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Human stem cells for the treatment of motor neuron diseases: regenerative potential, translatability and development of new biotechnologies

Coordinator: Prof. Andrea Biondi
Tutor: Dr. Giorgio Battaglia

Dr. Laura Canzi
Matr. No. 576998

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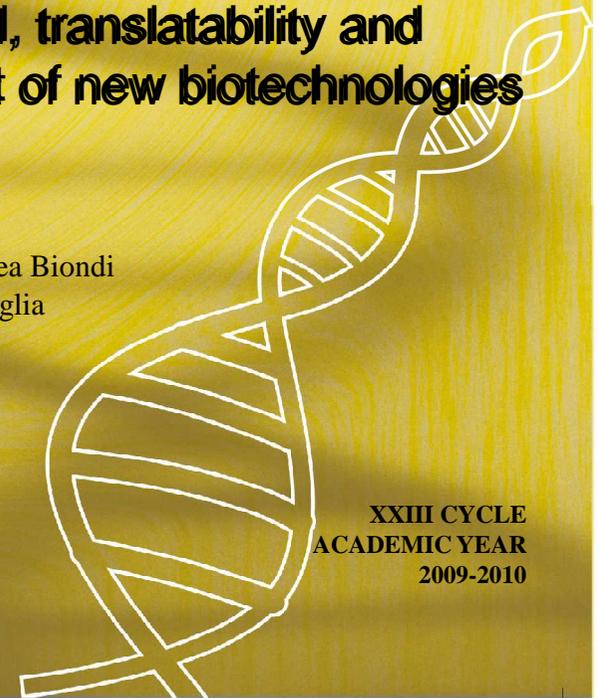
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Chapter 1   General Introduction

1.1. Stem Cells

The flurry of recent studies touting the near-miraculous properties of stem cells underscores the need for more rigorous definitions.

A true stem cell must satisfy several operational criteria like clonogenic capacity, must be capable of unlimited self-renewal by symmetric division. Then it must be able to divide asymmetrically, one daughter resembling its mother, the other daughter giving rise to multiple types of differentiated cells representing all three primitive embryonic germ layers, at the end it must originate from an embryonic or adult stem-cell source.

There are two different sources for the extraction of stem cells: the embryo or the adult tissues.

1.1.1. Embryonic Stem Cells

Embryonic stem cells (ESCs) are stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst at the stage of 4-5 days post fertilization, at which time they consist of 50-150 cells; in fact, after fertilisation, an embryo consists of identical cells which are totipotent. That is to say that each could give rise to an embryo on its own producing, for example, identical twins or quadruplets. They are totally unspecialised and have the capacity to

differentiate into any of the cells which will constitute the fetus as well as the placenta and membranes around the fetus.

At four to five days after fertilisation (morula storage), the embryo is still made up of unspecialised embryonic cells, but these cells can no longer give rise to an embryo on their own.

At five to seven days after fertilisation (blastocyst stage), a hollow appears in the centre of the morula, and the cells constituting the embryo start to differentiate into inner and outer cells: the outer cells will constitute the tissues around the fetus, including the Placenta; the inner cells (20 to 30 cells) will give rise to the fetus itself as well as to some of the surrounding tissues. If these inner cells are isolated and grown in the presence of certain chemical substances (growth factors), pluripotent ES cells can be derived.

ES cells are pluripotent, not totipotent since they cannot develop into an embryo on their own. If they are transferred to a uterus, they would neither implant nor develop into an embryo. This means they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body.

One of the complex technical issues surrounding the isolation and propagation of embryonic stem cells in vitro is the identification of the proper culture conditions, which can keep the cells in an undifferentiated state or induce their differentiation into a particular type of cell. In addition to soluble factors, plastic surfaces can affect cell morphology, cell density can influence interactions between cells, and the transfection of differentiation-inducing genes can help guide the pluripotent embryonic stem cell to a specific cell fate.

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Studies involving human cells date only from 1998, when human embryonic stem cells were first successfully propagated, (Shamblott MJ *et al*., 2001; Thomson JA *et al.,* 1998). This milestone relied heavily on the gradual progress made in the previous 20 years (Thompson S et al., 1984; Bongso A et al., 1994), when mouse and primate embryonic stem cells were extensively characterized (Evans MJ and Kaufman MH, 1981).

1.1.2. Adult and Fetal Stem Cells

The other stem cell source is dictated by an adult stem cell (ASCs). It is an undifferentiated cell found among differentiated cells in a tissue or organ, it can renew itself and differentiate to yield the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found.

The history of research on adult stem cells began about 50 years ago. In the 1960s, researchers discovered that the bone marrow contains at least two kinds of stem cells - Hematopoietic stem cells (HSCs), which form all the types of blood cells in the body and bone marrow stromal cells (BMSCs). Stromal cells are a mixed cell population that generate bone, cartilage, fat, and fibrous connective tissue. In the field of adult stem cells the specific area of hematopoietic stem cells is better understood and more researched than any other aspect of stem cells biology. CD34+ hematopoietic stem cells isolated from bone marrow that are capable of producing cells of the lymphoid and myeloid lineages in blood are the most

studied. Such cells are the only currently available therapeutic application of stem cells and are used for a variety of purposes usually entailing the replacement or the reestablishment of the immune system of a host after a disease or toxic therapy.

In the 1960s, researchers studying rats discovered two regions of the brain that contained dividing cells which become nerve cells (Becher H and Knoche H, 1962). Despite these reports, most scientists believed that new nerve cells could not be generated in the adult brain. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types: astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells (Noble M *et al.,* 1990). In 1998 Brüstle and colleagues found that human cells differentiate into neurons, astrocytes, and oligodendrocytes, which populate the host fore, mid-, and hindbrain. They generated neural chimeras composed of human and rodent cells to provide a unique model to study human neural cell migration and differentiation in a functional nervous system (Brüstle O *et al.,* 1998).

During development and throughout lifetime neuronal death, maintenance and neurogenesis occur in a tightly controlled manner (Tavazoie M. *et al*., 2008; Doetsch F. *et al*., 1997). The neurogenic zones, replete with neural stem cells (NSC) are primarily located in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus. These neurogenic zones are intimately associated with their local microvasculature beds. Further, the behaviors of the neuronal stem cells and microvasculature in these zones are thought to be

reciprocally co-regulated, in part, via oxygen tension, local NSC and several molecular factors including neurotrophins, vascular growth factors, cytokines and nitric oxide (fig 1) (Li Q. et al., 2006; Li Q. *et al.,* 2008).
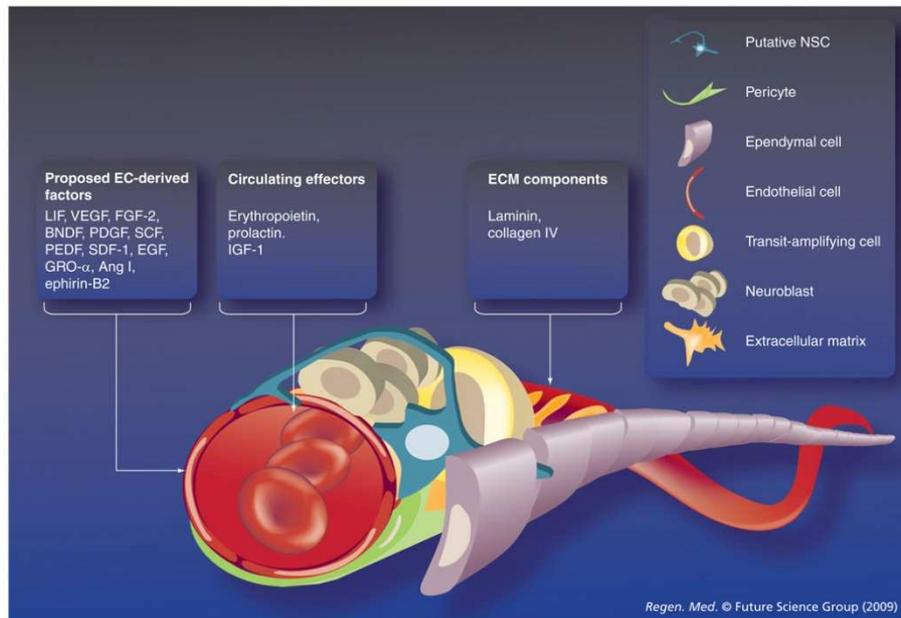
Figure 1: Proposed endothelium-derived regulators of the adult neural stem cell niche. Vascular endothelium-derived factors known to regulate the adult NSC niche can originate from the endothelial cell itself, the circulation or the ECM surrounding the blood vessel. Ang: Angiopoietin; BDNF: Brain-derived neurotrophic factor; EC: Endothelial cell; ECM: Extracellular matrix; GRO: Growth-related oncogene; LIF: Leukemia inhibitory factor; NSC: Neural stem cell; SCF: Stem cell factor; SDF: Stromal cell-derived factor.

Following brain lesion , NSC, supported by their local vasculature, are thought to proliferate, migrate to and differentiate at injury sites, affecting variable degrees of structural and functional recovery (Fagel DM. *et al.,* 2006). Nevertheless, endogenous neurogenesis are not able to recover the damaged neural tissue.

A notable exception to the tissue specific potential of stem cells is the mesenchymal stem cell or what is more recently called the multipotent adult progenitor cell derived from bone marrow stroma. Such cells have been shown to differentiate *in vitro* into numerous tissue types and similarly differentiate developmentally in blastocyste injection into multiple tissues including neuronal, adipose, muscle, liver, lungs, spleen and gut.

Adult stem cells have been identified in many organs and tissues. It is important to note that there is a very small number of stem cells in each tissue.

Stem cells are thought to reside in a specific area of each tissue where they may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury.

Scientists do not agree on the criteria that should be used to identify and test adult stem cells.

However, they often use one or more of the following three methods:

(1) labeling the cells in a living tissue with molecular markers and then determining the specialized cell types they generate;

(2) removing the cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to determine whether the cells repopulate their tissue of origin;

(3) isolating the cells, growing them in cell culture, and manipulating them, often by adding growth factors or introducing new genes, to determine what differentiated cells types they can become.

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Because of these generative properties, it is thought probable that stem cells will find use in the therapy of degenerative diseases or injuries.

Another source of stem cell is fetal tissue obtained after pregnancy termination. Multipotent stem cells such as neural stem cells derived from fetal neural tissue, can be multiplied in culture, though they have a limited life span. Fetal tissue has unique characteristics that make it especially valuable in some treatments. Fetal cells develop much faster than do adult cells, hastening their therapeutic effect — a potentially significant benefit for gravely ill patients. They are also less likely to be rejected by transplant recipients because they are less antigenic than adult cells. This reduces the need for exact tissue matches that can be so difficult to obtain. Fetal tissue is also easier to culture and proliferates more readily than comparable adult tissue. Furthermore, it is in greater supply, due to the number of elective abortions.

Fetal tissue can also give rise to pluripotent embryonic germ cells (EGCs) isolated from the primordial germ cells of the fetus. Such cells are interesting not only because of their renewal and differentiation potential, but also because such progenitor stem cells tend to be easily isolated based on anatomic location of dissection. Induction into mature tissue utilising these cells tends to be more straightforward as compared with using ESCs.

Although ESCs seem the most flexible type of stem cell, the ethical constraints and safety issues surrounding their potential clinical use have encouraged the search for other sources of stem cells.

Using fetal tissue in biomedical research and in transplantation is not a new practice.

As early as 1928 unsuccessful attempts were made to transplant fetal pancreas cells into diabetics (Fichera G, 1928).

Fetal tissue was used effectively in biomedical research during the 1950s, and was instrumental in the culture of the polio virus, which led to the development of the polio vaccine. Fetal tissue cultures were also essential in the development of the rubella vaccine, and continue to be used in virology research. Transplantation of fetal thymus cells into patients with DiGeorge Syndrome has been recognized as effective therapy since the late 1960s.

Many of the therapeutic applications involving fetal tissue are still in the experimental stage, so it is difficult to pinpoint its transplantation potential. One promising application is the transplantation of human fetal brain cells into the *substantia nigra* of patients with Parkinson's disease to restore motor function. Fetal neural transplants have also shown promise for patients suffering from Alzheimer's disease, spinal cord and other neural tissue injuries, and possibly some forms of cortical blindness. Fetal liver cells may be useful for treatment of some kinds of bone marrow disease seen in leukemia and aplastic anemia patients. Fetal tissue transplantation may also help those suffering from blood clotting disorders, such as sickle cell anemia, thalassemia, and hemophilia. Fetal pancreatic tissue has potential applications in the treatment of diabetes, especially juvenile onset diabetes. Human gene therapy may employ early fetal cells.

However, the use of adult stem cells is not without problems (Verfaillie CM, 2002).

First, expansion of adult stem cells in culture is hindered by the paucity of molecular markers by which they can be identified and quantified. In addition, the expression of markers to distinguish populations of stem cells appears to be variable and occasionally inconsistent; cell surface antigens vary according to the culture preparation, culture duration or plating density. Second, it is now recognised that cells with different stem cell activities can coexist within some tissues and, more importantly, within implanted cell populations. For example, there might be several varieties of each type of adult stem cell, and only one cell in a colony might have the capacity for a particular haemopoietic or mesenchymal fate (Phinney DG, 2002; Vogel W *et al*., 2003).

The quantitative and qualitative effects of ageing on stem cells are not well understood, although it is believed that stem cells from a younger individual should have greater potential (Van Zant G and Liang Y, 2003). Thus, many investigators suggest that fetal stem cells should have an advantage over adult stem cells in cell replacement therapies. All stem cells invest heavily in self-protective mechanisms and can self renew, but whether or not they age over the lifetime of an individual is the subject of much debate (Roccanova L and Ramphal P, 2003; Van Zant G and Liang Y, 2003).

There is some evidence from murine models that the homing efficiency of older Hematopoietic Stem Cell (HSC) is less than that of younger HSC, and adult stem cells are at a competitive disadvantage when transplanted with fetal cells. (Harrison DE, 1983).

Older HSCs have diminished self-renewal capacity, less developmental potency and give rise to decreased numbers of progeny

when subjected to haemopoietic demands, and this decline in function is even more apparent when older stem cells undergo increased stress (Wynn R *et al.*, 1999).

Similar qualitative effects of ageing are seen with Mesenchymal Stem Cell (MSC) (Bruce SA *et al.*, 1986; Mets T and Verdonk G, 1981); older bone marrow stroma blunts haemopoietic responses after transplantation and increases post-transplant autoimmunity (Doria G et al., 1997; Ordemann R et al., 2002).

Most, if not all, stem cells produce telomerase, which lengthens telomeres, protects against genotoxic damage and correlates with cell immortality. The self-renewal and replicative potential of stem cells probably depends on telomerase to maintain stable telomeres, as most evidence indicates that telomere length is a biomarker of the replicative history of cells (Hayflick L, 2000).

Cells of the germline have very long telomeres, which do not shorten with ageing of the organism, and fetal stem cells could be expected to have an advantage over adult stem cells in this regard. Comparative studies of fetal liver and adult bone marrow HSC have confirmed that fetal liver HSC have higher telomerase activity and adult bone-marrow-derived HSC shorter telomeres, which again implies that the proliferative potential of HSC is limited and declines with age.

There are, of course, ethical issues associated with the collection and use of fetal tissues for stem cell research. Nevertheless, it can be argued that fetal stem cells are currently obtained from terminated fetuses, thus using tissue that would otherwise be discarded. Fetal blood and liver, however, also represent potentially harvestable

sources of autologous stem cells, which might be amenable to genetic manipulation and reinfusion in ongoing pregnancies (Campagnoli C *et al.,* 2001). Considerable research now suggests that fetal stem cells are more plastic than adult stem cells, and hence have greater therapeutic potential. In addition, there is evidence that they have important differences in gene expression. Some fetal stem cells have higher replicative potential than their adult counterparts, a concept that argues in favour of the use of such cells for transplantation.

In addition, the pre-immune status of early fetal stem cells might be important in mismatched transplant situations (Gotherstrom C *et al.*, 2004). There is also a rationale that fetal treatment is better than adult treatment, especially in anticipated postnatal disease. Fetal stem cells are intrinsically primed to develop in the fetal environment and an obvious advantage is using autologous stem cells or even allogeneic fetal stem cells (Michejda M, 2004).

Culturing of human stem cells offers insights that cannot be studied directly in the human embryo or understood through the use of animal models. For instance, basic research on stem cells could help to understand the causes of birth defects, infertility and pregnancy loss. It could also be useful to give a better understanding of normal and abnormal human development (chapter 3).

Another area of interest includes studies of human disease on animal models. For example, mouse ES cells can be engineered to incorporate human mutated genes known to be associated with particular diseases and then used to make transgenic mouse strains. If such mice express the pathology of the human disease, this confirms the hypothesis that the gene is involved with the aetiology of the

disease. This strategy also yields an animal model of the human disease which has, in most cases, a much better predictability for the human situation than more conventional animal models. One of the most illustrative examples of this method is its use in order to address the potential causes of Alzheimer's disease.

Research also studies the use of stem cells in gene therapy. Stem cells could be used as vectors for the delivery of gene therapy. One current application in clinical trials is the use of haematopoietic stem cells genetically modified to make them resistant to the HIV (virus responsible for AIDS).

The last but not the least use of stem cells is the production of specific cell lines for therapeutic transplantation. If feasible, this would be the most promising therapeutic application of stem cells. Research is being actively pursued, mostly in the mouse, with the aim of directing the differentiation of stem cells to produce pure populations of particular cell types to be used for the repair of diseased or damaged tissues. For instance, the aim would be to produce cardiac muscle cells to be used to alleviate ischaemic heart disease, pancreatic islet cells for treatment of diabetes (juvenile onset diabetes mellitus), liver cells for hepatitis, neural cells for degenerative brain diseases such as Parkinson's disease, and perhaps even cells for treating some forms of cancer. The transplantation of stem cells could also help, for example, to repair spinal cord damage which occurs frequently, mainly following trauma (for instance car accidents) and is responsible for paraplegia. Results of this kind of cell therapy on animals are promising, but are still years away from clinical application. Even more remote (possibly decades away) is the

prospect of being able to grow whole organs *in vitro*, but if tissues for the repair of organs become available, it would greatly relieve the existing unsatisfied demand for donated organs for transplantation. In providing a potentially unlimited source of specific clinically important cells such as bone, muscle, liver or blood cells, the use of human stem cells could open the way to a new "regenerative medicine".

In fact, based on the positive results of experimentation on rodents and primates, clinical trial in patients with Parkinson's disease have been performed on around 200 patients over the last 10 years, especially in Sweden and the USA. They have shown that the transplantation of neural cells derived from the human fetus can have a therapeutic effect, with an important reduction of the symptoms of the disease in the treated patients. The clinical improvement among these patients has been observed for 6-24 months after transplantation and in some cases for 5-10 years. It has recently been shown that 10 years after the transplantation surgery, the transplanted neural cells were still alive and producing dopamine, the compound which is deficient in the brain of patients with Parkinson's disease. However, this therapeutic approach still remains experimental. In addition, the availability of neural fetal tissue is very limited. Five to six aborted fetuses are needed to provide enough neural tissue to treat one Parkinson's patient. That is why new sources of neural cells have been explored in some countries such as the US and Sweden. Their aim is to derive neural stem cell lines from fetuses with unlimited growth potential and that could be cryopreserved, providing much greater amounts of neural tissue for transplantation.

1.1.3. Stem Cells and Legislation

The use of stem cells, in particular, embryonic stem cells is governed by laws.

In Europe Article 18 of the Convention on Human Rights and Biomedicine establishes that each country should decide whether to authorize embryo research or not. Each nation is only obliged to respect two conditions: "to ensure adequate protection of the embryo", that is to say to adopt a legislation fixing the conditions and limits of such research; and to prohibit "the creation of human embryos for research purposes". The Convention is binding only for the States which have ratified it.

At the EU level, although there is no legislative competence to regulate research, some directives allude to the issue of embryo research and use. For instance, Directive 98/44/EC on the legal protection of biotechnological inventions (patenting on life) stipulates that "processes for cloning human beings" and "uses of human embryos for industrial or commercial purposes"… "shall be considered unpatentable".

Directive 98/79/EC on *in vitro* diagnostic medical devices (including the use of human tissues) provides that "the removal, collection and use of tissues, cells and substances of human origin shall be governed, in relation to ethics, by the principles laid down in the Convention of the Council of Europe for the protection of human rights and dignity of the human being with regard to the application of biology and medicine and by any Member States regulations on this matter".

The situation in the USA contrasts with that in Europe. In September 1988 the National Institutes of Health (NIH) in the US convened a panel of non-government experts, ethicists, lawyers, theologians, physicians and biomedical researchers, representing varied outlooks on the use of fetal tissue, and on abortion. The conclusion of a substantial majority of the panel members was that though "it is of moral relevance that human fetal tissue has been obtained from induced abortion," fetal tissue transplantation research is "acceptable public policy", provided that certain safeguards are in place. They recommended that there be anonymity between the donor and the recipient, and that special consent procedures be used to separate the decision to abort from the decision to donate tissue. These recommendations were in keeping with other guidelines established in other countries and "located squarely in the middle" of international consensus (U.S. National Institutes of Health, 1988).

All committees specified that no one should benefit economically from donating or distributing tissue, and most required some form of record keeping. Many advisory panels stated that fetal tissue research should be permitted if it is consistent with local laws and subject to approval of local institutional review boards.

The issue of stem cell research, particularly embryonic stem cell research, became a high-profile political issue in the U.S. during the first year of President George W. Bush's term in office. On August 9, 2001, Bush enacted a ban on federal spending for the purpose of deriving new embryonic stem cells from fertilized embryos. He argued that performing research on embryos is destroying human life, and should therefore be avoided. Both the 109th and 110th Congresses

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passed bills overturning the ban, but both were vetoed by Bush. During the 109th Congress, both houses passed and Bush signed a bill banning the creation of human fetuses with the sole purpose of destroying them and harvesting their body parts. The Senate also passed a bill encouraging research into the creation of stem cell lines without destroying human embryos.

President Barack Obama removed the rules limiting research on human embryonic stem cells in the United States. The National Institutes of Health (NIH) in Bethesda, Maryland, is now working out policies that will allow researchers to apply for grant money from the agency to study some of the hundreds of cell lines created after President George W. Bush limited federal funding to research on lines in existence at that time. President Barack Obama signed the executive order on 9 March 2009. The new order asks the NIH to develop guidelines and regulations to govern federally funded human embryonic stem-cell research.

Various commissions have been established in the United States and abroad to study the question of using fetal tissue. Some early commission reports examined research on live fetuses and pregnant women, paying little attention to the transplantation of fetal tissue. Later reports have focused more on the use of tissue from dead fetuses. Most committees have made distinctions between different categories of fetuses, depending on age and weight of the fetus, viability (the likelihood of the fetus surviving outside the uterus), whether the research was to take place *in utero* or *ex utero*, whether the research was to directly benefit that fetus, and, finally, whether or not the fetus was alive.

The Center for Biomedical Ethics at the University of Minnesota reports that more than 1000 patients have received transplanted fetal tissue worldwide. Countries where fetal tissue transplantation has occurred include: Australia, Canada, China, the Commonwealth of Independent States (formerly the U.S.S.R.), Cuba, Czechoslovakia, Finland, France, Germany, Great Britain, Hungary, India, Italy, Mexico, Norway, Spain, Sweden, and Yugoslavia (Vawter DE and Caplan A, 1992).

Recently, a UK stem cell research company has received approval from the UK Medicines and Healthcare Products Regulatory Agency (MHRA) to commence a first-in-man clinical trial for the treatment of patients who have been left disabled by an ischaemic stroke, the most common form of the condition. The company has used its c-mycERTAM technology to generate genetically stable neural stem cell lines isolated from human fetal brain.

Another innovation, described by Shinya Yamanaka's team from the University of Kyoto in Japan, worked on skin cells from the face of a 36-year-old woman. After collecting these fibroblasts, the scientists injected them with genes coding for four transcription factors. This genetic manipulation enabled them to activate certain genes in the skin cells that are normally active only at the embryonic development stage (Takahashi K *et al*., 2007).

In the USA, James Thomson's team showed that four factors (OCT4, SOX2, NANOG, and LIN28) are sufficient to reprogram human somatic cells to pluripotent stem cells that exhibit the essential characteristics of embryonic stem (ES) cells. Such induced pluripotent human cell lines should be useful in the production of new disease

models and in drug development, as well as for applications in transplantation medicine *(*Yu J *et al.,* 2007).

Although the results of these recent iPS studies are of enormous biological and clinical interest, the research has also triggered an ethical debate. What is the advantage of such a technique over current techniques? While some people present iPS as the solution to many ethical issues posed by embryonic stem cells and therapeutic cloning, not all the experts are in agreement. The technique does not require the use of embryos and produces cells that are genetically identical to the patient's own cells.

This recent discovery of the American and Japanese research teams does not seem to challenge the scientific worth of embryonic stem cell's debate. However, they do hold out new hope for treating ailments like cancer, diabetes, arthritis, spinal cord lesions, heart disease, burns, Parkinson's disease and Alzheimer's. With regard to organ transplants, the new technique could enable doctors to create stem cells using the patient's own DNA, thus eliminating the risk of rejection.

It is easy to understand why this innovative method is being greeted with so much fervour. However, there is still a long way to go before the method can be tested for clinical applications. Above all, scientists will need to determine how identical iPS are to embryonic stem cells.

About the human fetal stem cells there is a different way to improve the research from the government policy.

1.2. Human Adult Skeletal Muscle Stem Cells

1.2.1. Skeletal Muscle Stem Cells (Skmscs)

The primary functions of skeletal musculature are locomotor activity, postural behavior, and breathing. However, skeletal muscle is susceptible to injury after direct trauma (e.g., intensive physical activities, lacerations) or resulting from indirect causes such as neurological dysfunction or innate genetic defects. If left unrepaired, these injuries may lead to loss of muscle mass, locomotive deficiency, and in the worse cases lethality.

The maintenance of a working skeletal musculature is conferred by its remarkable ability to regenerate. Indeed, upon muscle injury a finely orchestrated set of cellular responses is activated, resulting in the regeneration of a well-innervated, fully vascularized, and contractile muscle apparatus.

The advances of molecular biology techniques combined with the identification and development of rodent models for muscular dystrophy have contributed to the identification of molecular pathways involved in muscle regeneration. In particular, the identification of muscle satellite cells has led to major advances in our understanding of muscle regeneration. Significant research into the biology of satellite cells has elucidated the cellular and molecular mechanisms during muscle regeneration. These studies have also led to insight regarding the development of therapeutic strategies that may alleviate some of the pathological conditions associated with poor

muscle regenerative capacity, such as the one observed in muscular dystrophy patients and in the course of normal aging. More recently, the identification of multipotent stem cells capable of myogenic differentiation in the course of muscle regeneration has extended our view on the muscle regenerative process and opened new perspectives for the development of novel therapies.

Muscle satellite cells have long been considered a distinct myogenic lineage responsible for postnatal growth, repair, and maintenance of skeletal muscle. Skeletal muscle satellite cells were first described in frog muscle by Mauro (Mauro A, 1961) based on their morphology and position relative to mature myofibers and were later identified in adult avian and mammalian muscle (Armand O *et al.*, 1983). Satellite cells adhere to the surface of myotubes prior to the formation of the basal lamina, such that the basal lamina surrounding the myofiber and satellite cells is continuous (Armand O *et al.*, 1983; Bischoff R and Heintz C, 1994).

Satellite cells mediate the postnatal growth of muscle and are the primary means by which the mass of adult muscle is formed (Schultz E and Jaryszak DL, 1985). The overall population of satellite cells decreases with increasing age. Satellite cells in adult skeletal muscle are normally mitotically quiescent but are activated (i.e., initiate multiple rounds of proliferation) in response to stress induced by weight-bearing exercise or trauma (Rosenblatt JD et al, 1995; Schultz E and Jaryszak DL, 1985; Bischoff R and Heintz C, 1994; Grounds MD, 1999). The descendants of activated satellite cells, called myogenic precursor cells (mpcs), undergo multiple rounds of division prior to fusing with existing or new myofibers.

Satellite cells appear to form a population of stem cells that become activated, proliferate and and express myogenic markers (satellite cells expressing myogenic markers are also termed myoblasts) in response to stimuli such as myotrauma. Ultimately, these cells fuse to existing muscle fibers or fuse together to form new myofibers during regeneration of damaged skeletal muscle. (Bischoff R and Heintz C, 1994; Schultz E and McCormick KM, 1994).

Since the original description of the myogenic satellite cell, considerable interest and research efforts have focused on myogenic satellite cell biology. These research efforts have enhanced our understanding of muscle growth, remodeling, and regeneration. In addition, new paradigms have been proposed regarding the regenerative capacity and the plasticity of the myogenic satellite cell population (Lemischka I, 1999; Megeney LA *et al.*, 1996.; Ordahl CP, 1999; Seale P *et al.*, 2000).

These paradigms suggest that the satellite cell population not only has a remarkable capacity for muscle regeneration but may also contribute to alternative muscle and non-muscle lineages and may have clinical applications.

Until a few years ago, skeletal-muscle satellite cells were thought to be capable of regenerating muscle fibres only. They are normally quiescent but become activated and start to divide in response to muscle trauma. However, studies in the past few years have shown that adult skeletal-muscle tissue contains progenitors or pluripotent stem cells able to differentiate into several phenotypes of mesenchymal cell (Young HE *et al*,1999; Young HE *et al.*, 2001) which suggests that the pool of skeletal-muscle-derived stem cells

includes distinct subpopulations of precursors with different differentiating capacities. Satellite cells may represent only one of these subsets (Heslop L *et al.*, 2001). Furthermore, a study in rats confirmed that skeletal muscle stem cells consist of a mixture of progenitors and pluripotent stem cells able to differentiate not only into different types of mesodermal cells, but also into cells of neuroectodermal origin such as astrocytes and neurons (Romero-Ramos M *et al.*, 2002). One of the limitations of most of these studies is that they used animal models, and there have been very few human studies.

Specific cells within skeletal muscle exhibit apparent stem cell-like plasticity and multipotentiality (Orkin SH and Zon LI, 2002; Romero-Ramos M *et al.*, 2002). This implies that there is something unique about the skeletal muscle environment that allows it to support the survival of primitive progenitors. Indeed, we have recently demonstrated that adult skeletal muscle derived stem cells (SkmSCs) may also differentiate into neurogenic cell lineages (Alessandri G *et al.*, 2004).

The number of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, suggesting a capacity for self-renewal within the satellite cell compartment. Beside this interesting potential, skeletal muscle contains cells with similar biological characteristics to mesenchimal stem cells (MSCs) (Asakura *et al.*, 2001).

MSCs can differentiate into multiple mesoderm-type cells (e.g. osteoblasts, adipocytes and chondrocytes (Kassem *et al.* 1993; Justesen *et al.* 2002; Johnstone *et al.* 1998). Recently, unexpected

plasticity has been attributed to MSCs, as they have been demonstrated to differentiate into a number of non-mesoderm-type cells, including neuronal-like cells (Jiang *et al.* 2002; Sanchez-Ramos *et al.* 2000). The emerging field of regenerative medicine holds the promise of treating a variety of degenerative diseases where no specific or effective treatment is currently available by transplanting biologically competent mature cells and organs. In particular, there are several investigations focused on the transplantation of MSCs in neurodegenerative diseases. What is encouraging is the presence of an accumulating evidence of the versatile differentiation potential and hypo-immunogenic nature of MSCs .(Le Blanc *et al.* 2003).

1.2.2. Satellite Cell Identification

Anatomic identification. Resident within adult skeletal muscle is a pool of undifferentiated mononuclear cells termed satellite cells because of their anatomic location at the periphery of the mature, multinucleated myotube. The defining characteristic of the satellite cell is that the basal lamina that surrounds the satellite cell and the associated myofiber is continuous (Schultz E and McCormick KM, 1994). Other distinguishing morphological features of the satellite cell population include a relatively high nuclear-to-cytoplasmic ratio with few organelles, a smaller nuclear size compared with the adjacent nucleus of the myotube, and an increase in the amount of nuclear heterochromatin compared with that of the myonucleus. These morphological features are consistent with the finding that satellite cells are relatively quiescent and transcriptionally less active. These

distinguishing features are absent following activation or proliferation of the satellite cells in response to growth, remodeling, or muscle injury. After activation, the satellite cells are more easily identified as they appear as a swelling on the myofiber with cytoplasmic processes that extend from one or both poles of the cell (Schultz E and McCormick KM, 1994). The number of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, demonstrating an inherent capacity for self-renewal (Gibson MC and Schultz E, 1983; Schultz E and Jaryszak DL, 1985).

The mechanism by which satellite cells undergo self-renewal in adult skeletal muscle represents an outstanding issue still poorly understood.

1.2.3. Morphological Characteristics of Skeletal Muscle Regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei. Minor lesions inflicted by day-to-day wear and tear elicit only a slow turnover of its constituent multinucleated muscle fibers. . Whether the muscle injury is inflicted by a direct trauma (i.e., extensive physical activity and especially resistance training) or innate genetic defects, muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase The initial event of muscle degeneration is necrosis of the muscle fibers. This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. The early phase of

muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells. Recent reports suggest that factors released by the injured muscle activate inflammatory cells residing within the muscle, which in turn provide the chemotactic signals to circulating inflammatory cells. Muscle degeneration is followed by the activation of a muscle repair process. Numerous nuclear radiolabeling experiments have demonstrated the contribution of dividing myogenic cells to regenerating myofibers, and it is well accepted that following the myogenic proliferation phase, new muscle fibers are formed much as during bona fide embryonic myogenesis; myogenic cells differentiate and fuse to existing damaged fibers for repair or to one another for new myofiber formation.

Aging is associated with a significant decline in the mass, strength, and endurance of skeletal muscles in both human and animals (Karakelides H and Nair KS, 2005). It has been proposed that compromised satellite cell function contributes to this age-linked muscle deterioration. Whether satellite cell numbers decline with age is controversial and probably varies among different muscles and species (Schafer R *et al.*, 2005).

1.2.4. Heterogeneity of Satellite Cells

The expression pattern of several molecular markers has indicated that there may be heterogeneity within the satellite cell pool of young mice (Beauchamp JR *et al.*, 2000). This could simply be due to dynamic expression of some of the antigens expressed by satellite and

could be related to the length of time that the cells have been quiescent. Various functional observations suggest that the satellite cell pool may be composed of a heterogeneous population: differences between myogenic progenitors are also seen in culture where cells exhibit heterogeneity in proliferation rate, clonogenic capacity and morphology.

Whether this satellite cell heterogeneity is linked to multipotency is unknown. Until recently satellite cells were considered unipotent and their function restricted to supplying myoblasts for muscle maintenance and repair but we found that cells isolated from muscle tissue are able to differentiate into both myogenic and neurogenic lineages (Alessandri G et al, 2004) while Tamaki and colleagues demonstrated that they can adopt myogenic and endothelial fates (Tamaki T *et al.*, 2002).

1.2.5. Muscle Stem Cell Plasticity

Interestingly, while traditionally thought to be committed to the skeletal muscle fate, it is now evident that muscle stem cells, including satellite cells, are multipotent. For example, bone morphogenetic protein (BMP) treatment activates osteogenic markers in a myoblasts line (Katagiri T et al, 1994; Katagiri T et al, 1997). Moreover, satellite cells derived from single fiber cultures spontaneously form adipocytes and osteocytes when cultured on a soluble basement membrane matrix lacking strong osteogenic or adipogenic signals (Asakura A et al, 2001).

The finding that undifferentiated cells in adult myoblast cultures co-express key regulators for myogenesis, osteogenesis, and

adipogenesis, supports the hypothesis that satellite cells have a multipotential predisposition (Wada MR et al, 2002). In order to extend these findings, we designed new experiments and confirmed that adult human SkmSC can also differentiate into myogenic and neurogenic cell lineages (Alessandri G et al, 2004).

1.3. Cell Therapy

Cell therapy - also called live cell therapy, cellular suspensions, glandular therapy, fresh cell therapy, embryonic cell therapy, and organotherapy - refers to various procedures in which processed tissue from animal embryos, fetuses or organs, is injected or taken orally. Products are obtained from specific organs or tissues said to correspond with the unhealthy organs or tissues of the recipient. Proponents claim that the recipient's body automatically transports the injected cells to the target organs, where they supposedly strengthen them and regenerate their structure.

The term "cell therapy" identifies one way of treatment in which drugs are replaced with cells. Recently, this definition has been used primarily to indicate procedures involving the use of well characterized cell subsets, subject to specific treatments, such as cell selection, and *in vitro* expansion, creating clones with anti-infective or anti neoplastic proprieties. In the area of cell therapy, it is preferable to use the term "implant" to define an enrichment of cellular entities in the host organism for therapeutic purposes since transplantation is the replacement of damaged biological structures with other intact

structures taken from the same patient or from a donor (homologous or heterologous graft).

The source from which the cells are obtained defines the category of type of implant used:

- Autologous implant: cells taken from the same patient.
- Allogeneic implant or heterologous: cells obtained from an immune-compatible donor.
- Singenic implant: cells of monozygotic twins (rare).

Cell therapy is the treatment of human diseases by the administration of cells that have been selected, multiplied and pharmacologically treated or altered outside the body (*ex vivo*). The aim of cell therapy is to replace, repair or improve the function of damaged tissues or organs or cells.

The reason for the great of interest in cell therapy is its ability to do a job better than any chemical could: cells can secrete hormones (e.g. insulin) or other therapeutic substances, they can form connections with host tissues (e.g. formation of neuronal connection in the brain) and they can proliferate to replace injured tissue and to provide structural support (e.g. in the case of musculoskeletal disorders).

1.3.1. History of Cell Therapy

The theory behind cell therapy has been in existence for several hundred years. The Swiss doctor and alchemist Philippus Aureolus Paracelsus (1493-1541 recorded the first discussion of the concept of cell therapy in his *Der grossen Wundartzney* ("Great Surgery Book") in 1536 that "the heart heals the heart, lung heals the lung, spleen heals the spleen; like cures like." Paracelsus and many of his contemporaries agreed that the best way to treat an illness was to use living tissue to restore sickly people.

The first form of cell therapy, in particular a blood cellular therapy, was made by Jean-Baptiste Denis (1640-1704), court physician to King Louis XIV. He attempted to transfuse lambs' blood into human subjects.

The first cell therapy experiment recorded, occurred in 1912, when German physicians attempted to treat children with hypothyroidism, or an underactive thyroid, with thyroid cells.

However, "The father of cell therapy" was Dr. Paul Niehans (1882-1971), a Swiss physician. In 1931, after a colleague erroneously removed parathyroid glands from a patient, Niehans attempted to transplant in this patient the parathyroid glands of an ox. Immediately, the patient began to improve. Niehans made more trials, even experimenting on himself, and reported that live cells extracted from healthy animal organs could cure illnesses. He believed adding new tissue stimulated rejuvenation and recovery.

Swedish researchers have successfully transplanted human fetal stem cells into Parkinson's patients, and the procedure is being investigated further as a possible treatment for repairing damaged

brain by neurological disorders such as Parkinson's disease, but also Alzheimer's disease and epilepsy. However, because the cells used in these applications must be harvested from aborted human fetuses, there is an ethical debate over their use.

Currently, applications of cell therapy in the United States are still in the experimental and clinical trial stages. The U.S. Food and Drug Administration has approved the use of a cellular therapy technique for repairing damaged knee joints. The procedure involves removing healthy chondrocyte cells from the patient, culturing them in a laboratory for three to four weeks, and then transplanting them back into the damaged knee joint of the patient.

In addition, cell therapy has been used successfully to repair spinal cord injuries, strengthen a weakened immune system, treat autoimmune diseases such as AIDS, and a wide range of chronic conditions such as arteriosclerosis, congenital defects, cardiac disorders and sexual dysfunction.

1.3.2. The Choice of an Appropriate Stem Cell Type for CNS repair.

The choice of an appropriate stem cell type is only one step in the complex decision process for designing a rationale for therapy.

Stem/progenitor cells can be administered in an undifferentiated state or in a pre-differentiated state. While undifferentiated stem cells have the potential advantage of combining, for example, vascular and neural contribution useful for central nervous system (CNS) repair, transplantation of undifferentiated cells may result in unwanted side

effects such as uncontrolled proliferation and/or differentiation into unwanted cell types. For instance, embryonic stem cells require pre-differentiation in order to avoid teratoma formation (Li Z *et al.*, 2007). Pre-differentiation of the stem cells could be advantageous since the environment in the sick organ may have lost the molecular cues necessary to induce correct differentiation. On the other hand, differentiated cells may be more sensitive to oxygen and nutrient deprivation and may therefore undergo significantly more apoptosis in an ischemic environment, such as in cerebrovascular diseases. Although it is possible that higher immunogenicity of the pre-differentiated cells contributed to their elimination, these results may well be compatible with the hypothesis that pre-differentiated stem cells are more prone to cell death in an ischemic environment, a question that is currently under investigation.

Recent preclinical work investigating the role of cell therapies for CNS repair has shown potential neuroprotection in the different animal models of neurodegenerative disorders and CNS injury. Mechanisms currently under investigation include engraftment and transdifferentiation, modulation of the locoregional inflammatory milieu, and modulation of the systemic immunologic/inflammatory response. While the exact mechanism of action remains controversial, the growing amount of preclinical data demonstrating the potential benefit associated with cell therapy for neurological diseases and injury warrants the development of well-controlled clinical trials to investigate therapeutic safety and efficacy.

For instance, regeneration of the CNS involves various concepts, including i) the Regrowth of disrupted neuronal axons, ii) the

Replacement of lost neural cells, and iii) the Recovery of lost neural function, all of which are essential components of CNS regeneration.

Although pioneering clinical experience with stem cell-related therapy seems promising, it is too early for general clinical use of this technique, since many questions remain unanswered. Indeed, while questions about safety, dose, and administration route/timing/frequency are the first ones to be addressed when designing a stem cell-based clinical approach, there is accumulating evidence from recent pre-clinical studies that other issues may also be at stake. For instance, the choice of stem cells to be used and their precise mechanism of action, the need/possibility for concurrent tissue regeneration in case of irreversible tissue loss, the differentiation degree and specific vascular identity of the transplanted cells, and the long-term survival of engrafted cells in the absence of a normal supportive tissue environment should be well considered.

Many researchers have focused their attention on certain types of cells that are potential candidates for their characteristics in regenerating the CNS. These cells can be classified into three categories:

1. Stem cells: adult stem cells derived from different tissues such as neural stem cells or bone marrow-derived mensenchymal stem cells and potentially, embryonic stem cells.
2. Progenitor cells: neuronal or glial progenitor cells.
3. Differentiated or precursor cells: neural precursor cells and specific neuronal subtype *in vitro* differentiated from progenitor cells or from adult or embryonic stem cells.

All three cell types have already been implanted into damaged CNS in animal experiments showing different properties and efficiency that must be appropriately marked to obtain the best results.

Stem cells (SCs) are immature cells endowed with self-renewal potential that are able to differentiate into various cell lineages, regardless of their source (pluripotent).

Progenitor cells are a type of cell already commissioned to a specific lineage and like stem cells have a capacity to differentiate into a specific type of cell but their self renewal and potentiality *in vivo* and *in vitro* are limited (unipotent, sometimes multipotent).

Differentiated or precursor cells are a more specialized cell type that have a specific physiological role and are not able to proliferate.

The optimal candidate for cell-based CNS regeneration must: proliferate efficiently, differentiate appropriately, survive in the recipient, integrate into the host tissue, work appropriately and not be harmful to the recipient. A variety of cell types have been examined and tested for their capacity. *In vitro* studies have examined the ability of stem cells to differentiate into all cell types of neural tissue investigated the functional characteristics of these cells. *In vivo* studies in animal models of neurological diseases have examined the capacity of stem cells to graft into the host neural tissue and then assayed the functional recovery of the diseased CNS.

These research has shown that cell therapy is a promising option for the treatment of CNS diseases. Therefore, strategies to augment cell function, survival, and homing could be crucial to improve success rates for cell therapy.

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1.3.3. The Development of Cell-Based Therapies

Stem cell research has the potential to transform disease treatment, reduce costs to society and enhance the quality of life for millions of patients. Stem cells also hold the promise of important business opportunities – with new directions for industry innovation and product development. The development of cell-based therapies is delayed by ethical and policy barriers as well as by technological and scientific obstructions. Generic methods for the derivation and establishment of new cells and cell lines and for the propagation, characterization and differentiation need to be established and validated. More specifically, some development processes suffer from the lack of viable and up-scaled techniques, the limited control over the expansion processes, the limited control over the directed differentiation process, the absence of biological markers to identify the specific cells intended for therapy and to monitor the differentiation process, the absence of reliable preservation methods (long term and short term), the lack of standardization in the field, and the lack of human embryonic cell lines free of contamination by animal components.

The advanced therapy products such as living, non-germ-line somatic cells administered to humans for therapeutic purposes are regulated as medicinal products, and require premarket approval. The regulatory, ethical and quality control levels have been addressed in official documents published by regulatory bodies in various Countries such as the Food and Drug Administration (FDA) of the United States or the European Medicines Agency (EMEA). These

regulatory bodies provide guidelines necessary to design a medical team in charge of a phase I/II somatic cell therapy clinical trial. The main points for implementing a clinical trial are:

- Patient Safety
- Quality Product Assurence:
1. Safety
2. Purety
3. Effectiveness
4. Correct Identity
5. Correct Strength
- Facilities
- Maintenance and testing

1.3.4. Patient Safety

When developing cell therapy, proponents of this treatment must accept the responsibility to ensure that participants are not exposed to known unreasonable risks and that the experimental products are as safe as possible. It is critically important that clinical trials assure the safety of human subject participants by using quality controlled products, and by practicing good clinical medicine.

Patients should approach these treatments with extreme caution, should inquire about their proven efficacy and legal use in the country where they live, and should only accept treatment from a licensed physician who should educate the patient completely on the risks and possible side effects involved with cell therapy. These same cautions apply for patients interested in participating in clinical trials of cell therapy treatments.

1.3.5. Quality Product Assurance

Cell therapy has the potential to revolutionize the management of diseases that are currently incurable or have inadequate treatments. Cell therapy products represent a rising area of therapeutic intervention and their relative newness and complexity present considerable challenges in accomplishing product release.

It is indispensable to maintain an high level of safety and quality in preparing cellular products for therapy. GMP (Good Manufacturing Practice)-graded cell processing such as cell preparation, culture, and manipulation is obligatory for the delivery of such advanced cell therapy.

Good Manufacturing Practice (GMP) is an international set of regulations, codes, and guidelines by which drugs (known as medicinal products in Europe), medical devices, diagnostic products, foods and Active Pharmaceutical Ingredients (APIs) are manufactured. The purpose of GMP is to ensure a quality product. In the European Union and USA, the GMPs are enforced, with more compliance requirements than those stated in the WHO GMPs, are in force; while in the USA, FDA's version of GMPs, including requirements over and above those stated in the WHO document. Similar forms of GMP are used in other countries, such Australia, Canada, Japan, Singapore and where highy sophisticated GMP standards are required.

In general, cell culture operations should be carefully managed in terms of quality of materials, manufacturing controls, and equipment validation and monitoring:

- Both manufacturing and testing procedures should be implemented which ensure the control of cell cultures with regard to identity and heterogeneity.
- Cell culturing practices and facilities should be designed to avoid contamination of one cell culture with another.
- Characterization of therapeutic entity which requires the manufacturer to prove scientifically that the product is safe, and efficacious.
- The essential characteristics of the cultured cell population (phenotypic markers such as cell surface antigens, functional

properties, activity in bioassays, as appropriate) should be defined, and the stability of these characteristics established with respect to time in culture. This profile should be used to define the limits of the culture period.

- Materials used during *in vitro* manipulation procedures should be clearly identified and a qualification program with set specifications should be established for each component to determine its acceptability for use during the manufacturing process. When using reagent grade material, the qualification program should include testing for safety, purity, and potency of the component where appropriate.

Quality control is imperative in order to confirm the conformity, sterility and the function of cells. The normes require that the quality control be independent and objective.

1.3.6. Facilities

In the case of pharmaceutical products, bacterial contamination is prevented by sterilization by heat or filtration after manufacturing. However, in order to maintain living cells for cell therapy cells cannot be sterilized after processing. Therefore, a dedicated GMP facility, conforming to specified standards in order to avoid microbial contamination of the product and the environment and/or to protect the operator, is obligatory.

Thus, the Italian Guidelines for Somatic Cell Therapy, and also other guidelines from other contries, prescribe that all operations be performed in Biosafety Level 3 (BL3) laboratories, with controlled

access to the laboratories and high efficiency particulate filtered air (HEPA filter). Specific training of the personnel and standard operating procedures must be provided.

1.4. Human ALS disease and animal models: Preclinical studies for development of new cell-based strategies

Amyotrophic lateral sclerosis (ALS), described in 1869 by the French neurologist Jean-Martin Charcot, is a fatal neurodegenerative disease whose cause is still unknown. ALS is sometimes referred to as 'Lou Gehrig disease' after the famous American baseball player who was diagnosed with the disorder. The clinical distinctiveness of the ALS pathophysiology is characterized by the progressive Motor Neuron (MN) degeneration in the spinal ventral horns, in the most brainstem motor nuclei, corticospinal and corticobulbar systems. The loss of MN leads to progressive atrophy of skeletal muscles. The fatal outcome caused by ALS is usually within 2–5 years after onset and it is due to the acute muscular atrophy leading to the respiratory failure with the denervation of the respiratory muscles and diaphragm. (Bradley WG. *et al.*, 2001). The annual incidence of ALS disease is about 2–6 cases/100,000 with a lifetime risk of developing ALS of 1 per 800 (Cleveland and Rothstein 2001). The median age of onset is 55 years (Adult-Onset ALS form), although it can start at younger ages (Juvenile-Onset ALS form) (Chancellor AM, 1996).

To date many attempts have been made to understand the cellular and molecular ALS pathophysiological mechanisms. However, little is known about the genetic defects or environmental factors that cause or predispose to ALS.

The 90% of ALS cases occurs in sporadic form (sALS) whereas the familial form of ALS (fALS) accounts for 5–10% of cases.

The fALS cases exhibit significant phenotypic and genetic heterogeneity (table 1). Among them, about 20% are caused by missense mutations in the SOD1 gene that codes for the enzyme Cu2+Zn2+ superoxide dismutase 1 (SOD1). To date it has been characterized about 70 different mutations in the *SOD1* gene that span all *SOD1* gene exons. Although most cases of SOD1-related fALS follow autosomal dominant inheritance, autosomal recessive inheritance has been rarely reported.

Recently, the genetic linkage studies have identified the loci for fALS juvenile-onset subtypes: Two recessively inherited loci map to chromosomes 2q and 15q whereas one dominant juvenile onset locus maps to chromosome 9q34 (Hentati A. *et al*., 1994; Hentati A. *et al.*, 1998; Chance PF. *et al*., 1998). In a recent study Hosler et al have identified the locus for an adult onset subtype of fALS with frontotemporal dementia (FTD) that maps to chromosome 9q21-q22 (Hosler BA. *et al*., 2000).

Although specific genetic alterations do not appear to cause sALS, a number of potential susceptibility and modifier loci have been identified. In the 1999 Cleveland et al reviewed the contributions that genetic linkage studies had made to found the deletions within the C-terminal KSP repeat region of the neurofilament heavy-subunit (*NFH*)

gene in a 1% of sporadic ALS patients. These sALS genetic linkages suggest that NF-H variants may represent risk factors for ALS disease (Cleveland DW. *et al.,* 1999) .

The fALS and sALS forms show very similar clinical characteristics and common histopathological features such as ubiquitinated inclusions and axonal swellings with the abnormal accumulations of neurofilaments in degenerating motoneurons (Ince PG. *et al.,* 2000). These evidences suggests that common mechanisms may be implicated in both fALS and sALS pathogenesis. On the other hand the genetic mutations described so far in fALS and sALS patients affect proteins involved in a wide range of cellular mechanisms, including free radical scavenging, energy metabolism, axonal transport, RNA processing, DNA repair, vesicular transport, and angiogenesis (table 1 and 2). Many hypotheses have been formulated to explain the ALS pathogenic mechanisms involved in the selective vulnerability of the affected MNs: these include mitochondrial dysfunction, Glutamate-mediated excitotoxicity, oxidative stress, protein misfolding, proteosomal dysfunction, aberrant growth factor signaling, microinflammatory process and glial activation. Nevertheless the exact mechanisms that trigger the focal MN degeneration of spinal cord brainstem and cerebral cortex in ALS patients remain to be elucidate. For instance it is believed that the development of the ALS disease is caused by an interplay between endogenous (genetic, metabolic) and exogenous factors (environmental, lifestyle).

Currently, the management of ALS is essentially symptoms-based, and many different drugs have been tested on their capacity to

alleviate or delay the symptoms of ALS patients and to extend their survival, but none has proved to be successful.

The only currently Food and Drug Administration-approved treatment for ALS that slightly slows disease progression and prolongs the survival of ALS patients, with no improvement in muscle function, is riluzole (Bensimon G *et al*., 1994).

This drug is an antiglutamatergic agent and limits glutamate release from nerve endings possibly by stabilizing the inactive state of voltage dependent sodium channels and by a G protein-coupled intracellular pathway. However the effect size of riluzile treatment was small, as the median increase in ALS patients survival is about two to three months.

Table 1: Familial ALS Loci.

fALS Gene/Loci	Onset	Comments	References
SOD1	A	Dominant and Recessive type of inheritance 20% fALS.	Shav CE. *et al*., 1998
Ch 18q21	A	Dominant type of inheritance	Sapp PC *et al*., 2003
Ch 16q12	A	Dominant type of inheritance	Abalkhail H *et al*., 2003
fALS Gene/Loci	Onset	Comments	References
TDP-43	A	Dominant type of inheritance. The TDP-43 protein was identified as a component of ubiquitinated inclusions in FTLD-ALS	Maekawa S *et al*., 2009
Ch 20ptel-p13	A	Dominant type of inheritance	Sapp PC *et al*., 2003
VAPB	A	Dominant type of inheritance.	Nishimura AL *et al*.,

		The vesicle-associated membrane protein/synaptobrevin-associated membrane protein B (VAPB) involved in the ER-Golgi transport systems.	2004
MAPT	A	Dominant type of inheritance. ALS with dementia, Parkinsonism. The mutations of the microtubule-associated protein tau (MAPT) have been associated with several neurodegenerative disorders.	Hutton M. *et al.*, 1998
Ch 9q21-22	A	Dominant type of inheritance 1%-4% fALS.	Hosler BA. *et al.*, 2000
Ch Xp11-q12	A	fALS X-linked Dominant type of inheritance	Siddique T *et al.*, 1998
Alsin (ALS2)	J	Recessive type of inheritance	Hadano *et al.*, 2001
SETX	J	Dominant type of inheritance	Chen YZ *et al.*, 2004
Ch 15q15.1-q21.1	J	Recessive type of inheritance	Hentati A. *et al.*, 1998

A= Adult; J= Juvenile. FTLD-ALS= Fronto Temporal Lobar Dementia-ALS

Table 2: ALS susceptibility/ modifier Loci.

ALS susceptibility/ modifier loci	Onset	Comments: variants and associations	References
NFH	A	The NFH gene encodes for the neurofilament heavy-subunit. The codon deletions in KSP repeats are found in 1% cases of sALS.	Figlewicz DA *et al*., 1994
EAAT2	A	Suppressed expression of the glial glutamate transporter EAAT2/GLT-1 in 60% of sALS patients.	Jackson M. *et al.*, 1999
VEGF	A	Vascular Endothelial Growth Factor gene Promoter SNPs in sALS cases	Lambrechts D. *et al.* 2003
SMN1	A	Copy number variants. sALS	Corcia P *et al.*, 2006
SMN2	A	Copy number variants. sALS	Veldink JH *et al.*, 2005
CNTF	J	Ciliary Neurotrophic Factor , a potent survival factor for motor neurons. Null allele in fALS	Giess R *et al.,* 2002
ApoE ε4	A	ε4 genotype	Drory VE *et al.*, 2001

A= Adult; J= Juvenile.

Several studies of pathogenic mechanisms of ALS relies in the analysis of ALS autopsy samples, but these data have not yielded reliable information about the pathophysiological mechanisms of MN degeneration from disease onset to the death of the patients.

In the last century the advanced Magnetic Resonance (MR) neuroimaging applications to patients suffering from ALS have become an indispensable methods for the understanding of ALS pathophysiology under in-vivo-conditions, mainly because of its noninvasive nature. The wide technical advances in MR imaging-based studies should be enhanced more extensively for monitoring treatment efficacy of the ALS-modifying or neuroprotective agents in the context of clinical trials. At present, the assessment of Riluzile treatment effects on 1H-MR spectroscopy measures in ALS is only preliminary.

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Under these circumstances, biomedical research is the only expect to develop possible therapeutic strategies.

In this sense experimental in vivo models have been developed to improve the knowledge of the SLA pathophysiology and the development of new pharmacological treatments. These include animals with spontaneous MN degeneration, transgenic rodents, and animals in which spinal MN death was produced with pharmacological tools (table 3).

Table 3: Overview of ALS animal models.

Animal Models	Description	Pathological changes	References
wobbler mouse	The vps 54 spontaneous mutants	Upper and lower MN degeneration, progressive muscular atrophy of the forelimbs.	Ref. tab. 5
Transgenic mutant SOD1 rodents	ALS-linked SOD1 mutations	Mitochondria swellings, vacuoles, SOD1 aggregates, neurofilament accumulations, MN loss. The severity of the ALS phenotype is directly proportional to the mutant SOD1expression levels.	Rosen DR . et al., 1993
Mutant NF-L rodents	ALS-linked NF disorganization	Perikaryal and axonal NF accumulations. Massive degeneration of spinal MN	Lee MK. et al., 1994
hNF-H overexpressor rodents	ALS-linked NF disorganization	Perikaryal accumulations and axonal atrophy Altered conductivity but no neuronal loss	Cote F et al., 1993
Peripherin overexpressor rodents	ALS-linked NF disorganization	Age-dependent IF aggregates in perikarya and axons 40% loss of spinal MN	Millecamps S et al., 2006
Dynamitin overexpressor rodents	ALS-linked microtubule-based transport defects	Abnormal gaits and decrease in strength. Axonal IF swellings 25% loss of MN axons at 16 months	LaMonte BH et al., 2002
kinesin heterozygous Knockout rodents	ALS-linked microtubule-based transport defects	Staggering gait after 1-year of age Progressive muscle weakness	Zhao C. et al., 2001
Dynein mutations heterozygous rodents	ALS-linked microtubule-based transport defects	Progressive motor dysfunction Loss of 4–70% of motor neurons at 16 months	Bommel H et al., 2002
Dynactin overexpressor rodents	ALS-linked microtubule-based transport defects	Defects in vesicular transport in cell bodies of motor neurons, axonal swelling and axo-terminal degeneration	Laird FM et al., 2008
Alsin knockout mice	ALS-linked endosomal	Defects of endosome trafficking, late-onset	Hadano S. et

	trafficking defects	degeneration of cerebellar Purkinje cells and of corticospinal axons	*al*., 2006
Transgenic mutant TDP-43 mice	ALS linked TDP-43 mutations	Gait abnormalities at 3 months of age and an average survival of 153 days.	
wild-type TDP-43 overexpressor rodents	ALS linked TDP-43 mutations	Lack of cytoplasmic TDP-43 aggregates Dose-dependent degeneration of cortical and spinal motor neurons and subsequent development of spastic quadriplegia	Wils H *et al*., 2010
Mutants TDP-43 overexpressor rodents	ALS linked TDP-43 mutations	Develop paralysis and death as early as 12 days.	StallingsNR. *et al*., 2009
Rodent Models of non-genetic spinal MN degenerations	Acute and chronic spinal MN degeneration models induced by excitotoxic agents, sciatic axotomy or sindbis virus	The main objective of these experimental studies is to test the different MN-proective agents for testing potential therapeutic approaches.	Deshpande DM *et al*., 2006

As a result, scientists can now carry out pre-clinical experiments aimed to test various approaches to ALS therapies in ALS animal models such as anti-glutamate agents, neurotrophic factors, antioxidant, antiapoptotic factors, cell and gene therapy strategies.

In the face of extraordinary advances in the knowledge of the stem cell biology that will likely hold remarkable potential for therapies and cures, Several recent lab studies have focused on ALS cell therapy approaches.

There already exists evidence from ALS pre-clinical studies that stem cells have the capacity to alleviate or retard the symptoms and to prolong the survival of the ALS animal models. (table 4)

Table 4: Overview of preclinical cell therapies in animal models of ALS

Animal Models	Cell source	Injection sites and cell numbers	Effect on onset and/or survival	References
Presymptomatic SOD1 G93A rats	Cervical sc hNSCs (8 w old fetus)	Bilateral lumbar SC injections, 4 sites, 50 000 cells/site	Delay in onset Increase in Life Span	Xu L *et al.*, 2006
Presymptomatic SOD1 G93A mice	LeX+/CXCR4+ mNSCs from CBA-eGFP or Hb9-eGFP mouse brains (6–8 w old) treated with bFGF, laminin, Shh and RA	Bilateral lumbar SC injections, 1 site, 1×10^4 cells	Delay in onset Increase in Life Span. Delayed loss of lumbar motor neurons.	Corti S *et al.*, 2007
Presymptomatic nmd mice (P1-2)	ALDH^{hi} SSC^{lo} mNSC from Thy1-YFP SC treated with Shh, RA, cAMP and NGF	Intrathecal delivery, 2×10^4 cells	Delay in onset Increase in Life Span. Delayed loss of lumbar motor neurons. Preservation of large axons (ventral root)	Corti S *et al.*, 2006
Presymptomatic irradiated SOD1 G93A mice (8 w old)	hUCB cells	R.o. injection, 34.2–35×10^6 cells	Delay in onset Increase in Life Span	Ende N *et al.*, 2000
Presymptomatic, irradiated SOD1 G93A mice (8 w old)	mBM cells	R.o. injection, $5 \cdot 10^6$ cells	Delay in onset Increase in Life Span	Ende N *et al.*, 2000
Symptomatic SOD1G93A mice (P114)	hNT cells	Bilateral lumbar SC injection, 1 site, $7.5 \cdot 10^4$ cells/site	No effect on average survival	Garbuzova-Davis S *et al.*, 2008
Adult rats with sciatic axotomy	K048 hNSCs treated with Shh	Unilateral lumbar SC injection, 1 site, $1 \cdot 10^5$ cells	Partial recovery from paralysis	Gao J *et al.*, 2005
5–7 w old rats with chronic, bilateral motor neuron deficiency	mES cells treated with Shh and RA	Bilateral lumbar SC injections, 1 site, $6 \cdot 10^4$ cells	Partial recovery from paralysis	Deshpande DM *et al.*, 2006

Animal Models	Cell source	Injection sites and cell numbers	Effect on onset and/or survival	References
Presymptomatic, irradiated SOD1 ^{G93A} mice (4 w old)	mBM cells from Thy1-YFP or CBA-eGFP or SOD1 ^{G93A} mice	I.p. injection, $30 \cdot 10^6$ cells	Delay in onset and increase in life span of wildtype BMCs. No effect of SOD1 ^{G93A} BMCs	Corti S *et al.*, 2004
Presymptomatic SOD1 ^{G93A} mice (P56)	hNT cells	Bilateral lumbar SC injection, 1 site, $7.5 \cdot 10^4$ cells	Slight delay of onset	Garbuzova-Davis S *et al.*, 2003
Symptomatic SOD1G93A mice (P114)	hNT cells	Bilateral lumbar SC injection, 1 site, $7.5 \cdot 10^4$ cells / site	No effect on average survival	Garbuzova-Davis S *et al.*, 2008
Adult rats with sciatic axotomy	K048 hNSCs treated with Shh	Unilateral lumbar SC injection, 1 site, $1 \cdot 10^5$ cells	Partial recovery from paralysis	Gao J *et al.*, 2005

BDNF, brain-derived neurotrophic factor; ChAT; choline acetyltransferase; CMAP, compound muscle action potential; CNTF, ciliary neurotrophic factor; CSF, cerebrospinal fluid; CXCR4, chemokine (C-X-C motif) receptor 4; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hMSC, human mesenchymal stem cell; hNPC, human neural progenitor cell; hNSC, human neural stem cell; HNu, human nuclear antigen; hUCBC, human umbilical cord blood cell; L1CAM, L1 cell adhesion molecule; MAP2, microtubule-associated protein 2; mBMC, mouse bone marrow cell; MN, motor neuron; mOB-NPC, mouse olfactory bulb neural precursor cell; NeuN, neuronal nuclei; NF, neurofilament; O4, oligodendrocyte marker; rAAV, recombinant adeno-associated virus; rMSC, rat mesenchymal stem cell; SC, spinal cord; SOD1, superoxide dismutase 1;Tuj1, antibody against β-III tubulin; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein.

1.4.1. The Wobbler mouse.

The original wobbler mouse mutation (*wr*) was first described by Falconer (1956) as a autosomal recessive genetic modification that appeared spontaneously in the congenic C57BL/6J stock and caused a wobbly gait characteristic of the mice (Falconer *et al*., 1956). Subsequently, the original *wr/wr* mouse strain was crossbred with a high fertility NFR/N stock to improve productivity. Since then, mice of the new wobbler (Wr) lineage have been well characterized (Mitsumoto, H. *et al*., 1988; Mitsumoto, H., and W. G. Bradley., 1982). This hybrid strain presents the same phenotypic characteristics as mice of the original Wr line (Takeo Ishiyama *et al*., 1997). The autosomal recessive mutation is pleiotropic, causing progressive motorneuron degeneration and defective spermiogenesis (Duchen L.W. and Strich S.J., 1968; Heimann, P. *et al*., 1991). A more accurate characterization of the motoneuronal degeneration in the ventral horn of the cervical spinal cord, in the brainstem, and in the cortical regions of the wobbler (Wr) brain have prompted researchers to consider the Wr mouse as a good model of amyotrophic lateral sclerosis (ALS) (Pioro E.P., *et al*.1998).

The statistical analysis of the Wr strain progeny by Falconer and colleagues, has provided the genetic mapping of the wr mutation to proximal mouse chromosome 11 in a region homologous to human chromosome 2p13–14. A recent study by Thomas Schmitt-John has refined the genetic localization of the *wr* locus, identifying a missense mutation in the *Vps54* gene (Schmitt-John, T. *et al*., 2005). This locus encodes for the vacuolar protein sorting 54 (Vps54), a subunit of the Golgi-associated retrograde protein (GARP) complex involved in the

retrograde transport of endosomes from the periphery to the Golgi apparatus. Until now the precise mechanism by which the missense mutation in Vps54 causes motoneuron disease is not known. Chimera analysis has shown that the Vps54 mutation exerts a pleiotropic action through a tissue or cell-autonomous mechanism in the affected organs, spinal cord and testis (Augustin *et al*., 1997). Up to now, there is no evidence for mutations of Vps54 human homologus in patients with ALS. Because of the clinical symptomatology and neuropathological Wr findings fit closely with human ALS it possible that common pathways to cell death, perhaps including impaired vesicle traffic are involved (Meisler MH, *et al*., 2008).

The evolution of motor neurodegeneration in the Wr line develops into three clinical phases: the presymptomatic phase, the evolutionary phase and the stationary phase (table 5) (Duchen *et al*., 1968).

Table 5: Overview of the clinical Wr phases and neuropathological Wr findings.

Clinical Phases	Time Slot	Clinical Characteristics	Pathological Findings	References
Preclinical Phase	The first mo of age	Subtle alteration in the righting reflex. Slight reduction in the normal weight ranges	Subtle Morphological Changes of spinal cord and brain stem MN	Bose *et al.*, 1999.
Evolutionary Phase	1–3 mo of age	40-50% of reduction in weight. Progressive Muscolar atrophy.	Loss of beetwen 20% to 40% of the MN in brainstem and cervical spinal cord. Neurodegeneration in the cerebral cortex and cortical hyperexcitability	Nieto-Gonzalez *et al.*, 2010; Pioro et al 1998;
Stabilization Phase	3–6 mo of age	Preserved motility only in the hindlimbs	Arrest of MN Degeneration. Severe muscular denervation of the forelimbs.	Melki J. *et al*., 1991

The presymptomatic phase occur in the first 4 weeks of postnatal life, in which the mice that are homozygous for the *wr* mutation do not display severe clinical signs of the motor neuron disorder unless there is a subtle alteration in the righting reflex at three to seven postnatal days of age and an impaired performance during grid walking at 2 wk of age.

Moreover, prior to 3 week of age, the Wr mice show a slight reduction in the normal weight ranges(Bose *et al., 1998; Bose et al., 1999).

The histological and molecular studies of the presymptomatic phase have revealed subtle morphological alterations of the motorneurons in the cervical spinal cord, in the brainstem, in the ventral reticular magnocellular nucleus, and in the motor nuclei of the cranial nerve V and VII of the mutant. These anomalies are characterized by an enlargement of motorneuron soma and a weak labeling of Nissl bodies. Sporadic neurons with a faded Nissl staining or chromatolysis have also been described in thalamic nuclei of the extrapyramidal system at 2 week of mutant age (Schmitt-John T., and Bartsch J.W., 1999). During the third week of Wr age the affected neurons show the first evidence of mild axon degeneration with ultra structural alterations of the cytoskeleton, abnormal invagination of the myelin sheaths, dilation of the endoplasmic reticulum, and the presence of large and dense secondary lysosomes. (Mitsumoto, H., and W. G. Bradley., 1982)

Alterations in the synthesis of neurofilaments, Chaperone Protein neuropeptide, Growth Factors and Growth-Factor-Receptor in the affected Wr Central Nervous System regions between the late presymptomatic phase and the early evolutionary phase have also been reported (table 6)

From the third to the fourth week of Wr age, Wr weight is between 40-50 % less than that of their control littermates and remains lower thereafter. At this juvenile stage, Wr mice show the first overt symptoms of the progressive neurological syndrome that develops

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until the second to third month of life. This stage corresponds to the evolutionary phase of the motor neurodegeneration (Coulpier M. *et al*.,1996; Bose *et al.,* 1999).

The clinical abnormalities of the Wr mice result in muscular atrophy and motor impairment that are particularly conspicuous in the forelimbs, e.g., biceps and triceps muscles. The symptoms in the early evolutionary phase are a tremor of the head, weakness of forelegs and unsteady gait. These clinical onsets progress in later stages until motility is retained only in the hindlimbs (Andrews J.M. *et al*., 1974).

The major histological features of the early evolutionary phase are characterized by a cytoplasm vacuolization of the motoneurons in the cervical spinal cord, brainstem and deep cerebellar nuclei followed by a progressive loss of affected motoneurons (Mitsumoto H and Bradley WG 1985; Duchen L.W. and Strich S.J.,1968; Campbell MJ *et al.,* 1972).

At 6 week of mutant age there is a loss of beetwen 20% to 40% of the motoneurons in brainstem and cervical spinal cord accompanied by extensive astrogliosis (Baulac *et al*., 1983; Pollin M.M., *et al.,* 1990; Junier *et al*., 1994). The neuronal loss is then stabilized but the vacuoles, which are present in the cell body of the surviving affected motoneurons, become increasingly large and numerous, until they completely fill the soma. These vacuoles originate from the dilation of the endoplasmic reticulum (Duchen *et al*., 1968; Andrews J.M. *et al*., 1975).

The morphological changes of the motorneuronal soma are accompanied by axonal degeneration, an event explained by the

reduction of axonal number or diameter and the alteration of axonal transport systems.

The slow axonal transport, fast anterograde, and fast retrograde transports display a reduction of the proteins transported (Bird M.T. *et al*., 1971; Mitsumoto *et al*., 1986; Mitsumoto *et al*., 1990; Mitsumoto *et al*., 1993).These phenomena may be related to the accumulation of abnormal neurofilaments observed during the presymptomatic period.

The most relevant molecular alterations observed in the Wr cervical spinal cord during evolutionary phase concern the molecular factors belonging to the pathways of neurotrophism, motorneuronal differentiation, astrogliosis and oxidative stress.

During the Wr evolutionary phase there is an increased expression of proteins believed to be involved in motoneuronal development and gliosis, such as brain-derived neurotrophic factor (BDNF) and its associated receptors, trkB and p75 NTR, c-Jun, transforming growth factor alpha (TGF α), and the growth-associated protein GAP-43 (Table 6). These evidences indicate that the Wr mutation may stimulate the production of molecules endowed with glio- or neurotrophic properties in an attempt to delay degeneration, considering that these factors promotes neuronal survival after axotomy and differentiation of embryonic neurons (Henderson *et al*., 1986).

However, the Wr motorneuronal degeneration progress and there is a decline of expression of choline acetyltransferase (ChAT) protein, the limiting enzyme for the synthesis of acetylcholine, a main neurotransmitter released by motoneurons.

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Astrogliosis with strong expression of the glial fibrillary acidic protein (GFAP) are found in Wr gray and white matters of the cervical spinal cord, primary motor cortex, and subpial regions. This phenomena is in common with ALS patients. To date the cause of astrocytosis in Wr mice and ALS patients are unknown. Some authors consider the astrogliosis event a secondary response to neurodegeneration In the other hand, available evidences suggest that the Wr mutation cause the abnormal astrocytic differentiation. In this sense, the dysfunction of astricyties could have a role in the motorneuronal degeneration.

In the 2002 study Corvino et al demonstrated the Wr astroglia hyperexpression of the S100B, a calcium-binding protein. These authors have show that the high concentrations of S100B protein is correlate with the increased immunoreactivity for the indicator of lipid peroxidation 4- hydroxynonenal (HNE) in the Wr spinal cord, providing the direct evidence of the occurrence of oxidative stress in Wr pathology (Corvino *et al.*, 2002).

The higher expression of nitric oxide synthase (NOS) with chronic free radical production found in Wr mice cervical spinal cord is one of the indirect evidences on the participation of oxidative stress in Wr neuropathology (Clowry and McHanwell., 1996).

Whether astroglia dysfuncitons are responsible for oxidative stress or not is yet a controversial question;

Recently, several studies have shown the mitochondrial dysfunction in the Wr motor cortex and spinal cordat the early stage of evolutionary phase. These findings lead to the hypothesis that

production of free radicals by affected mitochondries and its subsequent impact on lipid peroxidation, may play a key role in causing the early pathology in the Wr motor neurons (Xu G.P. *et al*., 2001; Kunjan R. D. *et al*.,2003).

Links between oxidative stress and mitochondrial dysfunctions in human ALS disease have also established (Bowling A. *et al*.,1993; Shaw, P.J., Eggett, C.J., 2000; Sasaki S and Iwata M., 2007).

Several studies have demonstrated that caspase-dependent apoptosis or necrosis does not account for Wr motoneuron death (Blondet B. *et al*., 200; Coulpier M. *et al*.,1996; Popper P. *et al*., 1997; Chu-Wang I.W. *et al*., 1978; Pilar G. and Landmesser L., 1976; Clarke P.G.H., 1990; Bigini *et al*., 2007).

In the 2007 study, Bigini and colleagues have shows the lack of apoptotic markers, such as caspase activation and DNA fragmentation, in the degenerating motorneuron of the Wr cervical spinal cord. Moreover Bigini et al characterize the intense vacuolization and the morpho-functional alterations of mitochondria in the cytoplasm of degenerating motor neurons suggesting the possible involvement of autophagic cell death in the Wr cervical motor neurons (Bigini *et al.*, 2007). However the identity of the components of the death pathways that are mobilized in Wr mice has not yet been identified.

The Wr cervical spinal cord motordegeneration, muscular denervation, astrocytosis, overexpression of trophic factors and oxidative stress with hyperexpression of NOS and S100B are the characteristics that fit with the spinal cord of ALS patients. Despite

these correlations, the Wr spinal cord neuropathology differ from the human ALS in fact of the selective Wr vulnerability of the cervical spinal cord compared to the lumbar tract. This Wr pathophysiological characteristic is a particularly interesting tool for further investigating the cellular determinants of motor neuron degeneration. In this line of purpose Bastone and colleagues have recently identified different pattern of proteins expression between the affected cervicaI tract and the Iumbar tract of the Wr spinaI cord. These authors show the selective Wr lumbar tract overexpression of proteins involved in vesicular and axonaI transport and proteins involved in mitochondriaI function. The hyperexpression of these proteins may have a neuroprotective effects and significantly contribute in maintaining integrity and functionality of motor neurons in the lumbar tract of the Wr spinaI cord (Bastone *et al*.,2009).

Several ultrastructural abnormalities have been found in the evolutionary phase of the Wr brain closely resembling those observed in the brain of patients with ALS.

In the 1998 study, Pioro and colleagues demonstrated the neuronal pathology in the neocortex of the mutant wobbler mouse by *in vivo* magnetic resonance spectroscopy 1H-MRS. These analysis have revealed a reduction in the ratio of *N*-acetylaspartate (NAA), one of the major metabolites localized to neurons, and creatine phosphocreatine (Cr) which serves as an internal standard   in the cerebral cortex of the wobbler mouse during the early evolutionary phase (8-12 wees of mouse age). The decrease of NAA/Cr signal in 1H-MRS analysis is a typical characteristic finding in ALS patients,

indicating neuronal dysfunction and/or loss (Giroud M. *et al.,* 1996; Gredal O. *et al.,* 1997) .

Pioro et al analyzed the metabolic alteration in the wobbler neocortex ex vivo by immunocytochemistry. These studies revealed the accumulation of ubiquitin and phosphorylated heavy neurofilament (P+NFH) in the wobbler cerebral cortex (Pioro E.P. *et al.,* 1998).

Similar immunocytochemical changes have also been reported in cortical neurons of patients with ALS (Leigh PN. *et al*.,1991).

Recently Nieto-Gonzalez and colleagues have demonstrated the cortical hyperexcitability of the Wr mice and their correlation whit alterations in inhibitory gammaaminobutyric acid (GABA)ergic system (Nieto-Gonzalez *et al.,* 2010).

Similar cortical hyperexcitability has been demonstrate in the ALS patients, but the cause is still unknown (Caramia MD *et al.,* 2000; Vucic S *et al.*, 2008). The studies of Nieto-Gonzalez et al raises the possibility that alterations in inhibitory gammaaminobutyric acid (GABA)ergic system could be explain this human ALS cortical symptom.

The evidence of neuronal pathology in the Wr mouse brain shows that neuronal target cells of the *wr* mutation are not restricted to motoneurons, but extend to upper brain areas,supporting the validity of Wr mice as an animal model of ALS where cortical motor neuron degeneration is usually present.

Table 6: Overview of Molecular Alterations in the Wr MN and Their Glial Environment.

Molecule	V	Tissue	Method	References
BDNF	+	MN	RNA: RT-PCR Protein: ICC	Junier M.P. *et al*., 1998
BDNF	+	MN, SC	Protein: ICC	Tsuzaka K *et al*., 2001
TGFα	+	dMN	RNA: RT-PCR Protein: ICC	Junier M.P. *et al*., 1998
SMDF	0	dMN	RNA: RT-PCR	Junier M.P. *et al*., 1998
NT-3	0	dMN	RNA: RT-PCR Protein: ICC	Junier M.P. *et al*., 1998
TNF-α, IL-1β	+	SC, brainstem, cerebellum	RNA: RT-PCR	Schlomann U. *et al*., 2000
IL-2, IL-6, IL-10, IL-12, IL-18 IFN-γ	0	SC, brainstem	RNA: RT-PCR	Schlomann U. *et al*., 2000
Trk B	+	dMN	RNA: RT-PCR	Junier M.P. *et al*., 1998
Trk C	0	SC dMN	RNA: RT-PCR	Junier M.P. *et al*., 1998
C-Jun	+	dMN	RNA: RT-PCR	Junier M.P. *et al*., 1998
P75NTR	+	dMN	RNA: RT-PCR Protein: ICC	Junier M.P. *et al*., 1998
NF light	0 + +	SC SC dMN	RNA: RT-PCR Protein: Western RNA: RT-PCR	Pernas-Alonso R. *et al.,* 1996
NF medium	+	SC	RNA: RT-PCR Protein: Western	Pernas-Alonso R. *et al.,* 1996
GAP-43	+	MN, dhSC	RNA: ISH Protein: ICC	Gonzalez D. M.C *et al.,* 1999
Adhesion molecule F3	0	SC	RNA: RT-PCR	Pernas-Alonso R. *et al.,* 1995
peripherin	0	SC	RNA: RT-PCR	Pernas-Alonso R. *et al.,* 1995
ubiquitin	+	Cortical neurons	ICC	Pioro E.P.*et al.,*1998
NOS (NAPDH diaphorase)	+	dMN	Activity: histochemistry	Gonzalez D. M.C *et al.,* 1999
PAR-1	+	SC	RNA: RT-PCR	Salcedo R.M.*et al*., 1998
TRPM-2	+	SC, MN	RNA: ISH, northern	Krieger C. *et al*., 1991
ADAM-8	+ 0	SC, brainstem cortex	RNA: RT-PCR Protein: ICC, western	Schlomann U.*et al*., 2000

The variations (V) as compared to control litter mates are symbolized with + (increase), – (decrease), 0 (no change). MN, motoneurons; dMN, motoneurons

presenting histological signs of degeneration; astro, astrocytes; glia, microglial cells; SC, spinal cord; dhSC, dorsal horn of the spinal cord; ICC, immunocytochemistry; ISH, *in situ* hybridization; SMDF, sensory and motor neuron-derived factor; TRPM-2, testosterone-repressed prostatic message 2. TGF-α and TNF-α are putative candidates for a role of motoneuronal inducers of astrogliosis (Junier M.P. *et al.,* 1998¸Krieger C. *et al*., 1991). Activation of the cell-surface receptor of the activated protease (PAR-1) by the serine protease thrombin may underlie an eventual reciprocal action of the reactive astrocytes upon the MN. Activation of PAR-1 by thrombin has recently been shown to result in apoptosis of various neuronal populations and of avian MN in particular. In Wr spinal cords, PAR-1 mRNA levels are slightly increased at birth, and multiplied by 5 in 4-wk-old mutants. Its cellular sources correspond to spinal MN and in vitro Wr astrocytes release enhanced levels of thrombin-like activities (Salcedo R.M.*et al*., 1998).

The progression of the motorneuron degeneration during the Wr evolutionary phase is associated with progressive physiological muscular denervation atrophy of the head, the neck, the shoulders, and the forelimbs and less significantly the posterior muscles. At the end of the Wr evolutionary phase the affected muscles show several anomalies of the neuromuscular synapses whit a severe reductions of the neuronal-cell adhesion molecule (N-CAM) expression, acetylcholine esterase activity and a reorganization of the neuromuscular contacts (Melki J. *et al*.,1991).

The Wr evolutionary period is followed by a stabilization phase, in which the clinical symptoms appear to reach a steady state. During this stage the Wr motoneuron loss stops and the valcuolization of the motorneuron disappear at 14 months of Wr age. The survival of affected mice is approximately 1 year when they are maintained together with normal littermates (Duchen *et al*., 1968).

The terminal stage of the Wr muscular atrophy is reached at the stationary state with the most severe symptoms that appear in the forelegs muscle. In fact the electromyographic recordings of the

forelimbs muscles at the Wr stationary state display characteristics of muscular denervation, whereas, the hind limb muscles are electromyographically normal (Andrews J.M. *et al.*,1974).

In summary the refined investigations of the Wr neurodegeneration at the clinical, cellular, and molecular levels reveal that motoneuron degeneration is accompanied by the classical etiopathological mechanisms that are common to other types of neurodegenerative illnesses excitotoxicity, defective free radical detoxification pathways, and accumulation of toxic protein aggregates. Thus the studies of the effects of potential therapeutic agents in Wr model, enable the researchers to develops new efficient therapies, such as cell therapy, also for other human neurodegenerative diseases.

Scope of the thesis

Stem cell research has the potential to transform disease treatment, reduce costs to society and enhance the quality of life for millions of patients(Chapter1).

The stem cell technology for neurological disorders such as Amyotrophic lateral sclerosis disease (ALS) promise to provide potentially curative treatments for this and other progressive and debilitating conditions.

In this thesis we analyze the neuroprotective and neurorestorative proprieties of human SkmSCs transplanted in an animal model of ALS diseases (Chapter 2). Moreover we demonstrated the utility of human neural stem cells, isolated from LND tissues, for studying disease and identifying potential therapeutics (Chapter3).

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Chapter 2 Innovative Stem Cell Therapy using human skeletal muscle derived stem cells in a mouse model of motor neuron disease

Laura Canzi MSc[1,4], Stefania Navone Ph.D.[1], Valeria Castellaneta[2] , Ileana Zucca Ph.D.[3], Gloria Invernici Ph.D.[1] , Silvia Cristini Ph.D.[1], Tiziana Mennini Ph.D.[2] , Paolo Bigini Ph.D.[2], Eugenio A. Parati M.D.[1].

[1]Department of Cerebrovascular Disease, IRCCS Foundation, Neurological Institute "C. Besta". via Celoria, 11 20133 Milan, Italy. [2]Department of Molecular Biochemistry and Pharmacology, Mario Negri Institute for Pharmacological Research, via La Masa, 19 20156 Milan, Italy. [3]Department of Scientific Direction, IRCCS Foundation, Neurological Institute "C. Besta". Via Celoria, 11 20133 Milan, Italy. [4]Ph.D. Program in Translational and Molecular Medicine (DIMET) University of Milano-Bicocca.

submitted

2.1. Abstract

Stem Cell Therapy represents a promising approach for treating incurable and fatal motor neurodegenerative disorders associated with progressive muscular atrophy such as Amyotrophic Lateral Sclerosis (ALS). Various sources of Stem Cells have been used in pre-clinical cell therapy research to evaluate their therapeutic potential on different neurodegenerative disorders. As candidates for the treatment of neurodegenerative diseases, mesenchymal stem cells possess numerous protective features as well as the capability to differentiate into functionally specialized neurons.

To determine the potential of non-neural stem cells for Central Nervous System (CNS) cell therapy, we investigated the usefulness of human Skeletal muscle derived stem cells (SkmSCs) transplants in the oldest known rodent model of the naturally occurring ALS disease, Wobbler (Wr) mouse.

In order to perform effective cell therapies, SkmSCs'homing was evaluated in a noninvasive manner. Using MRI longitudinal studies, we verified the location, distribution and long-term viability of human SkmSCs labeled with the contrast agent Endorem™, a superparamagnetic iron oxide (SPIO) associated with dextran, and transplanted in the lateral ventricles of the Wr brain. Remarkably, using histology, we show that, upon transplantation, SPIO-labelled hSkmSCs migrate and integrate in a manner appropriate for their location. In order to identify the colocalization between SPIO signal

and human SkmSCs engraftment, we performed histology with Anti-dextran and human specific neural marker antibodies.

Moreover, in order to determine the functional outcomes of SkmSCs engraftment, we performed clinical evaluation of ALS-related Wr motorneuropathological progression and muscular atrophy. Interestingly, the behavioural tests and histological analysis seem to suggest a functional motor recovery and a significant slowing of disease progression. Although further experiments to elucidate the human SkmSCs beneficial effects are required, the possibility to link the interactions between transplanted cells in host tissue to behavioural studies represents an original approach, likely to be associated with future clinical translation. Taken together, our findings demonstrate that human SkmSCs transplanted into the Wr brain survive and can improve behavioural deficits associated with widespread ALS disease pathology.

2.2. Introduction

Cell based therapies holds great promise for the treatment of acute and chronic central nervous system diseases that currently lack effective therapies. The main idea of stem cell therapy is based on the mainstream concept of protecting the parenchymal cells from degeneration and replacing those that have already failed in their function. As candidates for the treatment of neurodegenerative diseases, mesenchymal stem cells (MSCs) are emerging as an effective therapeutic approach to a wide range of neural insults.

Indeed, recent reports have shown that administration of MSCs also produced beneficial effects in animal models of neurodegenerative diseases, such as Parkinson's disease, experimental autoimmune encephalomyelitis, and stroke (Rina Aharoni et al., 2009). Human MSCs niches reside in various tissues, from which different amounts of human MSCs can be isolated.

Among them, Skeletal muscle derived stem cells (SkmSCs) have been functionally defined as multipotential cells with the capacity to transdifferentiate into many types of cells including neurons and glial cells (Alessandri *et al.,* 2004; Mignon *et al.*, 2005; Romero-Ramos *et al.*, 2002; Schultz *et al.*, 2006; Tamaki *et al.*, 2007; Vourc'h *et al.*, 2004; Baek YS *et al.*, 2009). The plastic ability of human SkmSCs to differentiate into neurogenic cell lineage and their neuroprotective effects make them a good candidate for autologous stem cell-based therapy purposes.

Amyotrophic lateral sclerosis (ALS), described in 1869 by the French neurologist Jean-Martin Charcot, is a fatal neurodegenerative disease whose cause is still unknown. ALS is sometimes referred to as 'Lou Gehrig disease' after the famous American baseball player who was diagnosed with the disorder. The clinical distinctiveness of the ALS pathophysiology is characterized by the progressive motor neuron degeneration in the spinal ventral horns, in most brainstem motor nuclei, corticospinal and corticobulbar systems. The loss of motor neurons leads to progressive atrophy of skeletal muscles. The fatal outcome caused by ALS is usually within 2–5 years after onset and it is due to acute muscular atrophy leading to respiratory failure with denervation of the respiratory muscles and diaphragm. (Bradley,

1996). The annual incidence of ALS disease is about 2–6 cases/100,000 with a lifetime risk of developing ALS of 1 per 800 (Cleveland and Rothstein, 2001). The median age of onset is 55 years (Adult-Onset ALS form), although it can start at younger ages (Juvenile-Onset ALS form). To date, many attempts have been made to understand the cellular and molecular ALS pathophysiological mechanisms. However, little is known about the genetic defects or environmental factors that cause or predispose persons to ALS.

In 90–95% of instances, there is no apparent genetic linkage (a form of the disease referred to as sporadic ALS), but in the remaining 5–10% of cases, the disease is inherited in a dominant manner (a form referred to as familial ALS). The hallmark of both forms is progressive muscle weakness, atrophy and spasticity. Denervation of the respiratory muscles and diaphragm is generally the fatal event. Development of an effective therapy to ALS is complicated by the multiple events considered to contribute to the selective loss of motor neurons. Many different drugs have been tested for their capacity to alleviate or retard the symptoms of ALS patients and to prolong their survival, but none has proved to be effective. The only currently Food and Drug Administration-approved treatment for ALS that slightly slows disease progression and prolongs the survival of ALS patients, with no improvement in muscle function, is riluzole. (Wokke J *et al.,* 1996).

Under these circumstances, biomedical research is the only hope to develop possible therapeutic strategies.

In this sense experimental in vivo models have been developed to improve the knowledge of the ALS pathophysiology and the

development of new pharmacological treatments. These include animals with spontaneous motoneuron degeneration, transgenic rodents, and animals in which spinal motoneuron death was produced with pharmacological tools.

Among them, Wr mice are one of the most valuable models for the study of ALS syndrome progression and therapeutic treatment effects.

The original wobbler mouse mutation (*wr*) was first described by Falconer (1956) as a autosomal recessive genetic modification that appeared spontaneously in the congenic C57BL/6J stock and caused a wobbly gait characteristic of the mice (Falconer *et al*., 1956). Subsequently, the original *wr/wr* mouse strain was crossbred with a high fertility NFR/N stock to improve productivity. Since then, mice of the new Wr lineage have been well characterized (Mitsumoto, H. *et al*., 1988; Mitsumoto, H., and W. G. Bradley., 1982). This hybrid strain presents the same phenotypic characteristics as mice of the original Wr line (Takeo Ishiyama *et al*., 1997). The autosomal recessive mutation is pleiotropic, causing progressive motorneuron degeneration and defective spermiogenesis (Duchen L.W. and Strich S.J., 1968; Heimann, P. *et al*., 1991). A more accurate characterization of the motoneuronal degeneration in the ventral horn of the cervical spinal cord, in the brainstem, and in the cortical regions of the Wr brain have prompted researchers to consider the Wr mouse as a good model of amyotrophic lateral sclerosis (ALS) (Pioro E.P., *et al*.1998;).

A recent study by Thomas Schmitt-John has refined the genetic localization of the *wr* locus, identifying a missense mutation in the *Vps54* gene (Schmitt-John, T. *et al*., 2005). This locus encodes for the vacuolar protein sorting 54 (Vps54), a subunit of the Golgi-associated

retrograde protein (GARP) complex involved in the retrograde transport of endosomes from the periphery to the Golgi apparatus. Until now the precise mechanism by which the missense mutation in Vps54 causes motoneuron disease is not known. Chimera analysis has shown that the Vps54 mutation exerts a pleiotropic action through a tissue or cell-autonomous mechanism in the affected organs, spinal cord and testis (Augustin *et al*., 1997). Up to now, there is no evidence for mutations of Vps54 human homologus in patients with ALS. Because the clinical symptomatology and neuropathological Wr findings fit closely with human ALS, it possible that common pathways to cell death, perhaps including impaired vesicle traffic are involved (Meisler MH, *et al*., 2008).

The evolution of motor neurodegeneration in the Wr line develops into three clinical phases: the presymptomatic phase, the evolutionary phase and the stationary phase (Duchen *et al*., 1968).

The presymptomatic phase occur in the first 4 weeks of postnatal life, in which the mice that are homozygous for the *wr* mutation do not display severe clinical signs of the motor neuron disorder. (Bose *et al.,* 1998*;* Bose *et al.,* 1999).

From the third to the fourth week of Wr age, Wr weight is between 40-50 % less than that of their control littermates and remains lower thereafter. At this juvenile stage, Wr mice show the first overt symptoms of the progressive neurological syndrome that develops until the second to third month of life. This stage corresponds to the evolutionary phase of the motor neurodegeneration (Coulpier M. *et al*.,1996; Bose *et al.,* 1999).

The clinical abnormalities of the Wr mice result in muscular atrophy and motor impairment that are particularly conspicuous in the forelimbs, e.g., biceps and triceps muscles. The symptoms in the early evolutionary phase are a tremor of the head, weakness of forelegs and unsteady gait. These clinical onsets progress in later stages until motility is retained only in the hindlimbs (Andrews J.M. *et al.*, 1974).

At 6 weeks of mutant age, there is a loss of beetwen 20% to 40% of the motoneurons in brainstem and cervical spinal cord accompanied by extensive astrogliosis (Baulac *et al.*, 1983; Pollin M.M., *et a.,l* 1990; Laage *et al.*, 1988; Hantaz-Ambroise *et al.*, 1993; Junier *et al.*, 1994).

Muscle weakness and contracture in the forelimbs progress rapidly until eight weeks of age. Subsequently, these neuromuscular deficits progress slowly. Motor function of the forelimbs decline to the terminal stage at 10-12 weeks of age.

The Wr evolutionary period is followed by a stabilization phase, in which the clinical symptoms appear to reach a steady state.

Noninvasive monitoring of stem cells, using high-resolution molecular imaging, will be instrumental to improve clinical neural transplantation strategies. Neurotransplantation holds great promise for the treatment of acute and chronic central nervous system diseases. The ability to track stem cell transplants in the brain by in vivo neuroimaging will undoubtedly aid our understanding of how these cells mediate functional recovery after transplantation.

In order to reach this goal, SPIO-labeled human SkmSCs were transplanted into the lateral cerebral ventricles of 4 weeks Wr mice. We then used MRI to analyze migration patterns of SPIO-labeled

transplanted SkmSCs. Using MRI, we were able to detect the SPIO-related signal into the Wb CNS until sacrifice. Moreover, using histology, we show, interestingly, that upon transplantation, SPIO-labelled SkmSCs migrated and integrated in a manner appropriate for their location.

Additionally, our results of behavioural tests in SkmSCs transplanted Wr mice seem to suggest a functional motor recovery and a significant slowing of disease progression. Results of the present study confirm and extend our previous findings, showing that human SkmSCs are able to differentiate into neural cell lineage. The SkmSCs, when transplanted into the lateral ventricles of Wr mice of 4 weeks of age, home along ventricles and integrate into the region of severe motor neurodegeneration, ameliorating the clinical outcome.

Knowledge of hSkmSCs migration patterns and the possibility to link the interactions between transplanted cells in host tissue to behavioural studies represents good strategies to improve the design of future clinical transplantation efforts.

2.3. Results

2.3.1. In vitro Characterization of Human SkmSCs

We isolated, expanded and characterized five human SkmSCs lines in order to verify their proliferative and differentiative potential.

Our protocol to isolate and culture human SkmSCs is previously described (Alessandri et al., 2004).

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After the digestion of Fetal muscle specimens (2gr) lead to obtain a large number of cells (6x107), with a heterogenic population of differentiated and immature cells, are obtained. The suspended cells, containing differentiated and more immature cells, are seeded in tissue culture flasks using serum-free growth medium.

After three passages (P3), these primary cultures are depleted of differentiated cells due to the serum-free conditions and only the more immature cells survive and grow. Under these circumstances, we are able to isolate an homogeneous human SkmSCs population with human mesenchymal stem cells-like morphology that grow as adherent cultures. As human SkmSCs gradually continue to grow and reached confluency, they are constantly collected in order to count the cell number with Trypan Blue Assay. A result, we observed that human SkmSCs kept in culture for several weeks (up to P20) maintain a stem cell-like'proliferation profile (fig. 1 A) The immunophenotypic analysis by FACS of this cell population showed that a high percentage of the human SkmSCs (P8-10) expressed characteristic CD markers of mesenchymal stem cells, e.g., CD105 (89.18 ± 4.44%), CD73 (98.27 ± 2.57%), CD90 (96.59 ± 2.57%), CD44 (98,9 ±0,75); CD166 (69.25 ± 5.95%) and CD56 (69, 7±11, 6).

However hematopoietic stem cell CD markers seem to be present in a lower fraction of human SkmSCs (P8-10), e.g., CD34 (44,8 ± 27,8), and CD133 (33,5±19,1) (fig. 1 B) This suggested a common precursor between the hematopoietic and mesenchymal lineages (Yin A H et al., 1997).

For neural differentiation assays, morphologically homogeneous human SkmSCs, (P10) were plated into matrigel-coated dishes in presence of growth factor deprived medium + 2% of FBS.

Human SkmSCs, when induced with neural differentiation media for seven days, started showing morphological changes suggestive of cells of astrocytic/neuronal and oligodendroglial lineages by day 4. At seven days post neural differentiation conditions, the Human SkmSCs samples stained positive and for early neural markers such as Nestin, Tuj1/Beta tubulin-III, GFAP on immunocytochemistry with a reproducible percentage of all neural markers, respectively: Nestin 56% ± 3,6; Tuj1/Beta tubulin-III 78% ± 7,5 and GFAP 76% ± 7,2 (fig.1 C and D)

These cells are cryopreservable and retain both proliferation and differentiation capacities even after long term in culture.

2.3.2. In vitro labeling of SkmSCs with SPIO and PLL transfect agent

In order to perform longitudinal studies with MRI of transplanted Wr mice, we labeled human SkmSCs (P10) with SPIO contrast agent.

We observed that SPIO labelling of human SkmSCs is feasible and does not impair cell viability or biological characteristics in vitro. We achieved a 100% labelling efficiency as determined by Anti-Dextran and Hoechst 33258 staining (fig. 2 A).

Moreover, there was no difference in the FACS analysis of CD expression markers and differentiation behaviour between SPIO-labeled and unlabeled Human KmSCs (data not show).

2.3.3. In vivo-ex vivo correlation of SkmSCs tracking Hoechst/SPIO- double labeling

The double labeling of SkmSCs with fluorescent (Hoechst 33258) and SPIO tracers allowed us to evaluate the possible anatomical correspondence between the hypo-intense signal associated with the presence of iron oxide and the fluorescence related to the nuclear marker.

MRI analyses, performed 1 day after cell transplantation, showed hypo-intense areas corresponding to the ventricular system. These results were similar to those observed in animals only receiving the SPIO-labeled SkmSCs (Fig 3A; Fig 3B) and were observed for all transplanted Wr mice. The visualization of brain slices from the two Wr sacrificed one day after SkmSCs transplantation, confirmed the almost selective localization of fluorescent nuclei along the different compartments of ventricular system. Figure 2B shows four different representative images of axial MRI slices, six days after cells transplantation, corresponding to the anterior body of lateral ventricles (Fig. 2B upper-left panel), the body of lateral ventricles close to the hippocampus (Fig. 2B upper-right panel) and the periaqueductal grey and cistern magna in the cerebellum and brainstem (Fig. 2B lower-left panel). Quite interestingly, the observation of spinal cord slices revealed that, already 24h after transplantation, a relevant amount of Hoechst positive nuclei is localized in the external surface, likely associated with meningeal layers (Fig. 2B lower-right panel). The MRI analysis confirmed the pattern of SPIO distribution observed one

day after cells transplantation. In addition highly magnified pictures, revealed the presence of Hoechst 33258 positive nuclei in correspondence of ventricular compartments characterized by a strong decay of signal intensity (Fig. 2B lower-left insert). These results further strengthen the correlation between the two exogenous tracers internalized in observed SkmSCs before transplantation and account for the presence of cells far from the site of administration and close to the site of motor neuron degeneration. Although several spinal cord sections analyzed from Wr mice 1 and 6 days after transplantation, displayed a relevant number of Hoechst 33258 positive nuclei, we did not find a migration of fluorescent cells to the gray matter and, in particular in the ventral horns.

In spite of a small decay of SPIO-induced hypo-intense signal detected by MRI, a drastic reduction in both the intensity and the overall number of Hoechst 33258 positive nuclei was observed in the ventricular system of Wr mice 42 days after SkmSCs transplantation. This apparent dichotomy among the two different approaches of cell tracking could be likely due to the bleaching of fluorescent dye or the detachment of bysbenzimide molecules from the minor groove of DNA. The hypothesis that these results were related to an actual loss of transplanted cells (reduction of fluorescent nuclei) and a release of SPIO nanoparticles inside the CSF (maintenance of hypo-intense signal by MRI) can be excluded by the presence of cells immunoreactive for the human-nuclei and carboxy-dextran (Fig. 4 A-D).

2.3.4. Clinical outcome

From the 4th to the 7th week of life, the body weight increase was almost similar between the Wr mice transplanted with SkmSCs and the group receiving PBS alone. In the last week of clinical observation the group of treated mice showed a higher trend than that of vehicle-treated mice but this difference did not statistically differ among the two groups (Fig. 5A). The rapid and progressive alteration of forepaw adduction observed in Wr mice receiving PBS by i.c.v. was greatly attenuated by SkmSCs transplantation (Fig. 5B). The beneficial effect was already found at the 1st week after transplantation and was maintained along the whole duration of clinical observation. The attenuation of the degree of forelimbs muscle atrophy was accompanied by a significant improvement of muscular strength in Wr mice transplanted with SkmSCs (Fig. 5C). The significant difference in terms of muscular strength between the two experimental groups occurred at the 6th week of life and was protracted until the end of the study.

2.3.5. Histopathology of human SkmSCs transplanted Wr mice

Figure 5 D-E shows a representative immunohistochemistry of Choline acetyltransferase (ChAT) positive motor neurons in cervical spinal cord from 9 weeks-old healthy mice (Fig. 5D) and from age-matched Wr mice. A reduction of about 60% of ChAT positive neurons was found in the cervical spinal cord Wr mice receiving PBS

alone (6.3±0.42 meanMNs±S.E.) compared to age-matched healthy mice (32.6±2.7 meanMNs±S.E.). The transplantation with SkmSCs did not lead to a reduction in the rate of motor neuron death at the cervical spinal cord level of 9-week-old Wr mice (6.19±0.22 meanMNs±S.E.). Histograms showing the difference of the amounts of ChAT positive motor neurons in the three experimental groups considered in the present study are reported in Figure 5 F.

2.3.6. Molecular characterization of SkmSCs regenerative proprieties

In the aim to characterize the molecular factors involved in therapeutic proprieties of SkmSCs in the Wr mice, we performed a human gene expression profile of the regulation of key neurogenesis processes in total brains and spinal cords of Wr mice 2 weeks after SkmSCs transplant. We focused our analysis on changes in gene expression higher than fourfold. A deeper analysis revealed that of the 84 genes analized, more than one showed a statistically significant increase in expression. The identified genes were classified into different groups: neural development, synaptic plasticity, neuronal transmission, myogenic differentiation and cell cycle/proliferation, depending on their function and the pathway in which in they were implicated (Table 1).

2.4. Discussion

The treatment of incurable neurodegenerative diseases with allogenic or autologous cells are becoming a reality in the field of regenerative medicine (Mathiasen A.B. et al., 2009; Arbab A.S et al., 2009). In particular, stem cells have unlimited self-renewal capacity, high multilineage differentiation potential, and injury site homing capability (Deans R.J., Moseley A.B., 2000).

As results, there is a massive and widespread interest in the ALS stem cell therapy, both in how it is helping people with ALS today and how it may benefit ALS patients in the future.

Therefore the regenerative capacity of different types of stem cells are under intensive pre-clinical investigations with ALS animal models.

With promising results from these studies, an important topic is how to monitor the temporal and spatial migration, the homing and the engraftment efficiency of the administered cells, along with the functional improvement of the target organs/tissues.

Recent attention has focused particularly on the role of the bone morphogenetic proteins (BMPs) inhibitors, in promoting neural induction and differentiation (Mabie PC, Mehler MF, Kessler JA, 1999; . Li W, LoTurco JJ, 2000; Lim DA et al., 2000). One of the earliest identified BMP antagonist/neural-inducing factors is Noggin. For the neuroregenerative purposes we have chosen to supplement the culture medium with Noggin to improve the neural commitment (Wada T, et al., 2009).

Several results have shown that transgenic expression of Noggin by MSCs, or culturing MSCs in a defined medium with Noggin, promote neural differentiation (. Kohyama J, et al., 2001). Thus, we speculated that SkmSCs may be primed *in vitro* towards neurogenic lineage by the addition of Noggin.

Although non-invasive methods for tracking various types of cells in real time with SPIO nanoparticles for in vivo MRI studies have been shown, there is a lack of information concerning the ability to track human SkmSCs.

Here, we prove that the biology of SPIO labelled-SkmSCs is not altered in vivo, allowing us to monitor the fate of these cells in vivo over extended periods of time (up to 18 weeks). These findings may have significant impact on the eventual translation of SkmSCs regenerative medicine to the clinic.

Histological examination of brain sections showed the presence of donor labeled cells in the area of decreased MR signal intensity, confirming a correlation between MRI findings and conventional histological evidence. These findings have important ramifications for the eventual transplantation of stem cells into the human brain and the role of MRI in facilitating the process and tracking stem cell migration.

Here we show cell tracking studies, along with functional outcome analysis of SkmSCs transplants in Wr motor neuron pathology. These approaches are essential for the translation of stem cell regenerative strategies from experimental studies to clinical therapies.

Current scientific evidence supports the claims that MSCs can provide a predominantly supportive and trophic function instead of a

replacement capability in neurodegenerative diseases (Mahmood A, Lu D, Chopp M., 2004).

The RT profiler analysis of SkmSCs-transplanted Wr CNS revealed that one of the genes overexpressed is human CDK5R1. This gene encodes for the Cyclin-dependent kinase 5, regulatory subunit 1 (p35).The complex of Cdk5 and p35, the neuron-specific regulatory subunit of Cdk5, plays important roles in brain development, such as neuronal migration, neurite outgrowth and neurotransmitter release in the presynaptic terminal (. Tomizawa K. et al., 2002). The overexpression of p35 in human SkmSC two weeks after transplantation, may suggest a possible effect of these cells in reducing the functional decay affecting spinal cord motor neurons of Wr mice.

Another gene overexpressed in transplanted Wr mice, is FGF-13. Quite interestingly, has been reported that the treatment with FGF-13 increases ChAT activity for seven days in neuronal cultures from rat embryonic cortex (Greene JM, et al., 1998).

ChAT activity is considered an indirect marker of motor neuron functionality and its significantly reduced levels have been found in different models of motor neuron degeneration and motor nerve pathology. A marked decrease of ChAT activity was reported by our group in the cervical spinal cord and in forelimbs biceps muscles of Wr mice already at the early symptomatic stage. This reduction proportionally decreased by the evolution of clinical progression thus indicating a direct association between the functional activity of ChAT and the degree of denervation and muscular atrophy (Bigini P. et al., 2001). Quite interestingly the percentage of the decrease of

ChAT activity at the different stages of the disease progression in Wr mice, was lower compared to the percentage of loss of cholinergic cervical motor neurons measured by immunohistochemistry (Bigini P et al., 2004). This difference could be due to an hyperactivity of ChAT activity in the remaining neurons, likely as a compensatory effect . The ChAT activity can be influenced by a wide range of players (Wu D, Hersh LB., 1994). The overespression of FGF-13 in the transplanted Wr CNS may suggest that FGF-13, expressed and secreted from human SkmSC even far from the pathological areas, was able to increase the activity of the remaining motor neurons thus not producing a direct effect on motor neuron survival.

Finally, we have seen that the gene MEF2C, involved in myogenesis is expressed up to 2,600 fold in respect to the control. Myocyte enhancer factor 2C (MEF2C) is a member of the MEF2 family which cooperates with the MyoD family to drive skeletal muscle development during embryogenesis and to maintain sarcomere integrity during the postnatal maturation of skeletal muscles. Knockout mice for MEF2C showed hypoplatic myofibers, defects in the M line, marked reduction of muscular mass and muscle fibers degeneration that caused perinatal death (Potthoff MJ, et al., 2007). A relevant disorganization of myofibers has been described in forelimbs muscles of Wr mice at the first stages of muscle atrophy (Mitsumoto H, Bradley WG., 1982)). The overexpression of MEF2C from transplanted SkmSC may therefore improve or maintain the sarcomere integrity during the first phases of muscular atrophy thus slowing the morphofunctional decay and significantly ameliorating paw function and muscle strength scores observed in Wr mice receiving SkmSCs.

However, investigators need to know the migration and accumulation status of the administered cells and the functional improvement of the target organs/tissues.

2.5. Conclusion

In conclusion, the issue of whether human SkmSCs can integrate, differentiated in neural lineage and improve clinical outcome of rodent animal model for ASL disease, is only one of the several aspects that must be clarified by rigorous laboratory experimentation in order to translate our results of pre-clinical stem cells therapy from bench to bedside.

2.6. Materials and Methods

SkmSCs isolation, culture, and differentiation

SkmSCs were derived from muscles of 12-16 week-old fetuses obtained following the ethical guidelines of the European Network for Transplantation (NECTAR) and were processed as previously reported (Alessandri G. *et al,* 2004). Briefly, These tissues are dissected into small pieces and enzymatically and mechanically dissociated. Single cells are plated in the presence of 20 ng/ml EGF and 10 ng/ml bFGF in a serum-free stem-cell medium (SCM) optimized for neural stem cell growth supplemented with rhNoggin

(100ng/ml, R&D systems, Minneapolis, MN, USA) (Growth Medium).

The experimental protocol was approved by the ethics committee of the Fondazione IRCCS Istituto Neurologico 'Carlo Besta' and Fondazione IRCCS Policlinico-Mangiagalli- Regina Elena.

Immunocytochemistry

For differentiation assay, human SkmSCs were plated on matrigel-coated glass-chamber slides (Nunc, Naperville, IL, USA), in basal medium with 2 % of FBS for 7 days and fixed in 4% paraformaldehyde. After that, cell composition was analysed by means of immunostaining with lineage-specific antibodies: such as GFAP (rabbit, 1:500), NG2 (rabbit, 1:200), b-III tubulin (mouse, 1:100) (purchased from Chemicon, Temecula, CA, USA) and Nestin (mouse 1: 100 R&D systems, Minneapolis, MN USA). After several washings, the cultures were incubated with secondary antibody, conjugated with either Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) and finally were mounted with Fluorsave and were viewed under a Zeiss Axiophot-2 microscope.

FACS analysis

The phenotypic characterization of SkmSCs were evaluated by flow cytometry (FACS) as described previously (Alessandri, G. *et al.,* 2004). Anti-CD44, CD166, CD73 were purchased from BD Pharmingen, (San Jose, CA, USANJ, USA) CD56, CD133/2, CD34 were purchased from Miltenyi Biotec (Bisley, Surrey, UK), CD105, was purchased from ABDSerotec (Raleigh, NC, USA) and CD90 was

purchased from Millipore (Temecula, CA,USA) The controls were isotype-matched mouse immunoglobulins.

Magnetic Labeling of human SkmSCs

SPIO particles Endorem (AMI-25; Guerbet; particle size, 80–150 nm; stock solution, 11.2 mg Fe/ml) and poly-L-lysine transfection agent were incubated for 30 min at 37° in culture medium before being added to the cells for 24 h as previously reported (Neri M. *et al.,* 2008). After incubation, the cells were washed off to remove the medium with un-internalized SPIOs and resuspended in fresh media.

Hoechst 33258 dye Staining of SPIO-labeled human SkmSCs

SPIO-labeled human SkmSCs cultures in 12-well plates were washed twice with phosphate buffered saline (PBS) and incubated with 2 µM Hoechst 33258 reagent (Molecular Probes, Eugene,Oregon, USA) for 1 hour at 37°C in the dark. After 1 h of incubation the cells were three washed with PBS and were plated on matrigel-coated glass-chamber slides (Nunc, Naperville, IL, USA), in Growth Medium for 2 days and fixed in 4% paraformaldehyde. After that, SPIO efficiency was analysed by means of immunostaining with anti-dextran antibody (StemCell Technologies, Sigma, St Louis, MO, USA marca). The Hoechst 33258 reagent is taken up by the nuclei of the cells, and apoptotic cells exhibit a bright blue fluorescence, whereas living cells are dark blue.

Animals

Mice were maintained at a temperature of 21±1°C, relative humidity 55±10% and 12h of light. Food (standard pellets) and water were supplied ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are

in compliance with national (D.L. No. 116, G.U. Suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize the number of animals used and their suffering. When mice showed the first pathologic symptoms (reduced hind-limb adduction after tail suspension), food and water were supplied inside the cage. The protocol for the use of laboratory animals was approved by the Italian Ministry of Health and by an internal ethical committee.

Wr mice were originally obtained from NIH genetics and then bred at Charles River Italy, Calco (LC), Italy. Since heterozygous mice do not show any phenotypic difference compared to homozygous healthy littermates, heterozygous founders were designed by genotyping.

Genotiping of Wobbler mice

Genotyping of Wobbler mice were based on restriction polymorphism in Cct4 gene (Rathke-Hartlieb S. *et al.*, 1999). The PCR reactions were performed as follows: initial denaturation step 4 min at 94°C followed by 35 cycles of 30 s at 90°C denaturation, 30 s TAnn and 30 s 72°C polymerization. Restriction enzymes were obtained from Roche. PCR products (15µl) were digested in a total volume of 25 µl with 1µl of AluI for 45 min at 37°C, separated by 2% agarose gel electrophoresis and stained with ethidium bromide. All animals received food and drinking water ad libitum. Mice showing the Wobbler phenotype (genotype wr/wr) (the phenotype appears at

about 30–40 days of age) were used. Littermates having normal phenotype (genotype +/+) were used as control

Experimental schedule

The recruitment of wobbler mice for our studies was carried out after clear diagnosis of the disease based on phenotype analysis (4th week of life). Age-matched healthy littermates, homozygous for the wild-type form of the VPs54 gene, were selected after genotyping (Rathke-Hartlieb S *et al*., 1999).

Thirty wobbler mice were recruited and randomly assigned two experimental groups. The first experimental group (n = 15) received unlabelled hSkmSCs (see next paragraph), the second group (n = 15) received the same volume of sterile PBS. Both groups were weekly examined by standardized behavioral trials (see below) in order to monitor their clinical progression and motor dysfunction. In order to verify that, at the starting point, both groups had a similar clinical condition, the first behavioural trial was carried out one day before transplantation. Behavioural trials were stopped at the 8th week of life, corresponding to the peak of clinical progression, and animals were sacrificed by intracardial perfusion (see below) cervical spinal cord was collected for immunohistochemical experiments (see below).

To track the route of cells in the brain and in spinal cord at different times, 4-week-old wobbler mice (n = 6) received 5x 10^5 SPIO-labeled hSkmSCs into the lateral ventricles (ICV). For both groups, the first MRI analysis was carried before cells transplantation. The following MRI were performed 1 day after SPIO-labeled hSkmSCs and then all animals were weekly monitored until the 18th week of life. At the end of MRI experiments all animals were

sacrificed by intracardial perfusion, brains and spinal cords were collected for immunohistochemical studies.

For the cell localization in different brain areas and at three different times transplantation, a group of 4-week-old wobbler mice (n = 6) were transplanted with 5x 10^5 hSkmSCs loaded by SPIO and incubated by the nuclear fluorescent vital dye Hoechst 33258 (Sigma-Aldrich,MO, USA). MRI experiments were carried 1 day before cell transplantation and 1 day, 6 days and 42 days after transplantation. One third (n = 2) of animals, randomly selected before the first MRI, were sacrificed just after the first (1 day), the second (6 days) and the third (42 days) MRI analysis, respectively. Brain and spinal cord were carefully removed and rapidly frozen by dry ice. Twenty µm thick serial coronal sections were observed under UV lasers to reveal the presence of Hoechst positive nuclei.

Finally, 4-week-old wobbler mice receiving unlabelled hSkmSCs (n = 4) were sacrificed twoo weeks after transplantation for the detection of the gene expression of several human genes (see below). To exclude fouls positive results, the same panels of genes was evaluated in a second group of 4-week-old wobbler mice (n = 4) sacrificed two weeks after ICV administration of the same of saline solution utilized to suspend hSkmSCs during for the transplantation. For each experimental group the same number of males and females were recruited, since no sex-related difference in the evolution of the wobbler disease has been reported.

Human SkmSCs Transplantation in ALS animal models

For injection of SkmSCs, mice were anesthetized using isoflurane and placed in a stereotaxic frame. The head was shaved and a ~1 cm

incision was made to expose the skull. Using a microdrill, two 1–2 mm holes were produced to allow two i.c.v. injections (stereotaxic coordinates of 4-week-old control mice +/-1.0mm lateral to bregma, +/-0mm rostro-caudal to bregma and 3.0mm deep from the skull surface; stereotaxic coordinates of 4-week-old Wr mice +/-0.8mm lateral to bregma, +0.5mm rostro-caudal to bregma and 2.5mm deep from the skull surface). As sham controls, animals were injected with 10μl PBS without cells. All injections were performed with a Hamilton syringe. The syringe was left in place for 2 min after injection to allow dispersion of the cells. A total of $5x10^5$ cells with a total volume of 5μl was injected per animal. No immunosuppression was given since human fetal cells were utilized. Following surgery, the skin over the skull was sutured closed and animals were placed in separate, heated cages and monitored until fully recovered.

MRI experiments

MRI scanning was performed by a 7 Tesla and 30cm bore Bruker specttrometer (BioSpin 70/30 USR) equipped with a surface mouse brain RF coil. A 3-orthogonal plane gradient echo tri-pilot scan was used as a geometric reference to locate the olfactory bulbs and to choose the sections from. T2 weighted images (Axial, sagittal, coronal; RARE factor=8, TR=3.3sec, TE=54ms, inter echo time=13.5ms, FOV=2.2x2.2cm2, data matrix 256x256, slice thickness=1mm) were acquired to visualize anatomical details. T2* weighted images (Axial; FLASH Flip Angle=30deg,TR=1sec, TE=7ms, FOV= 1.8x1.8cm2, data matrix 128x128, slice thickness=0.6mm) were acquired to visualize the susceptibility artefacts due to the presence of iron. In order to acquire the image of

the spinal cord with a Gray-White Matter contrast T1 weighted images (Axial; Spin-echo, TR=300msec, TE=11.7ms, FOV=2.7x2.7cm2, data matrix 256x256, slice thickness =0.5mm) were acquired.

Disease progression assessment

Behavioural trials were performed by the same operator blind to the treatment and were performed according to the procedure previously described (Mennini T, et al., 2006).

The following tests were employed to evaluate the neuropathological progression affecting wobbler mice:

1) Paw abnormality: Paw abnormality is an observational test. The operator assigns a score to these parameters, scaled from 0 to 4, on the basis of the severity of abnormalities. The paw position is graded as follows: 0, normal; 1, retracted digits; 2, curled digits; 3, curled wrists; 4, forelimb flexed to body.

2) Grip strength: Mice are lifted by the tail and allowed to grasp with both forelegs to a horizontal bar, which is connected to a mechano-electric transducer (Basile). The grip strength of the front paws is measured at the point when the mouse releases the horizontal bar as a result of a gentle traction applied by the operator. Healthy mice can record values higher than 100 g, whereas values recorded by wobbler mice are very low (< 20 g) and drastically reduced during symptom progression. When animals are no longer able to grip the bar, grip strength is recorded as 0 g. Values of grip strength were normalized by dividing each value by body weight to control for weight differences between wobbler and healthy mice.

Because wobbler mice develop early and severe atrophy of forelegs without a clear impairment of hindleg muscles, the classical rotarod test cannot be considered a reliable tool to evaluate the clinical progression in these mice.

Motor neuron counting

Animals were perfused transcardially with 4% paraformaldehyde (w/v) in 0.1M PBS (Sigma-Aldrich) and following steps were carried out according to the procedure previously described (Bigini and Mennini, 2004). Immediately after perfusion, spinal cords were rapidly removed and post-fixed for 4 h in the same fixative (4°C) and then dehydrated and cryoprotected with serial steps in 10%, 20% and 30% sucrose in 0.1 M PBS at 4°C until they sank. Subsequently, spinal cord were included in OCT (Tissueteck, Netherlands) and frozen in n-pentane at -45°C. Serial 30 µm thick coronal sections were cut and immunohistochemistry against choline acetyl transferase (ChAT) (mouse monoclonal antibody, Immunological Science, Italy, 1:1000) was performed according to the protocol described by Houser et al. (1984). ChAT positive neurons counting was carried out in the cervical region corresponding to C2-C5 from 9-week-old wobbler. At least 25 sections for each animal were stained by ChAT. The number of MNs for each animal was expressed as the mean number ± S.D of large-sized ChAT neurons (>250 µm2 of surface) calculated in each single section. Olympus-DB software, coupled to the microscope camera, was used to count the ChAT positive cells > 250 µm2. The number of large-sized ChAT neurons was also performed at the same spinal cord level (C2-C5) of healthy mice homozygous for the Vps54

gene (n 0 3) to assess the extent of MN loss in both groups of wobbler mice (n = 14 for each group).

Immunohistochemistry

At different times after transplantation, the isolated-CNS of Wobbler mice were equilibrated for 24 h in 30% sucrose and fixed in 4% PFA. The Serial sections, cut at the vibratome or cryoste, or wholemount spinal cords were processed by immunofluorescence with GFAP, Anti human nuclei, AHN (Chemicon, Temecula, CA, USA) and DAPI.

Real Time Quantitative Polymerase Chain Reaction

RNA extractions were performed on three total brains and spinal cords of Wr mice 2 weeks after transplanted with SkmSCs and on controls of SkmSCs cultured as described above.

For the detection of genes related to the regulation of key neurogenesis processes such as the cell cycle and cell proliferation, differentiation, motility, and migration, Real-time PCR was performed using the Neurogenesis and Neural Stem Cell RT² Profiler™ PCR Array according to manufacturer's instructions (SuperArray Bioscience Corporation, Frederick, MD, USA). Pathway-focused gene expression analysis was performed with the PCR Array System and the PCR Array Data Analysis Web Portal. Each Neurogenesis and Neural Stem Cell RT² Profiler™ PCR Array was done on separate cDNAs at least three times.

Statistical analysis

Numerical values are expressed as mean±SD. Comparisons of parameters were performed with Student's t test. Comparisons of

parameters among 3 groups were made with a one-way ANOVA test. A value of P<0.05 was considered significant.

2.7. Figures and Legends

Figure 1: *In vitro* characterization of human SkmSCs
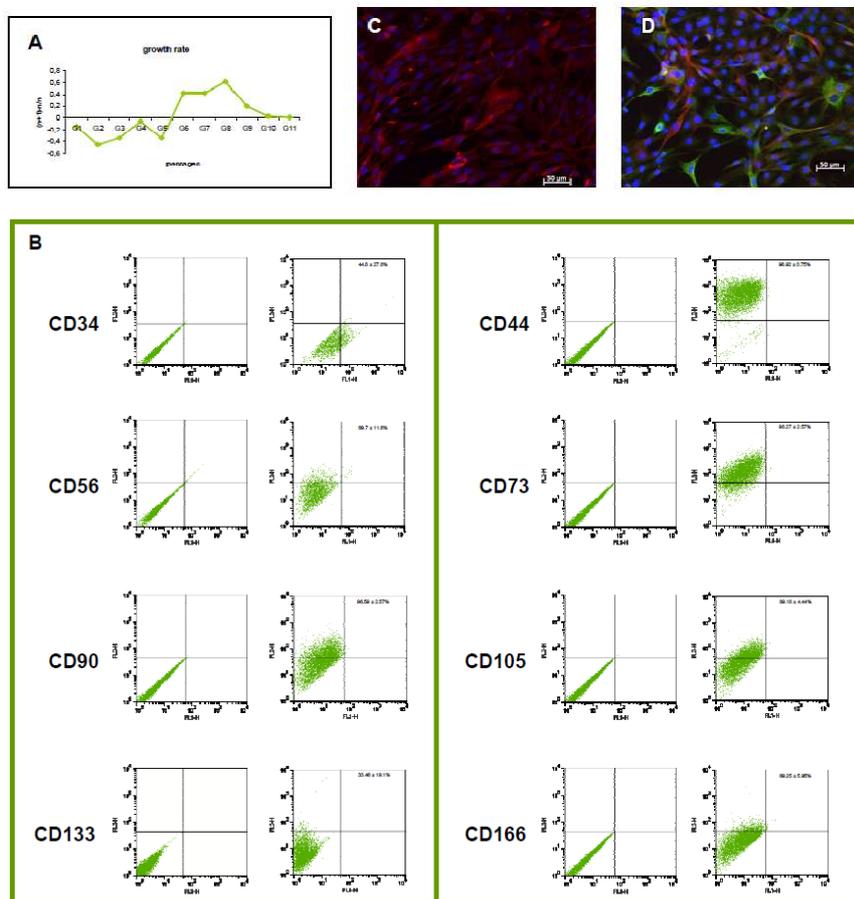
(A) Growth Rate of SkmSCs: the cell number within the individual passages was determined by the use of phase-contrast microscopy and trypan blue exclusion test. Growth rate=(n+1)-n/n

values; n=number of passage. (B) FACS analysis of SkmSCs: representative flow cytometry dot plots show the high percentage of expression of MSCs markers. (C-D) Immunofluorescent analysis of neural markers upon differentiation of SkmSCs. (C) Differentiated SkmSCs stained positively for b-tubulin III (red) and GFAP (green); (D) Immunostaining for Nestin (red). DIV, days in vitro. All nuclei were counterstained with DAPI (blue). Results are representative of five different SkmSCs lines. Results are displayed as mean ±SD.

Figure 2: human SkmSCs SPIO internalization and *in vivo* MRI tracing.
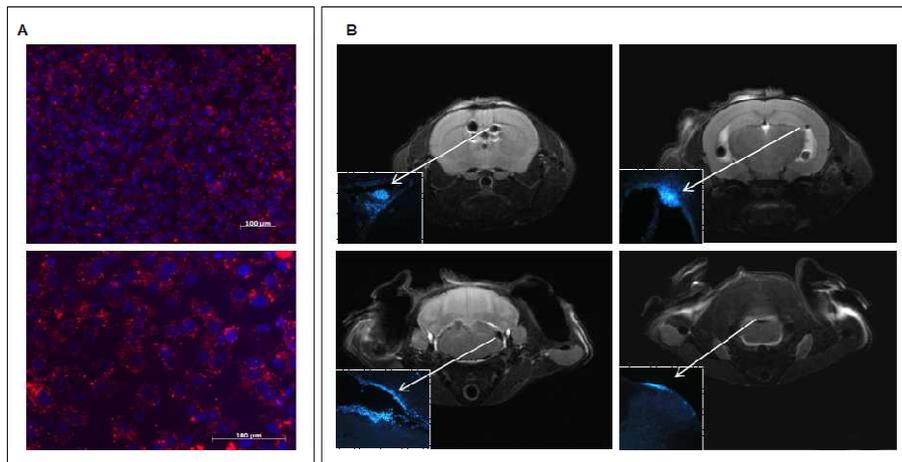
SkmSCs SPIO internalization and *in vivo* MRI tracing. SPIO labeling was efficient and did not affect cell viability of SkmSCs *in vitro*. (A) SPIO-labeled SkmSCs stained with an anti-dextran antibody (in red) and counterstained with a viable cells dye Hoechst 33258 (blue) for quantitative and qualitative analysis of SPIO labeling efficiency. (B) MRI of control mice brain postgrafted with hoechst/SPIO-double labeled SkmSCs was performed on living Wr mice one week

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postinjection. Axial MRI shows hypointensities representing Hoechst/SPIO-labeled SkmSCs in the lateral ventricle of the brain (upper panels), and in the pial surface of the brain stem (lower panel left) and cervical spinal cord (lower panel right) (Insert.) Corresponding histological section show the colocalization with MRI hypointense signal and Hoechst staining of SkmSCs.

Figure 3: Long-term monitoring of SPIO-labeled human SkmSCs
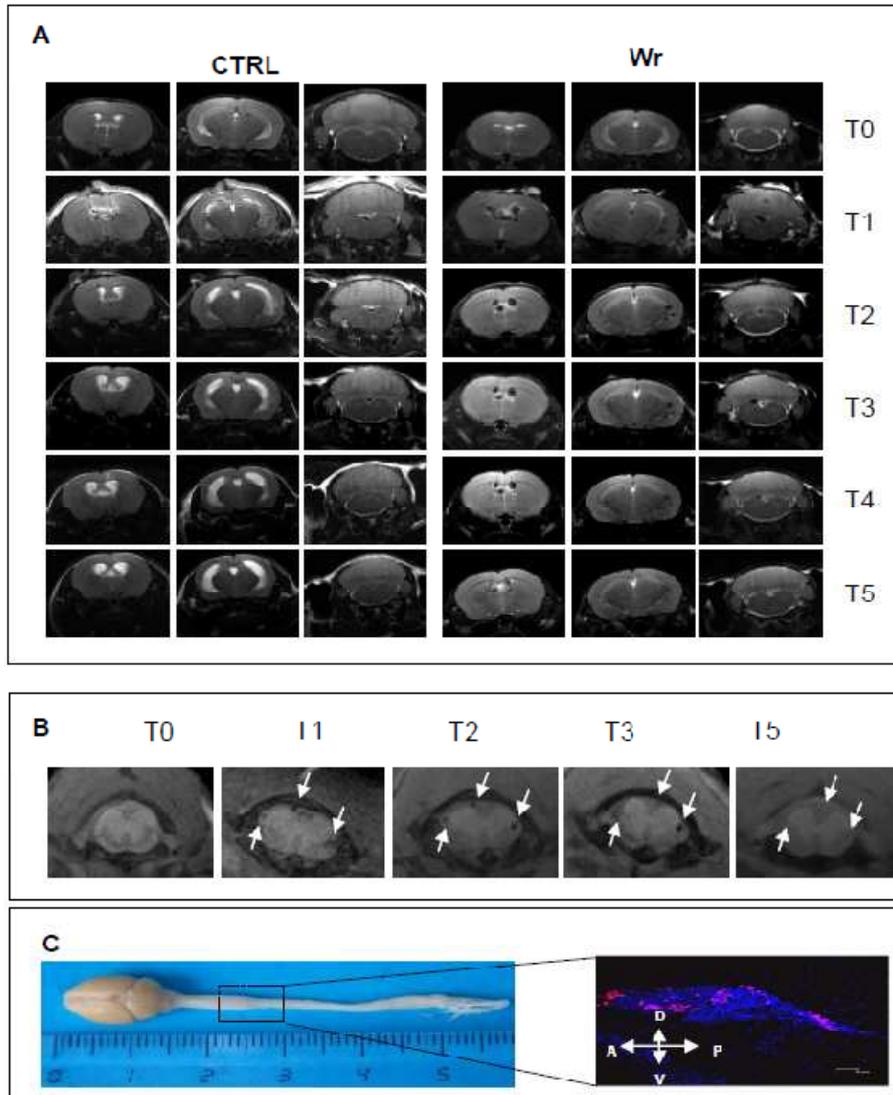
(A-B) Representative MRI axial sections of (A) T2-weighted images mouse brains and (B) T1-weighted images cervical spinal cord. (C) Photograph of Wr CNS. The immunostaining of Wr wholemount spinal cord cervical region with AHN (red) 18 weeks post-transplant.

All nuclei were counterstained with DAPI. T0=MRI acquisition of pre-transplanted mouse brain; T1=0 h post-transplant; T2=2 weeks post-transplant; T3=4 weeks post-transplant; T4=8 weeks post-transplant; T5=12 weeks post-transplant.

Figure 4: SPIO-labeled human SkmSCs integration in Wr brain
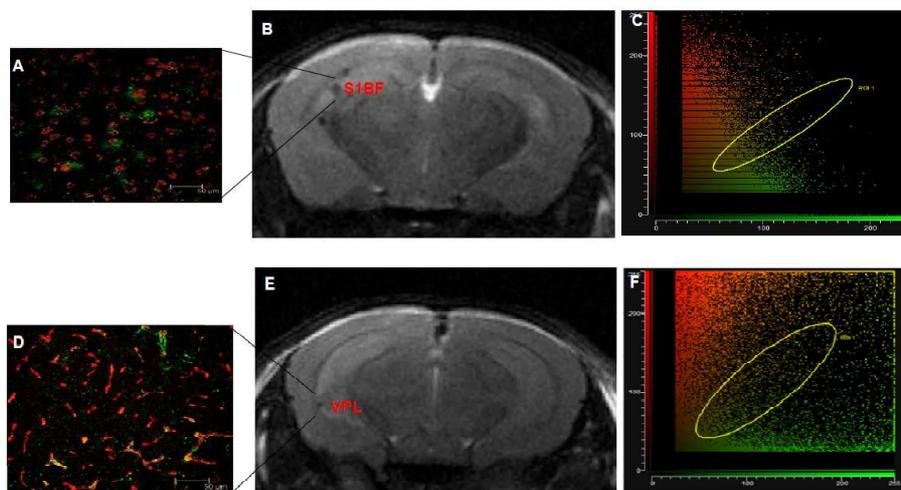
(A) Immunostaining of Wr brain medial region 18 weeks post-transplant with AHN (red) and NG2 (green). (B) Corresponding mouse brain axial T2-weighted MRI. (C) Scatter Gram. (D) Immunostaining of Wr brain medial region 18 weeks post-transplant with anti-dextran antibody (red) and GFAP (green). (E) Corresponding mouse brain axial T2-weighted MRI (F) Scatter Gram. S1BF= somatosensory 1 barrel field; VPL= Ventral Posterolateral Thalamic Nucleus.

Figure 5: Clinical outcome and histopathology of SkmScs trasplanted Wr mice.
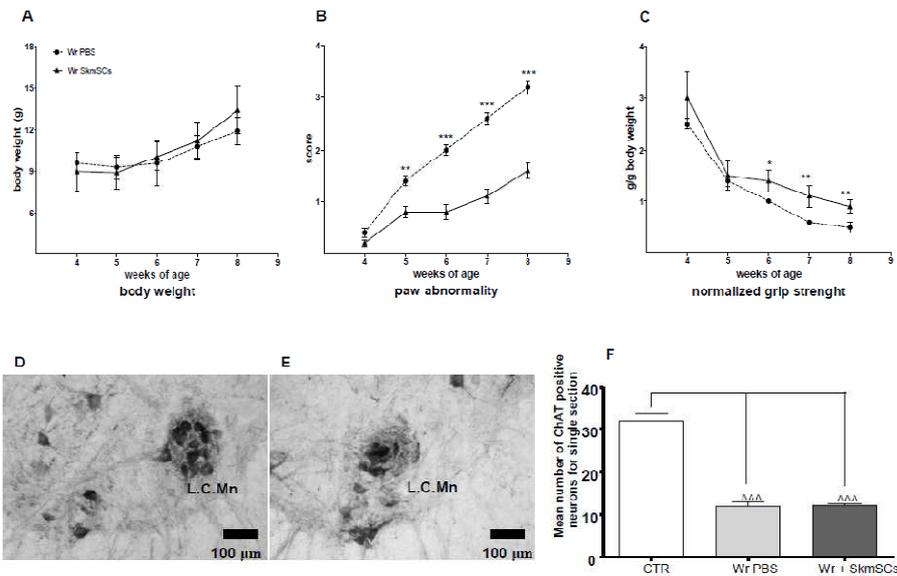
(A-C) Behavioral scores of Wr mice that received human SkmScs or veichle. Each point represents the mean ± SD of 15 animals per group. (D-E) Representative immunohistochemistry of ChAT positive motor neurons in cervical spinal cord from 9 weeks-old healthy mice and from age-matched Wr mice. (F) Effects of human SkmScs transplant on Motoneurons survival. Data represent mean ±SD of 14 replications. ^^^p<0.0001.

Table 1. Fold differences in gene expression profile of human SkmScs trasplanted Wr CNS

Gene ID	Fold difference	p value
Decreased gene expression		
S100B	16.6	0.0025
S100A6	21062.4	0.0001
Increased gene expression		
APBB1	646.9	0.002
CDK5R1	64.7	0.001
DVL3	146.7	0.042
GNAO1	1445.7	0.004
FGF-13	20.1	0.003
MEF2C	2660.7	0.005

Results are expressed in fold change signal between in vitro SkmSCs and SkmSCs-transplanted Wr CNS. Values are expressed as mean+SD. *P<,0.05 SkmScs trasplanted Wr CNS versus SkmScs cultured in vitro.

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Chapter 3 Human Neural Stem Cells: a model system for the study of Lesch-Nyhan Disease neurological aspects

Silvia Cristini[1], Stefania Navone[1], Laura Canzi[1,4], Francesco Acerbi[2,3], Emilio Ciusani[4], Uros Hladnik[5], Paola De Gemis[5], Giulio Alessandri[1], Augusto Colombo[6], Eugenio Parati[1] and Gloria Invernici[1]

[1]Laboratory of Cellular Neurobiology, Fondazione IRCCS Istituto Neurologico "Carlo Besta", 20133, Milan, Italy; [2]Department of Neurosurgery, Fondazione Istituto Neurologico Carlo Besta, 20133, Milano, Italy; [3]University of Milan, 20133, Milan, Italy; [4]Laboratory of Clinical Investigations, Fondazione IRCCS Istituto Neurologico "C. Besta", 20133, Milan, Italy; [5]Medical Genetics Unit, "Mauro Baschirotto" Institute for Rare Diseases - B.I.R.D., Costozza di Longare (Vicenza), Italy; [6]Department of Obstetrics and Gynecology, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, 20122, Milan, Italy; [4]Ph.D. Program in Translational and Molecular Medicine (DIMET) University of Milano-Bicocca

3.1. Abstract

The study of Lesch-Nyhan-diseased (LND) human brain is crucial for understanding how mutant HPRT might lead to neuronal dysfunction. Since Lesch-Nyhan disease is a rare, inherited disorder caused by a deficiency of the enzyme HPRT, human neural stem cells (hNSCs) that carry this mutation are a precious source for delineating the consequences of HPRT deficiency and for developing new treatments.

In our study we have examined the effect of HPRT deficiency on the differentiation of neurons in hNSCs isolated from human LND fetal brain.

We have examined the expression of a number of transcription factors essential for neuronal differentiation and marker genes involved in dopamine (DA) biosynthetic pathway. LND hNSCs demonstrate aberrant expression of several transcription factors and DA markers. HPRT-deficient dopaminergic neurons also demonstrate a striking deficit in neurite outgrowth. These results represent direct experimental evidence for aberrant neurogenesis in LND hNSCs and suggest developmental roles for other housekeeping genes in neurodevelopmental disease.

Moreover, exposure of the LND hNSCs to retinoic acid (RA) medium elicited the generation of dopaminergic neurons.

The lack of precise understanding of the neurological dysfunction in LND has precluded development of useful therapies. These results evidence aberrant neurogenesis in LND hNSCs and suggest a role for

HPRT gene in neurodevelopment. These cells combine the peculiarity of a neurodevelopmental model and a human, neural origin to provide an important tool to investigate the pathophysiology of HPRT deficiency and more broadly demonstrate the utility of human neural stem cells for studying disease and identifying potential therapeutics.

3.2. Introduction

Lesch-Nyhan disease (LND) is a X-linked recessive disorder that occurs as frequently as one in every 380,000 live births in Canada and 1/235,000 live births in Spain (Crawhall,J.C. *et al.*, 1972). This disorder involves a virtually complete absence of activity of the enzyme hypoxanthine-phosphoribosyltransferase (HPRT). The HPRT gene consists of 9 exons and 8 introns. An opening reading frame of 654 nucleotides corresponds to the protein-encoding region (Wilson,J.M. *et al.*, 1983). The HPRT gene is well known for its clinical and genetic heterogeneity. At least 2,000 different mutations have been reported (O'Neill,J.P. *et al.*, 1998). The human HPRT amino acid sequence is similar to that of rodents, implying that it is very conservative and sensitive to any single base substitution (Konecki,D.S. *et al.*, 1982). Molecular defects may result in partial or entire gene deletion, insertion, duplication, splicing error, or formation of a stop condon, leading to an alternation in the size of the translated protein. Some molecular defects cause only a single amino acid substitution. Any changes that alter the three-dimensional shape of the protein or reduce its active sites, at least partially decrease enzyme

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stability, functional activity, and enzyme synthesis (Mak,B.S. *et al*., 2000).

The lack of HPRT activity results in an excessive overproduction of uric acid and related symptoms of gout and renal dysfunction. In addition, patients with Lesch-Nyhan syndrome have relatively uniform motor phenotype that consists of severe generalized dystonia superimposed on hypotonia, sometimes with less prominent choreoathetosis or spasticity (Jinnah,H.A. *et al*., 2006), mental retardation, dysarthria and very dramatic compulsive self-mutilation habits (Lesch,M. and Nyhan,W.L., 1964).

It is documented that dopaminergic neurons are damaged in affected patients (Visser J.E *et al*., 2000; Nyhan,W.L. *et al*., 2000). However, the mechanism by which features of Lesch-Nyhan syndrome result from impaired purine metabolism is still not well understood.

In an attempt to study the underlying causes of the complex phenotype observed in persons with Lesch-Nyhan syndrome, at least two mouse models have been created that exhibit HPRT deficiency (Hooper M. *et al*., 1987; Kuehn,M.R *et al*., 1987). Mouse models contribute to our understanding of biology with respect to the similarities and differences between species and various organ systems, and provide models for testing therapeutics. Even though the HPRT-deficient mice have failed to fully recapitulate the phenotype in the human disease, they do have an abnormal phenotype at the molecular, biochemical, morphological, and pharmacological levels (Elsea S.H. and Lucas R.E., 2002). Some studies have sought to more closely model the neurobiological consequences of the enzyme defect

by studying HPRT-deficient dopaminergic neurons in cultures prepared from the knockout mice (Boer P. *et al*., 2001; Smith D.W. *et al*., 2000). HPRT-deficient mouse dopaminergic neurons are valuable for exploring the purine synthesis pathways with regard to the interactions with the dopaminergic pathways and the resulting effects on the basal ganglia. The recent development of induced pluripotent stem (iPS) cell technology provides models of human genetic diseases. iPS cells give a new opportunity to analyze the pathways that lead to disease pathogenesis based on a particular genetic trait at the cellular level. Park and colleagues (Park I.H. *et al*., 2008) generated an iPS line from a female carrier of Lesch-Nyhan Syndrome (LNSc-iPS2) that will be a valuable resource for studies of homologous recombination in iPS cells, and for analysis of X chromosome reactivation during reprogramming and random inactivation with differentiation.

Dopaminergic neuronal cultures were prepared also from HPRT-deficient PC12 (Bitler,C.M. and Howard,B.D., 1986; Yeh J. *et al*., 1998), HPRT-deficient neuroblastoma mutants (ShirleyT.L. *et al*., 2007; Lewers J.C. *et al*., 2008) and HPRT2 MN9D sublines (Ceballos-PicotI. *et al*., 2009). Another model of LND in human cells was created by Urbach and colleagues (Urbach A. *et al*., 2004) by inducing the mutation in human embryonic stem cells (ES).

Since LND is a developmental disorder, it is not at all clear that the knock-down of the gene comes close to recapitulating the more complex developmental changes that clearly occur in this disease as defined in the literature.

A limitation of cell models is that the findings may be restricted to the *in vitro* environment, with little relevance for the *in vivo* state, especially in the brain. This limitation can be addressed by examining brain tissue from affected Lesch-Nyhan patients.

To test the hypothesis regarding a defect in neurodevelopment we isolated, from LND human fetal brain specimens, human neural stem cells (hNSCs). These cells have two main properties: self-renewal capacity and pluripotent differentiation potential (Vescovi A.L. *et al*., 1999; Parati E.A *et al*., 2004). It has been suggested that these cells could play a major role in transplantation and be able to advance our knowledge in human development. Our *in vitro* model provides an alternative approach to study HPRT deficiency effect since LND hNSCs are capable of differentiation into dopaminergic neurons under controlled inducing conditions *in vitro* and could provide new insights into LND by permitting analysis in a human system, using a large number of genetically modifiable cells but without any genetic manipulation.

To our knowledge, we isolated and studied for the first time hNSCs from brain specimens of LND human fetuses and tested the hypothesis that HPRT deficiency can adversely affect specific processes in neural development, including differentiation and proliferation. The study of the differentiation of LND hNSCs to dopaminergic neurons may offer an opportunity to delve into the developmental processes that lead to the malfunction of the dopaminergic neurons in LND.

3.3. Results

In order to generate an *in vitro* model for Lesch-Nyhan disease, to our knowledge, we isolated, for the first time, hNSCs from two 14-week Lesch-Nyhan-affected fetuses according to the procedure previously described (Vescovi A.L. *et al.*, 1999). This technique uses a serum-free medium containing mitogens (EGF and bFGF) which facilitate the expansion of stem cells, while differentiated cells generally become apoptotic and display poor growth capacity (Vescovi A.L. *et al.*, 1999).

Characterization of the genetic mutation accounting for the deficient enzyme activity reveals that one fetus carries a r.319_402 deletion, while the other one carries a missense point mutation (c.208G>T, p.Gly70Trp). No other significant difference between the two LND hNSC lines was detected in all the analyses performed in this study.

Under these culture conditions, the morphological appearance of the Lesch-Nyhan hNSCs after 7 days of culture was typically neurospherical and showed no apparent morphological differences when compared to healthy ones. All pathological cells appeared healthy without overt signs of toxicity, such as a high frequency of non-viable cells, debris or apoptotic figures (Figure 1A).

We analysed *HPRT1* levels by Real-Time PCR and found that LND hNSCs express *HPRT1* at mRNA level.

The lack of HPRT function in LND hNSCs was confirmed by measurement of live culture incorporation of ^{14}C-hypoxanthine into

IMP. The healthy hNSCs readily incorporated ^{14}C-hypoxanthine, while LND hNSCs exhibited enzyme activity that was below detectable limits (Figure 1B).

HPRT malfunction is responsible for the accumulation of uric acid; to further analyse HPRT presence in hNSCs isolated from developing Lesch-Nyhan brains, we analysed the levels of uric acid in our hNSCs (Figure 1C). Indeed, the accumulation of uric acid was significantly higher in LND hNSCs than in healthy cells.

In addition, we compared the proliferation rate of healthy and LND cells under the selection of 6TG and HAT to confirm that the HPRT protein is not expressed in the LND hNSCs. HAT medium inhibits the *de novo* pathway of purine synthesis and prevents growth of cells that lack an active salvage pathway. The LND cells proliferated in the presence of 6TG and died in HAT medium while healthy cells behaved in an opposite fashion (Figure 1D). These results confirmed the deficiency of HPRT in LND hNSCs.

Human NSCs generated from LND fetal tissues have gross morphology that is similar to hNSCs derived from unaffected fetal tissue (Figure 1A). On the basis of appearance, LND hNSCs grown as neurospheres do not differ from healthy neurospheres.

To determine if HPRT deficiency impaired cellular proliferation, we analysed their growth rate and the frequency of neurosphere formation. To this end, we compared the behaviour of the two LND hNSCs to five healthy hNSCs lines with similar gestational age and culture history. Proliferation rates of LND hNSCs were assessed at different passages (from passage 2 to passage 10). The growth of LND hNSCs was not significantly different from normal at all passages. As

shown in Figure 2A, Lesch-Nyhan hNSCs proliferated well in serum-free conditions and showed no significant alterations.

Next, to determine the frequency of neurosphere formation, we initially plated single-cell suspensions at low cell density in Growth Medium containing methylcellulose to ensure that distinct colonies were derived from single cells and therefore clonal in origin. We found that LND hNSCs form neurospheres at the same frequency as those from unaffected individuals (Figure 2B).

RT-PCR revealed the expression of neural precursor and stem cell markers such as GFAP, nestin, β-tubulin III, NG2, SOX2, FLT4 and CD133 in healthy and LND undifferentiated neurospheres. As far as the expression of GFAP, NG2, SOX2 and CD133 was concerned, the data showed no particular alteration when compared to healthy hNSCs (Figure 2C), but regarding β-tubulin III and FLT4 expression, LND hNSCs appeared to express smaller amounts than healthy ones. Western blot analysis of neural markers GFAP, β-tubulin III and nestin, performed on undifferentiated hNSCs, evidenced nestin and GFAP protein levels comparable to healthy cells and confirmed a reduced level of β-tubulin III in LND hNSCs (Figure 2C).

Since one of the hallmarks of LND is neurological alteration, we induced hNSCs to differentiate as previously described (Vescovi A.L. *et al*., 1999) and performed immunofluorescence to test the morphology and the number of typical neural precursors such as nestin, GFAP, β-tubulin III and NG2 at different times (Table 1). We found interesting differences between pathological and healthy cells. In fact, LND cells revealed significant reductions for β-tubulin III

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positive cells already after 3 days *in vitro* (DIV) of differentiation and this discrepancy increased during time, whereas no significant difference was observed for GFAP expression (Figure 3A). Oligodendrocyte precursors, still present at 5 DIV, became greater in number at 7 DIV in LND cells with respect to those in healthy ones. The data at 7 DIV were confirmed by Real-Time PCR (Table 2).

To determine whether the reduction in the number of LND neurons was due to an increase in cell death or to a block in differentiation, cells were stained by using the TUNEL method and by using corresponding markers for the individual cell types, at different stages of differentiation. Only a few TUNEL-positive cells were observed in the healthy differentiated hNSCs while, the number of LND TUNEL-positive cells from 3 to 7 days of differentiation (Figure 3B) was significantly higher.

To identify the cell types of the TUNEL-positive cells, double staining was performed using β-tubulin III, GFAP and NG2 as markers for neurons, astrocytes and oligodendrocytes (Figure 3B, C).

Interestingly, the most apoptotic processes in LND differentiated cells were charged to astrocytes as demonstrated by double labelling with TUNEL plus immunostaining with GFAP. The percentages of LND TUNEL-positive astrocytes at 3, 5 and 7 DIV were respectively 20%±1.5, 18%±1.7 and 16.87±0.9 while the values of healthy differentiated cells were significantly lower: 0%, 1%±0.3, 2.30%±0.6 at 3, 5 and 7 DIV respectively.

LND TUNEL-positive neurons were significantly higher than healthy ones and the values ranged from 2.98% at 3 DIV, 2.15% at 5 DIV and 4.35% at 7 DIV.

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No TUNEL-and-NG2 double-positive cells were found either in LND or healthy cells at 3, 5 and 7 days upon differentiation.

We hypothesized that the differences in the extent of neuronal differentiation between Lesch-Nyhan and healthy hNSCs were probably due to dopamine (DA) dysfunction, a distinctive aspect of LND. To investigate these findings, we performed analysis to detect, in undifferentiated hNSCs, the presence of mRNA transcripts, relevant in dopaminergic neuron development and function, such as tyrosine hydroxylase (TH), D2 receptor (D2R) and nuclear receptor-related 1 (NURR1), determined by RT-PCR. This analysis did not indicate any relevant difference (Figure 4A). To further delve into the dopaminergic differentiative pathway of LND hNSCs, these cells were induced to differentiate towards dopaminergic neurons and immunofluorescence was performed to test the presence of key dopaminergic enzymes, such as dopamine transporter (DAT) and TH (Figure 4B and 4C). No significant differences existed between LND and healthy hNSCs regarding the percentage of cells positive to dopaminergic markers even if the physical appearance of Lesch-Nyhan dopaminergic neurons was significantly different when compared to healthy ones. Statistical analysis to examine the overall effect of HPRT deficiency in LND dopaminergic neurons revealed a significant reduction in soma area (Figure 4D). Although the number of neurites per cell could not be clearly evaluated at this developmental stage, LND dopaminergic neurons' neurite length was significantly reduced (Figure 4D).

During human fetal development, multiple transcription factors participate in the induction and maintenance of many aspects of the

neurochemical phenotype of LND neural cells (Lin J.C. and Rosenthal A., 2003). Although the proliferative potential of LND hNSCs appears unaltered, it is possible that the LND stem cells have distinctive gene expression patterns that have implications for later steps in development. To detect any alterations in the expression of genes related to the identification, growth and differentiation of stem cells, we performed a gene expression profile of stem-cell specific markers as well as stem cell differentiation markers. The comparison between LND and healthy hNSCs revealed 10 genes between the analyzed 84 genes, whose mRNA expression was more than three-fold different in LND hNSCs (Table 3). A deeper analysis revealed that five of these genes analysed showed a statistically significant decrease in expression and play a role in neural cell differentiation (ALDH1, CD44, NCAM1, NEUROG2 and TUBB3) while PPARδ, a transcription factor expressed during neuronal *in vitro* maturation (Cimini A. *et al*., 2007) and BMP2, key determinant of neural crest induction and development (Gossrau G. *et al*., 2007), were upregulated. All of the other genes misexpressed in LND hNSCs are involved in transcriptional pathways (Kurooka H. and Honjo T. 2000) (Table 3). Expression levels of ALDH1 in hNSCs were compared at the cellular level among controls and LND. Quantitative PCR confirmation (Table 3) revealed a thirty-fold decrease in the expression of the ALDH1 gene.

The major biosynthetic pathway of retinoic acid (RA) from retinol is the irreversible oxidation of retinal to retinoic acid catalyzed by a cytosolic aldehyde dehydrogenase 1 (ALDH1) (Elizondo G. *et al*., 2000). To determine whether RA synthesis by ALDH1 has a role in

the development of LND dopaminergic neurons, we aimed to compensate for ALDH1 downregulation by *in vitro* RA administration. The LND and healthy hNSCs were cultured in Growth Medium containing 5 μM RA. Seven days later, we first examined morphological changes during RA-induced neuronal differentiation (Liour S.S., *et al.*, 2000) and found that RA restores normal neurite formation and soma area (data not shown). Characteristic patterns of TH expression were observed in LND cells cultured in RA-enriched culture medium (Figure 4E).

In order to address the role of nitric oxide (NO) in the dopamine deficit that develops in HPRT-deficient brain, the formation of NO was evaluated by measuring the stable NO and its products, nitrite and nitrate (Table 4). These results revealed that LND hNSCs' NO production was significantly higher than that of healthy hNSCs and was prevented by the addition of L-NAME (100 μM). As the majority of NO produced in the brain is from the predominant NOS1 isoform, we determined its expression at mRNA level in healthy and LND hNSCs. Our results (Table 4) indicated that as far as LND hNSCs were concerned, NOS1 was not responsible for NO overproduction. To further investigate the idea that increased NO levels inhibit dopaminergic differentiation of LND hNSCs, these cells were treated with L-NAME (100 μM) upon differentiative conditions. After 7 days we examined TH (Figure 5C) and β-tubulinIII (data not shown). Positive cells were counted in a blind manner and no significant difference was observed between LND hNSCs with and w/o L-NAME addition upon differentiative condition.

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3.4. Discussion

HPRT activity in patients with classic LND is typically absent or less than 1.5% of normal values. This genetic defect leads to a typical pathological pattern which includes characteristic neurological symptoms. It is still not clear how the deficiency in HPRT activity leads to these symptoms, although it is documented that dopaminergic neurons are damaged in affected patients (Visser J.E *et al*., 2000; Nyhan,W.L. *et al*., 2000).

In an attempt to study the underlying cause of the complex phenotype observed in persons with Lesch-Nyhan syndrome, HPRT-deficient mouse models have been created. These mice are useful for studying the biochemical and physiological pathways especially involved in embryonic and fetal development, and specific functions in various organs. They provide a useful model system for the development of new therapeutic techniques.

An alternative approach is the generation of primary cultures from patients' cells. This methodology is limited due to the specific range of tissues from which cells can be obtained.

An innovative approach to study LND pathology is to examine the phenotype and possible aberrations of human fetal LND neural stem cells. By analysing human LND hNSCs, it is possible to study developmental abnormalities without regard to whether they are caused by direct action of *HPRT1* mutations, or, more likely, by a

combination of direct and indirect effects of *HPRT1* mutations and epigenetic factors.

As expected by prenatal diagnosis, LND hNSCs universally fail to incorporate hypoxanthine. When primary human fetal tissue is exposed to the mitogens EGF and FGF-2, aggregates of stem and progenitor cells form, which have been termed 'neurospheres'. These neurospheres can be expanded in culture for extended periods of time (Vescovi A.L. *et al.*, 1999) and as such provide an ideal alternative model to study human brain development. The neurosphere culture system allows the study of specific processes in neural development, including proliferation, migration and neuronal differentiation.

The results in this paper demonstrate that human fetal Lesch-Nyhan brain contains a population of neural stem cells which shows the same capacity to proliferate and form neurospheres as healthy hNSCs and which has the ability to express typical neural markers (Vescovi A.L. *et al.*, 1999). Cells within the neurospheres differentiate when exposed to an appropriate substrate and when deprived of growth factors to consistently produce neurons and glia. Therefore, human neurospheres offer two major advantages that make them ideal to study processes of neural development in LND: (i) tissue can be derived from human LND brain and neural stem cells can be expanded in culture for extended periods of time; (ii) neurospheres can be manipulated both genetically and epigenetically.

Here to our knowledge we report for the first time the use of neurosphere cultures to reveal molecular and cellular differences between LND and normal hNSCs. The limitation of our study is that *in vitro* models are not capable of considering all the variables of the

human body. Nevertheless, this study yields relevant data useful in designing *in vivo* studies to explore such variables.

Human LND NSCs' morphology was similar to that of hNSCs derived from unaffected fetal tissue. Proliferation rates and the lifespan of LND hNSCs were not significantly different from controls at any passage; the ability to grow these cells for extended periods of time allows their banking and makes them a valuable resource for LND studies *in vitro*.

Previous results indicate that it is the process of differentiation of dopaminergic neurons that is particularly affected adversely by a HPRT deficiency. This is consistent with the special importance of HPRT in the brain during neonatal life as indicated by the substantial increase in HPRT activity in human and rat brain during the first few weeks after birth, a period of active neuronal development (Adams A. and Harkness R.A., 1976). Thus it is reasonable to suppose some general differences in function and expression pattern between LND and healthy hNSCs. Based on our experience in defining the pattern of neural stem cell markers expression in healthy hNSCs, we compared the expression profile of healthy and LND hNSCs and found interestingly differences. The expression of β-tubulin III and FLT4 resulted significantly lower in LND hNSCs than in healthy ones. These alterations could be implicated in the clinical picture of LND since, as previously demonstrated, the decrease in the expression of β-tubulin III could represent a neurodevelopmental abnormality (Shafey D *et al.*, 2008). Furthermore, VEGF-C is required by brain neuroepithelial cells during embryonic development and has a direct

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neuroprotective effect on various types of neural progenitors cell expressing FLT4 (Le Bras B. *et al.*, 2006).

We next assessed the rate of neuronogenesis by culturing hNSCs in differentiating medium and establishing the number of neurons. Interestingly, the percentage of LND cells positive to neuronal markers was significantly lower when compared to healthy ones. The reduced number of LND neurons could be due to a block in differentiation or to enhanced cell death of differentiated neurons. The TUNEL stain results revealed a striking percentage of apoptotic events in LND astrocytes even at early stages of differentiation. As previously demonstrated by Pelled and colleagues (Guibinga G.H. *et al.*, 2010), the HPRT-deficient astroglia cultures exhibited altered content of purine and pyrimidine nucleotides. In view of the quantitative dominance of glial cells in the brain, the abnormal nucleotide content in these cells, even if confined to a specific stage of development only, may be associated with the neurological deficit in LND. A hypothetical pathway involved in LND astrocytes apoptosis could be found in the accumulation of 5′-Aminoimidazole-4-carboxamide riboside (AICAR) which has a toxic effect on neural cells (Pelled D., *et al.*, 1999). AICAR may accumulate in those individuals in which an inborn error of purine metabolism causes an increase in the rate of de novo synthesis and/or an overexpression of cytosolic 5'-nucleotidase, that appears to be the enzyme responsible for AICAR hydrolysis.

5'-nucleotidase activity has been shown to increase in patients affected by Lesch-Nyhan syndrome. Moreover, astrocytes cell death may contribute indirectly to neuronal injury or other CNS pathologies

since astrocytes provide neurotrophic support, protect against excitatory amino acid neurotoxicity, and maintain the normal homeostasis of the extracellular fluid (Guibinga G.H. *et al*., 2010).

It is documented that dopaminergic neurons are damaged in affected LND patients (Visser J.E *et al*., 2000; Nyhan,W.L. *et al*., 2000). The severity of symptoms in Lesch–Nyhan disease remains constant once the neurobehavioral syndrome is fully expressed (Garcia Gil M. *et al*., 2003) suggesting a developmental rather than a continuing degenerative process. The early presence of dopamine in the embryonic brain of primates (Anderson L.T., *et al.,* 1992; Olson L., *et al.,* 1973; Freeman T.B. *et al.,* 1991; Silani V. *et al.,* 1994) suggests a role for this neurotransmitter in the neurogenesis of the dopaminergic system and maturation of the striatum.

The establishment of an *in vitro* system that mimics the differentiation of undifferentiated Lesch-Nyhan and healthy neural stem cells into dopaminergic neurons gave us the opportunity to study the developmental processes that lead to the malfunction of the dopaminergic neurons in LND. When challenged to differentiate towards dopaminergic neurons, LND neurons showed a reduced neurite outgrowth and soma area. Morphological abnormalities could be a consequence of HPRT deficiency on purine metabolism. Previous studies demonstrate that guanine nucleotide depletion in neuroblastoma cell lines promotes neuronal differentiation and reduces proliferation, especially in dopaminergic neurons showing an abnormal neurite outgrowth (Messina E. *et al*., 2005; Connolly G.P. *et al*., 2001; Korenevsky A.V. *et al*., 1999).

Although the proliferative potential of LND hNSCs appears unaltered, it is possible that the LND hNSCs have distinctive gene expression patterns that have implications for later steps in development. To identify pathways affected by HPRT deficiency during early cortical development, Stem cell RT^2 profiler PCR array was then used to compare gene expression patterns between LND and normal stem cells. The benefit of looking at gene expression in neurospheres, as opposed to using primary fetal tissue, is that these cultures represent a relatively pure population of dividing cells at a specific time in development. We have shown that gene expression patterns are dramatically changed in LND neurospheres. Many of the specific signalling pathways are important for proliferation and differentiation, so the absence of significant differences in the proliferation of LND hNSCs could be explained by the fact that misexpression of these genes causes slight changes in the behaviour of the cells that we have failed to detect. The proneural gene neurogenin 2 (Ngn2) is a member of a family of bHLH transcription factors (Sommer L. *et al*.,1996), and is important not only for neuronal differentiation (Fode C. *et al*., 1998), but also for neuronal subtype-specification in various regions of the nervous system (Scardigli R *et al*., 2001).

We suggest that the dysregulation of the expression of transcription factor genes crucial for the proliferation and differentiation of hNSCs and for the maturation of neurons may be associated with the severe neurological phenotype of LND.

Along with the transcription factors described above, we have also demonstrated an altered expression of ALDH1. The downregulation

ALDH1 suggests a possible involvement of aldehyde dehydrogenases (ALDH) in the pathogenesis of LND disorder. ALDH is involved in the synthesis of retinoic acid from retinal (Yoshida A. *et al.*, 1992). Retinoic acid and related retinoids are likely to be of importance for mesencephalic DA neurons since receptors and handling enzymes are expressed at high levels both in the substantia nigra neurons and in their target regions (Zetterstrom R.H., *et al.*, 1996). During development, expression of retinoic-acid-generating enzymes precedes TH expression in DA neurons (Wallen A *et al.*, 1999), indicating that retinoids may be important for the induction of the dopaminergic phenotype. In this study, we show that *in vitro* supplementation of RA counteracts the developmental defect in LND DA-neuron morphology differentiated from LND hNSCs. This implies that RA is crucial to induce proper DA neuronal differentiation. Most appealing, by linking local RA synthesis to LND DA neuronal development and morphology, a novel mechanism is proposed, with essential implications for LND clinical pathology.

Another important aspect of this pathology, described by Song and Friedmann (Song S. and Friedmann T., 2007), is that HPRT deficiency induces secondary transcriptional aberrations in other genes as observed in our study, and the expression of the corresponding secondary genetic defects could play an important role in the development of some aspects of the HPRT-deficiency phenotype, especially the neurological deficits; in fact a relatively small number of unrelated genes are aberrantly expressed in HPRT-deficient mouse striatal tissue. These data suggest the possibility that functionally multigenic mechanisms of pathogenesis underlie this

monogenic disease (Song S. and Friedmann T., 2007). The fact that physiological or developmental damage may occur very early in life could make it difficult to rescue the abnormalities by reintroducing a non-mutant HPRT cDNA. Nevertheless, the future development of this study will include the correction of HPRT deficiency.

Oxidative stress compromises dopamine neuron function and previous studies found that the oxidant sensitive mitochondrial enzyme, aconitase, was partially inactivated by the increased production of either NO, O_2^-, $ONOO^-$, or H_2O_2, in HPRT-deficient brains (Visser J.E., *et al*.,2000; Gardner P.*et al*., 2000). The present study revealed an overproduction of NO which was not correlated with NOS1 expression. Alternative sources of NO, however, should not be ignored. The inducible form of NOS, NOS2, while not normally expressed in the brain, can be induced under pathological conditions (Hunot S. and Hirsch E., 2003). It is possible that HPRT deficiency may cause an upregulation of NOS2 leading to toxicity through NO itself. LND hNSCs' exposure to L-NAME results in reduced levels of nitrites and nitrates (reflecting reduced NO) but did not influence the differentiation of neural stem cells to neurons.

To our knowledge our results present for the first time experimental evidence that HPRT "housekeeping" gene plays an important role in human fetal neurodevelopment by complex mechanisms that still need to be elucidated. Developmental neurobiology may throw light into the neurochemical and structural events that contribute to the clinical picture of Lesch–Nyhan disease. Such knowledge may then suggest therapeutic interventions.

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3.5. Conclusions

In summary, our analysis points to the relevance of using human Lesch-Nyhan NSCs in complementing murine models for the study of this human genetic disease. It also reveals possibilities of utilizing LND human NSCs in investigating the role of HPRT in processes of neural development. Developmental neurobiology may throw light into the neurochemical and structural events that contribute to the clinical picture of Lesch–Nyhan disease. Such knowledge may then suggest therapeutic interventions.

3.6. Materials & Methods

Cell Culture and Neurosphere formation assay

Human fetal tissue was obtained from two 14-week-old legally aborted Lesch-Nyhan fetuses and from five 12-14 week-old healthy fetuses, according to the ethical guidelines of the European Network for Transplantation (NECTAR). Deficiency of HPRT was diagnosed on the basis of characterization of the genetic mutation accounting for the deficient enzyme activity: one fetus carries a r.319_402 deletion, while the other one carries a missense point mutation (c.208G>T, p.Gly70Trp).

The experimental protocol was approved by the ethics committee of the Fondazione IRCCS Istituto Neurologico "Carlo Besta" and Fondazione IRCCS Policlinico-Mangiagalli-Regina Elena. HNSCs were isolated as previously described (Vescovi A.L. *et al*., 1999; Galli

R. *et al.*, 2000; Pagano S. F. *et al.*, 2000). HNSCs were cultured in serum-free human medium optimised for neural stem cell growth (Growth Medium) (Alessandri G *et al.*, 2004). Under these culture conditions, hNSCs grew as nonadherent cells and formed neurospheres.

For neurosphere formation assay, healthy and LND cells were dissociated at different passages and the resultant single cells were plated in six-well plates at a density of 2 x 10^4 cells per well in Growth Medium containing 0.8% (wt/vol) methylcellulose. The number of cell clusters with a diameter of over 2 mm was counted at 3 weeks after plating under a Nikon Eclipse TE300 inverted microscope equipped with a Zeiss Axiovision device camera.

Selection Media and Cell Proliferation

Selection media were added to healthy and LND cells to test for the activity of the purine salvage pathway. Thus, the hNSCs were grown with 6TG (1μg/ml, Sigma, St. Louis, MO, USA). Alternatively, the cells were incubated with HAT ($1x10^{-4}$M hypoxanthine, $4x10^{-7}$M aminopterin, $1.6x10^{-5}$M thymidine; Gibco, Invitrogen, Carlsbad, CA, USA). Cell viability was evaluated by trypan blue exclusion test after one to three days after adding the selection media.

Real-Time Quantitative Polymerase Chain Reaction

TaqMan Real-Time polymerase chain reaction was performed to investigate expression of HPRT, β-III tubulin, GFAP and NG2. Three separate RNA extractions were performed on the single LND hNSC line and the pooled controls, and processed separately. Each cDNA sample (corresponding to 100 ng total RNA) was amplified in triplicate using a GeneAmp 5700 Sequence Detection System

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(Applied Biosystems, Foster City, CA, USA) in a PCR volume of 20 μl containing the TaqMan Universal PCR Master Mix (with AmpliTaq Gold DNA polymerase) and the Target Assay Mix (all from Applied Biosystems) for 35–40 cycles. The relative expression of each gene examined was normalized to GAPDH and calculated according to the formula $2^{-\Delta\Delta Ct}$ as described in the manufacturer's instructions (Applied Biosystems).

For the detection of genes related to the identification, growth and differentiation of stem cells, Real-Time PCR was performed using the Stem Cells RT² *Profiler*™ PCR Array according to manufacturer's instructions (SuperArray Bioscience Corporation, Frederick, MD, USA). Pathway-focused gene expression analysis was performed with the PCR Array System and the PCR Array Data Analysis Web Portal. Each Stem Cells RT² *Profiler*™ PCR Array was done on separate cDNAs at least three times.

HPRT enzymatic determination

Activity of the enzyme HPRT was determined in LND and healthy hNSCs by a high-performance liquid chromatography-based method (Rylance H.J. *et al.*, 1982; Puig J.G. *et al.*, 2007). In brief, each cell line was incubated for 40 min at 37 °C in 10 mM HEPES, 125 mM NaCl, 2.6 mM KCl, 5.5 mM glucose, 1 mM $CaCl_2$, 50 mM $MgCl_2$, 18 mM NaH_2PO_4 and 10 μM [^{14}C] hypoxanthine. At the end of the incubation time, radioactive IMP was separated from hypoxanthine by high-performance liquid chromatography, and radioactivity in the IMP peak was measured. HPRT activity was expressed as incorporation of [^{14}C] hypoxanthine normalized to cellular protein and percent of

healthy hNSCs. Numbers are the means±SD of five independent experiments.

Under these assay conditions, normal values for incorporation of [^{14}C] hypoxanthine into [^{14}C] IMP in cells were 98%–100%.

Western Blot analysis

The single LND hNSC line and the pooled controls (5×10^5 undifferentiated cells each line) were suspended in 100 µl of cold RIPA lysis buffer (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor cocktail. Protein whole extracts (40□g) were separated by SDS-PAGE and then transferred to nitrocellulose membrane (Pierce, USA). Membranes were probed with primary antibodies for GFAP (mouse, 1:500), GAPDH (mouse, 1:500) (both from Chemicon, Temecula, CA, USA), β-III tubulin (mouse, 1:100, AXXORA, San Diego, CA, USA), nestin (mouse, 1:100, R&D Systems, Minneapolis, MN, USA), followed by secondary antibody horseradish peroxidase conjugated-anti-rabbit and anti-mouse IgG (Chemicon; 1:5000). The lysate *ex vivo* human fetal healthy brain was used as a positive control.

TUNEL assay

To detect apoptosis in human LND and healthy differentiated NSCs, a DeadEND fluorometric tunel system (Promega, Madison, WI, USA) was used to carry out TUNEL method according to manufacturer's recommendations. Approximately 4×10^4 hNSCs were plated on chamber slides upon differentiating conditions.

As a negative control, cells were incubated with reaction mixture without the enzyme terminal transferase. No stained nuclei were detected.

Quantification of Uric Acid and NO Production

Lesch-Nyhan and healthy hNSCs were grown in 4 ml media for 48 hours. The medium was concentrated to 1 ml by centrifugal filter devices (Centricon, Amicon, Millipore, Bedford, MA, USA) according to manufacturer's instructions. We assessed the level of uric acid using Cobas6000 (Roche, Mannheim, Germany) as described in the manufacturer's instructions.

NO production was quantified by the accumulation of nitrite in the supernatants of hNSCs cultures by the standard Griess reaction according to the manufacturer's instructions (R&D). Conversion of absorbance to NO micromolar concentrations was deduced from a standard curve. Treatment with the NOS inhibitor N-Nitro-L-arginine methyl ester (100 μM, L-NAME, Sigma) dissolved in culture medium of LND hNSCs and maintained for 72 h. At that time, NO production was quantified as described.

Immunofluorescence and Morphological analyses and Neural Induction

HNSCs were plated on matrigel-coated glass chamber slides (Nunc, Naperville, IL, USA), in media without mitogens (Vescovi A.L. *et al*., 1999). After that, cells were fixed at different times (3, 5 and 7 Days *in vitro* (DIV)) and analysed for the presence of neural markers such as GFAP (rabbit, 1:500), NG2 (rabbit, 1:200), β-III tubulin (mouse, 1:100) (purchased from Chemicon) by means of immunostaining as previously described (Alessandri G *et al*., 2004). Three separate immunofluorescence analyses were performed on LND and healthy hNSCs; positive cells were counted in a blind manner.

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Neuronal differentiation was induced by addition of *cis*-retinoic acid (RA, Sigma) to the differentiation media (Vescovi A.L. *et al.*, 1999) on matrigel-coated glass chamber slides (Nunc), at a final concentration of 5 μM.

Neuronal differentiation was investigated upon differentiative condition after addition of 100 μM L-NAME (Sigma). After 7 days, the samples were fixed for immunocytochemical studies as described above.

For the evaluation of Dopaminergic differentiation, LND and healthy proliferative hNSCs were treated as previously described (Wang X. *et al.*, 2004). Induced cultures were fixed and processed for dopaminergic markers immunostaining (mouse anti-TH, 1:500, rabbit anti-NF, 1:250; rat anti-DAT, 1:500, all from Chemicon).

Phase-contrast digital micrographs have been obtained by merging of single fluorescence digital images of random fields with a Nikon Eclipse TE300 microscope equipped with a Zeiss camera. Soma size and neurite length were examined using Axiovision software. At least 50 neurons were evaluated for each line.

Reverse Transcriptase-Polymerase Chain Reaction

Three separate RNA extractions were performed on two undifferentiated LND hNSC lines and the pooled undifferentiated controls and processed separately. Total RNA from Lesch-Nyhan and healthy hNSCs was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. β-III tubulin, GFAP, nestin, NG2, SOX2, FLT4, CD133, D2R, NURR1, TH and GAPDH primer sequences are available on request. To ensure accuracy, each reaction was repeated three times.

RT-PCR reactions were optimized for GAPDH to determine the cycle-kinetic and the cDNA concentration under non-saturating condition. The optimal number of cycles was 28 using 25 ng of cDNA in 20 μl of the PCR reaction volume (Supplemetary Figure 1). Under these cycle-kinetic and the cDNA concentration conditions, we tested the expression of GAPDH gene in LND and healthy hNSCs. GAPDH levels was comparable in the analyzed cell lines.

Statistical analysis

Data are presented as mean±SD. Statistical analysis was performed using Wilcoxon rank sum test ($P < 0.05$).

3.7. Figures and Legends

Figure 1. Characterization of LND human neural stem cells
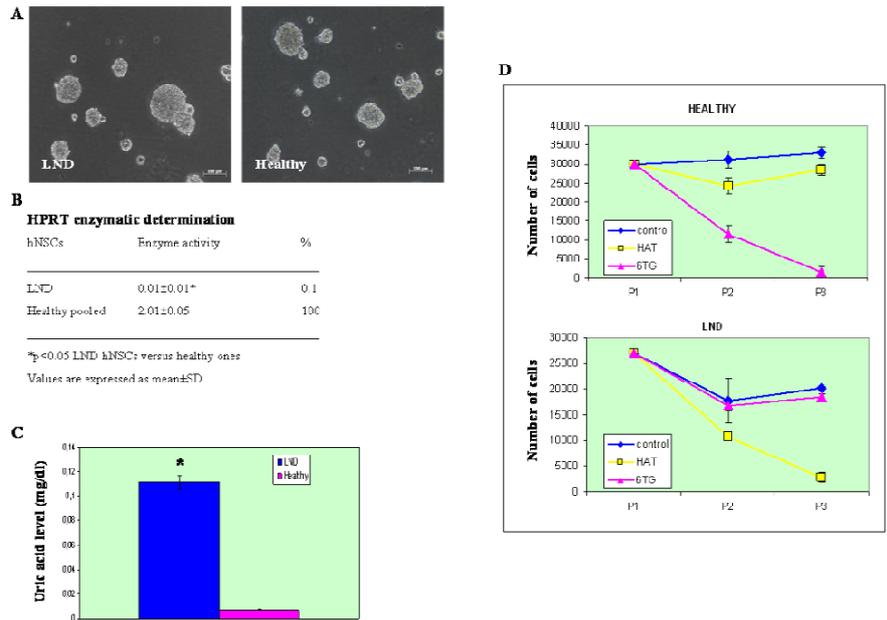
Figure 1

(A) Human LND and healthy hNSCs after 7 DIV. (B) Activity of the enzyme HPRT was determined in LND and healthy hNSCs by HPLC-based method. (C) Uric Acid Production in healthy and LND hNSCs. (D) Analysis of HPRT activity using selection media. Normal cells stop growing in the presence of 6TG, while the mutant cells stop growing in the presence of HAT.

Figure 2. Proliferation and gene expression profiles of LND and healthy undifferentiated hNSCs
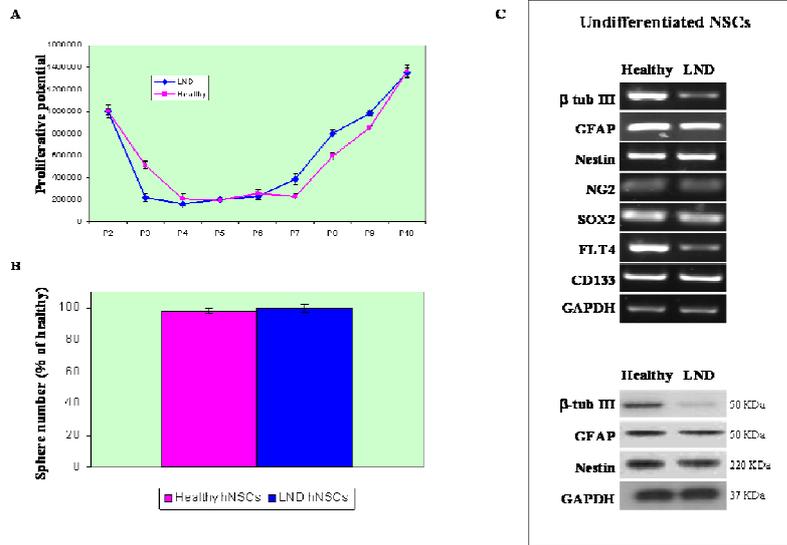
Figure 2

    (A) LND and healthy hNSCs proliferative potential. (B) LND and healthy hNSCs frequency of neurosphere formation. Data are means± SD (n=3).(C) RT-PCR analysis (upper) and Western blot (bottom) of neural markers in LND and normal undifferentiated hNSCs.

Figure 3. Differentiative potential of LND hNSCs
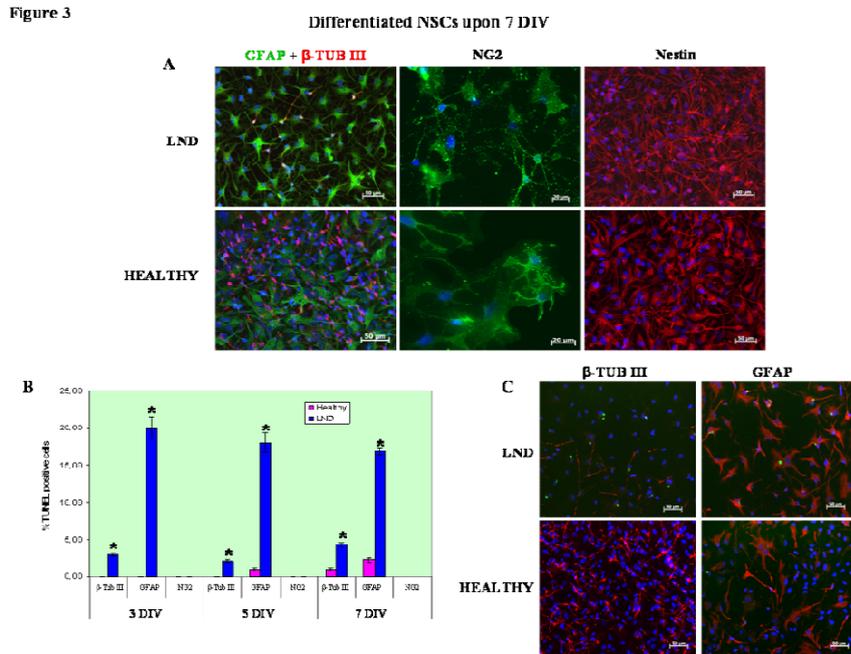
(A) LND (upper) and normal (bottom) hNSCs differentiated cells stained positively for β-tubulinIII (red) and GFAP(green), oligodendrocytes stained positively for NG2 (green) and for nestin (red). All nuclei were counterstained with DAPI (blue). DIV = Days *in vitro*.

(B) The data represent the percentages of TUNEL/GFAP or TUNEL/β-tubulinIII or TUNEL/NG2 double-positive astrocytes or neurons or oligodendricytes after double-positive cells from ten randomly chosen fields of four independent experiments.

(C) Double staining of TUNEL positive cells with specific cell markers. TUNEL/β-tubulinIII double-positive and TUNEL/GFAP double-positive were observed in LND hNSCs upon 7 DIV. All nuclei were counterstained with DAPI (blue).

Figure 4. Dopaminergic Differentiation and Gene expression in LND hNSCs
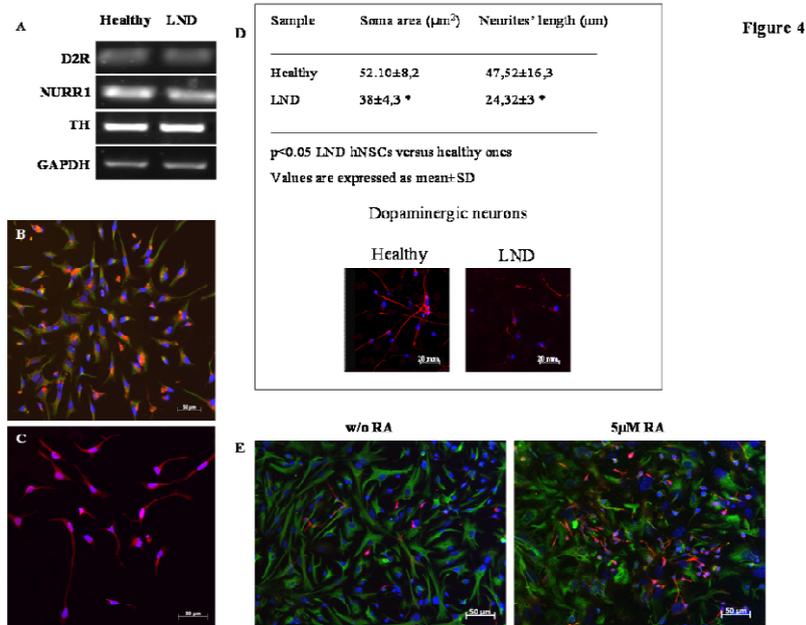
(A) RT-PCR analysis of dopaminergic marker in LND and in normal hNSCs. There is no difference between LND and healthy hNSCs. (B-C) Dopaminergic differentiation. LND hNSCs expressed DAT (red), NF (green) (in B), and TH (red in C). All nuclei were counterstained with DAPI (blue). (D) Morphometrical parameters after dopaminergic differentiation of LND and healthy cells. Bottom panel: morphology of LND and healthy dopaminergic neurons stained with NF. Nuclei were counterstained with DAPI (blue) (E) Immunostaining of LND hNSCs upon differentiation w/o RA (left panel) and with 5μM RA (right panel): RA induced dopaminergic differentiation in LND hNSCs as demonstrated by the expression of TH (red). Astrocytes

were stained with anti-GFAP (green). Nuclei were counterstained with DAPI (blue)

Figure 5: Optimization of RT-PCR conditions of the amplified GAPDH c-DNA
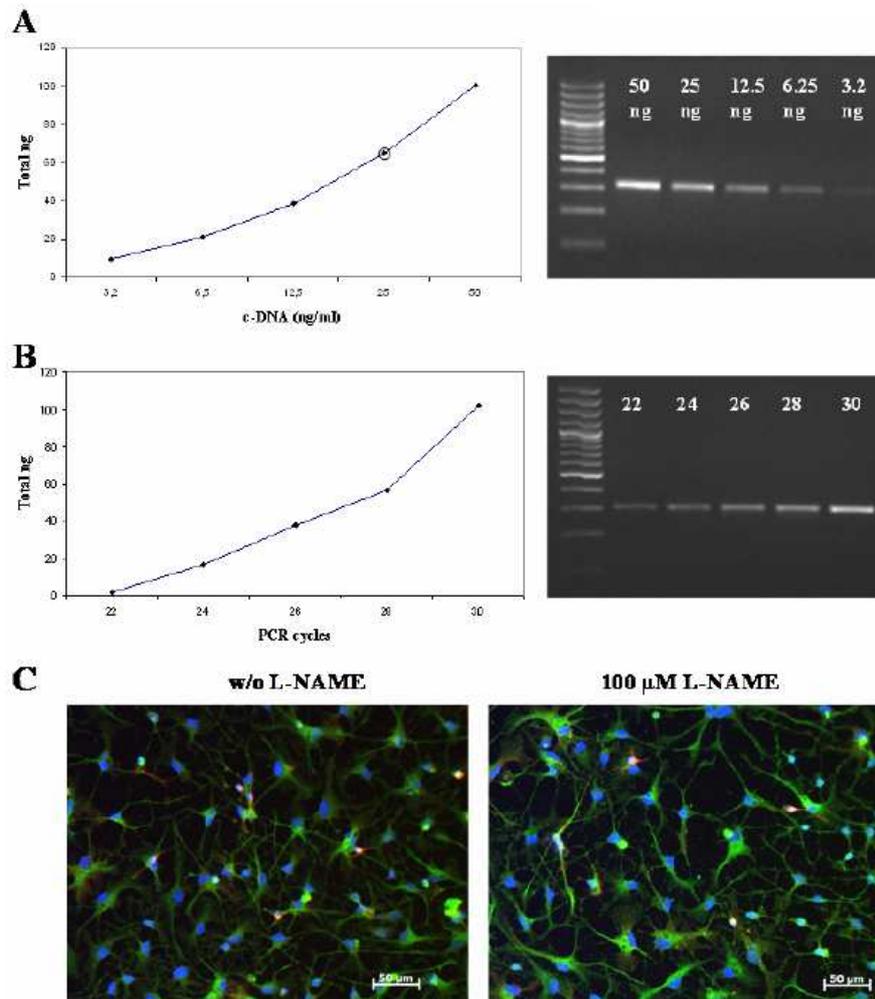
(A) Optimization of RT-PCR to determine cDNA concentration under non-saturating condition. The detection of non-saturating

conditions was carried out for GAPDH gene. The empty black circle indicates the c-DNA concentration (25 ng) utilized in the subsequent PCR of gene expression (right panel). In left panel the relative RT-PCR analysis of GAPDH (B) RT-PCR reactions were optimized for GAPDH to determine the cycle-kinetic. The optimal number of cycles was 28 using 25 ng of cDNA in 20 μl of PCR reaction volume (right panel). In left panel the relative RT-PCR analysis of GAPDH. (C) Immunostaining of LND hNSCs upon differentiation w/o L-NAME (left panel) and with 100μM L-NAME (right panel): L-NAME did not induced dopaminergic differentiation in LND hNSCs. TH positive cells are shown in red. Astrocytes were stained with anti-GFAP (green). Nuclei were counterstained with DAPI (blue).

Table 1

Immunofluorescent analysis of neural markers upon differentiation of hNSCs

	3 DIV		5 DIV		7 DIV	
	LND	Healthy	LND	Healthy	LND	Healthy
β-tubulin III	2%±0.1*	7%±2	5.2%±1*	14%±2.3	7%±0.2*	20%±1
GFAP	16%+3	18%+2	51%+4	49%+6	70%+2	67%+5
NG2	N.D.	N.D	1%±0.1*	N.D.	5.6%±0.4*	2%±0.6

Human neural stem cells (hNSCs); Lesch-Nyhan disease (LND); not determined (N.D.); days *in vitro* (DIV).

*p<0.05 LND hNSCs versus healthy ones

values are expressed as mean±SD

Table 2

Real Time PCR analysis of neural markers upon differentiation

	β-tubulin III	GFAP	NG2
LND hNSCs	0.6 ± 0.02%*	1.0±0.022%	1.24± 0.04%*
Healthy hNSCs (calibrator)	1±0.01%	1±0.015%	1±0.03%

Human neural stem cells (hNSCs); Lesch-Nyhan disease (LND)

* p<0.05 LND hNSCs versus healthy ones

Values are expressed as mean±SD

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Table 3

Fold differences in gene expression profile in LND hNSCs

Gene ID	Fold difference	p value
Decreased gene expression		
CD44	2.28	0.003
NCAM1	2.14	0.010
NEUROG2	9.49	0.013
TUBB3	1.40	0.008
ALDH1A1	39.02	0.001
Increased gene expression		
PPARDδ	11.99	0.001
BMP2	14.46	0.006
NOTCH1	11.11	0.042
MYST1	4.27	0.005
GCN5L2	12.41	0.039

Human neural stem cells (hNSCs); Lesch-Nyhan disease (LND)

Table 4

NO production and NOS1 expression in healthy and LND hNSCs

	NO production (µmol/L) Growth Medium	NO production (µmol/L) Growth Medium plus L-NAME 100 µM	NOS1 at m-RNA level
LND hNSCs	0,405±0.01*	no detection	no detection
Healthy hNSCs	0,197±0.008	no detection	no detection
Total brain (control)	not analysed	not analysed	positive detection

Human neural stem cells (hNSCs); Lesch-Nyhan disease (LND); nitric oxide (NO); nitric oxide synthase 1 (NOS1); NG-nitro-L-arginine methyl ester (L-NAME).

*$p<0.05$ LND hNSCs versus healthy ones

Values are expressed as mean±SD

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Chapter 4

4.1. Summary

Stem cells, irrespective of their origin, have emerged as valuable reagents or tools in human health in the past 2 decades. In this thesis, we have attempted to provide a preliminary account of human stem cells in the context of research in regenerative medicine.

Neurodegeneration refers to a great clinically and pathologically heterogeneous disease entity associated with slowly progressive neuronal loss in different anatomical and functional systems of the brain. Neurodegenerative diseases represent one of the leading causes of death and disability in the worldwide. Most of the Neurodegenerative disorders are thought to arise through a complex interplay between inherited genetic variation and environmental triggers. At the moment the most ordinary clinical Neurodegenerative diseases trials are based on pharmacologic strategies. The current pharmacologic and interventional strategies fail to regenerate dead neural tissue and are usually insufficient to prevent the impact of the neurodegeneration in the functional physiology of the CNS. For these reasons the developments of strategies that allow functional restoration of damaged CNS present a formidable challenge for the prevention of post-degeneration CNS functional failure. One strategy might be the use of cell-based therapies for both the prevention and treatment of

neurofunctional failure . The term of Cell Therapy identify one way of treatment in which drugs are replace with cells Recently, this definition has been used primarily to indicate procedures involving the use of well characterized cell subsets, subject to specific treatments, such as cell selection, in vitro expansion, creating clones of anti-infective or anti neoplastic proprieties. ALS is a progressive neurodegenerative disease with a multifactorial pathology; many causes are involved in the progressive degeneration and death of motor neurons and no effective treatment exist at the moment. Animal models of neurodegenerative diseases are useful to assess the effects of stem cell therapy and estimate the ability of stem cells to restore damaged tissues. Nowadays, an important limit in stem cell engraftment is the difficulty to detect transplanted cells. At present, it is possible to track the engrafted cells by labelling them with some commercial tracers, such as bromodeoxyuridine, or by utilizing transfected cells expressing a detectable marker. Although these systems are mainly utilized, they are not completely satisfactory. A particular labelling with SPIO nanoparticles allows to visualize the engrafted cells through magnetic resonance imaging. It is interesting to study human SkmSCs in vivo where cells can be engrafted in a defined area of the central nervous system in animal models to verify their distribution, differentiation and function. We studied the effect of transplanted human SkmSCs in pathological recipient animals to understand if they are able to produce trophic factors or some proteins useful as a defence against motor neuron death.

Nowadays it is possible to obtain stem cells of central nervous system from foetus (Piper DR. et al., 2000): neural stem cells are able to proliferate generating special clusters called neurospheres that posses a lot of plasticity. Under specific conditions neural stem cells can differentiate in neurones, astrocytes and oligodendrocytes in vitro. In our study we used human neural stem cells isolated from LND tissues to provide new insights into the mechanisms that underlie neurological diseases and neurodevelopment.

LND human neural stem cells allowed us to observe directly the effect of the HPRT deficiency on proliferation, migration and neuronal differentiation. We found that there is no correlation between HPRT activity and proliferation rate but their properties of differentiation, the expression of specific genes relevant in dopaminergic neuron development and function showed significant abnormalities.

These cells combine the peculiarity of a neurodevelopmental model and a human, neural origin to provide an important tool to investigate the pathophysiology of HPRT deficiency and more broadly demonstrate the utility of human neural stem cells for studying disease and identifying potential therapeutics.

4.2. Conclusions

Stem cell-based regeneration depends partly on the delivery of stem cells to the damaged area. Repair by stem/progenitor cells, whether endogenous or transplanted, may be limited in part by an inability to ensure a sufficient number of reconstituting cells in the damaged area at the opportune time. Optimising regenerative processes, therefore, may depend on first identifying the range of molecules that subserve trafficking. We intend to explore an aspect of this process by studying the interactions between human SkmSCs and microenvironment.

A key novelty of this project is the integration of advanced technological tools, such as nanotechnologies and neuroimaging, to study human stem cells cells' homing in animal models of ALS.

The failure of a noticeable number of experimental protocols based on neuroprotective drugs, which proved to be effective in rodents, indicates a clear demand for an additional experimental step between common rodent models and human patients in evaluating novel ALS therapies before entering clinical applications.

4.3. Future Prespectives

Stem cells and regenerative medicine is one of the most rapidly advancing area of biology today. Recent discoveries in the field of stem cell research have opened new avenues for the therapy of complex diseases, particularly those of the central nervous system (Nishino H et al., 2000; Ogawa Y et al., 2002; Silani V. et al., 2004).

The objective of this project is to promote a thorough exploration and characterization of the bi-directional communication between stem cells and the niche that they encounter in vivo under normal and compromised states, such as ALS. Of particular interest is the rigorous characterization of how interactions with localized cues in space and time regulate stem cell survival, migration, replication, and "plasticity" in the nervous system.

The main focus of this project is the study of human SCs homing and interactions within pathological microenvironment in order to identify the limits and the benefits derived from stem cell based therapies. To reach this goal, we will perform in vitro studies on human SCs trafficking, the critical initial step of the homing cascade. Our studies will compare different classes of stem cells or progeny at progressively more advanced stages of differentiation when placed in the same sites in vivo. The initial hypothesis governing these studies was that the migration of SCs to the affected areas and their transdifferentiation toward the

neuronal lineage could replace degenerated neurons and therefore restore the functionality of the motor system. However, it is now clear that the effect produced by these cells is likely related to an indirect action (e.g. induction of neurogenesis, growth factors release, anti-inflammatory activity) rather than an actual substitution of degenerating neurons (Mahmood A. et al., 2004).

Bruijn et al suggests that ''stem cells engineered to secrete growth factors or other factors required for neuronal survival'' or to ''stimulate endogenous stem cells in the brain to generate new neurons'' may be more feasible for ALS than neural replacement Bruijn et al., . Moreover, therapeutic strategies to retard disease progression ''seem to be a more realistic clinical approach as compared with neuronal replacement'' (Lindvall O. et al., 2004).

Using specific factors we will reprogram human primary cells to yield iPS; such an approach provides a unique clinical opportunity to use the adult cells obtained from a patient to genetically reprogram the cells according to the particular need of the patient.

To better understand stem cell biology and realize the full potential of stem cell therapy, we will monitor the trafficking of labelled stem cells by molecular and cellular imaging. The marriage of nanotechnology and stem cells will significantly advance our ability to understand and control stem cell-fate decisions and develop novel stem cell technologies, which will eventually lead to stem cell-based therapeutics for the prevention, diagnosis, and treatment of human diseases. Nanotechnology will allow us to label stem cells using magnetic, genetic or fluorescent

probes which can be monitored by magnetic resonance imaging (MRI) or fluorescence imaging.

Although numerous previous studies have investigated stem cell migration using fixed brain tissue, there is little knowledge based on live imaging in response to brain damage. Multiphoton microscopy is a key technology that will allow us to observe cell behaviour and cellular biological activity in live animals or living brain slices. This technique will provide insights in the mechanisms and dynamics of morphological remodelling in the pathological CNS following stem cell transplant. As secondary objective, the response of the surrounding cells to this specific perturbation will be studied as a model of the reactive and degenerative changes in microstructure observed in certain neurodegenerative diseases. Several different types of SCs, both mouse and human, have a capacity for precise migration to even widespread and distant areas of pathology in a number of experimental models of CNS disease. Because stem cell engagement with a degenerating environment is the first critical step in regeneration, realizing the therapeutic promise of the SC depends in part on understanding the mechanisms underlying its mobilization during degenerative progression of CNS disease .

Repair by stem/progenitor cells may be limited in part by an inability to ensure a sufficient number of reconstituting cells in the damaged area at the opportune time. Optimizing regenerative processes, therefore, may depend on first identifying the range of molecules that subserve trafficking .

In recent years, the application of nanotechnology in stem cell research and development have made great progress. For example, magnetic nanoparticles (SPIO) have been successfully used to isolate and sort stem cells, and all these advances speed up the development of stem cells toward the application in regenerative medicine (Song M et al.,2009). The use of nonlinear optical methods in biology is continually undergoing developments and refinements. Two-photon microscopy is particularly useful in neuroscience where in vivo imaging has shown great potential in studying the structural correlates of learning and memory. This technique will provide insights in the mechanisms and dynamics of morphological remodeling in the brain following stem cell transplant.

Although this project is focused on the treatment of ALS, it will provide an important therapeutic protocol for the treatment of other neurodegenerative diseases.

The approach described in this project represents an example of a state of the art strategy, combining 2 potentially therapeutic activities of human KmSCs: (i) replacement of degenerated neurons and (ii) delivery of therapeutic agents in vivo. This project will likely generate important information not only about the molecular details of the treatment of ALS, but also about the biology of transplanted stem cells.

The final goals of this project are:

- the development of innovative approaches that could contribute to the cure of several neurological disorders.

- to yield novel strategies for interfering with neuronal loss during neurodegenerative disorders.
- the translability of the results "from the bench to the bedside".

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Pubblications

-Silvia Cristini, **Laura Canzi**, *et al.* Human neural stem cells: a model system for the study of Lesch-Nyhan disease neurological aspects. Hum Mol Genet. 2010 Mar 4.

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