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HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells originate from the human preimplantation embryo. The derivation of the first human embryonic stem cells was reported in 1998. Since then we have learnt a great deal about how to isolate and culture these cells. Additionally, their stem cell phenotype and differentiation competence have been determined.

Although, it is expected that many basic biological properties, such as self-renewal and cell specification, are evolutionary conserved, at least from the mouse, we lack significant knowledge about the molecular events that regulate the unique stem cell features of human embryonic stem cells. The pluripotent nature of human embryonic stem cells have attracted great interest in using them as a source for cells and tissues in cell therapy. Recent progress in human somatic cell nuclear transfer suggests that the immuno-tolerance problems associated with the use of human embryonic stem cells in cell-replacement therapy may have a solution. Thus, human embryonic stem cells supply the research community with unique research tools to study basic biological processes in human cells, model human genetic diseases and develop new cell-replacement therapies.

INTRODUCTION

The definition of a stem cell is a cell that can both self-renew, in an undifferentiated state, and differentiate into one or more cell types. Embryonic stem (ES) cells are pluripotent stem cells that can be isolated from the inner cell mass of preimplantation embryos (1, 2) (Fig. 1A). The unique features of ES cells that distinguish them from other types of organ-specific stem cells, include the ability to maintain and expand the cells in culture for extended time while maintaining a normal karyotype and pluripotent nature. The latter was formally demonstrated for mouse ES cells by injecting the cells into host blastocysts and showing that they could contribute to all tissues of the adult mice (3). In addition to the multilineage commitment of ES cells in vivo, they show remarkable capacity to differentiate into a broad range of cell types in vitro (4, 5). The ability to commit to multiple cell lineages opens up a wide range of applications of ES cells in studying basic developmental biological processes. In particular, the use of human ES cells (hESC) will facilitate studies on early human development, which so far has been impossible. The isolation of hESC and development of somatic cell nuclear transfer (SCNT) (6) have generated increased interest in using hESC as an unlimited source for cells and tissues for transplantation therapy in various diseases and injuries. Furthermore, SCNT and further development of homologous recombination in hESC will enable unique new ways of modeling human genetic diseases. This review will cover basic features of hESC, such as their origin and properties, culture conditions and clinical applications. For additional details on hESC differentiation the reader is referred to recent reviews that supply substantial coverage of these issues (5, 7-9).

ORIGIN AND PROPERTIES OF HUMAN ES CELLS

Origin of hESC

Human ES cells are predominantly isolated from blastocyst-stage embryos derived from in vitro fertilized eggs (10, 11). The primate/human blastocyst consists of the surrounding trophectoderm epithelium and the inner cell mass (ICM) and extraembryonic endoderm (8, 12). The trophectoderm will participate in placentation and although not experimentally proven the inner cell mass presumably exclusively give rise to all cell of the body, whereas the extraembryonic endoderm may provide nutrients and patterning information to the embryo as in the mouse (13). In the mouse, three types of stem cells can be isolated from the blastocyst; ES cells, trophoblast stem (TS) cells and extraembryonic endoderm (XEN) cells (1, 2, 14, 15). Based on morphological and gene expression data it is thought that ES cells are epiblast derived, TS cells trophoblast derived and XEN cells primitive endoderm derived (10, 11, 14, 15). Furthermore, the fact that each stem cell contributes to its cognate lineage in chimeric embryos, suggested that the stem cells retain their appropriate developmental competence in culture (14-16).

In the mouse, important information regarding the molecular mechanisms determining the lineage decisions within the blastocyst has been reported. The POU domain transcription factor Oct4 is expressed throughout the early embryo, whereas it becomes restricted to the ICM in the blastocyst (17). Analysis of Oct4-deficient mice, which die around implantation, showed that Oct4 is required for ICM specification (18). The caudal-related transcription factor Cdx2 is expressed in the emerging trophectoderm (19), and Cdx2 mutant mice die before implantation due to failure to

maintain trophectoderm fate (19, 20). Furthermore, Oct4 is ectopically expressed within the trophectoderm of Cdx2 mutants (19). Based on these data it has been proposed that Oct4 and Cdx2 segregate the ICM and trophectoderm lineages by promoting respective lineage and negatively regulating each other. This model has gained further support from in vitro studies using ES and TS cells. Oct4 is expressed in ES cells (17), but not in TS and XEN cells (14, 15), and ES cells cannot be isolated from Oct4-deficient embryos (18). Notably, gene targeting of Oct4 in ES cells resulted in differentiation into trophectoderm lineages (21). Cdx2 is expressed in TS cells, but not in ES cells (15, 21), and TS cells cannot be derived from Cdx2 mutants (19). These data support the notion that these genes regulate ES and TS cell fates largely as they do in vivo.

Primitive endoderm differentiation is associated with downregulated expression of Oct4 and Nanog. The latter is another transcription factor expressed in the ICM/epiblast lineage (17, 22, 23). Based on the facts that Nanog-deficient embryos fail to form the epiblast and that Nanog-deficient ES cells differentiate into extraembryonic endoderm (22), it has been proposed that Nanog determines epiblast/primitive endoderm lineage decision. Furthermore, ectopic expression of Gata6, a transcription factor expressed within the primitive endoderm of the blastocyst (24), results in differentiation into extraembryonic endoderm. In accordance, Gata6 mutants exhibit late extraembryonic endoderm defects (24-26). These findings highlight an important function of Nanog/Gata6 in regulating epiblast/primitive endoderm lineage determination in the blastocyst.

Interestingly, despite the fact that ICM, trophectoderm and extraembryonic endoderm cells are present in the human blastocyst, only hESC, and not TS or XEN stem cells, have so far been isolated from the human blastocyst. To date, the

predominant proportion of the established hESC lines (>100) have been derived by methods previously used for isolating ES cells from other species, such as the mouse, i.e. combined enzymatic/acidic and immunosurgical (27) removal of the zona pellucida and trophectoderm, respectively. Human ES cells have also been isolated from spontaneously hatched blastocysts and zona-removed blastocysts (28, 29). Many differences between mouse ES cells and hESC exist, including morphology, growth rate, fibroblast growth factor 2 (FGF2) and leukaemia inhibitory factor (LIF) requirements (5, 7, 8). Another important difference is that hESC spontaneously and by exposure to BMP4 differentiate into trophectoderm-like cells (11, 30, 31), suggesting that the ICM of the human blastocyst may retain the ability to generate trophectoderm. In support of this concept are the findings that downregulation of Oct4 in hESC results in upregulation of trophectoderm markers, such as Cdx2, and of extraembryonic endoderm markers, such as Gata6 (32, 33). Inhibition of Nanog expression also resulted in an upregulation of trophectoderm markers (34). Altogether, these observations suggests a difference in the properties of stem cells isolated from the mouse and human blastocyst, in that hESC are endowed with a broader lineage potential that may represent the collective lineage capacity of ES, TS and XEN stem cells isolated from the mouse blastocyst. Does this imply a fundamental difference in the lineage restriction of the ICM in mice and humans, or does it simply reflect methodological aspects of how the stem cells are derived from the blastocyst? These are interesting questions to address in the future.

Properties of hESC

Human ES cells grow with a population doubling time of 24-48 hours in flat colonies with distinct cell borders and exhibit a high nucleo-cytoplasmic ratio and large

nucleoli (Fig. 1B), indicative of active transcription and protein synthesis during active cell proliferation. Undifferentiated hESC express characteristic markers on their cell-surface, such as the stage-specific embryonic antigen 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81, GCTM-2, and alkaline phosphatase, but not SSEA-1. Other characteristic markers for undifferentiated hESC include transcription factors, such as Oct4, Nanog and Sox2 (Fig. 1C), and DNA modifying enzymes, such as TERT (telomerase) and DNMT3 (DNA methyltransferase). Global comparative gene expression analysis of different hESC lines has generated conflicting data; some studies have reported similarities (35-39), whereas others highlight differences (40, 41).

Most hESC lines have been reported to have a normal complement of 46 XX/XY chromosomes. Chromosomal abnormalities are common in embryonal carcinoma cells and mouse ES cells, and karyotypic changes, such as trisomy and amplifications, which often is accompanied by proliferative advantages and shortening in the population doubling time are associated with prolonged culture of hESC (see C. Hanson) (42-46). These findings may on one hand give clues to the underlying genetic control of self-renewal of hESC, but they also warrant caution when designing new culture conditions, such as feeder-free systems, for future therapeutic applications of hESC.

To determine the full developmental potential of hESC, chimeric experiments that normally are used with mouse ES cells, can for obvious ethical reasons not be performed in humans. Therefore, alternative methods have been used to delineate the multilineage differentiation ability of hESC. In vitro, hESC differentiate into derivatives of all three germ layers (ectoderm, endoderm and mesoderm) and precursors to germ cells (5, 9-11). To examine the in vivo pluripotent features of

hESC, undifferentiated hESC are normally xeno-transplanted into mice where they develop into teratomas that typically consist of a wide array of cell and tissue types representing derivatives from all three germ layers (Fig. 2) (5, 9-11). Based on these findings it is assumed that the cell differentiation capacity of hESC is similar to mouse ES cells. However, a largely unanswered question is whether certain hESC lines are more prone to differentiate towards any particular germ layer or specific cell type. Systematic comparative analysis of the lineage potential of hESC lines derived in different laboratories should shed light on this question. Notably, when certain hESC lines that based on morphology, marker expression and in vitro differentiation assays behave like most pluripotent hESC are transplanted to mice the cells form a graft consisting of cystic structures instead of the characteristic solid multi-tissue structure of normal teratomas (29). Are these hESC equipped with a different cell lineage potential compared to hESC that generate typical teratomas, and if so, what is the underlying molecular mechanism for this difference? In summary, the realization that hESC can differentiate into a wide variety of cell types has opened up new avenues for studying human embryogenesis, such as gastrulation and organogenesis, modeling genetic human diseases and develop new cell therapies (see below).

MAINTENANCE OF HUMAN ES CELL

Human ES are typically cultured with animal-derived serum or serum replacement on mouse embryonic fibroblast feeders. In contrast to mouse ES cells, which can be enzymatically dissociated into single cells and grown at clonal density, the hESC are “social creatures” that survive poorly without their neighbors. Therefore, hESC are

passaged in small clusters either by manual dissection or by various enzymatic dissociation protocols. All currently available hESC lines have at some point during their derivation and/or maintenance been exposed to animal material. One potential consequence of using hESC that have been exposed to animal material in patients is an increased likelihood of graft rejection. For example, the animal-derived serum replacement and mouse feeders are sources of the nonhuman sialic acid Neu5Gc, against which many humans have circulating antibodies (47). Moreover, xeno-exposure increases the risk of transferring non-human pathogens in any clinical application (48). Thus, the predicted hazards associated with using xeno-exposed hESC in patients, makes these cells unsuitable for clinical applications. In an attempt to overcome these problems, several groups have used feeder-free systems (49-52), or feeder cells of human origin for derivation and culture of hESC lines (53-59). However, it has so far not been possible to derive and continuously culture hESC lines in a completely xeno-free system.

The development of new culture techniques for hESC, including the use of a chemically defined medium without serum or serum replacement, for efficient enzymatic expansion of hESC is not only relevant for developing clinically applicable hESC lines, but also for using the cells in basic research. Important progress in this direction was recently achieved with mouse ES cells. By replacing serum and feeders with recombinant LIF and BMP2 it is now possible to grow mouse ES cells at clonal density while maintaining their ability to contribute to all cell types in the body, including germ cells (60). Adopting a corresponding simplified culture system for hESC will not only facilitate their use in cell therapy, but also in studying the signalling pathways that regulate self-renewal and cell lineage determination in hESC.

MEDICAL APPLICATIONS OF HUMAN ES CELLS

Modeling genetic diseases

The generation of hESC lines that carry mutations conferring predisposition to certain diseases will supply valuable research tools for studying by which processes the diseases arise and potentially lead to development of new therapeutic strategies. Such hESC lines can be obtained by the introduction of mutations by homologous recombination (61, 62), by SCNT (6) from the affected patient's own cells, or by prenatal diagnostics. These hESC lines could then be differentiated into the afflicted cell type and used to study the onset and progression of the disease, screen for new drugs and test new therapies. For example, generation of pancreatic beta cells from hESC that carry a particular maturity-onset diabetes of the young (MODY) mutation (63) could assist in a better understanding of the development of the disease and facilitate the discovery of a cure for the disease.

Cell replacement therapy

The major advantage in using tissue-specific stem cells is that the patient's own cells could be expanded in culture and then reintroduced into the patient without immune rejection. However, several problems are associated with transplantation of tissue-specific stem cells, such as isolation difficulties, limited quantities, and the disputed existence of tissue-specific stem cells for some adult organs, such as the pancreas (see O.D. Madsen). These problems should however be possible to circumvent by the use of hESC. Developing strategies for coaxing hESC into different tissue types should theoretically create a source of cells that could be manipulated via cell and gene

therapy, to correct for particular defects in diseases, such as Type I diabetes, Parkinson's disease, and cardiovascular diseases, and injuries. Transplantation of ES cell-derived cells into pre-clinical models of human disease is already underway. For example, mouse ES cells were induced to differentiate into midbrain cells, including dopaminergic neurons, by exposing them to the signaling factors sonic hedgehog and FGF8. Importantly, these cells were functional when transplanted into a model of Parkinson's disease (64). Nevertheless, several outstanding issues need to be resolved before delivery of functional cell populations in vivo can be accomplished, including the type and number of cells to be delivered, site of engraftment, prevention of teratoma formation due to contamination of the graft with remaining undifferentiated ES cells and donor/recipient compatibility and graft rejection. To avoid the risk of teratoma formation the number of undifferentiated ES cells within the graft could be reduced by various methods to select for the lineage to be transplanted. Allogenic transplantation of ES cell-derived cells will require lifelong immunosuppression in order to avoid graft-versus-host disease (GVHD). However, different strategies may be used to circumvent this problem. For example, by cotransplanting hematopoietic stem cells with the tissue of choice can induce lifelong tolerance to the graft. This was shown to work in the NOD mouse model (65). Another way of avoiding immune rejection is by performing SCNT, because it will generate isogenic or "tailor-made" ES cells. Notably, Hwang et al recently reported the development of more efficient methods for human SCNT that were used to derive several hESC lines (6), suggesting that this alternative may be used in the near future.

FUTURE DIRECTIONS

Based on the stem cell phenotype and cell differentiation capacity of hESC, the potential applications of hESC in human basic developmental biology and regenerative medicine are apparent. However, to make progress towards the realization of the full potential of hESC in these research areas the solution to several methodological problems needs to be addressed. For example, it will be important to make further progress in the ongoing efforts to develop a culturing system where the cells ultimately can be grown without feeders in a chemically defined medium. In conjunction with these efforts, developing a stable enzymatic passaging technique for hESC that will enable up-scaling and efficient growth at clonal density seem urgent. Moreover, improved techniques for genetic manipulation of hESC will be important for most applications of hESC. Even though hESC can be genetically modified by transgenesis, including the use of conventional DNA delivery systems (66), lentiviral and adenoviral vectors (67, 68) and homologous recombination (61, 62) these techniques appears not to be as efficient as in mouse ES cells. Finally, based on the fact that little systematic comparison of cell lines derived in different laboratories has been carried out, it is presently unclear whether significant variation in the growth and differentiation properties of different cell lines exists. If hESC lines actually differ in these properties, some cell lines may prove more suitable for certain applications in basic research and therapy. The International Stem Cell Forum (<http://www.stemcellforum.org.uk>), is an international consortium, which is currently comparing the properties of 75 hESC lines (69). This initiative represents an initial important step towards integrating the advancement of hESC technology for research and regenerative medicine at an international level.

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FIGURE LEGENDS

Fig. 1. Human ES cells originate from the inner cell mass (ICM) of pre-implantation embryos. (A) A 6-day-old human blastocyst (courtesy K. Frej). TS indicates trophoctoderm. (B) Undifferentiated hESC colony grown on human foreskin fibroblasts (courtesy C. Ellerström). (C) Immunofluorescence staining of an undifferentiated hESC colony with anti-Oct4 antibodies (courtesy N. Heins).

Fig. 2. Image of a histological section of a teratoma derived from a pluripotent hESC line. Tissue-derivatives of ectoderm (neuroepithelium; NE), endoderm (gut-like epithelium; GE) and mesoderm (cartilage; C). Courtesy K. Emanuelsson.

FIGURES

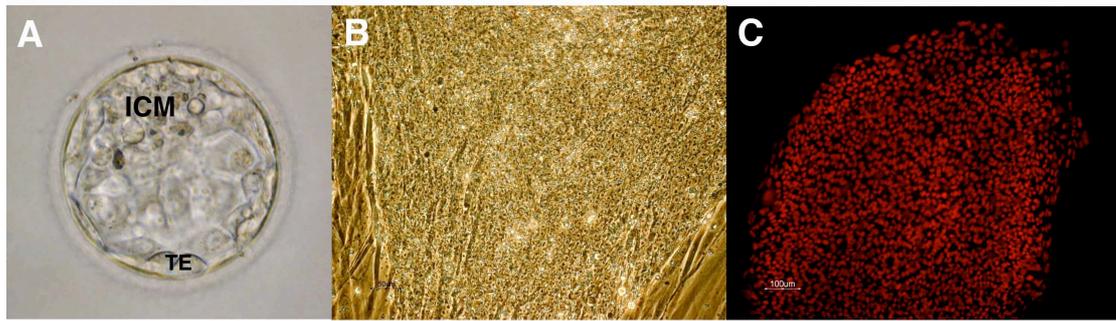


Fig. 1.

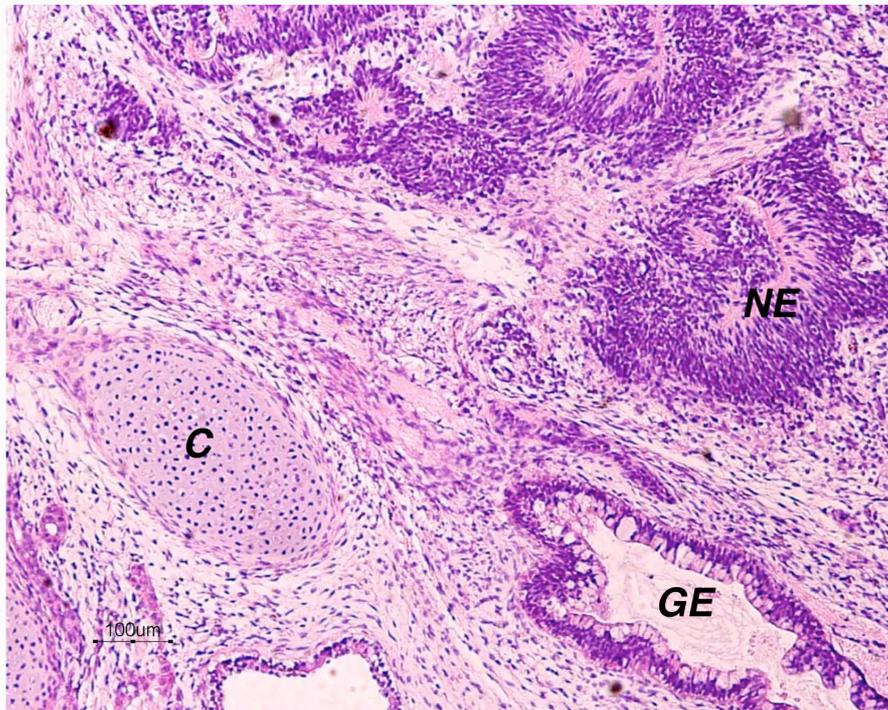


Fig. 2.