REVIEW ARTICLE

Stem cell-based cellular replacement strategies following traumatic brain injury (TBI)

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Abstract

Given the limited capacity of the central nervous system for self-repair, the use of stem cells holds an enormous potential in cell replacement therapy following traumatic brain injury and has thus received a great deal of scientific and public interest in recent years. During the past decade, several stem/progenitor cell types and lines from various sources such as embryonic rodent and human stem cells, immortalized progenitor cells, bone marrow derived cells or even post-mitotic neurons derived from human teratocarcinoma cells have been assessed for their potential to improve neurofunctional and behavioural outcome after transplantation into the experimentally injured brain. A number of studies indicate that cells engrafted into the injured brain can survive and, at least in part, may reverse behavioural dysfunction and histomorphological damage. Although these results emphasized their potential therapeutic role in traumatic brain injury, the detailed mechansim on how stem cells generate their mode of action, e.g. via integration into surviving neuronal circuits, local trophic support, or modification of the local mircoenvironment to enhance endogenous regeneration and potection remain yet to be identified. A review on current pre-clinical knowledge with respect to cellular replacement into the experimentally injured brain is presented.

Key words: *Traumatic brain injury, cell replacement therapy, stem cells*

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability world-wide. In the United States an estimated number of 1.6 million people sustain TBI each year accounting for 52.000 deaths and 80.000 patients suffering from permanent neurological impairment (1,2). Currently, more than 5 million individuals are living with the devastating emotional and economic costs (3,4). During the last two decades, improvements in acute pre- and inhospital care, time management, diagnostic procedures including sophisticated neuroimaging, and rehabilitation strategies have substantially improved the level of care and outcome in Europe (5) and have been summarized in clinical guidelines for the advanced treatment of patients with TBI (6). Despite these approaches, a significant number of patients with traumatic brain injury survive with significant brain damage and behavioral impairment, even after mild or moderate head injury (6,7). To date, no therapeutic approach has been proven effective in reversing the pathologic cellular sequelae underlying the progression of cell loss and in improving neurobehavioral outcome.

The pathophysiological sequelae following traumatic brain injury are known to be associated with

- primary injury, induced via direct physical and/or biomechanical impact on the brain, and
- secondary injury, initiated minutes after trauma and lasting for weeks, months and even years (8).

These secondary cascades are related to altered gene expression and dysregulation of various potentially damaging or restorative factors that interact in a complex network, thus leading to delayed cellular dysfunction and loss (9). The extended nature of

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these interactions provides unique possibilities for innovative therapeutic approaches with particular focus on limiting degeneration and enhancing regeneration (9,10). As the brain has limited capacity for self-repair restorative approaches with focus on replacement and repair of dysfunctional and dead cells within the traumatically injured brain have been studied for the last 15 years (11). It is currently believed that the stimulation of regenerative potentials within the injured adult central nervous system requires one or more of the following processes:

- Cellular replacement,
- delivery of neurotrophic factors,
- removal of growth inhibition,
- promotion of axonal guidance,
- adequate intracellular signalling,
- bridging and artificial substrates, and
- modulation of the host immunoresponse (12).

To date, the concept of cellular replacement has been based on the assumption that neurological dysfunction due to injury or disease may be improved via introduction of new cells that can replace lost neurons and/or glial cells or via trophic support to surviving cells to enhance survival rates, plasticity, and functional restoration (13). Pioneering experiments in other neuropathological states, for example in Parkinsons disease (PD), have indicated potential opportunities for the use of cellular transplants as a therapeutical option to repair and replacement of dysfuntional and/or dead cells within the central nervous system (14). Furthermore, the observation of ongoing neurogenesis in the adult brain (15,16) has indicated that one possibility for cellular replacement therapy may be promotion or enhancement of endogenous neurogenesis to treat central nervous system disorders. However, endogenous progenitor cells known to be present in various anatomical regions of the adult brain have been found to have only limited potential to generate new functional neurons in response to injury. Therefore, transplantation of stem cells may be a valuable option for repair of the injured central nervous system with the overall goal to compensate the function of cells lost due to injury. Experimental approaches using different models of stroke and PD have demonstrated that cellular replacement therapies may be successful (17) and, in some cases, these approaches have advanced into clinical investigation (18,19). In the present work the authors review and summarize recent results from transplantation studies performed in clinically relevant models of TBI and provide some rationale for cellular replacement strategies to treat traumatic brain injury.

Stem cell transplantation for traumatic brain injury

Stem cells are able to both self-renew and produce differentiated tissue. Pluripotent embryonic stem cells are isolated from the inner mass of the blastocyst and can be induced to differentiate into any tissue-specific cell type (20). An essential prerequisite includes the exposition to specific cues. In contrast, multipotent neural stem cells for example are primarily capable of differentiating into cerebral derived cell types such as neurons, astrocytes and oligodendrocytes (21). Neural stem cells have been isolated from the subventricular zones, the dentate gyrus, olfactory bulb, striatum and cortices of mammalian brains (13,22). Isolated stem cells from embryonic brains may supply an alternative source of embryo-derived stem cells. In addition immortalized progenitor lines from the developing central nervous system have been utilized as an alternative cell source for regenerative cell therapy. The immortalized cell lines offer the unique advantage of unlimited expansion in culture and the potential for genetic *in vitro* manipulation (23).

Immortalized and teratocarcinoma-derived stem cell lines

The most frequently used immortalized stem cell lines are progenitor cells from embryonic murine or rat brains.

The C 17.2 cell represents a clonal multipotent progenitor cell from the external germinal layer of the neonatal murine cerebellum immortalized via retroviral transduction of the avian gene myc (24).

The C17.2 cell line has been transplanted by stereotactic injection into ipsi- and contralateral cerebral tissue of mice and rats following controlled cortical impact (CCI) or lateral fluid percussion injury (LFP), respectively. Stereotactic implantation of C17.2 into ipsi- or contralateral hemispheres of the cortex hippocampus interface following CCI significantly improved motor function in immunocompetent C57BL/6 mice during the 12 week observation period (25). Cognitive dysfunction was unaffected by transplantation at either three or 12 weeks post-injury. Histological analyses showed that C17.2 cells survive for as long as 13 weeks after transplantation and were detected in the hippocampus and/or cortical areas adjacent to the injury cavity. At 13 weeks, the NSCs transplanted ipsilateral to the impact site expressed neuronal (NeuN; 60%) or astrocytic (glial fibrillary acidic protein; 40%) markers but not markers of oligodendrocytes (2'3'cyclic nucleotide 3'-phosphodiesterase), whereas the contralaterally transplanted NSCs expressed neuronal but not glial markers

(double-labeled immunofluorescence and confocal microscopy).

C17.2 cells transfected with the constitutively active epidermal growth factor receptor vIII survive predominantly when implanted into the ipsilateral corpus callosum of immunosuppressed rats following LFP (26). The authors concluded that the traumatically injured brain might release cytokines and trophic factors promoting graft survival. In fact, a marked elevation of nerve growth factor (pg/mg protein) was observed at 72 hours after injury in the injured hemisphere (x=80 +/− 10 pg/mg) compared with the contralateral uninjured hemisphere (35 +/− 0 pg/mg) (p<0.05). C17.2 cells transplanted into the injured hemisphere showed extensive process formation, expression of the progenitor cell marker nestin and NG2, a pro-oligodendrocyte marker that was not seen in cells transplanted into uninjured hemispheres. Migration of cells towards the injury cavity was observed two weeks after transplantation. At that time point cells injected into the contralateral hemisphere were observed to migrate across the corpus callosum. These results indicate that the environment associated with acute experimental traumatic brain injury can significantly modulate the phenotype and migratory patterns of the engrafted progenitor cells.

However, this environment may also impair graft survival after transplantation. Recently, Bakshi and co-workers demonstrated the caspase and calpain family of proteases to mediate apoptotic graft cell death in the acute post-traumatic period following TBI (27).

HiB5 cells represent conditionally immortalized progenitor cells from embryonic (E16) rat hippocampus that have been shown to integrate and differentiate into neuron and glia when engrafted into the neonatal hippocampus, cerebellum, and cortex, or into adult striatal tissue (28,29). Stereotactic injection of wild type HiB5 cells or HiB5 cells transfected with the mouse NGF (nerve growth factor) gene into three cerebral cortex sites adjacent to the location of LFP injury resulted in a significant improvement of neuromotor function and spatial learning behaviour in the Morris water maze of immunocompetent rats. A significant reduction in hippocampal CA3 cell death was observed in brain-injured animals receiving transplants of NGF-HiB5 cells, but not after injection of wild type HiB5 cells or vehicle control, indicating NGF release to be a major factor in the rescue of hippocampal CA3 neurons (30).

In accordance with these results a significantly improved learning pattern in the Morris water maze was also observed when MHP36 cells were injected into the ipsilateral or contralateral deep cortex of brain injured (LFP) rats (31). Cognitive deficits were also restored using MHP36 in comparative brain injury models (32,33). Regenerative approaches using MHP36 further demonstrated a reduction in lesion volume and neuronal damage when cells were transplanted following vascular injury (33) or diffuse brain injury in C57B1/6J mice (34), respectively.

The fibroblast growth factor 2 (FGF-2)-responsive Maudsley hippocampal cell line clone 36 (MHP36) cells are conditionally immortalized cells derived from E14 hippocampal neuroepithelium of the H-2Kb-tsA58 transgenic mouse and have the ability to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes. Transplantation of these cells following LFP resulted in occasional co-labelling for neuronal and astrocytic markers in the perilesional region (34). Migration of transplanted MHB36 has been demonstrated repeatedly. In host ischemic brain MHP36 cells survived robustly and migrated away from the injection tract towards the caudate nucleus and corpus callosum (34). Following LFP MHP36 cells were observed at the site of implantation, the subcortical white matter, the corpus callosum, perilesional regions and in the hippocampus ipsilateral to the injury, suggesting that these cells had migrated toward the injured brain regions. The migratory properties of MHP3 appear to be cell line-specific. Prestoz and co-workers (2001) investigated the cellular and molecular basis of the migratory property of MHB36 and MHB15 *in vivo* and *in vitro* (35). Using the same model of hippocampal damage the authors demonstrated that following transplantation MHP36 cells migrate and align within the damaged CA1 of the ipsilateral hippocampus. MHP15 cells, in contrast, migrated in a more indiscriminate pattern that did not reflect the anatomy of the region. Migration assays at a non-permissive temperature on the extracellular matrix substrates laminin, fibronectin, and vitronectin showed that MHP36 cells have a greater migration potential than the MHP15 cells. While the pattern of cell surface extracellular matrix receptors of the integrin family was identical in both cell lines, the different degrees of migration on vitronectin were both blocked by inhibitors of alphaV integrins. Differences in integrin signalling contributed to the greater migration potential of the repairing MHP36 cell line (35).

Furthermore, there seem to be limitations to the multifunctionality of MHP36 cells. For example, MHP36 cell grafts were unable to repair motor asymmetries in rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal dopamine system

(33). One may suggest that lines from different regions of the developing brain will be required to treat different brain diseases.

The NTera2 (NT2) cell line is a human derived teratocarcinoma cell line that terminally differentiates into postmitotic neuronal NT2N cells when treated *in vitro* with retinoic acid (36). Grafting of predifferentiated NT2N cells into the periinjured brains of immunocompetent rats following lateral fluid-percussion brain injury demonstrated survival and integration of these cells for up to four weeks. Engrafting of these cells at one month after lateral fluid-percussion brain injury during the chronic post-injury period also resulted in long-term survival (12 weeks), extended processes into the host cortex and expression of synaptophysin in the pre-differentiated cells (37). However, improvement of neurological function was not observed in any of the studies.

The long-term survival of this cell line *in vivo* seems to be a perfect prerequisite for the utilization of these cells as practical and effective platforms for delivery of therapeutic genes. Watson and co-workers demonstrated stable, efficient, and nontoxic gene transfer into undifferentiated NT2 cells using a pseudotyped lentiviral vector encoding the human elongation factor 1-alpha promoter and the reporter gene eGFP. Expression of eGFP was maintained when the NT2 cells were differentiated into NT2N neurons after treatment with retinoic acid (34). Following transplantation into the striatum of adult nude mice, transduced NT2N neurons survived, engrafted, and continued to express the reporter gene *in vivo*. Furthermore, transplantation of NT2N neurons genetically modified to express nerve growth factor significantly attenuated cognitive dysfunction following traumatic brain injury in mice.

In accordance with these observations, implantation of pre-differentiated NGF-expressing NT2N cells into the basal forebrain of brain-injured mice (controlled cortical impact) resulted in long-term survival of cells and expression of NGF *in vivo* (38). Furthermore, at one month post-transplantation, animals engrafted with NGF-expressing NT2N neurons showed significantly improved learning ability by Morris water maze testing, but no effect of NGF-secreting NT2N cells on motor function deficits was observed.

Existing evidence suggests that transplantation of these pre-differentiated human neuronal cells may be a promising approach for delivery of specific neuroprotective genes in both acute and chronic traumatic brain injury.

It is important to note that to date, no tumorgenesis has been observed following transplantation of immortalized progenitor cells or teratocarcinoma cell line in brain injury models, but additional studies are mandatory to better understand whether these lines improve post-traumatic dysfunction via replacement of neural/glial cells via local delivery of potentially trophic factors into the injured central nervous system.

Rodent embryonic neural stem cells

Neural stem cells derived from the germinal zone of E14.5 GFP-expressing mouse brains were predifferentiated into neurons, astrocytes, and oligodendrocytes and subsequently transplanted directly into the injury cavity or into the striatum below the injury cavity at one week following controlled cortical impact brain injury in immunocompetent mice (39). At all time points examined (one week to three months post-transplant), GFP-positive cells were confined to the ipsilateral host brain tissue. At one week, cells injected into the injury cavity lined the injury penumbra while cells injected directly into the striatum remained in or around the needle track. Striatal transplants displayed a lower number of surviving GFP+ cells relative to cavity injections at one week after transplantation ($p < 0,01$). At the longer survival times, i.e. three weeks to three months, 63–76% of transplanted cells had migrated into the fimbria hippocampus regardless of injection site, perhaps due to cues from the degenerating hippocampal structures. Furthermore, cells injected into the cavity within a fibronectin-containing matrix (collagen I (CnI)/FN gel) showed increased survival and migration at three weeks ($p < 0,05$ for both) relative to injections of cells only. The authors suggested that transplant location and environmental conditions may play an important role in neural stem cell graft survival, migration and integration after experimental traumatic brain injury.

In an extension to this work, the same group also assessed long-term survival, migration, differentiation and functional significance of the same cells up to one year post-transplant. Behavioural testing after neurospheres were injected into the ipsilateral striatum of adult C57BL/6 mice one week following unilateral cortical impact injury revealed significant improvements in motor abilities in verum-treated animals as early as one week, and recovery was sustained up to one year post-transplantation (40). Furthermore, mice receiving neural progenitor cell transplants showed significant improvements in spatial learning abilities at three months and one year, whereas an intermediate treatment effect on this behavioural parameter was detected at one month. At 14 months post-transplant, GFP+ cells

were observed throughout the injured hippocampus and adjacent cortical regions of transplanted brains. Immunohistochemistry demonstrated that the majority of transplanted cells co-labeled for NG2, an oligodendrocyte progenitor cell marker, but not for neuronal, astrocytic or microglial markers (40). The authors thus concluded from their experiments that transplanted neural progenitor cells can survive in the host brain up to 14 months, migrate to the site of injury, enhance motor and cognitive recovery, and may play an important role in trophic support after traumatic brain injury. The latter is obviously an important finding as neurobehavioural outcome after traumatic brain injury might be ameliorated via transplants even if the improvement is not achieved by neuronal replacement.

As indicated earlier, the trauma-associated cerebral milieu may represent some limitation for successful morphological and functional integration of transplants into the injured brain. In an approach to overcome potential restrictions to differentiation imposed to transplants by the altered environment, Hoane and co-workers pre-differentiated murine embryonic stem cells into neuronal and glial precursors and transplanted these cells into pericontusional areas of immunocompetent animals one week after exposure to unilateral controlled impact injury (41). Two days after transplantation animals were tested on a battery of behavioural tests. The authors found that transplantation of neuronal and glial precursors improved behavioural outcome by reducing the initial magnitude of the deficit on the bilateral tactile removal and locomotor placing tests. These cells also induced almost complete recovery on the vibrissae-forelimb placing test compared to control animals that failed to show any recovery at all. These neurobehavioural improvements were noted up to one month post-injury and were associated with reduced lesion volume at 40 days post-injury when transplanted cells, expressing neuronal and glial markers, were found in the pericontusional area, ipsilateral hippocampus and striatum, indicating migration into subcortical structures (41).

Rodent embryonic stem cells

Pluripotent embryonic stem cells (ESCs) derived from the inner mass of the blastocyst are able to differentiate into any tissue-specific cell type and may therefore possess great therapeutic potential in brain injury, since a variety of cell types are damaged or destroyed following cerebral trauma. In accordance with this assumption pluripotent murine embryonic stem cells (D3 ES cell line) have been shown to survive and differentiate following transplantation into rat brains in an experimental stroke model (42). Furthermore, enhanced green fluorescent protein (eGFP)-transfected D3 cells have been shown to migrate along the corpus callosum to the ventricular walls and to populate the border zone of the damaged brain tissue on the hemisphere opposite to the implantation site, indicating the highly migratory behaviour of implanted ES cells (42–44). It has to be taken into consideration that the highly proliferative characteristics (self-renewal potential) of embryonic stem cells combined with the ability to differentiate into all embryonic germinal layers (pluripotency) present a potential threat of tumor development (teratoma, teratocarcinoma) when they are transplanted into the adult central nervous system (CNS). Tumorigenesis has been observed after implantation of undifferentiated human ESCs into healthy rat brains, giving rise to teratomas and malignant teratocarcinomas (20). Accordingly, Erdo and co-workers compared the tumorigenic outcome after implantation of D3, clone BAC-7 ESCs in a homologous (mouse to mouse) vs. xenogeneic (mouse to rat) stroke model. In injured and healthy mouse brains, both transplanted undifferentiated and pre-differentiated murine ESCs produced highly malignant teratomas, while mouse ESCs xenotransplanted into injured rat brain migrated towards the lesion and differentiated into neurons at the border zone of the ischemic infarct, suggesting that tumorigenesis may be related to the host animal rather than to the differentiation status of the implanted cells (42).

Based upon these results, our own group recently implanted undifferentiated murine embryonic stem cells of the D3 line stably transfected with the pCX-(ß-act-)enhanced GFP expression vector (45) into ipsilateral cortices of immunosuppressed rats 72 hours after lateral fluid-percussion brain injury of moderate severity to examine their effects on neurofunctional recovery (46) (Figure 1). This strategy was based upon reports demonstrating that the initial inflammatory response decreases three days after the trauma to the nervous system and is succeeded by the development of astroglial scar within two days (47). The relatively early time point was chosen in order to avoid the peak of any inflammatory reaction and to allow for the migration and differentiation of stem cells that might be obstructed by the later formation of astroglial scar (47–50). Neuromotor function was assessed at one, three, and six weeks after transplantation using a Rotatrod and a Composite Neuroscore test. During this time period transplanted animals showed a significant improvement in the Rotarod Test and in the Composite Neuroscore Test as compared to
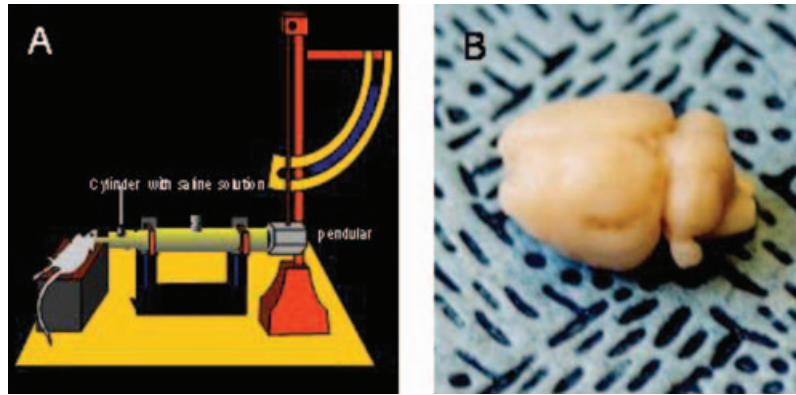
Figure 1. (A) Schematic representation of the Lateral Fluid Percussion Model; (B) Lateral fluid percussion induced cerebral lesion seven weeks post-injury.

controls. At one week post-transplantation, ES cells were detectable as distinct cell clusters in 100% of transplanted animals (Figure 2 A). At seven weeks following transplantation, only a few transplanted cells were detected in one animal (Figure 2 B).
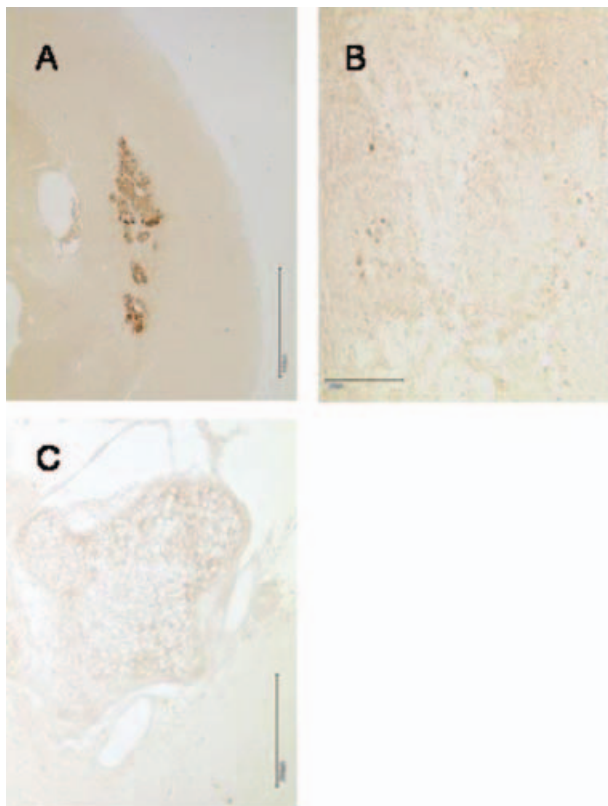

Figure 2. (A) Coronal section of the ipsilateral implantation site five days post-transplantation. Region stained with anti-GFP-ab reveals a cluster containing implanted cells. (B) Coronal section of ipsilateral implantation site, seven weeks post-transplantation; region stained with anti-GFP-ab reveals few GFP-positive cells residing at the former implantation site; (C) GFP-negative immunolabelling of a potential tumor (benign chondroma) located on top of the needle-track near the brain surface.

There was also tumor formation detected in this animal (Figure 2 C), although a tumor-suppressive effect of xenotransplantation has been suggested (42). Neither differentiation nor migration of cells was observed at any time point.

Due to the unexpected tumor formation, we critically investigated tumorigenesis and possible mechanisms of tumor-suppressive effects following xenotransplantation of D-3 murine ESCs into healthy adult rat brains. In 65% of healthy animals tumor formation was observed within two weeks of ES cell implantation, indicating the tumor-suppressive effect to mainly occur after transplantation of embryonic stem cells into injured rat brains (unpublished results).

This is in accordance with studies suggesting that low incidence of tumorigenesis following ESC xenotransplantation may be due, in part, to the removal of implanted cells by activated inflammatory cells in the injured brain (51).

Molcanyi and co-workers examined the time-dependent fate of these cells following ipsi- and contralateral implantation into rat brains injured via fluid-percussion injury. Double-staining for GFP and macrophage antigens revealed stem cell clusters embedded in and phagocytosed by infiltrated and activated macrophages, indicating the loss of implanted stem cells was due to an early post-traumatic inflammatory response (Figure 3). Macrophage infiltration was shown to be less pronounced when stem cells were implanted into completely intact healthy brains. The authors therefore suggested that the massive macrophage infiltration at graft sites might be ascribed to the combined stimulus exhibited by the fluid percussion injury and the substantial conglomeration of stem cells. Previous studies have also shown that the grafting procedure itself, i.e. needle insertion followed by pressure exerted by cell graft infusion, may provoke
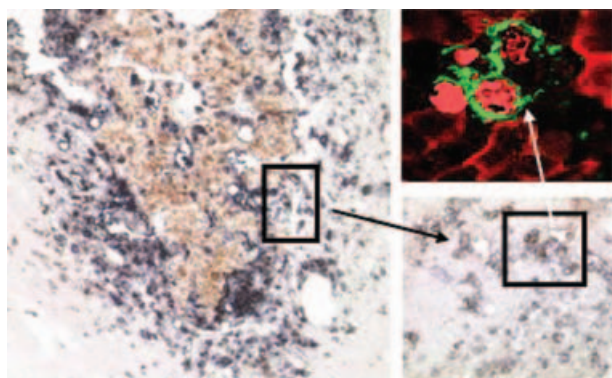
Figure 3. Phagocytosis of implanted cell clusters, five days post-transplantation. (A) Overview of the ES cell cluster surrounded by macrophages at the implantation site; cluster appears brown (GFP-DAB), macrophages are blue (ED-1–NBT/BCIP). (B) Lower section of the implanted graft embedded by macrophage population—detail. (C) Topographic three-dimensional reconstruction of phagocyting macrophage created by restacking all planar cross-sections in *x-y*-plane view, using AMIRA Silicon Graphics Software.

a notable cellular response in terms of macrophage invasion and microglia activation (52,53).

The massive loss of embryonal stem cells and the apparent lack of differentiation and migration of these cells following implantation into injured rat brains indicate that the significant improvement of motor function that was observed within five days of implantation is not due to the functional integration of stem cells into the neuronal cerebral network. *In vitro* studies demonstrated that the mechanism underlying stem cell-mediated functional improvement might be partially due to the release of trophic factors by implanted cells (54,55). Incubation of D3 stem cells with extract derived from injured rat hemispheres resulted in a rapid time-dependent and significant release of BDNF into the medium (54), indicating that similar to the observations made by Chen et al., the improvement of neuronal function following transplantation of embryonic stem cells might be triggered by the release of protective neurotrophic factors.

Human neural stem cells

The embryonic human brain contains multipotent central nervous system stem cells, which may provide a continuous, standardized source of human neurons that could virtually eliminate the use of primary human fetal brain tissue for intracerebral transplantation (56). Multipotential stem cells can be isolated from the developing human CNS in a reproducible fashion and can be exponentially expanded for more than two years. Vescovi and co-workers showed by clonal analysis, reverse

transcription polymerase chain reaction, and electrophysiological assay, that over such long-term culturing these cells retain both multipotentiality and an unchanged capacity for the generation of neuronal cells, and that they can be induced to differentiate into catechlaminergic neurons. When transplanted into the brains of adult rodents immunosuppressed by cyclosporin A, human CNS stem cells migrate away from the site of injection and express astrocytic and neuronal phenotype without tumor formation. The role of toxic immunosuppressive treatment to avoid rejection of transplanted cells is still on debate. Recently, Wennersten and co-workers demonstrated the importance of immunosuppression but also the possibility of shortening immunosuppression without impacting on the phenotype of the grafted hNSC (57).

In another study long-term growth-factor expanded human neural progenitors following transplantation into the adult rat brain was examined (58). Cells, obtained from the forebrain of a 9-week old fetus, propagated in the presence of epidermal growth factor, basic fibroblast growth factor, and leukemia inhibitory factor were transplanted into the striatum, subventricular zone (SVZ), and hippocampus. At 14 weeks, different migration patterns of the grafted cells were observed:

- Target-directed migration of doublecortin (DCX, a marker for migrating neuroblasts)-positive cells along the rostral migratory stream to the olfactory bulb and into the granular cell layer following transplantation into the SVZ and hippocampus,
- non-directed migration of DCX-positive cells into the grey matter in striatum and hippocampus, and
- extensive migration of above all nestin-positive/DCX-negative cells within white matter tracts.

At the striatal implantation site, neuronal differentiation was most pronounced at the graft core with axonal projections extending along the internal capsule bundles. In the hippocampus, cells differentiated primarily into interneurons both in the dentate gyrus and in the CA1-3 regions as well as into granule-like neurons. In the striatum and hippocampus, a significant proportion of the grafted cells differentiated into glial cells, some with long processes extending along white matter tracts. However, a large fraction of the grafted cells remained undifferentiated in a stem or progenitor cell stage as revealed by the expression of nestin and/or GFAP at 14 weeks survival time (58).

In an approach to evaluate the potential for human neural stem/progenitor cells to survive and differentiate following traumatic injury, Wennersten and co-workers transplanted long-term cultured and

cryopreserved neural stem/progenitor cells derived from 10-week-old human forebrain cells close to the injured site immediately after unilateral parietal cortical contusion injury in adult immunosuprressed rats (59). At two and six weeks post-intervention, the transplanted human cells were found in the perilesional zone, hippocampus, corpus callosum, and ipsilateral subependymal zone of the rats. Double labelling demonstrated neuronal and astrocytic, but not oligodendrocytic differentiation. At six weeks post-transplantation, the cells were noted to cross the midline to the contralateral corpus callosum and into the contralateral cortex. In another experiment, transplantation of human neural progenitor cells injected immediately following experimental cerebral contusion in the medial border of the lesion in immunocompetent rats resulted in a decrease of the number of degenerating neurons at six days post-injury (60). Control experiments revealed that host neuronal degeneration was increased in animals transplanted with non-viable human neuronal progenitor cells as compared to those receiving vehicle, indicating a bystander effect from introducing foreign antigen. In contrast, transplantation of viable progenitor cells attenuated neuronal degeneration, indicating that a potentially beneficial effect in progenitor cell transplantation is not limited to restoration by transplanted cells, but also improves survival of host cells by contributing essential trophic factors. These results suggest that expandable human neural stem/progenitor cells survive transplantation, migrate, differentiate, proliferate in the injured brain and may exert neuroprotective activity.

Human bone marrow-derived cells

Under experimental conditions, tissue-specific stem cells have been shown to give rise to cell lineages not normally found in the organ or tissue of residence. Neural stem cells from fetal brain have been shown to give rise to blood cell lines and conversely, bone marrow stromal cells (BMSCs) have been reported to generate skeletal and cardiac muscle, oval hepatocytes, as well as glia and neuron-like cells (61). The existence of stem/progenitor cells with previously unappreciated proliferation and differentiation potential in postnatal bone marrow and in umbilical cord blood opens up the possibility of using stem cells found in these tissues to treat degenerative, post-traumatic and hereditary diseases of the central nervous system. Recently, mesenchymal stem cells have been shown to express neuronal and glial markers *in vivo*. When transplanted into the naive adult rat CNS, these cells migrate throughout the brain and lose their immunoreactivity for mesenchymal markers and express neuronal and glial markers at 12 and 45 days post-transplantation (61). An advantage of these cells may be the fact that they can be harvested and cultured from the injured individual animal thus overcoming substantial issues associated with availability and toxic immunosuppression.

Mahmood and co-workers tested the hypothesis whether intracranial bone marrow (BM) transplantation would provide therapeutic benefit after traumatic brain injury (TBI) in rats (62). Bone marrow prelabeled with bromodeoxyuridine (BrdU) was harvested from tibias and femurs of healthy adult rats and injected adjacent to the contusion 24 hours after the controlled cortical impact. Histological examination revealed that BM cells survived, proliferated, and migrated toward the injury site. Some of the BrdU-labeled BM cells were reactive, with astrocytic (glial fibrillary acid protein) and neuronal (NeuN and microtubule-associated protein) markers. Transplanted BM expressed proteins phenotypical of intrinsic brain cells, i.e. neurons and astrocytes. A statistically significant improvement in motor function in rats that underwent BM transplantation was detected at 14 and 28 days post-transplantation.

In order to bypass relevant issues associated with direct intraparenchymal application of transplants, the same group administered BMSCs via intra-arterial (63) and –venous (64,65) injection at 24 hours following controlled cortical impact in rats. Marrow stromal cells injected intravenously into the tail vein significantly reduced motor and neurological deficits compared with control groups by day 15 after traumatic brain injury. The transplanted cells preferentially engrafted into the parenchyma of the injured brain and expressed the neuronal marker NeuN and the astrocytic marker glial fibrillary acidic protein. Marrow stromal cells were also found in other organs in female rats subjected to traumatic brain injury without any obvious adverse effects. For intra-arterial administration, Bromodeoxyuridine (BrdU)-labeled MSCs were injected via the ipsilateral internal carotid artery (ICA) at 24 h after TBI and the distribution of implanted MSCs was analyzed seven days after transplantation. Four groups were studied:

- group 1, animals transplanted with MSCs cultured with nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF);
- group 2, animals transplanted with MSCs cultured without NGF and BDNF;
- group 3, animals injected with a placebo (phosphate buffered saline); and
- group 4, rats subjected to TBI only.

In groups 1 and 2, BrdU-positive cells were localized to the boundary zone of the lesion, corpus callosum and cortex of the ipsilateral hemisphere. The number of BrdU-positive cells was significantly higher in the ipsilateral hemisphere than in the contralateral hemisphere. More MSCs infused intraarterially engrafted in group 1 (18.9%) than in group 2 (14.4%, $p < 0.05$). Using double staining, BrdU-positive cells expressed MAP-2, the neuronal marker NeuN, and the astrocytic marker glial fibrillary acidic protein (GFAP) in both groups 1 and 2, with this expression being greater in group 1 and the difference between two groups reaching statistical significance in the case of MAP-2. These results suggest that MSCs infused intraarterially after TBI survive and migrate into the brain. The addition of NGF and BDNF promote migration of MSCs into the brain and subsequent expression of neuronal protein MAP-2 (63). However, it is noteworthy that at one or two weeks following administration, bone marrow stromal cells were found to a substantial percentage in multiple organs (such as heart, lung, kidney, liver, muscle, spleen, and bone marrow), with only a limited percentage reaching the brain.

In another approach the effects of intravenous MSCs administered 24 hours after traumatic brain injury on the expression of growth factors in rat brain were assessed (66). The results from this experiment indicated that the post-traumatic neurobehavioral improvement observed following BMSCs intravenous injection was associated with an increased NGF and BDNF release at five and eight days post-injury in the injured hemisphere. The authors suggested that behavioral/histological improvement following stem cell transplantation might be linked to an up-regulation of neurotrophic factors.

Finally, the effects of intracerebral and intravenous administration of MSCs on endogenous cellular proliferation at 24 hours after controlled cortical impact brain injury in rats were assessed. In order to study newly generating cells all animals were injected with Bromodeoxyuridine (BrdU) intraperitoneally and coronal brain sections were stained immunohistochemically with diaminobenzidine to identify newly generating bromodeoxyuridine-positive cells at 15 days post-injury. To study the differentiation of newly generating cells into neurons, sections were also double-stained for neuronal markers (Tuj1, doublecortin, NeuN) with fluorescein isothiocyanate. Newly generating cells were mainly present in the subventricular zone, hippocampal formation, and boundary zone of contusion of both treated and control animals. Intracerebral MSC treatment significantly increased the progenitor cell proliferation in the subventricular zone and boundary zone compared with the controls, whereas intravenous MSC treatment enhanced this endogenous proliferation in subventricular zone, hippocampus, and boundary zone. In both groups, some of the new cells revealed positive staining for neuronal markers. A statistically significant functional improvement was observed in both the intracerebrally and intravenously treated groups. The promotion of endogenous cellular proliferation by intracerebral and intravenous MSC administration after experimental traumatic brain injury may be suggestive of an increased neurogenesis possibly mediated by the trophic factors released by the transplanted cells (66).

The same research group demonstrated that benefits associated with human bone marrow stromal cell (hMSC) administration that were observed 14 days after traumatic brain injury in rats can persist up to three months after injury, providing evidence that hMSCs injected in rats after traumatic brain injury survive up to three months and provide long-lasting functional benefit. This improvement may be attributed to stimulation of endogenous neurorestorative functions such as neurogenesis and synaptogenesis (67). In the search to further augment the benificial effects of MSCs administration in experimental traumatic brain injury, this group recently investigated the effects of a combination therapy of marrow stromal cells (MSCs) and statins (atorvastatin) after traumatic brain injury via controlled cortical impact in rats (68). Superior functional improvement was seen with two established testing modalities, i.e. modified neurological severity scores and Morris water maze, in animals receiving combination therapy. Microscopic analysis revealed significantly more MSCs in animals receiving combination therapy than in those receiving MSCs alone. Also, significantly more endogenous cellular proliferation was seen in the hippocampus and injury boundary zone of the combination therapy group than in the monotherapy or control groups.

In summary, the results from these experiments indicate that cell therapy for exprimental traumatic brain injury may also include the use of adult non-specific neural stem cells. As traumatic brain injury in humans usually represents a rather heterogenous disease with coexistence of focal and diffuse patterns of injury as well as multiple and multifocal contusional aereas the idea of multiple injections into multiple brain regions appears rather non-practical. Thus, the availability of a cell population to be harvested from the injured itself or genetically engineered *in vitro*, administered systemically with migratory properties towards the targeted site of the

lesion through the blood brain barrier makes bone marrow stromal cells an attractive option in the treatment of traumatic brain injury.

Discussion

The use of stem cells in cell replacement therapy has received a great deal of scientific and public interest in recent years. This is due to the remarkable pace at which paradigm-changing discoveries have been made regarding the neurogenic potential of embryonic, fetal, and adult cells (69). During the past decade, several stem/progenitor cell lines from various sources have been assessed for their potential to improve neurofunctional and –behavioural outcome after transplantation into the experimentally injured brain. A number of studies has indicated that cells engrafted into the injured brain can survive and, at least in part, may reverse behavioural dysfunction and histomorphological damage. However, the detailed mechanisms underlying the observed improvements are yet to be defined. It is assumed that cell-based strategies to treat traumatic brain injury may ideally act via

- differentiation and proliferation of engrafted cells into the phenotype of cells damaged or lost due to injury,
- local trophic support, and/or
- stimulation of the local microenvironment to enhance neuroprotection and –regeneration (11).

For functional integration of transplanted cells into the remaining cerebral network after injury, neurons need to

- develop polarity with the extension of axons and dedrites
- make functional synapses to other neurons in order to integrate properly into functioning circuitries,
- express and secrete functional neurotransmitter, and
- possess electrophysiological properties.

To date, the potential of engrafted cells to differentiate into neurons relies solely upon the expression and detection of specific neuronal markers and none of the above features has been observed following cell transplantation into the experimentally injured brain. The attenuation of neurofunctional and – behavioural dysfunction after traumatic brain injury, as frequently shown, has been observed in the order of days and weeks after transplantation. These observation periods are most likely too short for engrafted cells to develop and differentiate into mature und functional neurons.

Further work needs to be conducted to evaluate the low rate of cell survival after transplantation and the risk of tumorgenesis of embryo-derived cells (46). It has been shown that graft survival is greater in the acute phase after injury (70). However, the intracranial system may have a reduced ''compliance'' immediately after impact making the transplantation of excessive volumes into the brain rather unrealistic in order to prevent additional intracranial hypertension. A promising approach in cellular replacement is the pre-differentiation and/or genetic modification of stem cell cultures prior to transplantation. This strategy may provide the potential to generate sufficient cell quantities of the desired phenotype after grafting (71).

The normal brain is generally considered to be an immunologically privileged organ. However, this ''privilege'' may be lost in the acute phase after injury when the blood-brain-barrier is disrupted with subsequent influx of inflammatory cells into the brain. Our own experiments emphasized the presence of the local trauma-associated inflammatory response to substantially impair embryonic stem cell survival and integration after implantation into injured rat brain (51). Interestingly, own *in vitro* studies showed a cell-line dependent production of neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) after two different embryonic stem cell lines, i.e. BAC7 and CGR8, had been exposed to cerebral tissue extract derived from fluid percussion-injured rat brains (54). Thus, graft survival within the injured brain may be of particular interest in brain transplantation. Graft survival may be enhanced by selecting specific cell sources and by a better understanding of local microenvironmental sequalaes and graft-versus-host interactions including immunosuppression. Triggering specific factors and/or substrates in the culture media may additionally protect grafted neurons from oxidative and metabolic stress thus providing epigenetic trophic support. Transducing cells with factors that support survival in the local post-traumatic microenvironment may also confer protection on post-injury transplants (72). Transplant survival and integration into the injured brain may also be related to the severity of the injury sustained. Shindo and co-workers recently reported substantial differences in transplant survival, differentiation and functional outcome between mild and severe experimental traumatic brain injury (73). Following mild injury transplanted neurospheres were observed to survive in the hippocampus (CA3 region), but barely survived following severe traumatic brain injury.

Potential endogenous sources of cells to repair the injured brain include the transdifferentiation of various types of adult cells into neurons. Despite the excitement generated by examples of this phenomenon, additional work needs to be conducted towards the identification of instructive cues that generate neural stem cells from other tissue types and to evaluate whether these new cells are physiological with respect to their function (69). Furthermore, fundamental issues related to cell homogeneity and fusion need to be further addressed before the true potential of transdifferentiation can be understood. As known, endogenous stem cells also reside in neurogenic zones of the adult brain, for example ventricle lining and hippocampus. Basic understanding of the mechanisms that stimulate cell division and migration is required to learn how to amplify the small amount of new cells generated by the adult brain and to direct these cells to areas of injury or degeneration. Last but not least, a more fundamental understanding of brain injury and its sequelae is required to circumvent local brain environmental restrictions on endogenous cell differentiation and survival and to characterize the optimal time window after trauma when the generation of new neurons will lead to maximum restitution of neuronal circuits and functional recovery.

Future research with focus on cellular replacement after traumatic brain injury will benefit from multidisciplinary exchange between experts in the fields of neurotrauma and stem cell biology. Novel technologies, e.g. magnetic resonance imaging may be supportive to improve non-invasive approaches at high spatial and temporal resolution to document the survival, migration and differentiation of grafted cells (44,74). The repair of the traumatically injured brain is challenging due to the loss of a variety of different cell types and subsequent long-term atrophy (8). The clinical application of cellular replacement following traumatic brain injury remains a significant hope but requires additional and extensive pre-clinical research to demonstrate that neurons generated from stem cells

- can safely survive in brain injured animals,
- migrate to appropriate locations,
- show morphological and functional properties of neurons that have died, and
- establish afferent and efferent synaptic interactions with neurons that survived the insult.

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