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Adipose-derived mesenchymal stem cells: neuronal differentiation potential and neuroprotective action

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ABSTRACT

Adult mesenchymal stem cells derived from adipose tissue (ASC) offer significant practical advantages over other types of stem cells (SC) for potential clinical applications, since they can be obtained from adult adipose tissue in large amounts, can be easily cultured and expanded with a very low risk for development of malignancies. We investigated in vitro the neuronal differentiation potential of human ASC with a chemical protocol and a prolonged two-step protocol, which included sphere formation and sequential culture in brain-derived neurotrophic factor (BDNF) and retinoic acid (RA). After 30 days, about 57% ASC show morphological, immunocytochemical and electrophysiological evidence of initial neuronal differentiation. In fact, ASC display elongated shape with protrusion of two or three cellular processes, selectively express nestin and neuronal molecules (including GABA-A receptor and tyroxine hydroxilase) in the absence of glial phenotypic markers. Differentiated cells show negative membrane potential (−60 mV), delayed rectifier potassium currents and TTX-sensitive sodium currents, but they are unable to generate action potential. Considering the low efficacy and the not-fully mature neuronal differentiation, we evaluated if ASC display a neuroprotective effect. Using the H_2O_2-stressed neuroblastoma model in vitro, we show that ASC increase cell availability (compared to fibroblasts) and protect against apoptosis. A possible mechanism involved could be the secretion of BDNF, as reported for human BM-MSC: in this regard, we indeed find high levels of BDNF in ASC-condition medium. In addition to exert neuroprotection, soluble factors secreted by ASC promote neurite outgrowth, an additional mechanism that may favor neuroregeneration.

In view of these results and their immunosuppressive action *(Constantin et al, 2009)*, ASC may be a ready source of adult MSC to treat neurodegenerative diseases.

INTRODUCTION

Stem cell therapy is a cellular approach that aims to induce the neuro-restorative process essential for facilitating recovery after a loss of function. Among the different application, neurological diseases are interesting potential target, since the adult nervous system has a limited regenerative capacity.

A stem cell (SC) is an unspecialized cell that is able to self-renew indefinitely and can, under appropriate conditions, give rise to a wide range of mature cell types (*Herzog et al, 2003*). SC are defined by its 'potential' to generate more stem cells and differentiated daughters, rather than by its production of a SC and a differentiated daughter at each division, as classically defined as asymmetric cell division *(Morrison and Kimble, 2006).*

Two types of SC, embryonic (ESC) and somatic, can be distinguished according to their origin and their potential of differentiation (Fig. 1). ESC are derived from the early blastocyst and the inner cell mass of the embryo and are able to differentiate into cell types of the three germ layers *(Shuldiner et al, 2000).* Somatic SC are isolated from foetal (after gastrulation) or adult tissues, and traditionally are programmed to only produce cell types that belong to the tissue which they originate from. Currently, somatic SC have been isolated from different organs or tissues, including bone marrow (*Friedenstein et al, 1974*), brain *(Reynolds and Weiss, 1992),* blood *(Domen and Weissman, 1999),* epidermis *(Gandarillas and Watt, 1997)* and skeletal muscle *(Seale and Rudnicki, 2000).* It was classically admitted that somatic SC support tissue homeostasis by replacing damaged cells. However, while a great number of organs or tissues contains somatic stem cells, it appears that these cells are mostly quiescent or weakly active and are unable to efficiently repair the damaged tissue. This is especially true for neural stem cells (NSC) which are found in the sub-ependymal layer of the

ventricular zone and the dentate gyrus of the hippocampus in adult mammals *(Gage, 2002)*, within a cellular niche *(Rossi and Cattaneo, 2002)*. Those cells, while able to proliferate and differentiate in vitro into neurons, astrocytes and oligodendrocytes *(McKay, 1997)*, do not guarantee a successful brain homeostasis after external insults.

By contrast, adult BM is a potentially rich source of SC and contains two types of prototypical stem cell populations: hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). MSC, also referred as BM stromal cells, normally give rise to osteocytes, chondrocytes, adipocytes, during the whole life and thus guarantee homeostasis for these cell types and tissues. Moreover, such cells have been recently shown to display the ability to trans-differentiate, acquiring cell phenotype different from mesenchymal *(Wislet-Gendebien et al, 2005a)*.

ESC have seemed to be the most powerful tool for cell therapy in animal models, but several challenges need to be resolved before translate ESC application in humans. ESC are genetically and epigenetically unstable, and consequently human ESC lines can vary substantially with regard to differentiation potential. Furthermore, most differentiation procedures represent 'run-through' protocols, in which the cells are sequentially propagated in different media and growth factor conditions until they acquire the desired phenotype. Such approaches are prone to include undifferentiated ESC, which can result in formation of teratoma in the transplant recipients. A second risk factor for tumour formation is the protracted proliferation and differentiation times. This can lead to continuous cell proliferation after transplantation, resulting in neural overgrowth. Together, these concerns emphasise the need for more basic research into how to control proliferation and differentiation of ESC and their neural derivatives before these cells can be considered for clinical use. Despite the privileged immune status of the central nervous system (CNS), allogeneic grafts of stem cell-derived neurons and glia

remain susceptible to rejection; the establishment of ESC banks that contain ESC lines of different HLA haplotypes could overcome the problem *(Koch et al, 2009).*

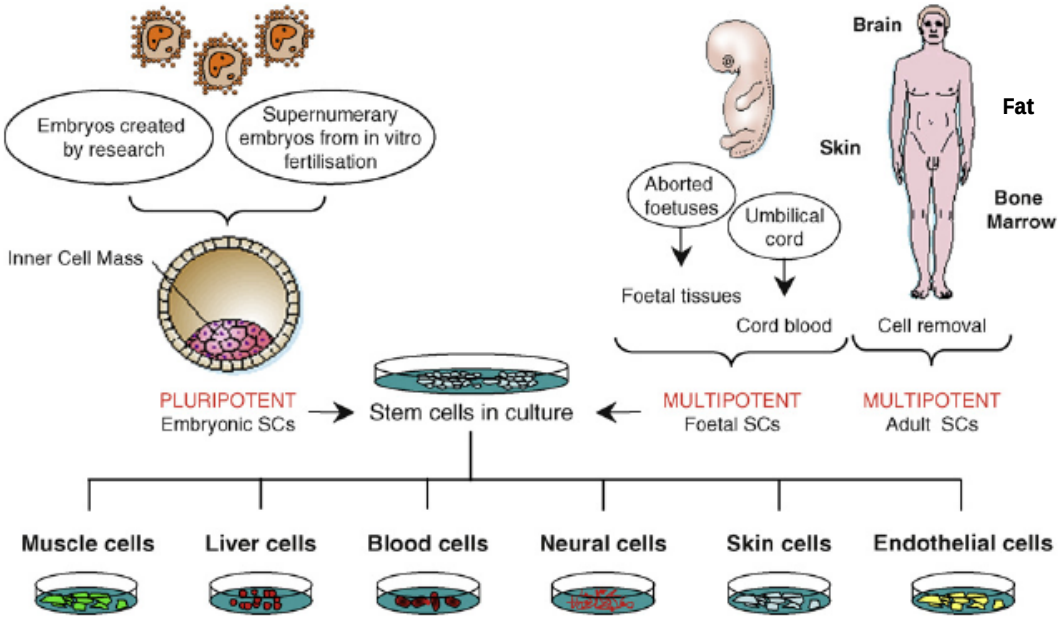

Figure 1. Sources of SC for therapeutic use. Pluripotent (namely embryonic) SC can be obtained from early stage embryos while somatic (or adult) SC – which are multipotent in nature and already partially committed into a certain types of cell lineages – can be virtually obtained from all tissue belonging to embryos, foetuses and adults. The therapeutic use of ESC is limited by the unlimited growth, the tumorigenic potential, and the undetermined differentiation. The therapeutic use of somatic (or adult) SC is restricted by the limited growth potential *(from Pluchino et al, 2009).*

Regarding the neural application, NSC probably represents the first choice as cell population to use for neural replacement. Although NSC have been very useful for proof-of-principle in animal studies and pre-clinical trials, it is unlikely that they will ever be clinically useful. The primary reason for this is that the very small numbers of donors would not represent a sufficient pool to allow immunological matches. These donors collectively provide immunological matches for approximately 70% of the possible recipients. A bank of NSC to approach this level of matching ability would be

extremely laborious, expensive and time-consuming and it is unlikely to be ever created. It is possible, however, that NSC posses only a weak capacity to stimulate immune rejection and, when implanted into an immune-privileged site, such as the brain, might evade the immune system altogether *(Schwartz, 2006).*

Thus, other somatic stem cells have been investigated, and growing interest has been paid to MSC because of their biological attractive features; in particular, they can be easily collected and extensively expanded in vitro and have the ability to seek out sites of tissue injury and repair the tissue by differentiating to replace injured cells or by creating an environment favorable for the repair of damaged tissue by endogenous cells *(Izadpanah et al, 2005*). Considering the different source of MSC, MSC derived from adipose tissue (ASC) are available in large quantities (hundreds of million cells per individual) and are easily isolated and can be cultured for several months in vitro with low levels of senescence *(Zuk et al, 2001; Zuk et al, 2002).*

Several neurological diseases are characterized by (primary or secondary) neuro-degeneration, combined with different degree of inflammation, as multiple sclerosis, stroke, Parkinson disease or traumatic injury. At present, therapy is mostly symptomatic and not restorative and the MSC therapy represents a powerful tool, considering their ability to immunomodulation, to support the brain parenchyma and their partial early trans-differentiation *(Pluchino et al, 2009)* (Fig. 2).

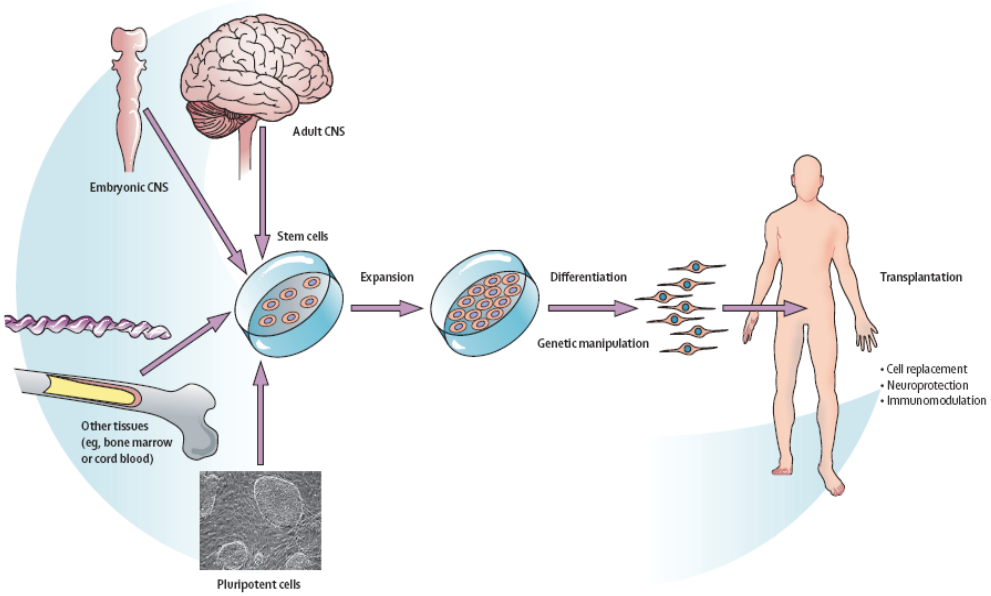

Figure 2. Whereas traditional approaches mainly used embryonic donor tissue, the current experimental choices include adult NSC, non-neural somatic stem cells and pluripotent sources such as induced pluripotent stem cells (iPSC). With regard to application, therapeutic benefits can occur through classic cell replacement, but also by transplant-mediated neuroprotective and immunomodulatory effects *(from Koch et al, 2009).*

1. MESENCHYMAL STEM CELLS (MSC)

MSC are adult non HSC, originally isolated by BM (BM-MSC). The very early hypothesis about their existence goes back to 1867, when Cohnheim showed as the intravenous dye could be absorbed by fibroblast-like cells and found at a distant injured tissue. One century later, Friedenstein et al. cultured BM-MSC in adherent plates *(Friedenstein et al, 1974).* Such cells can give rise to colony similar to cartilage or bone. Later, with different culture conditions, BM-MSC have been reported to differentiate in adipocyte, osteoblast and chondrocyte *(Pittenger et al, 1999; Benayahu et al, 1989; Colter et al, 2000; Jiang et al, 2002; Dennis and Charbord, 2002).*

Human BM-MSC are generally isolated from BM aspirate harvested from the superior iliac crest. Cells are cultured in basal medium such as Dulbecco's modified

Eagle's medium (DMEM) in the presence of 10% fetal bovine serum (FBS). BM-MSC in culture adhere to the tissue culture substrate, exhibiting a fibroblastic morphology, with large, flat body. When they reach the confluence they have fused morphology and they are no more able to differentiate in any kind of post-mitotic cell type. In vitro BM-MSC proliferate fast (duplication time: 48-72 hrs), with no phenotypic modification till 25 passages *(Izadpanah et al, 2008)*.

Despite increasing experimental and clinical interest in recent years, the understanding of MSC biology is not yet comprehensive. The absence of a consensual definition can make the results of MSC researches incomparable and it could be hinder progress in the field.

Recently, the International Society for Cellular Therapy (ISCT) defines the minimal criteria to be met for cells to be considered human MSC *(Dominici et al, 2006)*:

1. adherence to plastic in standard culture conditions;
2. phenotype positive (≥ 95%+): CD105, CD73, CD90;

   phenotype negative (≤ 2%+): CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR;
3. in vitro differentiation: osteoblast, adipocyte, chondroblast (demonstrated by staining of in vitro cell culture).

Such criteria show as the presumptive identification of the MSC remains mostly functional, lacking till now specific pattern of markers to prospectively and univocally isolate MSC. In fact, the basic question of how to define a MSC is still a point of discussion and controversy because of MSC are a heterogeneous population, and very recently some authors are trying to find new panel of markers *(Roche et al, 2009)* and to characterize the different subpopulations *(Bühring et al, 2009)*.

MSC, responding to their definition, are able to differentiate into to three mesodermal cells. The final proof of pluripotency is proven by clonal assay *(Zuk et al,*

2002; Guilak et al, 2006). Growing evidence has been showing as these cells are also able to change toward the neuro-ectodermal *(Sanchez-Ramos et al, 2000; Korbling et al, 2002; Grove et al, 2004)* and endodermal lines *(Petersen et al, 1999; Schwartz et al, 2002; Jiang et al, 2002; Ianus et al, 2003).* However, their capacity of mature differentiation and integration in the host tissue are not yet clear.

MSC have been isolated by tissues other than BM, as periostioum *(Nakahara et al, 1990),* trabecolar bone *(Noth et al, 2002),* synovium *(De Bari et al, 2001),* adipose tissue *(Zuk et al, 2001),* skeletal muscle *(Jankovski et al, 2002),* lung *(Sabatini et al, 2005),* thymus, spleen, pancreas, kidney *(Krampera et al, 2007; Da Silva Meirelles et al, 2006),* peripheral blood *(Roufosse et al, 2004),* umbilical cord blood *(Buzanska et al, 2002)* and placenta *(Yen et al, 2005).* All those cells are MSC, but show some differences related to proliferation and differentiation capacity *(Kern et al, 2006; Peng et al, 2008).*

Focusing on adipose tissue, multipotent adherent cells have been isolated, and called with many terms, including adipocyte precursor cells *(Hauner et al, 1989),* preadipocytes, adipose-derived adult stem (ADAS) cells *(Gimble and Guilak, 2003),* adipose-derived stromal cells *(Safford et al, 2002),* adipose-derived adherent stromal cells *(Katz et al, 2005),* processed lipoaspirate cells *(Zuk et al, 2001)* and adipose-derived stem cells (ASC) *(Miranville et al, 2004).* In accordance with a consensus reached by investigators attending the Second Annual International Fat Applied Technology Society meeting (2004, Pittsburgh, PA), we will refer to these cells as ASC.

A typical harvest of adipose tissue can collect ~2 x 10^8 nucleated cells per 100 ml of lipoaspirate, yielding around 4 x 10^6 ASC; at variance, the adult BM aspirate volume is generally limited to no more than 40 ml and yields approximately 1.2 x 10^9 nucleated cells, containing approximately 2.4 x 10^4 ASC. Fat aspirate is less invasive method and contain higher number of MSC compared to BM aspirate *(Strem et al, 2005).*

Approximately 400,000 liposuction surgeries are performed in the U.S. each year and these procedures yield anywhere from 100 ml to >3 L of lipoaspirate tissue. This material is routinely discarded *(Katz et al, 1999).* Moreover, ASC possess larger rate of proliferation compared to BM-MSC *(Peng et al, 2008).*

Although very similar to BM-MSC, ASC phenotypically differ regarding the expression of some adhesion molecules (ASC are intensively positive to CD54 and CD49d and weakly to CD106) and the lack of Stro-1 and genetically in around 1% genes *(Gronthos et al, 2001; Zuk et al, 2002; De Ugarte et al, 2003; Romanov et al, 2005; Katz et al, 2005; Lee et al, 2004; Kern et al, 2006).* ASC show greater plasticity and ability to conversion from one cell type to another than BM-MSC *(Dennis and Charbord, 2002)* and the same immunosuppressive activity of BM-MSC *(Yoo et al, 2009).*

In brief, the advantages of using MSC in cell therapy, compared to ESC, include:

a. the high availability, especially considering some cell sources, as adipose tissue;

b. the easy culture method, with no need of feeder, and their ability to proliferate till 25 passages with no signs of senescence *(Izadpanah et al, 2008)*;

c. the low immunogenicity, making them useful also in allo-transplant without immunosuppression *(Javason et al, 2004; Barry and Murphy, 2004)*;

d. no transformation in teratoma (*Jiang et al, 2002; Dezawa et al, 2004; Tropel et al, 2004)*;

e. immunomodulant action, useful in inflammatory diseases as well as in graft-host versus disease prevention *(Bartholomew et al, 2002; Krampera et al, 2003; Le Blanc et al, 2004; Zappia et al, 2005; Aggarwal and Pittinger, 2005; Le Blanc and Ringden, 2005)*;

f. no ethical concern.

Moreover, as ESC, they can secrete trophic molecules that can support the damaged host tissue *(Crigler et al, 2006; Zhao et al, 2004; Kang et al, 2003b)*; they can home into damaged tissues *(Chamberlain et al, 2007)* and can be transfected with therapeutic gene *(Lee et al, 2003b; Zhao et al, 2004)*.

All these features make ASC the ideal candidate for very different neurological diseases associated with degeneration and inflammation. Anyway, the complexity of the mechanisms involved in survival, differentiation and immunomodulation is not yet completely understood. The most vivid controversy is about the role of MSC in tumor: some works report their anti-tumoral effect, contrasting with other evidence that show pro-tumor activity, especially related to their pro-angiogenic and trophic properties *(Bexell et al, 2009; Sasportas et al, 2009; Karnoub et al, 2007)*. The ASC experimental application in tumor is much more limited *(Muehlberg et al, 2009; Sun et al, 2009; Cousin et al, 2009; Yu et al, 2008)*. The MSC ability of homing is unique, and make them useful looking forward gene-therapy also in the most common adult CNS tumor, the glioma *(Nakamizo et al, 2005)*. Concerning ASC, they have been used with success as cellular vehicles of prodrug-activating enzymes in models of glioblastoma, melanoma and prostate cancer *(Matuskova et al, 2010; Kucerva et al, 2008; Cavarretta et al, 2010)*.

2. NEURAL MSC DIFFERENTIATION

'From marrow to brain' titled the first article that reported as transplanted BM cells have been found in brain, expressing neural markers *(Brazelton et al, 2000; Mezey et al, 2000),* and later Cogle et al. described similar data in human *(Cogle et al, 2004),* showing as BM elements are able to cross the blood brain barrier and to exhibit a neural-like phenotype. These phenomena, however, were not evident in other studies *(Castro et al, 2002; Wagers et al, 2002).* Contemporaneously two pioneering works reported early trans-differentiation of MSC into neurons in vitro *(Woodbury et al, 2000; Sanchez-Ramos et al, 2000).* Such evidence gave rise to great interest, and in this decade several authors have studied the ability of MSC to differentiate into neural cells *(Abouelfetouh et al, 2004, Deng et al, 2001, Kim et al, 2002, Kohyama et al, 2001; Qian and Saltzman, 2004; Woodbury et al, 2002).*

The ability to manipulate MSC in vitro serves two logical purposes. First, pre-differentiation of MSC into more restricted neural cell types could enhance their ability to anatomically and functionally integrate into particular parts of the brain, with presumably higher efficacy in ameliorating lesions. Second, differentiated neural cells (especially when they become post-mitotic) are generally considered to have a lower chance of the eventually malignant transformation as compared to actively dividing precursors.

The studies attempting to differentiate MSC in vitro into neurons have used different approaches. Some involved rather simple chemicals, others used growth factor cocktails. Still others have more elaborate strategies involving transgenic expression of particular genes or co-culture. It should be kept in mind that MSC are by nature a heterogeneous population of cells with sub-populations with different morphology,

proliferative capacity and multipotency, and then different ability to trans-differentiate *(Chen et al, 2006; Bühring et al, 2009).*

The first approaches using chemical agents as, for example, β mercaptoetanol (BME), butylated hydroxyanisole (BHA), isobutylmethylxanthine (IBMX), make MSC modify into cells with contracted soma and elongated processes, staining for neural markers. Reports using different species and source of MSC described the morphology suggestive of neuron and the expression of neural markers *(Woodbury et al, 2000; Woodbury et al, 2002; Deng et al, 2001; Rismachi et al, 2003; Yamaguchi et al, 2006).* But some authors hypothesized that such phenomenon may be caused, or at least accompanied, by cellular events other than differentiation. First, the morphological changes did not come with clear observations of neurite growth cones, and were associated with a high degree of cell death. Second, and more important, this apparent trans-differentiation occurred in a seemingly unrealistic time frame of hours, compared with the days required for MSC to differentiate into cells of mesodermal origin, suspecting an underlying disruption of the actin cytoskeleton. Treatment with low pH or high salt produced similar morphological changes, which could also be observed when other cell types, such as fibroblasts, were subjected to BHA/DMSO treatment *(Lu et al, 2004; Neuhuber et al, 2004; Bertani et al, 2005).* Conversely, a plethora of cell stress such as EDTA, cytocalasin D and detergents could also elicit similar changes in MSC. Although the expression of classical neuronal markers (Neu-N and the 200-kDa neurofilament) is judged to be increased by immunostaining, these appeared to be aberrantly localized in the cell, and elevation of protein levels could not be confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Another point to note is that MSC appeared to already express varying levels of neural and astrocytic markers, and inhibition of protein synthesis with cycloheximide did not prevent cells from adopting neuron-like morphology after chemical induction. This

apparent lack of a need for transcriptional or translational change undermined the suspected trans-differentiation nature of the observed changes and reinforced the doubts on the effective neural differentiation.

More convincing evidence (morphological and phenotypical) of prolonged mature neural differentiation can be obtained using growth factors and signalling agents as brain-derived neurontrophic factor (BDNF), basal fibroblast growth factor (bFGF), FGF-8, nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), cAMP, dibutiril cAMP (dbcAMP), retinoic acid (RA) or forskolin *(Sanchez-Ramos et al, 2000; Dezawa et al, 2004; Kohyama et al, 2001; Tonderau et al, 2004; Long et al, 2005).*

Specific protocol using growth factors has been translated from the NSC culture method. NSC are traditionally grown in EGF and bFGF (the so-called neurosphere media), forming floating aggregation called neurosphere (*Reynolds and Weiss, 1992*). In such milieu, NSC can highly proliferate. Neurospheres are variably constituted by SC (10-50%), progenitors and differentiated cells; and represent the optimal solution of environmental adaptation in vitro conditions (*Bez et al, 2003*). Culturing the MSC in the neurosphere-media represents a potential way to get neural progenitor cell-like in vitro (*Bunnell et al, 2006)*. BM-MSC can form neurosphere-like in vitro, and then they can be induced by different agents (RA, dbcAMP, bFGF or BDNF) to differentiate in the neural lineage, with no selective phenotype. Wislet-Gendebiens et al. show that MSC neurosphere-like co-culturing with embryonic NSC express selectively astrocytic markers (*Wislet-Gendebiens et al, 2003*). Hermann et al. report more exhaustive results: human BM-MSC can form neurosphere-like and later, induced by N2, RA e BDNF, they can acquire mostly neuronal phenotype and produce dopamine. Such modifications do not happen without the neurosphere-step. Unfortunately technical limitations make no possible to get functional data (*Hermann et al, 2004*).

Independently from the factors inducing the differentiation, the neuron is a polarized, post-mitotic cell, with an axon and multiple dendrites, able to give rise to the action potential and to communicate through synapses with the others (*Reh, 2002*), and the electrophysiological data should support any type of 'differentiation'. Recently, some preliminary promising data have been reported. BM-MSC treated with growth factor exhibit negative membrane potential and very low potassium and sodium currents (*Kohyama et al, 2001; Dezawa et al, 2004; Hung et al, 2002; Egusa et al, 2005*). More relevant results have been obtained transfected MSC with BDNF (*Zhao et al, 2004*) or Notch *(Dezawa et al, 2004)* or after co-culture with fetal murine astocytes (*Jiang et al, 2003*) or cerebellar granula (*Wislet-Gendebien et al, 2005b*). Among these works, only Dezawa et al. *(Dezawa et al, 2004)* use human cells.

Considering how complicated and long is the embryonic CNS development, it can be easily comprehensible why these studies achieved only an early neuron-cells in vitro (*Carleton et al, 2003*).

Among MSC, interest is growing about neural differentiation potential of ASC. Compared to BM-MSC, ASC show a higher tendency to differentiate into the neural line: they produce larger amount of BDNF and react to NB and B27 media expressing higher level of nestin and MAP-2; moreover, in basal condition they express, low levels of nestin, Neu-N, Ca^{++} α_1 channel, sinapsyn I *(Kang et al, 2003a; Kang et al, 2004; Ashjian et al, 2003; Safford et al, 2004; Yang et al, 2004).*

Safford and collegues were the first to induce neural differentiation by human ASC in vitro, by treatment with EGF and bFGF, followed by BHA and forskolin: the cells readily change in morphology and phenotype, expressing at the same time early and some mature neuronal (nestin, Neu-N), glial markers (GFAP) and neurotransmettitor receptors (glutamate and GABA receptors) and lacking the expression of tyrosine hydroxylase (TH), dopamine-decarboxylase and 5-hydroxytryptophan. Moreover, there

was a massive cell death at the 5th day of culture. Such differentiation was unspecific, incomplete and short-lasting *(Safford et al, 2002; Safford et al, 2004).* Other chemical treatments with BME induced more selective neuronal morphology and phenotype, with no data about the functional properties *(Zuk et al, 2002; Tholpady et al, 2003; Romanov et al, 2005).* Only Ashjian et al. in vitro showed K^+ outward rectified currents in modified ASC exposed to IBMX and indomethacin for 14 days: this data was not selective for neuronal differentiation (*Ashjian et al, 2003*). The ability of ASC to form neurosphere-like is not well studied. ASC treated by NB and B27 after neurosphere-step express at the same time neuronal and glial markers (nestin, MAP-2, GFAP); the percentage of differentiated cells increases after BDNF (*Kang et al, 2004*).

In vivo MSC have been shown to generate both migratory neurons and guiding radial glia and to specifically differentiate after intra-ventricular injection in embryonic murine brain *(Munoz-Elias et al, 2004)*. In animal disease models the data about neural potential are largely debated; in this regard, we and other authors have not shown in vivo trans-differentiation *(Constantin et al, 2009; Gerdoni et al, 2007; Kim et al, 2007; Cova et al, 2010)*, whereas others report early neural differentiation, although unselective, immature and not sustained by direct functional data (*Li et al, 2001; Chen et al, 2001a; Chen et al, 2001b; Zhao et al, 2002; Hess et al, 2002; Hofstetter et al, 2002; Lee et al, 2003a; Kassis et al, 2008*). In addition, some groups dispute the potential fusion of MSC with the host cells, that can misinterpret the results *(Spees et al, 2003; Terada et al, 2002).* At the other side, it is still contrasting if in vitro pre-neural differentiation of MSC can be advantageous to the long-lasting effect of MSC transplant *(Parr et al, 2007; Zhang et al, 2009).*

3. MSC NEUROPROTECTION

Although the neuroprotective effects of MSC may result from their ability to replace the diseased or damaged neurons via cellular trans-differentiation, this specific pathway is probably very limited, although the functional improvement in animal model can be dramatic after MSC transplant *(Hofstetter et al, 2002; Lanza et al, 2005; Constantin et al, 2009)*. In addition to the bystander effects reported mainly in the experimental autoimmune encephalomyelitis (EAE) model *(Gerdoni et al, 2007; Constantin et al, 2009)*, other factors have recently been hypothesized, such as their trophic support and antioxidant potential.

Neurotrophins are a family of proteins that promote the survival, development and function of neurons. Among others, NGF and BDNF inhibit death-inducing pathways and also activate a variety of cell survival pathways of neuron and oligodendrocyte *(Gravel et al, 1997; Thoenen, 1995)*; bFGF moreover promotes angiogenesis *(Meijs et al, 2004)*. They are endogenously synthesized in the CNS, and they can be unbalanced in several neurological diseases. The administration of neurotrophins has been proposed as potential approach to the therapy of neural disorders such as Parkinson, Alzheimer, Huntington diseases, stroke and spinal cord injury *(Gash et al, 1996; Nagahara et al, 2009; Jin et al, 2005; Schabitz et al, 2000; Cheng et al, 2002)*. However, issues relating to compound delivery and potential side effects have limited the clinical application of this treatment strategy.

Indeed, MSC are able to produce important neurotrophic factors that support neuronal cell survival and promote nerve fiber regeneration at the sites of injury. BM-MSC are known to produce NGF *(Labouyrie et al, 1999; Garcia et al, 2004; Jiang et al, 2010; Crigler et al, 2006)*, BDNF *(Labouyrie et al, 1999; Chen et al, 2002; Crigler et al, 2006; Neuhuber et al, 2005; Jiang et al, 2010)*, GDNF *(Garcia et al, 2004; Jiang*

et al, 2010) and bFGF *(Mahmood et al, 2004).* Regarding ASC, human type can secrete BDNF, NGF *(Qian et al, 2009 epub),* vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin growth factor 1 (IGF1) *(Rehman et al, 2004; Wang et al, 2006*). BDNF, HGF, IGF, bFGF and PDGF can be secreted by rat ASC *(Peng et al, 2008; Wei et al, 2009a; Wei et al, 2009b)* and BDNF, NGF, bFGF and PDGF by murine ASC *(Costantin et al, 2009; Zhang et al, 2009).* Interestingly, the growth factor levels change related to MSC passages and to the considered cell sub-population *(Crigler et al, 2006; Wilkins et al, 2009).*

Neural-induced MSC display remarkably decreased levels of neurotrophins, regardless the MSC source, including rat BM-MSC *(Jiang et al, 2010*), human ASC *(Qian et al, 2010)* and human umbilical cord MSC *(Koh et al, 2008).* Such data in vitro could reflect the effect of artificial manipulation as well as the physiological aging, exhibiting how highly dynamic is the neurotrophin expression profile. Thus, non-induced MSC provide a natural carrier for neurotrophins.

The homeostasis and regular function of the neuron is guaranteed from the niche which supplies, besides growth factors, the opportune conditions to balance the oxidative stress. Regardless of the etiology, a component of oxidative stress is present in all neurodegenerative disorders, although is not yet clear if the oxidative stress is the consequence or the early event of the disease. Moreover, nitric oxide (NO) is a common second messenger in inflammatory signaling, and reactive nitroxide species (RNS) as well as Reactive Oxygen Species (ROS) are capable of both oxidation and nitration chemistries. Thus the oxidative stress is involved, besides to degenerative diseases, in neuro-inflammatory pathologies, as multiple sclerosis *(Sayre et al, 2008).* The relevance of oxidative stress contribution to the progression of many neurodegenerative disorders has generated an interest in the potential use of oxygen and nitrogen radical scavengers

and their natural biological counterparts for protecting cells and tissues from oxidative damage *(Prokai et al, 2003; Sancivez et al, 2006).*

Recently MSC has been shown to exert antioxidant effect, although the underlying mechanisms are largely unknown. Uccelli and coworkers reported that human BM-MSC decrease the expression of non-enzymatic and enzymatic antioxidants in H_2O_2-stressed neuroblastoma model in vitro, as well as in EAE *(Lanza et al, 2009).*

Human BM-MSC have been shown to be able to protect cerebellar neurons in vitro via cell-cell contact and/or secretion of soluble factors (among others, SOD3) after trophic deprivation and NO exposure *(Kemp et al, 2009 epub).* Murine BM-MSC can decrease glutamate-induced apoptosis in neuron *(Hokari et al, 2008).*

As BM-MSC protect versus apoptosis *(Zhao et al, 2004),* very recently Wei et al. show as rat ASC protect neurons against low potassium or serum free-induced apoptosis, reducing caspase 3^+ cells and Akt phosphorilation *(Wei et al, 2009b).* It is largely unclear if and how MSC can resist to the stress condition that damages neuron. Oxidative stress does not activate caspase 8 and 9 in MSC *(Szegezdi et al, 2008 epub).*

The combination of growth factors and antioxidant molecules produced by MSC can partially explain some in vivo results: BM-MSC transplant into the injured spinal cord promotes tissue sparing, which is evidenced by smaller cavities and preserved host white matter *(Akiyama et al, 2002; Ankeny et al, 2004; Ohta et al, 2004; Zhang et al, 2009),* decrease the ischemic volume in stroke *(Zhao et al, 2004; Liao et al, 2009; Koh et al, 2009)* and reduce axonal loss in EAE *(Zappia et al, 2005; Kassis et al, 2008; Costantin et al, 2009).* More interesting, MSC promoted endogenous neural progenitor cell proliferation in chronic EAE *(Constantin et al, 2009),* stroke *(Yoo et al, 2008)* or brain injury *(Mahmood et al, 2004).* Some authors selectively inhibit growth factor in vitro or in vivo (in animal disease model treated with MSC) and show the decrement of the beneficial effect of MSC treatment *(Wilkins et al, 2009; Koh et al, 2008),* sustaining

the role of growth factors. At the other side, other studies in vivo reveal an increase of BDNF in the MSC-treated group compared to sham *(Nomura et al, 2005; Zhang et al, 2009*) and ASC-Condition Media (ASC-CM) improve stroke *(Wei et al, 2009a).*

Thus, it is likely that the mechanisms underlying the neuro-protective effect of MSC transplant are related to the cell ability to produce and secrete factors that either arrest and/or prevent the onset of cell-destructive events.

The use of ASC in CNS disease model is very limited: our group very recently has shown how ASC can exert immunomodulation and neuro-protection in EAE *(Constantin et al, 2009)*; three others used ASC in stroke model *(Kang et al, 2003a; Kim et al, 2007; Wei et al, 2009a)*; Huntington disease model and spinal cord injury have been moreover recently investigated *(Lee et al, 2009; Zhang et al, 2009).* Intravenous administration of ASC in chronic established EAE significantly ameliorates the disease course and reduces both demyelination and axonal loss, increases number of endogenous oligodendrocyte progenitors and induce a Th2-type cytokine shift in T cells *(Constantin et al, 2009)*; intraventricular injection of ASC ameliorate clinical outcome: partially reach the ischemic area and differentiate in neural line *(Kang et al, 2003a)*; also ASC-CM injection is beneficial for stroke, reducing the secondary brain atrophy and the apoptotic cells *(Wei et al, 2009a).* In all these studies the role of growth factors synthesized by ASC is stressed.

Therapeutic applications of MSC in human neurological diseases are still preliminary: the first results regard methacromatic leukodystrophy and Hurler syndrome *(Koç et al, 2002),* stroke *(Bang et al, 2005)* and amyotrophic lateral sclerosis *(Mazzini et al, 2006; Mazzini et al, 2010)* and trials in humans are ongoing on intrathecal or intravenous BM-MSC in multiple sclerosis (NCT00781872, NCT00395200), intravenous BM-MSC in stroke (NCT00875654) and stereotactic application in PD

(NCT00976430). Such clinical trials are mostly studying the safe profile, meanwhile experimental laboratory studies are deeply investigating the mechanisms involved.

This study aims to further investigate the potential neuroprotective and neural-regenerative effect of ASC. These cells have several advantages over ESC and NSC, including lack of ethical controversy, immunosuppressive effects demonstrated in clinical settings *(Le Blanc et al, 2004; Ringden et al, 2006)*, and readily availability through very low invasive procedure.

AIMS OF THE PROJECT

ASC are adult MSC, easily available and safe. Considering that MSC has been shown to have neural potential and trophic function, we evaluated in vitro the ability of ASC to differentiate in neuron and their neuroprotective action against oxidative stress.

The research includes:

a. chemical neural induction: morphological and phenotypic evaluation,

b. two-step neuronal protocol, including neurosphere-formation and RA and BDNF long-lasting induction: morphological, phenotypic and electrophysiological evaluation;

c. Neuroprotective effect of ASC-CM: proliferation and apoptosis assay considering the effect of ASC-CM on H_2O_2-stressed neuroblastoma line in vitro, as compared to fibroblast effect.

The adult cells should represent the powerful future therapeutic tool for the neurological diseases, regarding their multiple mechanisms of action, including the trans-differentiation potential, the antioxidant and trophic activity and the immunomodulant action that could overcome either the inflammatory and the degenerative component of the disease.

MATERIAL AND METHODS

CELL CULTURE

Human fibroblasts were collected from diagnostic muscle biopsy after informed consent, and grown in 10% FBS and DMEM (Gibco, Milan, Italy). SH-SY5Y human neuroblastoma line (courtesy gift from dr. Della Bianca) was cultured in monolayer in Royal Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS. The cell cultures were saved at 37°C in a humidified atmosphere of 5% CO_2/95% air.

ISOLATION AND CULTURE OF HUMAN ASC

Human ASC were obtained from 40 ml lipoaspirate samples of abdominal fat from female donors after informed consent *(Katz et al, 2005).* Extracellular matrix is digested at 37°C in Hank's balanced salt solution with 1 mg/ml collagenase type I. Cells are then maintained in basal medium (20% FBS/DMEM) and cells are centrifuged at 1,200 g for 10 minutes; the pellet is then resuspended in 160 mM NH_4Cl to lyse contaminating red blood cells and filtered through a 70-μm nylon mesh. The cells are cultured at 30 x 10^6 cells/cm^2 in 25 cm^2 flasks in DMEM with high glucose concentration, GLUTAMAX ITM, FBS, penicillin and streptomycin (all from Gibco). After 72 hrs, non-adherent cells are removed. When 70-80% confluent, adherent cells are trypsinized, harvested and expanded in larger flasks. A homogenous cell population is obtained after 3 to 5 weeks of culture. All the experiments are performed on cells at passages 7 to 16.

Human ASC were characterized by the expression of CD105 (endoglin), CD73, CD29, CD44, CD90 class I HLA and lack of haematopoietic (CD45, CD14, CD34) and endothelial (CD31) markers. All the above monoclonal antibodies (mAb) are purchased from Pharmingen/Becton Dickinson. For the immunophenotypic analysis, ASC are detached using trypsin/EDTA, washed with PBS and resuspended at 10^6 cells/ml. Cell

suspension is incubated in 15% FBS, followed by incubation with the specific mAb for 30 minutes. At least 10,000 events are analyzed by flow cytometry (FACScalibur, Becton Dickinson) using Cell Quest software.

MESENCHYMAL DIFFERENTIATION OF ASC

Multilineage differentiation potential was assessed by testing the ability of ASC to differentiate into adipocytes, osteoblasts, and chondrocytes, as previously described *(Pittenger et al, 1999)*. Briefly, adipocyte differentiation is achieved after 3-week culture of ASC with adipogenic medium, containing 10^{-6} M dexamethasone, 10 µg/ml insulin and 100 µg/ml 3-isobutyl-1-methylxantine (Sigma Aldrich). Osteoblast differentiation is achieved after 3-week culture with osteogenic medium containing 10^{-7} M dexamethasone, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma Aldrich). Chondrocyte differentiation is achieved after 3-week culture with chondrogenic medium, containing 10^{-7} M dexamethasone, 10 ng/ml TGF–µ1, 50 µg/ml ascorbic acid, 40 µg/ml proline (Sigma Aldrich) and ITS+ Premix (BD Biosciences). Oil-red-O, von Kossa and toluidine blue dyes are employed to identify adipocytes, osteoblasts and chondrocytes, respectively.

NEURAL DIFFERENTIATION: CHEMICAL PROTOCOL

Chemical neural induction was performed according to a modified protocol from Woodbury et al. *(Woodbury et al, 2000)*. Briefly, basal medium is replaced with DMEM and FBS supplemented with 5 ng/ml bFGF (Sigma Aldrich) for 24 hrs; after this pre-induction, cells are washed with PBS and induction medium including DMEM with N2 supplement, butylated hydroxyanisole (BHA), KCl, valproic acid and forskolin (all from Sigma Aldrich) is added for 2 to 16 hrs. Cells are either fixed for

immunocytochemistry or transferred to basal medium for other 7 days, and then assess their morphology and phenotype.

NEURAL DIFFERENTIATION: PROLONGED NEURONAL DIFFERENTIATION PROTOCOL

Neuronal differentiation was induced by culturing ASC for 72-96 hrs in serum-free medium with 20 ng/ml bFGF and 20 ng/ml human epithelial growth factor (hEGF) (all from Peprotech), with the formation of floating bodies. Such spheres are then subjected to immunocytochemistry or dissociated and seeded on poly-L-lysinated coverslips (Sigma Aldrich) at 1,000/cm^2 in DMEM, 2% FBS, 10 ng/mL BDNF (Peprotech) and 0.75 mM all-trans RA (Sigma Aldrich). Medium is replaced every 5-6 days up to 30 days, when the cells are subjected to morphological, immunocytochemical and electrophysiological analysis to assess the presence of neuronal features. Alternatively, neuronal induction medium is replaced with basal medium for 7 days and the stability of the neuronal features is assessed as above.

PREPARATION OF FIBROBLAST- AND ASC-CONDITION MEDIA (CM)

ASC-CM was collected after culturing ASC in sub-confluent monolayer for 48 hrs. CM is used pure or diluted 1:1. As control, we use basal media or fibroblast-CM, harvested by sub-confluent cultures. The preparation of ASC-CM and fibroblast-CM for the ELISA assay was as followed: 1 x 10^5 cells are grown in basal condition for 24 hrs in 24-well plate, the supernatants are harvested, centrifuged for 10 min to remove cell debris and stored at -80 °C.

NEUROBLASTOMA MODEL AND THE OXIDATIVE STRESS MODEL

Neuroblastoma cell lines are a classical model system to study neuronal responses to different type of insults *(Sancivens et al, 2006).* SH-SY5Y are cultured in 24-well dishes, containing 1 ml of ASC-CM, fibroblast-CM or basal medium. After 48 hrs, neuroblastoma cells are fixed and stained with βIII tubulin (see below). H_2O_2 is diluted with culture medium at 100 μM concentration immediately before use. SH-SY5Y, ASC or fibroblast are exposed to H_2O_2 stress. SH-SY5Y are exposed to H_2O_2 in ASC-CM, compared to fibroblast-CM and/or basal media. Cells treated are evaluated for proliferation or processed for immunofluorescence (caspase 3) (see below).

PROLIFERATION ASSAY

Cells are seeded in 96-well plate and exposed to 100 and 200 μM H_2O_2 at 37 °C and 5% CO_2. The oxidative stress is performed in 10% FBS/DMEM (control) or 1:1 ASC-CM, for 24 hrs. Then, 10 μl Reagent 1 is added to each well, incubated overnight. The day after Reagent 2 is added and within 20 minutes the plate is read at 655 nm.

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ASC cellular morphology was evaluated at light microscope after fixation in 4% paraformaldehyde and hematoxilin staining or at scanning electron microscope (DSM 950, Zeiss, Germany) after sequential fixation in glutaraldehyde and 1% osmium tetroxide for 15 minutes, dehydration and final fixation with colloidal silver and gold. The immunophenotype of ASC was evaluated with antibodies directed against the mesenchymal marker CD105 (1:500, Caltag Laboratories, Burlingame, CA); the neuronal markers microtubule-associated protein 2 (MAP-2) and neuronal nuclear antigen (Neu-N) (both 1:1,000); nestin (1:200), a protein of intermediate filament expressed by NSC; the oligodendroglial marker Gal-C (1:100) (all from Chemicon Inc.,

Temecula, CA), the astrocytic markers S-100 (1:5,000) and glial fibrillary acidic protein (GFAP) (1:10,000, Dako); the Schwann cell marker PMP-22 (1:5,000; Chemicon); tyroxine hydroxilase (TH), an enzyme of catecholaminergic neurons (1:2,000, Santa Cruz Biotechnolgy, Inc., Santa Cruz, USA); sodium channel (1:800), α subunit of GABA-A receptor (1:400; Sigma). After washing, appropriate biotynilated secondary antibody and ABC amplification kit (Vector Laboratories, Burlingame, CA) are added and the reaction visualized with diaminobenzidine. Negative control includes the omission of primary antibodies. Experiments are performed in triplicate and the percentage of positive cells is blindly calculated. To determine the mitotic activity of ASC before and at the 28th day of the neuronal induction protocol, cells are exposed to 10 μM BrdU (Sigma Aldrich) for 4 hrs, fixed with cold ethanol for 20 minutes, treated with 2N HCl and then with 0.1M NaBo, pH 9. Double immunofluorescence is performed for MAP-2 and BrdU, whose signals are detected with secondary antibodies conjugated respectively with Streptavidine Texas Red (Vector) and fluorescein isothiocyanate (FITC) (both from Boehringer). Nuclei are stained with 50 μg/ml DAPI (Sigma). Cells are observed at the fluorescent microscope (Zeiss MC80) and the rate of mitotic activity is calculated dividing the number of $BrdU^+/MAP\text{-}2^+$ cells and that of $BrdU^+/MAP\text{-}2^-$ elements by the total $DAPI^+$ cells.

SH-SY5Y are fixed in 4% paraformaldehyde, and then processed for the cytochemistry to evaluate the neurite outgrowth evidenced by anti-βIII tubulin mAb (1:300, Chemicon). The antibodies are incubated overnight and then revealed with the secondary fluorescent secondary antibody FITC. DAPI is used as nuclear marker. Cells are observed at the fluorescent microscope: to determine the mean number of neuritis we count an average of one hundred SH-SY5Y cell bodies for each condition in each experiment. The apoptotic activity is investigated by the immunofluorescence using the caspase 3 antibody (1:400, Cell Signaling Technology) and the secondary Ab FITC.

Nuclei are stained with DAPI. Stress-induced apoptosis are calculated as ratio between caspase 3^+ during H_2O_2 and caspase 3^+ in basal condition.

ELECTROPHYSIOLOGY

Coverslips with ASC are placed in the recording chamber (1 cm^3 volume) and mounted on Olympus BX50WI microscope. The cells are perfused at the rate of 2 ml/min with artificial cerebro-spinal fluid with the following composition (mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 15 glucose (all from Sigma). Saline is continuously bubbled with 95% O_2/5% CO_2; the osmolarity is adjusted at 305 mOsm with glucose. Cells are exposed to tetrodotoxin (TTX, Alomone, Jerusalem, Israel), tetraethylammonium (TEA), glutamate or GABA (all from Sigma Aldrich). The tight-seal whole-cell recording technique is used. Borosilicate glass pipettes (OD 1.5 mm; ID 0.86 mm; Hilgenberg, Malsfeld, Germany) with internal filament are adopted for recordings. The pipettes have a tip resistance ranging from 4 to 6 MΩ when fill with these solutions. Seal resistance is always greater than 2 GΩ. The solution used for the recording pipette-filling solution contains (mM) 120 KCl, 10 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 2 Na-ATP, 10 glucose; the osmolarity is adjusted at 295 mOsm with glucose, and pH at 7.2 with KOH. Membrane currents are recorded and acquired with Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA). The series resistance is around 15 MΩ; 60-70% compensation of the series resistance is routinely used. Data acquisition is performed by a Pentium-based computer using 12 bit A/D-D/A converters (Digidata 1200B; Molecular Devices).

Prior to acquisition, the signals are filtered at half the sampling frequency by a lowpass 4-pole Bessel filter and digitized with sample times ranging from 10 to 100 μs. Off-line analysis is performed using version 10.1 of pClamp (Molecular Devices). Data

are expressed as mean + SEM and are statistically analyzed using Origin 7.5 software (OriginLab, Northampton, MA).

ELISA ASSAYS FOR GROWTH FACTORS

To determine the production of bFGF, BDNF, CNTF and PDGF-AB by ASC and fibroblasts their supernatants were analyzed by Quantikine® ELISA Immunoassay (R&D, Systems, Inc.), following the manufacturer's instructions. Samples are added in 96-well pre-coated plates and incubated for 2 hrs at RT. After washing, a specific polyclonal antibody followed by substrate solution is added and the colour development is measured at 450 nm (BioRad Microplate Reader). The concentration of growth factors is calculated using the standard curve.

STATISTICAL ANALYSIS

The results obtained by immunocytochemistry and electrophysiology in basal conditions and after neuronal induction are evaluated by Student's *t*-test and the difference is considered statistically significant when $p<0.05$.

RESULTS

BASAL ASC MORPHOLOGY AND PHENOTYPE

In basal conditions, ASC express CD105, CD73, CD29, CD44, CD90, CD106, lack of hematopoietic (CD45, CD14, CD11c, CD123 and CD34) and endothelial cell markers (CD31), and display the ability to differentiate into adipocytes, osteoblasts and chondrocytes (Fig. 3).

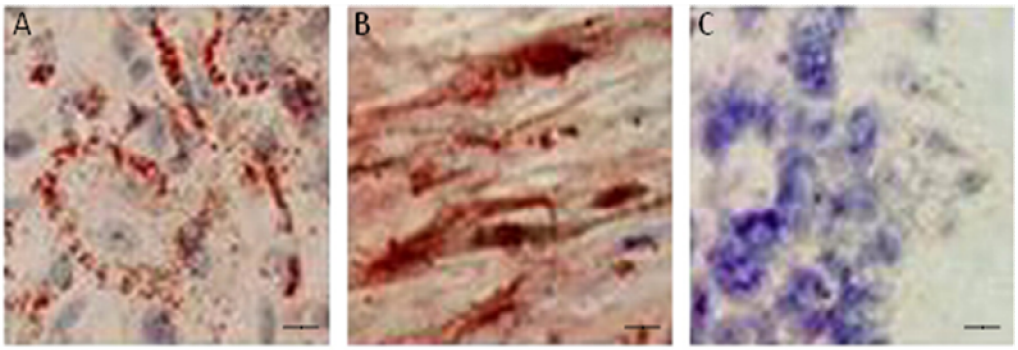

Figure 3. Multilineage differentiation of ASC. ASC are cultured for 14 days in flasks with adipogenic, osteogenic or chondrogenic media. Then cells are stained with Oil-red-O (A), von Kossa (B), and toluidine blue (C) methods, respectively, which confirm their multi-lineage potential. The figure shows a representative picture of three different experiments. Scale bar: 10 μm.

ASC in basal conditions form a monolayer of large and flat cells, assuming spindle-shaped morphology at confluence (Figs. 4A, B). As far as molecules of the neural lineage are concerned, ASC express in basal conditions low levels of GFAP and PMP-22 (Figs. 4C, D), and MAP-2 (not shown) and are negative for the other investigated markers (Neu-N, PSA-NCAM, nestin, GFAP, S-100 and Gal-C).

NEURAL DIFFERENTIATION: CHEMICAL INDUCTION

After exposure to neural induction medium, ASC exhibit very rapid morphological changes: most cells retract their cytoplasm, forming spherical cell body, emit cellular protrusions and completely stop to proliferate, as compared to ASC in basal conditions (13.9 ± 3.1% of ASC incorporating BrdU, 3 experiments). At the end of this process, we can identify three morphologically distinct subsets of ASC: 50-70% of ASC appears as sharp, elongated bi- or tripolar cells with primary and secondary processes (Figs. 4E-G), morphologically reminiscent of neurons or astrocytes and shows intense expression of PMP-22 (Fig. 4E1), GFAP (Fig. 4F1), Neu-N (Fig. 4G1), PSA-NCAM, nestin, S-100, MAP-2 and Gal-C (not shown); irrelevant mouse or rabbit IgG give no staining (Fig. 4K). The second population (about 15%) is characterized by elements with irregular shape and short, multibranched processes, morphologically similar to cells of the oligodendroglial lineage (Fig. 4H), and selectively express Gal-C (Fig. 4H1). The last population (15-20%) is similar to ASC in basal conditions, i.e., remains undifferentiated and does not up-regulate any neural markers. Irrespective of the morphological changes, all ASC retain CD105 expression (data not shown). These events start after 1 hr and reach their peak within around 2 hrs. With longer culture times, signs of cell toxicity are observed, with retraction of cell processes, detachment from plastic and eventually apoptosis of virtually all ASC.

The neural differentiation protocol induces a transient differentiation of ASC, as cells gradually recover basal morphology and phenotype within 48-72 hrs of culture with basal medium (Figs. 4I, J). Proliferation assay at 72 hrs shows that 7.5 ± 2.5% of ASC incorporate BrdU (i.e., half the proliferation rate of ASC in basal conditions); no relevant signs of cell death are observed. In addition, ASC multi-lineage differentiation potential into adipocytes, osteocytes and chondrocytes is still achievable (data not shown).

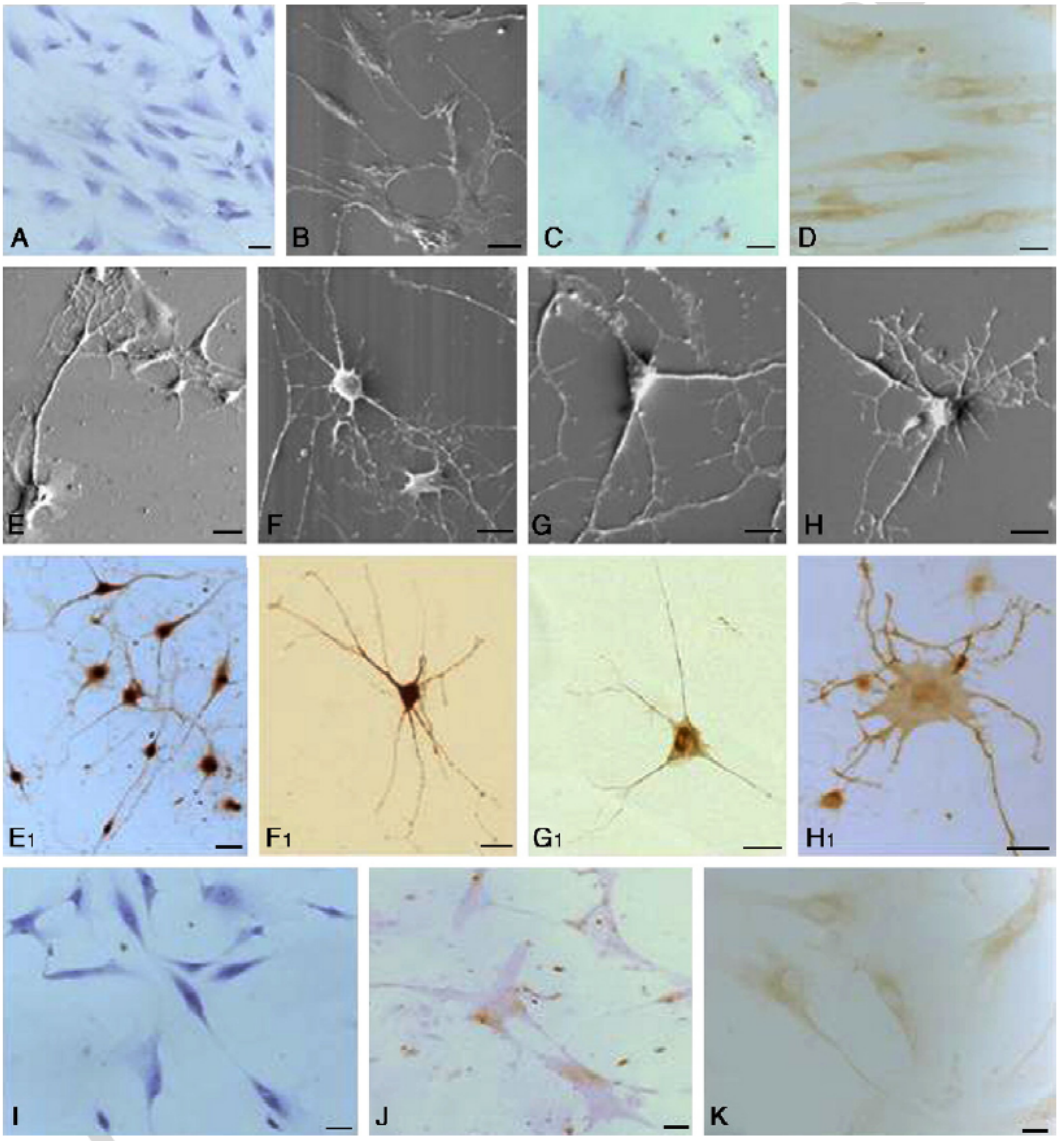

Figure 4. Neural induction medium. In basal condition ASC are spindle-shaped (A, hematoxylin staining at optic microscope; B, at scanning electron microscopy). Immuno-peroxidase for PMP-22 (C) and GFAP (D) on BM-MSC reveals low levels of expression in basal conditions. Dramatic changes after the exposure to neural differentiation medium in terms of morphology (E–H) and phenotype (E1–H1) are observed on ASC; the majority of ASC assumed an astrocytic/neuronal morphology (E–G) with bi- and tripolar shape with primary and secondary processes at scanning electron microscopy and intense immunoreactivity for PMP-22 (E1), GFAP (F1) and Neu-N (G1). About 15–20% of ASC displays oligodendroglial-like morphology with multi-branched, irregular shape (H) and selective up-regulation of Gal-C (H1). ASC regain their basal morphology (I) and faint immunoreactivity for GFAP (J) 72 h after removal of neural induction medium. Background immunostaining obtains with the omission of primary antibody on ASC (K) stimulated with neural chemical induction. Scale bar: 10 μm.

NEURAL DIFFERENTIATION: PROLONGED NEURAL DIFFERENTATION

As already reported for BM-MSC *(Hermann et al, 2004)*, in the presence of EGF and bFGF, ASC form spherical, floating aggregates within 2-5 days (Figs. 5A, B), which express nestin, Neu-N, and MAP-2 (Fig. 5C), but not the glial markers Gal-C, GFAP and S-100 (Fig. 5D) as well as CD105 (data non shown). The cells can be dissociated and culturing in the same media, forming no more than third-passage sphere. The neurospheres are then dissociated and single cells cultured in presence of BDNF and RA (neuronal induction). Two populations are clearly distinguishable based on morphological and immunophenotypical criteria; about half (56.81 ± 2.26%) of ASC shows the characteristic neuronal morphology with contracted cytoplasm, condensed nucleus and protrusion of two or three cellular processes as shown by electronic microscopy (Fig. 5E). By immunocytochemistry, these cells express nestin (Fig. 5F) and the neuronal markers MAP-2 and Neu-N (Figs. 5G, H). A very low proportion of these cells (about 1%) expresses also TH and α subunit GABA-A receptor (Figs. 5I, J), while we fail to detect immunoreactivity for the sodium channel. Interestingly, no signal for the glial markers S-100, GFAP, Gal-C is observed (Fig. 5K). The remaining ASC show no apparent response to the differentiation protocol and remain large and flat, with abundant cytoplasm and do not expressed any neuronal markers. Morphological and immunophenotypical changes suggestive of neuronal differentiation persist for at least seven days after the removal of differentiation medium (Figs. 5L, M). Exposure to the differentiation medium greatly reduces the proliferation rate of ASC. In fact, while 5 ± 1.5% of MAP-2$^-$ ASC with basal morphology is BrdU$^+$ (as compared to 7.5 ± 2.5% of untreated cells) (Figs. 6A, B), none of the ASC with neuronal morphology and MAP-2 staining show incorporation of BrdU (Figs. 6C, D).

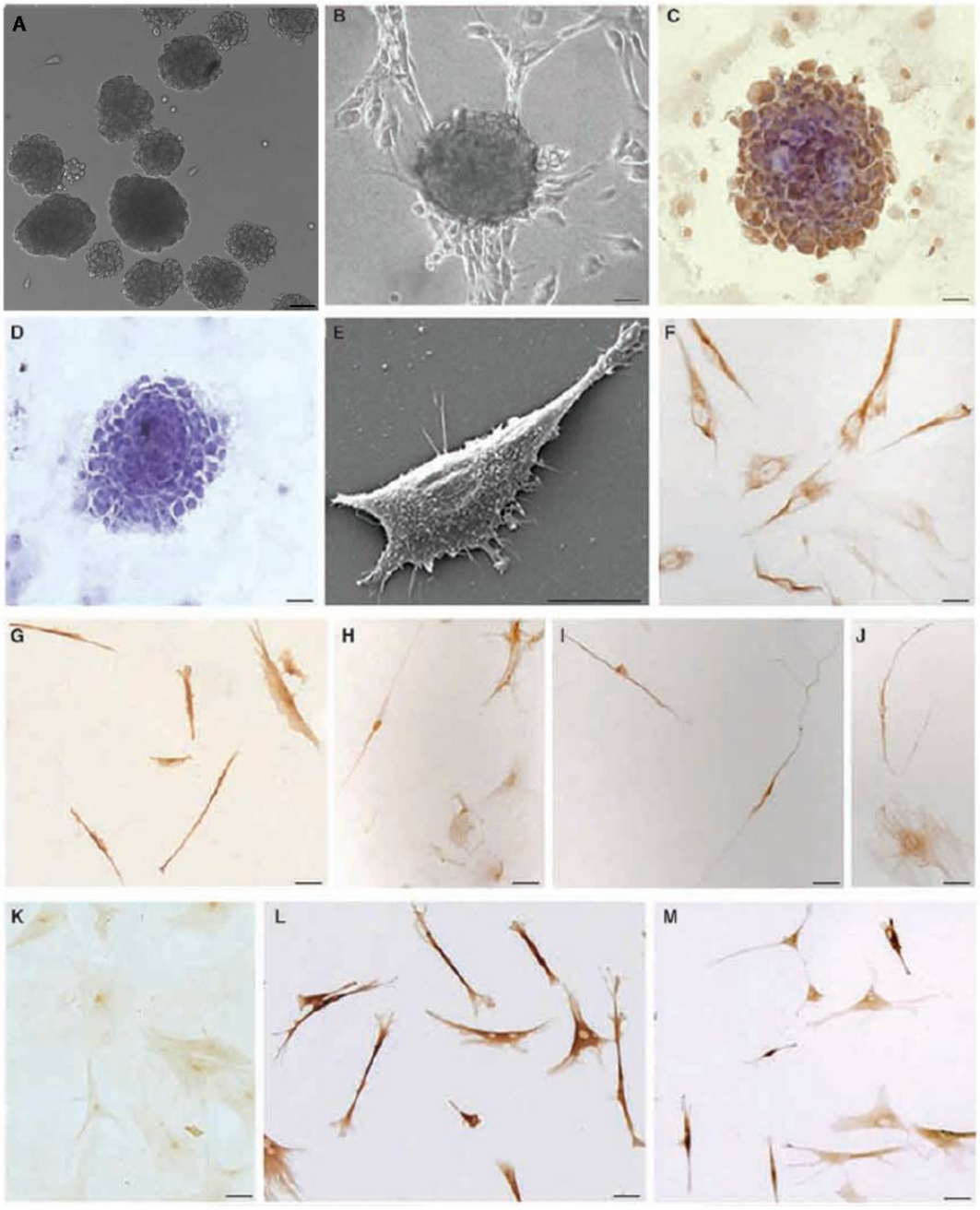

Figure 5. Morphology and phenotype of ASC subjected to neuronal differentiation. Treatment with EGF and bFGF induces the formation of floating aggregates (A, B), formed by MAP-2-positive (C), GFAP-negative (D) elements. After 28 days exposure to RA/BDNF, ASC exhibit contracted cytoplasm and long processes at scanning electron microscope (E), expressed nestin (F) and the neuronal markers MAP-2 (G) and NeuN (H). A small portion of the differentiated ASC express TH (I) and α subunit of GABA-A receptor (J), but not the glial marker GFAP (K). Morphological and phenotypical changes persist for at least 7 days after the replacement basal medium, with expression of nestin (L) and MAP-2 (M). Scale bars: 100 μm (A), 50 μm (B-M).

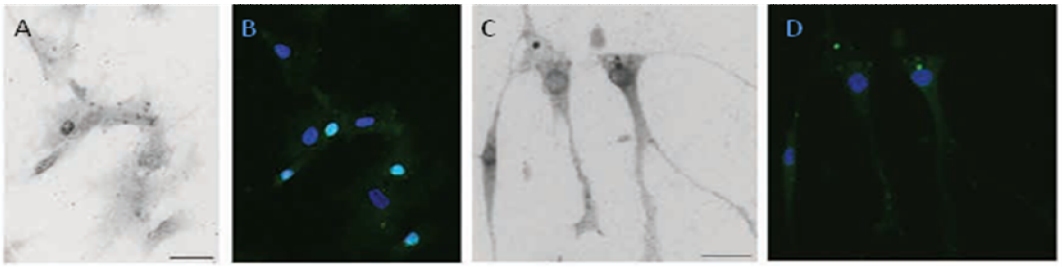

Figure 6. Proliferation after neuronal differentiation: the group of ASC not reacting to the induction maintain basal morphology and are $BrdU^{+}$ (A, B), in contrast with ASC with neuronal morphology and MAP-2 staining, which do not incorporate BrdU (C, D). Scale bar: 50 µm.

The formation of spherical, floating aggregates before the treatment with BDNF/RA is fundamental to obtain neuronal differentiation: in the absence of this step, ASC subjected to the differentiation protocol continue to proliferate and maintain the basal biological features (data not shown).

NEURAL DIFFERENTIATION: ELECTROPHYSIOLOGY

We evaluate electrophysiological properties in basal condition and after prolonged neuronal differentiation using the patch clamp technique in whole-cell configuration. Focusing on treated ASC with neuronal morphology, we estimate the membrane potential under current-clamp conditions and the presence of voltage-gated channels in whole-cell voltage-clamp experiments. The resting membrane potential of ASC with neuronal morphology (−59.75 ± 5.41 mV, n = 12) is significantly more negative than that of basal ASC (−33.54 ± 3.1 mV, n = 26; p = 0.001) (Fig. 7). Although not statistically different, also the mean value of membrane capacitance for differentiated ASC is lower (62.70 ± 9.67 pF, n = 10) than basal ASC (87.5 ± 9.51 pF, n = 28). A large outward current is isolated after blockage of inward currents with TTX.

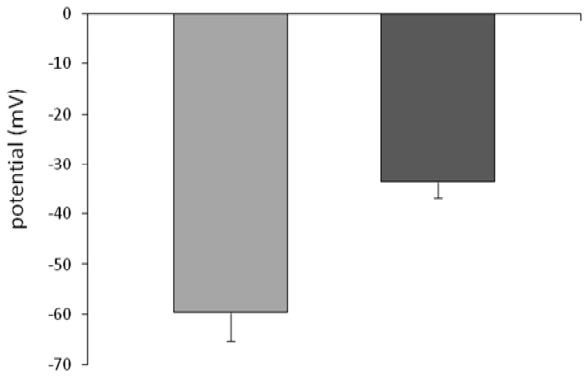

Figure 7. Resting membrane potential of ASC subjected to neuronal differentiation. Values of resting membrane potential of ASC recorded in basal conditions (grey) and at 28th day after exposure to neuronal differentiation protocol (light grey).

Depolarising pulses ranging from −50 to +40 mV in 10 mV increments from the holding potentials of −70 mV evoke a family of non-inactivating currents (Figs. 8A, B), significantly larger in differentiated ASC as compared to basal condition: at + 40 mV the mean amplitudes are 522.86 ± 117 pA (n = 10) in differentiated cells vs 223.34 ± 37.55 pA (n = 19) in basal cells (p = 0.005) (Fig. 8C). Their selective block by TEA indicates that these currents are carried by potassium ions. Differentiated ASC also exhibit a prominent inward current (Fig. 8D), which is virtually absent on basal cells. This is isolated by equimolar ion substitution of intracellular K+ with Cs+, and is evoked by voltage steps ranging from −50 to +40 mV after a complete removal of inactivation with a 200 ms step at −120 mV. At 0 mV the mean peak amplitude is −185.09 ± 4.65 pA (n = 7) in differentiated cells vs 4.65 ± 4.65 pA, n = 28 in basal cells (Fig. 8F). For their fast inactivation and their sensitivity to TTX these currents are definitely mediated by classical voltage-dependent sodium channels. The kinetic of the sodium current is investigated in detail. The development of inactivation is studied with a series of depolarising steps to the fixed potential of 0 mV after 180 ms conditioning pulses between −120 mV and −40 mV (Figs. 8G, H).

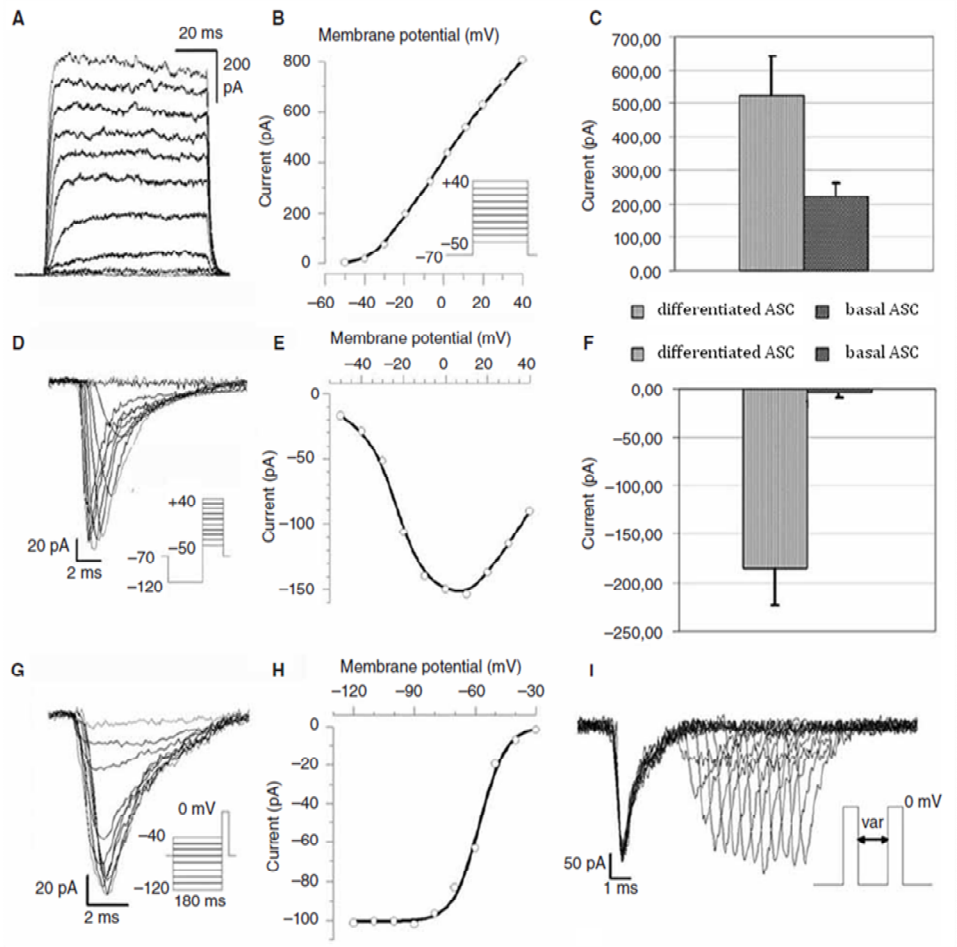

Figure 8. Electrophysiological features of ASC subjected to neuronal differentiation. Activation of potassium currents evoked by depolarizing pulses ranging between −50 and +40 mV and the corresponding current-voltage relationship in ASC with neuronal morphology (A, B). Voltage-clamp reveals a tendency for higher outward currents in differentiated ASC than in basal ones (C). Activation of sodium currents evoked by increasing depolarizing pulses as indicated in the inset and the corresponding current-voltage relationship in ASC with neuronal morphology (D, E). ASC with neuronal morphology exhibit inward currents significantly higher than basal cells (F). Inactivation of the sodium channels in ASC with neuronal morphology, obtained following the protocol shown in the inset. The corresponding current-voltage relationship, showing the peak amplitudes at the fixed test potential of 0 mV as a function of the conditioning pulse, has a midpoint centered at −57.7 mV (G, H). Removal of inactivation of the sodium current at −100 mV. Two pulses at 0 mV (inset) are separated by a variable delay to allow removal of inactivation, occurring in this case with a time constant of 2.0 ms (I).

The half-inactivation is at the potential of −57.7 mV and the time constant for the development of inactivation is 2.46 ± 0.13 ms at 0 mV (n = 7). The removal of

inactivation is studied using the protocol shown in the inset of Fig. 8I: two consecutive depolarizing pulses to 0 mV from different holding potentials (−100 mV for the case represented in Fig. 8I) are separated by an interval of variable length. The longer is the time spent at the holding potential between the two steps, the larger is the removal of inactivation and the amplitude of the current in response to the second depolarizing step; the time constant for the removal of inactivation is 2.0 ms at −100 mV.

To test for the presence of functional receptors, cells are maintained at a holding potential of −40 mV. Under these conditions, both glutamate and GABA fail to induce a current (data not shown).

ASC SUPPORT NEURITE OUTGROWTH IN VITRO

In addition to neurophysological features, we evaluated another biological parameter characteristic of neuronal commitment, i.e. the presence and the modulation by ASC-CM of neurite outgrowth.

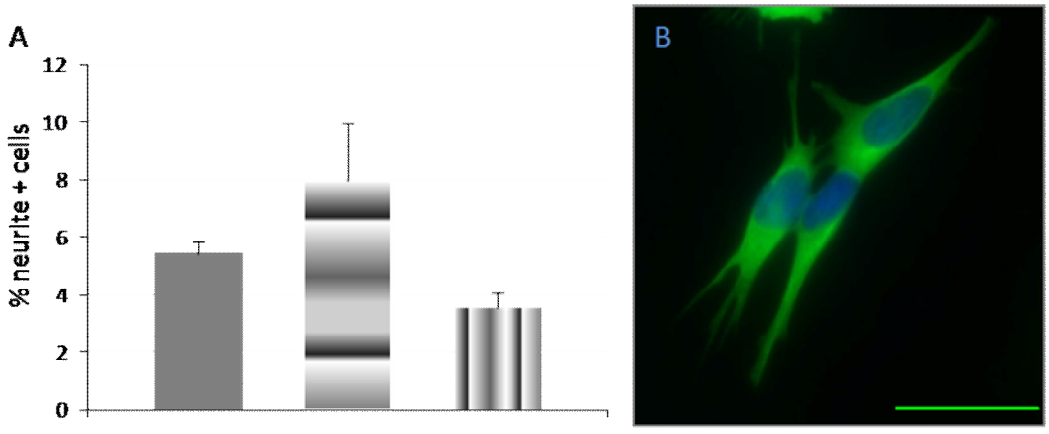

Figure 9. The percentuage of SH-SY5Y exhibiting the neurite after 48 hrs of culture in basal (grey), ASC-CM (horizontal filling) or fibroblast-CM (vertical filling) (A). An example of neurite-positve cells after ASC-CM culture, staining with βIII tubulin (green) and nuclear DAPI (blue) (B). Scale bar: 50 µm.

For this purpose, we used the human neuroblastoma SH-SY5Y cell model to study neuronal response in vitro *(Sancivens et al, 2006)*. We found that ASC-CM increased

the neurite outgrowth in SH-SH5Y (7.9 ± 1.9%) after 48 hrs of culture, as compared to fibroblast-CM (5.4 ± 0.4%) and basal medium (3.5 ± 0.5%) (Figs. 9A, B).

PROTECTION AGAINST OXIDATIVE STRESS

We then assessed whether ASC may exert a neuroprotective effect, by using an oxidative-stressed neuron model in vitro *(Haque et al, 2003)*. SH-SY5Y cells are exposed to H_2O_2, the main endogenous ROS which induces cytotoxic effect. H_2O_2 is added to basal medium or to ASC-CM (1:1) for 24 hrs. The cell viability is reduced after oxidative stress in basal group compared to ASC-CM group (92.1 ± 5.7% compared to 102 ± 2.9%). The protective effect was not evident at higher concentration (200 μM) of H_2O_2 (Fig. 10).

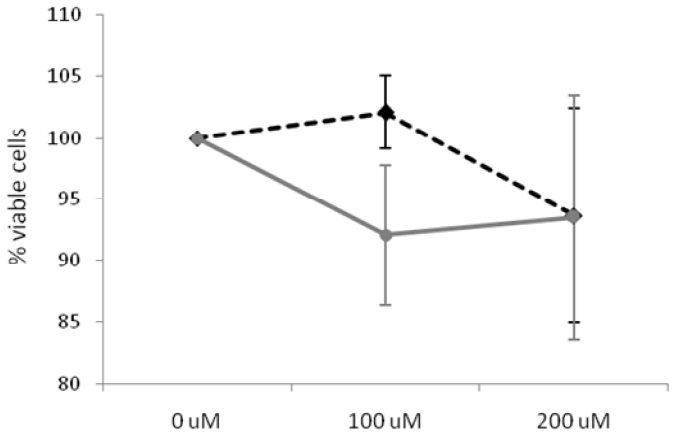

Figure 10. Cell viability assay in SH-SY5Y in response to H_2O_2, with or without ASC-CM. SH SY5Y cells are incubated with 100 μM and 200 μM H_2O_2 for 24 hrs. The control cells (grey, unbroken line) are incubated in basal media (10% FBS/DMEM). The ASC-CM cells (black, dotted line) are culturing for 24 hrs, as long as the interval of H_2O_2 exposure. The MTT assay is performed.

Then we evaluated whether the increased number of viable cells in the group treated with ASC-CM and 100 μM H_2O_2 was related to a decreased of apoptotic rate. SH-SY5Y are grown in the presence of 100 μM H_2O_2 in ASC-CM, fibroblast-CM or basal medium for 48 hrs. The presence of ASC-CM indeed protected SH-SY5Y results from

apoptosis, with a fold increase of caspase 3^+ cells of 1.89 ± 1.09% (while fibroblast-CM was 3.31 ± 0.46% and basal 2.86 ± 1.21%) (Figs. 11, 12).

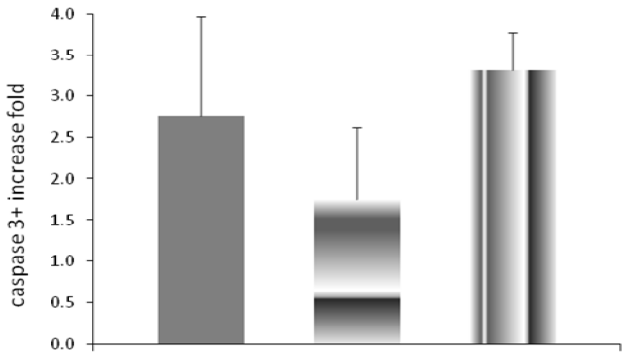

Figure 11. SH-SY5Y apoptosis evaluation after culturing in ASC-CM (horizontal filling) and fibroblast-CM (vertical filling) during 24 hrs 100 µM H_2O_2 exposition. Cell in basal media (grey). The caspase 3^+ cells are reported as the average of cell positive for one field. Each value represents mean ± SD of three independent experiments.

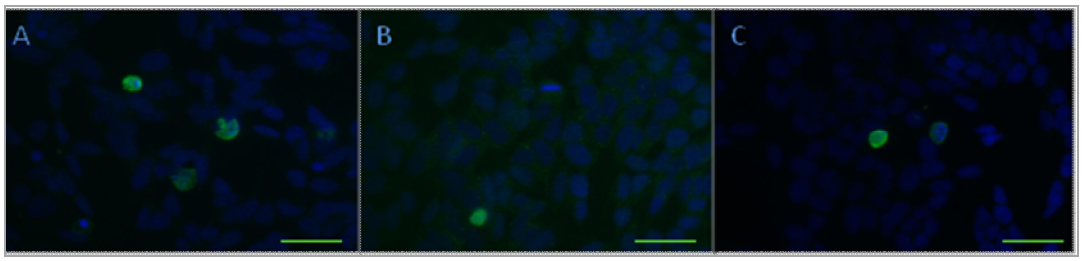

Figure 12. SH-SY5Y apoptosis evaluation after H_2O_2 exposition, culturing in basal medium (A), ASC-CM (B) and fibroblast-CM (C) for 24 hrs. The caspase 3 stains green; DAPI (blue) marks the nucleus. Scale bar: 50 µm.

PRODUCTION OF NEUROTROPHINS BY ASC

We next sought to determine whether BDNF, bFGF, CNTF and PDGF-AB are produced by human ASC and if this can explain the diverse neuroprotective effect from fibroblasts. ELISA assay in supernatants demonstrated significant amounts of BDNF secreted by ASC, but not from fibroblast (64.7 ± 10.58 pg/ml vs 0 pg/ml). Low amounts

of bFGF are synthesized by both cells types (Fig. 13), while CNTF and PDGF-AB are absent.

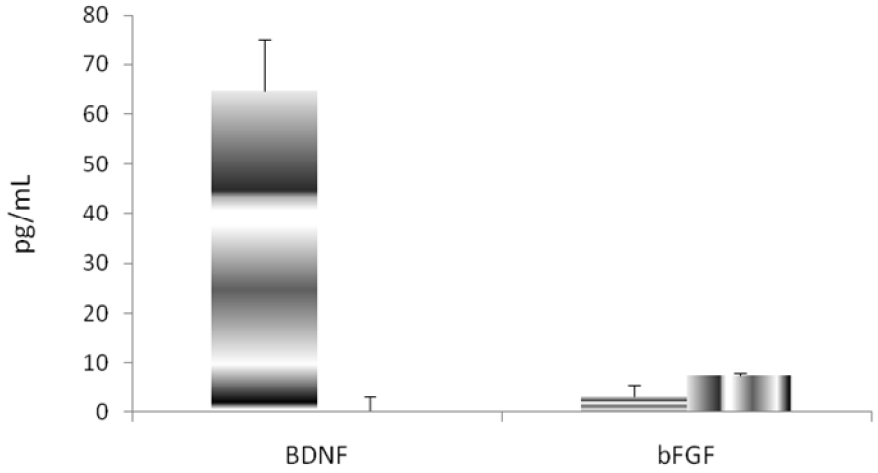

Figure 13. BNDF, bFGF, CNTF and PDGF-AB are quantified by ELISA, and reported as concentration (pg/ml) in ASC (horizontal filling) and fibroblast (vertical filling) supernatants.

DISCUSSION

This study raises from the interest of evaluating the ability of human ASC to differentiate in neuronal cells. Later on, we investigated their potential to exert neuro-protection. The source of these cells make them further attractive because they are easily obtained in large amount by human fat with liposuction and they are highly proliferative in culture (our data; *Dennis and Charbord, 2002; Zuk et al, 2002; Romanov et al, 2005; Katz et al, 2005; Lee et al, 2004; Kern et al, 2006; Strem et al, 2005*).

Although some authors have already studied the neural differentiation from ASC *(Safford et al, 2004; Kang et al, 2005; Ashiijan et al, 2003)*, the complete differentiation has not been demonstrated; rather, these studies show unspecific neural modification, with co-existence of neuronal and glial features. More data are available about ASC toward peripheral glial cell *(Radke et al, 2009; Xu et al, 2008; Kingham et al, 2007)*.

We first applied a chemical induction protocol *(Woodbury et al, 2000)* on human ASC, to consider if they behave similarly to BM-MSC: ASC modify into 3 sub-populations different for morphology and phenotype: one neuron/astrocytic-like expressing neural markers, the second oligodendrocyte-like, selectively reactive for Gal-C and the remaining sub-population is undifferentiated. The co-expression of neural markers of different lineages in the former group, some atypical immunochemical pattern (both cytoplasmic and nuclear staining for Neu-N) and the blunt onset and very short-lasting modifications raise doubts about the authentic modification (*Krampera et al, 2007*).

Our data are quite concordant with studies published from other groups, that alternatively report the immunocytochemical or the immunoblot profile (*Safford et al, 2002; Safford et al, 2004; Tholpady et al, 2003*). As we mentioned in the introduction,

DMSO/BHA treatment does not activate neural genes in BM-MSC, and it can induce very similar morphological change in keratinocytes and fibroblasts as in MSC (*Woodbury et al, 2000; Lu et al, 2004; Bertani et al, 2005; Neuhuber et al, 2004*). In a more in-depth probe of changes in the transcriptome (21,000-gene microarray analysis), Bertani et al. showed that the stimulation with the BHA/DMSO protocol for 6 hrs and 48 hrs has little effect on the overall transcription profile of BM-MSC cells. The treatment also does not significantly change the levels of certain neural-associated markers already expressed by MSC *(Bertani et al, 2005)*. These findings suggest that the morphological changes resembling neural differentiation observed with the chemical induction protocol can largely result from a fast-onset cellular response to chemical toxicity, and not from a differentiation program.

Then we decided to use a medium more similar to the natural CNS environment, considering either some of the fundamental molecules involved in neural survival and plasticity, following a previously described protocol *(Hermann et al, 2004)*.

RA plays a key role in neurogenesis during the embryogenesis and later on during the remodeling following a brain insult; moreover, it induces the expression of the BDNF receptor *(Glaser and Brustle, 2005)*. BDNF regulates the neuronal proliferation, migration and maturation; in addition it is involved in translating the activity signal into synaptic plasticity changes. Such neurotrophin is produced by both neural elements and endothelial cells of CNS, dependently to environment insults. MSC exhibit the specific receptors and are able to synthesize BDNF *(Zhao et al, 2004; Tapia-Arancibia et al, 2004; Crigler et al, 2006).* Neural induction media using different combination of RA and BDNF with or without other agents have been experimented on BM-MSC by several groups (*Sanchez-Ramos et al, 2000; Padovan et al, 2003; Zhao et al, 2004; Dezawa et al, 2004; Egusa et al, 2005; Cho et al, 2005; Kohyama et al, 2001; Jiang et al, 2002; Jiang et al, 2003; Long et al, 2005; Kondo et al, 2005)*, but only few studies

have been performed on ASC (*Kang et al, 2004; Romanov et al, 2005*). In particular, RA/BDNF do not dramatically modify BM-MSC phenotype after 14 days (*Sanchez-Ramos et al, 2000*), but the transfection of the BDNF gene in BM-MSC and the following treatment with RA induce non selective neural markers and, more interestingly, the formation of the voltage-dependent K^+/Ca^{2+} channel (*Zhao et al, 2004*).

Our prolonged neuronal protocol derived from the observation that neurosphere medium, traditionally used for NSC (*Reynolds and Weiss, 1992)*, can prompt also MSC toward very early neural commitment. Hermann and colleagues used sequentially neurosphere medium and RA/BDNF: the BM-MSC acquire neuronal morphology and phenotype, but there are no electrophysiological confirmation of such transformation (*Hermann et al, 2004*). Another group employed BDNF after the neurosphere-step to prime ASC, obtaining the concomitant expression of neuronal and glial markers at transcriptional and protein level (*Kang et al, 2004*).

To our knowledge, this is the first demonstration that human ASC, submitted to two-step protocol (neurosphere medium and consecutive RA/BDNF neuronal induction) show selectively early neuronal behavior in vitro. ASC in culture with EGF and bFGF aggregate in floating neurosphere-like, morphologically very similar to NSC neurosphere. ASC strongly exhibit nestin and MAP-2, as previously reported (*Bunnell et al, 2006*), suggesting that this step represents the neural pre-commitment necessary to obtain the response to the following induction medium. RA and BDNF medium induces a high proportion (57%) of ASC to acquire a neuronal phenotype: they are PSA-NCAM, Neu-N, MAP-2 positive and lack the expression of glial markers as GFAP and S-100, remaining weakly positive for nestin. In addition, they display a number of features of more mature neuronal phenotype, with expression of TH and α subunit of GABA-A

receptor, but not of Na^+ channel. These data confirm a previous study on the neurotransmitter enzyme profile of ASC (*Safford et al, 2004*).

In line with this immunophenotype, the electrophysiological analysis confirms the early selective neuronal differentiation: ASC with neuronal features displayed resting membrane potential close to −60 mV, outward currents blocked by TEA, suggestive of delayed-rectifier type K^+ currents, as well as voltage-dependent Na^+ currents selectively inhibited by TTX. We detected these features exclusively in the ASC with neuronal morphology and phenotype, and not in basal conditions or in ASC not responding to neural differentiating stimuli. Although K^+ currents are also related to proliferation and migration in other type of cells *(Fantozzi et al, 2006)*, the presence of Na^+ currents and the negative resting membrane potential are highly neuronal specific. Despite these features, the amplitude of the current is not enough to produce an action potential or to react to GABA or glutamate. These results are strongly meaningful, comparing to basal ASC features, that show resting membrane potential around –30 mV, very low K^+ current amplitude and virtually absent Na^+ currents. Basal electrophysiological ASC properties are not fully concordant with a previous report that describe up to 70% basal ASC with different K^+ currents and 7% with Na^+ currents, analyzing third passage cells (*Bai et al, 2007)*. The absence of immunoreactivity for Na^+ channel in differentiated ASC showing inward currents sensitive to TTX by patch-clamp is probably related to the different sensitivity between these two procedures, as already described *(Dezawa et al, 2004).* On the other hand, the induction of Na^+ current has been already described in BM-MSC treated with RA and/or other growth factors (*Hung et al., 2002; Egusa et al., 2005; Dezawa et al, 2004; Cho et al, 2005; Tropel et al, 2006*). The extent of the current was otherwise significant only after co-culture of BM-MSC with fetal astrocyte or cerebellar granule (*Jiang et al, 2003; Wislet-Gendebien et al, 2005b*) or Notch transfection followed by CNTF, NGF and BDNF treatment (*Dezawa et al, 2004*) or

very high concentration of RA (*Cho et al, 2005*). Nowadays the unique electrophysiological data regarding the neural ASC differentiation is related to the delayed-rectifier type K^+ currents induced by IBMX and indomethacin for 14 days (*Ashjian et al, 2003*).

The incomplete neuronal differentiation observed in our and other studies suggests that ASC might exert their therapeutic efficacy via mechanisms alternative to cell-replacement, as already proposed by BM-MSC. We and other authors have very recently reported that ASC in vivo model can not differentiate in neural cells *(Constantin et al, 2009; Kim et al, 2007*), in contrast with few others documenting the early neural differentiation in CNS *(Kim et al, 2009; Lee et al, 2009)*. Noteworthy, irrespective of the exact cellular mechanism, in all these studies MSC application was clinically beneficial.

An alternative explanation for this beneficial effect involves the ability of MSC to produce a wide range of potentially neuroprotective factors or by their anti-oxidant action *(Lanza et al, 2009; Crigler et al, 2006; Wilkins et al. 2009; Hokari et al, 2008; Parr et al, 2007).* It is conceivable that the final therapeutic effect comes from the complex interaction between the transplanted cell and the environment and that multiple mechanisms are involved. In this regard, we evaluated the ability of ASC to protect neuron using an in vitro model of oxidative stress, in which a neuroblastoma cell line is exposed to H_2O_2 *(Sancivens et al, 2006)*. Neuroblastoma cell lines are a classic model to study neuronal biological responses to different type of insults. H_2O_2 is the main endogenous ROS generated by several enzymes as peroxide dismutase, glucose oxidase and monoamine oxidase. The cytotoxic effect of H_2O_2 is thought to be caused by hydroxyl radicals generated from iron-catalyzed reactions, causing subsequent damage to DNA, proteins and membrane lipids. In particular, H_2O_2-induced cell death in neuroblastoma cell line involves an apoptotic mechanism dependent on caspase 3 and

calpain activation, mediated by AKT and JNK activity *(Sebastià et al, 2006; Sancivens et al, 2006).* The final effect of apoptosis in neural cells is mediated by p53 pathway *(McNeill-Blue et al, 2006).*

We tested the protective effect of ASC-CM on neuroblastoma, as compared to basal and fibroblast-CM. After 24 hrs H_2O_2 exposition, the number of neuroblastoma cells was significantly reduced, regardless H_2O_2 concentration. Interestingly, the ASC-CM reverted the number of cells exposed to 100 μM of H_2O_2 to the basal levels, thus displaying a true protective effect. We then investigated the death/survival pathway involved in neuro-protection against H_2O_2 stress, finding that ASC-CM exhibit anti-apoptotic function with reduction of caspase 3^+ cells, as compared to fibroblast-CM and basal media. Although the high proliferation rate of the neuroblastoma cell line does not reproduce the biology of neuronal cells, the data about the anti-apoptotic effect of ASC-CM are quite intriguing. In fact, neuronal apoptosis is critical for the pathogenesis of neurological diseases *(Prunell and Troy, 2004)*; and this process as well as neuronal proliferation and survival is highly regulated by neurotrophic factors, which are also particularly important in the pathogenesis of neurodegenerative disorders.

Such results are concordant with a very recent study *(Lee et al, 2009)*, supporting the role of secreted factors by human and rat ASC *(Wei et al, 2009a; Wei et al, 2009b)* and human BM-MSC *(Hokari et al, 2008; Wilkins et al, 2009; Kemp et al, 2010).* An indirect evidence of the anti-oxidant effect by ASC is remarked in therapeutic model of EAE reported by our group *(Constantin et al, 2009)*: 72 days after systemic ASC injection in EAE mice, spinal cord neurons show significantly lower apoptosis (caspase 3^+ cells) than the control ($13.28 \pm 1.41/mm^2$ vs $1.95 \pm 0.82/mm^2$). Such data are concordant with those from other groups, which reported the reduction of apoptotic neuron after ASC treatment in other animal models *(Kim et al, 2007; Lee et al, 2009; Wei et al, 2009a).* Similarly, other groups reported the modulation of the endogenous

anti-oxidant enzyme activity by MSC in vivo *(Lanza et al, 2009; Lee et al, 2009)*, in addition to their ability to release growth factors. To assess if such oxidative stress is detrimental for ASC, we treated the cells at the same conditions of neuroblastoma, and we report no increase of the apoptosis. From these data, we suppose that ASC can survive easier in neural stressful environment than the neuron, although the MSC long-term survival is variable in different animal models *(Kim et al, 2007; Lee et al, 2003; Gerdoni et al, 2007; Zhang et al, 2009)*.

We then test the potential effect of secreted molecules by ASC to induce an early neural differentiation on neuroblastoma cells. Neurite formation of neuroblastoma is significantly influenced by ASC-CM (as compared to fibroblast-CM or basal medium), in line with other studies on BM-MSC; in particular, human BM-MSC can induce neurite outgrowth on neuroblastoma cells (either RA-induced or not) *(Crigler et al, 2006; Croft and Przyborski, 2009)*. Noteworthy, the only study with ASC reported no difference between culturing neuroblastoglioma with ASC-CM or not *(Kingham et al, 2007)*. It is reasonable that such discrepancy may be related to different methodological approaches. We show that human ASC, but not fibroblasts secrete high amounts of BDNF, as reported for human BM-MSC *(Crigler et al, 2006; Neuhuber et al, 2005)*. Surprisingly, we detected higher level of BDNF compared to what mostly reported by literature *(Neuhuber et al, 2005; Jiang et al, 2010; Crigler et al, 2006; Wilkins et al, 2008; Wei et al, 2009a)*. Since BDNF receptors are present in neuron and in neuroblastoma *(Tapia-Arancibia et al, 2004; Brodeur et al, 2009)*, we hypothesize that the protective action of ASC may be partially mediated by BDNF *(Crigler et al, 2006; Wilkins et al, 2008)*. In addition to BDNF, moderate levels of bFGF, but not CNTF and PDGF-AB were detected in supernatants from ASC. In addition to the paracrine effect of growth factors, the communication with the resident cells and the cell-cell contact may strength the neuroprotective effect of MSC in vivo.

Taken together, our results suggest that neuronal differentiation of a proportion of ASC is possible, though incomplete and with low-efficiency in vitro. The full neuronal differentiation of MSC in vitro remains an intriguing challenge in the field of stem cells and CNS repair, particularly for neurodegenerative diseases. As a matter of fact, transplantation of MSC may promote CNS repair through the stimulation of endogenous neural precursors and via intrinsic neuroprotective bystander capacities; undifferentiated SC can release, at the site of tissue damage, a milieu of neuroprotective and immunomodulatory molecules, whose release is temporally and spatially orchestrated by environmental needs *(Constantin et al, 2009)*. Since these molecules, acting in a paracrine fashion, are pleiotropic and redundant and 'constitutively' secreted by SC – thus representing a sort of stem cell signature – we can easily explain data showing that stem cells other than NSC, with very low capabilities of neural (trans) differentiation may efficiently promote CNS repair.

The powerful aspect of ASC application is the possibility of autologous transplantation, avoiding any risk of rejection, although it is debated if MSC can be modified in human carrying genetic neurological diseases *(Boucheire et al, 2008; Ferrero et al, 2008).*

In 2008 the report of the induced Pluripotent Stem Cell (iPSC) has been the 'breakthrough of the year' *(Vogel, 2008)*. iPSC are pluripotent cell obtained by the reprogramming of murine skin fibroblast by retroviral expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc), that are able to give rise to all three germ layers *(Takahashi and Yamanaka, 2006).* The generation of iPSC would potentially allow for a source of cellular therapy for auto-transplantation and provide ethically acceptable routes to derive patient-specific stem cells. But the concern about the risk of cancer remains *(Okita et al, 2007)*, due to the use of integrating viruses which, for their oncogenic transgenes and insertional mutagenesis, are potentially tumorigenic.

Recently, alternative techniques for the derivation of iPSC have been developed, including the use of viruses other than retrovirus, non-integrating viruses, episomal expression systems or direct delivery of the reprogramming factors as bioactive proteins.

Wernig et al. have fascinatingly opened the door to specific neural application: iPSC from murine fibroblast, transfected with lentivirus with three neural-lineage-specific transcription factors can generate (up to 19.5%) functional neurons in vitro, displaying trains of action potentials and synapses with ex vivo cortical neuron *(Vierbuchen et al, 2010).* The fast conversion in neuron from iPSC can represent a promising strategy to get neural replacement, avoiding the tumorigenic pluripotent stem cells. Despite rapid progress in the field of reprogramming, several obstacles need to be overcome before iPSC can be considered in a clinical context. First, standardized protocols have to be developed. Second, genetic and epigenetic instability will also be highly relevant for the use of iPSC. Third, the optimum source of somatic cells used for reprogramming has not yet been identified. With these considerations in mind, we believe that cell therapy with iPSC will represent the new frontier for the regenerative medicine.

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ABBREVIATIONS

ASC	Adipose-derived Stem Cell
ASC-CM	ASC-Condition Media
bFGF	basal Fibroblast Growth Factor
BM	Bone Marrow
BME	βmercaptoetanol
BM-MSC	Bone Marrow-MSC
BHA	Butylatedhydroxyanisole
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
dbcAMP	dibutiril cAMP
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
EGF	Epidermal Growth Factor
ESC	Embryonic Stem cell
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem cell
IGF1	Insulin Growth Factor 1
IBMX	Isobutylmethylxanthine
iPSC	induced Pluripotent Stem Cell
ISCT	International Society for Cellular Therapy
mAb	monoclonal antibody
MAP-2	Microtubule-Associated Protein-2
MSC	Mesenchymal Stem Cell
NB	Neurobasal
Neu-N	Neuronal Nuclear antigen
NGF	Nerve Growth Factor
NSC	Neural Stem Cell
PD	Parkinson Disease
RNS	Reactive Nitroxide Species
ROS	Reactive Oxidative Species
RT-PCR	Reverse transcription-Polymerase Chain Reaction
SCI	Spinal Cord Injury
TEA	Tetraethylammonium
TH	Tyrosine Hydroxylase
TTX	Tetrodotoxin
VEGF	Vascular Endothelial Growth Factor