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Human embryonic stem cells: a potential source of transplantable neural progenitor cells

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Abstract

The primary therapeutic goal of embryonic stem cell (ESC) research is cell replacement therapy. During the last decade, great strides have been made in developing in vitro protocols for differentiating human ESCs into neuroepithelial progenitors. More recent progress has been made in further directing them into becoming cells with specialized regional and neurotransmitter identities, such as midbrain dopaminergic and spinal motor neurons. Along with directed differentiation, other current efforts are aimed at efficient enrichment, avoidance of immune rejection, demonstration of functional integration, genetic modification to regulate neurotransmitter and factor release, directed axon growth, in vivo cell tracking, and measures to ensure safety. This review will focus on the potential of ESCs as a source of transplantable cells for use in cell replacement therapy.

Keywords

cell replacement therapy; CNS cell transplantation; directed neural differentiation; embryonic stem cells; restorative neurosurgery

Cell replacement therapy is an exciting research area that offers potential treatment for several developmental, traumatic, and degenerative neurological diseases for which there is currently no cure. The requirement of the replaced cell can be as straightforward as synthesis and release of a protein, to something as complex as the establishment of lengthy projections and integration with host circuitry. A universal cell replacement strategy is unlikely to work.

Cells ultimately used for transplantation can be isolated from the donor organism at different lineage stages. If obtained later in development when their fate is restricted, they can be purified and sorted prior to transplantation. Alternatively, cells can be isolated at an early stage, expanded, specified, and differentiated prior to transplantation. The second option allows expansion and directed differentiation. Ideally, what is needed is an unlimited supply of expandable, stable cells with enormous potential that can be manipulated to give rise to specific cell types.

Significant strides have been made in designing cells with broad potential that can be directed in vitro down a given path to eventually become a specific phenotype. These cells have shown promise for brain repair in preclinical models of disease.⁶³ Major barriers include immune rejection, the potential of tumor growth, and incomplete understanding of the steps needed to manipulate human cells due to an incomplete understanding of the normal steps of human development.

Choosing the Stage to Harvest and Isolate Donor Cells

When designing a cell to use for replacement therapy, it is important to consider the lineage stage and commitment of the grafted cell so that its actions can be predicted after transplantation. Figure 1 demonstrates the predicted relationship between lineage position and cell behaviors following transplantation. In general, as a cell progresses from an ESC to a mature neuron, its capacity for cell division, migration, and differentiation gradually decreases, while the neurotransmitter phenotype predictability and safety increase.

Each human cell is isolated at a particular stage of development and will possess either broad or restricted potential. If stem cell technology is to become a practical therapy, there would ideally be a readily available stock of starting cells available that can be tailored for specific therapies. The ideal source of starting cells could be expanded to large numbers, stored for extended periods, is effective in cellular replacement, can be manipulated or tailored to fit the specific condition being treated, tolerant to immune rejection, and safe. In the case of neuronal replacement, the cell must possess the capacity to functionally integrate with existing circuitry. Presently, no cell meets all of these criteria. Many cells have been investigated in animal studies and clinical trials. Table 1 lists potential donor cells, their sources, and the advantages and disadvantages of their use in cellular replacement therapy.

Primary Neural Cells

Primary neural cells are fairly mature cells that are committed to a particular phenotype corresponding to their site of origin. An example would be the human fetal mesencephalic tissue used in clinical trials for PD.⁴ Advantages include predictable phenotype and low risk of tumor formation. The disadvantage is that these cells have a limited capacity to expand, and grafts must be prepared from several stage-specific embryos. If these cells were found to be effective in cellular replacement for neurological diseases, problems would arise due to their limited supply. The practical and ethical constraints that accompany the use of primary fetal brain tissue in cell transplantation are too numerous to be covered here.¹³

Adult NSCs

Adult NSCs can be isolated from the brains of adult animals and humans and expanded in culture.^{15,28,31} These cells can generate neurons and glia in vitro and after transplantation.^{18,41,45} Stem cells generated from adult neural and nonneural tissues are attractive because they avoid many ethical and immunological issues. They may prove effective for conditions that require synthesis and release of certain enzymes and factors. However, adult NSCs may not be optimal for neuronal replacement because they tend to become primarily interneurons when placed in neurogenic regions and astrocytes if placed in nonneurogenic regions.⁴⁵ The full potential of these cells has yet to be realized.

Neural Stem/Progenitor Cells

Neural stem/progenitor cells are isolated from embryonic CNS tissue and can be expanded in culture for prolonged periods using genetic or epigenetic approaches. Expanded cells have the capacity to differentiate into neurons, oligodendrocytes, or astrocytes.^{20,46} These cells are often confined to a specific regional identity at the time of isolation because they are frequently

isolated after rostrocaudal and dorsoventral specification. In this regard, they offer a good source for the cell replacement requirement in many general conditions, but may be limited as a source of specific neuronal replacements. Still, neural stem/progenitor cells isolated within a certain window of development appear to retain the ability to give rise to cells of other lineages. For example, coculture of NSCs isolated from the midbrain and hindbrain with ventral forebrain tissue induces the expression of ventral forebrain markers.¹⁹ Neural precursor cells isolated from the hippocampal dentate gyrus and grafted into the rostral migratory stream migrate to the olfactory bulb and become tyrosine hydroxylase-positive neurons, a type of dopaminergic neuron not normally found in the hippocampus.⁴⁵

Neural stem/progenitor cells can be immortalized with oncogenes. For instance, the clonal line C17.2, created by overexpression of *v-myc* in cerebellar granular cells, is capable of differentiating into a variety of neurons and glia in a site-specific manner.⁴² These cells differentiate into myelin-producing oligodendrocytes when placed into the dysmyelinated shiverer mouse brain,⁵⁸ but appropriately become neurons or glia when grafted into areas of injured brain or spinal cord,⁵⁵ suggesting that the fate of these cells is influenced by their environment. Because neural stem/progenitor cells have relatively restricted differentiation potential compared to cells of lower lineage stages, they appear to have a lower risk of tumor formation.

Embryonic Stem Cells

Embryonic stem cells possess several features that make them ideally suited for neuronal cell therapy. These cells, derived from the inner cell mass of a blastocyst,^{11,51,52} can be expanded in vitro for years while retaining the capacity to differentiate into any of the specialized cell types that make up an organism. Accordingly, they provide a useful model for understanding early mammalian embryonic development and also offer a source for generating specialized cells such as specific neurons and glia for therapeutic uses.^{44,63–66}

Directed Differentiation From ESCs

Neuroepithelial cells are specified from naive ESCs at approximately Day 7 in mice and near the end of the third week of gestation in humans. At this time, the CNS appears as a slipper-shaped plate of thickened ectoderm known as the neural plate. This plate is located in the mid-dorsal region in front of the primitive pit.^{3,17,66} These neuroepithelial or neural stem/progenitor cells generate neurons, astrocytes, and oligodendrocytes in a coordinated temporal and spatial fashion. These cells have positional identity, and their fate is influenced by local environmental cues from neighboring cells. In a reciprocal fashion, these cells influence their surrounding environment. Regionalization of these neuroepithelial cells is attained with morphogen gradients such as Shh, bone morphogenetic proteins, FGFs, retinoic acid, and Wnt proteins that control dorsal–ventral and rostral–caudal fate. When stem cells are isolated during various stages of development, their programmed fate will vary depending on the time and location of their isolation.

With this knowledge, naive neuroepithelial cells such as those generated in vitro from ESCs can be directed to a specific neuronal fate that is dictated by the presence of specific morphogens. For instance, ESC-derived neuroepithelial cells can be made to differentiate predominantly into dopaminergic neurons in the presence of FGF-8 and Shh (which confer midbrain dopamine neuronal identity), while the same cells can be made to generate spinal cord motor neurons in response to caudalizing signals such as retinoic acid. Cells will respond to these directive cues during a critical period, and if this period is passed, they tend to follow their intrinsic program of development.⁶⁵

The Therapeutic Potential of Directed ESCs

The potential benefit of ESC therapy was first noted in mouse studies. Mouse ESCs could be differentiated into neuroepithelial⁵⁴ and more specialized neuronal and glial subtypes such as midbrain dopamine neurons,^{2,23,25} motor neurons,⁵⁶ and oligodendrocytes.⁷ Moreover, these cells were used successfully in disease models. For example, mouse ESC-derived neurons and oligodendrocytes were shown to reverse the locomotive deficit of parkinsonian rats^{2,25} and produce myelin in dysmyelinated or injured rat spinal cords.⁷ This work in rodents led to experiments evaluating the potential of human ESCs in similar animal experiments. Human ESCs were initially isolated and shown to possess the potential to produce all cell tissue types of the body.^{35,36,51,53} Techniques were developed to efficiently differentiate them in vitro into NPCs^{10,34,67} and neuronal and glial subtypes.^{27,29,33,57} This source of synchronized neuroectodermal cells provides a starting source that can produce any neural cell as well as offering an in vitro model for scrutinizing the mechanisms of neural induction and cell lineage specification in early human development.

The Broad Potential of ESCs

Neural cells differentiated in vitro from hESCs exhibit broad cellular heterogeneity. The key in neuronal transplantation will be obtaining functionally effective cells that are restricted enough to “do the right thing” and resist doing harm, yet unrestricted enough that they can adapt to and integrate with the host environment in which they are placed. The neural differentiation process of hESC-derived NPCs appears to follow the intrinsic programmed temporal course of human neuronal and glial generation, while the ultimate neural subtype fate is largely influenced by the brain region in which the grafted cells reside.^{1,12,14,16,50,60} For instance, grafted cells residing in neurogenic brain regions continue to divide, while those in nonneurogenic regions cease cell division.¹⁶ Grafted cells dwelling in gray matter mostly become neurons, whereas those in the white matter differentiate exclusively into glial cells. No matter when the cell was isolated (embryonic or neural progenitor stage), neuronal subtype differentiation is also influenced by its final brain location, suggesting an environmental influence on neuronal differentiation.^{12,14,16,47,50}

One primary objective in neuronal replacement is functional integration of the transplanted cell with the host’s endogenous neuronal circuitry, leading to improved function. Recent strides have been made in achieving these goals in preclinical animal studies. There is evidence that hESC-derived NPCs can mature, form synapses, and functionally integrate following transplantation. After injection into the brain of neonatal SCID (severe combined immunodeficiency) mice, synaptic proteins, expressed by differentiated human neurons derived from hESCs, can be demonstrated by confocal immunohistochemistry and immunoelectron microscopy,¹⁶ suggesting potential functional communication between the transplanted cells or between endogenous and exogenous cells. Gage and colleagues²⁸ have shown that, after transplantation into the embryonic mouse brain, hESC-derived neural progenitors differentiate into mature neurons and functionally integrate into the host brain as demonstrated electrophysiologically. Recently, hESC-derived dopaminergic neurons have been shown to reverse the functional deficit in parkinsonian rats.⁵⁹

Together, these findings support the notion that hESC-derived NPCs produced in vitro have the potential to functionally integrate with host neuronal circuits and restore functional neurological deficits. The site-specific migration and differentiation of hESC-derived neuroepithelial cells in the neonatal and adult brain environment presents an opportunity for directed functional differentiation prior to and/or after transplantation.^{34,67}

Custom-Designed Neural Cells: Directed Differentiation

Directing cell differentiation down a given path requires an attempt to recapitulate the temporal and spatial environment that the cell would normally experience in becoming that final cell type. This means exposing cells to proper concentrations and sequences of factors. To achieve this goal would require a complete understanding of development of the nervous system. Human ESCs, while offering a potential source for cell replacement therapy, are at present proving to be more beneficial in adding to our understanding of human neurodevelopment.

One disease priority in cell replacement research has been PD. Electrophysiologically active dopamine neurons have been generated from hESCs through strategic and systematic application of FGF-8 and Shh.^{33,57} Importantly, these cells contribute to locomotive functional recovery in the 6-hydroxydopamine lesioned rat striatum 5 months after transplantation with improvement correlating to the dopamine neurons present in the graft.^{37,59} Underscoring the importance of temporal exposure to a given morphogen is the observation that when cells are treated prior to expression of Sox1, they generate dopaminergic neurons with characteristics of midbrain projection dopamine neurons (large cell bodies, complex processes, and coexpression of En1) while treatment after Sox1 expression results in production of dopaminergic neurons that lack En1 expression. Dopaminergic neurons can also be differentiated by coculture of ESC-derived cells with stromal cells such as PA6,^{8,24,32,48,62} MS,^{33,39} and HepGII.⁴⁰

Amyotrophic lateral sclerosis is a disease associated with the loss of motor neurons. Motor neurons can be specified from hESC-derived neuroectodermal cells in a manner similar to that for PD. Prior to, but not after, Sox1 expression, the neuroectodermal cells can be efficiently posteriorized by retinoic acid and, in the presence of Shh, can differentiate into spinal motor neurons that express HB9, HoxC8, choline acetyltransferase, and vesicular acetylcholine transporter, and are electrophysiologically active.²⁷ Transplantation of these cells into the developing chick embryo results in robust engraftment, maintenance of motor neuron phenotype, and long-distance axonal projections into peripheral host tissues. Transplantation into the spinal cords of adult rats yielded grafts with a large number of human motor neurons and outgrowth of choline acetyltransferase-positive fibers.²⁶

Other cells produced from hESCs include serotonin neurons,³⁸ γ -aminobutyric acid neurons, glutaminergic neurons,^{10,67} and myelinating oligodendrocytes.²⁹ Current efforts are directed towards developing reproducible protocols for directing cells to the appropriate phenotypes.

Immune Rejection and Somatic Cell Nuclear Transfer

Embryonic stem cells derived from in vitro fertilized embryos differ genetically from the patient's own cells and thus could be rejected. One common method for overcoming immunological rejection after transplantation is immunosuppression. Long-term immunosuppression, however, is associated with multiple problems, including opportunistic infection. An alternative strategy involves generating ESCs that are genetically identical to the patients' own cells.^{22,63,65}

Somatic cell transfer allows trans-acting factors present in the mammalian oocyte to reprogram fully differentiated somatic cells to pluripotent stem cells that exhibit the essential characteristics of ESCs. Takahashi et al.⁴⁹ induced pluripotent stem cells from mouse fibroblasts by retroviral introduction of Oct3/4, Sox2, c-Myc and Klf4, and these iPSCs were similar to ESCs in morphological characteristics, capacity for proliferation, and tendency for teratoma formation. Okita and colleagues³⁰ were able to generate similar mouse iPSCs with increased ESC-like gene expression and DNA methylation patterns by selecting for Nanog

expression. Successful reprogramming of differentiated human somatic cells into a pluripotent state may allow creation of patient- and disease-specific stem cells.

Recently, *Rhesus macaque* blastocysts were produced from adult skin fibroblasts using a modified somatic cell nuclear transfer approach, with successful isolation of 2 ESC lines. Deoxyribonucleic acid analysis confirmed that nuclear DNA was identical to donor somatic cells and that mitochondrial DNA originated from oocytes. Both cell lines exhibited normal ESC morphological characteristics, expressed key stem-cell markers, were transcriptionally similar to control ESCs, and differentiated into multiple cell types in vitro and in vivo.⁹

In a recent paper, Yu and colleagues⁶¹ were able to reprogram human somatic cell nuclei to pluripotent stem cells that exhibit the essential characteristics of ESCs using Oct4, Sox2, Nanog and Lin28. These human iPSCs have normal karyotypes, express telomerase activity, express cell surface markers and genes that characterize hESCs, and maintain the potential to differentiate into all three germ layers. These results, taken together, demonstrate proof-of-concept for so-called “therapeutic cloning.” In the future, iPSCs may be produced without the need for viral transduction, and instead via nongenetic approaches.

Safety of ESCs for Cellular Replacement

A serious problem with the use of ESC-derived neural precursors for cellular replacement is the known tendency of undifferentiated ESCs to form tumors. Tumor formation by a transplanted cell population derived from hESCs is thought to be due to the presence of undifferentiated ESCs in the transplanted graft and the inherent ability of ESCs to produce all cell types in the body.⁵ Brederlau and colleagues⁶ reported “severe teratoma formation” in rats grafted with hESCs predifferentiated in vitro for 16 days. There have been other reports of cell overgrowth and tumor formation in rats receiving grafts derived from hESCs within a short period (8–13 weeks).^{37,43} However, in similar investigations by the senior author (S. C. Z.), no evidence of tumor formation was found, as evidenced by lack of rosette structures within the graft and scant Ki 67 expression even 5 months posttransplantation.⁵⁹ We believe that the lack of tumor formation in these studies is secondary to the absence of contaminating ESCs in the original tissue graft. In other words, all grafted cells were committed to neural lineage, with no potential to form tissues of other germ layers. This laboratory uses a unique differentiation protocol that essentially eliminates undifferentiated cells.⁶⁷ Also, in these studies the hESCs are differentiated in vitro for a total of 7 weeks, a much longer time period than in other reports. Still, concerns over tumor formation are valid, and much work needs to be done before hESC-derived precursor cells will be considered safe for clinical studies.

To eliminate the possibility of tumor formation from ESC-derived progenitor cells, steps would need to be taken to ensure that grafts are free of undifferentiated ESCs. In other words, all grafted cells would be of a lineage stage similar to the NSCs transplanted in other described clinical studies. Strategies to achieve this include positively sorting out the target cells using cell surface markers or by using cell type specific transcription factors through homologous recombination or by use of promoters.^{56,68} Alternatively, pluripotent stem cells could be removed using stem cell surface molecules.

In addition, human ESC-derived neuroepithelial cells are at a primitive stage of development. These cells tend to undergo many more cell cycles than fetal-derived NSCs. Therefore, human ESC-derived neural transplants are often associated with an overgrowth of neuroepithelia^{37, 43} but not with teratoma formation. To avoid such overgrowth, the number of primitive neuroepithelial cells used must be minimized. This may be achieved by expanding the neuroepithelia before transplant, breaking the neuroepithelial rosettes (which keep cells in a primitive stage), sorting the dividing population out, or eliminating the dividing cells with pharmacological agents.

Future Directions

Problems in addition to tumor formation and immune rejection that must be addressed include promotion of migration and dispersement of cells after transplantation, directed long-distance axonal projection, and controlled transmitter release.

Growth cone migration and synaptogenesis are part of a complex 2-way developmental process that cannot be recreated in the mature adult brain environment. Even with establishment of synaptic connections, replaced neurons may not secrete neurotransmitters in a controlled or regulated fashion due to faulty afferent inputs. Efforts are directed toward regulation of neurotransmitter release with means such as genetic manipulation. Another potential problem is the lack of dispersement and/or migration of cells after engraftment. Many of the described diffuse neurological conditions would ideally require dispersement of cells to cover virtually all regions within the brain. No cell has been shown to accomplish this well in mature animal models that are near human size. Human ESC-derived NPCs have perhaps the greatest capacity for dispersement and appear to preferentially migrate throughout the white matter.¹⁶ Efforts to promote cell dispersement are aimed at manipulation of the transplanted cell and/or its environment.

Conclusions

Great strides have been made in the isolation, purification, and directed differentiation of hESCs. Neural progenitor cells derived from hESCs and produced in vitro show promise in animal models with the potential for functional integration and behavioral improvement. Investigations using ESCs have greatly improved our understanding of human nervous system development, brain tumor genesis, and pharmaceutical screening. The use of hESC-derived neural cells in patients is a distant goal. Much work still needs to be done in producing a purified source, promoting dispersement and integration, and ensuring safety.

Abbreviations used in this paper

CNS, central nervous system; ESC, embryonic stem cell; FGF, fibroblast growth factor; hESC, human ESC; iPSC, induced pluripotent stem cell; NPC, neural progenitor cell; NSC, neural stem cell; PD, Parkinson disease; Shh, sonic hedgehog.

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FIG. 1.

Relationship between cell behaviors and lineage position of a cell. As a cell progresses from an ESC to a neuron, production efficiency (in vitro expansion capability) and ability to self renew, proliferate, migrate, and differentiate progressively decreases, while neuronal phenotype predictability and safety of the transplant increases. ES = embryonic stem; N = neuron; NE = neuroepithelial; NP = neural progenitor. *Adapted from original: Guillaume DJ, Zhang SC: Neuronal replacement by transplantation, in Bottenstein J (ed): **Neural Stem Cells: Development and Transplantation**. Norwell, MA: Kluwer Academic Publishers, 2003, p 309. With kind permission of Springer Science and Business Media.

TABLE 1

Summary of the advantages and disadvantages of potential donor cells*

Cell Type	Donor Source	Advantages	Disadvantages
ESC	embryo	unlimited expansion in undifferentiated state, enormous differentiation potential	difficulty in directing in vitro differentiation, potential of teratoma formation
neural stem/ progenitor cells	fetal brain	long-term in vitro expansion, large differentiation potential, safe	lack of ability to differentiate into projection neurons
neuronal-restricted progenitor cells	embryo or fetal brain	predictable differentiation to neuronal phenotype, safe	restricted neuronal differentiation potential, very limited expandability
primary neural cells	fetal brain	predictable phenotypic differentiation, safe	lack of expandability, restricted differentiation potential, require stage-specific embryonic source, mixed/inconsistent cell types
adult stem cells	adult “self” neural or nonneural	avoids ethical & immunological constraints, expandable in culture, possible autologous transplant, safe	greatly restricted potential in neural differentiation, questionable functional differentiation & integration
xenotransplant cells	nonhuman	readily available, possible genetic modification	tendency of immune rejection, possible zoonoses
iPSCs	adult somatic cells with nuclear transfer	immunosuppression avoided, large differentiation potential, expandable	difficulty in directing in vitro differentiation, potential of teratoma formation

* Adapted from original: Guillaume DJ, Zhang SC: Neuronal replacement by transplantation, in Bottenstein J (ed): **Neural Stem Cells: Development and Transplantation**. Norwell, MA: Kluwer Academic Publishers, 2003, p 309. With kind permission of Springer Science and Business Media.