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เซลล์ต้นกำเนิดจากตัวอ่อนมนุษย์และแนวทางการใช้ประโยชน์

สุพัชญ์ สีนะวัฒน์
หน่วยชีววิทยาการเจริญพันธุ์ ภาควิชาสูติศาสตร์และนรีเวชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

Human Embryonic Stem Cells and Their Potential Applications

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ความก้าวหน้าทางเทคโนโลยีการเจริญพันธุ์ทำให้นักวิทยาศาสตร์สามารถเพาะเลี้ยงตัวอ่อนมนุษย์ในห้องปฏิบัติการได้เป็นผลสำเร็จกว่าสองทศวรรษมาแล้ว ความรู้และประสบการณ์ในการเพาะเลี้ยงตัวอ่อนของสัตว์เลี้ยงลูกด้วยนมทำให้นักวิทยาศาสตร์สามารถสร้างเซลล์ต้นกำเนิดจากตัวอ่อนของสิ่งมีชีวิตหลายชนิดรวมทั้งของมนุษย์ พัฒนาการในการสร้างเซลล์นำลู่ที่พัฒนามาจากเซลล์ต้นกำเนิดของตัวอ่อนมนุษย์ (embryonic stem cell lines) ก่อให้เกิดก้าวกระโดดในการสะสมองค์ความรู้ด้านเทคโนโลยีชีวภาพ เภสัชวิทยา วิทยาศาสตร์พื้นฐาน ตลอดจนการรักษาด้วยเซลล์บำบัด (cell-based therapy) ในปัจจุบันนี้มีความก้าวหน้าในการสังเคราะห์ ES cell lines ขึ้นมากแต่ยังขาดความรู้พื้นฐานในการพัฒนาให้เซลล์เหล่านี้เปลี่ยนแปลง (differentiate) ไปเป็นเซลล์ที่ต้องการเพื่อใช้ประโยชน์ทางคลินิก ความร่วมมือของแพทย์และนักวิทยาศาสตร์ตลอดจนความตระหนักในประโยชน์อันเนื่องจากวิทยาการแขนงนี้จากสังคมจะช่วยให้เกิดความต่อเนื่องในการพัฒนาองค์ความรู้ด้านเซลล์ต้นกำเนิดจากตัวอ่อนมนุษย์ซึ่งจะยังผลให้เกิดความก้าวหน้าในการดูแลรักษาด้วยเซลล์บำบัดต่อไป

Progress in reproductive technologies provided opportunity for scientists to be able to grow human embryos *in vitro* for more than two decades. Skills and knowledge derived from *in vitro* fertilization and *in vitro* culture of mammalian embryos opened the chance for scientists to develop the strategies to derive embryonic stem cell lines from mammalian and human embryos. This achievement has initiated a new era in the fields of biotechnology, pharmacology, basic scientific research and cell-based medicine. To date, scientists have made some progress in optimizing regimens in deriving ES cell lines from human embryos but much more research and development are still required especially in the aspect of directing stem cells into the specific cells of potential clinical use. Collaboration among clinicians and scientists from diverse fields, together with the public awareness of how useful this technology could offer to modern medicine, will result in the accumulation of knowledge in this field and ultimately a progress in cell-based therapy in the future.

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Introduction

Stem cell is a cell that has ability to divide (self-replicate) for indefinite periods. Under the right conditions, or given the right signals, stem cell can give rise (differentiate) to many different cell types that make up the organism. Stem cells, therefore, have the potential to develop into mature cells that have characteristics, shapes and specialized functions, such as muscle cells, neurons or skin cells.

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A pluripotent stem cell is an undifferentiated cell that has the potential to develop into virtually any cell types in the body. Pluripotent stem cells are transiently present during embryogenesis, in preimplantation embryos and fetal gonads. They can also be maintained as established cell lines, derived either from preimplantation embryos, primordial germ cell or germ cell tumors.

Embryonic stem cells

Embryonic stem (ES) cell lines are certain types of pluripotent stem cell lines which have been derived by the isolation and propagation of inner cell mass (ICM) cells of blastocyst stage embryos. These unique cell lines can develop into a wide range of cell types *in vitro* and *in vivo.* In addition, they are immortal. They can be grown continuously in culture without losing their properties or their wide development potential. These two features, pluripotency and unlimited self-renewal, have made ES cells extremely interesting and important to basic and applied research, especially to cell-based therapy and the study of early embryonic development.

The derivation of ES cell lines in mammals was first demonstrated in mice[1, 2] in which basic methods for their isolation, propagation and genetic manipulation were established. The accumulated experience in the mouse has allowed scientists to better define the properties of ES cells, that:

- derive from ICM/epiblast of blastocysts,
- are capable of undergoing unlimited number of symmetrical cell divisions without differentiating
- maintain a normal karyotype,
- can give rise to differentiated cells of ectoderm, mesoderm and endoderm origin *in vitro* and *in vivo* within teratoma/teratocarcinoma tumors following engraftment into immunodeficient mice,
- can colonize all fetal tissues, including the germ line, during embryonic development following their injection into host blastocysts,
- are clonogenic; each single cell can give rise to many other genetically identical cells that share the same properties and potentials as the original,
- specifically express the transcription factor Oct4, a regulatory molecule characteristic of pluripotential cells at different developmental stages.

Based on the accumulated experience both with mouse ES cells and with human embryonal carcinoma (EC) cells[3, 4], which are pluripotent and resemble ES cells in many apects, ES cell lines were successfully derived from nonhuman primates (common marmoset and rhesus monkeys).[5, 6] These studies have set the stage for the derivation of human EScells in human, first by Thomson et al. [7], Reubinoff et al[8]. and later by other groups. The described cell lines were derived from ICM cells of normal surplus blastocysts donated by couples undergoing in vitro fertilization (IVF). The human ES cells proliferate for extended periods *in vitro,* maintain a normal karyotype, differentiate spontaneously into somatic cell lineages of all three primary germ layers and form teratomas when injected into immunodeficient mice. Moreover, they express a panel of markers which are typical to nonhuman primate ES cells as well as to other types of human pluripotent stem cell lines (embryonic carcinoma (EC) cells and embryonic germ (EG) cells)[9]. As human ES cell research advances, scientists and clinicians now better appreciate the far-reaching potential of these cells. It is, thus, not surprising that many of the IVF clinics worldwide are now aiming to set the required system and skills for the establishment of new ES cell lines from human embryos.

Derivation of human ES cells

The same principles that were developed for the derivation of mouse ES cell lines are used for the establishment of human lines, with some modifications. Human embryos are cultured to the expanded blastocyst stage by using the standard commercially available sequential media. Based on data from the first groups that derived fully characterized human ES cell lines, a success rate of 34% was documented (10 lines from 23 embryos)[7-9].

The zona pellucida of the blastocysts is first removed by either enzymatic8 or chemical digestion[9]. To isolate ICM, the outer trophectoderm layer is removed, most commonly by immunosurgery[7, 8], although gentle mechanical removal (using 27G needles) is also possible[9]. The ICM is then plated on mitotically-inactivated feeders that support the proliferation and prevent the differentiation of the stem cells.

So far, mouse embryonic fibroblasts (MEFs) were most commonly used in the derivation of human ES cells, though human fetal muscle fibroblasts10 and mouse embryonic fibroblast cell line (STO cells) were also utilized[11]. Similar to the mouse ES cell system, in order to maintain the potential of the fibroblasts to support

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undifferentiated proliferation of human ES cell, it is important to avoid overcrowded cultures[3]. Only low passage cells (up to passage 5) are used to prepare feeder layers within gelatin treated tissue culture dishes[3]. Mitotic inactivation of feeders may be accomplished either by irradiation or by treatment with mitomycin C[8].

Within several days following plating of ICMs on feeders, groups of small, tightly packed cells may be identified proliferating from the ICMs. Seven to eight days after plating, clumps of these small cells may be mechanically isolated from outgrowths of differentiated cells by using the sharp edge of a glass micropipette. Following replating on fresh feeders, they give rise to round flat colonies of cells with well-defined borders. The cells within the colonies have distinct borders, a large nucleus, a high nuclear cytoplasmatic ratio and prominent nucleoli. The colonies are further propagated about every 7 days.

Maintenance of human ES cells in culture

Human ES cell cultures usually include a variable level of background spontaneous differentiation. To minimize this process, selective propagation of predominantly undifferentiated colonies or of undifferentiated areas (usually in the periphery of the colonies) may be required to maintain the culture at an undifferentiated state. Human ES cells are highly sociable cells and the survival of single cells is low, therefore, propagation of clumps of 50 cells is most commonly used.

In addition to MEFs, as mentioned above, STO cells[11] and feeders from various human adult and fetal tissues including fetal muscle[10], foreskin[12, 13] and marrow cells[14] can also support the derivation and/or propagation of human ES cells. Human serum rather than of bovine origin, combined with human feeders, may be used to develop human ES cells in an animal-free culture system[10].

Characterization of human ES cells

An international scientific consensus regarding the exact uniform criteria and standards that should be used to characterize and define human ES cells has not been established[15]. So far, the human ES cell lines that were derived by a number of groups were characterized by demonstrating the key properties of ES cells that were applicable to the human system. Given the potential unlimited self-renewal capability of human ES cells, an important part of the characterization process is to repeatedly demonstrate the key properties during, prolonged propagation of the cells in culture. Unfortunately, the majority of cell lines that were reported to date have not been available for sufficient time and have not been fully characterized. This article will summarize the properties that were most commonly used to define the reported human ES cell lines in the literature.

Colonies of human ES cells are flat with well-defined borders distinct from the surrounding fibroblasts. In the presence of serum, undifferentiated cells have distinct borders, a large nucleus with prominent nucleoli and a high nuclear cytoplasmatic ratio[7, 8]. In serum-free culture conditions, the colonies tend to become more tight packed, with less distinct borders between the cells[16].

Similar to mouse pluripotent cells, human ES cells express alkaline phosphatase activity[7-17]. A panel of surface markers that was mainly developed for the characterization of mouse ES cells and human embryonal carcinoma (EC) cells is used to characterize the immunophenotype of undifferentiated human ES cells. Human ES cells express specific globoseries glycolipids, which carry stage-specific embryonic antigen 3 and 4 (SSEA-3 and SSEA-4)[7-18]. In contrast to mouse ES cells and similar to human EC cells, undifferentiated human ES cells do not express SSEA-1[7-9] although its expression is upregulated following differentiation.[7] Similar to human EC cells, undifferentiated human ES cells also express high molecular weight keratin sulphate/chondroitin sulphate pericellular matrix proteoglycans,[7-17] that can be identified by antibodies against the core protein (GCTM-2) 19 or carbohydrate epitopes (TRA-1-60, TRA-1-81)[20].

A key marker of pluripotent cells, whose expression was demonstrated in undifferentiated human ES cells, is the transcriptional factor Oct4[8]. In the mouse. Oct4 is expressed in various pluripotential cell populations including blastomeres and germ cells in vivo and ES and embryonic germ cells in vitro. Oct4 expression is essential for the establishment of pluripotential cell lineages during mouse embryonic development[21]. The precise level of Oct4 governs the fates of mouse ES cells, and a critical level of Oct4 is required to sustain stem cell self-renewal[22].

As the knowledge of human ES cell biology expands, additional markers which are expressed consistently in undifferentiated cells and are down regulated upon differentiation are uncovered[18-20]. It is anticipated that with time the repertoire of markers that

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will be used to characterize undifferentiated human ES cells will be refined and standardized.

The characterization of human ES cells further includes the demonstration of key properties of ES cells. Standard cytogenetic analysis methods are used to show that the stem cells retain a normal karyotype along propagation in culture[7-23]. Pluripotency is demonstrated by showing the potential of the cells to differentiate into progeny representing the three germ layers both in vitro and in vivo within teratoma tumors.

Clonal expansion of a pluripotent cell population from a single cell is required to verify that the cultures are not mixtures of early progenitors of multiple lineages but truly include pluripotent cells.

In vitro differentiation

I. Spontaneous differentiation

It is possible to trigger the differentiation of human ES cells in vitro by growing them in suspension culture. In suspension, the cells tend to aggregate, forming multicellular structures termed embryoid bodies (EBs)[24]. As these cell structures form, they undergo spontaneous differentiation to produce terminally differentiated cells of mesoderm, ectoderm and endoderm origin. The formation of EBs is a gradual process and is accompanied by morphological changes. It begins with the formation of small bodied of densely packed cells (simple EBs), which by day 7 begin to cavitate (cavitated EBs) and eventually accumulate fluid within cysts. By day 20, the cystic EBs, which are a product of spontaneous and disorganized differentiation, are considered to be mature. They are composed of various terminally differentiated cell types, including nerve[25], blood[26], endothelial[27], heart [28] and pancreatic[29] cells. Some have even been shown to be functional as in the case of cardiomyocytes 28 and nerve cells[25].

II. Induced differentiation

Spontaneous differentiation of ES cells in vitro is a stochastic process, which results in the production of heterogeneous cell populations. However, the development of a highly purified population of a specific cell type is required for most of the scientific and therapeutic applications of human ES cells. Thus, it is necessary to direct the differentiation of the cells in vitro and/or to combine it with a lineage-based selection approach. There are several strategies that can be utilized for this purpose:

Growth factors

Exogenic factors can augment the process of differentiation towards a specific cell fate[30]. For example, it has been well established that the addition of retinoic acid (RA) induces the differentiation of ES cells into neurons[30] and that bone morphogenetic protein 4 (BMP4) can direct their differentiation into trophoblast cells[31].

The growth or differentiation inducing factors can be supplemented continuously or sequentially to the media, according to the requested, cell type and protocol. Since the cultures that are obtained following treatments with differentiation-inducing factors are still relatively heterogeneous, at present, this approach should be combine with additional strategies such as lineage selection, manipulation of the culture conditions and over expression of key transcription factors.

Lineage selection

The lineage selection approach allows obtaining a highly purified population of cells by performing selection for or against a specific cell type. Cells of a specific type may be sorted form heterogeneous populations of differentiated cells based on the expression of lineage-specific cell surface markers[25-27], or by genetic selection. The latter approach is based on the genetic introduction of a selectable marker gene under the regulation of a tissue specific promoter. The marker gene may either be a selectable reporter, such as green fluorescent protein (GFP), which can be selected for by fluorescence activated cell sorter (FACS)[32, 33], or the insertion of a drug resistance gene such as the neomycin resistance gene, which allows the direct isolation of the desired cells by the presence of G418 in the media[34].

Overexpression of key regulator genes

It is possible to force the differentiation of ES cells into specific lineages by overexpressing transcription factors which play major roles in early commitment of cells into specific lineages. This has been previously demonstrated to be feasible in the mouse ES cell system, where overexpression of Myod resulted in the induction of skeletal myocytes, which fused to create multinuclear contractile myotubes[35]. Similar experiments demonstrated the effect of hepatocyte nuclear factor (HNF) on the generation of hepatocytes[36], and of Nurr1 in the production of dopaminergic neurons[37].

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Potential applications of embryonic stem cells

1. Cell source for transplantation

Since human ES cells can be grown indefinitely in culture without losing their basic characteristics, and have the potential to develop into practically any cell types *in vitro,* they may be used as an unlimited cell source for cell transplantation. Once efficient protocols for induced differentiation will be established, it will be possible to generate specific cell types in large numbers for the repair of degenerating or damaged tissues in humans. This will reduce the current supply problems of tissues available for transplantation. Indeed, it has been demonstrated in mice, and to certain extent in humans, that ES cell-derived progeny can proliferate and integrate, following their transplantation into adult animals[25-30]. Moreover, in the mouse ES cell system, transplanted progeny were shown to be functional and could improve behavioral deficits in animal models of diseases. Mouse ES cell derived cardiomyocytes were able to form stable functioning intra-cardiac grafts[34], and glial precursor derivatives formed myelinating transplants in the brain and spinal cords of myelin deficient rats[38]. Also insulin secreting cells derived from ES cells normalized glycemia in strepto-zotocin-induced diabetic mice[39] and, in addition, transplanted functional dopaminergic neurons corrected motor asymmetry following transplantation into the animal model of Parkinsonís disease[37].

While these results are promising, many more experiments are required to test the functionality and safety of human ES cell differentiated derivatives in animal models before they can be considered appropriate for clinical use. In addition, there will be a need to overcome the difficulty of graft rejection as a result of the immune response[40]. There are several possibilities that can be applied for minimizing graft rejection of ES cell derivatives. One possibility is to establish a bank that will include a large number of ES cell lines that differ in their major histocompatibility complex (MHC) expressed molecules, thus allowing major histocompatibility complex matching between the donor cell line and the recipient. Alternatively, it may be possible to generate a "universal" donor cell line by "knocking out" the genes that are responsible for graft rejection. Finally, it might be feasible in the future to generate genetically identical nuclear transfer-derived ES cell lines, to provide the patients with autologous grafts.

2. Cell-based delivery system

ES cell-derived progenitor cells may be used as delivery vehicles for the regulated release of drugs or therapeutic proteins, by introducing genetically modified cells which express the therapeutic gene or protein at the site to the damaged tissue. Such a cell-based delivery system will permit the production of a therapeutic agent at a steady state level and in consistent physiological concentrations, thus overcoming current limitations caused by incomplete drug accessibility. The use of genetically manipulated stem cells as therapeutic vectors has previously been shown to be feasible in mouse models of genetic disorders[41].

3. Drug screening and toxicology

Human ES cells may have great value in the discovery and the development of pharmaceutical compounds. As these cells can form distinct populations of terminally differentiated cells in vitro, they may be used in the discovery of new compounds as well as for the optimization of currently available drugs by carrying out improved screens that are disease oriented. Furthermore, they may be used as cellular assays in the study of drug toxicity and teratogenicity.

4. Model developmental processes

The study of early human development is restricted by ethical constrains on research of human embryos. Human ES cells allow access to study the events occurring during early human development. It has been proposed that expanding EBs mimic, to some extent, early embryonic development, thereby allowing the investigation of processes as complicated and diverse as morphogenesis, differentiation and apoptosis. It has been demonstrated in the mouse that some temporal and spatial relationships between developmentally regulated genes that exist in the embryo are maintained *in vitro*[42]. Moreover, it should simplify the study of complex processes that occur during early embryonic development by isolating single events such as pre-amniotic cavitation and cell lineage selection.

5. Tool for gene manipulation

One of the great advantages of ES cells over other cell types is their accessibility for genetic manipulation. They can easily undergo genetic modifications while remaining pluripotent, and they can be selectively propagated,

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allowing the clonal expansion of genetically altered cells. Since the first isolation of ES cells in mice, many effective techniques have been developed for gene delivery and manipulation. These techniques both include transfection and infection protocols, as well as various approaches for inserting, deleting, or changing the expression of genes in the genome. These methods have been extremely useful for monitoring and directing differentiation, discovering unknown genes and studying their function. Similar approaches were recently successfully applied to the genetic modification of human ES cells[43, 44].

Genetic manipulation of human ES cells can be applied to the expression of either foreign or cellular genes, allowing the study of gene function as well as the isolation or elimination of specific cell types in culture[32-34]. It may also be used to direct the cell fate, as described earlier in this article[45]. Obviously, the ability of directing in vitro differentiation, isolating pure populations of specific cell types and eliminating undifferentiated cells prior to transplantation, may have great importance in cell-based therapy.

Apart from tagging, selecting and directing the differentiation of specific cell types, it is possible to inactivate endogenous genes and study their function. This can be achieved by several methods. The most widely used technique for this purpose has been site-directed mutagenesis. This procedure involves the replacement of a specific sequence in the genome of the cell with a mutated copy, through homologous recombination. By targeting both alleles, it is possible to create "loss of function" or so-called "knock out" phenotypes in ES cells that can be used for functional studies of specific genes. This technology has been well practiced in mice, to generate animals that are homozygous for the desired mutation. The creation of human ES cells with a null genotype for specific genes may have great importance in the modeling of human diseases, as recently demonstrated in Lesch-Nyhan syndrome. These in vitro models should be most valuable to basic research, but more importantly to the exploration of new therapeutic protocols, specifically to the development of gene therapy-based treatments and to drug discovery.

Conclusions

It has been generally accepted that the derivation of ES cell lines from human embryos has initiated a new era in the fields of reproductive biology, biotechnology, pharmacology, basic scientific research and regenerative medicine. It is well established that human ES cell lines can be readily derived in a reproducible manner. However, there still exists a need to increase the number of cell lines that are available to the research community and to generate more lines with a broader genetic and ethnic background. New lines from genetically abnormal embryos are also required, as well as lines suitable for clinical purposes. Much more research and development is required to exploit the remarkable potential of human ES cells. Appropriate public support and adequate legislation are crucial for the realization of the far-reaching applications of human ES cells. Collaboration among clinicians and scientists from diverse fields are also necessary for the development of cell-based therapy and reparative medicine using cells derived form human ES cells.

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