Concise Review: Self-Renewal in the Central Nervous System: Neural Stem Cells from Embryo to Adult

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ABSTRACT

The recent discovery of neural stem cells (NSCs) in the adult mammalian brain has fostered a plethora of translational and preclinical studies to investigate future therapeutic approaches for the cure of neurodegenerative diseases. These studies are finally at the clinical stage, and some of them are already under way. The definition of a bona fide stem cell has long been the object of much debate focused on the establishment of standard and univocal criteria to distinguish between stem and progenitor cells. It is commonly accepted that NSCs have to fulfill two basic requirements, the capacity for long-term self-renewal and the potential for differentiation, which account for their physiological role, namely central nervous system tissue homeostasis. Strategies such as immortalization or reprogramming of somatic cells to the embryonic-like stage of pluripotency indicate the relevance of extensive self-renewal ability of NSCs either in vitro or in vivo. Moreover, the discovery of stem-like tumor cells in brain tumors, such as gliomas, accompanied by the isolation of these cells through the same paradigm used for related healthy cells, has provided further evidence of the key role that self-renewal plays in the development and progression of neurodegenerative diseases and cancer. In this review we provide an overview of the current understanding of the self-renewal capacity of nontransformed human NSCs, with or without immortalization or reprogramming, and of stem-like tumor cells, referring to both research and therapeutic studies. STEM CELLS TRANSLATIONAL MEDICINE *2012;1:298–308*

INTRODUCTION

Neural stem cells are the most primitive cells in the central nervous system (CNS). Since most mature neural cells, with particular reference to neurons, are very specialized cells and are quite sensitive to environmental changes, such as oxygen conditions or excitotoxic molecules, the importance of neural stem cells (NSCs) in sustaining the development and homeostasis of nervous tissue is essential [1, 2]. The slow replenishment of degenerating cells with newly generated neuronal cells under physiological conditions in vivo has suggested that NSCs basically rest in a state of quiescence, which allows them to maintain a balance between the ability to undergo self-renewal and to differentiate without depleting the stem pool. When dividing, the NSC gives rise to other neural stem cells and/or to transient-amplifying cells called progenitors, which display a decreased proliferative potential and which progressively acquire a more restricted differentiation capacity into neurons, astrocytes, and oligodendrocytes. The dynamic equilibrium between self-renewal and differentiation is critical to both the maintenance of the stem cell pool and active neurogenesis. In this regard, a key role in the regulation of stem cell behavior is played by the niche [3], the CNS-specific compartment where somatic adult neural stem cells reside and are self-maintained throughout life. The isolation and characterization of multipotent NSCs from multiple locations within the mammalian brain has represented one of the most significant advancements in neuroscience and has provided accruing evidence of endogenous NSC potential to respond to neurological injuries [4]. Thus far, germinative zones have been identified in the subgranular zone (SGZ) of the hippocampus [5], the olfactory bulb [6–10], the subventricular zone (SVZ) surrounding the ventricles [11], and the subcallosal zone underlying the corpus callosum [12]. Recent evidence has reported the presence of active neurogenesis even in the adult cerebellum [13]. Among this evidence is the fact that the SVZ is the adult brain region with the highest neurogenic rate from which NSCs were first isolated [14, 15] and characterized. Following brain injury, NSC proliferation in the SVZ is enhanced to provide novel neural precursors migrating to the lesion [16, 17], thus suggesting

that the mechanisms regulating self-renewal are pivotal to NSC-mediated tissue repair.

SELF-RENEWAL OF NSCs

The self-renewal of NSCs is currently explained by two main theories: the stochastic model and the deterministic model. According to the former, the probability that a stem cell divides symmetrically into two stem cells or into two differentiating progenitors is the same [18]. Conversely, the deterministic model is based on the predetermination of the chances that a stem cell will self-renew or differentiate by the occurrence of extrinsic events. According to this theory, which is the most reliable in vivo, a stem cell can divide asymmetrically into one stem cell and one progenitor cell, or into one stem cell and one apoptotic cell, in response to specific environmental cues, such as the extracellular matrix, cytokines, oxygen, chemokines, and so forth [19–22]. However, the precise mechanism by which NSCs and progenitor cells self-renew and generate neurons at the same time is unclear. Because adult NSCs maintain a glial identity resembling that of embryonic radial glia [17], many studies suggest that the principles elucidated during development can be considered valid also for adult neurogenesis. During early neurogenesis of the embryonic mouse brain, most NSC divisions are symmetric to expand the NSC pool and to establish a stable niche compartment. With the progression of neurogenesis from the dorsal and ventral telencephalon, an increasing fraction of asymmetric divisions gives rise to progenitor cells (radial glia), which migrate to form the neocortical layers. An active reorientation of the mitotic spindle, according to vertical, horizontal, or oblique axes, has been proposed as being responsible for the asymmetric segregation of specific transcription factors or regulators with cell-fate determining function, although the precise correlation between spindle orientation and the kinetics of stem cell division remains unclear [22]. In the mammalian brain, as in *Drosophila*, heterotrimeric G proteins have been identified as master regulators of the mitotic spindle orientation, whereas multiple factors called segregation determinants, such as Numb and Numb-like [23–26] and the ubiquitin ligase protein Neuralized (Neur) [27], have been shown to be involved in the regulation of asymmetric versus symmetric divisions. Whereas most NSC divisions in vitro seem to be asymmetric [28], in the adult NSC niche, self-renewal and multipotency are mutually regulated through the secretion of molecules by surrounding nonstem cells [29–31]. Hence, the elucidation of regulatory mechanisms, which underpin the polarized distribution of these factors within the NSCs, could contribute to manipulating the balance between self-renewing and differentiating cells either in vitro or in vivo and target neural precursors for a specific cell fate.

One of the most highly conserved and efficacious mechanisms involved in regulating NSC function and development throughout an individual's lifetime is the family of Wnt and bone morphogenetic protein (BMP), which have broad roles in stem cell biology by regulating the balance between quiescent and actively proliferating cells [32–34]. As a matter of fact, the large family of Wnt proteins regulate neurogenesis [35, 36] by influencing proliferation and lineage decisions of neural progenitor cells and their progeny [37, 38]. Furthermore, BMPs, for their part, cause differentiation of neural progenitors from the subventricular zone and olfactory bulb, inhibiting neurogenesis and promoting exit from the cell cycle, which may be a result of dif-
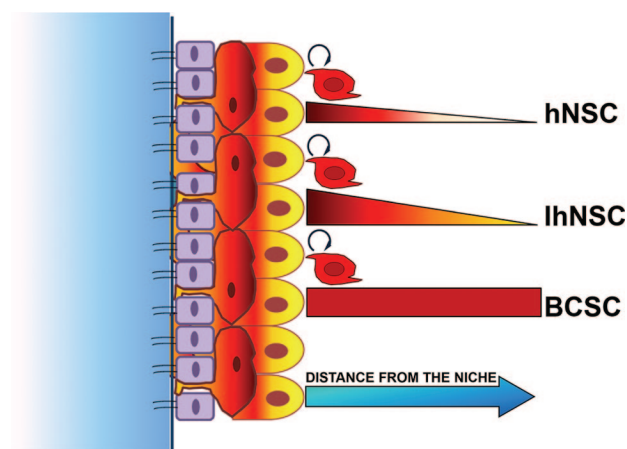

Figure 1. Model of the niche-dependent self-renewal capacity of neural stem cells and BCSCs. Untransformed cells, namely hNSCs or lhNSCs, which display a growth factor-dependent self-renewal in vitro, are able to self-renew in vivo only if implanted in proximity of a stem niche area, whereas BCSCs maintain a cell-autonomous self-renewal capacity both in vitro and in vivo. Red bars measure self-renewal capacity of hNSCs, lhNSCs, and cancer stem-like cells by two parameters: the kinetic and the long-term maintenance. In particular, red color gradations correlate with proliferation rate, which is higher in lhNSCs and BCSCs than in hNSC. The thickness of the red bars indicates the maintenance (for BCSCs) or the shortening (for hNSC and lhNSC) of self-renewal capacity, dependent on the distance from the niche. Abbreviations: BCSC, brain cancer stem cell; hNSC, human neural stem cell; lhNSC, immortalized human neural stem cell.

ferentiation [39–41]. It has been demonstrated that in vitro cultured NSCs exposed to BMPs show age-dependent disposition in terminal fate choice that mimics the in vivo developmental differentiation process [42]. In addition, self-renewal of NSCs residing either in the SVZ or in the SGZ of the hippocampus is regulated by the dynamic interplay of Notch- and epidermal growth factor (EGF)-activated signaling pathways [43, 44]. In particular, Notch regulates the NSC's identity and self-renewal, whereas EGF receptor modulates proliferation and migration of the transiently amplifying progenitors (neural precursor cells [NPCs]) [34]. Other factors, such as CXCL12, basic fibroblast growth factor, and pigment-epithelium derived factor, are known to contribute to NSC self-maintenance through non-cell-autonomous effects or can modulate the proliferation and differentiation of NPCs [45, 46].

Recent studies have highlighted the novel definition of the SVZ as a neurovascular niche consisting of two specific sublocalized compartments within the SVZ niche: the apical ependymal niche, which lines the ventricles and consists of ciliated ependymal cells intercalated with stem cells; and, proximal to it, the basal vasculature niche, a rich plexus of blood vessels [47]. Endothelial cells have been shown to secrete factors that enhance self-renewal and neuron generation from progenitor cells [48], suggesting that the vascular niche harbors mostly activated progenitors and suggesting the concept of a niche-dependent self-renewal. NSCs spontaneously arrest to proliferate and differentiate with increasing distance from the niche or, under restrictive conditions, they even die. This behavior is likely due to the absence of factors promoting self-renewal and also to the presence of signals fostering NSCs toward differentiation (Fig. 1).

ISOLATION OF BONA FIDE NSCS EX VIVO

Following the acceptance of the existence of multipotent NSCs in the adult mammalian brain able to self-renew and differentiate in vitro [14, 49], questions arose about specific selection criteria to establish stable NSC lines for either basic research or therapeutic purposes. NSCs are characterized by the expression of markers such as Sox2, glial fibrillary acidic protein (GFAP), nestin, and Musashi 1 and 2, but no specific combination of markers has so far been identified to definitely distinguish an NSC from an NPC. Much debate is now focused on the marker CD133 (also called prominin-1). It is currently unclear which portion of the CD133 population defines the relationship of CD133 to somatic NSCs and the selectivity of this relationship. This transmembrane glycoprotein, with a largely unknown function, has been used as one of the major markers, although it has been shown that clonogenic multipotent cells are also found in populations negative for CD133 [50–53]. However, lineage negativity remains as important as other criteria in identifying bona fide NSCs; indeed, cerebellar NSC have been isolated as CD133+ and lin− (lack of lineage marker expression) cells [13]. Alternatively, the neurosphere assay is based on the self-renewal capacity of NSCs, which is dependent on the presence of the mitogenic factors fibroblast growth factor 2 and EGF [54, 55], and it was developed as a procedure for the isolation and expansion of NSCs in vitro. Under these culture conditions, NSCs grow in suspension as floating cell clusters, called neurospheres, which are heterogeneously composed of stem, progenitor, and differentiating cells. A caveat of this method is that actively proliferating progenitors also generate neurospheres in vitro [56–58]. The neural-colony forming cell assay [59], by which only large colonies are enumerated as clonally derived from the extensive proliferation of NSCs, has been designed to exclude the possibility that an NSC population is composed exclusively of transient-amplifying progenitors.

Alternatively to the neurosphere assay, a number of groups have established NSC cultures on adhesive substrates mimicking the extracellular environment in the stem cell niche [60–62]. However, when establishing a renewable source of NSCs in vitro, expansion of NSCs on adhesion should be carefully evaluated. Monolayer cultures present the advantage of appearing as very homogeneous cell populations thanks to the continuous exposure of all the cells to the culture medium, but the adhesion substrates are molecules expressed in vivo by the extracellular matrix of the niche, such as laminin or collagen, which alter the expression of key receptors associated with proliferation, adhesion, migration, and differentiation of progenitor cell subtypes [19, 20, 63, 64]. Under these conditions, long-term proliferation of transient progenitors is most likely facilitated, and a neural progenitor culture can be erroneously mistaken for a neural stem cell culture. Soluble factors (such as growth factors, cytokines, and chemokines) and cell density have to be considered [19, 65–68].

EMBRYONIC STEM-DERIVED NSCS

During development, before the formation of the neural stem cell niches, the cells of the inner cell mass (ESCs) generate the three germinal layers of embryos and will then give rise to tissue-restricted stem cells [69]. Starting from these pluripotent ESCs, it is possible to test, in vitro, the conditions by which ESCs can be induced to generate a specific population of tissue-restricted stem cells able to grow and differentiate. Several protocols have been described for the derivation of a neural progeny from ESCs, some of them allowing for a long-term expansion of transient progenitors followed by terminal differentiation upon changes in culture conditions. In summary, three methods have been reported to generate neural progenitor cells from ESCs. The first method leads to the formation of embryoid bodies (EBs) [70]; the second generates neural rosettes under adhesion conditions and by transient supplementation of Noggin to the culture medium, without passing through the EB stage [71]; and the third is based on the progressive differentiation of EBs into neural rosettes under sequential exposure to retinoic acid, Sonic hedgehog (Shh), and cAMP [72]. These protocols have been widely used to originate a variety of differentiated neural cell types (such as astrocytes; oligodendrocytes; and glutamatergic, GABAergic, and dopaminergic neurons), thus providing a basal platform of tools for preclinical studies. Nevertheless, the characterization of the in vitro-derived neural progenitors still remains elusive, and their single-cell clonogenic potential over time needs further investigation.

FETAL AND ADULT NSCS

Somatic NSCs can be retrieved from the fetal neural tissue (8–12 weeks postconception) or from the neurogenetic regions of the adult brain (described above), and they can extensively self-renew in vitro as neurospheres and differentiate into neuronal and macroglial cells without altering their functional properties over passaging. Given the slow-dividing feature of adult NSCs in the mammalian brain, the isolation, in vitro, of stable human NSC lines still remains infeasible. Sanai et al. were able to isolate NSCs from different regions of the SVZ of a cadaveric autoptic brain, but their expansion in vitro was far from generating a number of cells amenable to preclinical studies [73]. Conversely, human NSCs have been successfully isolated from the telencephalic-diencephalic region [55] or from the SGZ [9] of the fetal human brain. Their self-renewal features have been widely characterized (Table 1).

INDUCED PLURIPOTENT STEM CELLS

Renewable sources of normal human neural cells with the functional characteristics expected of bona fide NSCs have significantly facilitated basic studies on human neurogenesis, as well as drug discovery. Their use in clinical applications will most likely eliminate the need for fetal human tissue [74]. A major obstacle to the progression of neural transplantation from the experimental level to clinical applications is the source of donor material [75]. In addition to the significant moral and ethical issues surrounding the procurement of human fetal tissue, other parameters, such as age, storage, viability, and contamination, must be standardized, making elective surgery difficult to schedule [76]. To further compound the problem, multiple fetuses are usually required for a single transplant, thereby introducing heterogeneity in the donor tissue and increasing the probability of immunological rejection or contamination [76]. Awareness of these difficulties has driven the search for alternative donor sources. Thus, immortalized brain precursors [77], xenogenic tissue [78, 79], and genetically engineered cells [80] have been

Table 1. NSC basal properties

NSC type	Tissue source	Properties	Caveats	References
ES-derived NSCs (ESCs)	Inner cell mass of blastocyst	High rate of proliferation	Ethical issues	[70–72]
		Pluripotency: high differentiation potential and plasticity	Tumorigenic potential	
			Multiple passages are necessary to commit ESCs to the neural lineage	
			Risk of immunorejection	
Fetal NSCs (hNSCs)	Telencephalic-diencephalic region from 8–12-week-postconception fetal brain	Stable profile of growth and differentiation in vitro	Slow rate of proliferation	[9, 55]
		Multipotency: default differentiation to astrocytes, neurons, and oligodendrocytes	(Low) risk of immunorejection	
		No ethical limitations (obtained from spontaneous miscarriages)		
		Low immunogenic potential		
iPSC-derived NSCs	Skin fibroblast from adult tissue	Optimal source for disease modeling and drug screening	Tumorigenic potential	[83–85]
	Newborn cord blood and adult peripheral blood mononuclear cells	Candidates for future autologous transplantation	Low efficiency of transduction	
		No risk of immunorejection		
IhNSCs	Fetal NSCs	High rate of proliferation	Viral recombination leading to tumor formation	[97–101]
	ES-derived progenitors	Large-scale availability	Risk of "leaky" events in conditional vector-mediated immortalization	
		Multipotency		
		Low tumorigenic potential		

Abbreviations: ES, embryonic stem; hNSC, human neural stem cell; IhNSC, immortalized human neural stem cell; iPSC, induced pluripotent stem cell; NSC, neural stem cell.

used in experimental neural transplantation, although autologous transplantation of non-neural stem cells or of somatic cell-derived NSCs (induced pluripotent stem cells [iPSCs]) actually represents the most desirable solution in overriding most of the limitations described above.

Recently, iPSCs have been proposed as an alternative source of neural cells, since they share embryonic stem characteristics (i.e., self-renewal) and the potential to differentiate into any somatic cell type, and they could provide an escape from the risk of immunorejection. *OCT4*, *SOX2*, and *NANOG* are among the few of the most notable primitive master genes involved in the maintenance of the undifferentiated phenotype, through the simultaneous activation of genes promoting proliferation and repression of genes promoting cell cycle arrest and differentiation [81]. The combinatory overexpression of the four master genes *OCT4*, *SOX2*, *C-MYC*, and *KLF4* has successfully reprogrammed adult human fibroblasts to the pluripotent stage [82], thus triggering a series of exciting studies mostly aimed at reprogramming somatic cells from patients affected by genetic or sporadic neurodegenerative diseases. Indeed, reprogramming of fibroblasts from two elderly amyotrophic lateral sclerosis (ALS) patients has allowed the generation of motor neurons potentially available for autologous transplantation [83, 84]. Moreover, a recent study by Chou et al. [85] has successfully established a method to generate integration-free human iPSCs from blood mononuclear cells. iPSCs currently offer multiple advantages: first, the ability to derive stem cells from skin fibroblasts could override the limit imposed by the need for human embryos to generate ESCs; second, with respect to traditional stem cells, they are able to gen-

erate neurons, astrocytes, and oligodendrocytes from adult patients; third, they provide the opportunity to elucidate how different cell types may be involved in a specific pathobiology through either cell-autonomous or non-cell-autonomous effects; fourth, they can be exploited to identify and characterize the cellular and biomolecular mechanisms that underpin the development of a chronic or progressive disorder; and finally, they represent an optimal tool for the discovery of novel drugs and high-throughput screenings [86].

One roadblock to the promotion of these cells to the clinical stage is represented by the genetic manipulation implied by the procedure, that, in principle, can drive the iPSCs to final tumorigenic modifications (Table 1). Accruing studies are currently investigating novel protocols for a higher grade of safety [87], so that, besides the traditional Yamanaka reprogramming, other methodologies are currently used: recombinant protein reprogramming [88, 89], consisting of administering each reprogramming factor to the cells as recombinant proteins, and cut-in/cut-out reprogramming by the use of the piggyBac transposon [89], synthetic RNA [90], and episomal DNA method [91]. However, when considering iPSCs as a source for autologous transplantation in human patients affected by neurodegenerative disorders, it still has to be determined to what extent the original affected microenvironment in the tissue from which iPSCs are derived is able to condition the novel regenerated cells and whether these are particularly sensitive when implanted back into the patient. Alternatively to full reprogramming of terminally committed cells into a pluripotent primitive stage, direct conversion without

reverting cells to a pluripotent state is being used to generate neurons and NSCs [92, 93].

IMMORTALIZED STEM CELLS

Several clonal, genetically homogeneous human neural stem cell (hNSC) lines have been obtained by genetic perpetuation methods [94–96]. Taking advantage of their nontransformed nature, human origin, multipotency, fast but conditional growth, unlimited availability, and suitability for molecular manipulation, these cell lines offer a great opportunity for the development of cell replacement and/or gene transfer-based therapies, such as using assays for pharmacological studies, drug discovery, and investigation of specific intracellular regulatory pathways, which require large amounts of human brain cells to be generated in a rapid and reproducible fashion. In particular, we have used stem cell lines from the telencephalic-diencephalic region of the human fetal brain as a source for generating cell lines immortalized with *V-MYC* or with the single-mutated variant *C-MYC T58A*, which could be routinely available for extensive studies aimed at elucidating the mechanisms of regulation of NSC development [97, 98]. Immortalized human neural stem cells (IhNSCs) provide an available source of human neural stem cells for pilot experiments of transplantation into animal models of neurodegenerative diseases [60, 61, 99, 100]. Recent studies have shown that conditional induction of *V-MYC* gene expression suffices to enhance the self-renewal of neural progenitors derived from fetal human brain with no tumorigenic potential either in vitro or in vivo [101].

CNS CANCER STEM CELLS

One of the most prominent topics in the field of cancer biology and therapy is that transformed stem/precursor cells, with the cardinal properties of stem cells, such as the ability of self-renewal and to generate large numbers of progeny, are responsible for the origin and maintenance of solid malignancies [102–104]. The idea that transformed stem cells initiate tumors was initially confirmed in the 1990s, based on studies of acute myeloid leukemia [105, 106], and has been strengthened by findings related to breast cancer [107]. A similar involvement of tumor stem cells in brain cancer was also supported by the fact that neural stem cells are nestin- and GFAP-positive precursors [108] and the discovery that, when targeted to nestin- or GFAP-positive cells, alterations of critical G_1 arrest regulatory pathways cause the onset of high-grade gliomas [106, 109]. The ensuing view that tumor stem cells underpin the development and/or maintenance of brain cancer has been confirmed, once and for all, by their identification in tumors of the central nervous system [102, 110–113]. Like stem cells in normal tissues, these brain cancer stem-like cells are relatively rare but have the capacity to establish and maintain glioma tumors at the clonal level and thus are thought to be tumor-initiating cells (Fig. 2) [114, 115]. It is currently unknown whether the molecular and functional characteristics of these cancer stem cells are a reflection of their origin from mutated normal NSCs or, rather, they emerge from the acquisition of stem-like features by more mature CNS cells following transformation [116–120].

By the application of the same culture system developed for neural stem cells (i.e., neurosphere assay), long-term expanding stable lines were isolated, propagated, and characterized in a reproducible fashion from human high-grade gliomas, named human glioblastomas (hGBMs) [55, 111]. Upon differentiation, these hGBM stem-like cells gave rise to the three major neural lineages in a way that is rather similar to NSCs [55], and they demonstrated self-renewal capacity. These cells also bear tumor-specific features, such as an exacerbated growth rate, highly unbalanced karyotypes with a high degree of hyperdiploidy and telomerase reactivation, a dysfunctional segregation of neural markers, and the activation of an aberrant differentiation program (Fig. 2). Nonetheless, following either subcutaneous or intracranial implantation, hGBM stem-like cells displayed the essential requirement for a cancer stem cell, that is, the capability of generating new tumors. Importantly, when injected into the brains of immunocompromised mice, the resulting tumors recapitulated the morphology, genotype, and gene expression patterns of primary glioblastomas (GBMs) and had extensive migratory and infiltrative capacity [114], thus being superior pathological models of human disease. These findings indicate that the in vitro cells, tentatively called hGBM stem-like cells or stem-like tumor initiating cells, faithfully preserve the in vivo key features of human pathology.

The complementary parallelism between the healthy behavior of wild-type NSCs and the uncontrolled expansion of transformed cancer stem-like cells has paved the way to the identification and the study of future cancer therapies. Administration of epigenetic factors, which are able to dampen or, hopefully, to arrest tumor progression, represents the most feasible strategy. The role of stem cells in the origination (i.e., initiation) of gliomas remains controversial, but the identification of brain cancer stem-like cells has led to new and specific cellular targets for therapeutic intervention in primary brain cancers, raising the possibility that specifically killing or blocking the proliferative potential of the tumor-initiating stem cells may increase treatment efficacy. Furthermore, it has stimulated the development of new hGBM cancer stem-like cell-based preclinical experimental models with the potential to improve mechanistic and preclinical therapeutic research [104, 121].

Nonetheless, the absence of maturity in the knowledge of somatic NSC biology and the transition of stem cell progeny into functional cells has stymied the study and characterization of the complexity of cellular interactions in human gliomas, making it difficult to develop targeted therapies if a specific target is still missing. Despite this controversial issue, however, signaling pathways that regulate self-renewal and cell fate in normal neural stem cells have been shown to be active and associated with oncogenesis in cancer stem cells of GBMs and brain tumors [122–124]. In addition to the well-known *NOTCH*, *SHH*, and *WNT*, tumor suppressor genes such as *PTEN* (phosphatase and tensin homolog on chromosome 10) and *TP53* (tumor protein p53) have been implicated in the uncontrolled self-renewal of brain cancer stem-like cells, which might generate tumors that are resistant to conventional therapies [125, 126]. Therefore, it has been suggested that therapeutic agents targeting these pathways might effectively deplete cancer stem-like cell populations in GBM as well [127–129].

An alternative approach is to activate specific differentiation pathways in a small population of tumor-initiating stem-like cells that drive proliferation and resistance, causing them to lose their stem and proliferative qualities. This regimen would make tumors less aggressive and more sensitive to cytotoxic treatment [130].
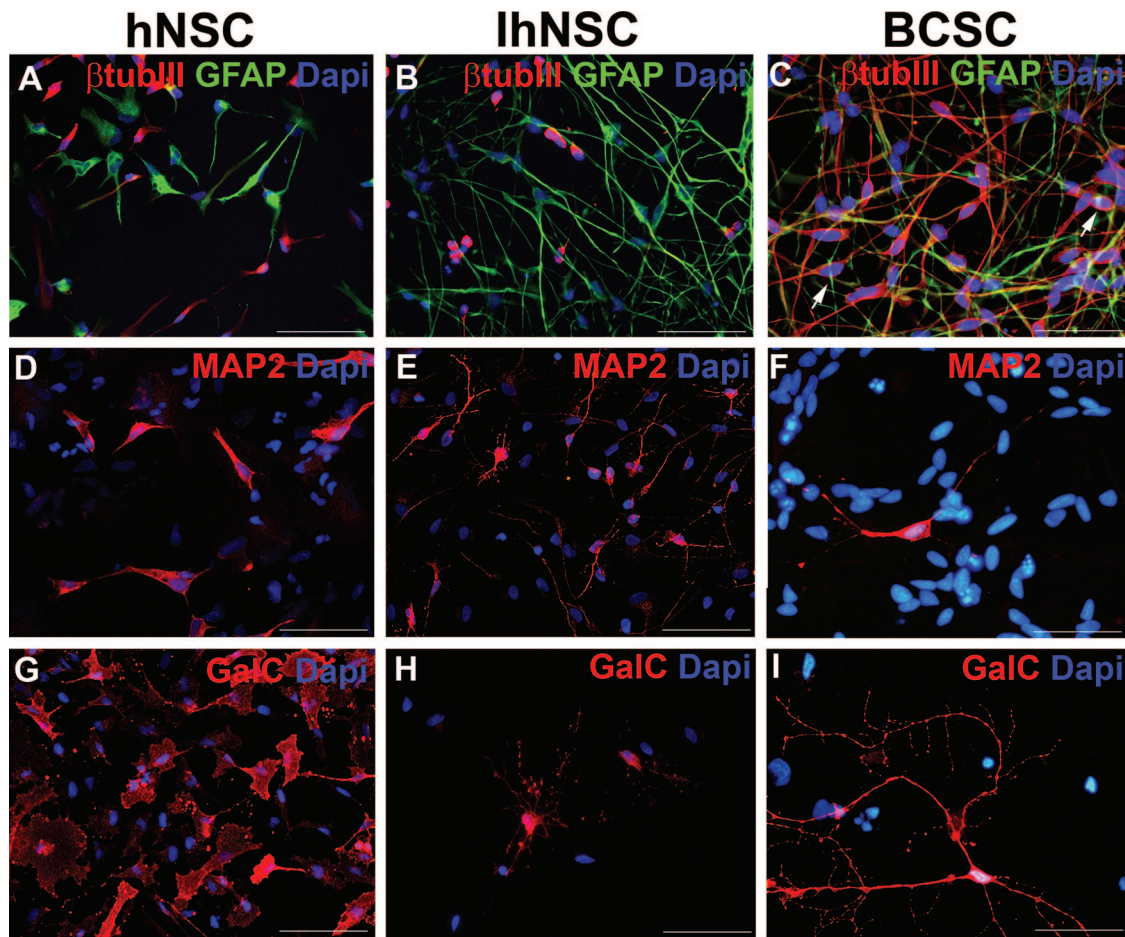
Figure 2. Expression of differentiation neural markers by normal (hNSC), v-myc-immortalized (IhNSC), and human glioblastoma (hGBM)-derived stem-like cells. hNSCs, IhNSCs, and hGBM-derived stem-like cells (indicated as BCSCs) were differentiated onto an adhesive substrate in the absence of mitogenic factors for 10 days. **(A–C):** Immunostaining showing the definite segregation of the early neuronal marker βtubIII (red) and the astroglial marker GFAP in hNSCs **(A)** and IhNSCs **(B)** and, conversely, a partial colocalization of the two markers in BCSCs (arrows in **[C]**). **(D–I):** Immunostaining of hNSCs, IhNSCs, and BCSCs with antibodies recognizing late neuronal marker MAP2 **(D–F)** and oligodendrocyte marker GalC **(G–I)**. Total nuclei are shown by Dapi staining (blue). Scale bars = 50 μm. Abbreviations: βtubIII, β-tubulin III; BCSC, brain cancer stem cell; Dapi, 4′,6-diamidino-2-phenylindole; GalC, galactocerebroside C; GFAP, glial fibrillary acidic protein; hNSC, human neural stem cell; IhNSC, immortalized human neural stem cell; MAP2, microtubular-associated protein 2.

A member of the bone morphogenetic protein family, BMP4, has been reported to be a potential heterogeneous/targeted strategy that appears to block tumor initiation and progression in a xenogenic in vivo murine model [42]. In culture and in vivo, the delivery of this protein to hGBM stem-like cells blocks proliferation and induces the cells to differentiate, as opposed to killing them [131]. This result is similar to the outcome of exposing somatic NSC to BMPs, which induces their differentiation down an astrocyte pathway [40].

Finally, it has also been documented that endogenous NSCs are naturally attracted to gliomas [132–134] and have the ability to migrate into the tumor mass, even contributing to the tumor bulk. As a matter of fact, Aboody et al. from the City of Hope's Department of Neurosciences demonstrated this inherent propensity (also known as tropism) of neural stem cells to home in on invasive tumor cells [135], even migrating from the opposite side of the brain or across the blood-brain barrier, and they harnessed the tumor tropism of neural stem cells to deliver therapeutic agents to invasive tumor sites. Because of the attraction of NSCs to gliomas, NSCs can be used as cellular vehicles for the delivery of therapeutic agents [135, 136]. In 2010, the FDA ap-

proved a phase I clinical trial for NSC-mediated therapy of high-grade gliomas, by a genetically modified human NSC line, generated by Seung U. Kim (Division of Neurology, University of British Columbia), which delivers a prodrug-activating enzyme (cytosine deaminase) to brain tumor sites. It is imperative to design new strategies based upon a better understanding of the signaling pathways that control aspects of self-renewal and survival, both in normal and cancer stem cells, to identify novel therapeutic targets.

THERAPEUTIC RELEVANCE OF NSCs

As of now, thanks to the development of paradigms for the isolation and expansion of NSCs ex vivo from different tissue sources, NSCs have been transplanted in different animal models as a tool for the cure of neurodegenerative diseases [137–140]. Irrespective of their specific etiology, neurodegenerative disorders eventually lead to loss or functional alteration of mature cells of the brain parenchyma. Symptoms vary significantly, depending on many parameters, such as
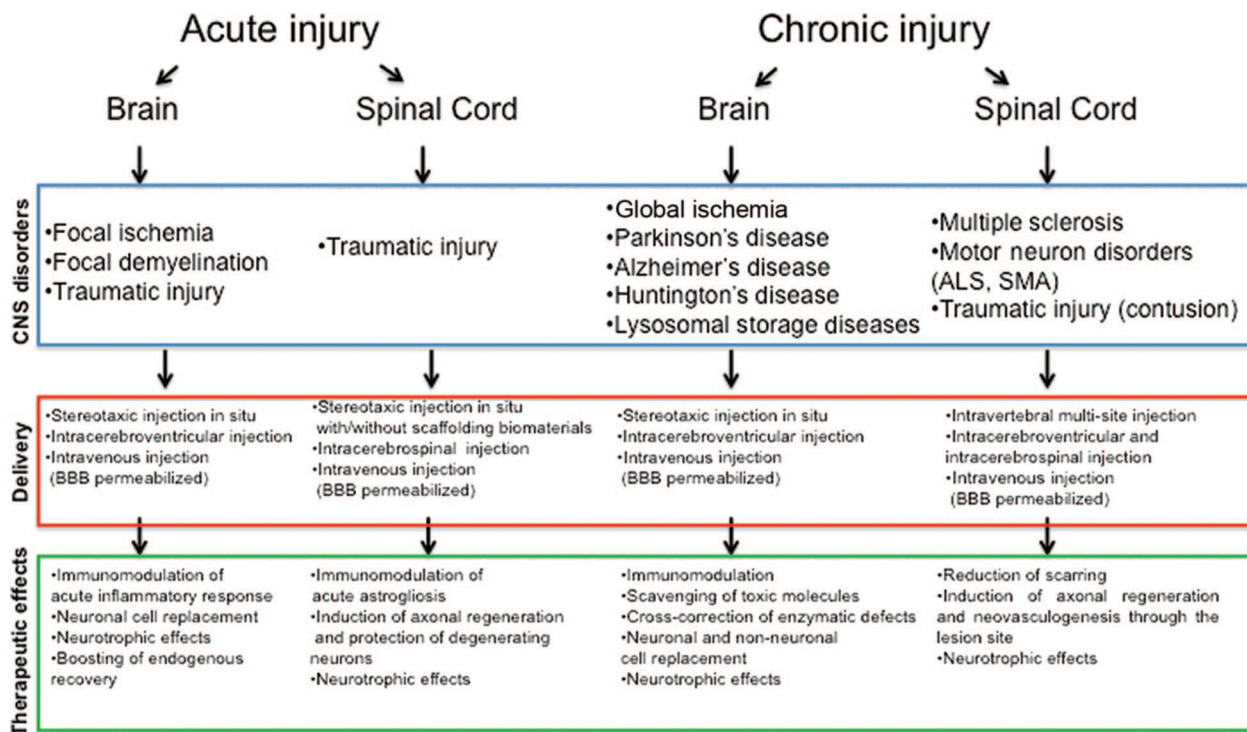
Figure 3. Neural stem cell-mediated therapeutic effects and their multiple clinical applications. Abbreviations: ALS, amyotrophic lateral sclerosis; BBB, blood-brain barrier; CNS, central nervous system; SMA, spinal muscular atrophy.

age of onset, region of the brain, the type of cells being damaged, and the origin and nature of the *noxa*—genetic, toxic, traumatic, ischemic, hemorrhagic, infectious, or immunological and inflammatory—to highlight the major examples. Several studies have demonstrated that, following brain injury, endogenous NSC proliferation is enhanced, and soon after it is followed by an increased migration of progenitors from the niche to the lesion site [16, 17]. However, given the inherent (and perhaps functionally essential) resilience of the postnatal and, particularly, adult mammalian brain, in addition to new cells of preexisting circuitry, repair of the damaged brain tissue by endogenous cell replacement is very limited. One of the current, most valued therapeutic hypotheses is to accomplish neuroregeneration by transplantation of exogenous cells, that is, by cell-mediated therapy [141]. Perhaps counterintuitively, it has now emerged that transplanted cells may actually boost endogenous recovery, besides replacing and partially repopulating damaged areas. One of the most promising approaches in cell therapy may exploit a plethora of healing actions that the introduction of new and healthy cells may elicit on damaged brain tissue. In the current scenario, NSCs may act as a reservoir, providing trophic support to surviving cells and synapses at various levels, perhaps by scavenging toxic compounds in genetic and metabolic disorders, such as Tay-Sachs, Sandoff, Canavan, and Batten diseases, or by releasing trophic factors at the level of post-traumatic or ischemic injury in neurodegenerative diseases, such as ALS [142]. Transplantation experiments in animal models of brain lesions or neurodegenerations have revealed that NSCs are capable of integrating into the host brain and ameliorate functional defects. Local environmental cues produced by the stem niche or by a damaged area, soon after injury, have been identified as promoting exogenous cell survival and migration

to the lesion (pathotropism) [143]. Consistently, several studies have shown that donor NSCs also display a tropism intrinsic to the recipient stem niche (homing), matching signals resembling their naive site of origin [100] (Fig. 1). The mechanisms determining the survival and migration of exogenous NSCs into healthy or lesioned adult brain have still to be identified and characterized, but extensive self-renewal of exogenous NSCs appears dependent on their proximity to the recipient stem niche (Fig. 1). Therefore, in view of future clinical applications of NSCs for the cure of neurodegenerative diseases, these findings suggest accurately evaluating the site of transplantation, according to specific therapeutic aims. NSCs are able to provide a multitude of therapeutic effects, and the extent to which and for how long these effects are needed depend on the specific neurological disorder [141]. In addition to the establishment of protocols for the extensive culture of NSC lines ex vivo, the nontumorigenic self-renewal of transplanted cells into the recipient CNS would be an auspicious goal in chronic disorders. Contrary to this, upon acute injury, such as stroke or focal demyelination, morbidity develops within a few days, together with the development of an acute inflammatory reaction. In this case, a limited or even absent self-renewal in vivo of transplanted cells together with an inherent or epigenetically induced tendency to differentiate into the proper phenotype could be the most appropriate therapeutic strategy. The transplantation of multiple subpopulations of neural precursors or of specific NSC-derived progenitors next to the site of injury offers the chance for a rapid and targeted therapeutic effect [100, 143] (Fig. 3). Several clinical trials harnessing various sources of neural stem cells have been started in the last few years (Table 2) [61, 144–148]. These important breakthroughs in the clinical application of NSCs demonstrate that a continuous and standardized clinical-grade source of normal human CNS cells (hNSCs), combining the plasticity of fetal tissue

Table 2. Clinical trials of neural stem cells

Stem cell source	Disease	Delivery	Year	References	Current state of the art
Fetal neural stem cells (Stem Cells Inc.)	Batten disease, or neuronal ceroid lipofuscinosis	Brain neurosurgery single dose	2006	[146–148]	Phase Ib, discontinued because of lack of enrollment Phase I, ongoing
Embryonic stem cell-derived oligodendrocyte progenitors (GRNOPC1 from Geron)	Spinal cord injury	Spinal cord injection	2009	[144]	Withdrawn because of risk of economic failure (too-late payoff)
Fetal neural stem cells (8-week-old fetus) (Neuralstem)	Amyotrophic lateral sclerosis	Multisite injection into the spinal cord	2010	[145]	Phase I, ongoing (14 patients transplanted)
Human embryonic stem cells (CTX0E03 line from Reneuron)	Stroke	Brain neurosurgery (stereotaxic injection in the putamen region)	2010	[61]	Under way
Genetically modified human neural stem cells to produce cytosine deaminase enzyme (Seung U. Kim, University of British Columbia)	Glioma	Intravenous delivery	2010	[135]	Recruitment of patients
Neural fetal stem cells (Stem Cell Factory, Hospital S. Maria, Terni, Italy)	Amyotrophic lateral sclerosis	Multisite injection into the spinal cord	2012	[55]	Phase I, recruitment of 18 patients

with extensive proliferative capacity and functional stability, is of paramount importance.

cultured on a large scale in vitro, have been proven to be optimal candidates.

CONCLUSION

The development of methods to establish NSC lines in vitro has been one of the main goals of researchers since the discovery of active neurogenesis in the adult mammalian CNS. Current pre-clinical studies strongly suggest that the therapeutic efficacy of stem cell transplantation mostly relies on NSC-mediated neuro-protection, rather than replacement of damaged cells. For clinical application, it is important that these protective strategies are proven safe and effective in humans. Several clinical trials using human embryonic stem-derived NSCs or fetal NSCs are currently under way, and their outcomes will contribute to improved transplant-based therapies.

Our greatest limitation in treating many neurodegenerative disorders is the lack of understanding of what causes the onset or drives the progression of sporadic and idiopathic pathologies. In this regard, one of the most significant advances in neural stem cell biology has been the use of stem cells for understanding pathobiological mechanisms and for the screening of novel therapeutic drugs. To this end, both ESCs and iPSCs, which can be

AUTHOR CONTRIBUTIONS

L.D.F.: conception and design, manuscript writing, provision of study material, final approval of manuscript; E.B.: manuscript writing, provision of study material, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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