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Manufacturing neurons from human embryonic stem cells: biological and regulatory aspects to develop a safe cellular product for stroke cell therapy

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Abstract

Demographic trends, particularly those related to longer life expectancy, suggest that the demand for tissue and organ transplants will further increase since many disorders result from degeneration, injury or organ failure. The most urgent problem in transplantation medicine is the shortage or lack of suitable donor organs and tissue, leading to ethical and societal problems such as organ trafficking. The discovery of stem cells in the inner cell mass of developing embryos and in adult tissue has revolutionized the medical field by introducing new therapeutic dimensions to consider for previously untreatable diseases and injuries. The unlimited self-renewal ability and pluripotent capacity to become any cell type of the organism make human embryonic stem cells (hESCs) a compelling source of cells to study tissue histogenesis and to apply in a wide array of tissue engineering, cell transplantation therapy and drug discovery applications. In this article, we will focus on hESCs and address the derivation of therapeutic neural stem cell lines from hESCs, as well as the biological and regulatory aspects to developing a safe cellular product for stroke cell therapy.

Keywords

cell therapy; cGMP; good manufacturing practice; human embryonic stem cells; human neural stem cells; investigational new drug application; manufacturing neurons; master cell bank; neuroplasticity; product development; regulation of stem cell products; stroke

Human embryonic stem cells & potential applications in regenerative medicine

Embryonic stem cells (ESCs) are totipotent, genetically normal and possess unlimited self-renewal ability. Totipotency is tested by implanting ESCs into the inner cell mass of a host

embryo and creating a chimera, whereby all tissue is derived from both the ESCs and the host cells. Ethical considerations preclude testing totipotency of human ESCs (hESCs); however, their pluripotency has been demonstrated by their ability to differentiate into all three embryonic tissue types: endoderm, mesoderm and ectoderm [2]. Unfortunately, these stem cell properties come with a teratogenic potential. Indeed, transplantation of a sufficient number of undifferentiated hESCs into animals induces formation of a teratoma [3,4], containing undifferentiated and differentiated cells of all the germ layers. This safety concern has grown in urgency, as progress has been made in the development of cellular products from hESCs for cell therapy. Stringent and universal *in vitro* and *in vivo* assays must be developed to monitor and set limits for the expression of pluripotency markers and tumorigenicity to ensure safety of the final product.

The isolation of tissue-specific self-renewable stem cells from hESCs is the most promising application for regenerative medicine. These multipotent stem cells could be used directly for tissue repair or building *ex vivo*, when multiple cell types are lost to injury or disease. They can also be instructed to terminally differentiate into specific cell types when a single lineage species is associated with an injury or disease. The terminal differentiation is induced by culturing the multipotent stem cells in media containing specific instructive molecules or by overexpressing lineage-specific gene(s). However, further studies are needed in this area to generate cellular phenotypes that are stably expressed *in vitro* and after grafting into diseased or injured tissue. Harnessing mechanisms governing tissue histogenesis that take place during early embryogenesis is a promising strategy for engineering specific cell types or tissues. Likewise, the same developmental pathways could also be induced *in vitro* using a set of instructive cues different from those normally involved during embryonic development.

Derivation of neural cells from hESCs

Neural induction from ESC cultures is the predominant default pathway and occurs in the absence of instructive signals [5]. However, under these conditions the induction is not purely neural and cannot be efficiently maintained *in vitro*. Reliable protocols have been developed to generate neural lineage from hESCs. Although there are differences in processes and instructive signals used (Figure 1), all of these approaches can be divided into two main types: enrichment for neural lineage, or isolation of self-renewable multipotent neural stem cells (NSCs).

Enrichment of neural lineages from hESCs

This process consists of directly inducing neural lineage elaboration from pluripotent hESCs, followed by a short-term perpetuation of the neural precursors to generate a batch of neural progeny. Every time a new batch of neural progeny is needed the original hESC culture is tapped into and the risk of contamination with pluripotent cells increases. The neural precursors may be induced from hESCs by using one of the following protocols:

- Promote embryoid body (EB) formation through aggregation of undifferentiated hESCs in suspension cultures [6,7];

- Co-culture hESCs with a feeder layer such as stromal cell lines MS5, PA6 or S17 [8,9];
- Inhibit TGF- β signaling and allow the default neural induction pathway to take place [10-12];
- Expose the hESCs to HepG2-conditioned medium or neural-inducing media [13,14].

The next step consists of selectively growing the neural precursors in a serum-free media supplemented with a mixture of nutrients F12, N2 or B27 and mitogenic growth factors, including one or more of EGF, bFGF and LIF. These conditions select for immature neural precursors expressing the intermediate neurofilament nestin. Lastly, the nestin-positive precursors are further expanded as neurospheres and induced to differentiate into neuronal and glial fates.

Selective Isolation of neural stem cells from hESCs

An important issue often not fully addressed in neural induction by enrichment is whether or not the neural cells derived exhibit the fundamental three properties of NSCs:

- Self-renewal ability and maintenance of long-term cultures through multiple passages under clonogenic conditions;
- Generation of a large number of progenies;
- Differentiation into the three principal neural lineages such as, neurons, astrocytes and oligodendrocytes.

Three recent studies directly address this issue [15-17]. Elkabetz *et al.* isolated specific subpopulations of neural precursors based on the cell surface marker *Forse1*⁺ expression from the neural rosette structure [16]. These rosette-NSCs (rNSCs) exhibited clonogenic potential and were expanded up to four passages in the presence of sonic hedgehog, DII4 and jagged-1. rNSCs differentiate into neurons of the CNS and peripheral nervous system and cannot be perpetuated *in vitro* by the use EGF and bFGF. Of interest, the transplantation of rNSCs into adult rodent brain induced tumor formation, suggesting that the cells maintained their pluripotent traits. One possible process to rid the tumorigenic property from the rNSCs is to perpetuate them in media containing EGF, bFGF and LIF.

Conti *et al.* isolated homogenous and clonogenic populations of NSCs from mouse and human ESC-derived rosettes [15]. The rosettes were mechanically transferred into serum-free media in the presence of EGF and bFGF. The NSCs isolated under these conditions were capable of long-term propagation (5 months), expressed nestin, vimentin and the radial glial marker 3CB2, and differentiated into neurons and astrocytes. Further differentiation of these NSCs toward oligodendrocytes required exposure to PDGF, forskolin and thyroid hormone, T3 [18]. After transplantation into adult mouse hippocampus and striatum, these NSCs engrafted and differentiated into neurons and astrocytes. More importantly these NSCs did not show signs of overgrowth in the brain. Nor did they form tumors after transplantation into the animal's kidney capsules [15].

Daadi *et al.* have recently reported the isolation and perpetuation of a homogenous population of NSCs [17] and developed a specific process to isolate growth factor-responsive primary neurospheres from hESCs in a serum-free media supplemented with EGF, bFGF and LIF and replated to eliminate other non-neural cells (Figure 2). The cumulative cell number and population doubling analysis demonstrated the continuous and stable growth of the human NSCs (hNSCs). These hNSCs were clonogenic and expressed the neural precursor cell markers nestin and vimentin, and the radial glial cell marker 3CB2. Under differentiation conditions, the SD56 hNSCs gave rise to neurons, astrocytes and oligodendrocytes, expressed transcripts for the neural-specific genes nestin, Notch1 and neural cell adhesion molecule, and for the lineage-specific markers β -tubulin class III, medium-size neurofilament and microtubule-associated protein 2 for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and myelin basic protein for oligodendrocytes. Transplantation of these cells into adult rodents did not lead to tumor formation.

From a therapeutic perspective, it is critical to determine whether the neurally induced cells possess all attributes to qualify for a potential clinical use.

Therapeutic attributes of NSC lines

In addition to proof-of-concept in animal models, a NSC line needs to possess key characteristics in order to be clinically and commercially relevant:

- Stable growth rate with short doubling time: to generate sufficient quantities in a reasonable and predictable time period in order to meet the demanding quality control/quality assurance (QC/QA) testing, preclinical testing and future clinical trials;
- Multipotent: to generate the three neural lineages with multiple neuronal phenotypes and to allow the cells to differentiate *in vivo* according to the local microenvironment. This is quite relevant in cell therapy for stroke, where multiple cell types are lost in the infarcted area;
- Stable differentiation profile: cell line to be regularly tested at late passages for multipotency and for potential drift in cellular composition from the initial passages. This is a critical property of cells destined for clinical use;
- Amenable for large-scale production: to scale-up the cell production and generate master and working cell banks from a therapeutic cell line in order to meet the demand to treat a large number of patients in multicenter clinical trials. Large studies are key to eliminate any variability between centers;
- Clinical or licensed product batch: in compliance with the regulatory agency the size of batches required for manufacturing a clinical product is at least 10,000-times the cell dose intended for the clinical use. Starting with a dose of 10 million per patient, one cell batch contains at least 100 billion (10^{11}) cells;
- Cryostability: good survival and recovery, with no change in properties after cryopreservation. This is a highly desirable property for collaborations, clinical use

and commercialization. A large batch of cells could be tested, released for clinical use or frozen in a microvial ready to be shipped for use at any geographical area.

Stroke

Stroke is a vascular disorder caused by a transient or permanent reduction of cerebral blood flow that leads to neurological consequences. Vascular thrombosis and embolus are responsible for ischemic strokes. However, loss of vascular integrity during focal cerebral ischemia leads to hemorrhage. Stroke has the highest annual incidence of any neurological disorder, including Alzheimer's disease, traumatic brain injury, epilepsy and Parkinson's disease. In the USA, more than 750,000 individuals suffer from stroke every year. There are approximately 5.4 million stroke survivors, of whom a third are unable to care for themselves and almost 75% are impaired in some activities of daily living. The estimated economic burden for stroke exceeds US\$56.8 billion per year in the USA.

Current effective therapeutic approaches, such as the use of thrombolytics, target acute stroke. Although acute thrombolysis has a significant impact on the management of stroke, it benefits only 1-4% of patients. This is due to numerous exclusion criteria, including a restrictive 3-h therapeutic window and a significant risk of hemorrhage [19]. Consequently, the vast majority of stroke patients experience progression of ischemia associated with debilitating neurological deficits.

There are no effective treatments to target residual anatomical and behavioral deficits resulting from stroke. Nevertheless, as illustrated by the large body of work, there is a concerted effort in the global research community to find strategies or methods to replace cell loss and to promote reorganization of the undamaged tissue and neural circuitry to restore the lost function. Stem cell therapy is one of the more promising approaches to alleviate or reverse stroke disability. However, to develop this potential therapy, careful consideration will need to be paid to the choice of animal model, cell preparation and delivery.

Animal model of stroke

Experimental models of cerebral ischemia include global and focal ischemia. As the name indicates, in global ischemia there is no cerebral blood flow to any area of the brain. In this model, neuronal injury is selective to vulnerable brain areas. Focal cerebral ischemia involves the occlusion of the middle cerebral artery (MCA), either at the distal or proximal part, and the occlusion may be transient or permanent. The MCA occlusion (MCAO) is the most common and extensively studied animal model for producing experimental focal cerebral ischemic lesion in animals because of its relevance to human thromboembolic stroke. The lesion produces an initial and secondary cascade of cell death in the brain that leads to acute and chronic impairments of sensorimotor and cognitive functions. The secondary damage results from: neuroinflammation, excitotoxicity, mitochondrial dysfunction, disruptions of calcium homeostasis and other severe alterations in basic cellular function. The clinical features modeled are hemiplegia, deficits in the contralateral skilled forelimb movements, somatosensory impairment, spatial neglect, and learning and memory deficits. The variability in stroke models is often not taken into consideration, specifically

with regard to the temporal profile and method of the vascular occlusion, the size and severity of the lesion and the extent of acute versus delayed effects [20]. Therefore, it is critical to be cautious when choosing the behavioral end point measures that are sensitive to the residual deficits and to correlate them with careful histological assessments.

Cell preparation, formulation & delivery

The cell preparation, formulation and delivery are critical steps for the success of the cell therapeutic approach. Once proven in the preclinical setting, these steps should be modeled in the clinic, as they affect the survival, engraftment and efficacy of the cells. Standardizing these three parameters when trying to replicate preclinical findings or in a clinical setup is the ideal situation to compare outcomes and improve the therapeutic approach. Frozen ampoules of cells is the preferred method of storing the cellular product as this allows for conducting QC/QA tests on a large lot of cells at once and to be able to meet all the necessary release tests and the need for large studies. This is again paramount in comparing outcomes between clinical sites. Various solutions may be used for cell formulation. Unfortunately, there are no studies systematically addressing the suitability of solutions for cell formulation and transplantation. Isotonic buffers are most often used in clinical setting [21-23]. Limits of acceptability need to be established to address the viability after thaw, the stability of the cells in the surgery room and the cryostability, as determined by periodic test-retests of cryopreserved ampoules (shelf-life).

The delivery impacts the cellular behavior and graft-host interactions. Studies suggest that the dispersion of grafted cells in the diseased or lesioned area, rather than an ectopic bolus of a high cell-density clump, may lead to better outcomes [24]. In this regard, it is important to highlight that the transplantation of a single cell suspension is a more desirable delivery method than clusters or pieces of tissue into the brain. This allows for precise dose-escalation studies to identify the potency of the cells, better cell-cell interaction between graft and host and better engraftment and less inflammation. Furthermore, in a single cell suspension, the cellular composition is more precisely identified and the *in vivo* behavior better predicted. Clusters may contain undesirable cells that may overgrow or influence ectopic regions and cause deleterious side effects. Among critical factors that influence functional engraftment and need to be established in animal studies is the optimal timing for cell transplantation. After stroke, excitotoxicity, peri-infarct depolarization, inflammation and immune response unfolds through a complex network of cytokines. This response is maximal during the first days/weeks postischemia and results in cell death [25]. In parallel, endogenous neuroprotection and regenerative repair is optimal during this period. Therefore, for a therapeutic strategy aimed at enhancing ongoing neuroplasticity and integration of grafted cells, an early cellular delivery would be desirable. Depending on the properties of the cell line, the timing of cell transplantation may vary. Thus for each cell line, a systematic analysis encompassing at least early and late time points for transplantation is necessary to determine the effects on functional recovery, cell engraftment and endogenous repair.

ESC-based therapy for stroke

In patients disabled by stroke, the concept of restoring function by transplanting hESC neural derivatives into the brain is simple and innovative. It consists of replacing the dead cells with new healthy ones. Although the prospect may seem remote, given the complex structure and function of the human brain and the various cell types lost to injury, the data currently available on animal model of stroke are promising. Cells transplanted into the ischemia-damaged areas survive, integrate and promote recovery of function through multiple mechanisms, such as attenuating the inflammatory reaction that takes place after stroke, making new neural cells and synaptic connections with the host cells, enhancing vascularization in the remaining brain tissue and increasing native axonal reorganization, sprouting and dendritic branching. All of these phenomena synchronize with the spontaneous neural plasticity that takes place poststroke to reorganize the brain in a functionally appropriate manner (Figure 3).

To date most ESC transplantation studies published have used mouse or primate ESCs. Early studies have demonstrated that in stroke rodent models the transplantation of heterogeneous neurally induced cell populations containing contaminating pluripotent stem cells leads to graft overgrowth and the formation of teratomas. Erdo *et al.* used undifferentiated mouse ESCs (D3) expressing green fluorescent protein (GFP), transplanted into mouse and rat stroke models [26]. Rats were grafted 2 weeks poststroke with $2 \times 40,000$ ESCs into the contralateral corpus callosum and striatum. After a 3-week survival period two out of 22 rats developed small microscopic tumors near the graft. The grafted cells migrated along the corpus callosum toward the lesioned hemisphere. In stroke mice, however, the transplantation of $2 \times 25,000$ ESCs into the same location, with the same timing, produced large macroscopic tumors in 10 out of 11 animals. Interestingly, there was no migration of these cells toward the stroke-lesioned hemisphere. These data demonstrated that undifferentiated ESCs develop aggressive tumors in the brain as early as 3 weeks poststroke and that it is more likely that tumors develop in an allogeneic transplantation paradigm than in a xenogeneic graft model.

Takagi *et al.* induced neural lineage from mouse ESCs (G4-2) by co-culture with PA6 cells [27]. Focal ischemia was induced in BL6 mice using a suture occlusion for 30 min. Whole 'neurospheres' (2 μ l) were transplanted 2 days after ischemia. The analysis of graft size demonstrated that there was an 8% increase in graft size between 2 and 4 weeks post-transplant. No data are available for longer time points. The differentiation profile of grafted cells showed that 60 and 40% expressed the neuronal markers NeuN and TuJ1, respectively, 22% becoming astrocytes and 0.4% oligodendrocytes. However, the transplanted cells formed a mass with signs of overgrowth and deformations. These results highlight the tumorigenic potential of transplanting not fully differentiated clusters of neurally induced ESCs.

To derive neurons from the mouse ESC lines EB3 and ES-enhanced GFP (EGFP), Nonaka *et al.* induced the formation of EBs using the hanging drops method for 4 days with a 100 nM retinoic acid treatment [28]. The predominant cell type on the culture was nestin⁺. Intracerebral hemorrhage was induced by stereotaxic injection of collagenase VII into the

striatum of rats followed 7 days later by the transplantation of 100,000 neural cells (5 μ l) into the lateral ventricle. After 1 month post-transplant survival time, grafted cells migrated cross the ventricular wall toward the lesioned area and engrafted in the boundary of the hematoma cavities. Some cells were GFAP⁺ and others were microtubule-associated protein (MAP)2⁺, no quantitative analysis was reported. This study also reported uncontrolled overgrowth of nodules containing transplanted cells. The tumorigenic property of the grafts is not surprising, since approximately 90% of the neurally induced cells expressed nestin, suggesting that 10% of cells may contain pluripotent cells. These studies clearly highlight the heterogenous cellular composition in the generation of neural cells under these protocols and suggest that other measures, such as cell sorting [29,30] or genetically engineering cells with a suicide gene [31-33] would reduce the risk of teratomas.

Wei *et al.* used mouse ESCs genetically modified to express the human anti-apoptotic gene *bcl-2*, grown without LIF for 4 days with a trypsination after 2 days then treated with RA for an additional 4 days [34]. It is important to note that these cultures showed variability in cell viability between different platings and that electrophysiological parameters were used to select for healthy cultures destined for transplantation. Rats were subjected to a 2 h suture occlusion of the MCA. The transplantation was performed 7 days after stroke with 4 μ l (25,000 cell/ μ l) injections into four striatal sites. There were more surviving neurons in the *bcl-2*-expressing cells than in nontransfected cell transplants. The survival rate and the proportion of astrocytes and oligodendrocytes were not analyzed. No tumor formation was reported after the 35-day survival period. This study also showed a significant functional recovery in transplant groups using a neurological severity score.

Buhneemann *et al.* investigated the neurophysiological properties of neural cells derived from mouse ESCs ubiquitously expressing EGFP [35]. Neural cells were derived using the EB method followed by expansion of the neural precursors in FGF2-containing media. Stroke was induced using a transient occlusion of the MCA by infusing the vasoconstrictor agent endothelin-1 next to the MCA. Neural cells suspended at 50,000 cell/ μ l were transplanted into the infarcted zone 1 week later. Grafted cells were highly proliferative during the first month, as demonstrated by the expression of Ki-67. No teratoma formation was reported. Grafted cells differentiated into NeuN-expressing neurons (30%), astrocytes (7%) and oligodendrocytes (<1%). Electrophysiological properties were investigated in cells located in the center and periphery of the graft within the infarcted zone. Patch-clamp recordings demonstrated that 27.7% of grafted cells expressed action potentials and voltage-gated Na⁺ and K⁺ currents typical of the neuronal phenotype. Interestingly, graft-derived neurons showed spontaneous postsynaptic current between 4 and 7 weeks post-transplant, demonstrating that they receive synaptic inputs. This study suggests that neuronal replacement could support the behavioral recovery observed in animal models of stroke.

Li *et al.* transfected mouse ESCs with the myocyte enhancer factor 2, a calcium-dependent transcription factor that enhances cell survival and differentiation of muscle and neural cells [36]. The authors used mice subjected to 1 h transient MCAO and transplanted 1 day poststroke. This study reported that the transplantation of 50,000 transfected neural progeny was sufficient to induce behavioral recovery in the fear-conditioning test. Electrophysiological recording demonstrated that the grafted cells developed synaptic

connections with host brain, suggesting that cell replacement had positive effects on extinguishing the conditioned behavior.

Theus *et al.* incubated mouse ESCs in a low-oxygen environment as a hypoxic preconditioning approach to increase cell survival after transplantation into ischemic brain [37]. The rats subjected to MCAO were transplanted 48 h after stroke with 100,000 cells into each of four trajectories. A total of 3 days after transplantation there was a higher number of TUNEL and caspase-3-positive cells in grafts not treated with either low-oxygen incubation or erythropoietin. Furthermore, grafted animals showed functional benefits, as demonstrated by neurological severity scores and the rotarod test.

Daadi *et al.* recently isolated a homogenous self-renewable hNSC line from hESCs [17]. The initial tumorigenicity tests were conducted in both normal and immunodeficient rats. A total of 1 million of these cells were grafted into the brain and 2 million into the flank of immunodeficient nude rats. After 2 months, the data demonstrated no tumor growth in all animals transplanted. Although these data suggest the lack of tumorigenic cells, further long-term tumorigenicity studies in other immunocompromised animal models, such as the severe combined immunodeficiency (SCID) beige mice, are necessary for a conclusive report. The efficacy of these cells was tested in the rat MCAO model of stroke. Grafted cells demonstrated a robust cell survival and differentiated into neurons, astrocytes and oligodendrocytes. The behavioral analysis showed that the transplantation of hNSCs significantly enhanced the independent use of the impaired contralateral forelimb in comparison to the pretransplant group and to the vehicle-treated group at 8 weeks.

Mechanisms of functional recovery after cell transplantation

The mechanisms behind the behavioral improvement after cell transplantation are not completely understood. Particularly in stroke there is a host-endogenous component; neuroplasticity, the inherent ability of the brain to remodel itself after a traumatic injury to compensate for the functional deficit. One component of this neuroplasticity is the endogenous neurogenesis that is stimulated in response to stroke. Newly generated cells actively migrate to the lesioned area [38-43]. However, at the injury site and under homeostatic conditions, the long-term survival of cells is jeopardized [44,45] and currently it is not clear how much, if at all, these newly generated cells contribute to spontaneous functional recovery after stroke. Nevertheless, the *in vivo* manipulation of this endogenous pool of neural precursors, either by growth factor infusion or stem cell transplantation, could lead to a successful therapeutic intervention in the future. The donor source of stem cells and their profile of differentiation after transplantation dictate the mechanism of action. Evidence has implicated NSCs in multiple mechanisms that support brain repair, including anti-inflammatory actions, cell replacement, neurotrophism, vasculogenesis, axonal sprouting, dendritic branching, neurogenesis and synaptogenesis (Figure 3) [46-54]. Neuroinflammatory response and excitotoxicity cause pronounced damage during the secondary cascade of cell death after stroke. NSC transplantation, especially using cells with a dominant tendency to differentiate into glia, could reduce the inflammation and excitotoxicity by secreting neurotrophic factors and buffering toxic concentrations of chemicals in the environment. However, evidence of humoral mechanisms responsible for

the anti-inflammatory effects has not yet been proven. Studies described above agree that transplantation of ESC-derived neural progeny lead consistently to neuronal and glial differentiation. Recent evidence demonstrated that ESC-derived neurons integrate into the local network, receive synaptic input and generate action potentials in a stroke-lesioned brain [35,36]. This suggests that cell replacement and new synaptic contacts of grafted cells could participate in re-establishing lost neural connections and function. Transplantation of NSCs could restore the integrity and function of the stroke-damaged tissue by cell replacement and enhancement of neuroplasticity. Further studies are needed to determine the direct involvement of grafted cells in these mechanisms of action.

Development of cellular product for clinical use: scientific & regulatory challenges

The regulatory agency or US FDA, Centre for Biologics Evaluation in the USA, has the mission to provide guidance, inspections and surveillance, to ensure the safety, purity, potency, and effectiveness of biological products for the treatment of human diseases or injury and to evaluate the scientific basis of manufacturing, preclinical and clinical studies. Before initiating a clinical trial, an investigational new drug (IND) application is submitted to the FDA. The IND application encompasses detailed information and documentation regarding the origin and manufacturing of the cellular product, detailed and comprehensive results from the pharmacology and toxicology testing, and the clinical protocol.

Cell manufacturing

The principles of current good manufacturing practice (cGMP) are outlined by the FDA in the code of federal regulation: 21 CFR 210, 211, 600, 610 and 820. The goal in GMP is to eliminate all animal-derived proteins or xeno-products from the culture system. However, this is not always the case; fetal bovine serum, for example, is commonly used in cell manufacturing. In these cases, adequate testing for bovine-related bacteria, mycoplasma and viruses, and sourcing of the fetal bovine serum lots must be managed appropriately in a qualification program. Thus, the key elements of GMP are the raw materials used for the manufacturing, the characterization of banked cells, in-process controls, specifications of the cells, and the validation that the manufacturing and testing end points consistently meet specifications. This is to ensure safety of the product by eliminating risks of contaminating the cells with animal pathogens, such as prions or unknown retroviruses. Currently, any potential clinical cell line derived from hESCs that had been exposed at some point to mouse feeder layer is classified as a xenogeneic cell product.

It is critical that all potential therapeutic cell lines follow these FDA guidelines to maintain consistency and predictability of cellular products and avoid potential set-backs in the field of ESC-based therapies. Documentation, such as standard operating procedures and accurate detailed records on the origin of cells and materials used to isolate and expand the cells, is critical for clinical development. This is highlighted by the recent revelation that some of the federally approved cell lines for research and development in the USA do not have the appropriate and standard consent forms and may not be eligible for either research or potential commercialization [55]. Nevertheless, the NIH Stem Cell Task Force stated that all

the registered lines have met the 2001 criteria and that the NIH will not be taking any lines off its registry [55].

Quality control & assurance

The QC/QA exert control over the manufacturing facility, the manufacturing process, the validation process and all the testing of the raw materials, in-process material, bulk products and the final formulated product. This controls for contamination with adventitious agents and for inadvertent changes in the cell properties or stability.

The quality of the media component used to grow the cells affects the safety, potency and purity of the final product. Therefore, all media components are carefully selected, tested for activity and qualified for GMP usage. The manufactured cells are then banked using a system that consists of a master cell bank and a working cell bank. The working cell bank is derived by expanding one or more vials from the master cell bank. Batches of cells are then produced from the working cell bank for human use. The critical step towards obtaining a final product is the lot-to-lot consistency and freedom from adventitious agents.

Specifications of the product are chosen to verify the quality of the product that will ensure safety and efficacy. Product-specific assays are developed with appropriate acceptance criteria that ensure acceptable levels of biological variations, loss of activity or degradation through the product's shelf-life. These limits of acceptability are set to control raw materials, cell banks, in-process testing, process evaluation and validation, stability testing and the consistency of lots.

Storage & stability

Storage conditions are selected to preserve the purity and potency of the product so that the specifications of the product are maintained throughout storage, shipping and handling at the clinic. Stability program provides the assurance that the cell product is stable within the specified shelf-life. The shelf-life depends on the specific attributes of the cells, recommended storage, packaging, shipping conditions and the intended clinical use. Stability studies are designed on the basis of scientifically sound approaches and in-depth understanding of the final cellular product and intended therapeutic use. Stability studies insure that the storage conditions maintain the purity and potency of the product and that the original release specifications are met.

Preclinical safety & efficacy studies

Undifferentiated hESCs that could contaminate the final neural cell population destined for clinical use pose a significant threat for tumor formation. Here, again, adopting manufacturing practices that eliminate or minimize the number of undifferentiated hESCs present in the final formulated preparation is paramount. One such approach is the use of fluorescent-activated cell sorting or magnetic-activated cell sorting for positive selection of the desired cell population or negative selection to eliminate unwanted cells based on the expression of unique markers. Specifications for lot release have to include unacceptable levels of pluripotency markers such as Oct4 or Nanog. Sensitive and robust preclinical tests

are critical and must be sufficient to provide a reasonable assurance of safety for clinical use. Reverse transcription-PCR may be used to ensure that the NSCs do not express detectable levels of the pluripotency markers Oct4 and Nanog [17]. For the IND, it is necessary to carry out the pharmacology and toxicology studies on the final cellular product manufactured under the cGMP. The toxicology studies should be designed for 6-12 months post-transplant survival time and conducted by independent contract companies under good laboratory practices.

The efficacy study should be carried out in the appropriate animal model that closely mimics the human condition. Careful considerations should be taken in the choices of the animal models and behavioral tests to detect the *in vivo* functionality of the investigational cells. The efficacy studies should be carried out with the GMP product's final formulation. A dose-escalation study is necessary to accurately determine a safe first-in-human starting dose level and to optimize both safety and potential benefits of early phase and subsequent trials.

Immunological considerations for hESC-based therapy for stroke

The immune system has the ability to distinguish between self and nonself. Immune responses against transplanted cells are elicited by genetically determined cell surface antigens. These antigens include the ABO blood group antigens, MHC molecules, minor histocompatibility antigens, polymorphic proteins, retrovirally encoded antigens, mitochondria protein and male-specific gene product. In the clinical setting, hESC-based therapy for stroke is allogeneic since it involves different members of the same species. Grafts of hESC-derived NSCs will be immunologically incompatible and therefore at risk of rejection in an immunologically competent host. Immunosuppressive treatment will be necessary in hESC-based therapy for stroke. Immunosuppressive drugs and immunomodulators such as cyclosporine A, tacrolimus (FK506), immunophilins, azathioprine, tacroazathioprine and steroids have been used in intracerebral cell transplantation therapy [56]. These drugs interfere with the activation of T cells and proliferation of T and B cells. Their chronic use in both preclinical and clinical settings is accompanied by controllable side effects. Nuclear transfer technologies or therapeutic cloning may solve the problem of immune rejection, since the ESCs derived will be genetically identical to the patient's cells. However, there are scientific and ethical problems with this approach that have so far prevented its application [57,58]. An alternative approach is the induction of immune tolerance by host conditioning with nondepleting monoclonal antibodies specific for the T-cell coreceptors, CD4 and CD8 [59]. The increased TGF- β 2 synthesis and activity, and the absence of the direct presentation of alloantigen by endogenous dendritic cells facilitated the polarization of infiltrating T cells toward a regulatory phenotype and the acquisition of immune tolerance. To investigate the humoral and cellular immune response, Drukker *et al.* transplanted undifferentiated hESCs into the kidney capsules of immunocompetent and immunodeficient mice [60]. Teratomas developed in the immunodeficient mice only 1 month after transplantation, suggesting a role of T cells in xenorejection of transplanted hESCs. Using different strains of mice, the nonobese diabetic/SCID versus SCID/Beige, as host for hESCs transplantation, Tian *et al.* demonstrated that the natural killer cells play a role in the rejection of differentiated hESC grafts [61]. Pretreatment of mice with antibody that deplete natural killer cells showed a

three-to tenfold increase in cell engraftment. Thus, immunosuppressive drugs are currently used in clinical setting and improved alternative noninvasive approaches to enhance cell engraftment are in the development stage.

The clinical protocol

The initiation of clinical trials will be based on the characteristics of the hESC-derived product and comprehensive preclinical pharmacology/toxicology studies in relevant animal models. Phase I clinical trials are designed as open-label pilot safety and tolerability studies with a single dose on a small number of patients. Phase II studies are usually short-term and double blinded to assess safety and, to a lesser extent, efficacy in a dose-escalation study. Phase III clinical trials are long-term, double-blinded, randomized, multicenter studies with a control group in a larger patient population (>300).

The clinical protocol includes ethical considerations and informed consent, defining the subject population, criteria for patient selection, route of administration, dosing regimen and patient monitoring.

The determination of patient suitability includes an evaluation of medical history, genetic, biochemical and immunological testing, baseline physical and functional measurements, laboratory tests and brain imaging. The functional evaluation of stroke patients encompasses inclusion and exclusion criteria based on standard scales, such as the NIH and the European stroke scales. The enrollment criteria are chosen to permit maximum possible benefit to patient, given the potential risks.

MRI or CT of the brain are used to identify the stroke region and determine the stereotaxic trajectories for cell transplantation. Post-administration monitoring of patients is a critical component of the clinical protocol. It includes written policies and procedures for monitoring patient outcomes and reporting adverse effects. Management of adverse reactions includes procedures for ensuring prompt medical evaluation and treatment and a system for reporting and evaluating adverse effects that could relate to the cell transplantation. Owing to the tumorigenic potential of hESC-derived products, long-term patient follow-up using analytical tools and noninvasive safety monitoring technologies, such as MRI, CT and PET imaging, testing immune responses to cellular product and other clinical and laboratory modalities, are necessary. MRI using metal-based contrast agents, such as superparamagnetic iron oxide, has been used to track grafted neural progeny in stroked animals [62,63] and grafted cells in dendritic cell therapy in humans [64]. Novel multimodal cellular and genetic imaging technologies to track and monitor the fate and function of cells after transplantation into the nervous system are currently in the research and development phase [65] and will soon reach the clinical arena.

Future perspective

hESCs provide an exciting opportunity to understand the basic mechanisms controlling tissue histogenesis, mechanisms that will translate into prospective applications in tissue engineering and biomedicine in general. A multidisciplinary approach will prove necessary and fruitful for nervous system tissue engineering and cellular therapy. Substantial progress

is expected in the discovery of novel instructive cues and gene networks involved in cell-fate specifications that will allow the generation of pure cell populations for further gene-discovery platforms and for the development of safe and predictable cell-therapy products. The research and development focused on stem cell-based therapy for stroke have advanced considerably during the last few years. Expectations are that within the next few years, we will have a better understanding of the applicability, limitations and mechanisms mediating stem cell repair. With sound cellular products and solid preclinical pharmacology and toxicology data, in addition to increases in the number of IND applications, a significant increase in stem cell-based clinical trials for stroke is anticipated.

Executive summary

Derivation of neural stem cells

- Neural progeny are derived from human embryonic stem cells (hESCs) by multiple processes employing defined media and growth factors.
- Two major strategies: enrichment for neural lineage or isolation of self-renewable neural stem cells may be used to generate neural progeny.
- Rosette-derived neural stem cells (NSCs) display primitive traits and maintain pluripotency with an overgrowth tendency after grafting.
- Growth factor-isolated and perpetuated clonogenic NSCs display definitive traits, maintain multipotency and are not tumorigenic.
- Growth factor-isolated NSCs are amenable for current good manufacturing practice (cGMP) manufacturing.
- Expanding NSCs by co-culture with feeder layers or as clusters of cells rather than single-cell dissociation represents a hurdle for cGMP production and cell delivery.

Stroke & neural repair

- Stroke is a promising target for a successful cellular therapy intervention since it induces brain plasticity and evokes *de novo* early developmental traits that enhance the appropriate integration of NSC grafts.
- The experimental stroke model and the choice of behavioral end point measures are critical for preclinical development.
- Grafts of ESC-derived neural progeny improve sensorimotor and cognitive functions in experimental model of stroke.
- Studies suggest that the mechanisms mediating the functional engraftment are cell replacement, neurotrophic support and synergy with ongoing neuroplasticity.

Product development & regulatory compliance

- Following regulatory steps is critical to cellular product development.

- Working together with regulatory agencies in early phase of the technology facilitates the translational process to clinical trials.
- Product- or NSC-specific assays are developed with appropriate specifications or acceptance criteria that ensure acceptable levels of biological variations.
- Specifications of the product are chosen to verify the quality of the product that will ensure safety and efficacy.
- Sensitive and robust preclinical tests are critical and must be sufficient to provide reasonable assurance of safety and predictability for clinical use.
- In clinical trials, the enrollment criteria of stroke patients are critical for the success of the therapeutic intervention and are chosen to permit maximum possible benefit to patient, given the potential risks.
- Immunological aspects of graft rejection must be carefully considered.
- Postsurgery monitoring of patients and long-term follow-up are a necessity for hESC-based cell therapy.
- Development of noninvasive multimodal cellular and genetic imaging technologies to track and monitor fate and function of cells after transplantation into patients will be critical for the successful progress of cell therapy.

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Figure 1. Procedures for generating neural stem cells from human embryonic stem cells

Through various processes hESCs give rise to neural precursors. Defined media contain supplements such as F12, N2, B27, insulin, transferin, selenium and growth factors bFGF, EGF, and in one protocol LIF. NSCs may be directly isolated from hESCs by exposure to defined media supplemented with EGF, bFGF and LIF. Rosette, an intermediate structure in the neural-induction pathway towards definitive NSC state, is lost following exposure to the mitogenic growth factors bFGF and EGF. EBs, an early intermediate stage, may be derived with media containing retinoic acid, conditioned media, serum or serum-free media. In the default pathway, neural lineage may be induced in low-density cultures of hESCs in the presence of inhibitors of the TGF- β signaling molecules. The induction of neural lineage may also be initiated by co-culturing with a stromal cell line leading to rosette formation. 1°Sph: Primary spheres; 2°Sph: Secondary spheres; CM: Conditioned media; Dll1: Notch ligand Delta-like1; EB: Embryoid body; hESC: Human embryonic stem cell; ITS: Insulin, transferin and selenium; Jag: Notch ligand jagged; LIF: Leukemia inhibitory growth factor; NSC: Neural stem cell; RA: Retinoic acid; SHH: Sonic hedgehog.

Figure 2. Schematic representation of the isolation and perpetuation processes of the SD56 human neural stem cells

NSCs were derived from hESCs and propagated using defined media supplemented with EGF, bFGF and LIF. The developmental progression of the *in vitro* neural specification and patterning was monitored by the expression of lineage markers, as indicated at each stage. DIV: Days *in vitro*; DMEM: Dulbecco's modified Eagle medium; hESC: Human embryonic stem cell; LIF: Leukemia inhibitory growth factor; NSC: Neural stem cell. Taken from [17].

Figure 3. Mechanisms mediating stem cell repair in stroke

Neural stem cell progeny may improve functional recovery in experimental stroke models via various potential mechanisms, including cell replacement and secretion of neurotrophic factors and cytokines. Grafted cells differentiate into neurons, astrocytes and oligodendrocytes. As reported in the text, studies have demonstrated that grafted neurons establish synaptic connections with host cells, suggesting replacement of cell loss. Astrocytes could participate in the repair of infarcted tissue through multiple mechanisms, including decreasing the extracellular excitotoxicity, secretion of neurotrophic factor, stabilizing synaptic formations and BBB, supporting axonal remodeling and synaptogenesis. Oligodendrocytes could protect axons and improve conduction by replacing myelin sheath lost after stroke. In response to stroke, neuroplasticity or endogenous regenerative processes are activated to compensate for the loss of function. For instance, in addition to vasculogenesis and axonal remodeling, there is a significant mobilization of endogenous neural precursors that takes place from the subventricular zone towards the stroke zone. Grafted stem cell progeny secrete neurotrophic factors and cytokines that may enhance neuroplasticity and stimulate axonal sprouting, neovascularization and synaptogenesis, as well as modulate the immune and inflammatory reactions. Together these mechanisms allow for structural repair of stroke-damaged tissue and recovery of function.