PGD) that is performed to couples carriers of a genetic defect. (C) Derivation of hES cells from a cloned blastocysts. In this procedure, a nucleus from a somatic cell of an affected individual is introduced into an enucleated oocyte, resulting in an affected cloned embryo. This embryo can now be used for the derivation of a mutant ES cell line. The advantage of this strategy would be in cases of multifactorial diseases and other disorders with yet unidentified genetics.

unidentified genetics. An example of its application is in the immune-deficient Rag2(−/−) mice, which were used as nuclear donors for transfer into enucleated oocytes to generate blastocysts, for the isolation of isogenic ES cell lines (Rideout et al., 2002).

5. hES cells derived from PGD-affected embryos

5.1. Preimplantation genetic diagnosis

PGD allows genetic analysis of embryos before their implantation in the uterus (Yaron et al., 2001; Sermon et al., 2004; Harper, 2002). It offers an alternative to prenatal diagnosis by chorionic villus sampling (CVS) or amniocentesis, which, in the case of an affected fetus, lead to therapeutic abortion. PGD is currently the state-of-the-art technique for ensuring a disease-free baby for couples at high risk (25–50%) of transmitting a genetic disorder to their offspring (Malcov et al., 2004, 2007; Yaron et al., 2005; Verlinsky et al., 1997; Verlinsky and Kuliev, 1998).

For PGD, the couple has to undergo the entire IVF process. In the usual protocol, cleavage-stage embryos are biopsied 3 days after fertilization and single blastomeres are genetically analyzed. This methodology is based on the concept that all blastomeres comprising the embryo are genetically identical and therefore allow deduction of the genotype of the entire embryo according to the analysis of a single biopsied blastomere.

PGD is used mainly for individuals at high risk of having a child with a genetic disease, e.g., carriers of a monogenic disease or of chromosomal structural aberrations such as translocations. PGD protocols are currently available for almost all mutations that can be diagnosed by amniocentesis or CVS during prenatal diagnosis (i.e., Fragile X syndrome, Duchenne muscular dystrophies, cystic fibrosis, Tay–Sachs, Bloom syndrome, etc.). Another clinical application for PGD is the selection of embryos suspected of being carriers of late-onset disease (e.g., Huntington disease), including inherited cancer predisposition mutations such as APC, NF1, and BRCA1 (Menon et al., 2007; Kastrinos et al., 2007; Spits et al., 2007). Moreover, it is now possible to perform PGD for single gene disorders combined with human leukocyte antigen (HLA) typing. These may prove to be beneficial therapeutic tools in situations where an affected child can be cured only by a bone marrow transplantation of an HLA-matched healthy sibling (Devolder, 2005; Kuliev et al., 2005; Kahraman et al., 2007). In addition, PGD for aneuploidy screening (PGS) is suggested to enhance the chances for a healthy baby among IVF patients with a low genetic risk but who have had repeated implantation failures or recurrent miscarriages (Verlinsky et al., 2004; Staessen et al., 2004)

Two main technologies are used for analyzing PGD: polymerase chain reaction (PCR) for monogenic disorders and fluorescence in situ hybridization (FISH) analysis for diagnosing structural or numerical chromosomal aberration, including translocations and sex-determination for X-linked disease with unidentified mutation, as well as for PGS (reviewed by Coonen et al., 1998). The procedure by which PGD-dervied embryos is used for the derivation of new hES cells lines, is illustrated in Fig. 2.

5.2. Potential applications of mutant hES cells

Derivation of human ES cell lines which harbor a specific gene defect may have great importance in the study of certain inheritable disorders, especially in those for which no good animal or cellular models are available. Moreover, it may allow examining the abnormal phenotype in an autonomous cell system, independent of external signals of neighboring cells, which are naturally present in the multisystem animal model. Another advantage of hES cells as an autonomous cell system is the ability to better manipulate and control the cells. Once efficient

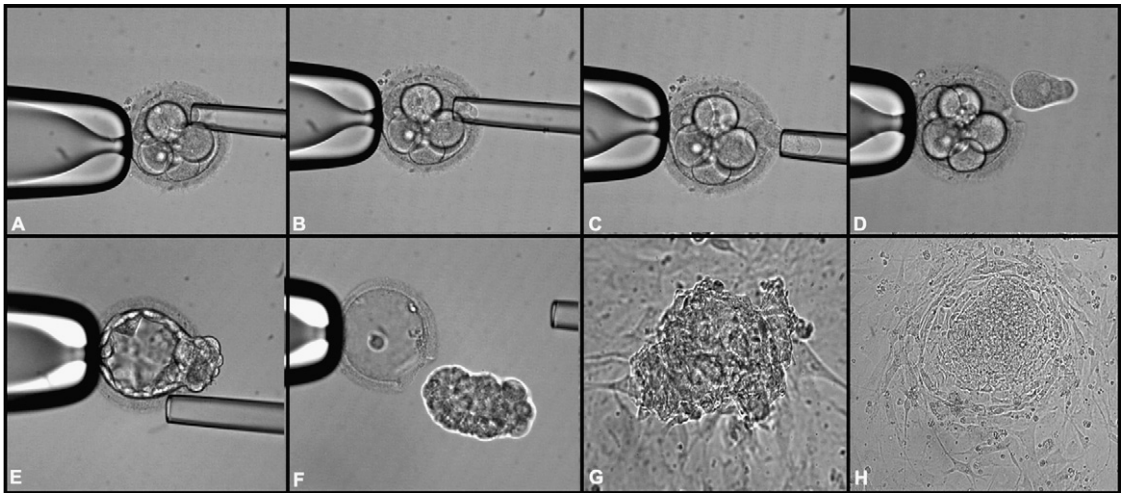

Fig. 2. Human embryonic stem (hES) cell derivation from preimplantation genetic diagnosed (PGD)-affected embryos. (A–D) Blastomere biopsy from an 8-cell embryo at day 3 of development. Note the visible nucleus within the biopsied blastomere which is later subjected to polymerase chain reaction/fluorescence in situ hybridization (PCR/FISH) analysis as part of the PGD (D). (E) Zona pellucida removal by micromanipulation of day 6 expanded blastocysts. (F) Collapsed blastocyst following zona removal. (G) Initial proliferation of the isolated clump of inner cell mass (ICM) cells on a feeders of inactivated mouse embryonic fibroblasts (MEFs) 48 h following immunosurgery. (H) First passage of mutated hES cells 72 h following mechanical splitting.

protocols for induced differentiation will be established, it will be possible to generate specific cell types in large numbers so that affected tissues of patients will be accessible for research. One example for implementing that protocol would be a genetic disorder that affects the brain and is most severely manifested in nerve cells. Since nerve cells cannot be cultured in vitro, the cell models used to study disorders such as these have been fibroblasts or endothelial or epithelial cultured cells obtained from affected individuals. The availability of hES-derived nerve cells carrying the specific mutation will provide the means to discover the molecular mechanism by which the mutation affects brain development at the cellular level and this will shed light on the factors that are critical in the pathogenesis of the disease. These cell lines may serve as an in vitro model for the disease, allowing greater understanding of its pathology by studying the abnormal phenotype at the cellular and molecular levels.

Another advantage of the availability of hES cells derived from mutant embryos is the possibility of investigating early stages in embryo development which are otherwise inaccessible for research. The molecular events which are crucial for the manifestation or lethality in some diseases occur very early during development. Since hES mimic human embryogenesis to some extent, it may be possible to study these genotypes in a better research model.

There are a number of chromosomal numerical aberrations (most trisomies and monosomies) which are lethal very early during embryo development. In such cases, it would be extremely useful to have cellular systems that will comprise the chromosomal constitution, thus enabling us to study the effect of those aberrations on cell viability.

Specific mutated hES cells can serve as an excellent cellular model for the study of predisposition to cancer. These mutated hES cell lines display the first critical molecular event that is required, but not sufficient, for cancer development. Therefore, they may allow following the multi-step process from its initial stage to its final endpoint as a cancer cell. A group of such mutations are those that can interrupt the cell response to DNA damage (DNA repair), leading to chromosomal instability. In some diseases, the presence of the mutation is not sufficient for gene silencing, and the epigenetic modifications that take place later during embryogenesis are crucial for gene inactivation and the development of the clinical manifestation of the disease.

Mutant hES cell lines would also have great value in in vitro gene therapy. One of the great advantages of ES cells over other cell types is their accessibility for genetic manipulation. They can easily be induced to genetic modifications, without losing their unique characteristics, and they can be selectively propagated, allowing the clonal expansion of genetically altered cells in culture. Genetic manipulation of hES cells can, therefore, be utilized for monitoring, selecting and even directing the differentiation of the cells into specific lineages. Moreover, the hES cell-derived progenitors may be used as delivery vectors for the regulated release of drugs and therapeutic proteins at the site of the damaged tissue. In addition, it may be possible in the future to repair the genetic defect by replacing the aberrant gene with an intact sequence. This can be accomplished by coupling embryo cloning with gene therapy, so that the genetic manipulation will be carried out on the genome of nuclear transfer (NT)-derived isogenic hES cell lines obtained from patients. Using this technique, it will be possible to cure the disease by providing the patients with genetically engineered autologous grafts, overcoming the difficulties in graft rejection as a result of the immune response. This approach, termed "therapeutic cloning", has been previously shown to be feasible in mice, where immune-deficient Rag−/− mutant mice were used as nuclear donors for transfer into enucleated oocytes, generating NT-derived blastocysts from which an isogenic ES cell line was isolated (Rideout et al., 2002). The Rag gene was then repaired in the mutant ES cells by homologous recombination. The genetically restored ES cell lines were used for generating live animals to be used as bone marrow donors. Alternatively, they were induced to differentiate in vitro into hematopoietic precursors. In both cases, the hematopoietic-committed cells were engrafted into the Rag−/− mutant mice, resulting in the rescue of the immunodeficiency (Rideout et al., 2002)

The clinical application of affected hES cell lines would be their readily accessible source for pharmacogenetic tests, and for screening and discovering drugs. Because these cells can form distinct populations of terminally differentiated cells in vitro, they may be used for large-scale screening of new pharmaceutical compounds, optimizing currently available drugs as well as examining their effect on cell toxicity and teratogenicity.

6. Summary

hES cells lines derived from PGD-affected embryos are important for the study of human genetics, especially in cases where no suitable cellular and/or animal models are available. Many investigators no longer see cell therapy as the first goal of hES cell research. Instead, it is considered that mutant hES may serve as an excellent research tool to study the mechanism of disease. Such in vitro systems may have great value in the study of human genetics, especially for the exploration of new therapeutic protocols, including gene therapy-based treatments and disease-oriented drug screening and discovery and, most importantly, in investigating the potential of gene therapy in curing disease.

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