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Stem Cell Therapy for Neonatal Brain Injury: Perspectives and Challenges

Running title: Stem Cells and Perinatal Brain Damage

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ABSTRACT

Cerebral palsy is a major health problem caused by brain damage during pregnancy, delivery, or the immediate postnatal period. Perinatal stroke, intraventricular hemorrhage, and asphyxia are the most common causes of neonatal brain damage. Periventricular white matter damage (periventricular leukomalacia) is the predominant form in premature infants and the most common antecedent of cerebral palsy.

Stem cell treatment has proven effective in restoring injured organs and tissues in animal models. The potential of stem cells for self-renewal and differentiation translates into substantial neuroprotection and neuroregeneration in the animal brain, with minimal risks of rejection and side effects.

Stem cell treatments described to date used neural stem cells, embryonic stem cells, mesenchymal stem cells, umbilical cord stem cells, and induced pluripotent stem cells. Most of these treatments are still experimental.

In this review, we will focus on the efficacy of stem cell therapy in animal models of cerebral palsy, and we will discuss potential implications for current and future clinical trials.

INTRODUCTION

Neonatal brain injuries and cerebral palsy (CP)

Brain injury in premature infants is a major health problem worldwide. The incidence of preterm birth has increased, and improvements in survival rates have led to increased numbers of disabled patients despite a concomitant decrease in long-term neurodevelopmental disability rates.¹⁻⁴

Perinatal stroke, intraventricular hemorrhage, and asphyxia are the most common causes of neonatal brain injury, with hypoxia-ischemia being the final common pathway of injury.

Periventricular white matter damage (PWMD, periventricular leukomalacia) is the predominant form of brain injury in premature infants and the most common antecedent of cerebral palsy (CP). Hypoxia/ischemia with or without systemic infection/inflammation is the main mechanism responsible for PWMD. Neuropathology studies show focal necrotic lesions within the white matter surrounding the lateral ventricles and/or the subsequent appearance of more widespread lesions⁵ related to apoptotic death of late oligodendrocyte progenitors.^{6, 7} The main neuropathological feature of PWMD is marked hypomyelination.⁸ In addition to the white matter damage, the gray matter exhibits abnormalities including neuronal loss and impaired neuronal guidance.⁹ These findings support the view that some of the dysfunctions seen in preterm infants reflect decreased brain connectivity with impairments in the integration of information arriving from different areas of the brain.^{10, 11}

Children assessed several years after preterm birth often perform less well in tests of cognition, attention, executive function, and perception than do children born at full

term.¹² The massive personal and economic burdens generated by long-term neurological morbidity, together with the high prevalence of perinatal brain damage, are of considerable concern, particularly as no effective treatment is available to date.¹³

Animal models of cerebral palsy

Because of the many developmental and functional differences between the neonatal brain and the adult brain, extrapolating adult data to neonates is generally unwise. Consequently, the efficacy of potential treatments should be tested in developmentally appropriate models. Several inflammation- or ischemia/hypoxia-based models of CP have been developed, mainly in rodents.¹⁴

Transient bilateral carotid artery stenosis leads to generalized brain ischemia, which selectively destroys specific cells. However, this model is associated with highly variable outcomes,¹⁵ and models of focal ischemia are therefore more commonly used. These models involve occlusion of the middle cerebral artery (MCA), which leads to infarction of the striatum and overlying cortex. Permanent occlusion is accomplished via laser-induced photothrombosis, ligation, or cauterization.¹⁶ Ischemia-reperfusion injury can be replicated by inducing transient occlusion¹⁷ using an intraluminal filament fed through the carotid artery into the proximal MCA. Animal models of hypoxia-ischemia have shown clearly that brain injury is followed by reduced myelination, ventricular enlargement, loss of neurons, damage to axons and dendrites, and alterations in neurobehavioral performance,¹⁸⁻²¹ thus reproducing common features of PWMD.

Experimentally induced inflammation has been used in a number of studies to model PWMD pathology and to test potential therapeutic interventions. The endotoxin lipopolysaccharide (LPS), a potent inducer of innate immune responses such as

inflammation,²²⁻²⁴ administered intracerebrally during the early neonatal period or intraperitoneally to the pregnant dam, results in (neuro)-inflammation and hypomyelination in the offspring.^{25, 26} Excitotoxicity is another key factor that contributes to the development of white and gray matter damage in the premature brain.^{9, 27, 28} Well-characterized animal models of excitotoxic damage involve activation of the excitotoxic cascade via the NMDA and metabotropic glutamate receptors. Ibotenate and quinolinate²⁹ induce white matter cysts, as well as cortical necrosis, reproducing the lesions observed in infants with PWMD.

Cell therapy

Cell therapy holds promise in various models of brain injury or disease.³⁰⁻³³ Over the last two decades, numerous studies have evaluated the efficacy and/or feasibility of transplantation within the injured brain of stem cells or specialized progenitor cells of both neural and non-neural origin, in order to replace lost cells or to prevent damaged cells from dying.^{34, 35} However, despite the wide availability of these cells and their common use in animal models of injury, few studies have focused on their protective/regenerative effect in models of perinatal brain damage.³⁶⁻⁴²

Stem cell types

Multipotency and self-renewal are the cardinal features of stem cells. Multipotency is the ability to differentiate into multiple cell types belonging to the same germ layer (endoderm, mesoderm, or ectoderm). Self-renewal is the ability to make identical copies via cell division. Stem cells have considerable proliferative potential. They can be derived from many human tissues (Figure 1).

Neural stem cells (NSCs)

NSCs are found in the adult human brain, mainly in the subventricular zone (SVZ) and hippocampus.^{43, 44} These cells give rise to the three cerebral cell types, namely, neurons, astrocytes, and oligodendrocytes.⁴⁵ NSCs from exogenous sources can be isolated from many regions of the central nervous system and cultured *in vitro* as neurospheres. Neurospheres are heterogeneous cell collections that include true stem cells, committed progenitors, and differentiated progeny. NSCs can also be derived from embryonic or fetal brain tissue, and they generate oligodendrocytes, neurons, and astrocytes when allowed to differentiate spontaneously in serum-free media.^{46, 47} NSCs can differentiate into functional cellular subtypes^{48, 49} such as cortical projection neurons,⁵⁰ interneurons,⁵¹ and hippocampal pyramidal neurons.⁵²

Embryonic stem cells (ESCs)

ESCs are derived from blastocysts during the 16-cell stage. They are pluripotent: they can give rise to all cell types within the developing embryo. ESCs can provide an almost unlimited supply of cells for transplantation. Unfortunately, ESCs form teratomas after *in vivo* transplantation.^{53, 54} Purifying neural cells by removing tumorigenic pluripotent stem cells is feasible,⁵⁵ but the resulting cell population is probably not different from NSCs derived from other sources.

Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells (iPSCs) can be obtained by inducing terminally differentiated somatic cells via nuclear reprogramming. Reprogramming has been

achieved by transducing mouse⁵⁶ or human⁵⁷ fibroblasts with retroviral vectors containing cDNA encoding four genes (Oct3/4, Sox2, c-Myc, and Klf4). Recently, similar results were obtained by transducing fibroblasts with Oct4, Sox 2, NANOG, and LIN28,⁵⁸ a strategy having the major advantage of not using the proto-oncogene c-Myc. An advantage of iPSCs is the ability to generate cellular products for autologous grafts. Thus, the administration of iPSC-derived progenitors is not followed by a graft-versus-host response.⁵⁹ A recent study established that functional neurons, of which a small proportion also expressed markers of GABAergic neurons, could be obtained in mice from adult somatic cells without the intermediate iPSC step.⁶⁰ Interestingly, the same procedure has been developed in humans, permitting for the first time the generation of hematopoietic progenitors and mature cells directly from human dermal fibroblasts, without establishing pluripotency.⁶¹ This strategy is ethically acceptable and, theoretically, eliminates the risk of tumor formation.

Mesenchymal stem cells (MSCs)

Bone marrow contains a non-hematopoietic stem cell, the mesenchymal stem cell (MSC). MSCs differentiate into mesodermal tissues, including bone, cartilage and fat. It has even been suggested that MSCs may differentiate into functional neurons.^{62, 63} *In vitro*, MSCs have been shown to differentiate into neurons, astrocytes, and oligodendrocytes.⁶⁴⁻⁶⁷ In these studies, *in vitro* neural transdifferentiation by MSCs was assessed based on the detection of neural-related mRNAs and proteins in the treated cells. However several neural-related mRNAs and proteins are expressed by undifferentiated human MSCs.⁶⁸ Moreover several studies have shown that *in vitro* neuronal differentiation protocols with chemical induction medium can produce

unexpected and misleading effects, with cell morphology changes caused by rapid actin cytoskeleton disruption, cell shrinkage, and cellular toxicity.⁶⁹⁻⁷² These studies indicate a need for a more cautious evaluation of MSC differentiation using dissection of molecular signaling and commitment events, in order to reliably assess the ability of MSCs to differentiate into neuronal lineages.

Apart from the bone marrow, non-embryonic tissues from which MSCs can be isolated include cord blood and the stroma of the umbilical cord and placenta.⁷³ MSCs can be expanded *in vitro*. This culture procedure usually takes at least 2-3 weeks, which is not consistent with early autologous MSC treatment after the insult. The immunogenicity of MSCs is extremely low, as MSCs lack expression of MHC Class-II antigens, which prevents the development of a graft-versus-host response. Moreover, MSCs have immunosuppressive activity and are even used to treat steroid-resistant graft-versus-host disease.^{74, 75} This last feature may facilitate the transplantation of allogeneic MSCs, thus considerably improving treatment feasibility by allowing the administration of stored *in vitro*-expanded MSCs. Apart from their regenerative capacity, MSCs also exhibit anti-inflammatory properties, a feature that underlines their potential for the treatment of cerebral injury.

Umbilical cord stem cells

Umbilical cord blood contains many stem cell types such as MSCs⁷⁶ and endothelial progenitor cells⁷⁷, and has been demonstrated as a viable alternative to bone marrow transplantation.^{78, 79} Pluripotent stem cells were recently isolated from cord blood.⁸⁰

The whole mononuclear cell fraction, containing all cord blood stem cells, is easily obtained from cord blood and has been transplanted in different animal models with the goal of assessing its possible neuroprotective effects.⁸¹⁻⁸⁴ Human umbilical cord blood mononuclear cells (HUCBCs) differentiate *in vitro* into virtually all mature cells⁸⁵⁻⁸⁹ and neural cells.^{80, 90, 91} In a few laboratories, iPSCs were recently generated from cord blood.⁹²

The many advantages of HUCBCs include ready availability with no harm to the baby or mother, a limited number of ethical issues, low immunogenicity,⁹³ and beneficial effects *in vivo* in animal diseases.^{81, 94, 95}

The connective tissue of the cord (Wharton's jelly) is also a promising source for regenerative treatments, as it can be used to generate relatively high numbers of MSCs, which exhibit greater proliferative activity compared to bone marrow.^{73, 96}

Strategies for stem cell therapy

Delivery routes and methods

Stem cells may be delivered either systemically into the vasculature or locally into the brain (intraparenchymally, intraventricularly, and intrathecally)^{52, 97} (Figure 2). Many stem cells are capable of migration toward a focus of injury.³⁹ NSCs are more frequently delivered directly into the brain, whereas MSCs are usually given systemically. Recently, we showed that intranasal delivery of stem cells was a useful and non-invasive method.⁴¹ The stem cells cross the cribiform plate and migrate through the brain via the rostral migratory route.⁹⁸

Cell dose

The appropriate dose of cells for transplantation depends on several factors, of which the most crucial is the host environment.⁹⁹ The goal is to obtain the greatest local benefits using the smallest possible number of viable transplanted cells in order to reduce the risk of toxicity.

In a hostile microenvironment, for instance in an area of acute brain injury, a large number of cells may be needed to achieve effective neuroprotection, neuroregeneration, and local repair modulation. However, implantation of large numbers of cells into a small intraparenchymal site may affect cell viability and differentiation. Moreover, when gliotic changes begin, the volume of cells that can be accepted physically at an injury site is limited, even when slow administration rates are used.¹⁰⁰ Strategies that are being evaluated in several animal models include multisite intracerebral injections; prolonged intraventricular, intrathecal, or intravenous infusion; and repeated cell grafting over time.^{52, 97, 101, 102} Our recent studies indicate that intracranial administration of 100,000 MSCs into the neonatal mouse brain 10 days after the insult is sufficient to improve outcomes.^{40, 103}

Timing of transplantation

Most neuroprotective agents have a relatively narrow therapeutic time window to be effective. The window for cell therapy remains ill-defined, and the effect of stem cells varies across disease models. This variability reflects the ability of stem cells to act as neuroprotective agents, as a source of cells for *in vivo* replacement (neuroregeneration), or as both.¹⁰⁴ Whereas neuroprotection may be suitable for the treatment of acute brain injury, the treatment of chronic neurodegenerative diseases requires both neuroprotective and cell replacement strategies. When using animal models of CP,

many groups transplant the cells 4 to 48 hours following acute injury. However, benefits have been observed with cell delivery up to 10 days after cerebral hypoxia-ischemia.⁴¹ Such findings may suggest mechanisms of action other than neuroprotection or in addition to neuroregeneration, such as enhancement of endogenous cerebral plasticity.

In acute human brain disorders such as stroke, the efficacy of therapeutic interventions is directly related to the time to implementation.^{105, 106} In animal models of stroke, administration of MSCs 3-24 hours after MCA occlusion diminished cell apoptosis by 50% in the ischemic penumbra.¹⁰⁷⁻¹⁰⁹ From a clinical point of view, whether stem cells are appropriate in newborns shortly after an acute brain insult is debatable. In this specific clinical situation, activated microglia/macrophages play a central pathophysiological role,^{110, 111} and inflammatory cytokine production by these cells may partly inhibit stem cell-mediated repair processes. In addition, stem cell transplantation may result in a systemic inflammatory response that may enhance microglial activation at the lesion site and worsen the white matter damage through immature oligodendrocyte death and oligodendroglial cell maturation arrest.⁷ Also, stem cell infusion has been associated with pulmonary thrombosis in children^{112, 113} and with a decrease in cerebral blood flow in rats.¹¹⁴ In acute hypoxic-ischemic injury, cerebral vessel occlusion after cell transplantation may dramatically worsen the effects of the perinatal brain insult.

Therapeutic effects of MSCs transplanted immediately after the insult may be chiefly ascribable to inhibition of neuroinflammation and apoptosis. When the cells are administered a few days or even a few weeks after the insult, functional improvements may be related to endogenous repair processes such as neurogenesis, angiogenesis, and synaptogenesis. For the development of future therapeutic interventions, working on

the therapeutic window is probably crucial. More specifically, we need to investigate the mechanisms of action of MSCs at a given time point after the insult. To obtain proof of concept in mice subjected to excitotoxic injury, we compared NSC therapy given 4 or 72 hours postinjury. The behavioral evaluation showed severe memory function impairments without treatment contrasting with normal memory in mice given NSCs 4 hours postinjury. This effect was mainly due to a neuroprotective mechanism.³⁹ Mice treated 72 hours after lesion showed partial memory function recovery. These data suggest that an intervention can be effective even when given late, after the inflammatory and apoptotic storm has subsided. As discussed above, inflammation leads to local microglial activation, inhibiting endogenous neurogenesis and suppressing the growth and survival of transplanted cells.¹¹⁵ On the other hand, inflammation also activates local repair, facilitating transplanted cell homing, growth, integration, and survival after acute injury.¹¹⁶⁻¹¹⁸

Better defining therapeutic windows in CP models, as well as the pathophysiological mechanisms involved, may help to achieve optimal integration of the newly formed cells into functional networks, thereby providing histological and behavioral improvements.

Monitoring

Clinical studies require noninvasive methods for monitoring the migration dynamics and viability of transplanted cells. Currently, there is no validated method for tracking transplanted stem cells in human patients, as bioluminescence and fluorescence techniques are not suitable for use in humans. In myelin diseases, myelin formation analysis may allow monitoring of *in vivo* NSC migration and engraftment.¹¹⁹⁻¹²¹

Alternatively, labeling of human NSCs (HuNSCs) grown with magnetic nanoparticles allows NSC detection by cerebral magnetic resonance imaging (MRI) in rodents^{122, 123} and would probably be useful also in humans. Metabolic markers have been tested and quantified in the human brain using proton nuclear MR spectroscopy.¹²⁴ Unfortunately, the amount of NSC metabolic biomarker decreases dramatically with increasing stem cell age, leading to potential misinterpretation of data. Other techniques are being evaluated such as the use of viral vectors to deliver genes to stem cells encoding proteins detectable on MRI scans.¹²⁵ However, before these techniques enter clinical trials, the use of genetically modified HuNSCs for *in vivo* tracking will have to be approved.

In animal studies stem cells can easily be labeled using a fluorochrome and traced throughout the period of regeneration.

Engraftment or stimulation of endogenous regeneration?

Originally, the positive effects of stem cell transplantation were attributed to donor cell engraftment in the lesion site. MSCs express neuronal proteins and differentiate *in vitro* to respond to depolarizing stimuli.^{64, 65, 126} Data indicate that MSCs can differentiate into functional neurons.^{62, 63} However, in a recent qPCR study of mice on postnatal day 9 (P9), we showed that after successful intracranial eGFP+-MSC transplantation 3 and 10 days after hypoxia-ischemia, less than 1% of the MSCs were traced 18 days after the last stem cell dose. Thus, at least in models of neonatal hypoxia-ischemia, MSCs may function merely as regulators of endogenous regeneration rather than as a substitute for damaged tissue (Van Velthoven et al., manuscript submitted). In this respect, it is worth noting that MSCs produce myriad growth and differentiation factors such as BDNF,

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BMP, CSF, NGF, EGF, interleukins, neurotrophin, Persepin, Spp1, TGFb, VEGF, and others. These factors may change the local milieu, thereby acting as inducing signals enabling endogenous regeneration (Van Velthoven et al. manuscript submitted and^{40, 41}).

Toxicity

Immune response

The central nervous system has been viewed as an immunologically isolated site protected by an impermeable blood-brain barrier (BBB). However, recent studies suggest that activated lymphocytes may cross the BBB at sites of injury and that resident microglia may have antigen-presenting capacity.¹²⁷ Although NSCs are minimally immunogenic, chronic low-grade rejection of transplanted NSCs remains possible. Furthermore, bone marrow-derived transplants can cause an inflammatory response and acute rejection,¹²⁸ especially when they contain CD34+ hematopoietic stem cells. Systemic administration of stem cells can also modify the immune response by modifying cytokine production.¹²⁹ Clinical investigators therefore believe that some form of immunosuppression is necessary to optimize donor cell engraftment and survival in humans, by blocking T-cell activation. This strategy has been studied in only a few patients with Parkinson disease^{130, 131} or Huntington disease^{132, 133} treated with fetal cell transplantation. Given the potentially serious side effects of immunosuppression, this topic will gain in importance in future clinical trials of stem cell treatments. The development of noninvasive means of monitoring cell engraftment will help to assess the need for immunosuppression.¹³⁴

Tumors

ESCs and iPSCs have substantial significant teratogenic potential after implantation into host tissues, due to their pluripotency.^{53, 54} Non-ESC-derived NSCs and MSCs are usually considered non-tumorigenic.¹³⁵⁻¹³⁸ Unfortunately, a recent report from Russia described a young patient with telangiectasia ectatica who developed several histologically proven neuroblastomas probably derived from HuNSC transplants.¹³⁹ Because the immunodeficiency associated with telangiectasia ectatica might have accounted for this complication, several methods were used to demonstrate that the tumor was not due to chromosomal and genetic instability in the patient. The results supported a nonhost origin to the tumors. The NSCs used in this patient for repeated intracerebral transplantation were derived from periventricular tissue isolated from several fetuses aborted at 8-12 weeks of gestation. Conceivably, the relatively poor purity of these cells may have contributed, at least in part, to tumor formation in this patient. This report confirms the need for a detailed safety assessment of stem cell treatments via well-conducted clinical trials.

Stem cell therapy in animal models of cerebral palsy

Stem cell treatments have been tested in various animal models of CP. Almost all types of stem cells produced beneficial effects in rodents (see Table). Brain injury in these models is not limited to a single cell type but instead affects heterogeneous cell populations. This heterogeneity is desirable, as it replicates observations in humans after neonatal brain injury. Research on stem cell therapy in this field has therefore focused on a mix of progenitor cells, whereas a single cell type has often been used in

other developmental disorders (e.g., oligodendrocyte progenitor cells in mouse models of congenital demyelination¹⁴⁰).

Stem cell therapy has been studied chiefly in focal lesions. Focal lesions are highly reproducible^{41, 141} and allow a histological comparison with the non-injured hemisphere. Another advantage of studying focal lesions is that injury to different cerebral areas causes different phenotypes.¹⁴²⁻¹⁴⁴ The efficacy of stem cell therapy in these models provides useful information about the pathophysiology of brain damage in full-term and preterm infants.

Embryonic stem cells (ESCs)

ESCs have been tested in postnatal day-12 mice with ligation of the right carotid artery.³⁸ NSCs derived from ESCs by retinoic acid-induced differentiation were injected into the striatum 2 or 7 days after ligation, and hemispheric brain atrophy was measured 4 weeks after ligation. Hemispheric brain atrophy was less severe in the pups treated 2 days postligation, but not in those treated 7 days postligation, compared to vehicle-injected ligated controls. In 3 of the 10 surviving stem cell-injected animals, the transplanted cells formed tumors that contained very abnormal structures.³⁸ Teratoma formation appears to correlate with the degree of cell differentiation and enrichment in culture.¹⁴⁵ It is possible that ESCs were transplanted with the ESC-derived NSCs, accounting for the tumor formation. Indeed, in transplants of cells derived from ESCs, the main challenge is elimination of tumorigenic cells from the progenitor populations.¹⁴⁵

Neural stem cells (NSCs)

The therapeutic potential of NSCs in acute neonatal brain injuries has been evaluated in rodent excitotoxic and ligature models. The mouse pup model of ibotenate-induced brain injury is a classic excitotoxic injury model for white and gray matter damage in human cerebral palsy.¹⁴¹ We recently reported that early (4-hour) and late (72-hour) neurosphere-derived precursor cell (NDP) implantation significantly reduced brain lesion size in this neonatal model.³⁹ The implanted cells, modified *in vitro* prior to transplantation toward the oligodendrocytic lineage, were capable of migrating toward the lesion site even when implanted contralaterally to the lesion, a feature similar to the long-distance migration of NSCs seen in a hypoxic-ischemic model of brain injury.¹¹⁸ At the lesion site, the NDPs underwent transient differentiation into neurons and oligodendrocytes but not astrocytes, suggesting that fate specification was achieved by the culture conditions. In parallel with the reduction in lesion size, the injured mice displayed a persistent and marked improvement in temporal and spatial memory at 3 and 6 weeks of age compared to littermates given intracerebroventricular injections of PBS or fibroblasts. The cells finally died *in situ*. Thus, even NDPs that do not survive can modify the functional impact of brain injury in neonates.

HuNSCs derived from ESCs were tested in mouse pups with excitotoxic lesions induced by the NMDA receptor agonist quinolinic acid.¹⁴⁶ Three days after the injury, the mice received intraparenchymal injections of HuNSCs, which were labeled *in vitro* for *in vivo* tracking. The cells migrated to the sites of injury, and subsets expressed neuronal and glial cell markers, partially restoring the striatal neurons in the brain-damaged mice.

More recently, HuNSCs derived from ESCs and genetically engineered for *in vivo* molecular imaging and histological tracking were tested in a rat model of permanent left carotid artery ligation followed by hypoxia.¹⁴⁷ HuNSCs were transplanted in the ischemic

left hemisphere in the newborn pups 24 hours after the brain injury. Transplanted animals had significantly better behavioral performances compared to the sham-operated controls. Bioluminescence imaging permitted real-time tracking of the grafted cells, which showed good survival, dispersion, and differentiation.

Mesenchymal stem cells (MSCs)

Transplantation of MSCs derived from bone marrow has been shown to promote functional neurologic recovery in various models of neonatal and adult cerebral damage and confers neurogenesis, oligodendrogenesis and axonal remodeling and rewiring.¹⁴⁸⁻

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The overall efficacy of stem cell treatment is greater in neonatal than in adult models of cerebral ischemia. This difference may be ascribable to the fact that the potential for endogenous neurogenesis continues throughout life but declines with age, so that neonates have a greater potential for regenerating lost neurons compared to adults.¹⁵¹⁻¹⁵³ We recently showed that two administrations of bone marrow-derived MSCs to neonatal mice 3 and 10 days after unilateral right carotid artery occlusion on P9 produced a 46% improvement in sensorimotor function as observed in the cylinder rearing test and a 60% decrease in neuronal loss, compared to vehicle-treated animals. Moreover, we observed cellular proliferation and differentiation of the proliferated cells into cells expressing neuronal, oligodendroglial and astrocyte markers. Interestingly, the number of proliferating microglia decreased after MSC transplantation, possibly as a result of the well-known anti-inflammatory effects of MSCs.^{40, 103} Finally, two MSC administrations induced extensive remodeling of the corticospinal tract with increased axon density and activity when analyzed by anterograde tracing with bovine dextran

amine or retrograde tracing with pseudo rabies virus (¹⁰³ and Van Velthoven et al., unpublished results). Interestingly, remodeling of the corticospinal tract correlated with sensorimotor improvement. These data demonstrate that MSC transplantation into human neonates with cerebral damage may constitute a promising and realistic treatment modality for regenerating the damaged neonatal brain.

Human umbilical cord blood mononuclear cells (HUCBCs)

When HUCBCs were injected into non-injured neonatal rodent brains, they showed relatively good engraftment with 20% of cells surviving for one month.¹⁵⁴ About 2% of the cells differentiated into astrocytes and neurons, indicating good adaptation to the host environment. In a rat model of spastic paresis induced by hypoxia-ischemia,⁸² HUCBCs injected intraperitoneally 24 hours after the insult were found exclusively in the damaged hemisphere and exhibited no evidence of differentiation. This cell homing was associated with an improvement in motor function.⁸² The chemokine SDF-1, which is secreted by astrocytes, has been suggested as an important player for attracting HUCBCs expressing the SDF-1 receptor (CXCR4).¹¹⁶ In the same model of neonatal hypoxic-ischemic brain damage (Rice-Vannucci model), intraperitoneal injection of HUCBCs 3 hours posthypoxia reduced microglial activation in the cortex and caspase-3 mediated cell death in the striatum.⁸¹ HUCBC transplantation resulted in improved functional outcomes as measured using negative geotaxis and cliff aversion reflexes. However, contrary to the previously cited work,⁸² very few cells reached the injured sites in the cerebral cortex and striatum. Furthermore, other authors showed no cognitive improvement or attenuation of structural damage after the intravenous administration of high HUCBC doses.⁸⁴ More recently, low doses of intravenously injected HUCBCs

induced behavioral improvements in the Rice-Vannucci rat model. This effect was potentiated by the use of the BBB permeabilizer mannitol and was associated with upregulation of brain neurotrophic factors.⁸³ In this work, only a few cells were found in the injured hippocampal dentate gyrus.

Altogether, these results indicate that experimental parameters relevant to HUCBC therapy, such as cell dose, timing of the injection(s), and delivery route, still need to be extensively investigated.

Mechanisms of action: mostly hypotheses

Although stem cells can differentiate into neurons, oligodendrocytes, astrocytes and, possibly, endothelium,^{35, 155} neuroregeneration by cell replacement after brain injury might not represent their main mechanism of action. As reported above, stem cells can survive after transplantation, tend to migrate toward injured areas,¹⁵⁶ and can generate functional neurons⁵⁰ that may form connections with host cells.¹⁵⁷ Neuroprotective^{39, 158} and immunomodulatory^{97, 159} effects are possible mechanisms in addition to cell replacement.¹⁶⁰ The effects of stem cells may include, without being limited to, attenuation of central nervous system inflammation, secretion of survival-promoting neurotrophic factors, stimulation of the plastic response or neural activity in damaged host tissue, and restoration of synaptic transmitter release via the provision and/or promotion of local re-innervation.¹⁶¹⁻¹⁶⁵ Promotion of central nervous system repair by these bystander effects is due to the extraordinary capacity of *in vivo* stem cells to find the best route to certain favorable niches, where they survive and act via interaction with various cell types in the micro-environment.^{166, 167} This interaction is mainly related to integrins, which are proteins that act via inside-out and outside-in signaling to control

many cell functions such as survival, differentiation, migration, and adhesion. The varied integrin expression displayed by stem cells of different tissue origins and the importance of integrin-mediated signaling and adhesion during development highlight the need for elucidating the signaling mechanisms involved.¹⁶⁸ Specific surfaces for stem cell derivation, maintenance, proliferation, and differentiation were developed recently. These surfaces mimic the complex cellular environment existing *in vivo*, allowing tailoring of tissue culture environments to individual stem cell lines, and are suitable for clinical applications.^{168, 169}

Although stem cells can survive after transplantation, long-term survival of engrafted cells does not seem needed to improve outcomes. Recent work by our group indicates that intracranially applied MSCs decline gradually, although their regenerative effects persist over time. Grafted stem cells induce activation of endogenous stem cell compartments and a global decrease in microglial activity.¹⁷⁰ Although all these mechanisms have been described, the mechanisms underlying the benefits of stem cell therapy may vary across animal models. It is worth noting that most studies did not show substantial changes in both morphology and behavior after stem cell transplantation.^{84, 156, 171} However, in our studies using bone marrow-derived MSCs in a mouse model of hypoxia-ischemia, MSC transplantation led to recovery of sensorimotor function; proliferation and differentiation into neurons, astrocytes, and oligodendrocytes; and rewiring of the corticospinal tract.^{40, 41, 103}

Stem cell therapy in neonates with brain injury

Clinical trials with NSCs

Human NSCs are becoming very attractive cells for transplantation, because of their stable expansion and *in vitro* differentiation into neurons and oligodendrocytes. Fetal tissue-derived NSCs have been used in preclinical mouse studies. When transplanted intracerebrally in mice with a disorder resembling infantile neuronal ceroid lipofuscinosis (NCL, Batten's disease), these NSCs showed robust engraftment, extensive migration, and production of sufficient enzyme levels to alter host neuropathology.¹⁷² The first FDA-approved open-label, dose-escalating phase I clinical trial with HuNSCs was completed in January 2009 (<http://clinicaltrials.gov/ct2/show/NCT00337636>). HuNSCs were transplanted into 6 patients in the advanced stages of Batten's disease, directly into the brain parenchyma. Two dose levels were tested (500 million and 1 billion cells), and patients received immunosuppressive therapy for 12 months. The safety profile was favorable and long-term survival of the transplanted cells was documented. A second phase I clinical trial in patients with Pelizaeus-Merzbacher disease (PMD), another central nervous system disorder characterized by defective myelination, has received FDA approval and is under way at the University of California in San Francisco. This clinical trial is expected to enroll 4 patients with congenital PMD, who will receive stem cell transplants and immunosuppression for 9 months. The expected primary completion date is December 2012 (<http://clinicaltrials.gov/ct2/show/NCT01005004>).

Clinical trials with HUCBCs

HUCBCs were used in a clinical trial to treat young infants with Krabbe's syndrome.¹⁷³ Krabbe's syndrome is a leukodystrophy due to a disorder in the metabolism of galactocerebroside, one of the main lipid components of myelin. This

disease usually begins during the first few postnatal months, causing severe loss of motor skills and often death before 2 years of age. In this trial, significant improvements with progressive central myelination and gains in neurodevelopmental functions were shown only in those infants given mononuclear HUCBCs before symptom onset. Very recently, a pilot study of autologous cord blood infusion was performed in 184 infants with acquired neurological disorders.¹⁷⁴ This retrospective study supported the safety of intravenous autologous HUCBC infusion. Finally, two randomized, blinded, placebo-controlled clinical trials designed to evaluate possible beneficial effects of autologous umbilical cord blood infusion in infants with established CP are ongoing at the Medical College of Georgia (USA, <http://clinicaltrials.gov/ct2/show/NCT01072370>) and at the Duke University (USA, <http://clinicaltrials.gov/ct2/show/NCT01147653>), respectively.

Based on animal models of hypoxic-ischemic encephalopathy, HUCBCs and MSCs may be the most promising stem cells, as they are effective and potentially available for human studies. HUCBCs have advantages over MSCs that may support their use for neonatal insults. First, HUCBCs can be harvested very easily with no harm to the baby or mother. They have better proliferative capacities compared to MSCs. As they are less mature than stem cells from adult tissues, they are probably less immunogenic. This feature is associated with a lower risk of graft-versus-host disease in recipients of transplants from unrelated donors, even when there is some degree of HLA mismatch.^{79, 93, 175} Moreover, HUCBCs have a low rate of viral infection,¹⁷⁵ and their use for newborns or children is particularly appropriate as one cord usually contains a sufficient number of cells for use in a low-body-weight patient. Finally, the use of cord blood improves the availability of stem cells for ethnic groups that are under-represented in bone marrow donor registries.

Perspectives

The most extraordinary promises held by stem cells are flexibility, adaptive growth, and improved host acceptance. Immunosuppression is a critically important aspect of moving stem cell treatments to clinical applications in humans. In the future, the need for immunosuppression may be obviated by the use of generated cells that are genetically identical to the patient.^{176, 177} Human mature neurons may soon be obtained from adult somatic cells without the intermediate iPSC step.^{60,61} Furthermore, given their ability to migrate toward areas of injury, stem cells may prove to be appropriate vehicles for delivering specific molecules that may not achieve sufficient concentrations in the injured area when given systemically.¹⁷⁸ Candidate drugs for such *ex vivo* stem cell-assisted gene therapy may include antiinflammatory, proangiogenic, and prosurvival molecules.¹⁷⁹

Current areas of concern include the risk for tumor formation, the lack of evidence that iPSC-derived neurons are functional, and the paucity of data indicating that stem cell transplantation provides clinically meaningful benefits in humans. Importantly, preclinical data showing neural protection should not be misinterpreted as evidence that these stem cell approaches are effective in patients with established CP. For the moment, clinical data are not available. Further studies are needed to fully understand the mechanisms involved in stem cell-mediated neuroprotection/neuroregeneration in animal models before cell therapy can be considered for newborns with CP.

Stem cells and perinatal brain damage

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FIGURE LEGENDS

Figure 1: Neural stem cell sources, cultured *in vitro* as neurospheres and giving rise to differentiated neurons, astrocytes, and oligodendrocytes.

Figure 2: Systemic and intracerebral routes of stem cell delivery to the brain.

Table: Stem cell types used in neonatal brain injury models.

<i>Cell type</i>	<i>Route; time after injury</i>	<i>Animal model</i>	<i>Functional outcome</i>	<i>Cellular effect</i>	<i>Reference</i>
Fetal cortex (ED 13)	Intracerebral; 7 days	P7 rats	NE	Graft survival	180
Fetal cortex (ED 16)	intracerebral; 3 days	P7 rats	Improved performance	NE	181
ESCs	Intracerebral; 2 days	P12 mice	NE	Decreased neural death	38
ESCs	intracerebral; 2 days	P7 mice	Improved performance	Increased number of neurons	182
NSCs	intraventricular; 24 hours	P7 rats	NE	Reduced brain damage	183
NSCs	intracerebral; and 72 hours	P5 mice	Improved performance	Reduced lesion size	39
HuNSCs	intracerebral; 3 days	P5 mice	NE	Decreased neural death	146
HuNSCs	intracerebral; 24 hours	P7 rats	Improved performance	Increased neurogenesis	147
MSCs	intracerebral or intravenous; 7 days	P7 rats	Improved performance	Reduced cell loss	148
MSCs	intravenous; 3 days	P7 rats	Improved performance	No effects	184
MSCs	intracerebral; 3 days	P9 mice	Improved performance	Increased neurogenesis	40
MSCs	Intranasal; 10 days	P9 mice	Improved performance	Reduced lesion size	41
MSCs	intracerebral; 3 and 10 days	P9 mice	Improved performance	Reduced lesion size	103
HUCBCs	intraperitoneal; 24 hours	P7 rats	Improved performance	No reduction in lesion size	82
HUCBCs	intraperitoneal; 3 hours	P7 rats	Improved performance	Decreased neural death	81
HUCBCs	intravenous; 24 hours	P7 rats	No effect	No effect	84
HUCBCs	intravenous; 7 days	P7 rats	Improved performance	Increased dendritic density	83

ESCs, embryonic stem cells; HuNSCs, human neural stem cells; MSCs, mesenchymal stem cells; HUCBCs, human umbilical cord blood mononuclear cells; NE, not evaluated; ED, embryonic day; P, postnatal day

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