Therapeutic Potentials of Human Embryonic Stem Cells in Parkinson's Disease

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Summary: The loss of dopaminergic neurons of the substantia nigra is the pathological hallmark characteristic of Parkinson's disease (PD). The strategy of replacing these degenerating neurons with other cells that produce dopamine has been the main approach in the cell transplantation field for PD research. The isolation, differentiation, and long-term cultivation of human embryonic stem cells and the therapeutic research discovery made in relation to the beneficial properties of neurotrophic and neural growth factors has advanced the transplantation field beyond dopamine-producing cells. The present review addresses recent advances in human embryonic stem cell experimentation in relation to treating PD, as well as cell transplantation techniques in conjunction with alternative therapeutics.
Key Words: Neurotrophic, microenvironment, cytokines, growth factors, chemokines, progenitor cells, precursor cells, neural stem cells, transplantation, central nervous system, injury, repair.

INTRODUCTION

Human embryonic stem cells (hESC) were successfully isolated and cultured in 1998, more than 20 years after the discovery of mouse embryonic stem cells. Now, after almost 10 years of research, scientists are still exploring the unique properties and functionalities of all types of human stem cells, with special emphasis on the hESC population. One of the most important therapeutic potentials for these cells encompasses their capability to treat the symptoms of Parkinson's disease (PD) and to halt the progression of disease. In general, the treatment option depends on the particular pathology of the disease. In the case of PD, this has historically been the replacement of neurons producing dopamine (DA)—preferentially, A9 neurons—which, from a transplantation prospective, is not considered as complex a task as for other neurodegenerative diseases.[1]

Since the first tissue and cell transplants to treat PD, the predominant assumption has been that there is a need to replace the dead or dying DA neurons with cells that have the same or similar properties and functionality. However, recent studies with hESC and other stem cell types have brought about new ideas on how these cells may be used to treat PD and other neurodegenerative diseases. In addition to the degeneration of the DA neuron, there is now clear evidence of alterations in cholinergic, serotonergic, GABAergic, and (most importantly) norepinephrine pathways, in which a direct relationship between degenerative neurons of the locus coeruleus and the degeneration of dopaminergic neurons in the substantia nigra (SN) in PD has been demonstrated.[2–10] The participation of these other pathways in PD changes the underlying premise of how cellular transplantation therapies are employed. Although restoring or replacing DA-producing cells has improved motor deficits, including these other cell types could be the difference between minimal improvement and reversing the progress of PD.

There are several converging lines of evidence suggesting that the effectiveness of stem cells, in general, may have more to do with protecting and repairing the degenerating or injured tissue than with the actual replacement of cells.[11] This may be accomplished either directly by the stem cells providing the protection or repair through their predetermined properties or genetic alteration of their properties,[12–14] or indirectly by the stem cells inducing the endogenous cells to repair or regenerate the damaged cells, or to release the needed neurotrophic or growth factors for repair and protection.[15]

There have been only a handful of well-designed studies, using either nonhuman primates or rodents, in which improved motor functions have been observed.[16–21] This

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review addresses the use of human stem cells in the treatment of PD, the clinical attempts at cellular and regenerative therapies for PD, and then, as the main component, presents a comprehensive deliberation on the therapeutic potential for hESCs in treating PD. An in-depth review of animal embryonic stem cells, however, is beyond the scope and limitations of the present article.

ADRENAL AND FETAL TISSUE CLINICAL TRANSPLANTS FOR PD: LIMITATIONS

The clinical trials of tissue transplants, to date, have used either adrenal medullary or fetal ventral mesencephalon (VM) tissue (both human and porcine). The double-blinded, randomized, sham-surgery controlled trials that used human fetal VM tissue may be responsible in part for researchers seeking other means and cell and tissue sources for transplantation in PD patients. Results reported by Freed et al.[22] and Olanow et al.[23,24] suggested that patients at early stages of PD received more benefits from the fetal VM tissue grafts than did patients at later stages. Among younger patients (≤60 years), the Unified Parkinson's Disease Rating Scale (UPDRS) revealed significant improvement in the transplantation group, compared with the sham surgery. There was no significant improvement in older patients (>60) within the transplantation group. The younger patients frequently had early-stage PD, and when VM tissues were transplanted into these patients, outcome from the graft was significantly better than the late-stage patients. A purely theoretical supposition can be drawn from these findings, in which the host microenvironment in the late stage of PD may be too hostile to accept fully the VM grafts, and possibly more tissue or one or more alterations of this unreceptive microenvironment are required in late-stage PD.

In addition, comprehensive reviews of methods and graft viability in both rodent and human PD transplant studies showed that with comparable methods there can be up to three orders of magnitude in variability in survival between grafts.[25] A multitude of problems may occur when using human fetal tissues, mostly because each organism is unique. Although some of these problems may be overcome, attempts to standardize studies will face drawbacks linked to the use of fetal VM tissue, such as 1) lack of sufficient amounts of tissue for transplantation in a large number of patients; 2) variable age, consistency, and viability, resulting in an inconsistency in functional outcomes, with some patients showing major improvement and others with modest, if any, clinical benefit; and 3) occurrence of troublesome dyskinesias in a considerable proportion of patients after transplantation. The intrinsic problems with the fetal VM tissue and the outcomes of the clinical trials have motivated researchers to find more reliable cell sources with fewer inconsistencies, better graft survival, and increased ability to reinnervate the host to prevent dyskinesias.[24,26–30]

In patients with PD, human pluripotent stem cells, whether of embryonic or adult origin, appear to be the most plausible cell source to consider for treatment. These cells have numerous advantages over terminally differentiated cells or progenitor and precursor cells; most important, they are self-renewing, can be readily available, can be produced to indefinite numbers under current good manufacturing practice (cGMP) conditions, and can be directed to become cells with distinct phenotypes that yield specific candidates for cell therapies for multiple diseases.

HUMAN STEM CELLS: DEFINITION AND TERMINOLOGY

A cursory review of the stem cell literature will reveal discrepancies in defining a stem cell. The various definitions of the term, *stem cells*, appear to depend on the particular scientific discipline one subscribes. Most scientists would agree the concept of a common ancestral stem cell was first proposed by Artur Pappenheim (1870–1916),[31] and that self-renewal is the necessary component of being a stem cell.[32] Stem cells are then immortal with self-renewing divisions (symmetrical leading to two cells, or asymmetrical leading to a stem cell and a progenitor) without cellular senescence or crisis. Whether or not differentiation is necessary for the cell to be a stem cell is the incongruence between the disciplines. Hematologists would define a stem cell as "cells with extensive self-maintaining (self-renewal) capacity, extending throughout the whole (or most of) the life-span of the organism. Differentiation potential is a property of some types of stem cells, but is not an essential feature of stem-ness."[33–35]

Other scientists assert that differentiation is a necessary function of stem cells, defining them as undifferentiated cells that are capable of self-renewal (immortal) and asymmetrical division to generate differentiated specialized cells or multipotent cells.[36–39] The main disparity is whether a stem cell needs to further differentiate or become a specialized cell to be defined as a stem cell. At some point, an asymmetrical division has to occur. With this in mind, it seems apparent that an organism may require some stem cells to be only self-renewing, without ever differentiating, thus creating other daughter cells that further differentiate and participate as specialized cells within the organism while the originating stem cell keeps proliferating at a slow cycle to protect the telomeres and genetic makeup and the new daughter cells fulfill the needs of the organism. In this instance, the microenvironment is almost certainly the influencing factor determining the behavior of the cells.

The discrepancy in defining stem cells also led to debates in defining their nomenclature as it relates to their hierarchy. To elucidate further, once a cell has differentiated and thus has limited potential for further differentiation or proliferation (self-renewal), what is this type of cell to be called? Is it a stem cell, a progenitor cell, or a precursor cell? This lexicon of words related to stem cells only adds to the confusion, and the use of the term "stem cells" has become quite free.[40,41] Although there is currently no formal, established nomenclature for all cells, there appears to be a consensus in the basic terminology of stem cells. Extensive reviews on defining stem cells and their nomenclatural hierarchy, beyond the scope of the present article, are available elsewhere.[34,36,40,42–47] We have, however, provided a table with our perception of the hierarchical terminology for the stem cell, along with a brief definition (Table 1). The terminology and definitions used in this manuscript are based on those in Table 1.

The most ancestral or primitive of cells is the zygote, which is the only totipotent or "totus" cell. To be precise, this is the only cell capable of giving rise to all tissue and cell types of the body, both ectodermal and extraectodermal; however, the self-renewing potential is limited or even nonexistent.[37] Pluripotent stem cells are of interest because of their ability to give rise to cells derived from the three embryonic germ layers and their inherent long-term capacity for self-renewal. Multipotent stem cells, progenitor cells, and precursor cells can be used in cellular therapies, despite their more restricted differentiation potential and limited lineage transdifferentiation. A cell lineage can be described as the embryonic origin of a specific cell. Through the use of fate maps that trace the developmental stages of a cell, the embryonic origin of that cell can be traced.[48–50] Accordingly, a stem cell that is more ancestral or primitive (higher in the hierarchy) is of greater interest because of that cell's ability to generate progenitors of more than one lineage. The progenitor and precursor cells are more limited (more restricted) than stem cells in their ability to give rise to cells of a different lineage; however, these cells are valuable for transplantation because of their unlimited self-renewal properties.

The genome integrity in the more primitive stem cells (parent cells) is protected because these cells only proliferate as necessary, thereby maintaining a slower cell cycle.[42] This is allowable because of their progeny, the progenitor and precursor cells (also referred to as *transit amplifying cells*), which are the workhorses responsible for the routine maintenance and regeneration of tissue in the organ.[51,52] In support of protecting the genetic material, this slow cycling has also been suggested as protecting the length of telomeres within the cells, thereby extending the life of these stem cells.

TABLE 1. *Definitions and Descriptions of Stem Cells**

Most Primitive Stem Cells

Totipotent Stem Cells. Cells from the inner cell mass of the embryo, which during the first few cell divisions, the cells differentiate into trophoectoderm, which give rise to the placenta as well as cells of the body.

Pluripotent Stem Cells

Embryonic Stem Cells. These cells originate from the inner cell mass of the embryo after development of the blastula, and occur after three or four cell divisions. They give rise to cells from the three embryonic germ layers (mesoderm, endoderm and ectoderm). They cannot, however, give rise to the tissue of the placenta (this is restricted to cells from the trophectoderm). Embryonic stem cells further differentiate into adult stem cells, also called somatic stem cells, and will give rise to germinal and somatic tissues. These cells have the ability to self-renew (proliferate) long-term (1 year or more) without differentiating (remain unspecialized) while still maintaining normalcy.

Stem Cells

A cell that is capable of self-renewal and self-maintained during the life of the organism. The stem cell may or may not produce cells that are further differentiated (become more specialized).

Ancestral Cell. Stem cells that yield further differentiated descendants.

Adult or Somatic Stem Cells. Undifferentiated cells located within certain tissues and organs of the body; they can self-renew, and differentiate to form cell types of the tissue in which they reside. These cells will remain quiescent until needed, thus reserving their renewing potential, and are thought to self-renew and differentiate in response to a tissue/organ that is damaged or injured, in which they reside. Adult stem cells may also be induced in culture to transdifferentiation.

Multipotent, Bipotent, and Unipotent. These terms are used to describe stem cells that are further differentiated daughter cells of pluripotent or totipotent stem cells. They may produce daughter cells that are further differentiated. However, they are restricted in the ability to naturally give rise to other cells of different lineages.

Progenitor/Precursor Cells

These are the progeny of stem cells, and have a limited self-renewal capacity (some have no ability to self-renew). These cells have a much more restricted differentiation potential and a limited lifespan, but these cells will cycle more than stem cells to provide the necessary cells for self-maintenance of the organism, thus conserving the stem cell or adult stem cell.

*This presentation demonstrates the inherent complexity in the terminology and interpretation of the hierarchy of stem cells. The definitions here are compiled from multiple sources referenced in the text.

INVOLVEMENT OF hESCs IN TREATING PD

Significant advancements in understanding of the biological and physiological properties of hESCs for their potential therapeutic use have been made over the last few years. However, there are issues concerning PD that

also should be addressed when developing the best treatment interventions. Depending on the stage of PD, treatment strategies may vary. For instance, the brains of early-stage patients may have host environments that are less hostile and more acceptable for differentiated hESCs with DA phenotype and for cells providing neurotrophic support.[53] The decision for cell transplantation is multifaceted, involving issues such as the host environment, type or types of cell to transplant, their functionality (e.g., A9 *versus* A10 phenotype), the cells' stage in the differentiation process, and pretransplant condition of the cells.

Although in PD the dopaminergic neuron is the primary cell responsible for the symptoms, it is not the only injured or degenerating cell involved in the disease process. Besides DA, other neurochemicals are involved in the disease process, which may include other neurotransmitters, neurotrophic factors, growth factors, cytokines, chemokines, and the like. Furthermore, greater therapeutic benefits could be realized from therapeutic regimes that are synergistic in their effects. For example, when DA cells are cotransplanted with cells producing growth factors, this could result in a combination of factors creating a synergistic effect.[1,54]

Very few studies have moved in this direction, but some have and these offer promising results. Superior graft survival and integration with the host tissue was seen in a clinical trial that transplanted human VM neurons along with human fetal striatal cells.[55] One neurotrophic factor that has shown positive results and enhances graft survival is glial-derived neurotrophic factor (GDNF). A number of cotreatment transplant studies with DA cells and GDNF have shown promise.[56–58] A synergistic effect was also seen when two cell types, testis-derived Sertoli cells (for trophic support) and DA neurons, were cotransplanted into the striatum of rats with PD induced by 6-hydroxydopamine (6-OHDA). This resulted in improved DA neuron survival rate, compared with the rats receiving only the DA neurons.[59] Thus, transplantation strategies that include other factors along with cells could allow for a synergistic environment that may prove to be beneficial in treating PD.

Although there are several new therapeutic strategies in which hESCs may be used, there are also established methods that require updating to reflect the advancements in this field. The following reflects the older and newer strategies, as well as how hESCs or their derivatives may be used now or in the future to treat patients with PD.

The first strategy would be use of the hESCs to replace the dead or dying VM dopaminergic neurons (specifically, A9) in the SN of patients with PD. The hESCs will need to replace the DA neurons, in the correct number, integrate into and be accepted into the host environment, and function as DA neurons by producing and releasing the appropriate amount of DA. The hESCs will need to be induced *in vitro* to a predetermined stage of the DA neuron in development.

The choice of cells to transplant should reflect the progression of the disease (stage of PD). The hESCs may be induced to form neural stem cells (NSCs), neural progenitors, neural precursors, or those that are fully differentiated into DA neurons. The cells to transplant should be determined as ones that are the most beneficial at that particular stage of the disease for that patient. The argument can be made that NSCs may provide more support for regenerating the damaged tissue in an early-stage patient, whereas in a late-stage patient the only hope is providing DA neurons to replace the lost cells. The second decision in this strategy is deciding on the area within the host brain in which to transplant the cells. The area of choice has been the striatum. The postcommissural putamen is the motor segment most affected in PD and the primary target. The microenvironment of the striatum is considered nonhostile, a place where transplanted cells can survive, and the dopamine taken up and used. Other sites need evaluation, and the SN is considered too hostile, with almost certain rejection occurring.

The second strategy would be to use hESCs to deliver neurotrophic factors, cytokines, chemokines, or growth factors to aid in the repair of the degenerating tissue, to slow or stop the progression of the disease, or both. The principle for the delivery of these factors by hESCs-induced cells is the re-establishment of a permissive environment within the degenerating tissue to facilitate transplant survival and reinnervations of the host. This should be achieved at a time when the injured tissue is still capable of self-regeneration. The hESCs would need to produce cells that express the neurotrophic and growth factors, cytokines, and the like, that are required by the host environment. These cells would then be propagated to the desired number and transplanted to the area or areas of the brain where they would be the most effective. The most beneficial transplantation could be one that requires cells that produce multiple neurotrophic factors and cells that produce DA. Cell transplantation could be used to deliver these factors directly or indirectly. Stem cells appear to be attracted to the site of injury by surrounding astrocytes that express the chemoattractant stromal derived factor-1 (SDF-1).[60–62] SDF-1 is known to attract various stem cells, and the receptor for SDF-1, CXCR4, is expressed on some hESCs. The transplanted hESCs once at the target site may recruit endogenous neurotrophic factors or release their own neurotrophic factors. These neurotrophic factors could in turn recruit endogenous stem cells to the site of injury to aid in the repair or to replace loss cells.

The third strategy would be the delivery of genetically modified hESCs or hNSC (gene therapy) to the host. One method is therapeutic cloning or somatic cell nuclear

transfer (SCNT), in which the nucleus of the unfertilized egg cell is removed and replaced with the material from the nucleus of an adult somatic cell, such as skin, heart, or muscle, followed by the cloned cell dividing.[63] After approximately 5 to 7 days, newly produced stem cells can be extracted from the cultures, cells that are genetically identical to the original somatic cell, providing a potentially unlimited source of selected cells for transplantation therapies. By this means, a selected primary cell of choice can be cloned and used. In future, the patient's own DA neuron may be harvested and cloned for this purpose, which would also avoid host immune rejection and apoptotic signaling.

Recently, a rather clever study showed that hESCs could become hematopoietic precursors by donor SCNT technique. The SCNT-h-ESCs differentiate to hematopoietic precursors when cocultured with donor bone marrow, with no xenogeneic material used, so that upon transplantation the cells would be tolerated. The derived cells resulted in an alternative source of CD34-positive stem cells to treat autoimmune disorders.[64]

A second method for delivering genetically modified cells would be the use of viral vectors with the hESCs (*ex vivo* gene therapy) that can be used before transplanting the cells into the brain to deliver GNDF, brain-derived neurotrophic factor (BDNF), or some other neurotrophic factor for neuroprotective or neurorestorative procedures. Altering the genetic make-up of the cells to be neurotrophic mini-pumps once transplanted, might be safer than direct, *in vivo,* gene therapy, because this avoids the introduction of unwanted vector proteins into the host brain and can induce the desired phenotype *ex vivo* without the risk of inflammatory response by the host.[65]

A third method would be to transplant the transfected mutated hESCs that have been used, or can be used, used to overexpress or underexpress one or more transcription factors, resulting in one or more specific cells for one or more specific purposes. The overexpression of genes that are instrumental in inducing cell fates can be used to produce a cell line required for particular use or need. For example, the dopaminergic neuron-associated nuclear receptor Nurr-1 is essential in the induction of A9 DA neurons and could be used to create a DA cell line for transplantation into PD patients. Other examples for DA neurons include transcription factors such as Mash 1, Lmx1a, and Ngn2.

The fourth strategy would be the stimulation of the host brain neural stem cells to proliferate and aid in repair of its own tissue. This may be achieved through genetically modifying hESCs so that upon transplantation they will induce endogenous NSCs to expand, differentiate, and repair tissue. The hESCs can be modified to deliver cytokines or chemokines that will induce endogenous NSC to the SN to differentiate into DA neurons. The hESCs may also produce signals in concert with endogenous signals, which will attract endogenous NSCs to migrate or arise from quiescent state. A similar protocol would be to transplant the genetically modified hESCs into SN to induce neurogenesis.

Although the SN is not usually thought of when considering endogenous neurogenesis, there is evidence from two studies reporting neurogenesis of NSCs in the SN of animals. The first study to show possible neurogenesis in SN was performed by Zhao et al.[66]; they showed that mice lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) had similar numbers of cells positive for tyrosine hydroxylase (TH) across their lifespan in the SN. Although the initial TUNEL assay showed a decrease in TH neurons within the SN at 12 weeks, the total TH neuronal number remained constant over the life of the animals. In support of this finding, Shan et al.[67] reported an increase, although a small one, in DA neurogenesis in the SN at basal levels; these cells were derived from neural progenitor. After lesioning, nestin-Lac-Z transgenic mice showed an increase in DA neurogenesis with cells derived from neural progenitor and BrdU labeled cells, thus suggesting that multiple cells are responsible for the DA neurogenesis.

The fifth and last strategy involves the endogenous stimulation of neurotrophins, cytokines, or growth factors that could aid in the repair of the injured brain. The method mentioned earlier takes advantages of induced or stimulated endogenous factors that are released at the site of injury. In addition, the hESCs can be preprogrammed to release the appropriate signals upon transplantation, which would induce the neurotrophic or growth factors to be released. However, this requires a detailed knowledge of the molecular pathway or pathways of interest and the necessary chemokines receptors and ligands involved. Another possible method is the homing of transplanted induced hESCs to the host bone marrow, in which the host stem cells are released, home to the degenerating tissue, and participate in the repair process.

A combination of these strategies may be the most useful, thus creating (as already noted) a synergistic effect. With the progress made thus far and with the knowledge gained, the idea that a single cell type will be sufficient to stop or slow the progression of PD no longer seems reasonable. The questions remain. What is the best way to use hESCs and to enhance their use? Is it genetically modified cells, epigenetic culturing, transplantation of multiple cells types, the use of viral vectors, or the use of SCNT, or yet some other approach? Each strategy discussed involves benefits and risks, and some may prove to be better than others, but the risk to the patient must be foremost in our thoughts and actions.

Limitations

The potential applications for hESCs in developmental biology and regenerative medicine are numerous, but there are basic methodological problems in their cultivation that should be addressed before certain applications may be practical. To avoid some of the cultivation dilemmas, regular karyotyping of the hESC line is required, as well as testing for possible mutations and the overall stability of the line, especially for long-term cultures with large number of passages.[68,69] Abnormalities in hESC karyotype have been reported in those cells that were cultured for long periods.[70] *Ex vivo* cultures with long duration of autologous stem cells may result in altered immunogenicity, which could lead to rejection of the cells upon transplantation.[71,72] This is likely because the self no longer recognizes its own cell once it has been immunogenically changed.

Further complications may arise when noncommitted (i.e., undifferentiated) stem cells are transplanted. Their lack of surface cell markers may hinder engraftment, and their differentiation may be negatively influenced by the degenerating host environment. Thus, the diseased environment may affect the transplanted cells further impairing tissue regeneration. The study performed by Wang et al.[73] is an example of how transplanted stem cells may become an unintended, unwanted cell type. They transplanted mesenchymal stem cells into damaged cardiac muscle, which resulted in some of these cells differentiating into fibroblast, with formation of scar tissue.

The newer gene therapies or genetic strategies have been inundated with both safety and efficiency concerns. There may be a necessity for concern. The overexpression of a single protein by the transfected cells could have unpredictable physiological consequences, both short and long term. Furthermore, the viral-based vectors could have unpredictable long-term safety issues. In addition, the stem cells that were genetically changed could, upon transplantation, become malignant. As yet, however, there is no substantiated proof to support any of these reservations. As is likely with any new method, false and positive claims will appear with no support behind them.

Use of animal products in human stem cells in cellular therapies

To date, all available hESC lines have at some point during their maintenance or culturing been exposed to animal byproducts, cells, or materials. Graft rejection is one of the potential consequences of using animal materials in hESCs that might be used in the treatment of patients. The most often cited potential problem is the nonhuman source of the sialic acid 5-*N*-glycolylneuraminic acid (Neu5Gc) obtained from animal serum and mouse feeder layers, against which many humans have developed antibodies.[74,75] In addition, there is an increased risk of transferring viral and prion disease from hESCs that have had any type of xenogeneic exposure and are then used in clinical applications.

The defined media cultures of hESCs contain some level of animal product, so that even in cultures in which cells are obtained by immunosurgery there is a small amount of animal product, resulting in cell lines with abnormal chromosomes—which may imply that culturing conditions were too rigorous and may jeopardize the integrity of hESCs genome.[76,77] Whether or not the minuscule amount of animal material may actually affect the properties of hESCs or their potential therapeutic benefit has not been determined. Furthermore, the results of treatment with these cells may not pose a risk to humans. We do not think that the decision to use hESCs or one of their unique applications should be based on the potential contact with animal material, but instead should be based on the therapeutic outcome and safety. Therefore, if hESCs deliver a therapeutic outcome in a safe manner, then the use of animal material should not be an issue that prohibits their use.

Transgenic modeling of PD with hESCs

We have discussed alternative ways in which hESCs may be used therapeutically to treat patients with PD, but these stem cells may also be useful in studying the etiology and pathology, along with new drug treatments, in models of *in vitro* PD. The hESCs can be induced to form DA progenitor that can be used for *in vitro* models of PD. The downregulation of one or more of the DA genes in these DA progenitor cells can create cells that are predisposed to die, creating an *in vitro* model of PD, and this would be an invaluable research tool. The investigation of PD as it arises and progresses could lead to new and enhanced treatment strategies. This mutation could occur by 1) introduction of mutation by the use of homologous recombination or 2) the mutation introduced by SCNT, and could be used to screen for new drug discoveries and to study progression of PD.

STEM CELLS FOR PD THERAPY

The ability to isolate and culture hESCs in 1998 was presented by Thomson et al.[78] in their landmark Science manuscript and then further described later by Reubinoff et al.[79] in 2000. Human embryonic stem cells are predominantly isolated from the inner cell mass (ICM) of embryo-stage blastocyst, from *in vitro* fertilized eggs, which consist of 30 cells capable of unlimited proliferation and retention of the potential to differentiate or generate cells of all types (making them ideal for regenerative medicine). The blastocyst consists of the ICM, the surrounding trophectoderm epithelium, and the extraembryonic endoderm.[80,81] Although it has not been

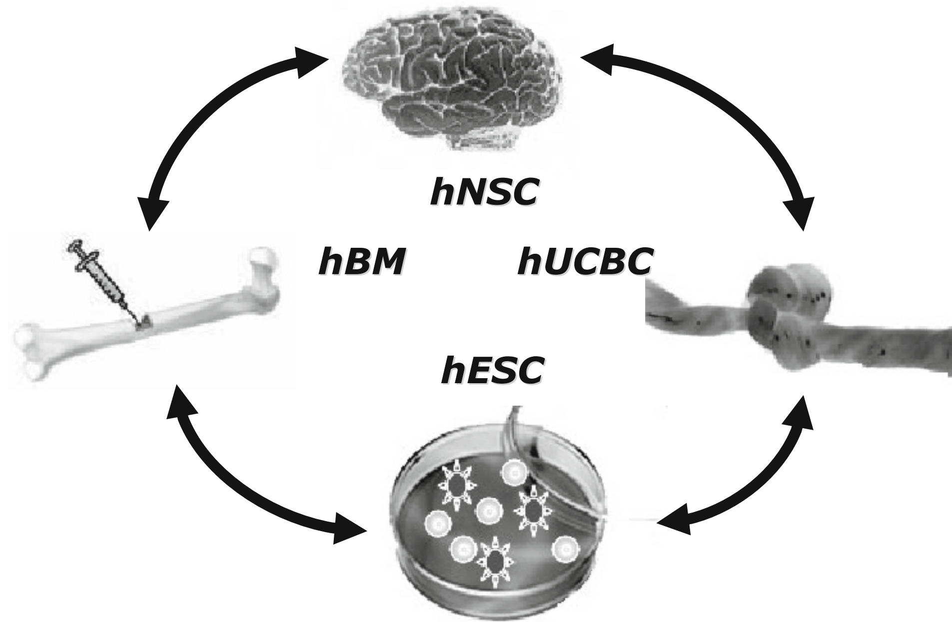

FIG. 1. This diagram presents the sources of the four types of human stem cells that we think hold the most promise in the treatment of PD: human bone marrow cells (hBM), human umbilical cord blood cells (hUCBC), human embryonic stem cells (hESCs), and human neural stem cells (hNSC). The stem cell may come from several sources, as represented by the bi-directional arrows. Transplantation of hBM cells and of hUCBCs has already been used for treatment of other diseases in humans, and other stem cells may be of use in the future (e.g., those found in adipose tissue). The hESCs are the donated cells remaining unused after *in vitro* fertilization procedures. The hUCBCs are obtained after delivery in either an open or closed system; the cord blood is gathered from the umbilical cord vein and is processed. Other tissues of the cord have also been investigated (e.g., Wharton's jelly). The hNSCs are usually obtained from aborted fetal ventral mesencephalon tissue or adult tissue that is acquired during autopsy. For transplantation, hBM cells are collected from either the patient or an HLA-matched donor (typically a sibling or other family member). For research, hBM cells are at present typically harvested and expanded by a private company and then sold to the scientist. Both hUCBCs and hNSCs (at least some) may also be available for purchase, but, to our knowledge, no hESCs as yet commercially available.

experimentally proven, the ICM is thought to give rise to all cells of the body.

In the mouse, in addition to the stem cell of the ICM, other stem cells residing within the blastocyst have been isolated in the trophectoderm (TS cells) and extraembryonic endoderm (XEN cells), and these stem cells have properties similar to those of the ICM. Although ICM, trophectoderm, and extraembryonic endoderm cells are all present in the human blastocyst, to date only hESCs of the ICM have thus far been isolated. An important difference between human and mouse ESCs is that hESC, when exposed to bone morphogenetic protein-4 (BMP4), spontaneously differentiate into trophectoderm-like cells,[78,82,83] suggesting the ICM of human blastocyst retains the ability to generate trophectoderm. Further support comes from the downregulation of octamer-binding protein (Oct4, POU domain transcription factor) in hESC that results in the upregulation of trophectoderm and extraembryonic endoderm cell markers Cdx2 and Gata6, respectively.[84,85]

The issue of other human embryonic-like stem cells residing in blastocyst at this time remains unresolved. It is highly likely, however, that this issue will be resolved in the near future. In addition, there are still ethical concerns surrounding the use of human blastocysts (similar to issues in using fetal tissue), which is limiting the progress of scientists. This issue is already very public, and beyond the scope of this paper, and we would like to think positively, with the belief that this issue will soon be resolved.

The other human stem cells

Besides the use of human fetal VM tissue and ESCs for PD therapy, there are other human stem cells under investigation for their therapeutic potential. The human stem cells depicted in Figure 1 represent those cells that have been, or could be, used in some manner to treat PD.

The VM tissue grafts and fetal dopaminergic cells are the same except the cells have been further separated and isolated from the VM tissue. Human neural stem cells (NSCs) may be one of the most popular alternatives for the treatment of PD. Although it may be possible for autografts of human NSCs to be placed into PD patients, it is more likely that NSCs will be obtained from allogeneic embryonic, adult, and even postmortem brain tissues (FIG. 1). The cells may be directed in culture to the desired neural phenotypes for the allografting. NSCs may also be derived from other human sources, including the pluripotent stem cells from embryonic carcinoma, embryonic germ, or ESC,

and from the hematopoietic stem cells of bone marrow and umbilical cord blood. The NCSs can, of course, be collected from the embryonic and adult brains from other species, but such xenografts are not desirable for cellular therapies. All of these choice have their own advantages and disadvantages as a cell source for use in cellular therapeutic treatments.

Identification of human embryonic stem cells and other stem cells

The hESCs can be identified by a morphology that typically exhibits flat colonies with distinct cell borders, by a high nucleocytoplasmic ratio and large nucleoli, and by a doubling time of 24 to 72 hours, depending on the culturing conditions and the scientist's hands.[86–88] The cells, their morphology, and proliferation rate are all highly dependent on their microenvironment (i.e., their culturing condition), in which cell clusters and embryonic bodies have been seen and are common.

The hematological area of the life sciences originally established one of the most widely used methods today for identifying and selecting hESCs and all types of stem cells. This includes cell surface antigens (epitopes) and cluster differentiation antigen markers, more commonly known as CD markers. More recently, the use of fluorescence-tagged antigens is being used for selecting cells by flow cytometric analysis and fluorescence-activated cell sorting. The particular cell surface marker may not be available for certain stages, however; hence, other means of sorting and identifying both hESCs and NSCs are required.

There are several markers (antibodies) and features that can be used to identify and characterize the undifferentiated hESCs. The regulation and expression of the stage-specific embryonic antigens (SSEA) that are globoseries glycolipids are probably the most well known for identifying hESC.[89] The expression of SSEA-4 on hESCs is at very high levels, with SSEA-3 expressed at low levels, and there is no expression of SSEA-1.[17,90–92] There are also high molecular weight glycoproteins expressed in hESCs and others, such as, the tumor rejection antibodies (TRA)-1-60, TRA-1-81, alkaline phosphastase (AP), and GCTM-2 (epitope on a 200-kD pericellular matrix keratin sulfate proteoglycan).[17,53,93] Transcription factors are also used to characterize and identify undifferentiated hESC, and these include Oct4, Nanog, and Sox2, along with the DNA-modifying enzymes TERT1 (telomerase) and DNMT3 (DNA methyltransferase).[94,95] Other biomarkers, less often reported, include CD9, CD24, LIN28, Rex-1, Cripto/TDGF1, EBAF, and Thy-1.[96]

Comparative gene expression analysis across different hESC lines in the undifferentiated state has resulted in controversy. There are reports of similar gene expression results[97,98] showing differences between hESC lines.[99] The cell surface epitopes for identifying and characterizing the undifferentiated hESCs are well proven and known, whereas the markers and methods for identifying the differentiated hESCs depend directly on their acquired phenotypic expression. Whether the discrepancy in transcription factors is the result of inherent difference the hESC lines or the methods use to obtain the results is not known at this time and requires further investigation.

Identifying differentiated hESCs and hNSCs

The hESCs and hematopoietic stem cells share the expression of some common surface markers. The best-known cell surface marker for the progenitor cells is the CD34, a glycophosphoprotein (type I transmembrane protein).[100,101] Although the biochemical activity of CD34 remains unknown, evidence suggests that one of the primary functions of CD34 is cell-to-cell adhesion, such as promoting hematopoietic cell adhesion to the stromal layer of bone marrow.[102] Although this is not true for the majority of antigens, the combinations of cell surface or other markers can be used to identify various types of cells. hHSCs that are younger or higher in hierarchy in the developmental process of cells are shown to express these surface markers: $CD34^{+}$, $CD38^{-}$ (a novel multifunctional ectoenzyme), $CD90^{low}$ (also known as Thy-1, and thought to be involved in the inhibition of proliferation and differentiation of cells), $CD117^{low}$ (also known as c-KIT and SCFR, a stem cell factor receptor), $CD135^{+}$ (also known as tyrosine kinase 3 and Flt3, a growth factor receptor), and HLA-DR^{-} (human leukocyte antigen).[103–108]

There is a subset of hNSCs that express CD133 (originally called AC133), which are induced from the hHSCs that express CD34. The expression of CD133 indicates the cells are NSCs of either hESC or hHSC origin.[98,109] Two forms of this antigen have been reported: the CD133/1, a cell surface glycoprotein antigen (cell membrane label), and CD133/2, an intracellular protein (perinuclear label) that reportedly is a marker for trophoblast (see[110,111] for CD133/1 and[112] for CD133/2). Although evidence suggests that these antigens recognize a more primitive subset of stem cells and have been recognized on several tissue types (human fetal liver, fetal and adult bone marrow, cord blood), there is special interest in CD133/1 and its usefulness in selecting, sorting, or recognizing human NSC.

In support of CD133/1 antigen expression on human NSCs, a study that isolated cells from human fetal brains and spinal cords (week 12) identified NSCs with CD133/1. More specifically, isolated cells were $CD133^{+}$, $CD34^{-}$, $CD45^{-}$ (leukocyte marker), $CD24^{-/low}$ (B cell marker) and upon culturing were shown to generate neurospheres, self-renew, and differentiate into neurons and glial.[113] In addition, neither CD34 nor CD45 positive cells could generate

neurospheres,[113] the neurospheres do not express these markers, and approximately 90% to 95% of the neurospheres' cells express the CD133 marker.

In addition to the CD133/1 antigen, FORSE-1 (forebrain surface embryonic) and SSEA-1 cell surface antigens are expressed at higher levels in hNSCs than in the human neural progenitors or neuronal-differentiated cells. In the differentiated hESCs, the stage-specific embryonic antigens 3 and 4 are found approximately at the same levels in both hNSC and hESC, and then are almost completely downregulated in the neural progenitors and neuronal differentiated cells. Although CD29, also known as integrin beta-1, is strongly expressed in hNSCs, neural progenitors, and neuronal differentiated cells, there is very little expression on undifferentiated hESC, which is also true for CD56, a neural cell adhesion molecule (NCAM), with no expression on hESCs, and with strong expression on hNSCs and neural progenitors cells, and the strongest expression on neuronal differentiated cells.[114]

One of the more prominent neural markers is A2B5 (a neuronal cell surface antigen), the expression of which is greater on neural progenitors and neuronal differentiated cells than on NSCs, for unknown reasons.[115,116] The same pattern of expression is true for the cell differentiation antigen CD24.[95,117]

The last precursor and progenitor cell marker to be discussed here is the intermediate filament protein, nestin, which has been used as a marker for neural precursors.[118,119] At one time, nestin was thought to determine neural lineage exclusively; however, this protein has also been found in endothelial cells, embryonic tissues, glioblastoma multiforme, and many melanomas.[119] Therefore, although nestin can be used as a marker for neural progenitors and precursors, its specificity is no longer accepted.

The hESCs have been shown *in vitro* to differentiate into derivatives of all three germ layers (ectoderm, endoderm, and mesoderm) and precursors to germ cells.[79,120,121] The need for developing standard protocols in the culturing and in (if required) neural induction of hESC is made relevant by the recent study by Pruszak et al.,[96] who used H7 and H9 hESCs (approved by the National Institutes of Health) *in vitro* and in a 6-OHDA rat model of PD. The researchers showed the development of a heterogeneous population of cells in neural-induced hESCs both *in vitro* and *in vivo*. The use of a multistep protocol and neural induction media resulted in nestin-positive neuroectodermal cells that matured at different rates, lacked a synchronized cell cycle, and consisted of heterogeneous population of immature cells, neural precursors, and neuronal process bearing cells that were identified by the expression of SSEA-4, OTX-2, and TuJ1, respectively.

Proliferation and differentiation of hESC

The realization of the therapeutic potential of hESCs will rely on our ability to maintain these cells, direct or induce specific phenotypes, support, and expand the cells—all before they are therapeutically applied. There are a vast number of proliferation or expansion and differentiation protocols, but very few that use only non-animal products, which may be required for therapeutic hESCs applications. The majority of neural differentiation protocols used embryoid body formation with the treatment of the morphogen retinoic acid.[122–124] These historic protocols are not refined enough for either government-regulated GMPs or good laboratory practices (GLP). New well-defined and proven protocols will be required to allow hESCs to move toward the clinic. Here, however, we will now discuss briefly the factors involved in proliferation and differentiation.

The endogenous factors that trigger proliferation and differentiation of stem cells and their progeny are beginning to be resolved. The proliferation, expansion, and differentiation of stem cells are regulated through intrinsic cellular factors, extrinsic cellular factors (cytokines, growth factors, cell adhesion molecules), and cell-to-cell interaction.[36] For example, mouse ESCs can be maintained, *in vitro,* in the presence of leukemia inhibitory factor (LIF), and these cells will produce NSCs when supplemented with fibroblast growth factor (FGF) or retinoic acid in culture.[125] Both basic fibroblast growth factors (FGF2) and epidermal growth factor (EGF) will keep the NSCs in a proliferative state,[126] whereas BDNF will direct neuronal progenitors to differentiation.[127] Although LIF is sufficient for mouse ESCs, the hESCs cannot survive with this growth factor alone.

Human ESCs have typically been cultured on mitotically inactivated mouse embryonic fibroblasts. For these cells and their progeny to move toward the clinic, however, the animal feeder layer must be eliminated from the equation. In fact, all animal products must be removed from the culturing condition, and there must be defined culture and media conditions in order to comply with GMP. Fortunately, both researchers[87,128,129] and biotechnology companies are producing media and supplements that are meeting this requirement. Unfortunately, the ingredients are proprietary, none of the companies will provide the ingredients (although the media are for sale), and very few of the scientists are willing at this point to share all of their knowledge.

What is general knowledge in culturing the hESCs for proliferation is that after the development of the basal media with the needed serum replacement (knockout serum) and other supplements, the human recombinant proteins sonic hedgehog (SHH), fibroblast growth factor 8 (FGF-8), and basic fibroblast growth factor (bFGF) are needed for these cells to proliferate. The induction of hESCs to neural progenitor requires the removal of

bFGF and, as with mouse ESCs, the addition of retinoic acid. The retinoic acid neural progenitors that spontaneously differentiate express polysialylated neural cell adhesion molecule (PSA-NCAM), and exposure to FGF-2 and EGF results in proliferation.[121,130]

Note that the hESC-neural progenitors induced by FGF-2 do not require EGF to proliferate, nor do they express PSA-NCAM.[123] In the matter of inducing hESC-neural progenitor to DA-like neurons, there are numerous protocols, each varying a little but all with the same premise of adding growth factors or taking others away (or some combination of the two) for a given amount of time. Eventually, repeated experimentation and time will provide the optimal protocols, and these may vary, depending on the stage of disease. Thus, the cells will be individualized to those that would be most beneficial for transplantation into a particular host environment.

Some stem cells have been reported to transdifferentiate—that is, to give rise or transit to a cell with properties distinct from the germinal layer in which they originated. Kondo and Raff[131] have shown that oligodendrocyte progenitor cells may transdifferentiate to pluripotent stem cells and give rise to neurons. However, caution is needed when determining the ability of a cell to transdifferentiate. Progenitor cells that were obtained from muscle, and consequently thought to be of muscle origin, were reported to transdifferentiate into hematopoietic stem cells. Upon further investigation, these stem cells were, in fact, determined to be hematopoietic stem cells residing within the muscle.[132] Although it is possible to direct the differentiation of pluripotent stem cells and lineage-restricted progenitor cells *in vitro*, we are only at the initial stages of learning how to direct stem cells to phenotypes that might be useful *in vivo*.

TRAFFICKING AND MIGRATION OF STEM CELLS AND THE INFLAMMATORY FACTORS OF THE MICROENVIRONMENT

For some time, stem cells were thought to be nonmigratory and to reside only within a bound area detected by signals. However, Snyder and Macklis[133] showed that stem cells retained migratory ability, thus allowing distribution of the stem cells over a larger damaged area. The migration of hNSCs toward the injured or degenerating tissue is known, and once at the target site the cells will begin to differentiate.[60,134,135] In addition, hNSCs have been shown to express the cognate receptor for SDF-1, CXCR-4, which is a primary migration chemoattractant, and SDF-1 itself also induces proliferation in hNSCs *in vitro*.[60] The CXCR-4 receptor, however, has not been shown on hESCs, and whether this receptor is expressed on more differentiated cells is currently unknown. This chemoattractant could be overexpressed in the induced hNSCs and transplanted to induce the NSC to endogenous aid in repair.

The brain has long been thought of as immunologically privileged; however, this status has been changing, as new evidence suggests donor-specific immunological responses to both allografts and xenografts at the implantation site within the brain. The immunological properties of the microenvironment within the host, the factors (e.g., cytokines, chemokines, neurotrophic) produced by the tissue at the transplant site, and the transplanted cells have not been fully investigated. However, inflammation and cytokine production is well known to occur both in experimental animal models of PD and in patients with PD (as shown from the brain autopsy).

In human PD brain, the proinflammatory agents tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-2, IL-4, IL-6, EGF, transforming growth factor (TGF)-alpha, and TGF-beta1 are present and increased above normal controls in the caudate and putamen.[136–139] Furthermore, in the region of the nigrostriatal pathway, the neurotrophins BDNF and nerve growth factor (NGF) are decreased.[138] These findings support the hypothesis of a hostile host environment and the need to provide neurotrophic growth factors, along with the neural-induced hESCs for transplantation. In addition, activated microglia in the SN are known to release proinflammatory agents and have been shown to be neuroprotective at first, but then as the PD progresses the activated microglia become more neurotoxic.[140] Although this is not new information, it does provide a starting point for applying one of the gene therapy strategies discussed, in addition to transplanting induced neural hESCs. Thus, targeting the microglial cells to either reduce the release of the major inflammatory agents or revert the microglia back to an early stage when they were neuroprotective may prove effective.

Immunogenicity of hESCs

The current literature reveals little or no concern for the immune properties of hESCs upon their transplantation. The possibility of immune rejection or the status of immune privileged cells needs to be determined before they are used clinically. However, even after the numerous clinical studies discussing the rejection of VM fetal tissues grafts, along with other immune responses, few studies have considered the immune properties of hESCs, or mentioned the use of immunosuppression therapies. Before these cells or their directed progeny can be advanced to the clinic, their immune properties must be determined as safe for use in humans.

Together, the innate and adaptive immune systems are directly responsible for the transplanted cells to be accepted or rejected from the host site. The human leukocyte antigen–major histocompatibility complex (HLA–MHC) class I is expressed at very low levels on

undifferentiated hESCs and, to date, the expression of HLA class II molecules have not been shown on these cells, with the former relating to low immunorejection properties. In addition, the expression of HLA class I can be upregulated on hESCs by interferon-gamma (IFN-γ), but not HLA class II.[141] The question remains as to whether hESCs can elicit an immune response.

The immune response of hESCs may be the major obstacle (or one of them) to be overcome, depending on the properties that these cells possess, and what the resulting or induced cells will possess. The undifferentiated cells have been shown to lack HLA class II and the costimulation molecules B7.1, B7.2, and CD40, which are important prerequisites for cells to function as antigen presenting cells (APC). The opposite is also true, however, in that hESCs fail to downregulate the established immune pathway by not expressing the transforming growth factor-β, CD95L (Fas ligand), and IL-10. At present, all indications are that hESCs are immune privileged; however, more work in this area, as well as the progeny of these cells, are required before they should be used in human regenerative medicine.[142–145] A significant area to consider when using these cells for transplantation is the immunoproperties of the host tissue and the hESCs and their progeny, both at the time of transplant and as they differentiate, and the interaction between the two.

One of our earlier studies in collaboration with colleagues,[146] showed the infiltration of mononuclear cells in MPTP-lesioned monkeys that received allografts (fetal mesencephalic), autografts (adrenal medullary and peripheral nerve tissue), or sham controls (surgery no grafts); in which the infiltration decreased throughout the 1-year period. The predominant cells types present were macrophages (CD68), CD2 T and natural killer cells, and CD3 T cells (helper antigen cells) at 1 week and at 1 and 3 months after transplantation. Low levels of MHC-I antigen were detected in tissue sections from graft sites and from areas of the needle tracks (control), which was speculated to be produced by macrophages and leukocytes residing at these sites. This study shows that the grafted cells and needle tracks produced at least a minimal cellular immune and inflammatory response. Because there was only minimal immune response, this may indicate that immunosuppression therapy is not needed; however, this was only one study, and further investigation is required in this area.

SUMMARY

We have shown that in the application of hESCs for any clinical purpose there are critical issues in culturing and expansion. It is imperative, before clinical application can be considered, to have the capability to predict the behavior of hESCs over a very long period in culture and after differentiation. Furthermore, the cells need to be monitored for stability regarding their karyotype, mitochondrial genome, cell cycle, and epigenetic gene development, especially after prolonged cultures.[147,148] Part of a successful transplant graft is the health and viability of the cells; the presence of unhealthy or low viability of the cells could easily jeopardize the graft. There are a few indications, besides visual inspection, that can assist in determining the status of hESCs and these, like the stability, should be checked periodically when cells are in culture: the pluripotent ability (differentiation into cells from all three embryonic germ layers); immortal ability (self-renewing or self-maintenance; requires proliferation assay), along with the expression of high telomerase activities; stable phenotype expression (primary cell surface markers are present: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and transcription factor Oct-4); and "clono-ability," or clonogenic character (the ability of individual cells to have the properties stated). Although we have not discussed the teratogenic potential of hESCs, it is real, and we must be aware of this deadly potential at all times. Even though there is not an immediate threat, a major technical challenge exists and a permanent solution is needed. Whether or not the induced hNSCs or precursors will revert back to pluripotent cells is debatable, whereas once the hESCs become terminally postmitotic DA-like neurons, the possibility of these cells reverting back ceases.

Tremendous hope exists for hESCs or one of their differentiated derivates, that they will be able to repair, replace, or provide support to the damaged tissue, cells, or pathways. In this review, we have presented some alternative strategies for human embryonic stem cells beyond transplantation of a single cell type, which has been the standard method. There are some exciting new concepts for cellular therapeutics. The SCNT technique, in which genetic material from the graft recipient somatic cell is placed in the nucleus that was removed from the oocyte,[63] is one of the more interesting methods, and one that could conceivably solve the immunological barriers to transplant without the use of immunosuppressants. For now, however, use of the method is allowed mouse ESCs in a few European countries, and not in the United States. Finally, it has become apparent the therapeutic cell transplantation for PD is more complex than just simple cell transplantation.

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