Stem Cell–Based Tissue Replacement After Stroke
Factual Necessity or Notorious Fiction?

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The increasing stroke incidence and the long-term, severe disability of survivors requiring complex nursing care over extended time periods cause an enormous social and economic burden to ageing societies. Unfortunately, all clinical trials on neuroprotectants have failed thus far. The only approved therapy is clot lysis (recombinant tissue-type plasminogen activator [r-tPA]), which is restricted to only 4.5 hours poststroke onset.

Although r-tPA application is statistically justified, it can result in severe adverse events, complicating individual r-tPA treatment decisions. Thus, the current strategies in stroke management are focused on prevention by identification of risk factors and intensive rehabilitation in the chronic phase. However, stroke outcomes remain poor, causing a strong but unmet demand for alternative therapeutic approaches. The failure of the neuroprotective paradigm and limited eligibility for thrombolysis spawned an interest in stem cell–based neurorestoration, which is characterized by a wide therapeutic time window and is highly congruent with rehabilitation.

However, stem cell–based tissue replacement may be aggravated by pathophysiological and anatomic features, whereas the beneficial effects of (stem) cells may not necessarily result from cellular restoration. Here, we review the state of the art of cell-based stroke therapies and balance arguments supporting and challenging the concept of poststroke tissue restoration. Moreover, we discuss the respective therapeutic mechanisms related to tissue restoration versus indirect means of regenerative support including practical issues such as transplantation time windows, routes of cell administration, and potential detrimental effects.

Part I: The Concept of Cell Replacement and Arguments for Its Benefit

The replacement of lost brain tissue by transplanted cells has fired the imagination of researchers for decades. Studies on lesion-induced axonal sprouting of catecholamine neurons devised the fundamentals for brain-regenerating strategies. Then, the functional connections of transplanted monoamine neurons were demonstrated, whereas fetal nigral transplants were able to reverse parkinsonism in animal models and patients. These early proof-of-principle studies supported the notion that replacement of lost brain cells by stem and progenitor cells may be viable option.

Neuronal Replacement for Stroke

The positive effects of dopaminergic neuron replacement in Parkinson disease are of great interest to stroke researchers. It was concurrently shown that fetal striatal grafts increased GABA release and reorganized GABA receptors in the infarcted striatopallidum, leading to improved spatial and conceptual learning. However, the integration of grafted cells in the host brain on the cellular level was not investigated, whereas fetal tissue was considered insufficient to counter a high-prevalence disease such as stroke. This resulted in a continuous search for unlimited sources of cells for replacement therapy.

Teratocarcinoma-derived neural progenitors are characterized by unrestricted expansion and were found to differentiate to postmitotic presumptive neurons (NT2N cells) in the presence of retinoic acid. The cells also showed molecular and structural polarity after transplantation into the rodent cerebrum, survived for 1 year in the nude mouse brain, and promoted behavioral recovery in ischemic rats. These positive preclinical findings represented a translational cornerstone for the transfer of the neuronal replacement paradigm to the clinic. The first clinical stem cell trial for stroke (phase I, only patients in the chronic stroke phase) proved the safety of the procedure. Detection of positive effects in a few patients missed statistical significance. However, serial [18F]fluorodeoxyglucose position emission tomography demonstrated a relationship between relative regional metabolic changes and the clinical performance of patients.

A neuropathological assessment of a
patient from this trial revealed neurofilament-immunoreactive neurons at the graft location and the absence of neoplasms. Functional integration of transplanted cells within host neuronal circuitries was not investigated. The subsequent phase II clinical trial resulted in improvements in some patients, but again no overall benefit was confirmed statistically. However, the limited statistical power of early stage clinical trials must be considered when assessing results from such studies. Given the primary focus on safety aspects and the relatively small number of patients enrolled, any therapeutic effect must be of notable size to be detected at statistical significance. Although statistical prove of a therapeutic effect of realistic size cannot be expected in phase IIa/b studies, efficacy end points are often included in the study design and are considered crucial when deciding on the continuation with a respective therapy, particularly in the industrial environment. Although understandable from an economic perspective, this practice clearly bears the risk for false-negative results and premature abandonment of otherwise promising experimental therapies.

Because the outcome of clinical studies using postmitotic, tumor-derived neurons has not been completely convincing, the strategy of using primary fetal-derived neural stem cells (NSCs) has been revived. Moreover, it is now speculated that true tissue restoration strategies will have to consider all major components of cerebral tissue, that is, neurons, astro- and oligodendroglia, which may come with different challenges (Figure 1A). One study convincingly showed neuronal differentiation, but integration of the graft was not studied. Another study revealed not only neural differentiation of grafted embryonic medial ganglionic eminence precursor cells but also robust

Figure 1. Poststroke tissue restoration in theory and practice. A. In theory, tissue restoration strategies will have to target all 3 major cerebral cell populations: neurons, astro- and oligodendrocytes. Replacement of each individual component may come with different challenges. Printed with permission of artwork creator David Rini. Copyright ©2015, Johns Hopkins University. B. In a recent study, medial ganglionic eminence (MGE)–grafted cells enhanced synaptophysin expression, which was quantified in the contralateral and ipsilateral sides (insets in brain slice scheme). Data are expressed as mean±SEM. *P<0.05 vs vehicle. C, Representative current clamp trace of an action potential from an MGE neuron, implanted into the dorsal striatum of a stroked rat, elicited in response to a 400 pA current injection (left). Sample trace of spontaneous excitatory postsynaptic currents in the MGE neuron held at −70 mV (native resting membrane potential for this cell was −56 mV; right). B and C, Adapted from Daadi et al. Copyright ©2009, Cognizant Communications Corp (see: http://creativecommons.org/licenses/by-nc/3.0/). Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
integration in host tissue, including synaptic connectivity and reorganized neuronal networks in the infarcted area, as evaluated by electron microscopy and electrophysiology on brain slices (Figure 1B). This translated into sustained behavioral improvement. In fact, the presence of vital cells exhibiting neuronal phenotypes that emerged from the graft and their meaningful functional integration into host brain tissue should be shown (Figure 1C) when claiming true tissue restoration by a given approach (see Table for examples).

The expression of neuronal markers has also been observed after transplantation of a human clonal stem cell line derived from the fetal neuroepithelium (ReNeuron Ltd), although the cells did not show mature neuronal morphology. Clinical trials were initiated with the Pilot Investigation of Stem Cells in Stroke (PISCES) study currently in long-term follow-up (NCT01151124). Transplantation of the NSI-566RSC line (Neuralstem Inc) into a rat stroke model revealed that most cells remained at the striatal transplantation site, but a small amount of human neuron-specific enolase+ fibers extending dorsally and ventrally from the graft were identified. The functional relevance of this sprouting remains unclear.

It was recently shown that human, induced pluripotent stem cell–derived, long-term neuroepithelial-like stem (human induced pluripotent stem cell–long-term self-renewing neuroepithelial-like stem cells) cells can differentiate into neuroblasts and mature GABAergic neurons. Induced pluripotent stem cells can be used in autologous scenarios, but a detailed knowledge of their integration is lacking. Inhibition of microglia/macrophage activation and mitigation of neuronal loss have been reported and potentially indicate indirect mechanisms of action.

Supplementation of Glia
Supplementation of glia has not attracted major interest thus far. This may be related to the so-called glial scarring after stroke, primarily considered a plasticity-impairing phenomenon. Although astroglia is present in abundance, there are scarce data on the fate of oligodendrocytes. We observed the extensive demyelination of still-viable axons after small striatal infarcts (unpublished data), which might cause clinical deficits. This stroke sequel could potentially be addressed by transplantation of glial/oligodendrocyte progenitors. The glial/oligodendrocyte precursors exhibit an excellent differentiation potential and could become an attractive supplement for stroke therapy (Figure 2).

Brain Tissue Engineering for Stroke
Although the above-mentioned approaches were focused on the delivery of cell suspensions to the living brain tissue, there have been attempts to repopulate the poststroke tissue cavity in the
chronic phase. Any physiological guidance structure such as radial glia or white matter bundles is likely to be absent in a lesion cavity, but may be crucially required for ensuring graft differentiation at the desired location. Moreover, when repopulating larger brain lesions, the neuroanatomical structure of the repopulated brain elements must be resembled, whereas larger transplants will have to provide vascular-like elements for adequate blood supply and a structural support of the tissue matrix. These aspects call for scaffolds to support the graft (Figure 2). It was demonstrated that injectable scaffolds, such as polyglycolic acid particles, can serve as a structural support for NSCs when delivered directly to the stroke cavity. Moreover, the efficient integration of such neuroscaffolds within host tissue has been observed. The various scaffolding approaches for brain tissue engineering have been extensively reviewed. Matrigel scaffolds were shown to provide a permissive environment for neuronal differentiation, with some transplanted cells revealing spontaneous action potentials or excitatory postsynaptic currents by post mortem whole-cell patch-clamp records. This coincided with behavioral improvements, but again, the existence of direct connections between the transplanted neurons and host circuits was not investigated. Polymer fibrous scaffolds have been shown to excellently support NSCs, and differentiated neurons probably reconstituted some anatomic connections, including long-distance projections. Some authors even propose channel-like, large matrices seeded with cells in vitro before neurosurgical implantation into the infarcted hemisphere (Figure 2).

Part II: Recovery Without Replacement and the Prevalence of Indirect Mechanisms

Functional recovery was originally interpreted as directly related to graft survival and integration. Thus, ensuring these aspects was thought important for therapeutic success, but eventually became a substantial challenge. In their pioneering study, Bühnemann et al reported initial engraftment and impressive subsequent expansion of murine embryonic stem cell–derived NSCs, leading to newly formed tissue 4 weeks after transplantation in the rat brain. Differentiation into all neural lineages, projections into the host tissue, and even appropriate electrophysiological activities of graft-derived neurons and astroglia were observed. However, most of the grafts disappeared between 4 and 12 weeks after transplantation and those that survived were subject to a significant decline in graft size of ≥90%. Despite thorough immunosuppression, enhanced microglial activity, rather than apoptosis and inappropriate metabolic graft support, was reportedly responsible for this loss.

However, neuronal replacement does not necessarily ameliorate functional impairments, whereas allogeneic transplantation leading to differentiation and structural integration may be successful in the healthy brain, but not necessarily after stroke. Experimental transplantation of various adult stem and progenitor cell–containing populations is frequently performed systemically to facilitate future clinical translation and has been repeatedly reported to improve neuronal function after stroke without neuronal replacement or prolonged graft survival, usually in a dose-dependent fashion. Mesenchymal stem cells (MSCs) are a prominent example. Although a differentiation capability into neuronal cells has been proposed, MSCs are thought to exert their therapeutic potential primarily by secretion of numerous paracrine factors, so-called bystander effects. Because MSCs were shown to robustly improve multiple outcome measures after experimental stroke, early stage clinical studies using MSCs are currently underway to investigate the approach in the clinical environment. The uncomplicated MSCs derivation and the possibility for autologous use make them even more attractive. Although optimal conditions of application remain to be defined for experimental therapies using adult cell populations, such therapies were also reported to have much wider time windows than r-tPA lysis and can address several degenerative or prerregenerative mechanisms after stroke apart from cell replacement (Figure 3; Table). The following paragraphs will discuss challenges for tissue replacement approaches and alternative strategies using bystander effects exerted by NSCs and selected non-neuronal, adult stem cell populations.

Considerable Challenges for Tissue Replacement Therapies in the Adult Lesioned Brain

Although replacement of lost cerebral tissue theoretically seems to be the most attractive strategy to achieve sustained restoration of neuronal function in the chronic phases after stroke, related therapeutic concepts may be hampered by practical difficulties even if graft survival was ultimately ensured. Stem cell differentiation must be perfectly synchronized with respect to functional, spatial, and temporal dimensions to ensure a permanent and sustained therapeutic effect. Functional aspects comprise the frequency and type of graft-derived neuronal, astro- and oligodendroglial cells, as well as the exact numeric ratio between those in a particular brain
area to be repopulated. Adequate access to cerebral blood supply and the neuroimmunological integrity of the graft must be ensured. Newborns further need to integrate at the correct place and at the correct time to interact meaningfully with each other, and, most importantly, with the remaining host brain tissue. Moreover, a plethora of pathophysiological responses take place after ischemic stroke, with some occurring simultaneously and most being detrimental, and all of which must be taken into consideration.

A perfect interaction of stem cell–borne neural populations is observed during embryo- and fetogenesis under physiological conditions, resulting in the human brain as one of the most complex organs and its neocortex as a masterpiece of evolution. Despite important advances in the understanding of brain and cortical development, our knowledge about the cellular, biochemical, and electrophysiological processes directing this interplay still remains fragmentary. However, even minor disturbances during this susceptible and delicate process can have significant consequences, and there is evidence for a strict regulation and temporal limitation of structured postnatal neurogenesis. This may, at least partly, account for the observation of teratomas or teratocarcinomas after transplantation of pluripotent stem cells into the adult mammalian brain. These tumors resemble rather primitive tissue formations, such as the neural tube, and typically emerge when precise regulatory processes of cell growth and differentiation are absent or have ceased. Thus, the assumption that the highly complex and incompletely understood pre- and early postnatal cerebral development can be locally recapitulated in the lesioned and adult brain only by placing pluripotent cells at a desired location may be simplistic. The risk of uncontrolled differentiation may be controlled effectively by ensuring a limited expansion potential of the grafted cells by previous differentiation, or the induction of suicide genes into transplanted cells. But, as yet, we can present neither a convincing concept of how to precisely orchestrate and control stem cell differentiation and integration in the adult and lesioned brain nor can we be confident that this regulation occurs both spontaneously and autonomously.

However, promising opportunities to beneficially influence the course of lesion development and maturation with stem cell–based therapies, which do not necessarily rely on cellular differentiation and integration and might therefore not require a detailed understanding of the underlying processes, could be used alternatively. This becomes especially relevant because our understanding of stroke as a focal event embedded in a systemic pathophysiological context is currently completing. Moreover, this approach can be considered advantageous from a pragmatic point of view: it may be easier to realize than complete deciphering of fundamental cerebral developmental biology.

### Extracerebral Causes of Neuronal Damage and the Impact of Stem Cell Transplantation

Recent data suggest that stroke, formerly recognized as a brain-specific disease, is accompanied by several pathophysiological processes throughout the entire organism. In particular, the interaction between the immune and the central nervous system was highlighted when immigrating splenic monocytes were identified as major contributors to delayed brain damage. A beneficial influence of adult stem cell–containing populations, such as human umbilical cord blood cells, was found countering these processes, and an interruption of the splenic response by intravenously injected NSCs has been described after hemorrhagic stroke. Systemically administered hematopoietic stem cells have been reported to accumulate in the spleen, downregulate inflammatory genes, and to attenuate deleterious brain inflammation after stroke. It was moreover reported in an animal sepsis model that intravenously injected MSCs could reprogram pulmonary macrophages to reduce inflammatory cytokine secretion. From these observations, it is tempting to speculate that immuno-modulatory effects of transplanted cells are independent of the cell type, but rather a result of an interaction with specific macrophage populations being responsible for the maintenance of daily immune tolerance.

### Neuroprotection, Growth Factors, and Angiogenesis: The Role of Bystander Effects

Interestingly, there is no study that convincingly shows that neuronal differentiation is a prerequisite for functional improvement after stroke, which is consequently discussed as strongly related to trophic effects rather than neuronal replacement. If the repopulation of the lesioned brain is too challenging, a promising alternative approach is the prevention of neuronal cell death beyond the acute phase. In fact, a formidably amount of tissue damage has been reported to occur in the subacute phase of stroke, providing excellent opportunities for therapeutic intervention.

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**Figure 3.** Pathophysiological aspects after ischemic stroke. A plethora of stroke sequelae contribute to immediate and delayed cell loss and impaired regeneration. Transplanted cells have been shown to affect relevant pathophysiological mechanisms, but successful treatment seems to be highly dependent on the time point of transplantation. When administered in the acute phase of stroke, cells can attenuate primary neuronal cell death, detrimental neuroinflammation and promote vascular repair and neovascularization. These early effects were frequently observed together with low or absent cell survival and integration, or even rely on cell scavenging. By contrast, the modulation of factors determining stroke outcome in the chronic phase seems to require prolonged presence of transplanted cells. Therapeutically relevant brain repair and attenuation of chronic inflammation may demand the secretion of sufficient amounts of trophic factors and continuous manipulation of perivascular spaces.
It has been suggested that the neuroprotective properties of NSCs may be a fundamental characteristic of their biological constitution. If the cells are a source of restorative processes, they must be more resistant against the detrimental influences that occur in the hostile micromilieu after central nervous system damage. NSCs can modulate the local environment to prevent such influences, thereby supporting the actions of neighboring neurons as a bystander effect. NSCs can, among others, also produce a broad spectrum of trophic factors, such as nerve growth factor NGF, brain-derived neurotrophic factor, and glia-derived neurotrophic factor, which play pivotal roles in neuroprotection, as they mitigate caspase-mediated apoptosis in the injured central nervous system. Glia-derived neurotrophic factor has been reported to be neuroprotective by promoting cell survival, but also enhances axonal outgrowth and synaptogenesis. To augment the growth factor–mediated neuroprotective potential of NSCs, therapeutic approaches that use the induced overexpression of factors such as brain-derived neurotrophic factor for the treatment of ischemic stroke or glia-derived neurotrophic factor for hemorrhagic stroke have been suggested. These strategies can even be combined with the use of cytoprotectants, making transplanted NSCs more resilient against oxidative stress and reperfusion injury. In all these cases, permanent survival of the graft is not a necessary condition to elicit the beneficial effect. Next to the growth factor–exerted neuroprotection in the subacute stage after stroke, the support of angiogenesis may contribute to the beneficial effects mediated by NSCs. In particular, the normalization of cerebral blood flow and blood–brain barrier integrity in perilesional areas is a relevant factor for functional restoration after stroke. Indeed, NSCs were shown to restore blood–brain barrier integrity and enhance angiogenesis in the postischemic brain. This is probably mediated by vascular endothelial growth factor, which can also help to preserve the microvasculature after cerebral ischemia. Thus, NSCs have the potential to preserve and restore an adequate cerebral blood flow in the poststroke brain. Importantly, bystander effects by secretion of immunomodulatory or (neuro)trophic paracrine factors as reviewed above are considered an important if not universal therapeutic mechanism which is also exerted by adult stem cell populations such as MSCs and umbilical cord blood mononuclear cells.

**Neurovascular Niche and Poststroke Brain Plasticity**

The preservation and recovery of the cerebral microvasculature are of importance beyond the maintenance of cerebral blood flow because of a close physiological interplay between angiogenesis and neurogenesis. This interaction, demanding spatial proximity, has been characterized as occurring in the neurovascular unit or neurovascular niche. It was further suggested that blood vessels represent directional structures for migrating endogenous neural stem and progenitor cells. Hence, it comes as no surprise that the potent proangiogenic factor, vascular endothelial growth factor, was shown to play a major role in the not only regulation of the neurovascular niche but also recruitment of NSCs into it. The neurovascular niche has been discussed as relevant for poststroke recovery, and NSCs may support this role by contributing to the preservation of the cerebral microvasculature.

Functional improvement has often been observed within a relatively short time after NSCs transplantation. De novo generated neurons, regardless of emerging from endogenous or exogenous stem cells, are unlikely to have induced this recovery. Recovery was therefore suggested to be caused by other processes than draft-derivated neuronal differentiation and the impact of NSCs on brain plasticity was investigated. An enhanced density in the network of corticostral, corticothalamic, and corticospinal connections was observed in stem cell–treated rats, which was related to functional recovery. Interestingly, vascular endothelial growth factor was again found to be a key mediator of these effects. Moreover, increased dendritic branching and increased synaptic plasticity have been reported, both related to swift functional recovery within 3 weeks after transplantation. This time is shorter than the time anticipated as being required for neuronal differentiation and integration, which indirectly supports the notion that functional recovery induced by NSCs transplantation is not dependent on neuronal replacement.

**Neuroimmunological Aspects**

Ischemic cell death leads to damage-associated molecular pattern signaling and sterile tissue inflammation that contribute to brain damage, but also orchestrate clearance and wound healing processes. Moreover, a persistent proinflammatory environment reduces the regenerative potential of the brain and impairs brain function. Immunomodulation by transplanted cells therefore represents a promising option to ameliorate acute and long-term stroke consequences. In fact, it was reported that intravenously transplanted NSCs exhibit a pathotropism toward the inflamed brain and could arbitrate long-term immunomodulatory effects. Both NSCs and MSCs hold a receptor and ligand machinery enabling them to follow chemogradients and transmigrate into the ischemic brain. Interestingly, the mechanisms of stem cell homing resemble in many aspects that of leukocytes that drive poststroke inflammation. In experimental stroke models, transplanted NSCs were found in the ischemic lesion border where they cause a downregulation of proinflammatory cytokines. MSCs were shown to effectively inhibit the infiltration of detrimental leukocyte populations, thereby improving functional outcome. Systemically transplanted NSCs and MSCs could further migrate toward draining lymph nodes and suppress antigen-specific T-cell responses, a process that may be highly relevant for long-term outcome of stroke.

**Transationally Relevant Aspects of Tissue**

Restoring and Bystander Cell Therapies: Time Windows, Administration Routes, and Potential Adverse Effects

The predominant modes of action (Table) exerted by a particular cell therapy will have a significant impact on its practical implementation. One of the clinically most important
features is the time window in which a therapy is effective. Given the narrow time window of r-tPA lysis, any significant extension of the time window would provide a clear benefit. Indeed, time windows between 4 hours and 7 days have been described for mononuclear cells from cord blood and bone marrow, whereas time windows of up to a month are reported for MSCs. However, bystander effects targeting poststroke pathomechanisms are most likely restricted to a time window because those processes damp as lesion maturing proceeds (Figure 3). In contrast, the time window of a therapy initiating a stem cell–based brain tissue restoration would be theoretically unlimited. Therapy induction in the chronic phase, when the hostile poststroke environment has ceased, may even be beneficial.

Another clinically important aspect is the route of cell administration and related safety concerns. Cell therapies exerting tissue restoration or relying on intracerebral bystander effects will require local (ie, intraparenchymal, intraventricular) or intra-arterial cell delivery, respectively. Although stereotaxic cell transplantation comes at a small, but considerable risk of secondary damage, intra-arterial administration of larger cells such as MSCs or the application excessive cell numbers may lead to secondary infarctions. Although this risk is not apparent for smaller cell populations such as mononuclear cells or glial/oligodendrocyte precursors, those may be in turn pass cerebral circulation in significant numbers without reaching their primary site of action. Intravenous cell administration is discussed as a clinically unproblematic approach, especially useful for cell populations exerting bystander effects that are thought to target also extracerebral causes of poststroke neuronal damage. In this scenario, larger cells can be trapped within so-called filter organs featuring an extensive capillary network such as the lungs or the spleen. This risk is particularly prominent for MSCs, but also for NSCs. Potential consequences of the trapping phenomenon including splenic or pulmonary microinfarction are currently unclear and may hence require further investigation. Next to the induction of cerebral microinfarction or secondary brain tissue damage, local and intra-arterial cell administration may come at the risk of tumor-like neoplasms or tissue overgrowth, which is prevalent for cell populations exhibiting a strong proliferation potential. These potentially detrimental side effects must be ruled out for a certain cell therapy approach before considering its clinical application.

Conclusions
Although the initial stem cell transplantation studies in stroke were aimed toward a cell replacement strategy, there is growing evidence that many of the beneficial effects are mediated by indirect mechanisms, such as trophic support and immunomodulation. This has been paralleled by an increasing understanding of the considerable difficulties that may challenge tissue replacement strategies in the adult lesioned brain. However, state-of-the-art, rapid technological advances, for example, in the field of biomaterials, may support or enable true neurorestoration by cellular replacement or even de novo formation of structured and functional brain tissue. This would allow us to capitalize on both functional recovery by neurorestoration and functional preservation and enhanced plasticity by indirect stem cell–mediated effects.

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