

Adult Stem Cells for Neuronal Repair

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Stem cell research offers great hope to patients suffering from neuronal damage. Stem cell-based regenerative medicine holds huge potential to provide a true cure for patients affected by a neurodegenerative disease or who have suffered a stroke.

A stem cell possesses two distinct traits that define it: self-renewal and differentiation capacity. Self-renewal is the process whereby a single cell gives rise to two daughter cells – one being identical to the original cell and the other further committed towards a more restricted phenotype. In turn, the further committed progenitor cell, upon specific cues and signals, may proceed and differentiate to a mature cell type, a process termed differentiation.

The stem cell population is comprised of two main cell types: embryonic stem cells and adult stem cells. The first are derived from the early blastocyte and are well known for their pluripotency, the ability to differentiate into virtually any specific cell type in the adult organism. The latter have been known for years for their capacity to differentiate along their lineage of origin (for example, hematopoietic stem cell differentiating into any mature blood cell type). However, there have been recent reports of the adult stem cell's ability to differentiate along different lineages than its original organ, showing multipotency and crossing lineages in a phenomenon known as "trans-differentiation."

The exact mechanism responsible for the beneficial effect of stem cell cellular treatment has yet to be elucidated. In principle there are three different approaches. The first is cell replacement; namely, the direct replacement of the degenerated cells with functional cells, e.g., implantation of differentiated dopaminergic neurons to replace lost cells in the denervated nigrostriatal pathway in patients with Parkinson's disease. The second approach is neuroprotection, where transplanted stem cells provide environmental support to the affected brain cells by secreting essential cytokines and neurotrophic factors, e.g., intravenous administration of undifferentiated bone marrow stromal cells in the treatment of stroke. The third is gene delivery, i.e., stem cells serve as a vehicle to deliver specific supportive genes in the affected area; e.g., implantation of engineered stem cells over-expressing glial-derived neurotrophic factor to the brains of PD patients will protect the remaining unaffected neurons and restrain the onset of the disease.

PD = Parkinson's disease

Cellular therapy for PD: clinical trials

Parkinson's disease affects more than 1% of the population over age 60 in the western world. PD is characterized by massive loss of specific dopaminergic neurons in the substantia nigra pars compacta and causes severe motor symptoms. Current treatments for PD comprise mainly dopamine replacement and symptom alleviation, rather than a cure for the disease. The type specificity of the damaged cells makes PD patients ideal candidates for cellular therapy strategies. Cell-based therapies for PD have until now focused primarily on transplantation of differentiated fetal nigral cells. From the beginning of the last decade, different groups have reported reasonably good results in a series of open label trials [1,2]. Benefits were associated with increased striatal fluorodopa uptake and evidence of graft-related dopamine release on positron emission tomography. Postmortem studies demonstrated long graft survival years after transplantation and a significant re-innervation in the patients' striata. These results encouraged the U.S. National Institutes of Health to sponsor two double-blind trials of fetal nigral tissue transplantation. In one study, Freed and colleagues [3] in Colorado implanted solid grafts of human embryonic mesencephalic cells derived from two donors per side into the striata of advanced PD patients. Despite achieving a modest improvement in motor function in patients under age 60, the study failed to meet its primary endpoint – to improve the quality of life.

In the second study, Olanow et al. [4] implanted fetal nigral transplants derived from one or four donors per side into the posterior putamens of PD patients. In this study, graft deposits were placed at closer intervals to try and obtain continuous innervation of the striatum and immunosuppression was employed for 6 months. In general, clinical outcome proved to be better than in the Colorado experiment. Post hoc analysis noted significant improvement, especially in patients suffering from milder symptoms prior to transplantation. In addition, transplanted patients improved as compared to placebo-treated patients in the first 6 months, but deteriorated afterwards. This deterioration coincided with the cessation of cyclosporine, and raised the possibility that immune rejection may have played a significant role in the long-term outcome of these patients. However, a substantial percent of patients in both trials suffered dyskinesias that persisted after stopping levodopa ('off-

medication' dyskinesia) that had not been described prior to transplantation.

More encouraging results were reported by Mendez and group in 2002 [5]. In that study, the graft was composed of fetal ventral midbrain cell suspension rather than intact tissue as previously described. Patients in these trials did not suffer from dyskinesias and showed some level of clinical improvement. Isacson et al. [6] recently conducted postmortems of brains of participants in that study and noted a 3 year survival of cells and a significant striatal reinnervation. The authors emphasize the importance of dopaminergic cell-type specification, stating that the transplanted cells would optimally acquire the A9 dopaminergic neuron phenotype in order to properly reinnervate the degenerated pathways causing the parkinsonian symptoms. The data obtained from the postmortems support the possibility of reconstructing the degenerated nigrostriatal pathways with functional dopaminergic cells, demonstrating that dopamine neuronal replacement cell therapy can be beneficial for PD patients.

Taken together, these studies highlight the therapeutic potential of cellular therapy for neurodegenerative diseases in general and PD in particular. However, while the need to establish the best transplantation strategy is evident, several questions remain unresolved. Choosing the right patients, finding the optimal cell source, using immunosuppression and controlling the side effects are just some of the issues still waiting to be addressed. Most of all, the controversial use of fetal tissues and the necessity to generate a reliable reservoir of dopaminergic neurons has led researchers to explore other stem cell resources for possible cell-based therapies.

Adult stem cells versus embryonic stem cells

Embryonic stem cells represent the classic manifestation of a stem cell population, showing great capacity of self-renewal and differentiation. Massive advancement in embryology and neurogenesis has helped ESCs researchers develop protocols that generate neural progenitors capable of differentiating into neurons, astrocytes and oligodendrocytes from human ESCs [7].

On the other hand, there are the adult stem cells, which are comprised of several subtypes of cell populations. The most studied adult stem cell population is the hematopoietic, which reside in the bone marrow and have long been known for their ability to replenish the blood system after immunologic irradiation of patients suffering from hematologic malignancies, by differentiating along the blood system hierarchy. However, in recent years a large body of evidence indicated that some subpopulations of adult stem cells are capable of differentiating into mature cells different from their original lineage, a phenomenon termed transdifferentiation. These multipotent adult stem cells reside in various compartments in the mature organism and display plasticity initially thought to belong exclusively to ESCs. Those cells have been isolated from brain [8], bone marrow [9], skin [10], fat [11], skeletal muscle [12] and other visceral organs.

These findings widened the spectrum of possible sources for

restorative medicine. Nonetheless, why should one go so far as to induce a blood cell to become a neuron rather than simply differentiate the more hymeneal ESC? Well, there are three major considerations to bear in mind. Firstly, the use of ESCs and fetal tissues raises major ethical issues. Secondly, implanting ESCs will always involve allograft transplantation, raising immunologic consequences while the ultimate use of adult stem cells will provide the possibility of autologous cell transplantation. Thirdly, adult stem cells, by their very nature of being more committed than ESCs, are of less risk than for teratoma formation [13]. Taking all of this into account, one must consider the different aspects of adult versus embryonic stem cells for clinical use, the first being more available and less risky, the latter more malleable and potent in terms of differentiating capacity.

Embryonic stem cells to neurons

The isolation of human embryonic stem cells has stimulated research aimed at the selective generation of specific cell types for regenerative medicine [14]. In recent years researchers have been relentlessly studying the mechanisms involved in the embryonic development in rodents and understanding the cues and signals directing the early inner mass cell towards the neuroectodermal lineage and onward towards maturing into fully differentiated nerve cells. In principle, stem cell researchers are attempting to apply that knowledge to develop protocols directing the uncommitted stem cell to become a neuron.

In the PD field of interest, McKay and colleagues [15] reported the generation of dopaminergic neurons from mouse embryonic stem cells *in vitro* after selecting central nervous system progenitors from the primary ESC population, expanding the cells in the presence of basic fibroblast growth factor and inducing dopaminergic differentiation by withdrawal of the mitogen and addition of cAMP and ascorbic acid. Moreover, their work showed that the addition of molecules previously known to be involved in the development of midbrain dopaminergic neurons, sonic hedgehog and FGF-8, significantly raised the expression of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. The induced cells expressed specific markers of dopamine neurons, secreted dopamine in response to depolarization and showed electrophysiologic properties similar to neurons. Since then several groups have been able to generate dopamine-producing cells from human ESCs by following or optimizing the differentiation protocol, achieving a higher yield of differentiated dopaminergic cells [16].

When transplantations of ESC-derived dopaminergic neurons were performed in animal models of PD, success was only partial. Takagi et al. [17] managed to generate dopaminergic neurons from primate ESCs and achieved behavioral improvement upon transplantation into MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) models of primates. Nevertheless, histologic analysis revealed a low rate of TH-positive surviving neurons after transplantations.

FGF = fibroblast growth factor

TH = tyrosine hydroxylase

ESC = embryonic stem cell

Xu et al. [18] reported a symptomatic improvement in a rat 6-hydroxydopamine model of PD after transplantation of mouse ESC-derived neural progenitors, again despite a low survival rate of cells 6 weeks after transplantation (2%). As for human ESCs, Reubinoff and co-workers [19] are the only group to date to report a behavioral improvement in a PD rat model after transplantation of hESC-derived neural progenitors. The authors point to a correlation between TH+ cell presence and the degree of behavioral improvement. But once again, the TH+ population was significantly reduced as compared to studies conducted *in vitro*, suggesting the need to direct cells to dopaminergic differentiation prior to transplantation. However, researchers who tried implanting ESC-derived dopaminergic neurons to animal models have not yet achieved a significant improvement in PD models [20,21]. Possible explanations for these disappointing results could be the low survival rate of TH+ neurons due to host immune response and apoptotic behavior of dopaminergic cells in the host brain, perhaps due to limited success of the differentiation protocol. Moreover, some of these studies have also reported ectopic non-neural protein expression of the transplanted cells in the brain, suggesting the possibility of teratoma formation [20,21].

Adult stem cells to neurons

The generation of differentiated cells with a mature neuronal or astroglial phenotype has been achieved using cells from the adult brain, bone marrow, skin, fat, muscle and more. It would be fair to say that a consensus is emerging among scientists today that there are stem cells in every compartment of the adult body, although the role of some of them in adult life is still unknown. The advantages of autologous stem cell transplantation are a great incentive for researchers to develop protocols that will eventually allow medicine to utilize adult stem cell for neuronal repair [Figure 1].

Brain-derived neural stem cells

It was long considered an axiom that the brain is deprived of the ability to produce new neurons. However, Gage [8] presented evidence for the existence of adult neural stem cells in the adult brain that are able to differentiate into all three neural cell types: neurons, astrocytes, oligodendrocytes. Those stem cells are characterized by their ability to form neurospheres, floating cell aggregates widely expressing the filament nestin, and their ability to expand extensively in the presence of the mitogens, epithelial growth factor and b-FGF. The subventricular zone and the dentate gyrus are known for their relatively abundant population of stem cells, but recent work suggests that even the striatum and substantia nigra contain a subpopulation of quiescent stem cells [22,23]. Neural stem cells have been of great interest to scientists seeking a cure for neuronal damage.

On the one hand, NSCs are a long way ahead of ESCs in their commitment to the mature neuronal or glial phenotype and are less prone than ESCs to form teratomas. On the other hand, they do not have to cross lineage borders in order to differentiate to mature CNS cells in comparison to adult stem cells residing in other compartments.

In rodents, Gritti and team [24] reported the expansion of NSCs from the olfactory bulb of adult mice following bulbec-tomy. NSCs proliferated extensively in the presence of EGF and b-FGF before being induced to differentiate into neurons, astrocytes and oligodendrocytes upon exposure to different growth factors and cytokines. Moe and colleagues [25] recently described the efficient formation of a functional neural network originated from a single cell of the adult human brain. NSCs were obtained from biopsies and expanded at the single cell level to form a large cell population. Upon withdrawal of mitogens and the addition of fetal calf serum, cells matured and expressed glutamate receptors, vesicular glutamate transporter and other mature neuronal proteins such as NeuN. Moreover, patch clamp analysis showed that the cells possess mature electrophysiologic properties. The authors argue that NSCs can easily be harvested following neuroendoscopy from the ventricular wall and may serve as a resource for autologous transplantation in the future.

Bone marrow-derived stem cells

The therapeutic potential of bone marrow transplantation has already proven itself in the treatment of hematologic malignancies. Whole bone marrow contains two distinct cell populations, the hematopoietic cells and the non-hematopoietic cells. Hematopoietic stem cells are well characterized as CD34+ cells and have been used in the clinic with major success for hematologic disorders. The non-hematopoietic cells are widely known for their essential role in nourishing the HSCs by secreting different growth factors and have also been used as a feeder layer supporting the development of ESCs. In the 1970s, Friedenstein et al. [26] found a stem cell population negative for HSC markers. Those cells showed the capacity to self-renew and differentiate along the mesenchymal lineage into bone, fat and cartilage cells. These cells are the bone marrow stromal cells, also referred to as mesenchymal stem cells. Recent studies have shown that under specific conditions, MSCs are capable of differentiating to other mesodermal lineages, extending their plastic potential.

Mesenchymal stem cells

Woodbury et al. [27] were the first to report the MSC capacity to break the mesenchymal lineage barrier and to differentiate into neurons. Rat and human MSCs were expanded before being induced to differentiate in a serum-free medium containing β -mercaptoethanol and other antioxidants. Upon exposure to induction medium, MSCs dramatically changed their morphology from long flat spindle-shaped cells to contracted rounded cells with long extensions resembling neurite outgrowths. The induced cells expressed typical neuronal proteins, such as

NSCs = neural stem cells

CNS = central nervous system

EGF = epithelial growth factor

HSCs = hematopoietic stem cells

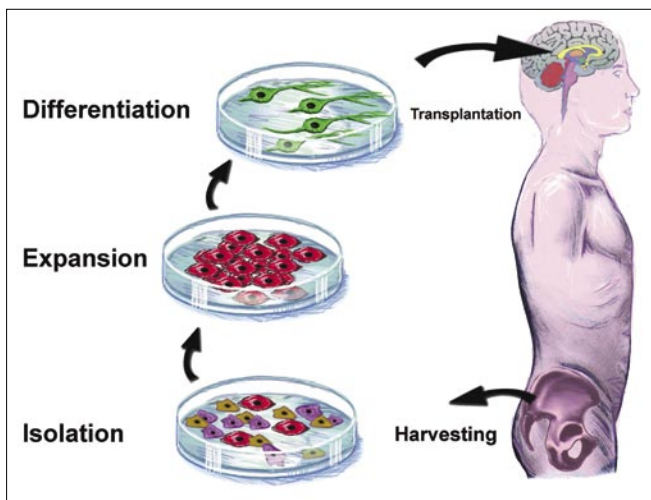


Figure 1. Schematic illustration of autologous bone marrow-derived mesenchymal stem cell transplantation for cellular replacement or neuroprotection.

neuron-specific enolase, neurofilament M and the neuronal nuclear-specific antigen NeuN. Sanchez-Ramos and collaborators [28] reported the differentiation of human and rat MSCs to both neuronal and glial cells in the presence of retinoic acid and brain-derived neurotrophic factor. Moreover, their experiments showed that co-culture of MSCs with NSCs derived from neonate mice or human fetal tissues promoted the neuronal differentiation of MSCs.

To determine whether trans-differentiation of MSCs to neurons could also occur *in vivo*, Munoz-Elias et al. [29] transplanted rat MSCs into the rat embryo. The transplanted cells integrated in the brain parenchyma and followed specific developmental cues and migratory pathways of the rat neurogenesis. Abundant donor cells were detected in the neocortex, hippocampi, cerebellar peduncles, colliculi, thalamus, midbrain, forebrain germinal zones, rostral migratory stream and olfactory bulbs. The cells expressed different gene products and morphologies in a region-specific manner, apparently exhibiting the plastic ability to respond specifically to different microenvironments.

Dezawa et al. [30] reported on the sciatic nerve regeneration induced by transplantation of schwann cells derived from MSCs. Rat MSCs were expanded and induced *in vitro* to express a schwann cell phenotype expressing S-100, O4 and GFAP following incubation with b-FGF, platelet-derived growth factor, forskolin and retinoic acid. The induced cells were transplanted into the proximal site of the dissected sciatic nerve. Within 3 weeks of the operation, transplanted cells elicited vigorous regeneration, accompanied by remyelination, compared

to rats transplanted with undifferentiated MSCs. The authors concluded that not only are MSCs capable of nerve regeneration but that differentiation *in vitro* prior to transplantation is essential to achieve optimal results.

The generation of dopaminergic neurons is of utmost importance in the quest to find a cure for PD. Storch et al. [31] described the generation of neural stem cells from human MSCs. These cells expanded in floating aggregates resembling neurospheres, displayed clonal capacity and were differentiated at the single cell level to both neurons and glial cells. Moreover, after induction with brain-derived neurotrophic factor, cells secreted dopamine in response to depolarization and showed electrophysiologic properties typical of dopaminergic neurons.

In our laboratory, Levy and co-workers [32] detailed the induction of the neuronal specific enolase promoter and the induced expression of other neuronal markers following neuronal differentiation of mouse MSCs. Moreover, when dopaminergic induced mouse MSCs were transplanted into a mouse 6-hydroxydopamine model of PD, we observed a reduction in rotational behavior as compared to mice injected with saline [paper in preparation]. As for human MSCs, we recently reported the efficient dopaminergic induction of human MSCs [presented at the Third ISSCR Conference]. MSCs were expanded and characterized for the typical MSC cell surface phenotype; the cells also ubiquitously expressed the typical neuronal progenitor marker nestin. Upon exposure to various differentiation media the induced cells acquired a typical neural morphology, expressed a variety of neuronal proteins including TH, and secreted dopamine in response to depolarization [Figure 2].

The mechanism whereby clinical improvement following MSCs transplantation is achieved has yet to be elucidated. One possibility is that the graft cells functionally replace the dam-

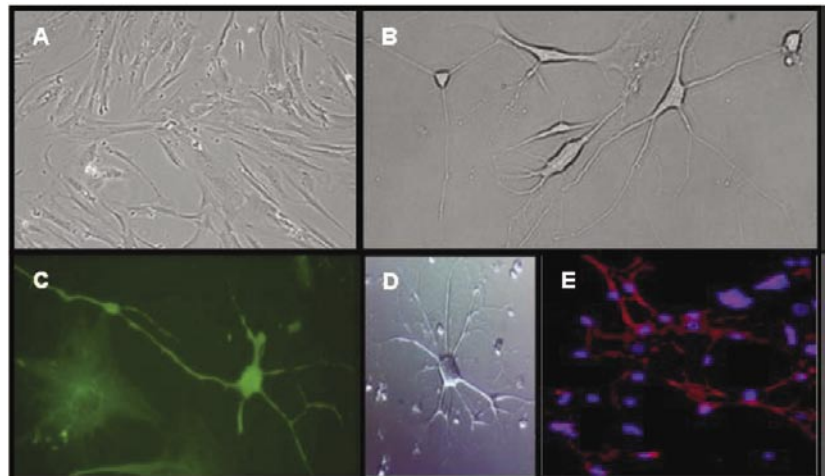


Figure 2. Neural induced human mesenchymal stem cells display neural morphology and express neuronal proteins. **[A]** Untreated human mesenchymal stem cells. **[B]** Mesenchymal stem cells following neuronal induction. **[C]** Mesenchymal stem cells following neuronal induction stained for Tuj1 (β -tubulinIII). **[D]** Mesenchymal stem cell following neuronal induction demonstrates astrocyte-like morphology. **[E]** Mesenchymal stem cells following neuronal induction stained for tyrosine hydroxylase.

MSCs = mesenchymal stem cells

aged cell population following trans-differentiation *in vivo* or *in vitro* prior to transplantation. However, some researchers claim that trans-differentiation is at most a marginal phenomenon that cannot account for the observed clinical improvement. Chopp and colleagues [33–35] administered human MSCs intravenously into animal models of various neurologic disorders. These included the MPTP mouse model of PD, a stroke model in rats, and recently in an experimental allergic encephalitis model in rats bearing symptoms similar to multiple sclerosis. In all three experiments a significant clinical improvement was observed. Intriguingly, immunohistochemistry revealed the abundant presence of administered MSCs near the damaged areas in the brain in comparison to unlesioned controls, suggesting that MSCs follow chemotactic cues and migrate through inflammatory pathways. However, Chopp and team attribute the clinical improvement to the supporting effect of the MSCs rather than to cell replacement following trans-differentiation. These investigators claim that although some cells stain positive to neuronal or astroglial markers, their level of expression is not sufficient to account for the clinical improvement. The therapeutic effect is rather related to a secretion of neurotrophic factors such as brain-derived neurotrophic factor, nerve growth factor and other factors such as vascular endothelial growth factor and inflammatory modulatory molecules.

Cord blood stem cells

Umbilical cord stem cells are considered an important source for autologous cellular therapies. Like bone marrow, cord blood consists of both a hematopoietic and a non-hematopoietic stem cell population. In recent years UCBSB transplantations have proved effective for treating children with hematologic diseases. However, the relatively small amount of cells harvested from a single cord makes it crucial to further develop new technologies that enable sufficient expansion for the treatment of adult patients. A review recently published by Sanberg et al. [36] sheds light on the possible potential of UCBSBs for neuronal repair. Like its counterpart stem cell population in the bone marrow, UCBSBs have shown plasticity *in vitro* by differentiating into both neurons and glia cells. Reports on the therapeutic effect of UCBSBs *in vivo* are still limited, but a couple of papers noted the beneficial effect of intravenous UCBSB transplantation in an animal model of stroke and an animal model of amyotrophic lateral sclerosis. However, the mechanism of the clinical improvement was not elucidated as there was no conclusive evidence of trans-differentiation, suggesting a more neuroprotective effect by inducing growth factor secretion or neovascularization.

Other adult stem cells for neuronal repair

A number of groups worldwide have observed trans-differentiation to neural cells in stem cells derived from tissues other than brain, bone marrow or cord blood. In a report published in the *Lancet* in 2004, Ioanides et al. [10] described the efficient generation of neural precursors from adult human skin. Dermis-

derived mesenchymal stem cells were isolated and propagated in the presence of EGF and b-FGF and expressed both neural stem cell transcripts and proteins. The skin-derived neural progenitors were then exposed to rodent astrocytes-conditioned medium. Upon induction the cells acquired typical neuronal morphology, expressed neuronal and glial proteins, and exhibited a reversible increase in intracellular calcium in direct response to potassium-mediated depolarization, which is consistent with the presence of voltage-gated calcium channels.

In another paper published in the *Lancet* in the same year, Alessandri and co-workers [12] described the neural induction of skeletal muscle-derived stem cell. Stem cells were obtained from brachioradialis muscle samples after tissue was minced and trypsinized. Cells were cultured in a typical neural stem cell culture medium containing EGF and b-FGF. Following massive cell death, the surviving cells proliferated and clonal analysis confirmed their stem cell qualities. Experiments *in vitro* indicated that the muscle-derived stem cells could be induced to express both neuronal and glial markers. However, an attempt to observe differentiation *in vivo* did not succeed because muscle-derived stem cells transplanted into an injured rat's spinal cord were found negative for neural specific marker staining.

Conclusions and future prospects

Stem cell therapy offers great hope for patients suffering from disease for which there is currently no cure. The notion of a scientist able to generate true cellular replacements to substitute defective cells in humans has sparked the imagination of doctors and researchers in all disciplines of modern medicine. Cellular treatment in cancer patients using hematopoietic stem cells has been used efficiently for more than 50 years and is now practiced routinely in the clinic. Stem cell transplantation in parkinsonian patients has proved feasible in several clinical trials.

In order to harness the full potential of stem cell therapy, science must overcome moral, logistic and clinical challenges. Adult stem cells appear to be the ideal candidates to serve as the cellular reservoir. In comparison to embryonic stem cells, adult stem cell harvesting does not involve the controversial use of embryos or eggs. They will optimally be transplanted autologously, decreasing to a minimum the risk of immune rejection and, because of their more restricted differentiation potential, are considered less prone to form tumors in the host.

Patients afflicted with neuronal damage, neurodegenerative disorders, spinal cord injury or stroke suffer the consequences of insufficient neurogenesis and cell renewal in their affected nervous system. Current therapies are mostly symptomatic and in some cases inefficient. The tireless efforts being invested in developing new technologies in stem cell research will hopefully yield the knowledge that will enable doctors to present patients with a cure.

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UCBSBs = umbilical cord stem cells

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