

Remodelling the injured CNS through the establishment of atypical ectopic perivascular neural stem cell niches

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ABSTRACT

Compelling evidence exists that somatic neural stem/precursor cell (NPC)-based therapies protect the central nervous system (CNS) from chronic inflammation-driven degeneration, such as that occurring in experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), cerebral ischemic/hemorrhagic stroke and spinal cord injury (SCI). However, while it was first assumed that NPC transplants may act through direct replacement of lost/damaged cells, it has now become clear that they are able to protect the damaged nervous system through a number of 'bystander' mechanisms other than the expected cell replacement. In immune-mediated experimental demyelination – both in rodents and non-human primates – others and we have shown that transplanted NPC possess a constitutive and inducible ability to mediate efficient 'bystander' myelin repair and axonal rescue. This novel mechanism(s), which may improve the success of transplantation procedures, is likely to be exerted by undifferentiated NPCs whose functional characteristics are regulated by both CNS-resident and blood-borne inflammatory cells releasing in situ major stem cell regulators. Here, we discuss some of these alternative 'bystander' mechanisms, while pointing at the formation of the atypical ectopic perivascular niches, as the most challenging example of reciprocal biologically sound cross talk between the inflamed microenvironment(s) and transplanted therapeutic NPCs.

Key words

Neural stem cells • Atypical stem cell niches • Stem cell therapies • Regenerative medicine • CNS repair

Neural stem cell/precursor cell (NPC) transplants and neurological diseases

The recent advances in stem cell biology have raised great expectations that diseases and injuries of the CNS may be ameliorated by the development and delivery of non-haematopoietic stem cell-based therapeutics. Most of the more classical experimental cell therapies – based on the focal injection of neural lineage-committed progenitor cells – have in fact failed to foster substantial tissue repair in disease models where the anatomical and functional damage

is widespread and an inflamed and/or degenerative microenvironment co-exists (reviewed in Martino and Pluchino, 2006). On the other hand, solid evidence has been accumulated from many laboratories and in many conditions that NPCs survive transplantation procedures within the host CNS, specifically migrate within the damaged tissue, and protect the nervous system from inflammatory and other forms of damage. However, irrespectively from the experimental disease course (acute vs. chronic), the neuropathological features (focal vs. multifocal) and type of inflammation (primary vs. reactive), the

overall functional recovery obtained following NPC transplantation is usually poorly correlated with the absolute numbers of the donor-derived progeny *in vivo* (reviewed in Martino and Pluchino, 2006). This has been shown for NPC transplants in experimental Parkinson's (PD) or Huntington's diseases (HD), where NPCs very rarely differentiate into neurons *in vivo* while mediating a significant clinico-pathological recovery (reviewed in Martino and Pluchino, 2006). Similarly, mice with acute cerebral ischemic stroke or intracerebral haemorrhage improve at both behavioural and pathological levels, in spite of the post-mortem evidence that most implanted stem cells have assumed an astroglial fate (or nestin immune reactivity) (Lee et al., 2008; Bacigaluppi et al., 2009). In line with these observations, others and we have provided strong evidence that the systemic injection of somatic – and more recently embryonic stem (ES) cell-derived – mouse and human NPCs ameliorates the clinico-pathological features of rodents and non-human primates with experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Pluchino et al., 2003; Pluchino et al., 2005; Aharonowiz et al., 2008; Pluchino et al., 2009a; Pluchino et al., 2009b). We have demonstrated that this is dependent on multiple mechanisms of action of NPCs within specific CNS *vs.* non-CNS microenvironments (reviewed in Martino and Pluchino, 2006).

The route of NPC administration

The route of cell administration represents a major issue for NPC transplantation and appears to be very much depending on the location and number of CNS lesion site(s) (focal *vs.* multifocal). The anatomic-pathological features of focal CNS disorders, might suggest that direct local (intralesional) cell transplantation would facilitate tissue regeneration, while the multifocality of certain others CNS disorders – such as MS and epilepsy – would represent a major limitation for intralesional cell-transplantation approaches. Following the first observation in experimental brain tumours, the systemic (e.g. intravenous, intrathecal) transplantation of NPCs can be therapeutically efficacious in multifocal CNS disorders, owing to the ability of transplanted cells to follow, once travelling into either the blood

stream or the cerebrospinal fluid, a gradient of chemoattractants (e.g. pro-inflammatory cytokines and chemokines) occurring at the site of inflammatory lesions (Pluchino et al., 2005; Martino and Pluchino, 2006). Specific homing of transplanted NPCs has been shown, so far, in spinal cord injury (SCI), epilepsy, and stroke. However, the exact molecular mechanisms sustaining this phenomenon have been detailed, so far, only in EAE. Tethering, rolling, and firm adhesion to inflamed endothelial cells and then trans-endothelial migration across the blood brain barrier (BBB) into the inflamed CNS areas are sequentially mediated by the constitutive expression of functional cell adhesion molecules (CAM) (e.g. CD44) (Rampon et al., 2008), integrins (e.g. $\alpha 4$, $\beta 1$), and chemokine receptors (e.g. CCR1, CCR2, CCR5, CXCR3, CXCR4) on NPC surface (Pluchino et al., 2005; Martino and Pluchino, 2006). Irrespective from the characteristics of the experimental disease, neuropathological features and type of inflammation, functional recovery obtained by NPC transplants scarcely correlates with the absolute numbers of the transplant-derived terminally differentiated progeny *in vivo*. Transplantation of NPCs into rodents with experimental PD or HD, very scarcely differentiate into tyrosine hydroxylase (TH)-immunoreactive neurons despite significant behavioural improvement (reviewed in Martino and Pluchino, 2006). Similarly, mice with SCI show remarkable locomotor recovery, despite the pathological evidence of preferential astroglial fate of transplanted NPCs (reviewed in Martino and Pluchino, 2006). On the other hand, the large majority of NPCs injected intravenously into mice with experimental cerebral haemorrhage or with acute ischemic stroke, retain, when detected at the boundaries of the ischemic brain tissue, the expression of undifferentiation markers (e.g. nestin) (Fig. 1) (Bacigaluppi et al., 2009). Also in EAE, the very rare differentiation of transplanted NPCs (e.g. into oligodendrocytes) is in apparent contrast with the evidence of significant axonal protection at a neurophysiological level. In the very same context, more than 20% of transplanted NPCs accumulate (and survive for months) at the level of perivascular inflammatory CNS areas, while retaining morphological and phenotypic features of suggestive of undifferentiation (Pluchino et al., 2005; Pluchino et al., 2009a).

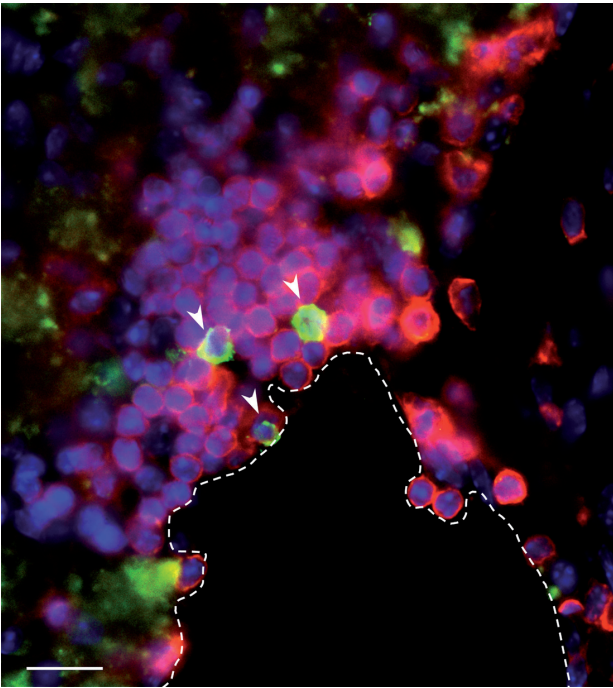


Fig. 1. - Perivascular area in the ischemic hemisphere of a mouse with experimental middle cerebral artery occlusion (MCAO) injected i.v. with GFP⁺ NPCs at 3 days post MCAO and sacrificed 30 days after cell injection. Shown are three individual NPCs (arrowheads; green) being found in close proximity to blood vessels (dashed contour), while being surrounded by numerous CD45⁺ blood-derived leukocytes (red). Nuclei are counter-stained with Dapi (dark blue). Scale bar: 40 μ m.

Interestingly enough, this scarce propensity to undergo terminal differentiation within (and functional integration into) the host tissue, might suggest that transplanted NPCs exert its therapeutic effects via a number of bystander mechanism(s) alternative to the initially expected cell replacement (Pluchino et al., 2009c). As such, significant reduction of scar tissue and increased survival and/or function(s) of endogenous glial and neuronal progenitors is also observed in rodents with experimental cerebral stroke after transplantation of somatic stem cell sources other than NPCs (Chen et al., 2003; Yoo et al., 2008). This tissue(trophic) effect is indeed accompanied by increased *in vivo* bioavailability of major neurotrophins (Lu et al., 2003; Yan et al., 2004; Muller et al., 2006). Moreover, transplanted NPCs promote bystander immune modulation, as they release soluble molecules (e.g. cytokines, chemokines and stem cell regulators), thus being significantly capable to changing the (inflammatory vs. ischemic) microenvironment into more favourable to repair/regenera-

tion (Pluchino et al., 2005; Bacigaluppi et al., 2009; Pluchino et al., 2009a; Pluchino et al., 2009b). More recent evidence suggest that transplanted NPCs contribute to down-regulate the effector functions of inflammatory T cells, macrophages as well as dendritic cells (DCs), also into bodily sites other than the CNS (e.g. within secondary lymphoid organs) (Einstein et al., 2007; Pluchino et al., 2009b). NPCs show remarkable propensity to migrate to and accumulate at the level of the site of injury also when injected intravenously in rodents with SCI. This observation is associated to significant amelioration of the clinico-pathological features (e.g. locomotor activity and volume of injury) of SCI (Takeuchi et al. 2007), with transplanted NPCs displaying propensity to differentiate mostly towards the astroglial lineage (Fujiwara et al. 2004). Also in this latter cases, indirect evidence is available that recovery of functions is related to up-regulation of RNA levels of neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and leukaemia inhibitory factor (LIF), as well as of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , *in vivo* (Bottai et al., 2008).

Transplanted NPCs have (tissue) trophic effects

The insufficient repair capacity of the adult CNS results from a number of failing repair programmes, including the apparent inability of endogenous progenitors and stem cells to properly respond to disease states, to replace damaged cells and from lack of regenerative capacity of injured axons (Franklin, 2002; Martino, 2004; Franklin and Ffrench-Constant, 2008; Pluchino et al., 2008). NPC therapy is therefore emerging as a mode of treatment that can enhance the host brain's ability to repair itself in both aspects (Einstein and Ben-Hur, 2008). Recent studies have focused also on several bystander (tissue)trophic properties of transplanted NPCs. NPCs seeded on a synthetic biodegradable scaffold and grafted into the cord of hemi-sectioned rats have induced significant clinical recovery while reducing the necrosis of the surrounding parenchyma and preventing extensive secondary cell loss, inflammation and glial scar formation. Moreover, the NPC graft has induced a permissive environment for axonal regeneration (Teng et al., 2002). Substantial

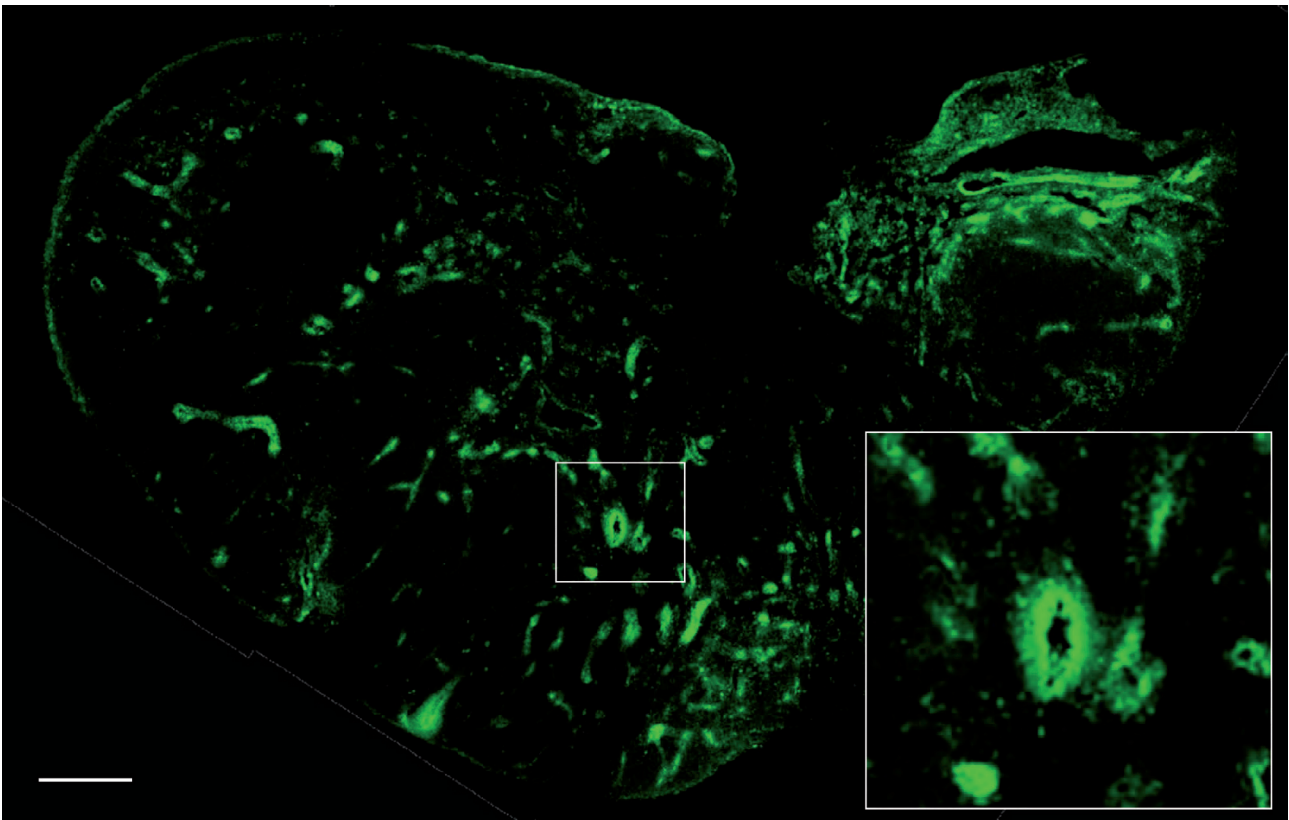


Fig. 2. - Reconstruction of a representative axillary lymph node from a R-EAE mouse injected s.c. with GFP⁺ NPCs at 3 and 10 days post immunization and sacrificed at 72 days after cell injection. Shown is a direct fluorescence signal depicting the distribution of NPCs (green) throughout the entire lymph node, with significantly predominant clusters of NPCs being mainly found around blood vessels. Scale bar: 500 μ m.

endogenous re-constitution of the brain structural connectivity has been found following injection of NPCs in biodegradable scaffolds into regions of extensive brain degeneration caused by hypoxia (Park et al., 2002a; Park et al., 2002b) or following intracisternal (i.c.) transplantation of NPCs after ischemia/reperfusion injury in mice (Capone et al., 2007). Transplanted NPCs have rescued endogenous dopaminergic neurons of the mesostriatal system in a PD model in rodents (Ourednik et al., 2002), while prevention of motor neurons from dying has been observed when NPCs were transplanted in models of amyotrophic lateral sclerosis (ALS) (Kerr et al., 2003; Ferrer-Alcon et al., 2007; Suzuki et al., 2007). NPCs-driven bystander tissue protection has generally meant as reduction of glial scar formation and increase of survival and/or functions of endogenous glial and neuronal progenitors surviving to the pathological insult. However, the underlying molecular

mechanisms by which transplanted NPCs have exert such a broad (tissue)trophic effects are still poorly understood, though they relate in part to increased *in vivo* bioavailability of major neurotrophins (e.g. NGF, BDNF, ciliary neurotrophic factor [CNTF], glial-derived neurotrophic factor [GDNF]) (Teng et al., 2002; Lu et al., 2003; Pluchino et al., 2003; Chu et al., 2004; Einstein et al., 2006) and to the modulation of the host environment into more permissive for repair/regeneration.

Indeed, the multiple roles of neurotrophins as mediators in cell cycle regulation, cell survival, and differentiation during development and adulthood, make them potential candidates for the regulation of endogenous stem and progenitor cell proliferation and differentiation following CNS injuries. For example, neurotrophins secreted by transplanted NPCs help to promote corticospinal axon growth after transplantation onto an organotypic co-culture

system, containing dissected brain cortex and spinal cord (Kamei et al., 2007). Moreover, several neurotrophins that may be released by NPCs inhibit EAE. Insulin-like growth factor (IGF)-1 and glial growth factor (GGF)-2 are neurotrophic factors that promote survival and proliferation in the oligodendrocyte lineage (Barres et al., 1992; Canoll et al., 1996; Canoll et al., 1999; Mason et al., 2000), while treatment with these very same factors is beneficial both clinically and pathologically in rodents with EAE (Yao et al., 1996; Akassoglou et al., 1998; Cannella et al., 1998). Interestingly, some of these neurotrophin-mediated effects enhanced the survival of endogenous oligodendrocytes (Butzkueven et al., 2002; Linker et al., 2002), others decreased neuroinflammation (Cannella et al., 1998; Villoslada et al., 2000; Flugel et al., 2001; Ruffini et al., 2001).

Recent studies show NPCs grafted into the striatum of rodents treated with 3-nitropropionic acid (3-NP) as a model of oxidative stress, exhibit higher survival of striatal neurons and preservation of the overall striatal cytoarchitecture, which both lead to reduced behavioural symptoms as compared to sham-treated controls. *In vitro*, increased viability of primary neural cells was observed in co-cultures with NPCs in the presence of 3-NP, and ascribed as dependent from interference with production of free radicals, up-regulation of the antioxidant enzyme superoxide dismutase 2 (SOD2) in neurons, and increased expression of neuroprotective factors such as CNTF and vascular endothelial growth factor (VEGF) in a network of NPCs and local astrocytes (Madhavan et al., 2008).

There is also attractive evidence that transplanted NPCs can enhance endogenous neurogenesis in certain physiological and pathological conditions (Munoz et al., 2005; Hattiangady et al., 2007). As such, mice exposed pre-natally to opioids display impaired learning associated with reduced neurogenesis, whereas the transplantation of NPCs improves learning functions, as well as host brain derived neurogenesis in the dentate gyrus (DG) of the hippocampus (Ben-Shaanan et al., 2008). A similar neurotrophic effect was also reported in physiological ageing. Again, while neurogenesis in the DG declines severely by middle age, transplantation of NPCs into aged rodents stimulates neurogenesis in the DG and leads to increased generation of dentate granule cells (Hattiangady et al., 2007).

Thus, transplanted NPCs may enhance the innate self repair capacities of the adult CNS, by restoring the ability of endogenous progenitors and stem cells to both respond properly to disease state and replace damaged CNS cells as well as the ability of severed axons to regenerate.

Transplanted NPCs have immune modulatory effects

In addition to cell replacement and (tissue)trophism (Pluchino et al., 2003), we have first described significant immune modulatory capacities for transplanted (*undifferentiated*) NPCs *in vivo* in EAE. It is in fact now established that NPC-mediated bystander actions may take place in the CNS, at the level of the atypical perivascular niches (Pluchino et al., 2005), as well as in secondary lymphoid organs (Einstein et al., 2007; Pluchino et al., 2009b). Nonetheless, following our own first report that membrane-bound Fas/CD90 ligands were regulating part of the NPC-mediated suppressive effect on encephalitogenic T lymphocytes in the CNS (Pluchino et al., 2005), other groups have generated data that describe the mechanisms responsible for these specialized stem cell functions. In general, this has been approached mostly by *in vitro* studies that have utilized immune cell/NPC co-cultures (Einstein et al., 2003). In support of this NPC mediated immune regulation, we have observed similar results in cerebral ischemic stroke. The systemic injection of syngeneic NPCs in sub-acute mice with transient middle cerebral artery (MCA) occlusion induced a marked reduction of the very same pro-inflammatory mediators (e.g. interferon [IFN]- γ , TNF- α , interleukin [IL]-1 β and IL-6) known to contribute heavily to the detrimental post-ischemic brain inflammation (Barone et al., 1997; Hallenbeck, 2002; Allan et al., 2005), as well as decreased the number of activated microglial cells accumulating at the boundaries of the ischemic hemisphere (Bacigaluppi et al., 2009).

On the other hand, others and we have also recently provided evidence of long-term persistence of systemically injected NPCs into peripheral bodily organs (Einstein et al., 2007; Pluchino et al., 2009b). Interestingly enough, transplanted NPCs – both intravenous and subcutaneous cell injection in EAE mice – display remarkable capacity to target (and

synergize with) immune cells also at the level of secondary lymphoid organs, where they survive over two months, while stably changing the perivascular microenvironment (Fig. 2). Interestingly, the ultrastructural analysis of lymph nodes from NPC-injected EAE mice showed the presence of numerous large-size NPCs, which were frequently found to establish consistent anatomical contacts with lymph node cells through either polarized *nanotubes*, secreted membrane particles, cytoplasmic expansions or elongated intercellular junctions. We also demonstrated that NPCs surviving in lymph nodes hampered the activation of myeloid DCs via the release of the stem cell regulator bone morphogenetic protein (BMP)-4. This BMP-dependent immune regulatory effect was highly specific for NPCs, and, in turn, led to the steady restraint of the expansion of antigen-specific (*encephalitogenic*) T cells (Pluchino et al., 2009b). Concurrently, other reports have begun to elucidate some of the paracrine factors that are responsible for mediating the immune suppressive *vs.* pro-survival capacity of other non-hematopoietic somatic stem cell sources; these include chemokines and the inducible nitric oxide synthase (iNOS) (Ren et al., 2008) and stanniocalcin-1 (STC-1), a peptide hormone that modulates mineral metabolism (Block et al., 2009). These and other evidence highlight the particular flexibility of major stem cell capacities upon exposure to precise *in vivo* microenvironments (reviewed in Martino and Pluchino, 2006). We have then provided robust evidence that most of the therapeutic effect of NPC transplants result from highly sophisticated mechanisms of *in vivo* intercellular communication, rather than cell replacement. Importantly though, the detailed molecular and cellular mechanism(s) responsible for this multifaceted *therapeutic plasticity* exhibited by stem cell transplants remain far from being fully elucidated.

NPC transplantation in CNS diseases: the atypical ectopic niche

Altogether these results challenge the concept that (somatic) stem cell transplantation therapeutically works throughout cell replacement. As a matter of fact, stem cell transplantation may also promote CNS repair via intrinsic neuroprotective bystander

capacities, mainly exerted by undifferentiated stem cells releasing, at the site of tissue damage, a milieu of neuroprotective molecules once temporally and spatially orchestrated by environmental needs.

Interestingly, once within inflamed CNS areas, systemically-injected NPCs accumulate (and persist) around the perivascular space where reactive astrocytes, inflamed endothelial cells and blood-borne infiltrating T cells co-reside. In these areas, named '*CNS atypical ectopic niches*', a highly sophisticated molecular cross talk is likely to take place between the different constituents of the atypical niche. On one hand then, the great majority of transplanted NPCs survive long term, while displaying undifferentiated features (e.g. round-shaped morphology and lack of major antigens of differentiation), owing to the focal release of stem cell regulators by immune cells and reactive astrocytes. On the other hand, NPCs promote neuroprotection by *in situ* releasing immunomodulatory molecules and neurotrophic factors (Pluchino et al., 2005; Martino and Pluchino, 2006). Via the release/expression of immune modulatory molecule (e.g. FasL, Apo3L, TRAIL) NPCs promote apoptosis of encephalitogenic Th1 effector cells expressing death receptors. Via the inhibition of the release of neurotrophic growth factor (e.g. transforming growth factor [TGF]- β , fibroblast growth factor [FGF]-II), NPCs also contribute to significant reduction of glial scarring (Pluchino et al., 2003; Pluchino et al., 2005; Martino and Pluchino, 2006). Last but not least, transplanted NPCs may also differentiate into myelin forming cells (Pluchino et al., 2003). As a net effect of these different mechanisms of neuroprotection, significant rescue of endogenous neural progenitors, as well as consequent substantial tissue protection/recovery is obtained (Pluchino et al., 2009c).

The intrinsic nature of most of these molecules as well as their 'constitutive' characteristics, represent a stem cell signature that also reconcile data showing that other sources of somatic stem cells (e.g. bone marrow-derived stem cells [BMSC] and mesenchymal stem cells [MSC]), with very low capabilities of neural differentiation, may efficiently promote CNS repair (*therapeutic plasticity*) (Pluchino and Martino, 2008). As such, in multifocal inflammatory CNS disorders, also therapeutic somatic stem cell sources other than NPCs (e.g. BMSCs, umbilical

cord blood stem cells [UCBSC], MSCs) are capable to travel into biological fluids (e.g. the blood, the lymph, the cerebrospinal fluid) and specifically reach the inflamed CNS areas, where they persist for months and promote functional and tissue recovery (Garbuzova-Davis et al., 2003; Chu et al., 2004; Xiao et al., 2005; Liu et al., 2006; Ziv et al., 2006). Thus, the formation of *CNS atypical ectopic niches* following somatic non-hematopoietic stem/precursor cell transplants can be postulated as the functional requisite for the therapeutic activity of transplanted cells (Fig. 3). Similarly, to what we have shown in EAE, also in ischemic stroke intravenous transplanted NPCs not only migrate specifically to the injury boundary zone but also establish anatomical interaction(s) with endothelial cells, blood-derived leucocytes and CNS resident immune cells (Bacigaluppi et al., 2009).

We envisage that the exact knowledge and the potential impact of non-conventional stem cell-mediated therapeutic mechanisms will result, in certain circumstances, in more efficacious curative alternatives.

Conclusions

Research carried out during the last decades revealed that NPCs persist within the adult brain, providing a source of neuronal and glial cell precursors throughout life. Besides restricted neurogenic sites harbouring bona fide stem cells that undergo regulation within their typical niches, multipotent progenitor cells capable of proliferation and self-renewal are likely to be present also in widespread areas of the mature CNS parenchyma. Emerging evidence indicates that these progenitor cells form a rather heterogeneous population with multifaceted properties that can be (re)expressed under experimental conditions *in vitro* although remaining mostly unknown *in vivo*. Further, while the replacement of lost/damaged cells was until few years ago assumed as the prime therapeutic mechanism of stem cells, it is now clear that transplanted somatic stem cells may simultaneously instruct several therapeutic mechanisms among which the sole cell replacement does not prevail.

The ‘therapeutic plasticity’ can therefore be viewed as a true functional signature of somatic stem cells. Strictly depending on when injected into a live host suffering from a tissue specific disease (inflamma-

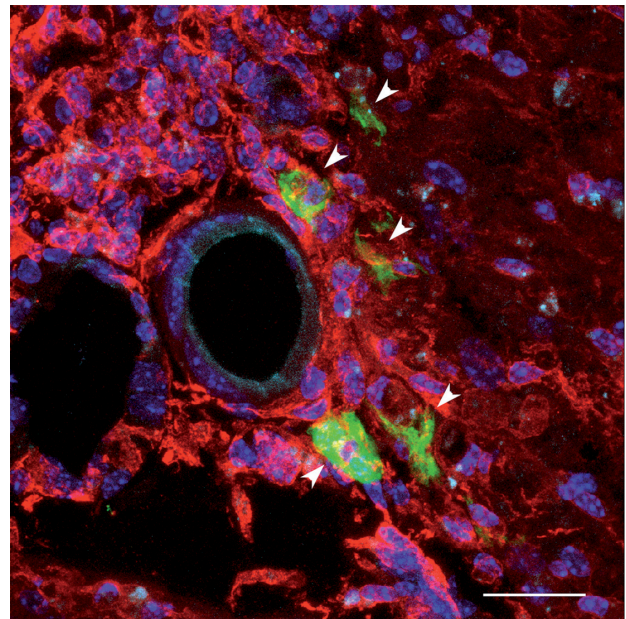


Fig. 3. - Representative confocal microscopy image of a perivascular area in the spinal cord of a mouse with experimental contusion spinal cord injury (SCI) implanted focally around the injury site with GFP⁺ NPCs at 7 days post SCI and sacrificed 60 days after cell injection. Shown are four individual NPCs (arrowheads; green) being found in close proximity to CD31-immune reactive blood vessels (cyan-blue), while being surrounded by numerous Iba1⁺ macrophage/microglial cells (red). Nuclei are counterstained with Dapi (dark blue). Scale bar: 20 μ m.

tory vs. degenerative), transplanted stem cells are likely to display some unique therapeutic adaptive functions. This is due to the fact that stem cells display an extraordinary capacity of finding *in vivo* the proper way(s) towards certain favourable atypical niches, where they survive and act as therapeutic weapons through the interaction with the different cell types of the (micro)environment (Pluchino et al., 2005; Jin et al., 2006; Calabrese et al., 2007; Einstein et al., 2007; Lee et al., 2008; Thored et al., 2007). Nonetheless, the recent demonstration that also other sources of somatic stem cells may display equally significant bystander capacities and promote CNS repair (Escobar et al., 2005; Zappia et al., 2005), further proves the relevance of stem cell-dependent therapeutic mechanisms alternative to cell replacement.

The next challenge for the future of stem cell-based therapies would be that of finding the way to carefully weight and tightly regulate the different therapeutic alternative mechanisms such cells may instruct

in vivo at the level of specific (possibly exclusive) therapeutically relevant microenvironments.

Acknowledgements

We thank J.M. Garcia-Verdugo and C. Alfaró-Cervello (University of Valencia) for the ultrastructural studies of NPC transplants in EAE lymph nodes and L. Peruzzotti-Jametti and E. Brambilla (INSPE), for the deconvolution image of NPC transplants in MCAo mice and the confocal image of NPC transplants in SCI mice, respectively.

This work was supported via the Italian Multiple Sclerosis Foundation (FISM, grants 2004/R/15 to SP; 2002/R/37 to GM), the National Multiple Sclerosis Society (NMSS, partial grants RG-4001-A1 to SP; RG 3591-A-1 to GM and RG 3762-A-1), the Italian Ministry of Research and University (MIUR), the Italian Ministry of Health (CS 118.1 to GM), the BMW Italy Group (BMW 2008 MART to GM), the Myelin Project, Wings for Life (WfL PLUCH to SP) and Banca Agricola Popolare di Ragusa (BAPR, unrestricted grant to SP).

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