Vol. VIII • Número 3 • Diciembre 2006

Artículo de revisión

Stem Cells a Hope for Cell Therapy and Regenerative Medicine

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Historical Overview

In the 1960´s when the idea of using isolated cells to treat sickness(1) and the observation of the existence of multi-potentiality in embryonal carcinomas(2), a new era in the history of medicine and cell biology began. The shortage of donors and the need to assist all patients that are organ transplantation waiting lists have inspired researchers and clinicians to look for the perfect and universal cell source for cell therapy(3). Cell therapy and regenerative medicine have adopted the concept of stem cells for over 100 years with the identification of tissue with the capacity of self-renewal postulated by Regaud et al. based on his studies of sperma-togenesis(4). At the same time, some hematologist suggested that all blood cells derive from common stem cells or ancestral cells(5). Thus, historically the concept of self-renewal, differentiation and stem cells became closely linked. Stem cells are defined as cells that have clonogenic and self-renewal capabilities and that differentiate into multiple cell lineages and have been classified into categories according to their developmental status and their

Abstract

Patients suffering from diseased and injured organs may be treated with transplanted organs. However, given the aging population, there is a severe shortage of donor organs which is worsening yearly. Scientists in the field of regenerative medicine and tissue engineering apply the principles of cell transplantation, material science, and bioengineering to construct biological substitutes that will restore and maintain normal functioning in diseased and injured tissues. Therapeutic cloning, where the nucleus from a donor cell is transferred into an enucleated oocyte in order to extract pluripotent embryonic stem cells, offers a potentially limitless source of cells for tissue engineering applications without immunological rejection. Also, the stem cell field is advancing rapidly, opening new options for therapy. This paper reviews recent advances in regenerative medicine and describes applications of these new technologies which may offer novel therapies for patients with end-stage organ failure.

Key Words; Stem Cells, Differentiation, Cell Therapy, Regenerative Medicine, Tissue Engineering.

Resumen

Pacientes que padecen de órganos dañados y enfermos podrían ser tratados mediante el transplante de órganos. Pero debido a la creciente población añosa existe una severa falta de donadores de órganos que empeora año tras año. Científicos y médicos en el área de la medicina regenerativa y la ingeniería tisular han aplicado los principios del transplante celular, ciencia material y bio-ingeniería para construir substitutos biológicos que restauren y mantengan la funcionalidad normal de tejidos dañados y enfermos. La clonación terapéutica, donde el núcleo de una célula donadora es transferida a un óvulo anucleado para extraer células madre embrionarias pluripotentes, ofrece un recurso potencialmente sin límite para la aplicación en la ingeniería tisular sin reacción inmunológica. También el campo de las células madre está avanzando rápidamente, abriendo nuevas opciones de tratamiento. Este articulo revisa los más recientes avances en la medicina regenerativa y describe las aplicaciones de esta nueva tecnología que ofrece nuevas terapias para pacientes con falla orgánica terminal.

Palabras clave; Células madre, Diferenciación, Terapia celular, Medicina regenerativa, Ingeniería tisular.

Artículo Recibido 9-05-06 • Corregido 16-10-06 • Aceptado 19-10-06

capacity to differentiated in different type of cells, by their degree of plasticity(6)(See table 1). Whereas, adult stem cells have been postulated to be part of tissue-specific cells of the post-natal organism into which they are committed to differentiate(6), embryonic stem cells are derived from mammalian embryos in the blastocyst stage and have the ability to generate any terminally differentiated cell of the body. With the work of Evans and Kaufman(7) over 20 years ago developing embryonic stem cells (ES cells), these cells have become a very important source of investigation. They established the pluripotent derive cells from the inner cell mass of blastocyst and the culture conditions for growing the mouse ES cells in vitro.

In 1998, human ES cell lines were derived from preimplanted embryos by Thomson et al.(8) frozen early stage human embryos, produced by in vitro fertilization for clinical purposes, were thawed and cultured to the blastocyst stage. Fourteen inner cell masses were isolated, and five ES cell lines were derived. During the first eight months of culture, no period of replicative crisis was observed in any of the cell lines. The principle characteristics were that the cells expressed high levels of telomerase activity, suggesting that their life span exceeds that of somatic cells. These cells also expressed surface markers typical of human embryonic carcinoma cells. ES cells could be used for a number of purposes such as early developmental research(9), toxicology and drug screening(10), and gene and protein screening. The most important use is in regenerative medicine.

Cell therapy refers to the transplantation of healthy, functional and propagating cells to restore the viability or function of deficient tissue(11). Although there are many kinds of cells that can be used for tissue repair and regeneration, the pluripotency and proliferative capacity of the ES cells make them one of the best hopes for the future treatment of human diseases. In this article, we will review the current status of ES cells research by characteristics and differentiation, with particular emphasis on the future possibilities and the progress towards the development of clinical application.

Somatic Stem Cells vs Embrionic Stem Cells

Understanding that the regenerative process is involved in many organs has opened millions of options and cell sources such as primary cells, adult stem cells, embryonic stem cells, and even the possibility of using cloning cells through nuclear transfer(12) or autologous transplantation. Each has its advantages and disadvantages such as insufficient donor organs in the case of primary cells. This sourcing problem could be resolved by in vitro generation of tissue-specific cells from stem cells. The main goal in stem cell therapy is mass propagation of the cells and then coaxed to differentiate into the desire lineage for transplantation. Theoretically, these cell grafts can be obtained from different types of stem cells. Ones derived from somatic cells or embryonic origin stem cells have particularly great interest. However, the study of stem cells has frequently given rise to many questions, such as which type of stem cells should be used? What quantity of cells should be used? What kind of mechanism do the stem cells use to engraft, grow and differentiate? What is the lifespan of the stem cells once transplanted in vivo? How safe is the therapy? What are the limits of the carcinogenetic potential of the stem cells? There are some challenges in the application of adult stem cells or embryonic stem cells. In order to use stem cell-replacement therapy, we need to meticulously select the best candidate in function of facilities to propagate, manipulate, and select the purest population of the desired cell type. We also need to be able to produce immune tolerance if allogenic cells are used for stem cell-derived replacement cell therapy.

Adult stem cells are a great potential source of autologous cells for transplantation therapy. They have been isolated from adult tissues such as brain, bone marrow, skin, liver and muscle, and they might have a broader developmental potential. However, it remains unclear whether the observed plasticity or transdifferentiation potential of the adult stem cells is inherent to the cells or the consequence of culture conditions, contamination or cell fusion(13).

Before determining the origins of stem cells, we have to analyze some characteristics that will allow us to understand why the development of embryonic stem cells offers greater advantages in comparison to somatic stem cells (See table 2).

An exceptional case is the isolation of pluripotent adult progenitor cells. These cells were isolated from an adult mouse, rat and human after a three-month protocol culture. These cells have the capacity to become any kind of cell of the three germ layer, both in vitro and in vivo when they were injected into blastocyst. But these cells have to show correction of a disease phenotype in animal models or humans(13).

Properties of Embryonic Stem Cells

Donated embryos have been used for research after being targeted for discarding. Thomson et al. performed initial derivations of hES cell lines at the University of Wisconsin (Madison, WI, USA) by isolating from the ICM of human blastocysts and placing them on inactivated murine feeder cells(8). Since that time, many laboratories have applied the same technique to derive hES cell lines. hES cells are currently evaluated by a set of markers and their capacity to differentiate. These criteria include the expression of several surface markers and transcription factors associated with an undifferentiated state. In addition, extended proliferative capacity, pluripotency and no less important, the euploid karyotype and the epigenetic status (See figure 1). To demonstrate that hES cells characteristics do not change over the time is crucial for future use in clinical trials.

A number of surface markers are currently used to characterize hES cells. Glycolipids and glycoproteins that were identified in human embryocarcinoma cells are present in hES cells such as SSEA-4, TRA-1-60 and TRA-1-81. Also, some surface antigens initially described in other stem cells populations such as AC133, CD-9, CD-117, CD-135 are used(14). Some studies have the stability of the expression of these surface markers following prolonged periods of time.

A number of critical transcription factors that play a critical role in maintaining stem cell self-renewal have been identified and their analysis is also useful for characterization. One is Oct-3/4, previously described and several target genes

Table 1. Classification of stem cells based on their plasticity

Totipotent	Able to give rise to the all the embryonic and extra-embryonic cell types
Pluripotent	Able to give rise to all cell types of the embryo proper
Multipotent	Able to give rise to a subset of cell lineages
Oligopotent	Able to give rise to a more restricted subset of cell lineages then multipotent stem cells
Unipotent	Able to contribute only one mature cell type

Characteristics of the therapeutic potential of adult and embryonic stem cells.

Table 2. Adult and Embryonic Stem Cells Characteristics

Somatic Stem Cells	Embryonic Stem Cells
Difficult to isolate	ES cells are derived rather easily
Hard to propagate in culture	Grow Indefinitely in culture
Can be genetically manipulated only through the introduction of retroviral transgenes	Can be manipulated genetically by homologous recombination
The differentiation potential seems to be restricted	Can be coaxed into becoming any type of cell

Classification of stem cells based on their developmental potential according to Wagers and Weissman.

for Oct-3/4 have been identified in hES cells including Utf-1, Rex-1, PDGF R, Otx-2, Lefty-1 and Nanog but their specific role is still unclear, and its expression has been reported being retained for over a year in culture(15, 16). Additionally, all hES cell lines express high levels of telomerase, the enzyme that helps to maintain telomeres, which protect the end of the chromosomes. Telomerase activity and long telomeres are characteristics of proliferating cells in embryonic tissue and germ cells. As cells divide and differentiate throughout the lifespan of an organism or cell line, the telomeres become progressively shortened and lose the ability to maintain their length. The function of telomeres and telomerase appear to be important in the cell division, normal development and aging(17)(See Fig 2).

Several reports have demonstrated that the addition of exogenous Leukemia Inhibitor Factor (LIF) to hES cells do not maintain pluripotency capacity like it does in mouse ES cells(18). Although complete signaling pathways have not been yet clarified, transcriptional profiling or gene expression technology has identified several genes and transcription factors including ligand/receptor pairs and secreted inhibitors of signaling pathways.

ES cells have been reported to be maintained in several different conditions such as using growth factors of the TGF- 1/BMP superfamily, Fibroblast Growth Factor (FGF) family and Wnt family.

Previous studies have demonstrated that prolonged propagation of undifferentiated ηES cells requires growth on embryonic fibroblast feeder layers. However, bFGF has become a common thread in the culture media formulations recently developed like feeder free conditions developed by Xu et al.(19) hES cells can be maintained in serum replacement-containing medium that has been supplemented with 36-40 ng/ml bFGF. In these conditions, the cells can retain expression of surface markers, transcription factors, normal karyotype, telomerase activity and pluripotency.

The mechanism of FGF activity remains unclear, but Xu et al. demonstrated that treatment with 40-100 ng/ml bFGF inhibits BMP signaling. A further role of bFGF in hES cell to maintain pluripotenciality is the finding that bFGF activates the phosphatidylinositol 3-kinase (P13K/Akt,PKB) pathway, which subsequently enhances expression of extra-cellular matrix molecules (ECM).

Initial culture conditions of hES cell lines were exposed to xenogeneic components. hES cell culture conditions contained high quantities of fetal bovine serum (FBS) or Knockout Serum Replacement (SR). The exposition of the hES cells may carry the risk of infection by nonhuman pathogens. In addition, the presence of the nonhuman sialic acid, Neu5Gc, on hES cells grown in SR medium and on murine feeder layer was shown to evoke an immune response with Neu5Gc-specific antibodies, which are present in most human serum(20). So, the necessity to humanize the culture conditions is extremely important for future clinical application. However, some reports have shown derivation of hES cell lines in animal-free culture conditions(21).

Differentiation Potential of ES cells

As mentioned earlier, pluripotency is one of the defining features of ES cells. Embryonic body formation (EBs) in vitro and teratoma formation after injection into immunocompromised mice are currently used to validate pluripotency in hES cell lines in culture. Once differentiation has begun, cells representing primary germ layers spontaneously develop in vitro.

Initially, an outer layer of endoderm-like cells forms within the EB, followed over a period of a few days by the development of an ectodermal layer and subsequent specification of mesodermal cells.

The generation of specific functional cell types from hES cells can also be demonstrated both in vitro and in vivo. But the majority of the studies have focused on in vitro differentiation protocols, which assess the differentiated

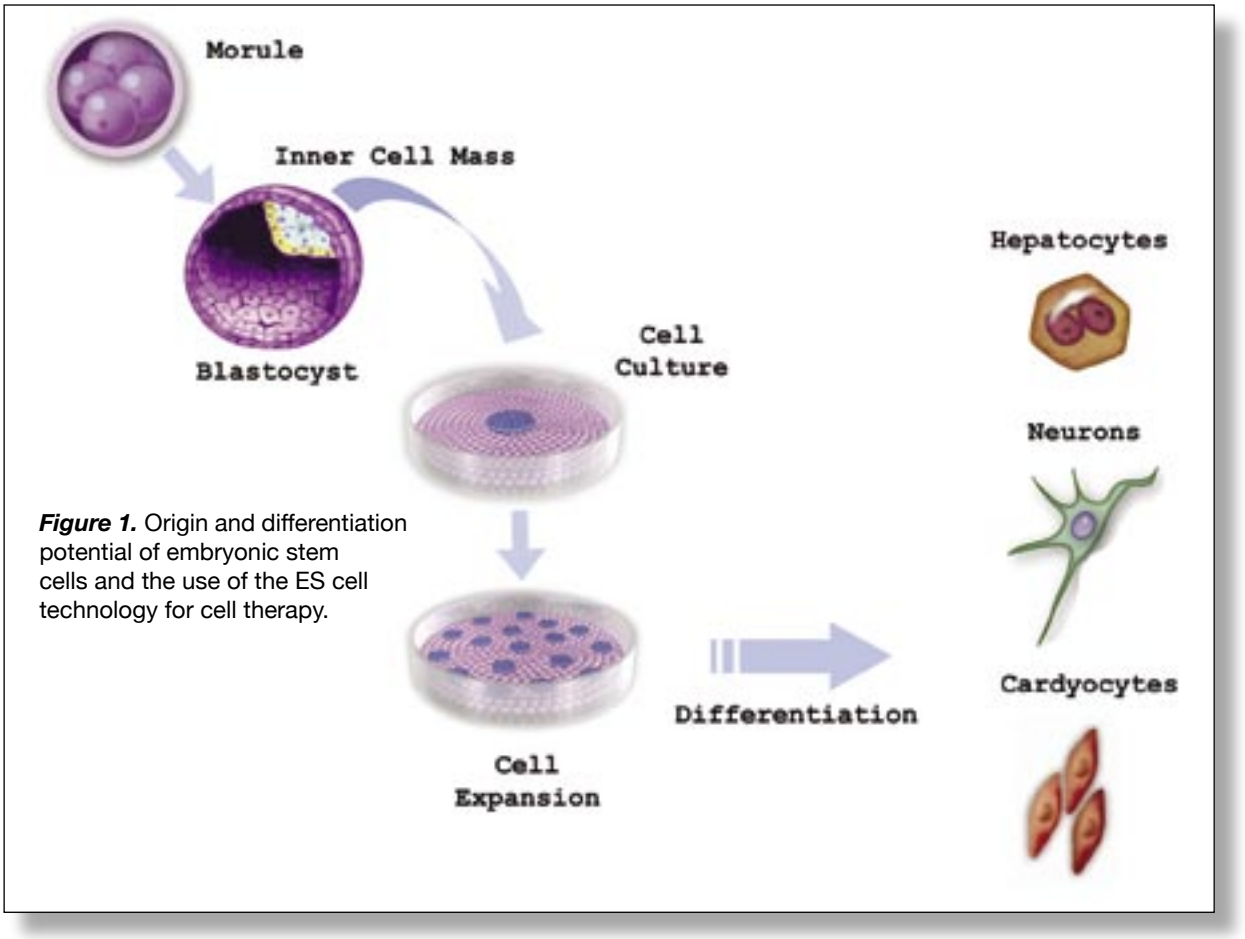

Figure 1. Origin and differentiation potential of embryonic stem cells and the use of the ES cell technology for cell therapy.

cells via expression analysis of cell-specific markers. Once the defined cells have been identified, functionality of the cells generated in vitro has also been assessed and in some cases the differentiated cells have subsequently been transplanted into animals. Although the generation of ectoderm, mesoderm and endoderm, as well as trophoblast and germ cells have demonstrated pluripotency and differentiation capacity, a full differentiative capacity of hES cells must be determined.

Endodermal Differentiation

During embryogenesis, the pancreas develops from dorsal and ventral regions of the foregut, whereas the liver originates from the foregut adjacent to the ventral pancreas compartment. The pancreas and liver are the derivatives of the definitive endoderm. Hepatic and pancreatic cells are of special therapeutic interest for the treatment of hepatic failure and diabetes mellitus and both can be developed from ES cells.

Researchers have demonstrated different strategies in vitro to differentiate mES cells into hepatocytes-like cells(22). These cells have showed specific transcription factors and proteins of hepatocytes, and have also shown the presence of the three lineages of the liver hepatocytes, bile duct epithelial and oval cells. Once transplanted, these cells can integrate and function in the host liver.

We have developed a new system of transplantation using an internal bioartificial device. By this procedure, we assure the safety of ES cell-derived hepatocytes in mice, which could represent a viable strategy for future clinical application of ES cell-based therapy while an effective system of differentiation can be developed(21).

The generation of ES-derived insulin-producing cells may stand for a critical cell source for the treatment of diabetes. In this field, ES cells hold a great hope as a source of β-cell, but might be a difficult goal and has proven to be more complicated than expected. Research to restore the β-cell deficiency of diabetes by differentiation of embryonic stem cells seems to have great promise. It is very difficult to predict which approach will cross the finish line to clinical application. Before that happens, we must focus on the development of new techniques for in vitro differentiation of a major and functional population. Further investigation is necessary for regenerating functional islet-like cells (23).

Ectodermal Differentiation

Embryonic stem cells give rise to various cell lineages but neuroectodermal development has offered promise. Also, the epithelial lineage and epidermal tissue development by ES cells have been studied. Epithelial cell differentiations from ES cells have been identified by the presence of cytokeratines and specific keratinocyte markers(24). Enrichment of kerati-

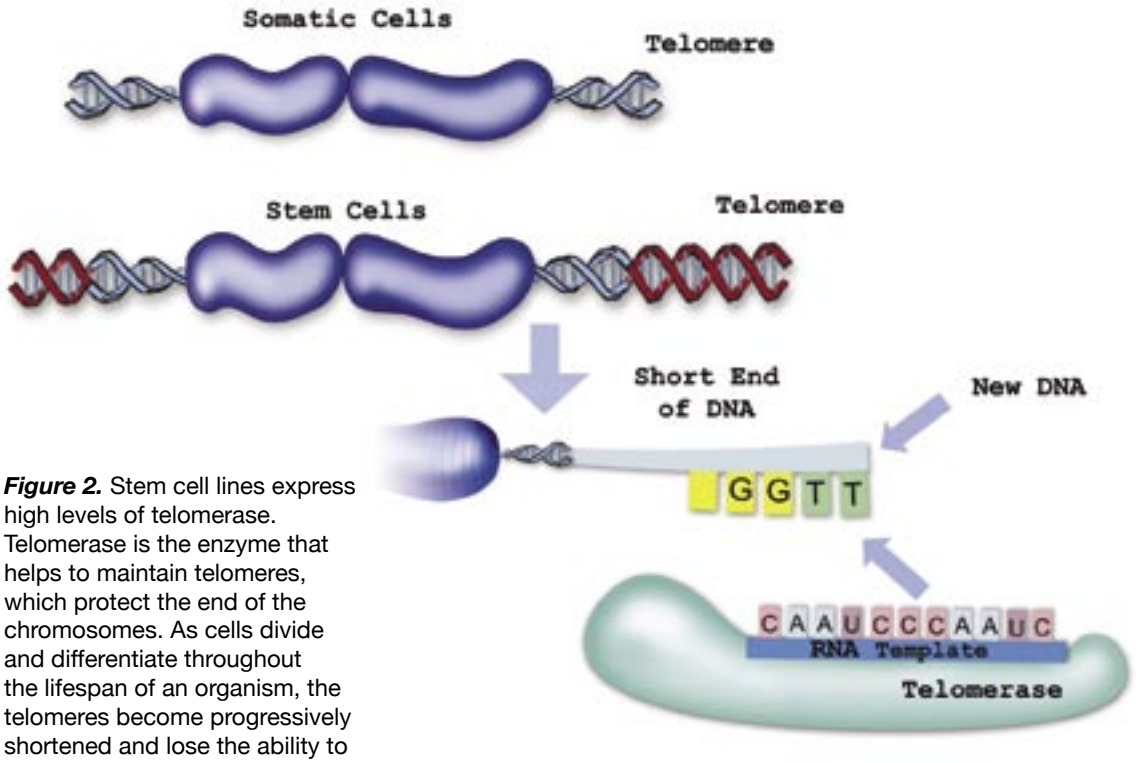

Figure 2. Stem cell lines express high levels of telomerase. Telomerase is the enzyme that helps to maintain telomeres, which protect the end of the chromosomes. As cells divide and differentiate throughout the lifespan of an organism, the telomeres become progressively shortened and lose the ability to maintain their length.

nocytes in vitro from ES cells has been achieved by seeding onto various extracellular matrix proteins in the presence of bone morpho-protein (BMP-4) and/or ascorbate. Such protocols promote formation of an epidermal equivalent. It has been reported that the resulting tissue displays patterns similar to embryonic skin. The cells express late differentiation markers of fibroblast. The data reported suggests that ES cells have the capacity to reconstitute in vitro fully differentiated skin(25).

Several groups have reported studies of the capacity of ES cells differentiation into neurons and glial cells. Since 1995, there are reports about differentiation of mES cells into neuronal cells(26). After that, there are reports that the neural rate differentiation has been improved significantly by the introduction of numerous strategies involving the use of retinoic acid, lineage selection (dopaminergic, serotonergic, -aminobutyric acid –GABA- ergic neurons, glutamatergic and cholinergic neurons(27), astrocytes and oligodendrocytes(28))and growth factors. The possibility of producing differentiation of dopaminergic neurons from ES cells was identified by Ye et al.(29), where fibroblast growth factor 8 (FGF8) and sonic hedgehog were implicated as tandem initiators of dopaminergic neurogenesis. Later, by the same method, Studer et al. generated dopaminergic neurons from ES cells, and achieved enrichment of dopaminergic neurons by mimicking the oxygen tension of the developing midbrain(30).Such cells when implanted into 6-OHDA-lesioned rats, where nigrostrial dopaminergics afferents are largely lost, restored functional normalcy to the dopamine-depleted animals(31).Similar results were observed using monkey ES cell-derived dopaminergic neurons allografted into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned adult cymolgus monkeys, in which treatment-associated behavioral improvement was noted by 10 weeks after transplantation(32). In this experiment, the result of in vitro differentiation was achieved using medium supplemented with FGF20 and conditioned by a permissive stromal cell line.

In general, neural differentiation can be supported by the addition of FGF, EGF and neural differentiation factors, like the glial cell line-derived neurotrophic factor (GDNF), neurturin (NT), TGF-β3 and IL-1 (32).

Mesodermal Differentiation

This germ layer has the capacity to differentiate into muscle, bone, cartilage, blood and connective tissue. ES cells also have been successfully used to reconstitute mesodermal developmental processes in vitro.

One of the cell lineage most studied from ES cells is the generation of cardyocytes. As with other differentiation protocols, the generation of cardiomyocytes from ES cells requires an initial aggregation step. Many factors are in-

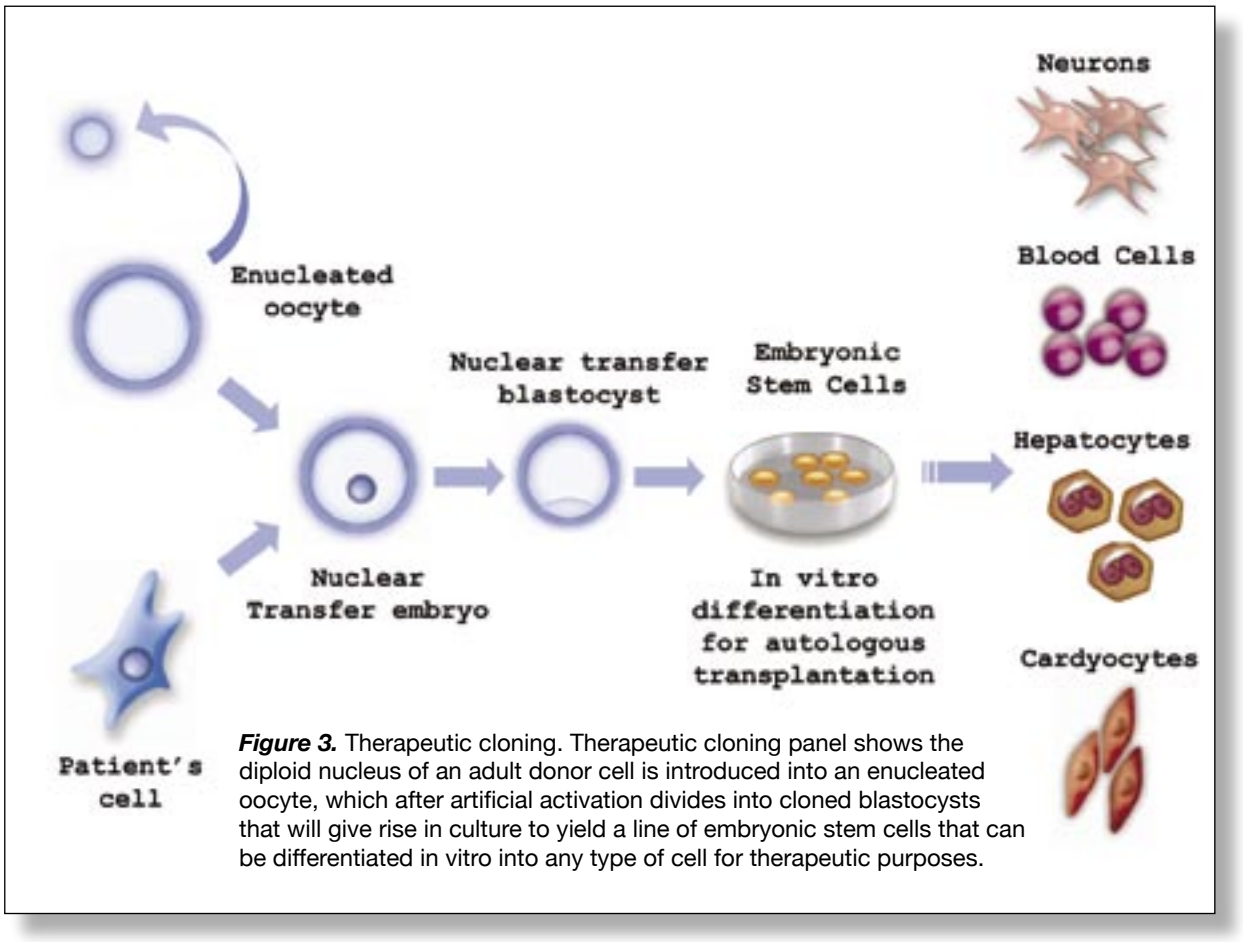

Figure 3. Therapeutic cloning. Therapeutic cloning panel shows the diploid nucleus of an adult donor cell is introduced into an enucleated oocyte, which after artificial activation divides into cloned blastocysts that will give rise in culture to yield a line of embryonic stem cells that can be differentiated in vitro into any type of cell for therapeutic purposes.

volved in influencing cardiomyocyte differentiation, such as the number of cells, special conditions of the culture medium, ES cell lines and the time of EB plating. Within the EBs, cardiomyocytes are located between an epithelial layer and a basal layer of mesenquymal cells(33). Cardiomyocytes are easy identifiable after 3-4 days of culture as they spontaneously contract. With continued differentiation, the number of spontaneously beating cells increases, and all the EBs may contain certain number of beating cells. The rate of contraction within each beating area rapidly increases with differentiation. Developmental changes of cardiomyocytes can be correlated with the length of time in culture. First, (pacemaker-like cells), and second,(atrail-, ventricular-, nodal-, His-, and purkinje-like cells).[72] Cardiomyocytes differentiation can be induced by differentiation factors such as dimethyl sulfoxide (DMSO), retinoic acid and some molecules such as Dynorphin B and cardiogenol derivatives(34, 35).

Human ES cell-derived cardiomyocytes have shown the expected molecular, structural, electrophysiologic and contractile properties of nascent embryonic myocardium. Human ES cell-derived cardiomyocytes show sustained cell cycle activity both in vitro and in vivo transplantation into the nude rat heart(35). In vivo studies with human ES cell-derived cardiomyocytes have increased recently Xue et al.(36) demonstrated engraftment and electromechanical integration with host myocardium within the uninjured hearts of immunosupressed experimental animals using mapping techniques to demonstrate that the site of human ES cell-derived cardiomyocytes implantation served as an ectopic pacemaker(36). This data, represent an exciting proof-of-concept evidence for the potential application of human ES cell-derived cardiomyocytes in the formation of biological pacemakers. But several important challenges remain such as long-term studies to follow the grafts to see if they maintain their pacemaking ability over time and development of efficient protocols for large-scale production of highly purified preparations.

ES cells in complex cystic EBs raise the generation of blood islands containing erythrocytes and macrophages, while differentiation on semi-solid medium is efficient for the formation of neutrophils, mast cells, macrophages, and erythroid lineages(37). Application of fetal calf serum (FCS), cytokines like IL-3, IL-1 or granulocyte-macrophage colony stimulating factor (GM-CSF) to ES cells generates early hematopoietic precursor cells expressing both embryonic z globin (H1) and adult major globin RNAs.

Future Perspectives in Embryonic Stem Cell–based Therapy

Considerable progress has been made toward the generation of more defined hES cell culture conditions since initial isolation and growth conditions were described. There have been many modifications of this procedure including growth of the ES cells, differentiation potential and cell lines establishment. The availability of human ES cells represents

an extraordinary opportunity for cell transplantation and regenerative medicine that may be applicable to humans. There are three points that make ES cells a great hope for cell therapy; 1) ES cells can be expand indefinitely in culture, 2) ES cells can be genetically manipulated and loss of function can be repaired by the introduction of transgenes, and 3) ES cells can be differentiated in almost any cell type.

Despite the exciting advances in the field of human ES cell research discussed in this review, many challenges have to be addressed in the near future. The culture conditions have to be improved and humanized. Cells cultured in xenogeneic conditions are likely to be considered and regulated. Infection from nonhuman pathogens is the most discussed issue for clinical application.

Furthermore, if therapeutic application is the final goal, culturing techniques need to be scaled up for mass production of clinically relevant quantities of the specified cells. Recent work from our group has tested the ability of ES cell-derived hepatocytes transplanted into mice with acute liver failure through an internal bio-artificial device that assures the isolation and functionality of the cells sufficient to improve the survival of the experimental animals(23). Such an approach may represent a potential option in using ES cell-based cell therapy while avoiding the risk of teratoma formation. Finally, immunological barriers must be overcome. Regarding this issue, some promising alternatives have recently been suggested such as nuclear transplantation with the introduction of a nucleus from an adult donor cell into an enucleated oocyte to generate a cloned embryo. This embryo has the potential to give rise to ES cells that may become any type of cell present in the adult body. Because the resulting ES cells by nuclear transfer are genetically identical to the donor and thus potentially useful for therapeutic applications, this process is called "therapeutic cloning" (See Figure 3). Therapeutic cloning might substantially improve the treatment of neurodegenerative diseases, blood disorders, diabetes, since therapy for these diseases is currently limited by the availability or immunocompatibility of tissue transplants. Experiments in animals have shown that nuclear cloning combined with gene and cell therapy represents a valid strategy for treating genetic disorders(38). Potential strategies for overcoming immune rejection of human ES cells, including traditional allogeneic transplantation with pharmacologic immunosupression, genetic manipulation of the major histocompatibility genes to produce a universal donor human ES cells line, and induction of immune tolerance through the transplantation of human ES cell-derived hematopoietic recursors or establishment of bone marrow chimerism are discussed in several reviews(39).

Conclusions

Fifteen years ago, the idea of regenerating tissues such as brain, heart, and pancreas was radical. Today, using stem cells to rebuild such tissues from its components parts is a mainstream clinical experimental concept. We are very optimistic that these approaches will eventually lead to an effective and routine clinical therapy. The cell therapy community must not follow the trajectory of clinical gene therapy, where serious clinical complications set the field back many years(40). Regenerative medicine in the meantime is bringing together basic scientists, clinicians, developmental biologists and engineers, to expand the field and give hope to improving the health of millions of people worldwide.

Reference

1. Berry MN, Friend DS. "High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study". *J Cell Biol* 1969;43:506-520.
2. Kleinsmith LJ, Pierce GB, Jr. "Multipotentiality of Single Embryonal Carcinoma Cells". *Cancer Res* 1964;24:1544-1551.
3. Kobayashi N, Okitsu T, Tanaka N. "Cell choice for bioartificial livers". *Keio J Med* 2003;52:151-157.
4. Regaud C. "The alternating rhythm of cellular mitoses and the radiosensitivity of the testis". *Int J Radiat Oncol Biol Phys* 1977;2:569-570.
5. Leblond CP. "Classification of Cell Populations on the Basis of Their Proliferative Behavior". *Natl Cancer Inst Monogr* 1964;14:119-150.
6. Weissman IL. "Stem cells: units of development, units of regeneration, and units in evolution". *Cell* 2000;100:157-168.
7. Evans MJ, Kaufman MH. "Establishment in culture of pluripotential cells from mouse embryos". *Nature* 1981;292:154-156.
8. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. "Embryonic stem cell lines derived from human blastocysts". *Science* 1998;282:1145-1147.
9. Pera MF, Trounson AO. "Human embryonic stem cells: prospects for development". *Development* 2004;131:5515-5525.
10. McNeish J. "Embryonic stem cells in drug discovery". *Nat Rev Drug Discov* 2004;3:70-80.
11. Strom TB, Field LJ, Ruediger M. "Allogeneic stem cells, clinical transplantation and the origins of regenerative medicine". *Curr Opin Immunol* 2002;14:601-605.
12. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P. "Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer". *Science* 2001;292:740-743.
13. Vassilopoulos G, Wang PR, Russell DW. "Transplanted bone marrow regenerates liver by cell fusion". *Nature* 2003;422:901-904.
14. Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, Lucero M, et al. "Properties of four human embryonic stem cell lines maintained in a feeder-free culture system". *Dev Dyn* 2004;229:243-258.
15. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, et al. "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4". *Cell* 1998;95:379-391.
16. Niwa H. "Molecular mechanism to maintain stem cell renewal of ES cells". *Cell Struct Funct* 2001;26:137-148.
17. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, et al. "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture". *Dev Biol* 2000;227:271-278.
18. Sato N, Sanjuan IM, Heke M, Uchida M, Naef F, Brivanlou AH. "Molecular signature of human embryonic stem cells and its comparison with the mouse". *Dev Biol* 2003;260:404-413.
19. Xu C, Rosler E, Jiang J, Lebkowski JS, Gold JD, O'Sullivan C, Delavan-Boorsma K, et al. "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium". *Stem Cells* 2005;23:315-323.
20. Martin MJ, Muotri A, Gage F, Varki A. "Human embryonic stem cells express an immunogenic nonhuman sialic acid". *Nat Med* 2005;11:228-232.
21. Simon C, Escobedo C, Valbuena D, Genbacev O, Galan A, Krtolica A, Asensi A, et al. "First derivation in Spain of human embryonic stem cell lines: use of long-term cryopreserved embryos and animal-free conditions". *Fertil Steril* 2005;83:246-249.
22. Yamamoto Y, Teratani T, Yamamoto H, Quinn G, Murata S, Ikeda R, Kinoshita K, et al. "Recapitulation of in vivo gene expression during hepatic differentiation from murine embryonic stem cells". *Hepatology* 2005;42:558-567.
23. Soto-Gutierrez A, Kobayashi N, Rivas-Carrillo JD, Navarro-Alvarez N, Zhao D, Okitsu T, Noguchi H, Basma H, Tabata Y, Chen Y, Tanaka K, Narushima M, Miki A, Ueda T, Jun HS, Tanaka N, Lebkowski J, Yoon YW & Fox IJ. "Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes". *Nature Biotech* 2006; (In press).
24. Bagutti C, Wobus AM, Fassler R, Watt FM. "Differentiation of embr-

yonal stem cells into keratinocytes: comparison of wild-type and beta 1 integrin-deficient cells". *Dev Biol* 1996;179:184-196.
25. Coraux C, Hilmi C, Rouleau M, Spadafora A, Hinnrasky J, Ortonne JP, Dani C, et al. "Reconstituted skin from murine embryonic stem cells". *Curr Biol* 2003;13:849-853.
26. Fraichard A, Chassande O, Bilbaut G, Dehay C, Savatier P, Samarut J. "In vitro differentiation of embryonic stem cells into glial cells and functional neurons". *J Cell Sci* 1995;108 (Pt 10):3181-3188.
27. Goridis C, Rohrer H. "Specification of catecholaminergic and serotonergic neurons". *Nat Rev Neurosci* 2002;3:531-541.
28. Angelov DN, Arnhold S, Andressen C, Grabsch H, Puschmann M, Hescheler J, Addicks K. "Temporospatial relationships between macroglia and microglia during in vitro differentiation of murine stem cells". *Dev Neurosci* 1998;20:42-51.
29. Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A. "FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate". *Cell* 1998;93:755-766.
30. Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, McKay R. "Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen". *J Neurosci* 2000;20:7377-7383.
31. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, et al. "Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease". *Nature* 2002;418:50-56.
32. Takagi Y, Takahashi J, Saiki H, Morizane A, Hayashi T, Kishi Y, Fukuda H, et al. "Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model". *J Clin Invest* 2005;115:102-109.
33. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. "Differentiation of pluripotent embryonic stem cells into cardiomyocytes". *Circ Res* 2002;91:189-201.
34. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, et al. "Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes". *J Clin Invest* 2001;108:407-414.
35. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, et al. "Electromechanical integration of cardiomyocytes derived from human embryonic stem cells". *Nat Biotechnol* 2004;22:1282-1289.
36. Xue T, Cho HC, Akar FG, Tsang SY, Jones SP, Marban E, Tomaselli GF, et al. «Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers». *Circulation* 2005;111:11-20.
37. Wiles MV, Keller G. "Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture". *Development* 1991;111:259-267.
38. Rideout WM, 3rd, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. "Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy". *Cell* 2002;109:17-27.
39. Odorico JS, Kaufman DS, Thomson JA. "Multilineage differentiation from human embryonic stem cell lines". *Stem Cells* 2001;19:193-204.
40. Couzin J, Kaiser J. Gene therapy. "As Gelsinger case ends, gene therapy suffers another blow". *Science* 2005;307:1028.

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Grant Support: This work was supported in part by the Ministry of Education, Science, and Culture, and the Ministry of Economy and Industry, and Life Science Project of 21st Century, Japan.

Carlos Larracilla/Luz de luna