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"Development of cryopreservation of human neural tissue as strategy to produce clinical-grade stem cells"

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“Le cellule nervose acquistavano ai miei occhi
una individualità che non siamo soliti attribure loro”

Rita Levi Montalcini

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ABSTRACT

The Advanced Therapy Production Unit of the Institute for Stem-cell Biology, Regenerative Medicine and Innovative Therapies (ISBReMIT), headed by prof. Angelo Luigi Vescovi, located in San Giovanni Rotondo (FG – Italy), is a Cell Factory authorized by the competent Ethic Committee to isolate human neural stem cells (hNSCs) according to a Good Manufacturing Practice (GMP) grade protocol, from brain specimens of fetuses died for natural causes in utero death or miscarriage, for research and clinical applications for neurodegenerative diseases. The very same hNSCs have already been used in a phase I clinical trial on Amyotrophic Lateral Sclerosis (ALS) patients, concluded and conducted in Azienda Ospedaliera Santa Maria di Terni (Mazzini L. et al., 2015). In this study hNSCs were delivered into the spinal cord showing no major complications due to transplant procedure; moreover, in a very similar study, conducted by prof. Boulis and colleagues in Atlanta, cells that were detected up to 30 months after transplant even in patients showed a transient improvement of motor function (Boulis et al. 2011; Riley J.P. et al. 2011, Riley J. et al. 2014, Glass J.D. et al. 2012, Feldman E. et al. 2014, Tadesse T. et al. 2014). We are now planning a Phase II clinical trial on ALS and a Phase I on SPMS patients is currently on-going. Considering the source of hNSCs, the starting material is very rare and precious; in addition, since the spontaneous abortion may occur at any and unpredictable times and our manufacturing method requires that fetal human brain tissue specimens should be received and transferred into GMP facility to be immediately processed, this could involve a heavy workloads and, if there is unavailability of suitably trained staff, waste of precious tissue donations. Therefore, it is really important to find a way to optimize the production process of hNSCs for the development of future clinical trials. We hypothesize that block of fetal human brain tissue can be cryopreserved and that neural stem cells with a high post-thawing viability could be recovered at later time. To our knowledge, currently there is no description of a specific clinical-grade protocol for the cryopreservation of human brain tissue. Dimethyl sulfoxide (DMSO) is the most frequently used as cryoprotectant agent in clinical setting but there is no consensus on its optimal concentration in cellular or tissue products. Defining the right concentration is important, because DMSO is cytotoxic. Historically, 10% of DMSO is widely used for cell cryopreservation. Our Translational Advanced Therapy Research Center follows the "standard" of 10% for cell cryopreservation (Gelati M. et al. 2013); however, the recent trend is reducing DMSO concentration. We report here a GMP protocol for successful freezing of

pieces of tissue derived from human fetal brain subventricular zone brain, testing three different percentages of DMSO in freezing medium. Cellular assays show that the cell cultures derived from frozen tissue are equivalent to those cultures derived from fresh tissue with no significant difference among 5%, 8% and 10% of DMSO solutions compared with fresh tissue. Moreover, our results argue that freezing of tissue up to 5 days at -80°C and up to 2 and 9 months in liquid nitrogen does not markedly alter cell viability and multipotency of NSCs. From our preliminary data, we conclude that cryopreservation tissue allows to create a system of bio-banking of stem cells for our restorative therapy, granting necessary safety and quality control standards.

1. INTRODUCTION

1.1 Neural Stem Cells: historical note

Over the last decades, number of studies have shown that the adult brain retains the ability to self-renew some of its neurons. It has long been thought that the adult mammalian central nervous system (CNS) does not regenerate after injury (Ramòn y Cajal S., 1913). In 1950 a new method was developed to mark cells in proliferation based on the detection, by self-radiography, of 3H-thymidine, which is incorporated into DNA in replication during the S phase of the cellular cycle (Sidman R.L., 1970). In the early 1960s, Altman et al., by combining the production of brain lesions in rats with intracranial injection of thymidine-H^3, showed the persistence of cell in proliferation in adult rats near the lateral ventricle wall and olfactory bulb (Altman J., 1962). In following years, another germinal zone near hippocampus was discovered (Altman J. and Das G.D., 1965; Altman J. and Das G.D., 1966; Kaplan M.S. et al., 1977; Bayer S.A. et al., 1982). Results on mammalians were carried out, later, on non-human primate. Within the past two decades, technical advances, particularly the fate mapping method using BrdU in animals, have allowed researchers to demonstrate that a large number of newly generated cells in the adult brain were indeed neuron. In 1992, the discovery of the existence of NSCs in the adult rodent brain by Weiss and Raynols (Reynolds B.A. et al., 1992) and the initial isolation of hNSCs by Vescovi lab (Vescovi A.L. et al., 1999), have eventually provided a solution to studies on neurogenesis, thus paving the way to the implementation of perspective cell therapy application using the brain's own stem cells derived from iPS produced with autologous fibroblasts.

1.2 Neurogenesis and characteristic of Neural Stem Cells

NSCs are immature cells present in the mammalian developing and adult CNS. Typically, NSCs are defined by three cardinal characteristics: *self-renewal potential*, *multipotency* and competence for *in vivo regeneration* (Conti L. and Cattaneo E., 2010). They have the potential to generate both neurons and glia of the developing brain and they also account for the limited regenerative potential in the adult brain. In the adult CNS, NSCs reside in defined regions "neurogenic niches" that sustain their multipotency and regulate the balance between symmetrical self-renewal and fate-committed asymmetric divisions (Fuentealba L.C. et al., 2012). These are specialized niches located in the SVZ of the lateral ventricle wall (Sanai N. et al., 2011; Wang C. et al., 2011) and in the SGZ of the dentate gyrus of the hippocampus (Knoth R. et al., 2010). Whether NSCs reside in other regions of the adult mammalian brain is still debated (Palmer T.D. et al., 1997; Weiss S. et al., 1996; Seri B. et al., 2006). Neuroblasts produced in the rodent SVZ migrate to the olfactory bulb following the RMS, an anatomic structure well characterized in the rodent brain. The NSCs located in the SVZ, also called type B cells, generate actively dividing intermediate cells, named type C cells (Doetsch F. et al., 1999), which further divide giving rise to neuroblasts, referred to as type A cells that migrate away from the SVZ. These migrating neuroblasts are organized in chains that connect the SVZ to the olfactory bulb (constituting the RMS) where they gradually mature into functional GABAergic granule neurons. Fate-mapping studies actually reveal that type B cells are not developmentally restricted to neuronal lineages but can give rise also to glial progenies, suggesting they are authentic tripotent NSCs. The second germinal zone of the adult mammalian brain is the dentate gyrus of the hippocampus. Astrocyte-like NSCs, called type I progenitors, have been identified within the SGZ facing the dentate gyrus hilus. They share several properties with the type B cells of the adult SVZ, although they apparently exhibit a narrower developmental potential. Type I progenitors likely divide asymmetrically to produce immature proliferating progenitors, type II cells. These gradually differentiate into migrating neuroblasts (Berg D.K. et al., 2015) that travel into the granule cell layer of the dentate gyrus, where they progressively mature into functional granule neurons. Differently from the type B cells of the SVZ, the progeny of type I progenitors does not migrate long distances, but remains localized in clusters closely connected to the parent cell. Additionally, hippocampal NSCs appear to be developmentally restricted to become granule neurons; currently, there is no evidence that type I progenitors can generate mature glial derivatives *in vivo* (Fig.1).

Moreover, it's known that the fate of NSCs is clearly under tight environmental control. In fact, intrinsic (Hack M.A. et al., 2005; Kohwi M. et al., 2005; Waclaw R.R. et al., 2006) and extrinsic signals play important roles for the regulation of neural stem cell fate (Galli R. et al., 2000). In the early '90s, the identification of EGF and basic FGF-2 in human and rodent as key mitogens for NSCs led to set up culture conditions that support extended cell division of cells with NSCs properties (Reynolds B.A. et al., 1992). It is noteworthy that there are significant differences in the overall pattern of epigenetic stimulation required to achieve extensive self-renewal in stem cell cultures from humans compared to rodents. While either FGF2 or EGF alone are sufficient to support the long-term proliferation and expansion of mouse and rat cells, the simultaneous presence of both factors is an absolute requirement for the extensive expansion of human CNS stem cells (Vescovi A.L. et al., 1993; Gritti A. et al., 1996), as well as the absence in culture of this factors directs to the differentiation of hNSCs in neurons (about 10-15%) in astrocytes (about 60-70%) and in oligodendrocytes (about 1-4%) (Johe K.K. et al., 1996).

Stem cells are notoriously difficult to identify. For a long time, human NSCs have been considered a quiescent population of undifferentiated and homogeneous cells. Instead, studies demonstrate that neurosphere forming cells are ultra-structurally heterogeneous and express different neural lineage-specific markers that indicate the existence of distinct cellular phenotypes. Besides nestin which is a marker of precursor neural cells (Kukekov V.G., et al., 1999), which were previously described only in extraneural tissue, have been associated with them. This implies variable developmental commitments of parental clone-forming cells (Lobo M.V.T. et al., 2003). For this reason, various methods have been developed to isolate NSCs and characterize their capacity to proliferate and differentiate (Alvarez-Buylla A. et al., 1995; Cattaneo E. et al., 1990; Gange F.H. et al., 1995): NSCs can be isolated by cell sorting based on expression of individual surface antigens such as CD133 (Uchida N. et al., 2000) transcription factors such as Sox-2 and Musashi 1 or considering physical proprieties such as scattering (cell size) and side scattering (granularity) (Murayama A. et al., 2002). So, there is much to be discovered about the specific characteristics of these cells.

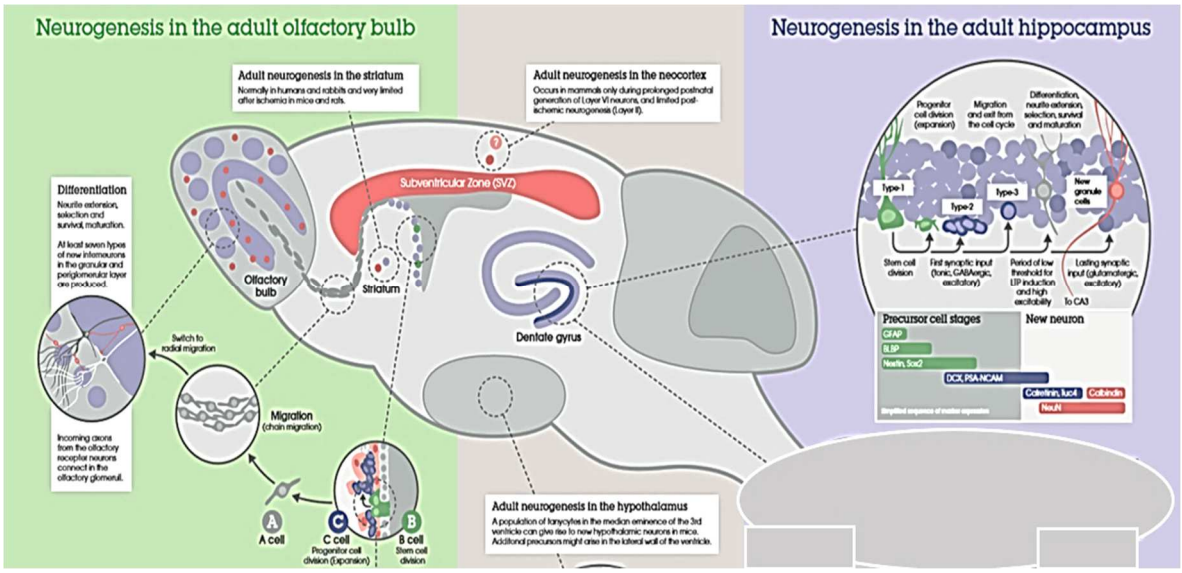

Fig.1. Schematic representation of neurogenic niches in the adult brain: sub ventricular zone neurogenic niche in the in the lateral wall of the lateral ventricles and in dentate gyrus of hippocampus (as well as the striatum and hypothalamus in same species). SVZ niche composed of type B1 cells, that corresponds to neural stem cells, type C cells that rapidly proliferate and type A neuroblasts, which migrate through the rostral migratory stream to the olfactory bulb where they mature into interneurons. Neurogenesis in the SGZ. Radial type 1 cells give rise to type 2 cells that differentiate into type 3 neuroblasts that become immature neurons and then mature into granule neurons that migrate into the granule cell layer. (Copyright © 2018 Modified from Poster *Adult neurogenesis* created by Abcam, 2018).

1.3 Neural stem cell-based treatment for neurodegenerative diseases

The discovery of the existence of NSCs throughout life in animals and then in humans led to rapid recognition of the therapeutic potential of these cells. Over the last decades, a great interest has arisen, overall, in the field of stem cell therapies, given their potential to treat many conditions, where present conventional treatments are inadequate. For example, clinical studies suggest that stem cell–based approaches could be used therapeutically to restore function in ND, which are a global burden, afflicting more than 20 million people worldwide (Brandi K. et al., 2008). Parkinson's, Alzheimer's, Huntington's, Amyotrophic Lateral Sclerosis's, Multiple Sclerosis's disease are the most dominant neurodegenerative diseases and have in common the progressive loss of structure, function or number of neurons, including death of neurons (Hung C.W. et al., 2010; Sakthiswary R. and Raymond A.A., 2012).

However, neurodegenerative diseases require a tailored approach to treat their unique pathology and progression, because each one affect specific brain regions and different cell types (Brandi K.O. et al., 2008), such as the selective loss of dopamine neurons in PD and motor neurons in ALS, or a widespread degeneration of many types of neuron such as occurs in AD (Lindvall O. and Kokaia Z., 2010). Different cell-based approaches can be applied to cure neurodegenerative disease, for instance it might be possible to replace lost neurons or glial cells by transplantation of stem cell–derived neural cells, or cell replacement might also be achieved by inducing endogenous stem cells in patients own brain to form new neurons and glial cells. Besides these mechanisms, grafted stem cells and their derivatives could induce functional improvement by releasing therapeutic molecules that are neuroprotective or modulate inflammation, the paracrine and by-stander effect that add up to cell damaged replacement. (Aboody K. et al., 2011; Brandi K. O. et al., 2008; Pluchino et al. 2009; Rota N. et al. 2010, Ferrari et al. 2012).

A wide range of stem cells from various sources have been widely studied to translate stem cell discoveries into the patient affected by neurodegenerative diseases, including hESCs, MSCs, iPSCS, fetal NSCs (Sakthiswary R. and Raymond, A.A. 2012) (Fig.2). However, many studies have shown that there is no a single stem ideal for all applications: cultured hESCs grow very efficiently and maintain pluripotency but cancer risk remains a significant deterrent to their clinical use; adult stem cells are an attractive source of cells for generating autologous cell transplants but are not practical because they rapidly senesce in culture. Non-neural cells, such as mesenchymal stem cells, can be easily isolated from patients to produce autologous cell transplants but we do not yet know how to convert them into authentic CNS cells, which is the

ultimate goal of restorative therapies. Fetal brain-derived neural stem cells, instead, should be easily expanded and maintained in culture, are not tumorigenic but there are ethical, regulatory and clinical problems in source tissues needed for all individuals that might benefit from their therapeutic use. (Brandi K.O., et al., 2008).

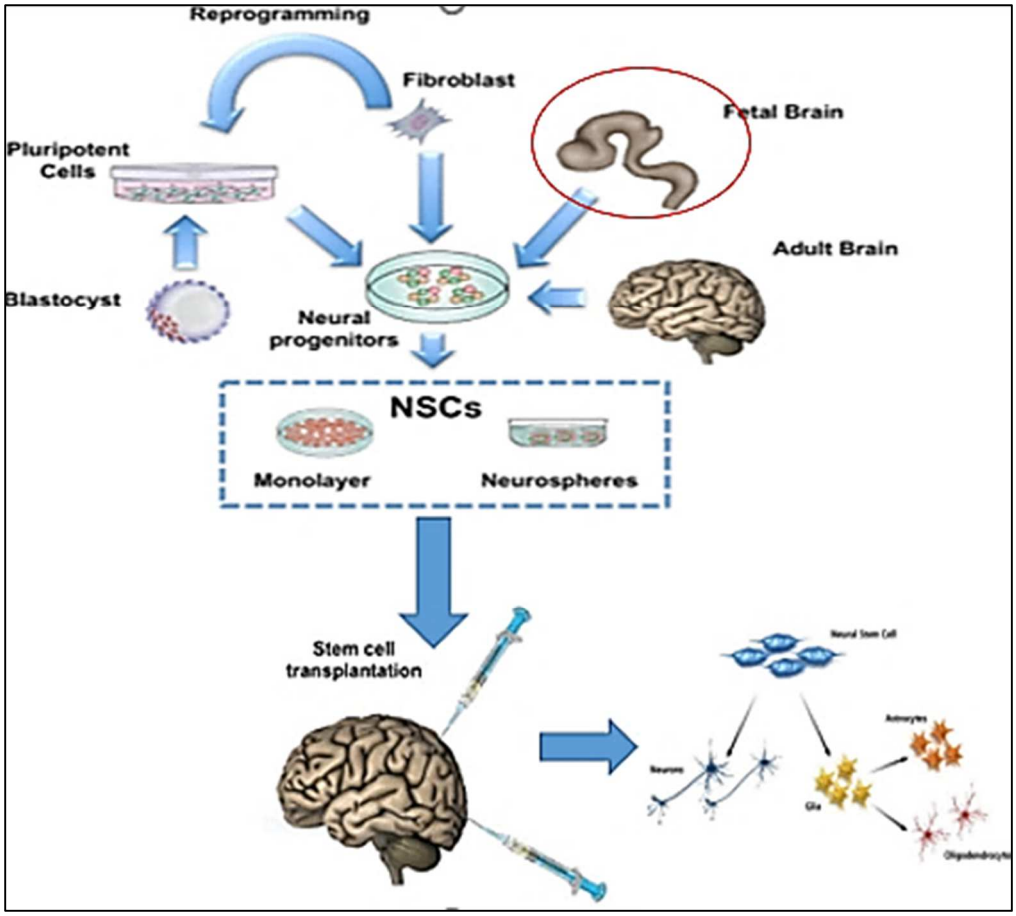

Fig.2. Schematic drawing illustrating the different stem cells sources and their origin that can be used in cell therapy of brain diseases. Human embryonic stem cell lines are derived from the inner cell mass of the blastocyst; fetal brain cells can be obtained from aborted fetuses; induced pluripotent stem cells are derived by reprogramming of differentiated cells such as human fibroblast; mesenchymal stem cells are harvested from cord blood or bone marrow. These different cell types can be differentiated into neuronal precursors that are transplanted into the diseased brain (Casarosa S. et al., 2014).

Therefore, the best type of stem cell pool for regenerative repair of the central nervous system is still yet to be determined and may vary according to the disease or injury.

However, given the high and no tumorigenic proliferative capacity of brain-derived neural stem cells, which permit the generation of cells for many patients from a single donor, it is important to focus on the outcomes of clinical trials testing fetal-tissue-derived allogenic NSCs products for neurodegenerative disease. A large number of studies have explored grafting behavior of several NSCs typologies (and their progeny) in a variety of preclinical studies and in some clinical investigations. According to the website *clinicaltrials.gov*, 880 international clinical trials are employing the use of stem cells for treatment of patients affected by several CNS disorders (Tab.1), among which 37 involving NSCs in the word (Casarosa S. et al., 2014; Barreau K. et al., 2016) (Fig.3). Restricting the search to non-tumor diseases, result only 51 studies with only 27 of these currently open. Interestingly, if we analyze these results more carefully, only 5 studies are actually on going to explore the potential clinical relevance of NSCs while the remaining are testing the regenerative potential of mesenchymal stem cells whose actual usefulness in brain diseases is far from being a solid preclinical reality. Recent in vivo studies have shown that transplanted neural stem/precursor cells display good survival and integration capacity into the damaged brain parenchyma, while also eliciting putative therapeutic effects in different pathological scenarios (Barreau K. et al., 2016; Casarosa S. et al, 2014). In these studies, in addition to integration and differentiation into neurons, astrocytes and oligodendrocytes, transplanted NSCs exerted their beneficial effects through an immunomodulatory action involving both innate and adaptive immune responses, as well secretion of trophic factors and cross correction of missing enzymatic activities.

To date, few Phase I and II clinical studies employing NSCs have been performed, with the main objective to demonstrate safety and practicability and to explore the potential effectiveness of the treatments (Casarosa S. et. al 2014). Among them, is reported the ALS Phase I Italian clinical trial using allogenic fetal brain-derived human NSCs sponsored by the Azienda Ospedaliera Santa Maria (Terni, Italy) in June 2012 and completed in December 2015 (*clinicaltrials.gov identifier no. NCT01640067*).

This clinical trial, thanks to an Italian technique, developed in 1996 by A. Vescovi, professor of cell biology at the University of Milan-Bicocca and currently Director of IRCCS “Casa Sollievo della Sofferenza” of Saint Pio (San Giovanni Rotondo), was the first treatment in the world that uses GMP-grade fetal human neural stem cells free from any ethical issue, since they are derived from a fragment of brain tissue taken from a single fetus died from natural causes,

using a procedure similar to that of the voluntary organs donation in the adult (Mazzini et al., 2015).

Even though this technique has now been freed of any ethical concerns arising from the use of human fetal tissue, limited availability of human fetal brain tissue represent still a barrier to further development in clinical trials.

NCT number	Title	Recruitment	Conditions	Interventions	Sponsor/Collaborators	Phases	Enrollment	Start date	Completion date
NCT00337636	Study of HuCNS SC Cells in Patients With Infantile or Late Infantile Neuronal Ceroid Lipofuscinosis (NCL)	Completed	Neuronal Ceroid Lipofuscinosis	Biological: HuCNS-SC	StemCells, Inc.	Phase 1	6	May 2006	September 2009
NCT01005004	Study of Human Central Nervous System (CNS) Stem Cells Transplantation in Pelizaeus-Merzbacher Disease (PMD) Subjects	Completed	Pelizaeus-Merzbacher Disease	Biological: HuCNS-SC cells implantation	StemCells, Inc.	Phase 1	4	November 2009	December 2012
NCT01151124	Pilot Investigation of Stem Cells in Stroke	Active, not recruiting	Stroke	Biological: CTX0E03 neural stem cells implantation	ReNeuron Limited	Phase 1	12	June 2010	March 2015
NCT01217008	Safety Study of GRNOPC1 in Spinal Cord Injury	Completed	Spinal Cord Injury	Biological: hES-derived GRNOPC1 implantation	Asterias Biotherapeutics, Inc.	Phase 1	5	October 2010	July 2013
NCT01238315	Safety and Efficacy Study of HuCNS-SC in Subjects With Neuronal Ceroid Lipofuscinosis	Withdrawn	Neuronal Ceroid Lipofuscinosis	Biological: HuCNS-SC cells implantation	StemCells, Inc.	Phase 1	0	November 2010	April 2011
NCT01321333	Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Patients With Thoracic Spinal Cord Injury	Active, not recruiting	Thoracic Spinal Cord Injury|Spinal Cord Injury|Spinal Cord Injury Thoracic|Spinal Cord Trauma	Biological: HuCNS-SC cells implantation	StemCells, Inc.	Phase 1-2	12	March 2011	December 2015
NCT01348451	Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis	Active, not recruiting	Amyotrophic Lateral Sclerosis	Biological: human neural spinal cord stem cells implantation	Neuralstem Inc.	Phase 1	18	January 2009	August 2014
NCT01640067	Human Neural Stem Cell Transplantation in Amyotrophic Lateral Sclerosis (ALS)	Completed	Amyotrophic Lateral Sclerosis	Biological: Human Neural Stem Cells implantation	Azienda Ospedaliera Santa Maria, Terni, Italy|Azienda Ospedaliero Universitaria Maggiore della Carita|Università di Padova Italy	Phase 1	18	December 2011	September 2016
NCT01725880	Long Term Follow Up of Transplanted Human Central Nervous System Stem Cells (HuCNS-SC) in Spinal Cord Trauma Subjects	Enrolling by invitation	Spinal Cord Injury	Observation	StemCells, Inc.	Phase 1-2	12	November 2012	March 2019
NCT01730716	Dose Escalation and Safety Study of Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis	Enrolling by invitation	Amyotrophic Lateral Sclerosis	Biological: Human spinal cord stem cell implantation	Neuralstem Inc.	Phase 2	18	May 2013	April 2014

Tab.1. Advanced therapies/regenerative medicine. Examples of international studies, phase I/II, using neural stem cells, last 6 years. Highlighted ALS Phase I clinical trial using allogenic fetal brain-derived human NSCs has been sponsored by the Azienda Ospedaliera Santa Maria (Terni, Italy) in June 2012. This study, under the direction of prof. A.L. Vescovi, has included a total of 18 ALS patients that was treated with intraspinal implanted allogeneic human fetal-derived stem cells. (*clinicaltrials.gov identifier no.NCT01640067*).

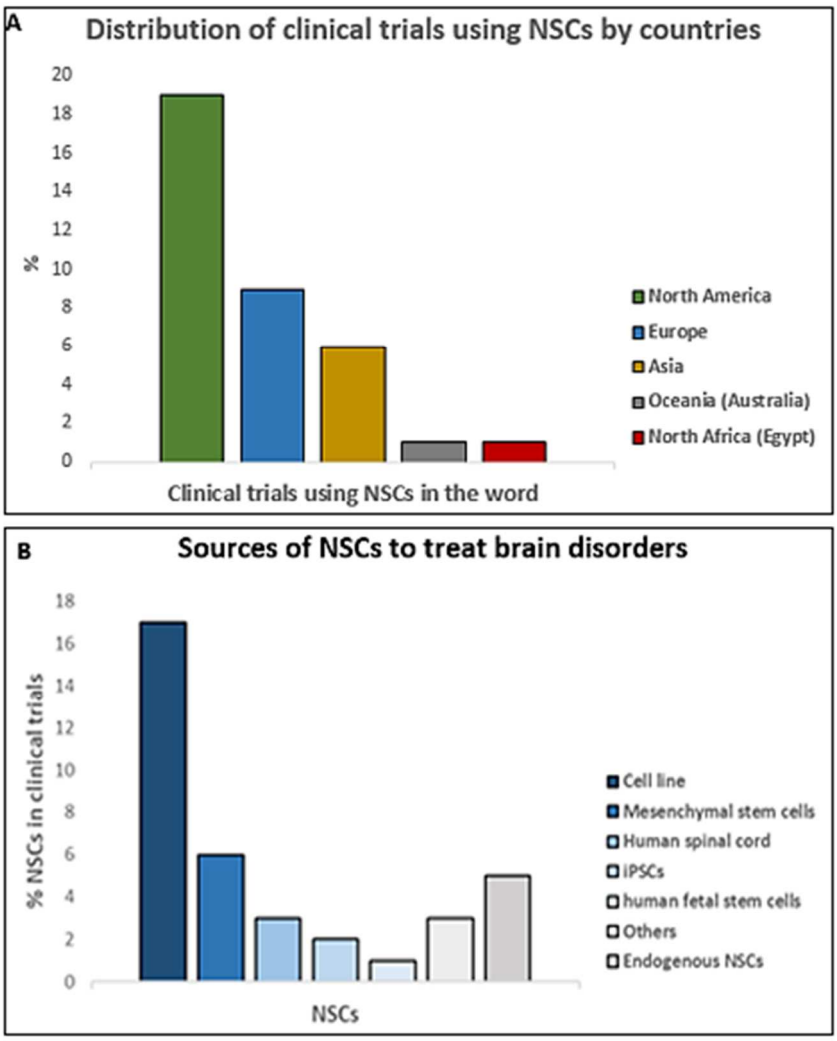

Fig.3. Clinical trials using NSCs in the word. (A) Distribution of clinical trial using NSCs in the word. (B) Trials using endogenous or derived-neural stem cells. Most clinical trials used NSCs derived from different cell lines (HuCNSSC®, CTX DP, HB1.F3.CD, hCE1m6, ISC-hpNSC, AST-OPC1). Other studies also used mesenchymal stem cell-derived NSCs, human spinal cord-derived NSCs, iPSCs or human fetal-derived NSCs. Only five clinical trials involved endogenous NSCs (Barreau K., et al., 2016)

1.4 Neural Stem cells as Advanced Therapy Medicinal Product: Regulation 1394/2007

Recently, pharmaceutical and biotechnology companies have taken an increased interest in stem cell biology, driving the promise of novel regenerative therapies into clinical trials. So, the advances in science and research have yielded a new class of innovative and complex biological medicinal products whose pharmacological activity is derived from modified somatic cells.

Somatic cell therapy products, tissue-engineered products or genes therapy products and tissue or cells associated to a device "combined products" are the frontier of medicine and were called ***Advance Therapy Medicinal Products***. According to Directive 2001/83/EC, harmonized by REGULATION (EC) No 1394/2007 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 13 November 2007 on advanced therapy medicinal products (amending Directive 2001/83/EC and Regulation (EC) No 726/2004), ATMPs are classified as follow:

sCTMPs contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor. Alternatively, is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues

GTPs contain genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting recombinant genes into the body, usually to treat a variety of diseases, including genetic disorders, cancer or long-term diseases.

CATMPs contain one or more medical device as an integral part of the medicine, such as cells embedded in a biodegradable matrix or scaffold.

TEPs are cells or tissues that have been modified for regenerating, repairing or replacing human tissue (Directive 2001/83/EC).

Nowadays, in Italy ATMPs represent a research field with excellent results. In fact, Italy is leader in Europe, since three ATMP at present on the European market have been completely developed by Italian researchers and are produced in Italy. Among those, Holoclar was the first Italian market-authorized ATMP in Europe based on stem cells.

While ATMPs represent a significant tools for efficacious treatments in patients suffering for diseases with limited or absent therapeutic options, this products involve specific risks that need

to be accounted for an appropriate regulatory framework. So, ATMPs have the same stringent conditions required for drugs therapies before they are placed on the market. Since the very beginning of the ATMP development, all the production entities have complied with applicable regulatory requirements and are regularly controlled by Competent Authorities. The legal and regulatory framework for ATMPs in the European Union (EU) was established by the EU Commission on October 2007 (Regulation EC No.1394/2007) and first applied in European Union since 30 December 2008 (Fig. 4).

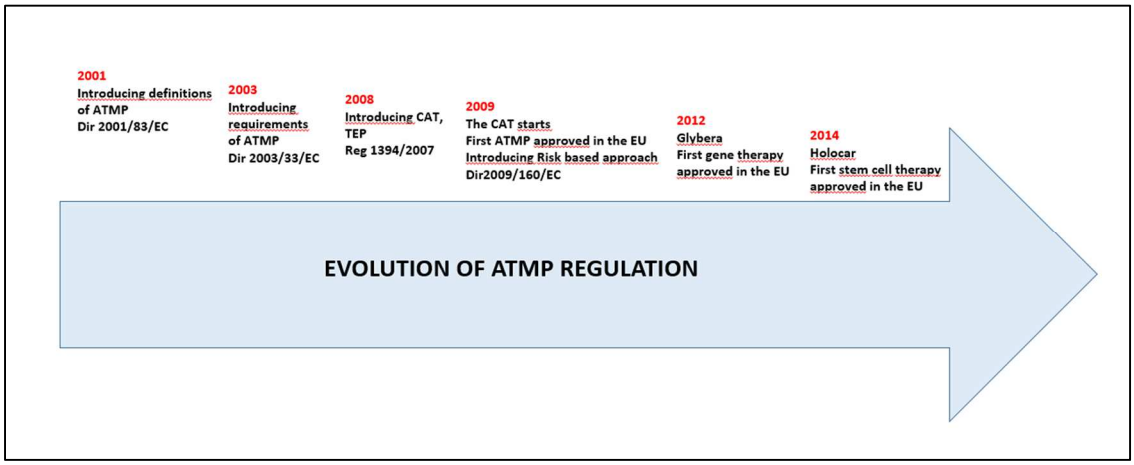

Fig.4. Evolution of ATMP regulation.

The main goal of the regulation is to facilitate the access of ATMP to the EU market, to ensure their free movement within Europe as well as to foster the competitiveness of European companies in the field while at the same time safeguarding the health of patients.

In the EU, market authorization of ATMPs encompassing gene, somatic and tissue engineered therapies is governed today by the EMA. Evaluation to market authorization is delegated to the national competent authority, i.e. in Italy to the AIFA. The so-called ATMP Regulation lays down specific rules for the MA, supervision and pharmacovigilance of ATMPs and set up the CAT. It formulate a draft opinion on the quality, safety and efficacy of an advanced therapy medicinal product for final approval by the CHMP (dossier evaluation), it provide advice, on whether a product falls within the definition of an advanced therapy medicinal product (classification); CAT advise on any medicinal product which may require, for the evaluation of its quality, safety or efficacy, expertise in one of the scientific areas (Scientific advice) and finally assist scientifically in the elaboration of any documents related to the fulfillment of the objectives of this Regulation (criteria and guidelines).