Adult Neural Stem Cell Therapy: Expansion *In Vitro*, Tracking *In Vivo* and Clinical Transplantation

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Abstract: Neural stem cells (NSCs) are present not only in the developing nervous systems, but also in the adult human central nervous system (CNS). It is long thought that the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus are the main sources of human adult NSCs, which are considered to be a reservoir of new neural cells. Recently adult NSCs with potential neural capacity have been isolated from white matter and inferior prefrontal subcortex in the human brain. Rapid advances in the stem cell biology have raised appealing possibilities of replacing damaged or lost neural cells by transplantation of *in vitro*-expanded stem cells and/or their neuronal progeny. However, sources of stem cells, large scale expansion, control of the differentiations, and tracking *in vivo* represent formidable challenges. In this paper we review the characteristics of the adult human NSCs, their potentiality in terms of proliferation and differentiation capabilities, as well as their large scale expansion for clinical needs. This review focuses on the major advances in brain stem cell–based therapy from the clinical perspective, and summarizes our work in clinical phase I-II trials with autologuous transplantation of adult NSCs for patients with open brain trauma. It also describes multiple approaches to monitor adult human NSCs labeled superparamagnetic nanoparticles after transplantation and explores the intriguing possibility of stem cell transplantation.

Key Words: Neural stem cells, large scale expansion, transplantation, adult stem cells, magnetic nanoparticles, superparamagnetic iron oxide, clinical phase I-II trials, MRI.

INTRODUCTION

Stem cells are the essential building blocks of multicellular organisms [1-5]. They have two defining properties—self-renew to produce more stem cells and differentiate to generate specialized cell types [6, 7]. The adult central nervous system (CNS) was long thought to be largely postmitotic with very limited ability to regenerate [8-10]. Thus, it came as a surprise when the existence of neural stem cells (NSCs) in the adult CNS was discovered [11-14]. Neurogenesis, the generation of neurons, has been found in specific regions of the adult CNS of all mammals examined [11-14], including humans [15, 16]. Within the last decade, multipotent NSCs have been isolated from diverse regions of the adult CNS of both rodents and humans [17-22]. These adult NSCs can be amplified *in vitro* through many passages without losing their multipotentiality, capable of giving rise to neurons, astrocytes, and oligodendrocytes both in culture and after transplantation to specific regions *in vivo* [12-14]. In the last few years, the functionality of neurons derived from adult NSCs has been demonstrated both *in vitro* [23, 24] and *in vivo* [25, 26].

Rapid advances in adult NSC biology have raised great expectations that these cells can be used as potential resources for neuronal replacement therapy after cerebral injury or neuro-degenerative diseases. In this review, we focus on the recent progress cellular transplantation therapy aimed at restoring function to the nervous system from the clinical perspective. Then we describe on-going clinical phase I-II trials with adult neural stem cells isolated from patients with open brain trauma and multiple approaches to label NSCs for tracking their fate *in vivo*.

EXPANSION OF ADULT HUMAN NSCS *IN VITRO*

Source of Adult Stem Cells

Active neurogenesis at first was found to be restricted to two specific regions in the adult CNS [12, 14, 16, 27, 28]: the subventricular zone (SVZ) surrounding the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). The SVZ is a remnant of the enlarged perinatal periventricular germinative area. During development, this germinative area narrows to the most rostral part of the lateral ventricle and forms the SVZ, which persists through adulthood [29, 30]. During development the SVZ generates the three major cell types (neurons, astrocytes, and oligodendrocytes) of the CNS. In culture, the adult SVZ stem/progenitor cells can be expanded in serum-free medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) and form neurospheres, which generate neurons and glia [31, 32]. However, *in vivo*, BrdU and retroviral tracing demonstrate that neuron generation most occurs in the olfactory bulb (OB), from SVZ cells migrating through the rostral migratory stream (RMS) [33-35]. The nature of the stem cells in the SVZ is a subject of controversy. According to the

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original theory, the SVZ stem cells originate from the subependymal layer of the lateral ventricle [36-38]. Electron microscopy studies show that SVZ stem cells have the ultrastructural characteristics of astrocytes, which extend a single cilia into the ventricle lumen through the ependymal barrier [29]. A nomenclature for the SVZ organization was established by the Tramontin *et al*. group. The slowly proliferating stem cells expressing glial fibrillary acidic protein (GFAP; type B cells) differentiate to become rapidly dividing immature progenitors (type C cells) and generate neuroblasts (type A cells), which migrate in chain through the RMS to the OB. It has recently been demonstrated that the RMS and the OB also contain stem cells and thus can be considered by themselves as germinative areas [39]. In this study, although all regions gave rise to neurons, astrocytes, and oligodendrocytes *in vitro*, the rostral part of the RMS generated more oligodendrocytes. Cells arising from the SVZ and migrating through the RMS to the OB were also described for the adult primate forebrain [40, 41]. Similarly to the case for rodents, these adult neurospheres give rise to functional neurons and glia [42, 43]. However, they seem to have a limited life span in culture and generate very few oligodendrocytes [42, 44]. Neural stem cells can also be isolated from the adult human OB [45]. The DG of the hippocampus has also been extensively studied. Stem cells originating from SGZ of the DG migrate into the granular cell layer (GCL), where they differentiate into granule neurons that extend axons to the CA3 region [46]. Gage and colleagues studied rodent SGZ cells *in vitro* and found that those stem cells retain the potential for self-renewal and the ability to differentiate into neurons, astrocytes, and oligodendrocytes [47].

Cells with neurogenic potentials can, however, be derived from other regions and exhibit multipotent stem cell properties in culture [21, 22]. NSCs have been isolated from the human cortex and amygdala [22]. Recently, resident glial precursors were isolated from human white matter [21]. These precursors appear to be multipotential cells retaining the ability to undergo both neurogenesis and gliogenesis *in vitro* and following transplantation. Gross and colleagues report the genesis of new neurons in the neocortex of adult macaque monkeys [48]. These cells arise from the SVZ and migrate through the white matter into the neocortex, where they differentiate into mature neurons. This ventricular-cortical migration may be a remnant of the waves of tangential migration observed from the lateral and medial ganglionic eminences to the neocortex during development [49]. However, two studies performed in macaques were not in agreement with these data and, in contrast, demonstrated that BrdU-positive cells detected in the neocortex were in fact satellite glial cells closely apposed to resident neurons [50, 51]. These studies underscore the necessity to perform detailed confocal analysis and three-dimensional reconstruction to establish unambiguously the origin of newly generated neurons and glia in the adult CNS. We have isolated adult human NSCs from brain debris that were obtained from frontal and temporal lobes in patients with open brain trauma [52]. Among the samples, the debris obtained from inferior prefrontal subcortex (IPS) generated more neurospheres per ml in the presence of EGF and FGF than those derived from other regions. Twice cloning assessment showed the cloning efficiency of NSCs derived from IPS was significantly higher than that of parietal and temporal lobe tissue [53]. Human IPS neurospheres can differentiate into glia fibrillary acidic protein (GFAP)-positive astrocytes, O4-positive oligodendrocytes and TuJ1-positive neurons. Primary neurospheres yielded 10.5±3.4% neurons, 5.8±1.5% oligodendrocytes and 68.8±3.1% astrocytes. And expression of TLX gene, an orphan nuclear receptor (Tailless homolog), was much higher in the neurosphere of IPS than that of cortex (Fig. **1**). This region is equal to the rostral extension, where the migration of NSCs comprise RMS in rodent and primate. Therefore, IPS maybe a new adult NSCs pool, which is composed of the NSCs not only migrated from SVZ, but resides in this region itself [54].

Unlike hematopoietic stem cells, which can be directly isolated with cell surface markers, the precise identification of NSCs occurs only retrospectively, and scientists are still in search of effective methods for prospective isolation of NSCs [12-14]. Nonetheless, many types of adult stem cells with neural potentials have been derived [11-14, 17-22, 55-58]. Strikingly, some appear to be derived from nonneural tissues [56-59], such as blood, bone marrow, or skin. In most cases, the neuronal identity is determined merely based on the expression of certain markers (e.g., Tuj1, NeuN), rather than their functional properties [56-58]. Interpretation of some of the transplantation experiments are further complicated by the potential fusion events that have occurred between the transplanted cells and the host cells [56-58]. Because functionality is the foundation for the success of neuronal replacement therapy, it is essential to characterize the physiological properties of neurons derived from different types of adult stem cells. For adult multipotent NSCs derived from human white matter [21] and multipotent adult progenitor from bone marrow of mice [60], electrophysiological studies have shown that the neuronal progeny of these stem cells is electrically active, capable of firing action potentials. For adult NSCs derived from hippocampus, extensive functional analysis showed that these cells retain the capability to give rise to electrically active and functional neurons with all essential properties of mature CNS neurons, even after extensive propagation and amplification in cultures [23].

Amplification and Differentiation of Adult Human NSCs

Proliferation of adult NSCs in the SVZ and SGZ is regulated by a variety of stimuli, including aging, stress, stroke, seizure, and physical activity [12-14, 61-67]. Neurotransmitters (e.g., serotonin, NMDA, nitric oxide) and steroid hormones are also known to adjust the proliferation of adult NSCs [12-14, 61]. Recent findings suggest that local vasculature [68] and astrocytes [69, 70] may serve as potential cellular sources for the signals. In culture, astrocytes from hippocampus or SVZ promote proliferation of adult NSCs [69, 70]. In the adult hippocampus, hot spots of cell proliferation have been found to be associated with vascular structures [68]and astrocytes [70]. Furthermore, factors promoting endothelial cell proliferation also increase neurogenesis [71], suggesting an important relationship between these two processes.

A	hF-NSC	h-IPS	h-Cort	m-IPS	m-Cort
1st cloning assessment	28	24	22	25	23
2nd cloning assessment	12*	9*	1	10*	2

* compared with h-Cort and m-Cort, $p<0.01$.

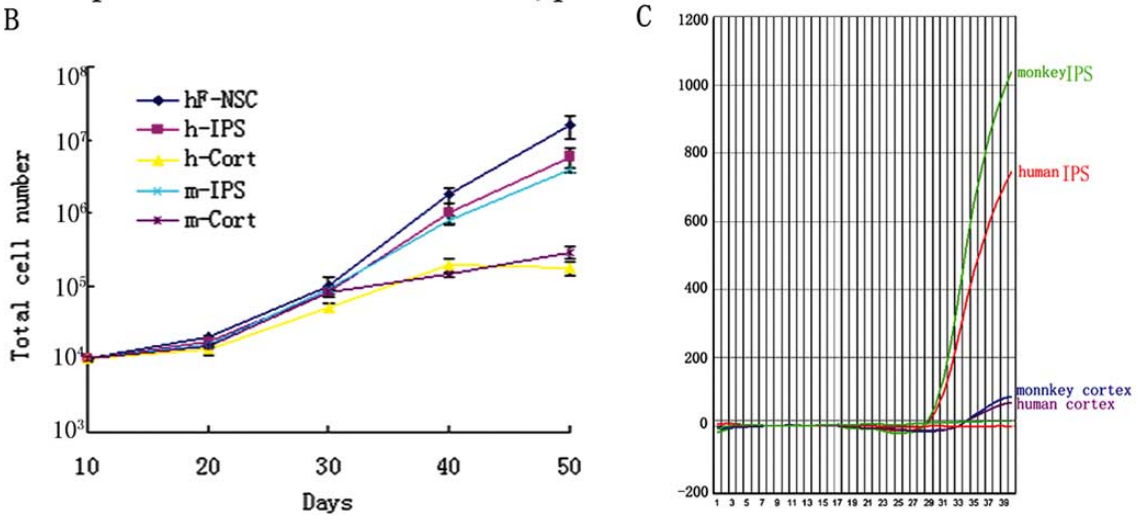

Fig. (1). NSCs derived from inferior prefrontal subcortex (IPS) are different from NSCs from cortex. Double cloning assessment show that both human and monkey NSCs in IPS are more efficient in cloning formation than that in cortex (A). NSCs in IPS have more growth advantage than those in cortex (B). Moreover, the expression level of TLX gene is much higher in IPS than that in cortex, which suggests IPS maybe a new adult NSCs pool.

While the *in vivo* mitogenic factors from vascular and astrocytic sources remain to be identified, several molecules have been shown to be effective in inducing proliferation of adult NSCs *in vivo* [12-14, 72-74]. Infusion of EGF, FGF-2, or transforming growth factor-a (TGF-a) into the brain has been found to promote proliferation of adult NSCs both under normal and injured conditions [13, 72, 75]. EGF and FGF-2 have also been used, almost exclusively, as mitogens for NSCs derived from the adult CNS, either in adhesive or in neurospheres cultures [12-14]. The mitogenic effects of FGF-2 for cultured NSCs at clonal densities appear to require a co-factor, recently identified as a glycosylated form of cystatin C [76]. Combined delivery of FGF-2 and cystatin C to the dentate gyrus in adult mice stimulates proliferation of NSCs in the SGZ [76]. Sonic hedgehog (Shh) signaling has been shown to regulate proliferation of adult NSCs [73, 74]. Loss of Shh signaling results in abnormalities in both the dentate gyrus and olfactory bulb [74]. Pharmacological inhibition or stimulation of Shh signaling in the adult brain also leads to decreased or elevated NSC proliferation in the hippocampus and SVZ, respectively [73, 74]. Shh is sufficient to maintain the proliferation of NSCs derived from adult rat hippocampus *in vitro* [73], while mouse SVZ progenitors that lack Smoothened, a key downstream effector of Shh, formed significantly fewer neurospheres [74]. Other factors that are not traditionally regarded as mitogens have also been implicated in regulating proliferation of adult NSCs [77]. For example, Eph/ephrin signaling has been shown to be involved in axon guidance, neural crest cell migration, establishment of segmental boundaries, and formation of angiogenic capillary plexi [78]. A 3-day infusion of the ectodomain of either EphB2 or ephrin-B2 into the lateral ventricle not only disrupte migration of neuroblasts, but also increase proliferation of NSCs in the SVZ of adult mice [77]. The proliferation signals for adult NSCs activate an array of interconnected cytoplasmic signal transduction pathways that eventually lead to the activation of gene transcription in the nucleus. While many of these pathways have been intensively investigated in other cell types, specific cytoplasmic pathways involved in proliferation of adult NSCs remain to be identified.

The molecular mechanisms underlying fate specification of adult NSCs are largely unknown [14, 62-64, 66, 79]. Members of the bone morphogenic protein (BMP) have been shown to be able to instruct adult NSCs to adopt a glial fate [80]. In the neurogenic SVZ, the BMP inhibitor noggin, released from the ependymal cells in the lateral wall, can block the gliogenic effects of BMP [80]. The instructive factors for neuronal differentiation of adult NSCs, including those released from hippocampal or SVZ astrocytes, remain to be identified.

Numerous attempts to transplant multipotent NSCs directly into the nonneurogenic regions of the adult CNS failed to generate significant numbers of new neurons [13]; however, transplantation of neuronal lineage-restricted progenitors did generate neurons [81], suggesting that the neuronal fate specification was the limiting step. The molecular mechanisms regulating neuronal fate specification and neuronal subtype differentiation will be an area of intensive investigation in the near future. Understanding how these developmental processes occur during embryonic stages will clearly facilitate our efforts. Protocols have already been developed to differentiate ES cells effectively

into different neuronal subtypes [82], including dopaminergic, GABAergic and motor neurons. It is expected that similar strategies will also be developed to coax the adult stem cells [60]. The functionality of neuronal progeny for stem cells, including membrane excitability and release of neurotransmitter, is an essential piece of the task, and should be rigorously examined.

Survival and Functional Integration of Newly Generated Neurons

While the functional roles of adult neurogenesis remain elusive [13, 61], recent studies provide convincing evidence that newly generated neurons are able to integrate into the existing neuronal circuits in the adult CNS [25, 26]. Functional studies of the maturation and integration process during adult neurogenesis have also revealed some unique features of neuronal development in the adult CNS that are different from that being observed during embryonic and neonatal stages [25, 26].

In the case of neurogenesis in the dentate gyrus, about half of new newborn cells die within 2 weeks after birth [61]. Four weeks after retroviral labeling, some of the remaining neurons become electrically active and start to receive synaptic inputs, as shown by electrophysiological recordings [83]. Whether these newborn granule neurons also make functional synaptic connections with their target neurons, thus actively involved in the information flow, remains to be demonstrated. The complexity of their dendrites and density of the dendritic spine, the major sites for excitatory synapses, continue to increase for at least several months [25]. Thus, the course of neuronal maturation for newborn granule neurons in the adult CNS appears to be much more protracted than those generated during embryonic stages.

In the case of neurogenesis in the olfactory bulb, electrophysiological recordings show that tangentially migrating neurons express extrasynaptic GABA (gamma-aminobutyric acid) and AMPA (alpha-amino-3-hydroxy-5-methyl- 4-isoxazole propionate) receptors, while NMDA (N-methyl-d-aspartate) receptors appear later in radially migrating neurons [26]. The sequential expression of receptors for neurotransmitters is different from what occurs during embryonic neuronal development, where expression of NMDA receptors normally precedes AMPA receptors. These newborn olfactory neurons become synaptically connected soon after migration has been completed [26]. However, spiking activity does not occur until the neurons are almost fully mature. This is also different from developing embryonic neurons, which can fire action potentials and release neurotransmitters even before they are connected. One hypothesis for this unique neuronal maturation process is that the delayed maturation of excitability can prevent the newborn cells from disrupting the function of circuitry already in place in the adult brain.

The mechanisms regulating maturation and synapse formation by adult NSCs are largely unknown. *In vitro* studies with adult hippocampal NSCs suggest that local hippocampal astrocytes play essential roles in the maturation and synapse formation process [23]. In the absence of astrocytes, the neuronal progeny of cultured adult NSCs remain immature, both morphologically and functionally. They display simple morphology, limited membrane excitability, inability to fire action potentials, and little functional synaptogenesis [23]. In contrast, adult NSC derived neurons in the presence of astrocytes acquired physiological properties comparable to those of mature CNS neurons [23]. Similar active roles of astrocytes in regulating synapse formation have been previously observed in neonatal neurons [84].

A significant percentage of neurons, either derived from endogenous NSCs [61] or from transplantation [85], died soon after in the adult CNS. Thus, strategies to support the survival of endogenous and/or transplanted cells are of apparent importance [85]. Adult NSCs and their progeny can be genetically modified either *in situ* or *in vitro* to become the cellular sources for growth factors and neurotrophins that may promote or support the survival of themselves and the surrounding neurons. To achieve functional integration by the newborn neurons from either endogenous NSCs or transplantation, we will have (It is important) to understand the mechanisms that control the neuronal migration, axon/dendrite guidance, and synapse formation in both normal and diseased adult CNS environment. Extensive characterizations are necessary for elucidating the expression of developmental guidance cues in normal and abnormal adult CNS. In addition, novel approaches will need to be developed to monitor the correct integration of newborn neurons.

Large-Scale Adult Human NSC Expansion

The use of NSCs has the potential to revolutionize the treatment of neurodegenerative diseases, but large numbers of cells arc required to treat the millions of afflicted individuals, which has fuelled the need to develop large-scale culture methods for these cells (Fig. **2**).

Owing to the difficulty of obtaining human neural precursor cells, as well as the potential commercial benefits, a great deal of the work related to the expansion of human cells is being carried out in non-academic settings. However, significant advances are being made at academic institutions, and the results are available in the literature. Human NSCs also form neurospheres when cultured in the presence of FGF-2 and/or EGF [86-89]. The extracellular matrix produced by these cells causes the cells to be much more tightly bound than murine NSCs [90-92] . As a result, the neurospheres are much more difficult to dissociate. Mechanical dissociation has typically been a necessary part of the cell passaging procedure. A solution to this dilemma is to slice the neurospheres mechanically when the diameter becomes large enough [87]. This procedure has been used successfully to expand human NSCs *in vitro*. An expansion of $1x10^7$ was obtained over a period of II passages (154 days) in stationary culture. During this time, the neurospheres were sectioned into quarters every 2 weeks (14 days) [88]. Studies found that disrupting the cell-to-cell contact while passaging NSCs decreased the ability of the cells to divide. The overall expansion was enhanced when the cell-to-cell contact was maintained by sectioning the aggregates instead of forming single-cell suspensions [93, 94].

Other researchers have also expanded human fetal NSCs using EGF, FGF-2 and LIF (Leukaemia inhibitory factor)

[86, 95], In the first study, the expansion rate did not depend on the gestational age of the donor (5-10.5 weeks) but did depend on the presence or absence of LIF [86]. However, the effect of LIF was only seen after 50-60 days *in vitro*. An expansion ratio of $x10^7$ was obtained after 175 days with LIF, compared to $x10^3$ without LIF. The NSCs were passaged by mechanical dissociation of the neurospheres into a single-cell suspension every 7-30 days. The maximum expansion was $x10^{14}$ over 350 days. In the second study, an expansion of 10^7-fold *in vitro* was obtained in stationary culture after 21 passages (every 7-10 days) (i.e. $x10^7$ over 147-210 days) [95]. NSC neurospheres were mechanically dissociated into single cells and re-inoculated at a cell density of 10^5 cells ml^{-1}in fresh medium. The obtained expansion was approximately equal to that obtained by Svendsen *et al*., using a neurosphere sectioning technique (i.e. $x10^7$ over 154 days) [87].

Research on immortalized NSCs has primarily been performed with a murine progenitor cell clone C17.2 that was created by transducing the constitutively downregulated v-myc gene into neonatal mouse cerebellar cells [96-98]. Murine fetal neural progenitor cells that have been expanded by the addition of the myc gene exhibit 'stem-like' properties and can integrate and differentiate when transplanted into mouse models of injury and Parkinson's disease [98-101]. As these cells are immortalized, there are no real challenges in culturing them for extended periods of time. More recently, Villa *et al*., have published results combining genetic and epigenetic means to develop a protocol for rapidly and continuously propagating human NSCs [102]. Human fetal neural progenitors have been similarly isolated, propagated and transplanted successfully into the murine brain [103]. However, much of the work with genetically modified human neural progenitors has not described in long-term passaging *in vitro* [103, 104].

The use of cell lines derived by oncogene expression facilitates the examination of neural cells at different stages of development, as well as the large-scale expansion and transplantation of those same cells. However, there are limitations to the clinical application of transformed cells. First, the transduced target cell is not completely defined. A mixture of cells obtained by tissue culture of embryonic brain tissue will be transfectcd by the oncogene. This means that the resulting cell line may be a multipotcnt stem cell line or a restricted neuronal or glial progenitor cell line. Secondly, the reversible oncogenes are inserted at random locations in the genome, resulting in unpredictable behavior [19]. These reasons, in addition to the public perception of oncogenes, may limit the clinical use of immortalized neural progenitor cells.

TRACKING OF NSCS *IN VIVO*

To determine the fate of transplanted cells, including their migration *in vivo,* cells are currently labeled *ex vivo* using a vital dye (e.g., a fluorochrome), a thymidine analog (e.g., bromodeoxyuridine, BrdU), or a transfected gene (e.g., LacZ or green fluorescent protein, GFP), for later visualization using immunohistochemical procedures following invasive and irreversible tissue removal. The use of progenitor and stem cells in clinical studies will require a technique that can monitor their fate noninvasively and repeatedly, so as to take a momentary "snapshot" assessment of the cellular biodistribution. Magnetic resonance imaging (MRI) has the capabilities of non-invasive whole-body *in vivo* imaging, with a resolution of 25 to 50 microns, approaching the resolution of single cells. For transplanted cells to be detectable by MR imaging, they need to be labeled with an MR contrast agent.

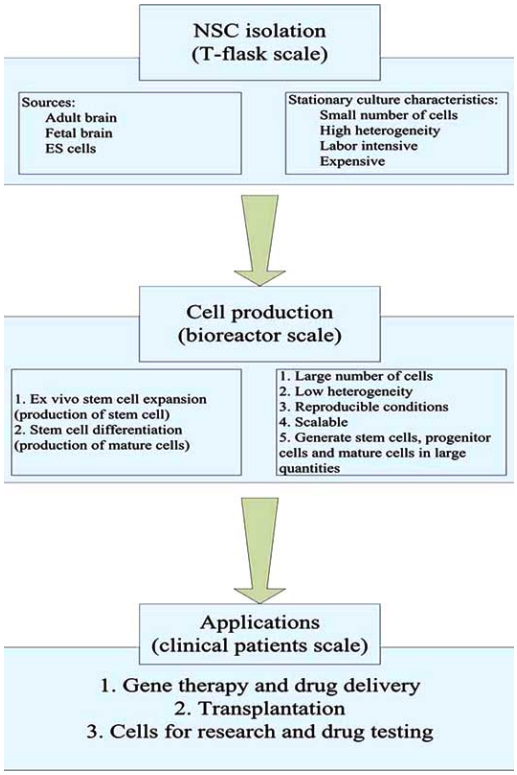

Fig. (2). Potential sources, production and applications of neural stem cell technology in treatment of neurodegenerative disorders and brain injuries.

The ability to localize or track specific cell populations *in vivo via* MRI has been pursued intensively over the past decade. A number of different contrast agents have been developed, all predicated on loading cells with paramagnetic or superparamagnetic compounds. Initially, attempts were made to label leukocytes, lymphocytes, and monocytes with superparamagnetic iron oxides. Strategies to prepare magnetically labeled cells included incubation with nonderivatized, dextran-coated iron oxide particles [105-108], incubation with liposome-encapsulated iron oxide particles [109], and lectin-mediated uptake [110]. The first such experiments used fetal rat cells harvested from sheets of cortical tissue. To label the cells, viral particles reconstituted to contain ultrasmall iron oxide particles were incubated with the fetal cell suspension. The cells were then grafted back into rat brains, with the grafts appearing dark in T2-weighted images [111]. Subsequent studies using impermeable paramagnetic compounds, such as Gadolinium (HI) (1,4,7, 10-tetraazacyclododecane-1,4,7,10-tetra(aceticacid)) (GdD OTA)-poly-D-lysine or dextran, required microinjection of the agent into individual cells. This method has useful

applications in embryology, with injection of large single cells in developing embryos, but has not been practical for tracking populations of cells in whole organisms [112]. Initial techniques to facilitate endogenous cellular uptake of superparamagnetic iron oxide particles included targeting to the transferrin receptor *via* monoclonal antibodies or liposomal coating and then membrane fusion, but neither resulted in efficient enough uptake for *in vivo* tracking, and there was significant cellular toxicity or impact on critical cellular characteristics [113, 114]. Small superparamagnetic iron particles coated with dextran were taken up into cells via endocytosis and allowed dynamic tracking of loaded T cells to a site of inflammation in the rat testicle [108]; this labeling enabled single cells to be detected *in vitro*. Using the same approach, oligodendrocyte precursors have been labeled with small dextran superpara-magnetic iron particles and localized after infusion into the brain of rats [115]. Recently, transferrin receptor-targeted dextran-coated iron oxide nanoparticles were shown to have very efficient cellular uptake and were used to follow *in vivo* migration of labeled neural progenitor cells after injection into rat spinal cords [116]; 50 000 labeled cells were injected at one site, and it is unclear what minimum number of cells localized in one area could be imaged using this contrast agent. This magnetic labeling approach is limited, because it requires the availability of an internalizing monoclonal antibody that recognizes a specific cellular surface antigen. Weissleder and coworkers linked small dextran-coated fluorescent iron oxide particles (USPIO) to the tat peptide from the human immunodeficiency virus. This translocation signal increased uptake of the particles up to 100-fold into lymphocytes and other hematopoietic cells, compared with particles without tat [105, 117, 118]. Human CD34+ cells labeled with these particles could be recovered from the marrow of immunodeficient mice following transplantation and detected via MRI in bones of these animals after removal of the bone from the whole mouse but not *in vivo* in real time following transplantation [118]. However, since the Tat protein has an affinity for the nucleus, it is possible that during biodegradation of the USPIO-Tat particle, reactive iron species might be released temporarily. Reactive iron species could then catalyze the formation of hydroxyl free radicals, which might initiate lipid per-oxidation and destroy membrane structure and function, as well as lead to damage of proteins and DNA within the nucleus [119].Most recently, Bulte and coworkers [120] have utilized a new contrast agent termed a magneto-dendrimer, suspending iron oxide particles within a dendrimer matrix that is efficiently taken up into cells and optimized for favorable magnetic properties for imaging; 50 000 neural stem cells labeled with these particles could be detected *in vivo* following injection into the rat brain and used to track migration of the cells for up to 6 weeks. Finally, mixing ultrasmall iron oxide particles with common lipofection agents has enabled efficient labeling of stem cells and *in vivo* tracking in the brain [121, 122].

Labeling of the cultured cells with superparamagnetic iron oxide nanoparticles and the use of (SPIO) provide a noninvasive method for studying the fate of transplanted cells *in vivo* [15, 120, 123-125]. Superparamagnetic contrast agents are formed by a superparamagnetic core, which is represented by iron oxide crystalline structures described by the general formula Fe_2O_3 Mn_2O, where M is a divalent metal ion ($M=Fe^{2+}$, Mn^{2+}). For the synthesis of the contrast agents, small crystals of magnetite Fe_2O_3FeO are predominantly used. During the preparation of the contrast agent, the crystals are covered by a macromolecular shell, formed by dextran, starch and polyol derivatives. In an applied magnetic field, SPIO particles create extremely large microscopic field gradients for dephasing nearby protons [123, 126]. This, in turn, dramatically shortens the nuclear magnetic resonance T_2 relaxation time, over and far beyond the usual dipole-dipole relaxation mechanism that affects both T_1 and T_2. Owing to the predominant T_2 effect, these "T_2 agents" usually create hypointense contrast on conventional spin-echo MR sequences, in particular when agglomerated within cells. On gradient-echo images, where T_2 effects dominate, these (intracellular) particles induce an even larger hypointense contrast effect. This in turn leads to a "blooming effect", that is, an amplification of signal changes. Given the greater sensitivity of MR imaging for detecting superparamagnetic nanoparticles, these contrast agents are a natural choice for labeling cells. In addition, these agents are composed of biodegradable iron that can theoretically be recycled within the body. For instance, radiolabeled studies on the use of a liver-specific SPIO have shown that the iron is metabolized by liver Kupffer cells, with subsequent reuse and incorporation into the normal iron blood pool as well as erythrocytes [127]. Since 1999, much effort has focused on exploring efficient techniques for incorporating the SPIO nanoparticles within cells. Using transfection agents to incorporate magnetic nanoparticles is a promising approach for labeling cells. Liposome agents, dendrimers, poly-L-lysine [PLL] and protamine sulfate all can efficiently incorporate the SPIO or USPIO into cells, and have no significant toxicity to labeled cells [128-131]. Moreover, the combination of two commercially available, FDA-approved agents, ferumoxides and protamine sulfate, are used to effectively label a variety of cells without short- or long-term effects on cell viability, proliferation, and differentiation [132]. Clinical experience with use of both agents should allow translation of this method from the experimental setting to clinical trials.

We reported the feasibility to labeling human NSCs with SPIO and tracking NSCs after clinical transplantation with 3.0 Tesla MR [133]. A 34-year-old male patient suffered with open brain trauma in left temporal lobe in February 2004. During emergency operation, exposed brain debris among the hair and cranial fracture bone were collected and transported immediately to a laboratory dedicated to the cultures of NSCs. The day before implantation, co-incubation of Feridex IV (a contrast agent based on dextran-coated SPIO) and Effectene (a lipofection reagent) in serum-free medium for 60 min led the contrast agent infusion into the cells to label NSCs (Fig. **3**). Preclinical experiments in monkey showed SPIO labeled NSCs present a patch of hypointense signal in MR imaging (Fig. **4**).

After harvesting, cells were diluted in patient's cerebrospinal fluid, and autologuously implanted at four points around damaged region, each point contained 40 ul volumes of cell suspensions ($5x10^4$/ul) with MRI guided stereotactic technique. Imaging was achieved in gradient reflection echo (GRE) with TR/TE 200 ms/20 ms and a flip

angle of 20° at 24 hour later and each 7 days after transplantation, with a MR imager (GE Signa 3.0 T). The SPIO labeling of NSCs led to a markedly susceptibility change change with powerful signal damping in T2-weighted MRI. It thus produces a strong contrast against the normal tissue background. The injection sites were visible as circular dark tissue areas on the first day after implantation, where no pronounced hypointense signals were found in the injection sites before implantation. The hypointense signal in the injection points faded during the follow-ups. From the first week, the implanted cells had accumulated and extended around the lesion, which intensified during the second and third week. The implanted cells massively populated the border zone of the damaged brain tissue and were localized in the injured tissue around the traumatic lesion, suggesting that NSCs migrating away from the primary implantation sites and gathered around the lesion. Feridex IV contrast agents approved by the FDA for use in hepatic reticuloendothelial cell imaging and in cancer imaging [134-137]. Our study demonstrates that non-invasive MR techniques can be used to detect magnetic labeled stem cells in human brain by using clinical 3.0T scanner, which can *in vivo* observe stem cell engraftment and migration after implantation. Hoehn, *et al*., have provided evidence that contrast released from lysed cells or freely injected into rat striatum led to rapidly dissipate MRI contrast with the contrast agent diffusing radially through the extracellular space in rats [121]. Such a dynamic contrast pattern was not

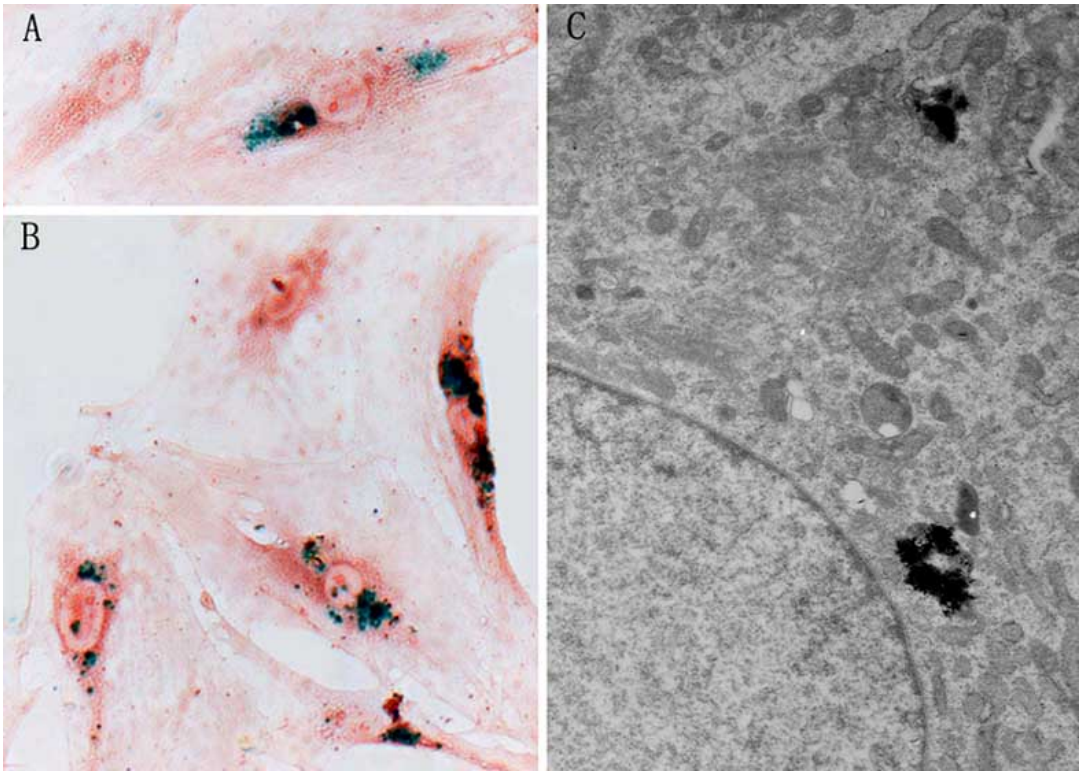

Fig. (3). A, B: NSCs in culture labeled with iron oxide nanoparticles, which is stained blue to dark-blue dots by Prussian blue. Findings at 24 hour (A) and 7 day (B) after labeling with SPIO are shown. The cells are counterstained with neutral red. C: Transmission electron microphotograph showing a cluster of iron nanoparticles located in the close vicinity of the Golgi apparatus_nuclear and cell membrane (arrows), confirming the presence of iron inside the cell.

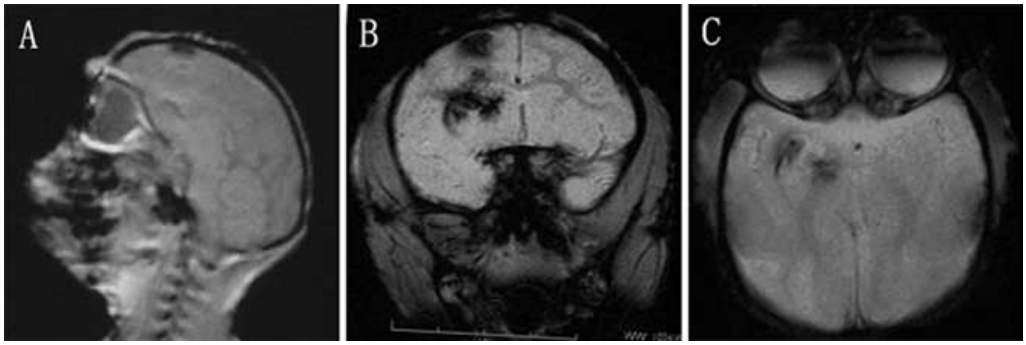

Fig. (4). Preclinical studies of SPIO-labeled NSCs in a monkey (A). The brain image of the SPIO-labeling NSCs implanted monkey at 24 hours after implantation (B-C). Some large, visible hypointense signals appeared at injection sites at 24 hours after implantation of SPIO-labeling NSCs, which were much larger than the regular cell implants.

observed after implantation of labeled cells. The SPIO-labeling NSCs implanted patient had no seizure, fever and deterioration of neurological function after progenitor cell implantation. The method opens a variety of field for clinical investigation of the therapeutic potential of stem cell transplantation strategies.

CLINICAL TRANSPLANTATION FOR BRAIN INJURY

Cell replacement is a vital step in CNS lesions, in which alternative endogenous systems cannot supplant the function of lost cells. Based on experimental data, several clinical trials have taken advantage of fetal tissue grafts to replace cells after a variety of CNS insults. Among those clinical trials, the investigation in Parkinson's disease (PD) patients treated with this approach is much ahead of other neurodegenerative diseases.

Based on a sound experimental background [89, 138-142], pilot studies in patients with human fetal nigral transplantation began to be undertaken in the second half of the 1980s [143]. These studies confirmed that lasting clinical benefits of up to 14 years are possible using this approach, and that these benefits are directly due to the grafted tissue as evidenced through functional positron emission tomography (PET) studies [143, 144]. Overall, the data from these ongoing, open label studies with moderately severe patients show motor score improvement of up to 40% in the Unified Parkinson's Disease Rating Scale (UPDRS) [145-148] with a striatal dopamine uptake increase of up to 60-70% using 18F-dopa PET [149]. Furthermore, in these studies there was little evidence for the induction of major dyskinesias, and in fact there was improved duration of time in the on state without dyskinesias [145-147]. Those open-label trials in patients with PD after intrastriatal transplantation of human fetal mesencephalic tissue have provided proof of principle that neuronal replacement can work in the human brain [150, 151]. The grafted neurons survive and reinnervate the striatum for as long as 10 years despite an ongoing disease process [144, 152]. The grafts are able to normalize striatal dopamine release2 and to reverse the impairment of cortical activation underlying akinesia [153]. Thus, grafted dopaminergic neurons can become functionally integrated into neuronal circuitries in the brain [153].

Two randomized, double-blind, placebo-controlled trials were performed using human fetal mesencephalic tissue. In the first of these studies, Freed *et al*., reported the outcome measures in one year after transplantation. Using a subjective global rating scale, no overall improvement was demonstrated, although [154], in the subgroup of patients under 60 years of age, a significant improvement was observed compared with the placebo group. However, the functional benefit reported in one year follow up may represent an underestimation of graft function, as many of the patients have reported ongoing clinical improvement at 2-3 years after transplantation [149]. Of concern, however, was the first report of levodopa-independent dyskinesias, which occurred in 15% of patients and the origin of which remains obscure. In the second trial, Olanow *et al*., [155, 156] reported no overall improvement following neural transplantation, although a subgroup of patients with less severe disease did appear to improve. Furthermore, in this study more than 50% of the patients develop dyskinesias which were only partially levodopa-dependent (i.e. removal of levodopa for extended periods aborted these dyskinesias). The reason for this is unknown but it is important to note that clinical studies have by and large targeted severely affected PD patients. In other words, stratification based on disease severity showed a significant treatment effect in the patients with milder disease, but not in those more severely affected.

To summarize, the results of a recent meta-analysis show consistent improvements on a number of clinical outcomes using fetal domaminergic allografts in patients with advanced PD. However, cellular transplantation therapy has been hampered by moral and ethical objections, inadequate availability of donor tissue (transplantation of primary tissue requires several fetuses per patient), and inadequate survival of grafted tissue, which leads to a re-evaluation of this approach and has catalyzed the search for alternative sources of cells.

For treatment of stroke, the application of human neural precursor cells from a human teratocarcinoma cell line NT-2 was carried out in the 12 patients with stroke affecting basal ganglia and fixed motor deficits. The patients received implants of NT2 precursor cells into the infarcted area [157]. NT-2 cells proliferate in culture and differentiate into pure, postmitotic human neuronal cells (LBS-Neurons) upon treatment with RetA. Thus, NT2 precursor cells appear to function as CNS progenitor cells with the capacity to develop diverse mature neuronal phenotypes. Improvements in motor performance among some affected individuals correlated with increased metabolic activity assessed by FDG-PET at the graft site [158]. This finding could be interpreted as graft function but might as well reflect inflammation or increased activity in host neurons. Autopsy in one individual who had suffered a stroke revealed a population of grafted cells expressing a neuronal marker 2 years after surgery [159]. Using human teratocarcinoma cell line as sources of grafted cells has some concern on tumorgenesis in the grafted area.

The discovery of the existence of active functional neurogenesis in the adult CNS and rapid advances in stem cell technology have fueled our hope to cure currently intractable CNS diseases by replacing damaged or lost neurons [139, 141, 160, 161]. Many types of stem cells with neurogenic potential have been identified, including pluripotent ES and EG cells and multipotent fetal NSCs [75, 154-156, 162-170]. Adult human NSCs have been isolated from brain debris of patients with open brain trauma or during minimally invasive neurosurgical procedure [171]. Importantly, these neural stem cells develop into functionally mature neurons in culture. Using these techniques, large numbers of neural stem cells can be generated from very small amounts of brain tissue harvested with minimal morbidity from living, adult patients. These cells represent a potential source of cells from transplantation therapy that may not raise as many objections as fetal tissue or human teratocarcinoma cells.

Preliminary studies in animal models of brain injury suggest that cells transplanted into the injured brain can promote recovery of lost function. NSCs transplanted into

the injured areas of rats with brain trauma resulted in improved memory and motor function [172-174]. Cognitive improvement was observed in animals that already had measurable memory impairment and not in unimpaired animals [175]. Several studies using animal models of ischemia have demonstrated success in transplanting exogenous cells into the damaged brain to produce new, presumably functional connections [75]. Most notably, such experiments have demonstrated functional recovery of motor skills in mice with brain injury. All of the studies suggested that transplanted NSCs improve functional recovery and paved the way for clinical study.

Usually, debris of brain tissue that exposed out of skull in patients with open brain trauma was discarded by neurosurgeons. We succeeded in isolating multipotent neural progenetor cells/neural stem cells in the 10 cases out of 14 adult patients from the injured brain tissues and propagate them *in vitro*, which indicates it is possible to make debris of brain tissue in open head injury as a source of NSCs in some cases (Fig. **5**).

Preclinical investigation was performed in monkey for assessing the safety and feasibility of NSC transplantation. Six months after transplanting human NSCs labeled with LacZ into monkey's brain, the human NSCs were spread along the parenchyma, and no significant lesion were found in any other tissues. Meanwhile, using fluorescence patch clamp, we examined the electrophysiological properties of the neurons derived from GFP labeled NSCs. Stimulation collaterals elicited field excitatory potentials in slices. The evidence has demonstrated that regenerated neurons are functional at least in certain aspect (Fig. **6**).

To assess the safety of NSCs, the adult NSCs were implanted into the brains of BALB/C nude mice and followed for 6 months or until development of focal neurological deficits. None of the NSCs developed into brain tumor. This suggested that NSCs themselves cannot form malignant tumors and is safe for transplantation therapy, because no tumors appeared in mice after implanting adult derived hNSCs. Further to test the safety of retrovirus infected NSCs, no brain tumor occurred in nude mice when hNSCs were transferred with retro-LacZ.

A Phase I-II trial of autologous multipotent neural progenetor cells (NPCs)/NSCs implantation for the treatment of brain trauma was approved by the local ethics committee. Patients or the next of kin of each patient provided written informed consent. We have implanted NPCs/NSCs into traumatic regions for 8 patients with open brain trauma. Meanwhile, we had other 8 untreated counterparts as case control. Within 2-year follow-ups, patients were investigated by functional MRI (fMRI), F-18-fluorodeoxyglucose-positron emission tomography (FDG-PET), somatosensory evoked potential (SEP) and Disability Rating Scale (DRS) for functional recovery [176].

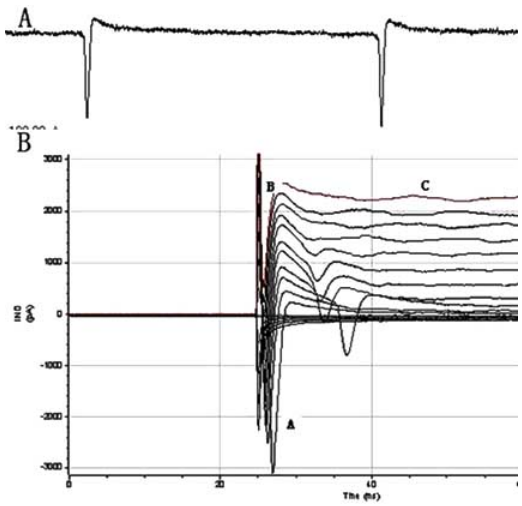

Fig. (6). Using fluorescence patch clamp, A. spontaneous action potentials in slices were recorded in the neurons derived from GFP labeled NSCs. B. field excitatory potentials were recorded in the GFP labeled neurons.

In all treated patients, FDG-PET was performed at baseline 2 weeks after injury and repeated at follow-ups to assess neural metabolism, which indicated the viability of neural cells, in the damaged area. ROI analysis with repeated measurement statistics showed mean tracer uptake in the damaged territory increased significantly in both groups, and the mean tracer uptake in implantation group was significantly higher than control groups. The mean tracer uptake in the damaged areas among the control group increased from 72.1±12.5% to 125±9.4% at 12-month follow-up, whereas it increased strongly from 71.4±7.9% to 161.3±4.6% in implantation group (Fig. **7 A-D**). In the patients with 2-year follow-up, the difference of the FDG-PET data between pre-implantation and post-implantation at different intervals were analyzed with SPM method. Using

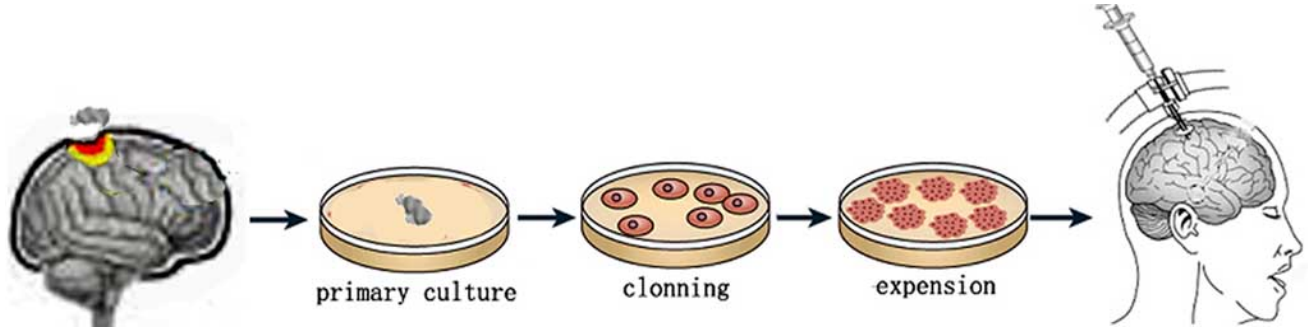

Fig. (5). The clinical protocol of autologous NSCs transplantation for treatment of open brain trauma.

paired t-test, the plots showed that FDG uptake in the damaged right frontal lobe was significantly increased in the patient who received the implantation of NPCs/NSCs, but no significant change in the patient as control (Fig. **7 E-F**). [18F] FDG PET was used to investigate accurate rates of cerebral glucose metabolism in conscious subjects and quantitative within subject metabolic changes induced by traumatic brain injury [177]. Through quantitative [18F] FDG PET, the study could demonstrate that longitudinal recovery from traumatic brain injury induced metabolic depression was significantly correlated to recovery of behavioral dysfunction, which suggests that [18F] FDG PET is quantitative, reproducible and sensitive to metabolic changes and provides a new approach to the longitudinal study in neuroscience research. Furthermore, PET allows the quantitative localization of expression of genes coding for membrane receptors and transporters or cellular enzymes by measuring the binding and transport of the respective receptor binding and membrane transporter compound or the accumulation of the specific enzyme substrate [178-181]. Important endogenous enzymes, receptors and membrane transporters in neuroscience, which are already non-invasively assessed on a routine basis in clinical applications by specific probes and PET, are: cellular hexokinase (HK), by [18F]FDG; cellular thymidine kinase (TK), by 3'-deoxy-3'- [18F]; fluoro-L-thymidine ([18F]FLT); aromatic amino-acid decarboxylase (AADC), by 2- [18F]fluoro-2-deoxy-dopa ([18F]FDOPA); acetylcholine esterase (AChE), by [11C]-N-methyl-4-piperidinylacetate ([11C]MP4A); dop-amine D2 receptors (D2R), by [11C]raclopride and 3-(2'-[18F]fluoroethyl)-spiperone; benzodiazepine receptors, by [11C]flumazenil ([11C]FMZ); amino acid transporters, by [11C]methionine ([11C]MET) . These tracers are clinically applied for early detection of Alzheimer's disease ([18F] FDG, [11C]MP4A), in the differentiation of Parkinson's disease from multiple systems atrophy ([18F]FDOPA, [11C]raclopride, [18F]spiperone, [18F]FDG), in the grading of gliomas and differentiation of radionecrosis from recurrent tumor ([18F]FDG, [18F]FLT, [11C]MET), in the exact localization of epileptogenic foci in partial epilepsy syndromes and in the assessment of neuronal integrity after stroke ([11C]flumazenil, [18F]FDG) [182-186].

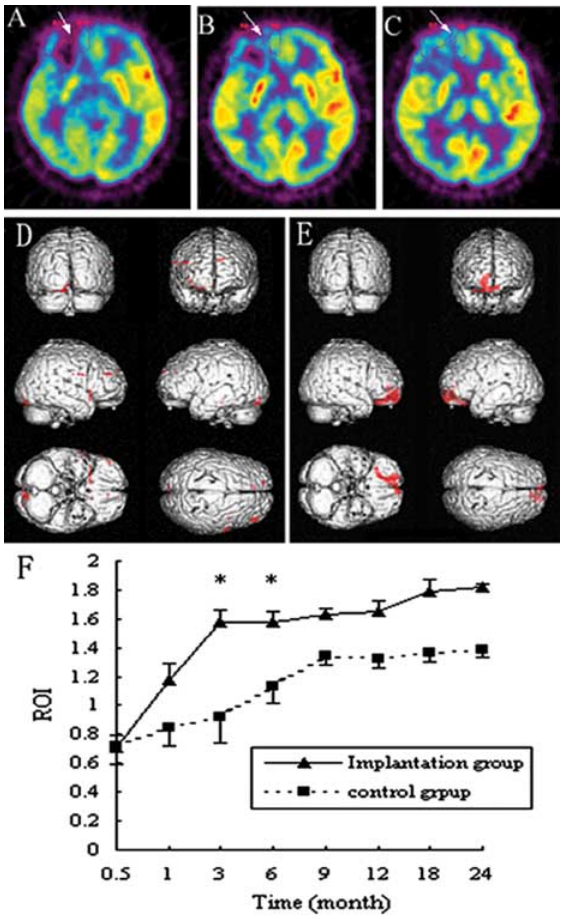

Fig. (7). FDG-PET brain metabolic scans. (A to F) Black, no perfusion; blue–green–yellow–red, increasing metabolism. Scans done before (A) and after neural progenitor cell injection (B, C). Arrows show location of cell injection. Note that there was no perfusion of the right frontal lobe before operation (A); metabolism was slightly restored 1 months after cell injection (B), and significantly restored 24 months after cell implantation. SPM plots showed that FDG uptake in the damaged right frontal lobe was significantly increased in the patient who received the implantation of neural progenitor cells (E), but no significant change of damaged right frontal lobe occurred in the patient as control (D). The time course of single intensity in damaged areas in FDG-PET plot (F). The mean tracer uptake in ROI reached a plateau at the third month in treated group, which persisted about nine months, and then went on elevating slightly. Whereas, tracer uptake in the damaged areas among control group increased slowly, plateaued at 9th month, and no longer increased. * indicates significant differences ($P < 0.01$) from values at follow-up points.

The activation in fMRI maps was seen in the damaged motor cortex since 3 month after implantation, whereas, no active signal appeared 3-month and 6-month follow-up among control group. Some investigations have utilized fMRI in assessment motor recovery. For example, Miyai and colleagues [187] have recently presented fMRI findings obtained during the rehabilitation phase of 12 patients who sustained hemiparetic strokes. The results indicated that patients who demonstrated improved use of the affected limb also had new or enhanced activation detected by fMRI in the supplementary motor area, the contralateral sensorimotor cortex, or the ipsilateral parietal cortex. By contrast, patients without significant recovery demonstrated lower relative fMRI activations. The findings suggest that successful brain reorganization during recovery after stroke involves multiple cortical areas, and that lack of activation might predict a poor recovery.

All patients with motor deficits caused by the injury of motor cortex received examinations of SEP. Among the first 3 months after injury, there was no significant change in the latency of SEP wave at the contra-lateral lambs from baseline to follow-up in treated patients. Similarly, few changes were observed in patients of control group. In contrast, from the 6 month follow-up, the latency of SEP in treated group recovered faster than that of control group (45.9±5.1ms in treated group versus 48.5±5.56 ms in control group at the 6th month, 45.44±4.93ms in treated group versus 48.16±5.61 ms in control group at the 9th month), which suggested in part that implantation of NSCs contributes to the recovery of neural function (Fig. **8A**).

Recruited patients were performed Disability Rating Scale during the follow-ups regardless of treatment. Among the first 3 months after injury, there was no significant difference in the DRS between treated group and control group. In contrast, from the 6-month follow-up, the DRS in treated group recovered faster than that of control group (6.63±2.33 in treated group versus 10.25±1.29 ms in control group at the 6th month, 5.43±1.62ms in treated group versus 9.57±1.99 ms in control group at the 9th month) (Fig. **8B**).

The results of this clinical trial demonstrate that autologous implantation of adult NSCs is feasible and safe in patients with open brain trauma. Contrast to control group, implantation of NSCs was associated with a significant improvement in patient's neurological function. The improved brain function was accompanied by partial recovery of activity in damaged areas as assessed by fMRI and SEP and by significant increases in neural viability within injured territories as assessed by FDG-PET. In this context, it should be pointed out that stem cell transplantation probably can lead to clinically valuable improvements through several mechanisms. First, the tissue damage per se can stimulate plastic responses or interfere with neural activity in the host. Second, the transplants can act as biological minipumps and release a missing transmitter or secrete growth factors. These factors can stimulate plastic responses and improve the survival and function of host neurons [188]. Third, the grafts can restore synaptic transmitter release by providing a local reinnervation. Fourth, and this is true neuronal replacement, the grafts can become integrated into existing neural and synaptic networks, and reestablish functional afferent and efferent connections [189]. Importantly, none of our patients had seizure, fever and deterioration of neurological function after progenitor cell implantation. In addition, injection of progenitor cells did not induce an acute inflammatory response as measured by blood examinations. Thus, the culture and expansion of NSCs followed by re-implantation appears to be safe for clinical application. The results of FDG-PET scan demonstrating increased metabolism of the damaged area during follow-ups argue against the hypothetical concerns that implantation of NSCs populations may enhance scar formation contributed by proliferation of astrocytes in the brain injury region.

SUMMARY

The remarkable progress in isolation of adult stem cells with neural capacities and understanding of basic mechanisms controlling functional neurogenesis in the adult CNS has reinvigorated our hope to repair the diseased CNS with adult stem cell-based neuronal replacement therapy. The possibility of autologous transplantation of adult stem cells expanded *in vitro* will circumvent the safety and ethical issues associated with other types of stem cells. However, these enthusiasms have to be coupled with challenges before the dream can be realized. The first is to identify and isolate stem cells from adult sources. With the success in isolation of adult stem cells, it is of immediate concern to determine the functionality of the progeny of these adult stem cells. Finally, the integration of new neurons in the existing neuronal circuits needs to be monitored to avoid adverse effects. Thus, both developmental neuroscientists, who are interested in the normal brain development, and clinical neuroscientists, who are interested in using stem cells for therapy, have to be united to bring stem cells research and therapy to a new height, enabling the dream of CNS repair to come true. Our initiated clinical trial with autologuous transplantation of NSCs for patients with open brain trauma presents an encouraging result, which may fuel the stem cell–based approaches in clinical practice. Although stem cell therapy is still a long way off, there is reason to be optimistic that stem cell–based approaches will eventually be developed.

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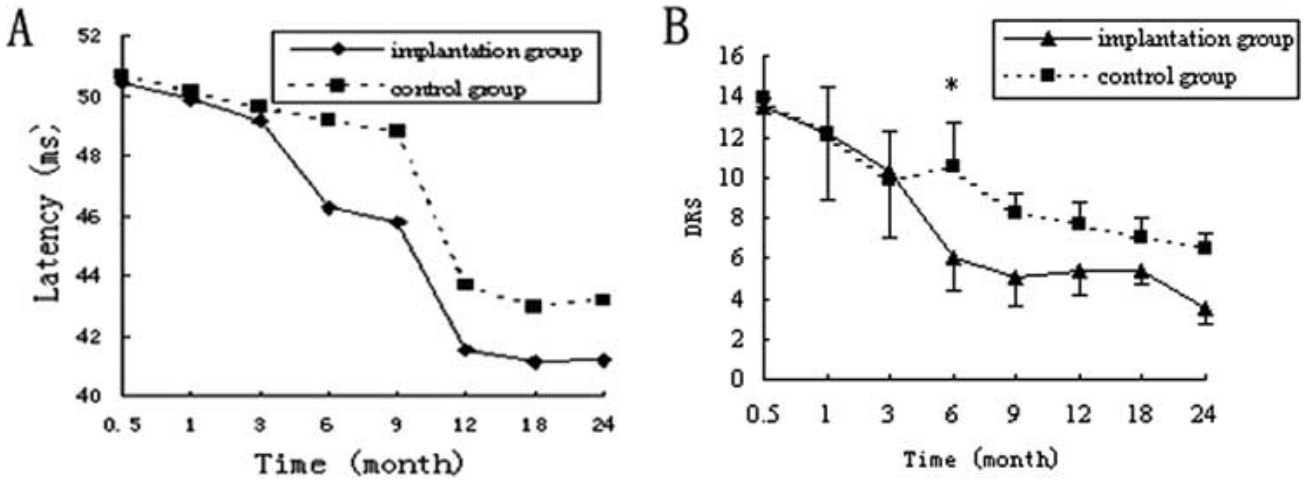

Fig. (8). The time course of latency alteration in SEP plot for contra-lateral lambs was showed in A. Time course of alteration of DRS during follow-ups was showed in B. * indicates significant differences ($P < 0.01$) from values at follow-up points.

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