Translational Research Strategies for Treatment of Acute Spinal Cord Injury

SECTION 1) Adipose Tissue-Derived Stem Cells

Summary

Cell transplantation and regeneration of damaged tissues may provide life-saving treatments for individuals affected by incurable neurological disorders. However, the use of transplanted embryonic stem cells (ESC) still poses problems since it is difficult to induce all ESC within a colony to differentiate into the desired cell phenotype. Spinal cord injury (SCI) results in paralysis below the site of injury. Although many cell types have been used, none of these procedures have been fully satisfactory. Adult stem cells hold potential for treating spinal cord and brain injuries, and human adipose tissue may be an alternative source. hASCs meet the required criteria (i) are abundant; (ii) can be collected and harvested by a minimal invasive procedure; (iii) could be differentiated into multiple cell lineage pathways in a reproducible manner; (iv) and could be safely and effectively transplanted to either an autologous or allogenic host. However, in order to avoid inappropriate gene expression in transplanting cells or tumorigenesis the role of genome wide CpG methylation in regulating the transition to specific differentiation programs must be investigated.

Specifics aims

1)To optimize the methodology for isolation and purification of hASCs from adipose tissue of patients (later those with SCI); 2)To establish in vitro adequate protocols for transdifferentiation of hASCs towards oligo and neural phenotype; 3)To evaluate the eventual role of epigenetics in the differentiation and tumorigenic potential of hASCs; 4)To study the degree of tissue preservation and functional recovery achieved by transplantation of hASCs in the model of spinal cord contusion injury in the mouse; 5)To assess the survival, differentiation, integration and migration of transplanted cells within the injured spinal cord, and their effects on inflammatory response, glial reactivity, cytokine production and axonal regeneration; 6)To establish the optimal conditions for hASCs transplantation after SCI, regarding number of cells, method of grafting and time after injury; 7)To assess MRI and electrophysiological features of hASCs transplants in SCI.

Background

Traumatic spinal cord injury (SCI) is devastating for the individual patient and costly to society by requiring substantial long-term health care expenditures. The response to SCI develops in two well defined steps, the first being the trauma itself leading to direct damage of the nervous tissue while the secondary injury is characterized by events that amplify the early primary lesion. SCI leads to complex responses spanning from massive inflammatory reactions and formation of the glial scar to changes in intracellular signalling. The administration of erythropoietin or IL-8 antagonists resulted in the attenuation of the inflammatory response, enhanced production of neutrophic factors and reduction of secondary degeneration with promotion of functional recovery after SCI (Gorio et al 2002, 2005, 2007). Similarly the i.v. administration of adult neural precursor cells (NPCs) or ES within 24 hours of SCI promoted functional recovery and enhanced the production of neurotrophic

factors (Bottai et al 2008, 2010); on the other hand their survival at lesion site lasted less than 3 weeks due to the phagocytosis by activated macrophages. This event somewhat stopped the progressive recovery of function, that did not improve further. The original reprogramming of specialized cells by Yamanaka changed the understanding of the scientific community by overturning the dogma that such cells retain an immutable identity. In this project we will show that mild induction of human pluripotent stem cells derived from adipose tissue (hASCs) may show a certain degree of flexibility, and represent a patient specific promise being less immunogenic for cell transplantation therapies. Mesenchymal stem cells (MSCs) are able to induce neuroprotective (10), immunomodulatory (18) and some regenerative effects in animal models (3, 23), pointing to the great potential of this type of cells as therapeutic agents for SCI and other neural disorders. MSCs are multipotent somatic cells able to differentiate into neural phenotypes, that can be derived from various tissues, most commonly from the bone marrow, and can be readily isolated and expanded (22). They have significant advantages, such as the lack of ethical controversy regarding their source and the potential for providing autologous transplants, thus avoiding risk of rejection or side effects associated with immunosuppression (19). For autologous transplantation, MSCs have several advantages over other adult stem cells, in that they are relatively easy to isolate and expand in vitro to increase numbers from the patients own bone marrow, without creating any further damage (13). Moreover, MSCs have been used for years in the treatment of hematopoetic diseases, so that protocols for isolation, application and safety are well established in clinical use (4). Nevertheless, the established criteria to define MSCs derive in a heterogeneity that explains the variable results regarding functional effects between studies, and also the ability of these cells to survive, integrate and differentiate. In the majority of recent studies in which MSCs were transplanted into injured adult CNS, functional improvement was moderate, but was not accompanied with replacement of lost neural cells. The inability of the MSCs to produce neural cells can be explained by the hostile environment of injured spinal cord, that also reduces the survival time of the transplanted cells. Therefore, both microenvironment and the intrinsic state of transplanted cells can play a decisive role in grafted cells survival and integration as well as in the functional outcome. The importance of pre-induction of MSCs previous to transplantation into the injured spinal cord has been suggested for promoting regeneration and functional recovery associated to cell fate and its relationship with tissue sparing (10, 12, 16). Previous studies reported poor survival of transplanted MSCs (as well as other cell types) within the spinal cord injury site at several weeks post-injection, suggesting a correlation between the degree of cell survival and the extent of functional recovery (2). Taken together, all the previous reports point to the hypothesis that increasing cell survival and inducing differentiation of transplanted MSCs will further improve neural replacement and functional outcome in SCI. In our study MSCs will be obtained from human fat processed with a special gadget (Lipogems®) that reduces liposuction fat particles to 500 micrometer so that hASC diffuse out without the need of any enzymatic treatment. These cells bear neural antigens, survive in the injured CNS for over a month after transplantation and trans-differentiate according to the area. Injected into the striatum all hASCs become Th-positive cells, and HADC inhibitor valproic acid exposed hASCs migrate very efficiently to the injured cord, differentiate and express neuronal antigens.

Aims

Cell transplantation for treatment of patients with spinal cord injury (SCI) is a novel and promising field of translational research that offers the prospect of developing curative approaches by regenerating or rejuvenating lost and/or diseased spinal cord tissue and potentially inducing restitution of lost neural cells or providing supporting structure and factors for axonal growth. In this context, human adipose mesenchymal stem cells (hASCs) are believed to have a particularly high potential, among the cell types that are currently available for cell therapy in clinical use. The initial hypothesis is that a transplant of hASCs constitutes a promising method for repairing the injured spinal cord and for modulating and reducing secondary effects after spinal cord injury, by combining neuroprotection, cell restitution and central regeneration of spinal pathways. To test this hypothesis the project comprises four subprojects to provide state-of-the-art research conducing to:

1)To optimize the methodology for isolation and purification of hASCs from adipose tissue of patients (later those with SCI).

2)To establish in vitro adequate protocols for transdifferentiation of hASCs towards oligo and neural precursor phenotype with the objective of enhancing cell survival and protection after transplant. Different factors will be assessed for increasing survival of the cells.

3)To evaluate the eventual role of epigenetics in the differentiation and tumorigenic potential of hASCs.

4)To study the degree of tissue preservation and functional recovery achieved by transplantation of hASCs in the model of spinal cord contusion injury in the mouse.

5)To assess the survival, differentiation, integration and migration of transplanted cells within the injured spinal cord, and their effects on inflammatory response, glial reactivity, cytokine production and axonal regeneration.

6)To establish the optimal conditions for hASCs transplantation after SCI, regarding number of cells, method of grafting and time after injury.

7)To assess MRI and electrophysiological features of hASCs transplants in SCI

Originality

These hASCs have significant advantages, such as the lack of ethical controversy regarding their source and the potential for providing autologous transplants, thus avoiding risk of rejection or side effects associated with immunosuppression. For autologous transplantation, hASCs have several advantages over other adult stem cells, in that they are relatively easy to isolate and expand in vitro to increase numbers from the patients own bone marrow, without creating any further damage. Moreover, hASCs have been used for years in the treatment of hematopoetic diseases, so that protocols for isolation, application and safety are well established in clinical use (4). Notably, these plastic-adherent hASCs can be easily cultured in vitro and expanded to a clinical scale. In addition, the multilineage differentiation observed in MSCs has enabled therapeutic trials to repair various kinds of tissue damage using ex vivo-expanded MSCs. These trials included cell therapy for regeneration of myocardium in ischemic myocardial infarct, regeneration of blood vessels in Buergers disease, regeneration of bones in osteogenesis imperfecta or in large bone

defects, as well as some immunological applications, such as amelioration of graft-versus-provided some encouraging results, the extent of functional improvement or the contributions of hASCs to the structure of the regenerated tissues have not been as satisfactory as initially anticipated. For example, while large numbers of experimental studies have demonstrated their differentiation into myocardium-like cells expressing myocardium- specific markers, few studies have demonstrated their successful differentiation into mature myocardium, or their functional integration into the damaged myocardium. Similarly, hASCs have been shown to express various types of neural markers such as nestin, Tuj1, Oct4 but very rarely acquire neuronal peculiarity even in vitro.
In the majority of recent studies in which MSCs were transplanted into injured adult CNS, functional improvement was moderate, but was not accompanied with replacement of lost neural cells. The inability of the MSCs to produce neural cells can be explained by the hostile environment of injured spinal cord, that also reduces the survival time of the transplanted cells. Nonetheless, some studies have reported that when late-stage precursors (neural progenitors) were transplanted into intact or injured spinal cord, neural differentiation could be observed (2). Therefore, both microenvironment and the intrinsic state of transplanted cells can play a decisive role in grafted cells survival and integration as well as in the functional outcome. The importance of pre-induction of MSCs previous to transplantation into the injured spinal cord has been suggested also as required for promoting regeneration and functional recovery (10, 12, 16). Another relevant issue is the transplanted cell fate and its relationship with tissue sparing and functional recovery. Previous studies reported poor survival of transplanted MSCs (as well as other cell types) within the spinal cord injury site at several weeks post-injection, suggesting a correlation between the degree of cell survival and the extent of functional recovery (2).

Taken together, all the previous reports point to the hypothesis that increasing cell survival and inducing differentiation of transplanted MSCs will further improve neural replacement and functional outcome in SCI. Thus, there is still need for more experimental assays using clinically relevant SCI models and, importantly, in chronic stages after injury (20, 25). Our non-enzymatically derived hASCs cells bear neural antigens, survive in the injured CNS for over a month being highly resistant to inflammatory states and trans-differentiate according to the area. Injected into the striatum all hASCs become Tyrosine Hydroxylase positive cells, and, after exposure to valproic acid, hASCs migrate very efficiently to the injured cord and , there, accumulate at the edges of the lesion site and express specific neuronal antigens.

Since treatment with HADC inhibitors greatly affects the in vivo properties of hASCs our project also pays attention to the epigenetic control of these cells. Although human adult stem cells represent a promising tool for new clinical concepts in supporting cellular therapy in spinal cord and brain injuries, it remains to be explored to many questions in order to certificate an appropriate quality assurance and control of these cells, in order to avoid inappropriate gene expression in transplanting cells. Gene expression potential in stem cell differentiation is regulated by epigenetic processes that confer a specific chromatin conformation of the genome, among which CpG dinucleotide methylation is the best characterized epigenetic modification. As a consequence, not only the genome must be preserved, and also the epigenetic profile must be strongly considered in order to certificate cell identity after reprogramming.

One of the main objectives of the project will be to investigate the implication of DNA methylation in neural differentiation from adipose-derived stem cells. The epigenetic patterns of neural cells derived from these stem cells will allow us to gain further insight in the understanding of the neurogenesis process. The mammalian "junk DNA" genome encodes many thousands of large non-coding transcripts including a class of large intergenic non-coding RNAs (lincRNAs), between them some have been functionally characterized for their biological role by Dr. Guttman (Nature 477, 295–300 Sept 2011 PMID: 21874018) who performed loss-of-function studies on lincRNAs expressed in mouse embryonic stem cells (ESCs) and characterized the effects on gene expression. Dozens of lincRNAs were identified which upon-loss-of-function caused an exit from the pluripotent state and dozens of lincRNAs acted to repress lineage-specific gene expression programs in ESCs. In Guttman's paper LincRNAs were integrated into the molecular circuitry of ESCs and was shown that lincRNA genes are regulated by key transcription factors and that lincRNA transcripts themselves bind to multiple chromatin regulatory proteins to affect shared gene expression programs at the epigenetic level.
The main molecular goal of this project is the identification of the mechanism by which hADSC are driven throughout differentiation after been drafted into the injured recipient in order to possibly pharmacologically help the process. Is known that *epigenetic* memory drives cells trough cell division and repair and moreover during the whole process of differenzation and tumorgenesis. The first point I want to investigate is the possible involvement of *epigenetic* memory in the process of hADSC cells differenzation after transplantation and the role of lincRNAs and chromatin modifying enzymes.
This knowledge could help the improvement and design of novel guides for assuring stability in stem cell- based therapies.

Preliminary Data

Lipogems®-derived micelles liberate hASCs, bearing typical mesenchymal markers; their growth rate is similar to those derived from enzyme-treated hASCs, but their differentiation towards neural or adipose phenotypes is much superior. Hedgehog receptor activation blocks adipogenic differentiation. Excellent survival after transplantation, far superior to our previous findings with dermal amd bone marrow MSCs, neural adult and embryonic stem cells since most of them die within 3-4 weeks (Gorio et al 2004, Bottai et al 2008, 2010). Our non-enzymatically derived hASCs cells bear neural antigens, survive in the injured CNS for over a month being highly resistant to inflammatory states and transdifferentiate according to the area. Injected into the striatum all hASCs become Tyrosine Hydroxylase-positive cells, and, after exposure to. HADC inhibitor valproic acid-exposed hASCs migrate very efficiently to the injured cord, and as expected accumulate at the edges of the lesion site and express specific neuronal antigens.
In attachment to this application you can find some preliminary results showing RNA-immunoprecititations (RIP) and Chromatin-immunoprecipitations (ChIP) performed in a cancer stem cell model (figure 1, 2 mammospheres and CD24low/CD44++ cancer stem cells) where I have detected the formation of a lincRNA core Ribonucleoprotein complex on placed regulatory regions of genes involved in development and tumorgenesis.
In figure 2 I show that some lincRNAs (MEG3 and CCND1linc) are overexpressed in mammospheres if compared with the parental mammary adenocarcinoma cell line (MCF7) and that HDAC6, a chromatin modifying enzyme (deacetylase), together with an RNA stabilizing protein, called HuR, are able to bind them specifically in the cancer stem cell sub-population CD24low/CD44++ (figure 3, 4). Moreover I show that HuR and HDAC6 are sited on the chromatin at the level of the regulatory regions of a gene (P21-cip) involved in tumorgenesis together with a

member of the polycomb group, ENX, and SOX2 a transcription factor essential for maintaining self-renewal (figure 5).
These preliminary results on cancer stem cells can be easily applied to the hADSCs in transplantation and the molecular interplayers will be all good targets for drug treatments hopefully leading to the modulation of the overall differenzation process.
As it was previously mentioned Spinal Cord Injury (SCI) results in functional deficits usually leading to severe and permanent paralysis. Cell therapy for SCI patients aims to induce reduction of tissue damage, restitution of lost neural connections and support for axonal growth. Among cell types currently available for clinical use, adipose tissue derived mesenchimal stem cells (hADSCs) have particular interest, since they were reported to induce neuroprotective, immunomodulatory and regenerative effects in animal models, and have promising results in a few clinical assays. The main objectives are to improve by drug coadministration transdifferentiated hADSCs transplants used for repairing SCIs in experimental models, and to translate these results into a pilot trial in SCI patients. Understanding the molecular mechanism by which hADSCs differentiate leads to an improvement in the rescue of the lesion by autologus transplantation.
RIP-on-chip and ChIP-on-chip microarray-based studies at genome/transcriptome-wide level will be performed. Profiles of adipose stem cells, primary and neuroblastoma cell lines derived neurospheres will be compared. In vitro models will be employed for validation of the tumor properties of neurons derived from stem cells.

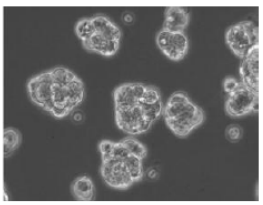

Figure 1. MCF7 derived mammospheres (phase contrast 40x)

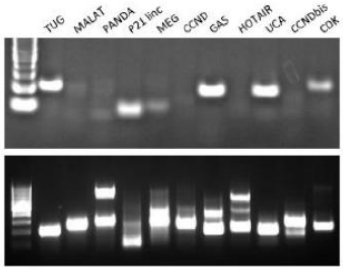

Figure 2. lincRNAs experession level in adenocarcinoma cell line (MCF7) and derived mammospheres. Picture shows a semiquantitative RT-PCR on total RNA. Malat, PANDA, MEG1 CCND1linc and HOTAIR are overexperssed in stem like cells.

PRELIMINAY RESULTS
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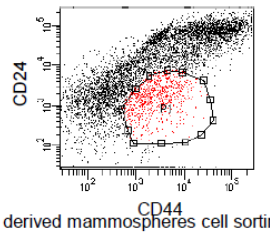

Figure 3. MCF7 derived mammospheres cell sorting of CD24low/CD44++ cancer stem cells (red gate P1)

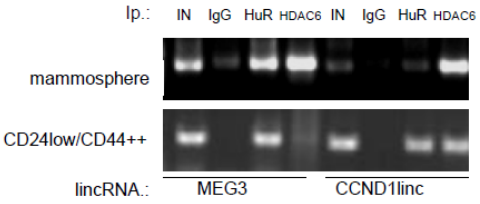

Figure 4. RNA-immunonoprecipitation (RIP) using antibodies against and RNA stabilazing protein (HuR) and a chromatin de-acetylaese (HDAC6). Preimmune serum (IgG) is the negative control and total RNA is the positive control (IN). Semiquantitative RT-PCR was performed on each sample looking for MEG3 and CCND1lincRNA. RIP was performed on mammospheres and CD24low/CD44++ cancer stem. MEG1 lincRNA loses the binding to HDAC6 in cancer stem cells.

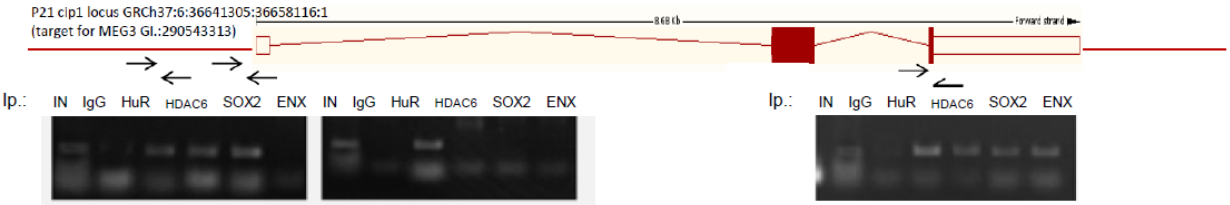

Figure 5. Chromatin-immunonoprecipitation (ChIP) using antibodies against and RNA stabilazing protein (HuR) a chromatin de-acetylaese (HDAC6) the transcription factor SOX2 and a polycomb complex protein ENX . Preimmune serum (IgG) is the negative control and total genomic DNA is the positive control (IN). Semiquantitative PCR was performed on each sample with primers mapping the P21 cip1 locus. ChIP was performed on CD24low/CD44++ cancer stem. There are positive signals for the presence on the locus of the different proteins. In the upper part the P21 cip1 locus is reported to map the used primers

Main Expected Results and Impact

Spinal cord injury (SCI) results in functional deficits usually leading to severe and permanent paralysis. Cell therapy for SCI patients aims to induce reduction of tissue damage, restitution of lost neural connections and support for axonal growth. Among cell types currently available for clinical use, adipose tissue derived mesenchymal stem cells (hASCs) have particular interest, since they were reported to induce neuroprotective, immunomodulatory and regenerative effects in animal models, and have promising results in a few clinical assays. The main objectives are to prove that transdifferentiated hASCs transplants can be used for repairing SCIs in experimental models, and to translate these results into a pilot trial in SCI patients.
To prove that a hASC transplant ameliorates SCI by reducing the injured tissue and inducing functional recovery in the experimental model, and to demonstrate that is a safe and feasible procedure for treating SCI patients. To develop methods for transdifferentiation of hASCs to neuronal or OPCs, and assess that they may increase the beneficial effects of hASCs transplanted into a SCI, thus constituting a promising alternative for future trials.

Microarray-based methylation studies and gene expression at genome-wide level will be performed using the most advanced technology. Profiles of adipose stem cells, neural cells derived from stem cells, normal primary neurons and neuroblastoma cell lines will be compared. In vitro models will be employed for validation of the tumor properties of neurons derived from stem cells. This knowledge could help the improvement and design of novel guides for assuring stability in stem cell- based therapies. Furthermore, the pathways through which deficient function of specific genes lead to an increased cancer risk could allow us to gain further insight in the tumorigenesis risk of stem cell therapy. The epigenetic patterns derived from hADSCs allow us to gain further insight in the understanding of the neurogenesis process. This knowledge could help the improvement and design of novel guides for assuring stability in stem cell-based therapies. Furthermore, the pathways through which deficient function of specific genes lead to an increased cancer risk could allow us to gain further insight in the tumorigenesis risk of stem cell therapy.

SECTION 2) Post-Mortem Erythropoietin-Releasing Neural Stem Cells

BACKGROUND. Cell transplantation and regeneration of damaged tissues organs holds tremendous promise for humankind. It may provide life-saving treatments for individuals affected by acute injuries as well as chronic disease conditions. Cell transplantation offers new and promising avenues to treat incurable neurological disorders. However, the use of transplanted embryonic stem cells (ESC) for neuron replacement therapy in the postnatal brain still poses problems since it is difficult to induce all ESC within a colony to differentiate into the desired cell phenotype. Although many cell types have been used in neurotransplantation, including pluripotent stem cells, none of these procedures have been fully satisfactory. For example, pluripotent embryonic stem cells have the potential to generate a wide variety of cell types but exhibit an increased tumorigenic potential when used for cell transplantation in the nervous system (1). In contrast, progenitor cells have reduced tumorigenic potential since they are mitotically active, but produce linage restricted precursor cells only transiently. Thus, the use of neural progenitor cells that have already undergone linage restriction reduces concerns about aberrant (non-neuroglial) phenotypes in the fully differentiated daughter cells, although their ability to generate a variety of neuronal phenotypes is less certain. Within the postnatal and adult CNS, neuronal progenitor cells situated in the anterior subventricular zone (SVZ) generate mainly neuroblasts and have several

characteristics that make them ideal for transplantation. Although postnatal SVZ progenitor cells normally generate a limited number of cells that migrate to striatum (2-4),in response to ischemia the number increases sustantially (5-9). *Lesions of the Central nervous system and in particular of the spinal cord are characterized by a secondary process of degeneration, that is a complex condition of ischemia-like syndrome and neuroinflammation leading to a large expansion of the early mechanical damage (10, 11. 12, 13, 14). A successful transplantation of neural precursors requires their survival in such an unfavourable environment.* We had previously reported that neural precursor cells (NPCs), accumulate at spinal cord injury (SCI) site and improve the rate of hind limb functional recovery. Their viability in the injured spinal cord, however, lasts 12- 20 days, then NPCs are phagocytated by macrophages (15). A similar outcome was observed with embryonic stem cells (16). *In view of such results we aimed at isolating adult neural stem cells from the subventricular zone (SVZ) after a prolonged global ischemia. The isolation of ischemia-resistant neural precursors may supply cells with properties different from NPCs, and able to survive in the unfavourable environment of the traumatically injured central nervous system.* Indeed we have recently described the isolation from the SVZ of adult neural stem cells resistant to the ischemic condition and collectable from mouse brain at several hours after mouse death (6 hours). These cells were called Post-Mortem Neural Precursor Cells (PM-NPCs). Their proliferation is similar to that of NPCs obtained at killing time (T0) (17,18), while their differentiation yields about 33 % β tubulin III- and 36% of MAP2- positive cells compared to 10-12% of NPCs (19). Differentiated PM-NPCs show higher HIF-1@ activation, express both EPO and EPO-R, and active voltage-dependent Ca^{++} channels (19). Such a higher differentiation requires the functionality of mTOR and MAPK systems and is prevented by exposure to anti-EPO and anti- EPO-R antibodies. *Thus, EPO-dependent adult neural stem cells purified from the brain of cadavers may represent a successful approach to the treatment of CNS degenerative conditions.*

RESEARCH STRATEGY AND SIGNIFICANCE. Traumatic spinal cord injury (SCI) is devastating for the individual patient and costly to society by requiring substantial long-term health care expenditures. The response to SCI develops in two well defined steps, the first being the trauma itself leading to direct damage of the nervous tissue while the secondary injury is characterized by events that amplify the early primary lesion. SCI leads to complex responses spanning from massive inflammatory reactions and formation of the glial scar to changes in intracellular signalling. The administration of erythropoietin or IL-8 antagonists resulted in the attenuation of the inflammatory response, enhanced production of neutrophic factors and reduction of secondary degeneration with promotion of functional recovery after SCI (Gorio et al 2002, 2005, 2007). Similarly the i.v. administration of adult neural precursor cells (NPCs) within 24 hours of SCI promoted functional recovery and enhanced the production of neurotrophic factors (Bottai et al 2008); on the other hand their survival at lesion site lasted less than 3 weeks due to the phagocytosis by activated macrophages. This event somewhat stopped the progressive recovery of function, that did not improve further. We reported that also embryonic stem cells die at the site of lesion within few weeks and promote only a quick small but significant recovery of function. However, these cells colonize and differentiate in healthy unlesioned parts of the same spinal cord (Bottai et al 2010).*Here, we propose to study a new clone of NPCs obtained 6 hours after donor mouse death (DR-NPCs). These cells differentiate mostly in neurons, and the event is regulated by the autosecreted erythropoietin (Marfia et al 2011). Preliminary results suggest their accumulation at injury site and promote recovery of function (FIGs 1 and 2).*

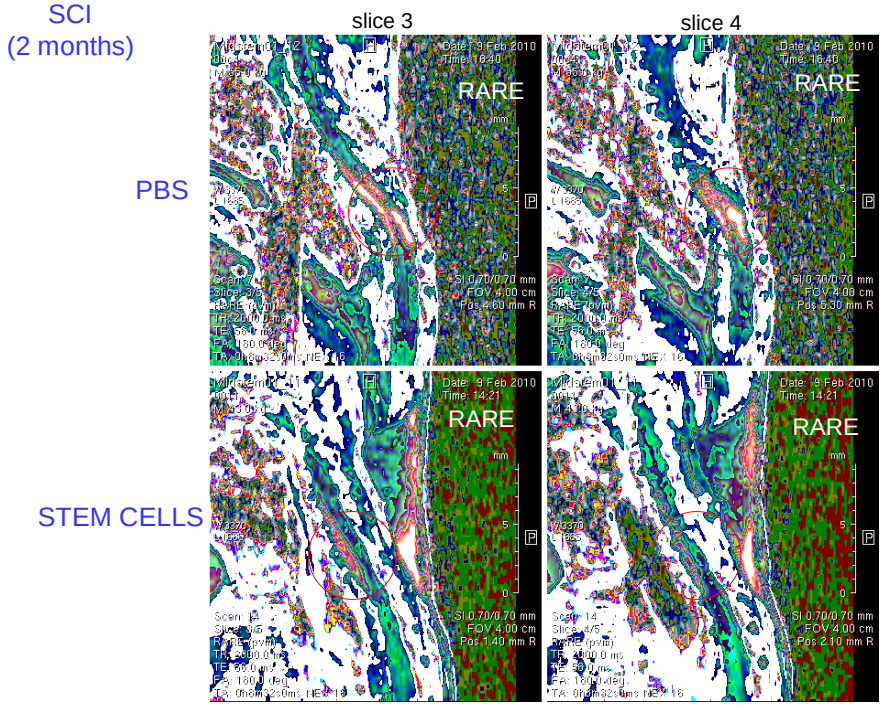

Fig 1. MRI image of spinal cord lesion site, 60 days after lesioning. The size of the spinal cord is much thinner at lesion site compared to rostral and caudal portions, when animals were treated with saline. Differently the i.v. supplementation of DR-NPCs preserved a larger amount of spinal cord, and the edema (hyper-intense signal: white colour) is markedly reduced. This image correlates well with the improved behavioural recovery.

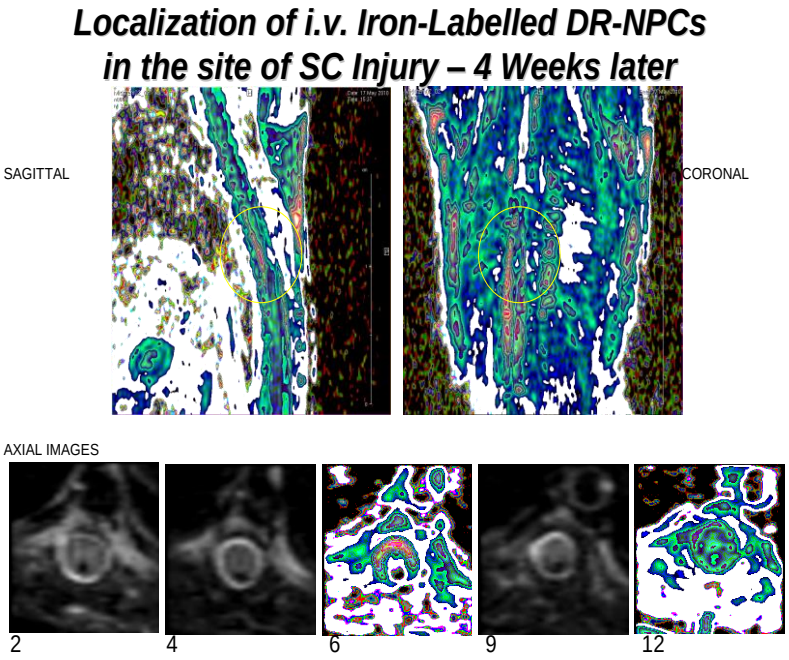

Fig 2. Accumulation of Endorem-labelled DR-NPCs at the site of injury, one month after lesioning and transplantation.

Thus it appears that DRNPCs are able to overcome the fundamental problems caused by SCI through the attenuation of secondary degeneration with enhanced survival of neural tissues at the injury site and the formation of new neural cells that might substitute those lost with the trauma. In addition, the creation of a favourable environment should enhance axonal regeneration across the lesion site. As observed with all treatments capable of reducing the extent of secondary degeneration, also DRNPCs should promote the preservation of white matter by reducing the oligodendrocyte apoptosis. ***In view of the DR-NPCs features and the stimulating preliminary results, we propose this study to determine the feasibility and efficacy of the use of adult neural precursors resistant to ischemia in a model of tissue repair and recovery of function such as SCI.***

RESEARCH DESIGN AND METHODS

Preparation of Neural Pecursor Cells Resisting to Ischemia (DR-NPCs) from mouse brain. DR-NPCs from sub ventricular zone of young adult mice CD1 either wild type, or GFP (Green Fluorescent Protein) transgenic animals at 6 hours after death. The animals will be killed following the indications required by the Ethical Committee of the University of Milan. The brain will be removed and kept in a PBS solution composed by, glucose (0.6%), penicillin (100 U/ml), streptomicin (100 U/ml). *Dissection of the regions of interest*. The brain will be removed and put in the Sol 1 at 4 ºC. Dissection of the SVZ. A 2 mm of the coronal section of the mouse brain at the level of the rostro/caudal length. The region around the lateral ventricle ventricle is dissected making attention avoiding the corpus callosum and the striatum. The tissue of interest is isolated and cut in small pieces to improve the following enzymatic digestion.
Enzymatic digestion . The enzymatic digestion is performed at 37 °C for one hour in 5% CO2 in a solution containing Cistein (Sigma) 0.2 mg/ml, EDTA (Sigma) 0.2 mg/ml Papain (Worthington DBA)1mg/ml named Sol2 in EBSS (Enhanced Balanced Salt Solution) (Gibco). There will be used 5 ml of sol2 for every brain region, at the end of the digestion the tissue is spun at 123 gs for 10 minutes and the supernatant removed and substituted with 1 ml of EBSS medium. The pellet is desegregated using a 1000 microl Gilson pipette. 7 ml of EBSS will be added and performed another centrifugation with the same parameters of the previous. The supernatant will be removed leaving 200 microl and the pellet will be further desegregated pipetting up and down with a 200 microl Gilson pipette. 5 ml of EBSS will be added and a further centrifugation at e 17 g for 10 minutes performed. Then the supernatant will be removed and the pellet re-suspended in 200 microl of medium containing EGF (20ng/ml)(Epidermal Growth Factor), bFGF (10ng/ml)(basic Fibroblast Growth Factor), DMEM/F12 medium 2 mM L-glutammine, 0.6% glucose, 9.6 gm/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin, and 2 mg/ml heparin. The pellet is dissociated with ten shots with a 200 microl Gilson pipette and the cells counted in a Burker chamber and plated as 3500 cells/cm2 in the appropriate volume of the aforementioned medium. In these conditions the stem cells start to proliferate forming the so called neurospheres and after 3–5 days they reach the appropriate 100- 150 microm to be processed further. With this aim the neurospheres will be transferred in a 15 mL falcon tube and centrifuged at 17 gs for 15 minutes. The supernatant will be discarded leaving 200 microL and the pellet mechanically dissociated with a 200 microl Gilson pipette. The repetitive passage of the neurospheres in the tip will determine the desegregation of the neurospheres in single cells. The cells will be plated in a concentration of 10000 cells/cm2 and kept at 37°C and 5% of CO2.
Isolation of ischemia resistant NSCs. Total Ischemia is obtained by dislocating the mouse cervical spine, with sudden death of the animal. Six hours later, brain is removed and SVZ isolated and placet in a cold solution containing 100 U/ml streptomycin and 0.6% glucose; then SVZ is enzymatically digested by 45 min. incubation with 1 mg/ml papain and 0.2 mg/ml EDTA. After centrifugation at 123 g, pellet is re-suspended in 1 ml EBSS e dissociated mechanically; the isolated cells are re-suspended in DMEMF-12 containing FGF (10 ng/ml) and EGF (20 ng/ml). Cells are

then plated at the density of 3500 cells/cm2, neurophere form and are collected within 7 days. Mechanical dissociation of neurospheres, allows the isolation of single cells, that are plated as such. This procedure is repeated 3 times. *Animal model.* Adult mice of 25 grams will be maintained in our animal facilities under standard housing conditions. A standard dry diet and water is available ad libitum. All experiments will be performed according to protocols approved by the Review Committee of the University of Milano. The traumatic SCI will be performed by means of the "Infinite Horizon Device" (H). We plan to apply a force 0.4 Newton for 1 s for mice, which is followed by an automatic return rod. Animals are under anaesthesia by halothane inhalation, and before awakening are treated with buprenorphine for pain and penicillin G as antimicrobial agent. After SCI, animals are housed two per cage and, if required, have manual bladder evacuation. Floating spheres, derived clonally and isolated at third passage, will be dissociated, and 1 million cells injected i.v. Cells will be administered within 30 min after TSCI. We shall use two control groups, one will be treated with live fibroblasts and the other with dead DR-NPCs. Each experimental group will be made up of at least 10 animals. This has been a standard procedure for the past years. The evaluations will be done in a blind fashion**.**

SPECIFIC AIMS AND METHODS

AIM 1) In Vitro Characterization.

Wells are coated with matrigel and incubated at 37°C for 2 hours. Then matrigel is removed and 25,000 NSCs/cm2 plated in presence of DMEM F-12 deprived of the proliferating factors and with the addition of 1% serum. Neural differentiation will be assessed by immunocytochemistry with appropriate antibodies for differentiation studies. Cells on coverslips will be fixed in PBS at pH 7.2 containing 4% paraformaldehyde for 30 min., followed by three washes (10 min. each) in PBS at pH 7.2. All cover slips will be incubated with the antibodies for 1 h at room temperature. After washing with PBS, cover slips will be exposed to the appropriate secondary IgG antibodies for 1h in PBS at room temperature and cell nuclei will be stained for 5 min. at room temperature with DAPI.

AIM 2) Transplantation and evaluation of migration and homing to the lesion site, and in vivo differentiation of DR-NPCs. Thirty, sixty or ninety days later, spinal cord will be removed, frozen in liquid nitrogen-cooled isopentane, stored at – 80 °C until sectioning. Some animals will be fixed with aldehydes by perfusion and then paraffin-embedded. Serial sections of 12-15 μ m thickness will be cut by means of a cryostat throughout T7-L1 spinal cord segments or by microtome in the case of paraffin-embedding. For immunostaining of neuronal markers, sections will be fixed in 4% paraformaldehyde then incubated with antibodies to nestin, MAP2, beta-tubulin III, neurofilament, and neurotransmitter markers. For astroglial cell detection we use anti-GFAP antibody. To evaluate the expression of inflammatory markers sections will bee fixed, then incubated with IL 1beta;, MAC-1, CD14, CD4 and CD8. Vimentin and PECAM-1 will also be used for assessing differentiation into fibroblast-like and endothelial-like cells. Cell nuclei will be stained with DAPI. Also the attenuation of apoptosis is a primary target of any pharmacological and transplantation treatment aiming at promoting recovery after SCI. We shall use the TUNEL technique as done previously. Evaluation will be performed 7, 14 and 28 days after SCI at the level of the gracilis and cuneatus fascicle (3 mm rostrally from the site of contusion injury) for the oligos and the periphery of the lesion for the neurons.

AIM 3) Evaluation of recovery from disability. Recovery from hind limb disability will be evaluated by means of behavioural tests, that will be performed 24 hours, 4, 7, and every 4 days thereafter following SCI. Evaluation of motor recovery. The 9 points of Basso Mouse Scale (BMS) will be used. Such a test allows the quantification of rat hind limb free locomotion deficits by observing their movements in an open space free of obstacles .

AIM 4) In vivo monitoring of site of lesion and cell fate. MRI multislice T2-weighted spin-echo images will be obtained in the anatomic coronal and longitudinal orientation. All sequences will be performed within 40-60 minutes per mouse. MRI images will be reviewed to assess the lesion area.

The same methodology may be applied to determine the localization of DR-NPCs transplanted. To this end, DR-NPCs are labelled with SPIO particles Endorem (AMI-25; Guerbet; particle size, 80–150 nm, stock solution, 11.2 mg Fe/ml). Labelling will be performed by incubating the cells at 37° for 24h with Endorem in presence of poly-L-lysine transfection agent in culture medium. After incubation, Endorem in excess will be washed off. A Bruker 7 Tesla/40cm horizontal bore MRI System, with a 400mT/m, 120mm inner diameter gradient insert is used with a laboratory-built 3cm diameter Helmholtz coil for transmitting and receiving the MRI signal. Mice will be placed in prone position (ventral recumbency) on a laboratory-built plexiglas holder that also incorporates the matching and tuning electronic circuitry of the radiofrequency (RF) coil. The dorsal region of the animal is placed between and parallel to the two loops of the RF coil, and fixed in place with maskingtape. Exterior landmarks (occipital bone and shoulder blades) are used to ensure T9 is placed at the center of the coil. Anesthesia is maintained in all animals with inhaled Isoflurane at 1–2%. Body temperature is maintained at 33 – 37°C using a warm water blanket (TP12; Parkland Scientific, Coral Springs, FL) connected to a circulator. Respiratory rate will be monitored continuously using the monitoring & gating system for small animals (Model 1024; S.A. Instruments Inc., Stony Brook, NY). Injury location (T9) is carefully positioned at the isocenter of both the RF antenna and within the magnet. Tuning and matching of the Helmholtz coil is performed for each animal, prior to and after insertion into the magnet. Acquisition of a three plane localizer for identifying the vertebral column is followed by a higher resolution 2.2 minute sagittal gradient echo, T1 weighted localizer (TR/TE=500/4.5ms; flip angle=90) that allowed depiction of vertebral bodies which are used as anatomical landmarks. Axial MRI studies cover a 16.4mm region of the spinal cord. Consecutive series of axial T1, T2 and PD weighted images will be acquired. T1 weighted (Gradient Echo: TR/TE/flip angle=500/4.9ms/90°, 5 averages) and proton density weighted (PD; Spin Echo: TR/TE=2000/15ms, 2 averages) images will be acquired with 175×175μm in-plane resolution and 1mm slice thickness with a 0.1mm gap between slices. The 3D T2 images (Spin Echo, TR/TE/RARE factor= 1629.2/59.7ms/16, 2 averages) will be acquired with 179×175× 625μm resolution.

Data Analysis. Axial MR images will be used to quantify the evolution of the pathology in the two SCI groups. Axial images will be reconstructed from the raw data, magnified 4 times, and cropped to retain just the vertebral column using IDL (Research System Inc., Boulder, CO, USA). Data will be analyzed blind to treatment condition. Seven consecutive T1 and PD weighted images around the lesion center will be overlaid and regions of interest will be manually traced using MetaMorph software (vs.6.3; Molecular Devices Corporation, Downingtown, PA, USA). This combination of images provides the most detail to clearly determine areas of the whole cord and areas of hypointense (commonly thought to reflect hemorrhage) and hyperintense (edema) signal within the lesioned cord. Pixel counts are converted into area units (mm^2) by scaling with the in-plane pixel size. Volume measurements (mm^3) are obtained by adding the individual slice areas and multiplying by 1.1mm slice plus gap thickness.

AIM 5) Assessment of myelination preservation in the site of injury. Myelin preservation will be evaluated comparing the levels of myelin in the ventral white matter at 0.4 mm from the lesion epicenter, since the preservation of descending and segmental motor pathways located in the lateral and ventral funiculi, which contains descending motor pathways, was shown to contribute substantially to locomotor function in rat which underwent SCI; we previously reported that the quantification of the spared ventral myelin evaluated in a semi thin section gave comparable results when fluoromyelin was used (Vitellaro-Zuccarello, et al., 2007). For homogeneous analysis the staining with fluoromyelin will be carried out on sections of control and treated animals placed on the same coverslip. The confocal microscope images will be obtained using the same intensity, pinhole, wavelength and thickness of the acquisition.

AIM 6) Evaluation of the reconstruction of the lesion site and axonal regeneration. Our lesion paradigm causes the complete degeneration of the grey matter and the partial destruction of the white matter with the partial sparing of the ventral component. Through quantitative techniques we

shall evaluate the ability of DR-NPCs to restore the lost tissue and improve myelin preservation. The regeneration across the lesion will be determined will be evaluated estimating the extent of 5-HT- and TH-positive axons in the cord caudally to the lesion. Also the dorsal injection at T1 of Red Ruby will allow the estimate the number of dorsal axons caudal to the lesion. The recovery of the connection between sciatic nerve and higher centers will be determined by injecting into the sciatic nerve the beta cholera toxin, 72 hours later the label should be found rostral to the lesion and eventually in the gracilis and cuneatus nucleus.

AIM 7) Site of injury, cellular infiltration, and inflammatory cytokines. The effects of the transplants upon glia scar formation and macrophage infiltration will be evaluated by immunocytochemistry with GFAP and ED-1 antibodies respectively. Neutrophils are of interest, their migration into the lesion site begins within 24 hours after injury being driven by the overproduction of IL-8. Evaluation will be performed 24, 48 and 72 hours, and 7 days after SCI. We shall assay by PCR data analysis and ELISA the in situ and systemic production of MIP-2 (IL-8), TNF-alpha and other major cytokines. Assays will be performed 6, 24 and 48 hours, and 7 days after SCI. PCR analysis is performed using the $\Delta\Delta$Ct, this procedure can be used since we have previously determined that the replication efficiencies (slopes of the calibration or standard curves) for our genes of interest and housekeeping gene are very close. The selection of the primers is performed using a DNASTAR Lasergene program.

AIM 8) Do pharmacological treatments affect fate and action of neural stem cells?
Administration of ischemia-resistant NPCs will be accompanied by concomitant treatment with daily methylprednisolone 35 mg/kg for 3 days after SCI. EPO may represent an interesting synergistic agent with neural stem cells, as it occurred when it was applied with dermal stem cells, and it will be administered i.p. once a day for the 3 days following the trauma at the dose of 5000 u/kg. Hind limb recovery of function up to 28 days after lesioning will be the standard monitoring tool to assess any interference of the drug with neural stem cell action, then morphology of the lesion site and stem cell differentiation paradigms will be evaluated.

AIM 9) Application of DR-NPCs to animals with Chonically Injured Spinal cord. Goals 2 through 8 will apply also to the use of DR-NPCs in mice with a chronic injury to the spinal cord. One month after lesioning the extent of hind limb functional recovery is rather stable, at this time cells (250.000 cells in 15 microl of PBS) will be applied stereotaxically into gracilis and cuneate fascicle at the lesion site or 1 mm caudally to the lesion. The lesion site will be monitored in vivo by MRI and behavioural tests will help in assessing whether the transplanted DR-NPC affect hind limb function. Thirty days after transplantation we shall assess gaims 2, 5, 6, and 7.

SECTION 3) NEUROGEL PREPARATION

Briefly, the matrix is produced by radical polymerization of methacrylate and methacrylamidebased monomers in the presence of a crosslinking agent and water (ISO 9001:2008 and ISO 13485:2003). pHPMA is a biocompatible synthetic hydrogel that displays no signs of toxicity, chronic inflammation or risks of transmitting disease. This hydrogel is a cross-linked hydrophilic macromolecular network that has a water fraction of 95.66%., and presents a multimodal pore size distribution from <2 nm to >50 <300 μm.

The hydrogel implant will be sized and adapted to the dimension of the lesion then placed to allow settling of the gel on the spinal tissue. The gel will hydrated with drops of sterile saline solution to ensure complete apposition of the surfaces between the polymer implant and the cord. HADSCs and Adult DR-NPCs will be applied into and above the hydrated neurogel. In addition, a crude preparation of Lipogem-treated fat will be applied over it. Preliminary results are quite optimistic.

GENERAL CONSIDERATIONS

The global impact of neurodegenerative disorders and their unsustainable social issues and costs are constant reminders of the urgent need for novel and effective strategies to attenuate and/or cure these conditions. In this direction, cellular therapies, stem cells and regenerative medicine could offer a definitive solution and an alternative to drug treatments with all their limitations. It is therefore imperative that we pursue alternative strategies. While the ultimate source of stem cells is yet to be defined, it is critically important that scientists worldwide continue to explore the potential of different sources of stem cells and related technologies, until the best sources for treating neurodegenerative diseases will be defined.

Budget for 3 years (EUROS)

Salary for Senior Research Associate	50.000 x year for 3 years	150,000.00
Postdoctoral fellow	25.000 x year for 3 years	75,000.00
Consumables (reagents, gas, glassware, standard lab equipment)		400,000.00
Lab Equipment		150,000.00
TOTAL	775,000.00	

GROUP organization and involvement

	Position	Involvement (Month/person)*
Alfredo Gorio	Full professor	6
Carelli Stephana	Fellow	12
Giallongo Toniella	Fellow	10
Messaggio Fanuel	Fellow	12
Danuta Hebda	PostDoc	12
Elisa Latorre	PostDoc	6