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Stem Cells and Stroke: Opportunities, Challenges, and Strategies

Terry C Burns, MD, PhD and **Gary K Steinberg, MD, PhD**
Stanford University School of Medicine, Department of Neurosurgery

Abstract

INTRODUCTION—Stroke remains the leading cause of disability in the Western world. Despite decades of work, no clinically effective therapies exist to facilitate recovery from stroke. Stem cells may have the potential to minimize injury and promote recovery after stroke.

AREAS COVERED—Transplanted stem cells have been shown in animal models to migrate to the injured region, secrete neurotrophic compounds, promote revascularization, enhance plasticity and regulate the inflammatory response, thereby minimizing injury. Endogenous neural stem cells also have a remarkable propensity to respond to injury. Under select conditions, subventricular zone progenitors may be mobilized to replace lost neurons. In response to focal infarcts, neuroblasts play important trophic roles to minimize neural injury. Importantly, these endogenous repair mechanisms may be experimentally augmented, leading to robust improvements in function. Ongoing clinical studies are now assessing the safety and feasibility of cell-based therapies for stroke.

EXPERT OPINION—We outline the unique challenges and potential pitfalls in the clinical translation of stem cell research for stroke. We then detail what we believe to be the specific basic science and clinical strategies needed to overcome these challenges, fill remaining gaps in knowledge and facilitate development of clinically viable stem cell-based therapies for stroke.

Keywords

stem cell; neurogenesis; neuroregeneration; stroke; ischemic brain injury; neural progenitor cell; neuroblast; neuroprotection; clinical trial; translational research; plasticity; subventricular zone; migration; differentiation

1. INTRODUCTION

With an incidence of almost 800,000 new victims per year in the US alone, stroke persists as the leading cause of disability and the third leading cause of mortality in the Western world. Stroke leads to rapid destruction of brain tissue over several hours, with an estimated 1.9 million neurons, representing approximately 14 billion synapses dying each minute[1]. It is important to recognize that each lost neuron was born at a specified time and location during development as a result of complex sequences of physical and chemical signals as well as intrinsic timing mechanisms guiding progenitor cell fate. After birth, the immature neurons were precisely guided into appropriate locations, from which they extended projections

Corresponding Author Gary K. Steinberg MD, PhD, Bernard and Ronni Lacroute-William Randolph Hearst Professor of Neurosurgery and the Neurosciences, Director, Stanford Institute for Neuro-Innovation and Translational Neurosciences, Chairman, Department of Neurosurgery Stanford University School of Medicine, 300 Pasteur Drive, R281, Stanford, CA 94305-5487, Phone: +1 650 725 5562, Fax: +1 650 723 2815, gsteinberg@stanford.edu.

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along intersecting gradients of diffusible, membrane and extracellular matrix-bound molecules. They then competed successfully for neurotrophic signals and established thousands of activity- and experience-dependant synaptic connections. In the wake of stroke, these intricate networks are swiftly reduced to an expanding necrotic milieu of dead and dying cells. Adjacent neurons teeter on the edge of viability with marginal blood supply, where mounting inflammatory responses may mediate additional cell death. In addition to neuronal cell loss, even greater numbers of glia with likely under-appreciated location-defining and regulatory as well as supportive roles are also destroyed. Restoration of blood flow within the first three to four hours of stroke onset enables measurable improvements in outcome. However, only a small minority of patients arrive early enough to receive effective therapy. Despite decades of work and promising animal data, neuroprotective strategies aiming to limit further exacerbation of cell loss within and beyond this timeframe have uniformly failed in human trials[2–5].

Stem cells have the potential to generate nearly unlimited numbers of neural cells. Given the complex fidelity of neuronal development and integration, however, true cell replacement has proven an elusive goal. During the past decade, dozens of cell types have been tested via multiple routes of delivery in numerous animal models of stroke; in many cases, markedly decreased lesion size and improved functional outcomes have been achieved. Though some have claimed “replacement” of neurons by transplanted cells, others have encountered poor survival despite functional benefits, suggesting indirect mechanisms of recovery. Others have sought to stimulate the brain’s own stem cells toward regeneration with promising preliminary results[6]. Below, we evaluate the preclinical and clinical progress of stem cell therapy to date. We discuss current evidence regarding mechanisms of action, and outline pertinent opportunities, challenges, and strategies for safe and effective translation of stem cell therapy into clinical practice.

2. CELL TRANSPLANTATION FOR STROKE

2.1. Exogenous Stem Cells

Preclinical studies of cell transplantation have identified a surprising variety of cells that promote functional recovery after stroke. Work to optimize delivery parameters such as route, timing, cell dose, and immunosuppression is ongoing. To date, demonstrated mechanisms of benefit have included direct inhibition of cell death, enhanced regeneration of vasculature, immunomodulation, induction of neuronal plasticity, and promotion of endogenous neurogenesis.

2.1.1. Human Fetal Brain Cells—Pioneering cell transplantation work focused initially on replacement of dopaminergic neurons for Parkinson’s disease (PD). Studies employing fetal midbrain demonstrated behavioral benefits from “cell replacement,” prompting several clinical trials with variable outcomes. In the mid 1980s, Polezhaev and Alexandrova performed transplantations of fetal brain tissue into rat brains after ischemic injury. Robust engraftment was observed with evidence of synaptic integration. Grafts also decreased cell death and promoted the restoration of “dysfunctional” neurons to their normal state[7]. Grafts, which seemed to survive best in the penumbra[8] improved local neurotransmitter levels and facilitated cognitive recovery[9]. Human fetal brain tissue is a limited and ethically challenging resource. As such, no clinical trials of fetal cells have been pursued for stroke and significant efforts have sought to develop alternate cell types that may be more readily amenable to widespread clinical application.

2.1.2. Human Teratocarcinoma Cells—A teratocarcinoma cell line, NT2, was shown in 1984 to generate pure populations of post-mitotic neural-like cells upon exposure to retinoic acid[10]. In 2000, based on preclinical evidence for functional improvements in

animal models of stroke[11], these became the first cells reported in a phase 1 clinical trial of a cell-based therapy for stroke[12] (see table 1). Cells were grafted stereotactically into patients with stable deficits after a basal ganglia infarct; immunosuppression was continued for 2 months. Overall, no adverse effects were noted, and surviving cells were observed post-mortem with no evidence of neoplasm at 27 months[13]. In 2005, the report of a phase 2 randomized controlled trial involving 14 treatment and 4 control patients revealed functional improvements in some patients. Given the very small group sizes, improvements based on a primary outcome measure of European stroke scale at 6 months did not reach statistical significance. Reported adverse outcomes included one seizure and one subdural hematoma requiring evacuation[14].

2.1.3. Porcine Fetal Neural Cells—A major challenge of adult neural cell therapy is the relatively inhibitory environment presented by the adult brain to neurite outgrowth. It has been suggested that molecular differences between species may permit better engraftment of xenograft than allograft neurons. In 2005, Savitz et al published results from a trial employing stereotactic delivery of up to 50 million anti-MHC1 antibody-treated fetal porcine cells for stable basal ganglia stroke[15]. Of five patients enrolled, one showed temporary worsening of symptoms and another had a seizure. Both had questionably concerning findings on MRI, prompting the FDA to terminate enrollment. Two of the five patients experienced improvements in symptoms over several months that persisted at four years[16].

2.1.4. Bone Marrow Mononuclear Cells (BMMCs)—Endogenous bone marrow-derived cells are swiftly recruited to regions of ischemic injury. Administration of supplemental bone marrow mononuclear cells has been under investigation in animal models of stroke since 2000[17], with benefits attributed to various trophic mechanisms, in spite of largely poor long term survival. To date, BMMCs have appeared well tolerated in multiple small clinical trials, mostly involving intravascular delivery[18]. However, Suarez-Monteafudo et al recently reported long-term, asymptomatic EEG abnormalities after intraparenchymal BMMC administration[19]. Interestingly, meta-analysis of BMMCs in clinical trials for acute MI indicated a 4.77% improvement in left ventricular ejection fraction after three months[20]. Granulocyte-colony-stimulating factor (GCSF), which also has direct neurotrophic effects, may in part replicate the action of BBMCs by promoting mobilization of bone marrow cells. GCSF is now under clinical investigation for stroke[21, 22], having previously enabled a 3% increase in ejection fraction in meta-analysis of clinical trials for acute MI[23].

2.1.5. Mesenchymal Stem Cells (MSCs)—By selectively culturing bone marrow-derived cells that adhere to a culture dish in serum-containing media, cells variably termed marrow stromal cells or mesenchymal stem cells (MSCs) are generated that have shown benefit in animal models of stroke[24]. Recent meta-analysis of IV-delivered cells in preclinical studies for stroke showed the benefit of MSCs on behavioral outcome to be roughly twice that of BMMCs, consistent with the same study's finding that cell lines and cultured or genetically modified cells are significantly more efficacious than primary cells[25]. Much literature has focused on conditions that may promote the neuronal differentiation of such cells either *in vitro* or *in vivo* after transplantation. However, few if any such claims withstand current standards of scrutiny[26, 27].

MSCs offer a somewhat more homogeneous and well characterized cell population for cell transplantation. These cultured cells are also amenable to genetic manipulation, allowing targeted delivery of specific therapeutic compounds. Both BMMCs and MSCs seem to have quite limited survival in the brain after either local or systemic delivery. Thus it is likely that benefits are mediated predominantly via trophic signals of variable duration. Though widely

regarded as safe, some reports of MSC-derived sarcomas have appeared, suggesting that limits on passage numbers and stringent standards of cytogenetic quality control will be required for clinical applications[28].

Lee et al recently published five year follow-up data from a previously reported[29] randomized open label trial of IV administration of two doses of 50 million autologous MSCs. Five year outcomes suggested significantly improved mRS scores, as assessed by blinded observers (p=0.046). Of interest, levels of stromal cell-derived factor-1 (SDF-1), which have been associated with MSC, as well as NSC homing, were found to correlate positively with clinical outcomes[30]. MSCs have been suggested to stimulate endogenous neurogenesis after stroke[31]. Thus, it is worth noting that patients in whom the subventricular zone (SVZ) was spared from infarct (n=5) uniformly improved with MSC therapy, though outcomes in MSC-treated patients with infarct extending to the SVZ (n=11) were more variable. Although no adverse effects were observed in MSC-treated patients within five years, recruitment was suspended due to the publication of concerns regarding use of xenogenic bovine calf serum in culture media for grafted cells[30].

2.1.6. Neural Stem Cells—Techniques for the *in vitro* culture of neural stem cells were first described in the early 1990s by Reynolds and Weiss[32]. With inherent neurogenic potential, demonstrated trophic benefit, and minimal risk of tumorgenicity, NSCs represent an excellent cell therapy choice and have been widely employed in pre-clinical stroke studies during the past 10 years with encouraging results[25, 33]. Recent meta-analysis of preclinical studies employing intravenous cell delivery indicated that NSCs yielded the greatest behavioral improvements when compared to bone marrow-derived or other cell types [25]. Due in part to regulatory delays, very few clinical trials have been initiated employing neural stem cells. NSC-like olfactory ensheathing cells from the olfactory mucosa have been employed in a clinical trial for spinal cord injury with early establishment of safety and feasibility[34]. Neural stem cells from Stem Cells Inc were employed in a recently completed phase 1 trial of 6 patients for Neuronal Ceroid Lipofuscinosis, also known as Batten Disease[35]. The results of this study remain to be published. An open label trial of the neural stem cell line CTX0E03, from ReNeuron, Ltd, began in June 2010 and plans to enroll 12 patients for intraparenchymal delivery of 2–20 million cells, 6–12 months following subcortical stroke (see table 2).

2.1.7. Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem (iPS) Cells —ESCs possess the defining capacity to generate all cell types of a developing embryo under appropriate conditions. ESCs have received particular attention as a source of cells for which no reliable tissue-specific progenitor is available, such as cardiomyocytes, as well as certain neuronal lineages not readily obtainable from NSCs, including dopaminergic neurons for PD and motor neurons for ALS. The recent development of techniques to generate ES-like cells via epigenetic reprogramming, termed induced pluripotent stem cells, or “iPS cells,” opens the potential for autologous neural cell therapy, thereby averting the need for immunosuppression. Safety concerns regarding the viral constructs used to reprogram iPS cells are being mitigated by the development of transient transfection techniques that leave cells genetically unaltered after reprogramming[36]. By definition, however, ESCs do generate teratomas. As such, the development of differentiation and culture techniques that eliminate any residual undifferentiated ESCs continues to be a high priority. Neural stem cell lines derived from hESCs have been generated that promote functional recovery in animal models of stroke without tumor formation, and are under development for future clinical applications[37, 38]. Wernig’s lab recently demonstrated that selective genetic reprogramming may enable direct trans-differentiation of fibroblasts to functional neuronal cells without need for an intermediate ES or iPS cell stage[39].

In 2009, Geron received FDA approval to initiate the first ever clinical trial employing hESC-derived cells. This trial, for treatment of spinal cord injury, is based on the observation that pure cultures of hESC-derived oligodendrocyte precursors cells (OPCs) promote functional recovery by remyelinating axons in spinal cord-contused rats. A clinical hold imposed shortly after initial approval by the FDA for further safety evaluations was lifted July 30, 2010, allowing the trial to proceed.

2.2. Delivery Variables for Exogenous Cell Therapy

For any given cell type, a number of options are available regarding when, where, and how to implant, and what adjunctive treatments should additionally be administered. The variety of protocols in use suggests that "right" answers to these questions are not easily determined; optimal parameters may vary depending on the model, cell type, extent of injury, and outcome measure being assessed.

2.2.1. Administration Route—Though not without risks, stereotactic delivery allows precise targeting of defined numbers of cells to desired sites, with best survival seen in the peri-infarct region. The first clinical trials of NT2 stem cells for chronic basal ganglia stroke involved 25 cell deposits along 5 stereotactic tracts throughout the infarct area[40]. A recent protocol involving up to 88 deposits targeted selectively to the peri-lesion area was recently described for administration of MSCs[19].

Most studies of bone marrow-derived cells to date have employed intravenous delivery. Meta-analysis of preclinical results suggests robust benefit, in spite of limited evidence for significant numbers of cells reaching the infarct site[25]. Intra-arterial or intra-carotid therapy has been advocated by several groups to facilitate delivery to the ischemic region and minimize cell sequestration in systemic tissues such as liver, lung and spleen[41]. With appropriate protocols to regulate cell density and allow continued blood flow during injection, risk of microembolic infarcts resulting from adherent cell clusters or vessel occlusion can be minimized[42]. Brain penetration of NSCs after intra-arterial delivery appears to be dependent upon upregulation of vascular cell adhesion molecule-1 (VCAM-) following stroke, which binds the cell surface integrin CD49 that is expressed on the NSCs[43] . Future studies may assess whether or not genetic manipulation of receptor expression enhances targeting to the ischemic region. By comparison with stereotactic implantation, intravascular approaches have the advantage of readily allowing repeated administrations of cells. Combinations of intraparenchymal and intravascular therapies may also be feasible.

2.2.2. Cell dosage—Recent meta-analysis of intravenously-delivered cells in animal studies showed a robust effect of cell dosage on lesion size, behavioral outcome, and molecular measures of outcome such as apoptosis, neurogenesis and angiogenesis[25]. Darsalia et al, recently demonstrated that the *percentage* of surviving cells is decreased with intraparenchymal delivery of larger numbers of cells. However, the total number of surviving cells trended upwards with incremental increases in cell dose[44]. The potential benefits of higher cell dose must be weighed against potential risks, including potential mass effects, theoretical risks of increased tumorgenicity in certain cells, and potential for embolic events with intra-arterial delivery. As such, potential toxicity must be evaluated via appropriate dose-response analyses in preclinical studies[2].

2.2.3. Immunosuppression—The use of immunosuppression for cell therapy in CNS disorders is controversial[45]. Erlandsson et al, recently demonstrated that immunosuppression promotes endogenous progenitor migration and tissue regeneration with enhanced accumulation of SVZ-derived cells at the site of cortical injury[46]. By contrast, in meta-

analysis of preclinical studies of IV-delivered stem cells, immunosuppression had no significant impact on behavioral outcomes, though a trend was noted towards more favorable outcomes without immunosuppression[25]. It should be noted that preclinical studies of human cell lines in animal models will almost always require host immunosuppression, which, along with nature of the xenograft model itself, may significantly influence results. To date, autologous cells have necessarily been limited to bone marrow, and clinical trials of allogeneic cells for stroke have employed temporary immunosuppression[13] Given the difficulty of accurately extrapolating immunosuppression results from animal studies, optimal protocols for allogenic cell grafts may be best derived in the setting of appropriately controlled clinical trials. Further development of iPS or nuclear reprogramming technologies may ultimately enable autologous human cells to be differentiated toward neural or neural stem cell lineages prior to grafting without need for immunosuppression.

2.2.4. Timing—The optimal timing for cell delivery is unclear, but may depend upon the predominant mechanism of action. Therapies aiming for neuroprotection will require earlier delivery than those targeting neuroplasticity. Some studies suggest optimal survival of transplanted cells at early time points (e.g. 48 hours) before inflammatory responses are maximal[44], though studies have demonstrated robust benefit even when delivered over one month after stroke[47]. Meta-analysis of animal studies employing IV cell delivery found that the degree of inhibition of apoptosis was the strongest predictor of functional outcome[25]. This same study demonstrated a non-significant trend towards improved benefit with earlier cell delivery[25]. Stem cells have yet to be evaluated as adjuncts to thrombolysis or thrombectomy. However, pre-banked allogeneic cells may be feasibly delivered at quite early time points in this setting. Autologous therapies and intraparenchymal delivery will predictably be associated with greater delays.

2.2.5. Adjuncts—Cellular grafts may be supplemented by extracellular matrix products to improve survival and neurite outgrowth[48, 49]. Cells may be genetically modified to secrete select growth factors, with significantly enhanced therapeutic outcomes[25]. Alternatively, cells may be grafted after specialized pre-treatment protocols ranging from relative hypoxia to cytokine pre-treatment or cellular co-culture. Delivery of cocktails containing multiple cell types, or adjunctive viral constructs is also feasible. Finally, cells grafted in clinical trials of stroke have predominantly been identified based on histopathological evaluation at autopsy. The modification of cells with appropriate transgenic or other cellular labels may markedly improve the capacity to track cells *in vivo* after transplantation, via MRI and/or PET[50].

2.3. Proposed Therapeutic Mechanisms

2.3.1. Integration—Graft-derived neurons can survive and mature, forming synaptic connections to host brain circuitry after transplantation of fetal[51], ESC-derived[52, 53], and NSC-derived[54] cells into stroke-lesioned rodents. However, what role such integration, plays, if any, in functional recovery is unclear. Benefits are frequently seen at early time points, well before grafted cells could mature and form synaptic connections. Benefits may also be seen in the presence of few, if any surviving cells, and after grafting of cells devoid of neurogenic potential. As such, popular consensus now favors trophic mechanisms as the predominant basis for functional gains after cell transplantation[55]. Selective ablation of grafted cells, e.g., via administration of diphtheria toxin in rodents after human cell grafting, would be needed to critically assess the requirement for ongoing graft survival for maintenance of functional gains[56]. No such studies in stroke-lesioned animals have yet been reported. In lesions involving cell death of select neuronal subpopulations with maintenance of surrounding cytoarchitecture, integration of grafted cells may be more feasible than after

focal infarct and cavitation[57]. Use of supportive extracellular matricies may further maximize integration potential[58].

2.3.2. Neuroprotection—Bone marrow-derived and neural stem cells produce an impressive array of neuroprotective compounds. In a meta-analysis of 60 preclinical studies of intravenously-delivered cells, Janowski et al demonstrated that outcomes were most strongly correlated with inhibition of apoptosis[25]. Less significant correlations were also found with neurogenesis and angiogenesis. Endogenous NSC-derived neuroblasts intrinsically migrate to the injured region following stroke and promote neuroprotection. This endogenous neuroprotection may be significantly bolstered by supplementation with exogenous cells. Careful attention to cell source may be important. Takahashi et al found that NSCs derived from embryos were more effective than those derived from adults in mitigating ischemic damage[59]. Neuroprotection may be direct via secretion of neuroprotective compounds or indirect, via immunomodulation, angiogenesis, or amplifying the endogenous NSC response.

2.3.3. Immunomodulation—After acute ischemic injury, secondary injury may occur as a result of inflammatory mediators. Microglia are among the predominant regulators of the local inflammatory environment and may be modulated by grafted cells[60]. It should be noted that meta-analysis of preclinical studies employing IV cell delivery failed to find a significant correlation between immunomodulation and outcome[25]. However, the interactions between inflammatory signals and stem cells are notoriously complex and conflicting literature abounds. As a general rule, although SVZ and hippocampal neurogenesis increases after stroke, inflammatory signals following stroke impair neurogenesis[61]. Anti-inflammatory treatments, such as indomethacin, can increase neurogenesis following focal stroke[62]. It should be noted, however, that ablation of activated microglia exacerbates infarct size[63], consistent with an additional role of inflammation in the reparative process[64]. The immunosuppressive and anti-inflammatory effects of multiple stem cell populations are well documented[65]. Exactly when and how these effects impact outcomes following stroke requires further study. Unlike simple anti-inflammatory treatments, it is conceivable that stem cells may respond dynamically to changing inflammatory signals over time and may adjust their regulatory activities accordingly. Ideally, future studies may be undertaken in humanized mouse models to maximize insights relevant to human clinical therapies[66].

2.3.4. Vascular Repair—The integral relationship between endothelial cells and neural progenitors is well established[67, 68]. Neural precursors promote endothelial proliferation in the peri-infarct region. Conversely, such proliferation appears to enhance the recruitment of SVZ-derived neuroblasts[68]. Bone marrow-derived stem cells similarly secrete multiple pro-angiogenic compounds including VEGF, EGF, and IGF-1 in response to signals from ischemic brain [69], promoting endothelial proliferation in the peri-infarct region[68]. Blockade of angiogenesis in BMMC-treated cells markedly impedes recovery[70].

2.3.5. Plasticity—The adult brain possesses much greater plasticity than previously appreciated. The spontaneous development of new host projections after stroke has been significantly correlated with behavioral recovery[71]. Moreover, mice devoid of Thrombospondin 1 and 2, important for synapse formation and plasticity, demonstrate poor functional recovery after stroke[72]. Recent studies demonstrate that plasticity, as evidenced by increased synapse formation and new neuronal projections, is significantly enhanced by treatment with bone marrow-derived or neural stem cells following stroke[60, 73].

2.3.6. Recruitment of Endogenous Neural Progenitors—Neural stem cells proliferate and give rise to neuroblasts that migrate toward the injured region following stroke. These neuroblasts exert neuroprotective and pro-angiogenic effects upon arrival in the peri-infarct region. Bone marrow-derived stem cells are known to secrete a variety of compounds that promote the proliferation and migration of endogenous neural progenitor cells, suggesting that exogenous cell therapy may act, in part, by augmenting the endogenous neurogenic response to stroke[30, 74].

3. STROKE-INDUCED NEUROGENESIS

In the adult brain, neural stem cells are located in the hippocampal dentate gyrus[75] and SVZ[76] that give rise to new functional neurons throughout life. Hippocampal NSCs modulate learning, memory, and spatial navigation as well as psychiatric states[77]. In the SVZ, slowly dividing stem cells generate transit amplifying cells which in turn generate neuroblasts[78]. Unlike hippocampal NSC progeny that remain in the dentate gyrus, SVZ neuroblasts migrate along the rostral migratory stream (RMS) to generate functional olfactory bulb neurons[79], though they can be redirected towards areas of injury.

3.1. Subventricular Zone Response to Focal Infarcts

After ischemic stroke, hypoxia-induced signals promote the proliferation of neural stem and progenitor cells. SDF-1 and angiopoietin redirect neuroblasts from the SVZ and RMS along blood vessels toward the infarct region[80]. Rare new neurons are generated, though most recruited cells die or remain undifferentiated in association with blood vessels near the infarct boundary zone[80, 81]. Increased progenitor cell proliferation and neuroblast recruitment may persist for at least several months after ischemic injury[82].

Given the very low numbers of new neurons generated, the relevance of stroke-induced neurogenesis to functional recovery has been controversial, and has been examined via several experiments in the past decade employing irradiation or chemotherapeutic agents to impede neurogenesis. These manipulations worsened stroke outcomes, but conclusions were tentative, given possible toxicity from the experimental treatment. In 2006, Won et al demonstrated that reelin mice lacking doublecortin (dcx), a protein required for neuroblast migration, had larger infarcts and worsened behavioral outcomes following stroke[83]. However, baseline behavioral deficits in these mice somewhat hampered interpretation. In 2009, Jim et al selectively ablated migrating dcx+ cells and similarly observed increased infarct size as well as substantially worsened behavioral scores within days following stroke[84]. These studies collectively imply that immature endogenous neuroblasts act locally at the peri-infarct region to promote neuronal survival, well before any new mature neurons could possibly be generated.

The exact mechanisms via which endogenous neuroblasts enhance outcomes are incompletely defined. However, NSCs are known to produce neurotrophic factors such as NGF and GDNF, to regulate the inflammatory environment, and to produce pro-angiogenic complexes including netrin-4, laminin and integrins[85]. Of note, NSCs constitutively secrete factors implicated in synaptic plasticity, including those that are considered anti-angiogenic, such as thrombospondins[72]. It is likely that the factors generated by NSC progeny vary dynamically as the infarct injury evolves. The specific factors generated by endogenous recruited neuroblasts and their temporal patterns of expression after ischemia remain to be evaluated. Such analysis may provide fundamental insights regarding the endogenous response to ischemic injury.

A plethora of signaling molecules including VEGF, BDNF, EPO, FGF2, noggin, notch, IGF-1, TGF-alpha, SCF, NO, EGF, angiopoietin, MIP 1-alpha, SDF-1, cell surface

molecules, including CXCR4, VCAM, integrins and extracellular matrix molecules, are now known to regulate NSC proliferation, migration and differentiation[86, 87]. Experimental manipulation of these pathways in rodents can substantially bolster the neurogenic response with subsequently decreased lesion size and improved functional outcomes[88]. The clinical implications of these findings may be substantial. Manipulations that increase progenitor proliferation and migration with improvements in functional outcomes may be observed *without* significant increases in the number of new post-mitotic neurons generated. However, increased numbers of number of new neurons may be observed with overexpression of bFGF[82]. Moreover, "filling" of the infarct cavity with new neurons may be attained with sequential administration of EPO and EGF[89]. While robust functional improvements are observed in these cases, the capacity of the new neurons to form functional connections with surrounding circuitry or contribute to functional recovery remains to be assessed.

3.2. Selective Neuronal Replacement by Endogenous Progenitors

In animal models of selective neuronal loss, endogenous neural progenitors appear more inclined to replace the lost cell type. CA1 neurons in the hippocampus are particularly susceptible to ischemic injury[90]. Spontaneous regeneration of CA1 cells was observed after hypoxic injury, with cells arising from the periventricular region. This regenerative response could be significantly augmented by intraventricular delivery of EGF and bFGF. Functional recovery occurred in a delayed manner, which correlated with the appearance, maturation, and electrophysiological integration of new neurons. In another example, Macklis' lab developed a selective photo-ablation technique to delete subsets of cortical projection neurons without hypoxia, ischemia, or other injury. Remarkably, SVZ-derived neurons appeared to migrate along the corpus callosum to the affected region, and then replace ablated neurons with subsequent generation of long distance corticospinal and corticothalamic projections[91, 92]. The signals responsible for recruitment and directed differentiation of the new cortical projection neurons in this case remain unclear, though would be of fundamental importance to any attempts to promote regeneration after cortical injury.

3.3. New Neurons from Cortical Progenitors?

Increasingly frequent reports of cortical progenitor cells with neurogenic potential have appeared in recent years. Ohira et al recently found that the rat layer 1 cortex contains Ki67+/GAD67+ cells that bear few of the usual NSC markers, and generate no new neurons under baseline conditions. Impressively, in response to hypoxic injury, these cells generated new inhibitory neurons throughout the cortex that appeared to integrate into local circuitry and survive for at least eight weeks[93]. These cells likely correspond to the mitotically active GFAP+ cells in cortical layer 1 described recently by Xui et al[94]. These cells expressed vimentin and nestin in response to a cortical insult, migrating into deeper cortical layers over subsequent days and assuming an immature neuron-like morphology. The tendency of these cells to generate GABAergic neurons suggests they may represent residual undifferentiated progeny from the medial ganglionic eminence where cortical interneurons originate during development. The factors regulating the behavior of this newly identified population of cells and their response to focal ischemic injury remain to be evaluated.

Certain astroglial cells from the cortex or subcortical white matter may also de-differentiate into *bona fide* neural stem cells after *in vitro* culture[95, 96]. Heinrich et al recently demonstrated that reactive adult astrocytes isolated after cortical injury can be differentiated into glutamatergic or GABAergic neurons after transduction with Neurogenein2, or Dlx2, respectively. These neurons formed mature synapses with electrophysiological properties of mature neurons *in vitro*. Whether or not a similar strategy may be employed to redirect glial

cells toward neurogenic fates *in vivo* after cortical injury remains to be determined[97]. Some have argued that small cortical infarcts that spare the striatum provide a poor stimulus for SVZ neuroblast migration and that neural progenitors surrounding such infarcts may be locally derived[98]. Lineage tracing studies will be needed to fully characterize the identity, behavior, and function of naturally occurring cortical progenitor cells.

3.4. Human Implications

Though distinct in structure from rodents, the human subventricular zone[99] and rostral migratory stream[100] has been characterized. Moreover, evidence for neuroblasts has been found in multiple studies of patients after ischemic and hemorrhagic stroke[101–103]. As such, strategies to augment the human neurogenic response may yield improved outcomes. As always, rigorous preclinical safety studies will be needed to ensure factors employed to mobilize endogenous neural stem cells are safe. For example, EGF and BDNF have both been implicated in the development of glioma-like growths from SVZ progenitors[104, 105], BDNF may induce spontaneous seizure activity[106], bFGF infusion has resulted in demyelination[107], and all cause mortality was elevated in a large clinical trial of EPO for treatment of acute ischemic stroke [108]. Nevertheless, appropriate dosing in animal studies has enabled marked benefits with several cytokines without clinically untoward effects.

4. CONCLUSION

Early recanalization remains the most effective treatment of acute stroke by minimizing infarct size. Neural and bone marrow-derived stem cells appear to function via multiple synergistic mechanisms to augment natural recovery mechanisms. In the acute setting, endogenous endothelial and neural progenitor cells work together to minimize neuronal cell death and both may be bolstered by signals from exogenous stem cells. Both neural and bone marrow-derived stem cells directly secrete pro-survival factors in addition to modulating the endogenous response to stroke, thereby maximizing the amount of original neural circuitry that survives the ischemic insult. Thereafter, stem cells and their progeny function to promote synaptic plasticity, optimizing functional recovery. Multiple cellular and molecular tools now exist to enhance endogenous responses to stroke. While much work lies ahead, ample proof of principle suggests that substantial benefits may reward an ongoing investment in the science and translation of stem cell therapies for stroke.

5. EXPERT OPINION

5.1. Unique Challenges of Stem Cell Therapy for Stroke

The failure of hundreds of neuroprotective compounds in clinical trials illustrates the sobering challenge ahead of translational therapy for stroke. Given the inherently heterogeneous nature of stroke, clinical trials will be prone to inadequate power, being based on preclinical data from models that imperfectly reflect human disease. They may also suffer from being based on a literature that is skewed towards the publication of only positive results. Collaborative strategies will be needed to ensure not only that scientifically well-founded studies are initiated, but also that such potentially beneficial therapies are not aborted prematurely due to Type II errors. Considerations of statistical practicality and therapeutic potential may be in conflict as therapeutic windows are selected for trials. Should cells be given early when there is potentially more to gain, or later, when the gains can be most accurately measured from a stable baseline? As a novel technology, stem cells offer new risks, not only to patients, but also to the field as a whole, should early complications undermine public support. Will randomized double-blind studies be acceptable for therapies that may be invasive and risky, including possible stereotactic intracerebral cell delivery and immunosuppression? Ongoing collaborative discussions

between experts on all sides of the negotiations will be essential. While stem cell therapies have attracted significant media hype and venture capital, transparency regarding realistic expectations is critical. Regulatory bodies, stake holders, and the public at large should all be prepared for a long term investment that is more likely to be marked by small steps, than home runs. Refinement of protocols related to cell preparation, delivery, and detection after the onset of human studies will require diligence and patience.

5.2. Pre-Clinical Directions

Several preclinical questions remain that are pertinent to translational efforts. How long must cells survive for therapeutic benefit? Simple timed ablation studies, for example, using diphtheria toxin, are needed and may guide decisions regarding immunosuppression and cell labeling in clinical trials. Selective ablation of specific graft-derived cell types using cre-lox technology may help to dissect mechanisms underlying long term functional benefits. Knockdown of putative therapeutic genes in transplanted cells may further illuminate molecular mechanisms of benefit. The endogenous neurogenic response remains poorly characterized. Lineage tracing labels based on selective markers such as dcx should be used for isolation and gene profiling of endogenous neuroblasts at various times post-infarct. Subgroups of newly born neurons should also be ablated after augmentation studies to assess their functional contributions to recovery. Studies employing humanized mice may yield clinically relevant insights regarding immunomodulatory effects of grafted and mobilized cells, while guiding decisions regarding immunosuppression.

5.3. Clinical Directions

In the treatment of stroke, it remains true that “time is brain.” Cell therapies should be developed in conjunction with optimized recanalization technologies to target residual areas of ischemia, combat reperfusion injury, and provide trophic support in areas of hemorrhagic conversion. Defined ex-vivo genetic modifications of grafted cell lines should be considered to introduce transgenic MRI-detectable labels, as well as inducible suicide genes as insurance against undesired proliferation or neoplastic transformation. Transgenes may also permit delivery of complementary therapeutic genes. Inducible viral constructs should also be prepared for *in vivo* or cell-based delivery of cytokines for mobilization of endogenous NSCs. Use of bicistronic constructs with MRI labels may facilitate monitoring for impacts upon transduced endogenous cells, while regulatory elements may improve safety in case of untoward side effects. Therapeutic candidates may be expanded to include intracerebral hemorrhage, with stereotactic cell delivery following stereotactic clot evacuation. Additionally, global ischemic injury after myocardial infarction may be amenable to cytokine augmentation of endogenous cell replacement. Upon establishment of safety, stem cell pretreatment may be indicated for high risk patients, such as in subarachnoid hemorrhage patients at risk for vasospasm, and patients undergoing embolizations, complex tumor resections or cerebral revascularization procedures.

In sum, although challenges abound, and while vigilance to safety and monitoring will be paramount, maximal efforts are indicated to ensure timely translation of the most promising therapies for the treatment of stroke.

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Table 1

Published trials of cell therapy for stroke

Reference	Sponsor / Location	Cell Number / Type / Route	Age	Infarct Severity/ Type	Treatment Window	n	Phase	Primary Outcome	Randomized?	Blinding	Follow up	Results	Comments
Kondziolka *et al.*, 2000 [12]	University of Pittsburgh, PA, USA	2 or 6 million / hNT cells / IC	44 – 75	Basal ganglia infarct	6 months – 6 years	12	I	Safety, feasibility	no	no	6 months	Safe. European Stroke Score improved at 6 months (p=0.046)	Adverse events in two patients, possibly unrelated
Kondiolka *et al.*, 2005 [14]	University of Pittsburgh, PA & Stanford University, CA, USA	5 or 10 million / hNT cells / IC	40 – 70	Basal ganglia infarct, ESS 10 – 45	1 – 5 years	14 + 4 controls	II	European Stroke Score	yes	Observer only	6 months	Feasible; primary outcome measure not met	Adverse events in two patients, possibly unrelated. Some measures improved.
Savitz *et al.*, 2005 [16]	Harvard MA and Cornell, NY, USA	Up to 50 million / fetal porcine cells / IC	25 – 52	NIHSS 5 – 11	1.5 – 10 years	5	I	Safety	no	no	4 years	FDA terminated trial due to possible side effects	Improvements in two patients remained stable for 4 years
Bang *et al.*, 2005 [29]*	Yonsei University, Souel, S Korea.	50 million ×2 / Autologous MSCs / IV	30 – 75	NIHSS >6	32 – 61 days	5 + 25 controls	I/II	Safety	yes	Observer only	12 months	Safe, feasible; Barthel index higher at 3 and 6 months only.	Decreased *exvacuo* ventricular dilitation at 12 months in cell group, p=0.019.
Suarez-Monteagudo *et al.*, 2009 [19]	Centro Internacional de Restauracion Neurologica, Habana, Cuba.	14 – 55 million / Autologous BMMCs / IC	41 – 64	NIHSS 10.6±0.92	1 – 10 years	5	I	Safety, tolerance	no	no	12 months	Safe; neuropsychiatric improvements in some patients	Epileptic-like activity on EEG at 6 and 12 months; no clinical seizures
Barbosa da Fonseca *et al.*, 2010 [18]	Universidad Federal, Rio de Janeiro, Brazil	125 – 500 million / autologous BMMCs / IA	24 – 65	NIHSS 4 – 17	8 – 12 weeks	6	I	Neurologic deficits	no	no	120 days	No neurologic worsening	Cells not seen in brain by SPECT beyond 24h

Reference	Sponsor / Location	Cell Number / Type / Route	Age	Infarct Severity/ Type	Treatment Window	n	Phase	Primary Outcome	Randomized?	Blinding	Follow up	Results	Comments
Lee *et al.*, 2010 [30]*	Yonsei University, Souel, S Korea.	50 million ×2 / Autologous MSCs / IV	30 – 75	NIHSS >6	5 – 7 weeks	16 + 36 controls	I/II	Safety	yes	Observer only	5 years	Safe, feasible; mRS improved (p=0.046), best if SVZ intact	Enrollment suspended due to concern regarding animal products in culture media

BMMC: bone marrow mononuclear cells, hNT: human teratocarcinoma-derived neural cell line, IA: intra-arterial, IC: intracerebral, IV: Intravenous, MSC: mesenchymal stem cells, na: not available, NIHSS: National Institutes of Health Stroke Scale, SPECT: single-photon emission-computed tomography.

*[30] Includes patients previously reported in [29]; mRS not significantly different between groups in [29]; Barthel index not reported in Lee [30]

Table 2

Ongoing and pending trials of cell therapy for stroke

Clinical Trials ID [Reference]	Sponsor / Location	Cell Number / Type / Route	Age	Infarct Severity/Type	Treatment Window	n	Phase	Primary outcome	Randomized?	Blinding	Start Date	End Date
NCT00473057 [109]	Federal University of Rio de Janeiro, Brazil	500 million / Autologous BMMC / IA versus IV	18 – 75	NIHSS 4 – 20	3 – 90 days	15	I	Neurologic deficits	No	Open Label	December 2005	June 2011
NCT00535197 [110]	Imperial College London, UK	na/ Autologous $CD34^+$ cells from bone marrow / IA	30 – 80	Total anterior circulation syndrome	<7 days	10	I/II	Toxicity	No	Open Label	September 2007	June 2010
NCT00593242 [111]	Duke University, NC, USA	5 million cells/kg / Autologous cord blood / IV	<14days	Neonatal hypoxic / ischemic injury	<14 days	12	I	Adverse events	No	Open Label	January 2008	January 2011
NCT01028794 [112]	National Cardiovascular Center, Osaka, Japan	25 or 50 cc bone marrow / autologous BMMCs / IV	20 – 75	NIHSS >9	7 – 10 days	12	I/IIA	NIHSS	No	Open label	May 2008	March 2012
NCT00761982 [113]	Hospital Universitario Central de Asturias, Spain	na / Autologous $CD34^+$ cells from bone marrow / IA	18 – 80	NIHSS >7; MCA	5 – 9 days	20	I/II	Adverse events	No	Single-blind (assessor)	September 2008	March 2010
NCT00859014 [114]	The University of Texas Health Science Center, Houston, USA	na/ Autologous BMMCs / IV	18 – 80	R: NIHSS 6 – 15; L NIHSS 6 – 18	24 – 72 hours	10	I	Safety, feasibility	No	Open label	January 2009	January 2014
NCT00950521 [115]	China Medical University Hospital, Taichung, Taiwan	2 – 8 million / Autologous $CD34^+$ cells from peripheral blood / IC	35 – 70	NIHSS 9 – 20	6 – 60 months	30	II	NIHSS	Yes	Open label	June 2009	December 2010
NCT01019733 [116]	Hospital Universitario, Nuevo Leon, Mexico	8 – 10cc bone marrow / $CD34^+$ cells post G-CSF / IT	1 month –18 years	Cerebral palsy	1 month – 18 years	10	I	Battelle Devel. Inventory	No	Open label	July 2009	August 2010
NCT01091701 [117]	Stempeutics Research Pvt Ltd, Malaysia	2 million cells/kg / adult allogenic MSCs IV	20 – 80	mRS <5 with motor weakness	<10 days	78	I/II	Adverse events, NIHSS	Yes	Double blind	May 2010	December 2011

Clinical Trials ID [Reference]	Sponsor / Location	Cell Number / Type / Route	Age	Infarct Severity/Type	Treatment Window	n	Phase	Primary outcome	Randomized?	Blinding	Start Date	End Date
NCT01151124 [118]	ReNeuron, Ltd, Glasgow, UK	2, 5, 10, 20 million / CTX0E03 neural stem cells / IC	60 – 85	NHSS>5; Subcortical white matter or basal ganglia;	6 – 24 months	12	I	Adverse effects, MRI, NIIHSS, antibodies	No	Open label	June 2010	na
NCT00875654 [119]	University Hospital, Grenoble, France	na / Autologous MSCs / IV	18 – 65	NIHSS>2	<14 days	30	II	Feasibility, tolerance	Yes	Open label	September 2010	December 2013
NCT00908856 [120]	University of California, Irvine, CA, USA	30 cc bone marrow / autologous BMMCs / IV	18 – 85	NIHSS 7 – 24; supratentorial	2 – 21 days	33	II	Mortality	Yes	Double blind	January 2011	December 2012
na	SanBio Inc., Mountain View, CA, USA	2.5, 5.0, 10 million / SB623 cells – modified* BMMCs / IC	18 – 75	mRS 3 – 4; ESS 40 – 50; Subcortical MCA or striatum +/− cortex	6 – 12 months	18	I/II	Adverse effects, acute and long-term safety	No	Open label	January 2011	na

BMMC: bone marrow mononuclear cells, ESS: European Stroke Scale, IA: intra-arterial, IC: intracerebral, IV: intravenous, MCA: middle cerebral artery, MSC: mesenchymal stem cells, na: not available, NIHSS: National Institutes of Health Stroke Scale.

*Transient Notch 1 transfection.

Table 3

Overview of exogenous cell types

Exogenous cell type	Tumor risk	Cost	Survival	Autologous	Comments	Progress of clincal trials
Human fetal brain cells	–	$$$	+	–	Limited availablility; ethical challenges	None planned
Teratocarcinoma cells	+	$$	+	–	First cell type in trials for stroke	Early trials completed. No more planned
Porcine fetal neural cells	–	$$	+	–	Complex immune considerations	Prior trial aborted by FDA. None planned.
Bone marrow mononuclear Cells	–	$	+/–	+	Most readily available cell type	Trials completed and in progress
MSCs	+	$$	+/–	+/–	Trophic effects despite poor survival	Trials completed and in progress
Neural stem cells	+	$$$	++	–	Robust efficacy data in animal studies	Trials now starting
Embyonic-stem-derived Cells	++	$$$$	++	+ (iPS only)	iPS cells now available. May be used to generate NSCs	Still in pre-clinical phase