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Editorial

Research Articles

EDITORIAL

A special issue devoted to STEM CELLS

Our Biological Research Journal of the Society of Biology of Chile is definitely one of the scientific publications of experimental biology in Latin America with greatest impact internationally. Recently our ISI Impact Factor increased significantly, to 1.17.

With the purpose of improving the visibility of Biological Research, the Society of Biology of Chile obtained a grant from the National Commission of Science and Technology (CONICYT), FP Project # 11021-2012 entitled "Strategies to increase the visibility of Biological Research". In this project the strategies chosen to accomplish greater visibility were: the inclusion in social networks (Twitter and Facebook), Google AdWords and dissemination by publishing a special issue devoted to a focal topic. This latter strategy has generated this special issue of Biological Research dedicated to stem cells. For this issue we invited renowned international and national scientists who are actively working in research on basic and applied biological aspects of biomedicine of stem cells. To all of them we extend our sincere thanks. Dr. Alejandro Erices participated in the preparation of this special issue; so we appreciate his initial your collaboration in the editorial work.

The 12 articles contained in this special number address a broad range of issues, from the general aspects of stem cells, through basic biological aspects of different types of stem cells including cancer stem cells, and covering topics on the application of stem cells in human disease therapies, and finally a discussion of bioethical aspects of basic and applied research on human stem cells. The contributions of the invited scientists are:

1. The group of Professor Carlo Redi, of the Fondazione IRCCS, Pavia, Italy, with an article that provides an overview of biological and applied in therapy of stem cells.

2. The group of Dr. M. Sogayar, of the Chemistry Institute, Dept of Biochemistry, University of São Paulo, Brazil, covering the topic of stem cells in embryonic skin development.

3. The group of Dr. V. Palma, of the Faculty of Sciences, University of Chile, covering the subject of Sonic Hedgehog in cancer stem cells and its novel link with autophagy.

4. The group of Dr. E. Rodríguez, of the Institute of Anatomy, Histology and Pathology of the Austral University of Chile addresses the relationship of neural stem cells to abnormal neurogenesis and hydrocephalus.

5. The group of Dr. F. Nualart, of the Faculty of Biological Sciences, University of Concepción, Chile, addresses the problem of typical and atypical stem cells In the brain, in relation to Vitamin C and its effects in neuropathology.

6. Drs. F. Rivera and Ludwig Aigner of The Institute of Molecular Regenerative Medicine, Paracelsus Medical University, Austria, address the topic of adult mesenchymal stem cell therapy for myelin repair in Multiple Sclerosis.

7. The group of Dr. F. Figueroa, of the Faculty of Medicine, University of the Andes, Chile, makes a critical review of mesenchymal stem cell treatment for autoimmune diseases.

8. The group of Dr. J. P. Rodríguez of the Institute of Nutrition and Food Technology, University of Chile addresses the problem of the differentiation of mesenchymal stem cells and the improvement of bone marrow adipogenesis in osteoporosis.

9. The group of Dr. P. Conget, of the Institute of Sciences, Faculty of Medicine, Clínica Alemana-Universidad del Desarrollo, Chile, addresses the issue of stem cell mesenchymal transplantation as a strategy for the treatment of diabetic nephropathy.

10. The group of Dr. Castellón, of the Institute od Biomedical Sciences, Faculty of Medicine, University of Chile, addresses biological aspects of cancer stem cells isolated from prostate carcinomas.

11. The group of Dr. F. Barriga, of the Department of Pediatrics and Hematology Oncology, Faculty of Medicine, Catholic University of Chile, reviews the situation and prospects of the clinical use of hematopoietic stem cell transplantation.

12. Drs. M. J. Santos and P. Ventura-Juncá of the Faculties of Biology Sciences and Medicine at the Pontifical Catholic University of Chile address the bioethical aspects of basic research and medical applications of human stem cells.

We are confident that the quality of these various articles contained in this special issue of Biological Research will cause them be read worldwide and cited nationally and internationally, thus helping to improve visibility and the impact factor of our Biological Research journal.

Manuel J. Santos, MD, PhD
Editor Biological Research

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Stem cells: sources and therapies

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ABSTRACT

The historical, lexical and conceptual issues embedded in stem cell biology are reviewed from technical, ethical, philosophical, judicial, clinical, economic and biopolitical perspectives. The mechanisms assigning the simultaneous capacity to self-renew and to differentiate to stem cells (immortal template DNA and asymmetric division) are evaluated in the light of the niche hypothesis for the stemness state. The induction of cell pluripotency and the different stem cells sources are presented (embryonic, adult and cord blood). We highlight the embryonic and adult stem cell properties and possible therapies while we emphasize the particular scientific and social values of cord blood donation to set up cord blood banks. The current scientific and legal frameworks of cord blood banks are reviewed at an international level as well as allogenic, dedicated and autologous donations. The expectations and the challenges in relation to present-day targeted diseases like diabetes mellitus type I, Parkinson's disease and myocardial infarction are evaluated in the light of the cellular therapies for regenerative medicine.

Key words: embryonic stem cell; adult stem cell; pluripotency; induced pluripotent stem cells; umbilical cord blood; spinal cord stroke; Parkinson' disease.

STEM CELL HISTORY: FROM PAPPENHEIM TO MICRORNAS

To understand the range of stem cell (SC) biology in technical, ethical, philosophical, judicial, clinical, economic and biopolitical issues, two pre-requisites must be clarified: 1) the derivation of the term "stem cell" or better, its root "staminal" and 2) anatomic SC sources. This is necessary for the psychological implications behind the daily use of scientific terms: for example, the colloquial use of the term "life". While appearing to have a scientific meaning, it actually refers to the experience spectrum of everyone that has lived.

Regarding the derivation of the words stem and staminal, only two contributions explain it. Ramalho-Santos and Willenbring (2007) suggested that: "One would be tempted to assume that the term stem cell has some relation to the term ''meristem'' because meristems are the stem cell compartments of plants." The term meristem was first used by the botanist Karl Nägeli, born in Switzerland, describing the areas of continual cell division in a plant (Nägeli, 1858). He derived the term from the Greek ''meristos'' and the suffix ''-em'' (as in ''phloem'' or ''xylem'').

Since the adjective *staminalis, -e* did not exist in Latin, we suggested (Monti and Redi, 2011a) the neutral noun *stamen, -inis* being the basis of the neo-Latin word "staminal", which was coined in an English-speaking scientific environment. *Stamen* indicates the warp of the cloth and metaphorically suggests "the fiber of life" with the idea of a "grounding" entity, which is the founding "stem cell".

SCs are then defined by their double capability to simultaneously perform two distinct processes: self-renewal and differentiation. The phenomenon that ensures this capacity is called "asymmetric division", but there is no agreement on the mechanism that sustains it. Another hypothesis, focused on the "immortal DNA strand", suggests that the daughter cell inheriting the old "template" strand keeps the stem capacity, while its sister cell inheriting the new DNA strand will undergo differentiation (Cairns, 1975; 2002; 2006). Notably, this hypothesis can account for the origin of cancer stem cells (CSC) since the mutations will continuously accumulate in the newly synthesized DNA strands that are always inherited by the differentiative-committed cell reaching the neoplastic (stemness) condition due to the inactivation of tumor suppressor genes (Cairns, 2006; Rando, 2007). Supporting this view, Reya and Clevers (2005) showed that different kind of tumors share the deregulation of the Wnt signaling network that is able to activate the self-renewal capacity. The European group "Migrating Cancer Stem Cells" (www.mcscs.eu), led by Riccardo Fodde, is continuously updating the CSC biology field. Recently, however, the hypothesis of the immortal template DNA was questioned by the evidence that it does not apply to intestinal epithelial SCs (Escobar et al., 2011).

An alternative is the SC hub-niche hypothesis presented by Schofield (1978). It is based on studies in the vertebrate's hematopoietic system and was corroborated by the *Caenorhabditis elegans* germ cell development (Kimble and White, 1981) and research with the *Drosophila melanogaster* ovary (Kiger et al., 2001; Tulina and Matunis, 2001; Fuller and Spradling, 2007). Several reviews illustrated how the SC niche represents a paradigmatic concept in SC biology (Scheres, 2007; Jones and Wagers, 2008; Knoblich, 2008; Morrison and Spradling, 2008). Proximal signals (cell surface molecules) as well as distal ones (secreted molecules) act in a specialized anatomical microenvironment (the hub-niche) where they control cell proliferation and differentiation. Whatsoever the explanation, the medical community has an intense interest in using SCs for cell replacement therapies in tissues that have been damaged by aging, trauma or specific pathologies.

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As for the SC sources: at first it was thought, naively, that only a few tissues posses SCs: blood, intestinal epithelia, bones, skin. Now we know that they can be found in any tissue of the body, surprisingly in the pulp of deciduous teeth (Gronthos et al., 2000), amniotic fluid (De Coppi et al., 2007) and breast milk (Cregan et al., 2007). Nerve tissue was believed to be completely differentiated, but it presents SCs, even within 18 hours after death. In mammals, all phases of preimplantation embryo development yield embryonic SCs (ESC). After implantation, the embryo affords somatic SCs (SSC), which unfortunately are also called "adult". Nearly 10^{15} ESCs in the human are pluripotent; a few ESCs of the early embryo are totipotent: only these are able to produce every type of body cell in any number.

The restricted SSCs are multipotent when they give rise to more than one cell type pertaining to a specific cell lineage. More restriction happens later on in development, and SSCs become only unipotent when they produce just a single cell type.

Until the beginning of the 1950s, the existence of SCs was conjectural. After the Second World War, autoradiography introduced time as the fourth dimension to histological investigation, along with the conversion of the nuclear energy physics into biological application. French-Canadians took the opportunity to follow the movements of labeled isotopes in time and thus discovered the renewing male germ cells (Leblond and Clermont, 1952) and the SCs at the base of the intestinal villi. From that moment on, there was *fast and furious* flourishing of SC discoveries that have lead us to revolutionize the SC concept. Examples were the induction of stemness in a terminally differentiated cell (Yamanaka, 2009) and the new way of looking at cancer stem cells (CSC; www.mcscs.eu). This important field of research is still fascinating in retrospect. Some SC bibliographic masterpieces are quite instructive for those entering the field. We recommend the 2006 EMBO summary *Stem Cell Research - status, prospects, prerequisites* (www.embo.org/index.php). This is still a valid document introducing SC biology and all problems related to what is undoubtedly considered the hottest topic in biology and medicine nowadays. The journal *Nature* offers a website devoted to SCs, which is constantly updated and particularly instructive (www.nature.com/stemcells/index.html). We also note the drawings by Artur Pappenheim who lived 13 Dec 1870 – 31 Dec 1916 (Dinser 2001). He unraveled the differentiation of hematopoietic cells while working at the Virchow Pathological Institute in Berlin (see Fig. 2 in Maehle, 2011). This is especially interesting when compared to the present-day view about the microRNA modulation in the hematopoietic lineage (Chen et al., 2004; Kluivert et al., 2006). This comparison teaches the lesson that sometimes, in science, it is reasonable and fruitful to believe in something invisible! The mammalian egg for example was not discovered until Karl Ernst von Baer found it in 1827. But it was supposed to exist already during the time of Johann van Horne (1621 – 1670), Niels Stensen (1638 – 1686) and Reignier de Graaf (1641 – 1673). Actually, we know perfectly the microRNAs, which control self-renewal and cell differentiation in the hematological system (Chen et al., 2004; Kluivert et al., 2006). Their identification is due to a fundamental change of biology from a historical (ontological) science to a hard science, *from the description to the synthesis of life*. Now we can biotechnologically produce these desired cells, thus – *there will be blood*! (Durand and Zon, 2012; literature quoted there).

STEM CELL TYPES: EMBRYONIC AND SOMATIC

ES cells have been first derived in the mouse by Martin Evans and Matthew Kaufman (1981) from the Dept. of Genetics, Cambridge University, UK, and by Gail R. Martin (1981) from the Dept. of Anatomy, University of California, San Francisco, who coined the term "embryonic stem cell". Later on, such cells were isolated from blastocysts in primates and humans (Thomson et al., 1998). These projects have been funded by large private pharmacy corporations like Geron (Menlo Park, California, USA). The USA Federal government under President George W. Bush's leadership never supported research with human embryos. However, President Barak Obama is promoting such studies, and new ES lines have been derived and are now freely available to the scientific community (http://stemcells.nih.gov/registry).

The primordial germ cells (PGC) appear at the 1st and 3rd developmental week in mouse and human, respectively. Once isolated from the embryo, these cells can multiply giving rise to pluripotent embryonic germ cells (EG, Shamblott et al., 1998). Anyway, difficult and time-consuming isolation restrains their use in therapy.

ES cells can be obtained from embryos. This is a legal practice where the ethical debate on the embryo status has reached a consensus either on a pragmatic-utilitarian view (Australia) or on an ideological-religious view (Singapore, Taiwan, Israel). In these countries, embryos come from in vitro fertilization clinics after the procedures have granted a baby. Both partners have to sign an informed consent form agreeing to donate embryos for ESC derivation. In other countries like Brazil, Spain and the UK, a special authority, like the Human Fertility and Embryology Authority (HFEA; www.hfea.gov.uk) rules on research applications. The green light for the derivation of ESC lines is given very rarely. The donor embryos must be destroyed mandatorily before the end of the second developmental week. In many more countries (for example, Austria, Germany, Ireland, Italy), embryos left after IVF have to be cryo-preserved. The international situation, the ethical issues and the philosophical themes are broadly discussed in "Biopolitics of the frozen embryos" by Monti and Redi (2011b).

INDUCTION OF PLURIPOTENCY: THE EGG AS THE *REPROGRAMMOME*

Embryos can be created *de novo* when nuclei of terminally differentiated cells are transferred into de-nucleated oocytes. The oocyte cytoplast enforces genetic reprogramming of the received nucleus, which acquires the embryonic developmental programme. Nuclear transfer is the pivotal technique in the cloning procedure with animals. The first success was *Dolly* the sheep (Wilmut et al., 1997). Even though this did not result from a proper procedure of nuclear transfer, *Cumulina,* the first cloned mouse, clearly did at least (Wakayama et al., 1998).

If the development of the composed embryo is stopped at the first mitotic divisions, ESCs can be derived from its inner cell masses. This fact opens the possibility of producing autologous SCs on demand for personalized medical treatments. Unfortunately, this theoretical possibility gave rise to the term *therapeutic cloning*. The term is often used, but should be avoided, since it calls to mind unacceptable practices, e.g., human cloning (Solter, 2002).

Nuclear transfer techniques show the capacity of the oocyte cytoplasm to induce activation of the stemness genes, but the responsible substances and their mechanism still remain unknown. Seminal studies by Hans Spemann and Hilde Mangold proved a cytoplast's capacity to induce re-acquisition of the stemness status, and *for his discovery of the organizer effect in embryonic development,* Spemann won the Nobel Prize 1935 (www.nobelprize.org).

Recent studies by Sir John Gurdon and colleagues worked out that the reprogramming process replaces somatic proteins by oocyte polypeptides and includes DNA demethylation and histone modification. Since these reactions obey a chronological order, the Gurdon group supports a deterministic view for the phenomenon (Jullien et al., 2011, especially Figures 1-4). Thus, the authors face the prevailing stochastic idea that, however, better justifies the very small yields of the process.

Extracts from oocyte cytoplasts, when added to differentiated cells in culture, will probably hugely increase the yield of reprogrammed cells. This promising consideration justifies the efforts put forward by the scientific community to find the best strategy. A "natural" approach will use de-nucleated oocytes or ESCs, whereas an artificial way will lead to the synthesis of molecules showing reprogramming capacity (Byrne, 2011). Using a cytoplastic strategy, it was possible to turn fibroblasts into ES-like cells by "growing" them in cytoplast extracts from mouse oocytes. Considering ≈ 0.01 - 0.1% efficiency, the yield of reprogrammed cells was good (Neri et al., 2007). A cocktail of the essential substances should theoretically be able to erase the epigenetic imprinting of the differentiated starting cells and to switch on the stemness network. It seems reasonable that stemness does not depend from a single gene, but hinge on a genetic cascade. The theoretical premises came forward from the group of Helen Blau who showed that a defined and specific methylation status of Oct-4 and Nanog genes is necessary to get a stemness status (Palermo et al., 2009). Recently, Michele Boiani and colleagues restricted the number of the candidate factors of the "reprogrammome" to 28 polypeptides (Pfeiffer et al., 2011).

INDUCED PLURIPOTENT STEM CELLS (iPS)

Some genes play a master role in activation and maintenance of the regulatory stemness networks. Their ectopic expression opens a "direct" route to pluripotency in target cells. The feasibility of this strategy was proved at Kyoto University by transfection of the four stemness genes Oct4, Sox2, c-myc and Klf4 into terminally differentiated fibroblasts. Takahashi and Yamanaka (2006) used retroviruses that supported the induction of pluripotency in a very small fraction of the fibroblasts (only 0.001 - 0.01% efficiency). All the reprogramming techniques for induced pluripotent stem cells (iPS) have still low efficiency, due to several reasons explained by Shinya Yamanaka (2009). However, his pioneering study allowed researchers to reduce, step by step, the required genes to one, Oct4, and to obtain high rates of reprogramming (Giorgetti et al., 2009; Kim 2009). Now we conceive that the elusive cytoplast substances are made of just some of the proteic products of the master stemness genes (e.g., Oct4, Sox2, Nanog).

The data are of social importance, because they testify to the good attitude and responsibility of the scientific community in public problems. The iPS cells liberate especially Catholics from the Roman disagreement on the use of ESCs. The iPS cells provide a positive perspective in science-society relation.

The theoretic background and the conceptual derivation of the physiological principles of cellular reprogramming have been reviewed by Thomas Graf and Tariq Enver (2009). Wolfgang Reik and colleagues presented a similar model within a Waddington's epigenetic landscape (Hemberger et al., 2009).

Ideal approaches avoid the use of foreign DNAs in reprogramming. This objective was aimed at by the use of chemicals or by biophysical stimuli at the Scripps Research Institute (La Jolla, California). The group of Sheng Ding produced iPS cells using purified proteins, which were derived from the four Yamanaka's genes (Xu et al., 2008; Li et al., 2009). Their protein mix contained valproic acid (a histone deacetylase inhibitor), synthetic small molecules and natural products, which are able to bind nuclear receptors, histone- and DNA-modifying enzymes, protein kinases and signaling molecules.

To get the story on promises and reality of iPS cells updated, see the *Special Insight* on regenerative medicine in Nature 453:301 (2008), Stadtfeld and Hochedlinger (2010), as well as Hayden (2011). After all, the *Nature* web focus is devoted to stem cells nowadays (www.nature.com/focus/stemcells).

STEM CELL SOURCES

A vast body of literature is devoted to the SC derivation from almost every organ (for information, readers can refer to the web sites mentioned above). In addition, we have acquired the ability to produce several cell types from the SSC isolated from different tissues of adults, fetuses and the umbilical cord blood (CB). SC isolated from preimplantation embryos only now entering the preclinical trials steps due to the necessary caution in their use (their clonogenic capacity can lead to tumors) and for the ethical and legal considerations on the embryo status (Monti and Redi, 2011b). No doubt, ES are a need for the scientific community, but actually for other uses than for therapies; in other words, we need them to advance our knowledge on the very first embryo developmental steps and to shorten the time of toxicological and pharmacological tests so that translational medicine can take profit of the iPS' ability to "draw in a test tube" the diseases. However, quite frequently the ethical concerns on the ES derivation from embryos (either from frozen or de novo created embryos) focus the debate on the attempts put forwards to accommodate this problem (Monti and Redi, 2011b) thus diverting our attention from alternative SC sources that intrinsically resolve the ethical concerns: in other words, the SSC that can be obtained from the umbilical cord blood (CB). Here we would like to draw the reader's attention on this partly neglected SC source in the hope to boost the creation of public CB banks.

STEM CELLS FROM UMBILICAL CORD BLOOD

Ethical concern about ESC derivation from embryos guides our attention to alternative sources. The cord blood (CB), also known as placental blood, contains SSCs, which are not subject to ideological restrictions. In 1988, Eliane Gluckman conducted the first successful transplantation of SCs from

CB. The recipient was a young patient suffering from Fanconi anemia, a rare congenital blood disease. The success invalidated the idea that hematopoietic SCs can be exclusively derived from bone marrow, and further that only these cells can be used for transplantation. Three research groups have combined their different skills to overcome the prevailing skepticism. Arleen Auerbach (Rockefeller University, New York) devised a reliable method for the prenatal diagnosis of Fanconi anemia. Hal Broxmeyer (Indiana University) deeply studied the characteristics of the CB hematopoietic SCs: their sufficient number and clonogenic potential to repopulate bone marrow. After all, Eliane Gluckman (Hôpital Saint-Louis, Paris University VII) devised the pre-transplant conditioning by reduced chemotherapy, which allowed the engraftment of CB SCs. The prenatal diagnosis had proved the CB SCs of the young patient and of his brother being healthy and compatible. CB was collected after birth, cryo-preserved at -180° C, and infused after rapid thawing. The first signs of the transplanted cells appeared twenty-two days later and finally brought complete hematologic and immunologic reconstitution. The patient is alive and in good health twenty years after the transplant. This exciting experience evoked many scientific, organizational and ethical questions.

Is the CB collection safe for both the mother and the baby? Does the volume of one umbilical cord (60-120 ml) contain sufficient SCs to ensure success of a transplant? Does the "contamination" of CB with maternal cells cause severe immune responses in the recipient? Can we obviate the dreaded graft versus host reaction? Can patients with onco-hematological diseases, such as leukemia, recover after transplant? Are CB SCs different from SSCs concerning immunological properties and the ability to repopulate bone marrow? What are the criteria for collecting, storing and monitoring samples of CB? Is the therapeutic benefit worth the preceding efforts?

In recent years, the worldwide cooperation of institutions yielded answers to all these questions. Eurocord, funded by the European Union, provides an international platform specialized in research in CB SCs and in the international registry of CB transplants. Thus, it brings together the European network of cord blood banks (NETCORD), the European Group for Blood and Marrow Transplantation (EBMT), the European Hematology Association (EHA) as well as the CB banks in Athens, Leiden, Madrid and Milan.

Eurocord has started an initiative called "On-line CME program in cord blood technology and transplantation", which provides scientific, technical and regulative information for healthcare. This program is available in several languages (www.eurocord-ed.org).

The Center for International Blood and Marrow Transplant Research (CIBMTR) is a private firm at the Medical College of Wisconsin Clinical Cancer Center in Milwaukee (Wisconsin, USA), which promotes research in hematopoietic cell transplantation and cellular therapy worldwide.

The Foundation for the Accreditation of Cellular Therapy (FACT) with headquarters at the University of Nebraska Medical Center (Omaha, NE; USA) deals with patient care and laboratory practice. The nonprofit corporation was co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for the inspection and accreditation in the field of cellular therapy.

Likewise, the National Marrow Donor Program (NMDP) is a nonprofit organization based in Minneapolis (Minnesota, USA) that registers volunteer donors of bone marrow and CB. As of February 2012, the NMDP had facilitated more than 50,000 transplants worldwide (wikipedia.org).

Without doubt, CB can be collected safely for both, mother and child, after delivery.

It was learned that one umbilical cord contains an adequate number of SCs for a successful engraftment in low body weight patients (up to 40 kg) to reconstitute their immune system. CB SCs are highly prolific; they can be used for transplants to patients affected by onco-hematologic and genetic diseases. The criteria for isolation and conservation are now well defined and internationally shared.

PARAMETERS OF UMBILICAL CORD BLOOD

Ian K. McNiece and Elizabeth J. Shpall were the first to show that CB shares similar cell types with bone marrow and peripheral blood. After growth stimulation, e.g. with the Granulocyte Colony Stimulating Factor, undifferentiated and slightly differentiated SCs were obtained (McNiece and Shpall, 2009). Such differentiated SCs and functional auxiliary cells are essential for SC engraftment. However, the total number of cells, comprising hematopoietic progenitors, collected from one umbilical cord, is significantly lower (roughly 5×10^6) than from donated bone marrow or from peripheral blood after mobilization (roughly 1×10^8 cells). Until few years ago, only little children (up to 40 kg) could be transplanted with SCs obtained from CB. A stringent code for quality and security determines the amount of a CB transplant. Donated CB must contain per kg recipient's body weight at least 3×10^7 nucleated cells or more than 2×10^5 specific cells with CD34+ phenotype. In each case, two disparities are tolerable between donor and recipient in their Human Leukocyte Antigen (HLA) systems. It is important to remember that in SSC transplants, donor and recipient must have a perfect HLA compatibility. For genetic diseases with a higher rejection risk, CB transplant requires, at least, 3×10^7 cells (as suggested by Gluckman's latest paper, even though this number will likely change in a few years).

Some specialized hospitals, including MD Anderson Cancer Center (Texas, USA) and Fondazione IRCCS Policlinico San Matteo (Pavia, Italy), try to overcome these stringent limits. Their novel schedule administers two similar, but not identical CB doses, which provide patients with a lot of cells during initial transplant phases. The donated cell populations compete in proliferation, and the most suitable prevail. Preliminary data show this approach promises to expand the possibility for transplantation in older and consequently heavier patients.

Impressive data certify CB as an important source of SCs for transplants. A matching dose with checked good quality can be identified in CB banks within 15-20 days. In contrast, 6 months are usually required to find in the donors' network a compatible sample of bone marrow.

A CIBMTR survey over the last 12 years estimated that 20% of transplants in young patients (under 20 yr) had been performed using SCs derived from CB. In Japan, 8,000 transplants from unrelated donors have used SCs derived from CB. This figure represents 50% of transplantations.

Data obtained from 233 European and 196 international transplant units through 1988-2008 showed intriguing results

for treatment of genetic diseases or leukemia. No substantial differences were found between the treatments with SCs from traditional sources or with SCs from CB, neither in engraftment ability nor in long-term survival. Preliminary transplant data from adult high-weight patients indicated SCs from CB and from classical sources equally good.

CB application may meet limitations sometimes, when an engraftment fails because of too few SCs in a sample. Another risk comes from the potential transmission of genetic abnormalities like malignant mutations occurring in young SCs.

In the last ten years, however, the cellular composition of CB has became more important. Researchers are looking for differences in cells that populate a given sample of CB.

BANKS AND RECIPIENTS OF UMBILICAL CORD BLOOD

Three aims for CB conservation may be discriminated according to the donor's intention: allogenic donation, dedicated donation and autologous donation.

Regarding the allogenic donation, CB is given to a specialized bank for anybody who is compatible and needs a hematopoietic SC transplant. Indeed, CB donation is a solidarity act regulated by law in Italy. Lombardy was the first to set up a regional bank for conservation, analysis, characterization and distribution of CB. Nowadays, Italy has 18 public banks and 200 birth centers able to collect up to 20,000 CB samples (www.trapianti.ministerosalute.it). The banks in Milan and in Pavia alone dispose of 7,000 and 4,000 CB doses, respectively. Currently, a total of some 300,000 CB doses are available around the world, where New York, Paris, Barcelona, Dusseldorf and London have the largest collection banks. Theoretically, a bank of 50,000 CB units is able to meet the transplants needs of a country as large as the U.K.

The challenge in the coming years is to collect and characterize CB with very high quality to meet regional and international requirements prescribed by FACT-NETCORD and JACIE (Joint Accreditation Committee-ISCT) and to achieve certifications by ISO (International Organization for Standardization) and EFI (European Federation of Immunogenetics). At the international level, only 16 banks got the prestigious international FACT accreditation and two of them are in Italy (Milan and Pavia).

The dedicated donation means that a child's sibling will get the CB infusion. In Italy, this procedure is restricted to families with high transmission risk of genetic diseases that can be treated with CB SCs transplant. CB conservation for this kind of use must be authorized by the hospital's expert committee.

The third intention is autologous conservation when CB of a newborn is determined for the exclusive use in favor of this child. The scientific community is against such conservation, because there are no established indications and protocols for this very special kind of transplant. Besides, CB SCs may carry genetic mutations. In Italy, the law prohibits CB conservation in private institutions and does not allow any advertising for autologous conservation; however, a specific authorization may be issued by the Ministry of Health if autologous CB has to be conserved abroad. In this last case, parents expecting a baby must choose the hospital to which the CB delivery will take place and must afford the costs without any administrative help. It is worth noting that the most respected scientific societies, like the medical institutions of the European Union, the Council of Europe and particular scientific authorities (French, Italian and others) take autologous conservation - rare cases excepted - for therapeutic futility. Unfortunately, some marketing campaigns try to convince VIP mothers from show business or sports to conserve their baby's CB. They appeal to emotions and spread naïve statements that autologous conservation is a "little treasure" for a child's future. This creates false expectations in the society, especially when famous actresses declare to conserve the CB of their babies in private banks. It must be remembered that if not counteracted this situation produces, just for Italy, the actual figure of nearly 10,000 CB units exported to private banks (Switzerland, San Marino).

This manifests an attitude directed against young patients who actually are waiting for transplants. Only allogenic CB donations make SCs available for everyone without any distinction of ethnicity or wealth.

Nowadays a new method for the collection of the large amount of CB that is commonly thrown away is emerging: actually, only 25-30% of collected CB units are cryo-preserved (those with high level standards for clinical use and the correct qualitative and quantitative parameters established by European regulations). By increasing the number of CB units, it will be possible to provide therapeutic support for a great number of new patients in a very short time. This opportunity is quite relevant considering that CB contains a highly heterogeneous mixture of cells: in addition to the hematopoietic SC there are both mesenchymal SC (MSC) and endothelial progenitor cells. MSCs have different biological functions: they're assisting the engraftment of hematopoietic SCs, they have immunosuppressive proprieties and especially they are able to differentiate and reconstitute several tissues like bones, cartilage and muscles. Endothelial progenitor cells can be isolated and cultured *in vitro* and thanks to the high potentiality to grow and differentiate they are able to form new blood vessels. Researchers still have to understand how these pluripotent SCs are able to repair and/or generate *ex novo* different tissues, even though data regarding their ability of repairing cardiac tissue affected by ischemia are available (Khoo et al., 2008). Other new therapeutic advancements are emerging under the name of medical bio-engineering (Conconi et al., 2005; Bian and Bursac, 2009; Zhang and Webster, 2009).

These important findings lead to develop specific programs for the capillary collection of this exclusive biological material that could be used for many different therapeutic needs.

STEM CELLS IN REGENERATIVE MEDICINE: CELLULAR THERAPIES

The possibility to substitute damaged or dead cells with new functional ones has shifted the focal point in medicine from traditional pharmacological and surgical methods to SC-based therapies. Indeed, "regenerative" medicine is rapidly evolving in research and application. The new approach comprises regeneration, repair and replacement of cells or tissue or organs with the aim to restore their impaired function.

Moreover, regenerative medicine could be useful for conditions where present therapies are unsatisfactory or not effective. The human body has an endogenous repair system, in which SCs play a fundamental role. In fact, SCs can be found in every tissue, even if the activation of the repair mechanism is not fully understood. The artificial SC

application, however, faces problems associated with extensive *in vitro* cell expansion, as well as cell death after implantation (Vacanti, 2006). There are also difficulties with the biomaterials employed as carriers, and extensive cell manipulations are still expensive. Lastly, ethical and moral questions compete with clinical translation.

The Medical Research Council published "a strategy for UK regenerative medicine" in March 2012 (www.mrc.ac.uk/index.htm). It presents clear objectives and a delivery plan how the increased understanding for SC biology can be converted into clinical practice. The strategy extends to the stimulation of the body's own repair systems, to SC transplantation and to the use of acellular products. Such practices of translational medicine, which should be mimicked by other countries, will benefit both patients and economy.

Very innovative are preliminary employments of differentiated ESCs, especially of nerve cells. On 23 January 2009, the Food and Drug Administration (FDA) approved a phase I multicentre clinical trial for transplantation of oligodendrocytes, normally present in brain and spinal cord. Eleven patients who sustained complete thoracic-level spinal cord injuries were treated with "GRNOPC1", a product by the Geron Corporation (Menlo Park, California, USA). This was a suspension of oligodendrocyte progenitor cells derived from human ESCs, which had shown an amazing capacity to remyelinate and to stimulate nerve growth in preclinical studies. Geron estimated that these SCs need several months to replicate and to determine that the treatment was successful or not. Unfortunately, the trial was put on hold in August 2009 and then continued to 30 July 2010. Finally, in November 2011, Geron announced it would abandon SC research for financial reasons, but would continue monitoring the patients. Hopefully, this anecdotal trial will become routine soon for the patients sake. A good sign comes from the FDA decision to unlock another trial, focused on the Lou Gehrig's disease, which is the most common form of motor neuron diseases. The occasionally interrupted project is run by Neuralstem (Rockville, Maryland, USA).

To complete the scenario, it is important to note that regenerative medicine disposes already of several admitted SC applications. The biological reagents, the SCs, are available and ready for use. Some approaches apply a patient's own SCs, e.g. with defects in bone growth. Autologous SCs, either circulating ones or those from certain histological niches, can be induced to move towards the area in need of repair.

Other approaches produce large quantities of differentiated cells in vitro thanks to an innovative culturing technique. The "3-D suspension culture system" is a reliable alternative to the adherent static conditions in dishes: SCs are stirred in suspension bioreactors containing molecules of variable composition (Fluri et al., 2012; Shafa et al., 2012). Thus, cell differentiation can be triggered to form a variety of tissues: renal epithelium, lung or liver constituents, cardiomyocytes, dopaminergic neurons, motor neurons, bone cells, etc. High yields of differentiated cells are useful in tissue engineering to obtain three-dimensional, transplantable constructs. The process uses biopolymer "skeletons" to produce the required "organs". Basic research is testing this technique by targeted experiments on animal models in the pursuit of ambitious goals. For example, human germinal cells, sperm and oocytes, are to be created from triggered SCs with the help of pliable biomaterials as "skeleton" using nude mice as intermediate hosts.

Research into organ cultivation is underway in several countries and covers the whole spectrum of tissue defects. The efforts aim at repair the nervous system after spinal injuries and neurodegeneration, the cardiovascular system with necrotic areas after heart failure or the substitution of blood vessels. The production of muscles, articular cartilage, collagen type I for skeletal diseases, tendons and ligaments is intentional. The endocrine system is aimed at by treating diabetes mellitus type I. Attempts to grow teeth and the heart are in early stages of development. To date, successful cultivation of transplantable organs is limited to the bladder (Atala, 2011).

STEM CELLS IN REGENERATIVE MEDICINE: TARGETED DISEASES

The ongoing trials with SC based treatments span nowadays some diseases that find therapeutic relief already. Despite being still in an experimental phase, the new treatments may be approved in as little as five years. Three diseases, diabetes, Parkinson's disease and heart necrosis, are shown as examples.

Diabetes mellitus type I. The treatment of this disease has seen great advancement from the transplantation of pancreatic islets. Expectations come from the ability to culture SCs and to differentiate them in pancreatic cells. However, a method would be most innovative, which could obtain *in vivo* genetic reprogramming of differentiated exocrine pancreatic cells into endocrine ß-cells. This strategy was realized in adult mice through the expression of Pdx1, Neurog3 and Mafa, which are key genes in ß-cells (Zhou et al., 2008). Thus, it was possible to turn exocrine cells into cells that are actually similar in morphology, size and ultra structure to ß-cells. Moreover, these cells lowered hyperglycemic levels thanks to the production of insulin.

Parkinson's disease: The treatment of Parkinson's aims to compensate for the lost neurons with new and efficient dopaminergic cells. Experimental cellular therapy in animals showed already the feasibility of this approach (Nishimura et al., 2003). But from clinical trials on this illness, a time-consuming controversy came about.

The first double-blind study transplanted human embryonic dopamine neurons in 40 cases with severe Parkinson's disease. The transplants survived, and some clinical benefit was found in younger, but not in older patients (Freed et al., 2001). However, an immediate meta-analysis applied the Unified Parkinson's Disease Rating Scale (UPDRS) to monitor disease progression in the 40 patients above (Isacson et al., 2001). It derived that Freed and colleagues had not correctly performed data analysis.

Then, more than 400 patients have been treated and rescued from the typical Parkinson symptoms (Lindvall and Hagell, 2002; Linazasoro, 2003; Lindvall and Bjorklund, 2004).

Scepticism arose because the primary end points (relief of the symptoms) did not match in these trials, even though the tomographic evidence showed the presence of active dopaminergic neurons in the midbrain of the treated patients.

For a recent update of the dopaminergic neurons production for Parkinson's therapy, see Lindvall (2012).

Myocardial infarction. Annually, nearly half million cases of myocardial infarction occur in the USA. Almost sixty million people bear cardiovascular diseases worldwide, among them eight million in Italy. These numbers indicate a pressing reality to provide a systematic amelioration for a panoply of diseases with a common denominator, the loss of cardiomyocytes. Ideally, the injured heart functions will be restored by recruitment of progenitor cells *in vivo* and *in loco*. But regenerative medicine meets a bottleneck in cardiovascular complaints due to the lack of significant regenerative capacity of the mammalian heart. Hoped-for future, a viable source of SCs or progenitor cells of cardiomyocytes will be identified to overcome these difficulties.

Recent findings showed a resident source of SCs in mice, which possess the potential to contribute *bona fide* terminally differentiated cardiomyocytes after cardiac infarction (Smart et al., 2011). In addition, the possibility of a direct reprogramming of fibroblasts into cardiomyocytes is contributing to the optimistic view we are getting (Efe et al., 2011). Till now however, we remain with first transplantation trials using autologous bone-marrow derived SCs (BMSCs).

Martin-Rendon and co-workers at the Stem Cell Research Laboratory of John Radcliffe Hospital (Headington, Oxford, UK) reviewed on clinical evidence the safety and efficacy of BMSC transplantation in acute myocardial infarction (AMI; Martin-Rendon et al., 2008). They evaluated thirteen trials of BMSC treatment for AMI with a total of 811 participants. Accordingly, SC therapy had improved left ventricular ejection by 2.99% and had reduced the area of myocardial lesion by 3.51%. Better results occurred when BMSCs had been infused within seven days following AMI and when the administered dose was higher than 10^8 BMSCs. The latter figure and the general outcome were confirmed in further meta-analyses now including thirty-three trials with 1,765 participants (Clifford et al., 2012).

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Stem cells in embryonic skin development

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ABSTRACT

The skin is a complex stratified organ which acts not only as a permeability barrier and defense against external agents, but also has essential thermoregulatory, sensory and metabolic functions. Due to its high versatility and activity, the skin undergoes continuous self-renewal to repair damaged tissue and replace old cells. Consequently, the skin is a reservoir for adult stem cells of different embryonic origins. Skin stem cell populations reside in the adult hair follicle, sebaceous gland, dermis and epidermis. However, the origin of most of the stem cell populations found in the adult epidermis is still unknown. Far more unknown is the embryonic origin of other stem cells that populate the other layers of this tissue. In this review we attempt to clarify the emergence, structure, markers and embryonic development of diverse populations of stem cells from the epidermis, dermis and related appendages such as the sebaceous gland and hair follicle.

Key words: Skin development, Embryonic skin, Epidermis, Dermis, Sebaceous Gland, Hair follicle.

1. INTRODUCTION

The skin is the primary barrier which protects the body from dehydratation, mechanical trauma and microbial insults, consisting of an outer epidermis and appendages separated from the underlying dermis by a basement membrane (Koster and Roop, 2007).

As a complex organ, the skin is composed of several tissues and a variety of accessory structures. The primary function of human skin, as well as that of other terrestrial animals, is to prevent excessive loss of water through the body, serving as a permeability barrier. Moreover, the skin is the first organ of the body's defense mechanism against external agents, providing protection against mechanical, chemical, thermal, and sunlight injuries in addition to infection by microorganisms. It also displays thermoregulatory sensory and metabolic functions (Fuchs and Raghavan, 2002). To repair damaged tissue and replace old cells, the skin depends on stem cell populations residing in the adult hair follicle, sebaceous gland, dermis and epidermis for continuous self-renewal (Fuchs, 2007).

The skin may be divided into three layers. The outermost layer, called the epidermis, with thickness varying from 0.06 to 1 mm, is a squamous stratified epithelium composed mainly of keratinocytes, in addition to attachments which are inserted into the dermis such as follicles, sweat and sebaceous glands. The dermis (1-2 mm deep), separated from the epidermis by an epidermal basement membrane and consisting of the extracellular matrix, is a support system in which there are nails, blood and lymph vessels and nerve endings. The hypodermis (1-2 mm) is composed of adipose tissue which is molded to muscles and bones underlying the skin (Koster and Roop, 2007).

The mammalian skin is one of the best-studied epithelial systems containing stem cells to date, however, the origin of most of the stem cell populations found in the adult epidermis is still largely unknown (Benitah and Frye, 2012). Far more unknown is the embryonic origin of other stem cells which populate the other layers of this tissue. In this review we attempt to clarify the emergence, structure, markers and embryonic development of diverse populations of stem cells not only from the epidermis, which has been explored in several high quality reviews (Benitah and Frye, 2012; Driskell et al., 2011; Fuchs, 2007; Watt and Jensen, 2009), but also from the dermis and epidermis-related appendages such as sebaceous glands and hair follicles.

2. STRUCTURE AND MORPHOLOGY OF THE EPIDERMIS

As a stratified epithelium, the interfollicular epidermis consists of several layers, each with its own characteristics. The cells from the basal layer display two main functions, adhesion of the epidermis to the underlying dermis through the basement membrane, and providing new cells to replace the ones shed from the cornified exterior of the tissue (Candi et al., 2005) (Dai and Segre, 2004). In order to accomplish these functions and maintain epidermal homeostasis, these cells must maintain a stringent balance between quiescence and proliferation (Simpson et al., 2011). The main structural proteins within the basal keratinocytes are keratins 5 and 14, which have long been known as hallmarks for cells displaying proliferative potential in this tissue (Fuchs and Green, 1980). After commitment to differentiation, certain basal keratinocytes (epidermal stem cells) migrate from the basal into the suprabasal layer, also known as the spinous layer. After reaching this layer, these cells progressively lose their proliferative potential and begin to synthesize a set of structural proteins and enzymes associated with the assembly of the cornified envelope, such as involucrin, envoplakin and periplakin (Candi et al., 2005). The specific and best known keratins present in this layer are keratins 1 and 10 (Fuchs and Green, 1980). In the next layer, the granular one, the keratohyalin granules, consisting of the

Abbreviations: 5-bromo-2'-deoxyuridine(BrDU) - 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) - Dermal-Epidermal Junction (DEJ) - Glial Fibrillary Acid protein (GFAP) - Follicular Dermal Papilla (FDP) - Dermal Sheath (DS) - Platelet-derived growth factor A (PDGF-A) - Sonic hedgehog (Shh) - Sebaceous Gland (SG)

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filaggrin precursor profilaggrin, group keratin fillaments into tight bundles, inducing the collapse of the cell to a flattened shape (Candi et al., 2005). This protein is used as a scaffold in the next stratum, the cornified layer, for its full maturation through the deposit and cross-linking of proteins such as loricrin and periplakin, among several others, by enzymes of the transglutaminase family. After addition of a set of lipids, the final differentiated cells are known as corneocytes (Serre et al., 1991).

The main epidermal appendages in mammals are the sweat glands, sebaceous glands and hair follicles, discussed in more detail in other sections. The sweat gland will not be explored here, but has been explored in several reviews elsewhere (Lobitz and Dobson, 1961; Richert et al., 2000) Other cells such as melanocytes, Langerhans and Merkel cells are important components of the epidermis, but will not be discussed here due to space and scope limitation constraints.

2.1 Epidermal specification and differentiation – the role of interfollicular epidermal stem cells

Interfollicular epidermal stem cells rely on an underlying basement membrane enriched in extracellular matrix (ECM) proteins and growth factors. Basal cells attach to this structure through adhesion complexes such as the hemidesmosomes, which contain a core of α6β4 integrins and focal adhesions of α3β1 integrins. These proteins also play a role in growth control and migration (Fuchs, 2007). The α6 and β1 integrins have been used as markers of epidermal stem cells (Kaur and Li, 2000), similarly to p63, a p53 homologue which is expressed throughout the basal layer of the epidermis (Pellegrini et al., 2001) and has a putative function in maintaining these cells in a slow cycling state. These stem cells are responsible for a rapidly dividing progeny referred to as transit amplifying, which undergo a limited number of divisions before withdrawing from the cell cycle, committing to terminal differentiation and migrating towards the surface of the skin, generating dead, flattened, differentiated keratinocytes (Fuchs and Raghavan, 2002). The filagrin and involucrin intermediate filaments, expressed during this process, are specific markers of epidermal differentiation (Fuchs and Raghavan, 2002). These cells were described in the epidermal basal layer by Jones et al. in 1995 (Fuchs and Raghavan, 2002); several enrichment protocols are available in the literature for the isolation of epidermal stem cells, based on β1 integrin expression (Kaur and Li, 2000), α6 integrin and CD71 (Tani et al., 2000) or Hoechst 33342 exclusion, combined with cell size (Dunnwald et al., 2001) (Reviewed in (Watt and Jensen, 2009)). The epidermis includes several other niches and populations of stem cells associated with the hair follicles, which are described in detail in another section.

2.2 Embryonic origins of the epidermis

In the mouse, after gastrulation a single cell layer of ectoderm is formed at embryonic day 9.5 (E9.5). Mesenchymal cells from the underlying layer begin to transmit signals that induce the stratification of the ectodermis, which will then generate the epidermis and also contribute to commitment of the several appendages present in this tissue (Koster and Roop, 2007; Millar et al., 1999). In response, the basal layer of the stratifying epidermis produces the basement membrane (Mikkola, 2007).

During the initial steps of stratification, which extend up to E12.5 to E15.5 in the mouse, proliferation is almost completely confined to the basal layer. During this period, a transient protective layer of tightly connected squamous endodermis-like cells (M'Boneko and Merker, 1988) called periderm covers the epidermis. The function of the periderm is still unclear, but it likely forms an early epidermal barrier to protect the developing skin from constant exposure to the amniotic fluid (Benitah and Frye, 2012). Once the stratification program is completed, the periderm is shed and the epidermis has fully stratified and differentiated (around E17.5) (M'Boneko and Merker, 1988). These events are summarized in Fig. 1.

2.3 Signaling pathways of the developing epidermis

After gastrulation, emergence of the neuroectoderm is a key event, since it will allow the development of the nervous system and the skin epithelium. Neural induction is reinforced by a positive balance between fibroblast growth factors (FGFs) and inhibition of bone morphogenetic proteins (BMPs) (Gaspard and Vanderhaeghen, 2010). In the opposite direction, the epidermal fate is driven by the expression of BMPs, with additional Wnt signaling (Wilson and Hemmati-Brivanlou, 1995).

Another important cell signaling component in the formation of the epidermis is the Notch signaling pathway (Kolev et al., 2008). The process of lineage commitment between hair follicles and epidermal interfollicular lineages is regulated by Notch 1 and 2, as shown by experiments in which these genes were deleted (Yamamoto et al., 2003).

3. HAIR FOLLICLE STRUCTURE AND MORPHOLOGY

The hair follicle structure, located above the skin surface, may be divided into two different parts, a permanent upper part which does not visibly cycle and a lower part which is continuously being renovated with every hair cycle (Schlake, 2007). Once a hair follicle is produced it may undergo many of these cycles, continually generating, growing, and losing the hair shaft. In mammals the hair growth cycle includes three stages: anagen (follicle generation and hair production), catagen (follicle regression), and telogen (resting phase) (Philpott and Paus, 1998).

In the mouse, one of the most studied models for mammalian skin, there are four different types of hair. The guard hair has low abundance (2-10%); it is straight, very long and contains two columns of medulla cells. This is also the only type of hair with two sebaceous glands instead of one as the other types have (Jones et al., 1994). Awl and auchene hair together comprise around 28% of the hair follicles of the mouse, being characterized as significantly shorter than the guard hair, with two or more columns of medulla cells. Auchene differs from the awl hair by a single sharp bend. Zigzag hair (~70%), the fourth type, contains a single column of medulla cells, and owes its name to three to four sharp bends in alternating directions. These different types of hair are considerably interspersed across the entire body of the mouse (Panteleyev et al., 2001).

3.1 Embryonic origins of the hair follicle

Development of the hair follicle is intrinsically related to the stratification of the embryonic epidermis. This process occurs in three phases, which are known as hair placode

formation, hair follicle organogenesis and cytodifferentiation, further subdivided into eight morphologically distinct stages (Schmidt-Ullrich and Paus, 2005; Stenn and Paus, 1999).

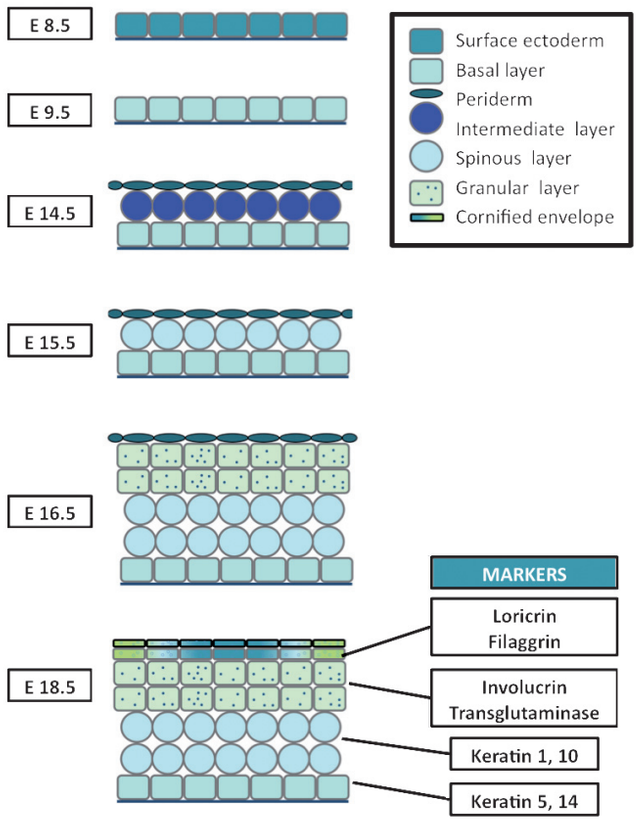

Figure 1: Timeline representing Epidermal Progenitor commitment steps during embryonic development

During the first stage, epidermal keratinocytes form clusters, which enlarge and elongate to generate hair placodes (E14). After this event, a cluster of specialized fibroblasts is formed just above the placode and the crosstalk of these two structures leads to increased proliferation of both (Michno et al., 2003). In the second stage, the enhanced proliferation leads to a downward growth of the epidermal component shaping the dermal papilla. The resulting structure, called the hair germ, is typically observed at E15.5. The keratinocytes continue to penetrate the forming dermis and envelop the dermal papilla, giving rise to the third-fifth stages, collectively known as the peg stage (E16.5-E17.5). The inner root sheath is then formed, triggering these cells to terminal differentiation, which generates the hair shaft (Stenn and Paus, 2001). Simultaneously, the outer root sheath starts to form a cylinder around the inner root sheath, and a bulbous peg structure is formed during stages 6-8 (E18.5).

The timelines described are for guard hairs, one of the most studied types of hair. Awl and Auchene hair follicles begin to form later on, at E15.5-E16, while zigzag hairs appear at E17 and do not reach the final process until postnatal life (Stenn and Paus, 1999). As illustrated in Fig. 2, the process of hair follicle formation is spatially and temporally controlled; the signals involved in this process are reviewed elsewhere (Andl et al., 2002; Benitah and Frye, 2012; Fuchs, 2007).

3.2 Hair follicle-associated stem cell populations – markers, specification and differentiation

3.2.1 Bulge Stem Cells

One reservoir of stem cells is the permanent lower part of the hair follicle, called the bulge. Bulge stem cells were originally identified as slow cycling cells through pulses of BrDU experiments, in what became popularly known as label-retaining cells (Bickenbach and Mackenzie, 1984; Braun et al.,

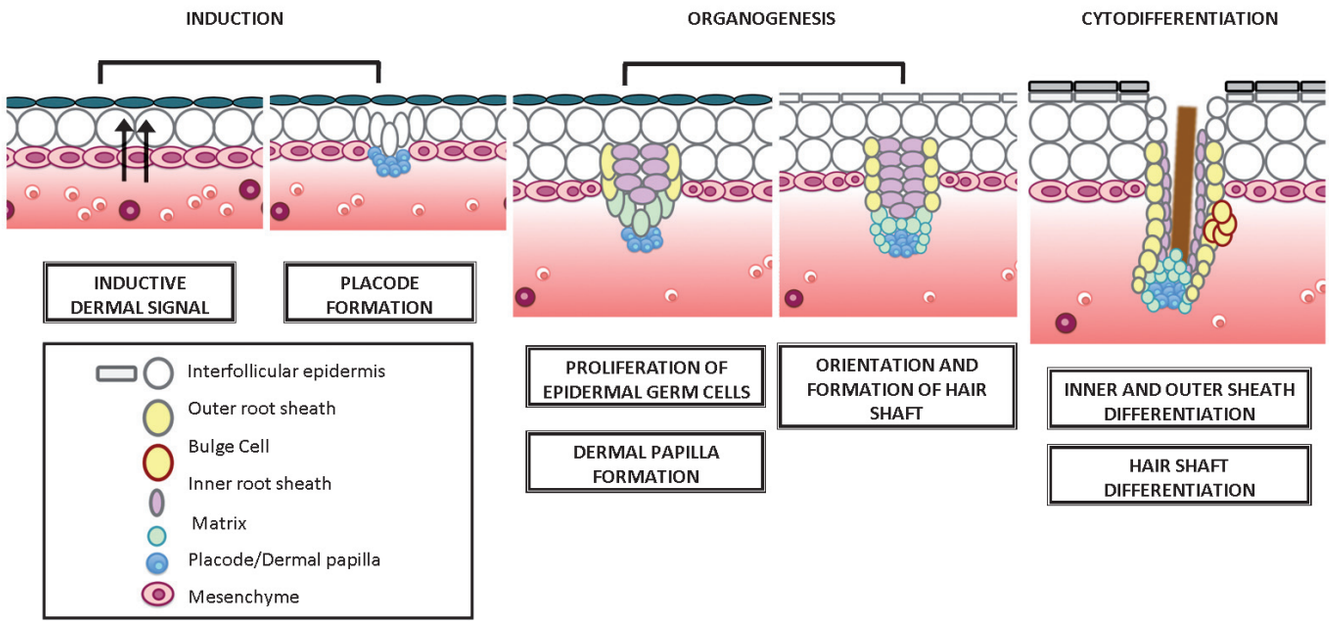

Figure 2: Embryonic developmental stages of the Hair Follicle.

2003; Cotsarelis et al., 1990). More recently a large number of markers have been described for these cells, such as the high expression of α6 integrin (Li et al., 1998) and ABCG membrane transporter proteins (Tumbar et al., 2004), but all of these are shared with the interfollicular epidermal stem cells. One promising marker which appears to be specific for the bulge is the CD34 cell surface glycoprotein (Blanpain et al., 2004; Trempus et al., 2003). Lineage tracing analysis revealed the role of the bulge progeny: under normal homeostasis these stem cells contribute to all lineages of the hair follicle, with minimal contributions to the interfollicular epidermis and sebaceous glands (Kasper et al., 2011; Snippert et al., 2010). In more recent reports, the Lgr5 stem cell marker was considered to be a marker of bulge stem cells (Panteleyev et al., 2001) .

3.2.2 Skps – skin precursors

In 2001 Toma et al. described another multipotent precursor cell population in adult mammalian dermis, more specifically in the follicle dermal papillae (Toma et al., 2001). These cells –termed SKPs, for skin-derived precursors– were isolated and expanded from rodent and human skin and differentiated into both neural and mesodermal progeny, including cell types never found in skin, such as neurons. These cells expressed neuronal precursor markers such as Nestin and mesenchymal markers such as Vimentin, but not Fibronectin. Later on, the same group proposed that SKPs represent a multipotent neural-crest-like precursor which arises in embryonic mammalian tissues, and is maintained into adulthood (Fernandes et al., 2004). This may explain why SKPs are capable of differentiating into neurons (βIII tubulin+) and glial cells such as oligodendrocytes (CNPase+) and astrocytes (GFAP+). *In vivo*, these cells were capable of generating myelinating Schwann cells, a fact of great impact in the spinal cord injury treatment area (Biernaskie et al., 2007).

3.3 The mouse hair follicle junctional zone cells

Recently it has been shown that cells of the junctional zone of the hair follicle, a region directly adjacent to the infundibulum and sebaceous gland, may contribute to the interfollicular and sebaceous lineages, presenting the Lrig1 protein as a specific marker (Jensen et al., 2009). Indeed, loss of Lrig1 leads to epidermal hyperplasia (Suzuki et al., 2002) in a mechanism most likely due to the lack of negative regulation by *c-myc* (Jensen et al., 2009). Therefore it appears that despite the fact that this population apparently does not contribute to the normal homeostasis of this tissue, these cells may act as stem cells in the case of injury.

4. STRUCTURE AND MORPHOLOGY OF THE DERMIS

The Dermal-Epidermal Junction (DEJ) is characterized by a basement membrane with components secreted by both basal keratinocytes and dermal fibroblasts. The DEJ acts not only by attaching the epidermis to the dermis, but also plays an important role in exchanging of signaling molecules between the two layers and allowing the transit of immune cells, and facilitates keratinocyte migration during wound-healing (Sorrell and Caplan, 2004). Almost imperceptible under regular staining, the DEJ undulating structure becomes clear with PAS or Giemsa staining. Its undulating feature is due to the presence of epithelial protuberances and dermal papillae (Prost-Squarcioni, 2006; Sorrell and Caplan, 2004).

DEJ has a complex and characteristic composition and may be subdivided into four regions by more detailed electron microscope analysis: (i) more superficially, the cell membrane of keratinocytes forming hemidesmosomes and melanocytes; followed by (ii) the *lamina lucida*, which is rich in pectin and laminin 5, 6 and 10; (iii) the osmiophilic *lamina densa,* composed mainly of type IV collagen and laminin 5 (electron dense); and more deeply (iv) the sub-basal lamina filamentous zone (Prost-Squarcioni et al., 2008; Sorrell and Caplan, 2004). Anchoring fibers are composed of collagen VII.

Located between the epidermis and hypodermis, the dermis is a connective tissue that acts supporting and protecting the epidermis. The dermal layer consists of fibroblasts, dendritic cells, macrophages/monocytes, neutrophils and lymphocytes, embedded in an extracellular matrix mainly composed of collagenous and elastic fibbers (Prost-Squarcioni, 2006; Prost-Squarcioni et al., 2008; Sorrell and Caplan, 2004). The width of the dermis varies according to its anatomic location, being thicker on the back of the palms and soles, for instance (Sorrell and Caplan, 2004). The dermal layers may be subdivided into the more superficial papillary dermis and the reticular dermis. Separating these two layers is the subpapillar vascular plexus, and at the lower limit of the reticular dermis, the *cutaneum vascular plexus* separates the dermis from the hypodermis (reviewed in (Sorrell and Caplan, 2004), (Kanitakis, 2002)). The limits between the papillary dermis and the epidermis show an undulating pattern due to the presence of dermal papillae which contain tactile corpuscles and vascular components (Kanitakis, 2002). The papillary and reticular dermis differ greatly in their extracellular matrix composition and structure. The papillary dermis contains collagen fibers, composed mainly of collagen type II and III in disorganized loose bundles, and thin elastic fibers composed of elastin stretching perpendicular to the DEJ (reviewed in Sorrell and Caplan, 2004). The reticular dermis displays more compact collagen fibers which tend to be parallel to the skin surface and thicker elastic fibers (Kanitakis, 2002). The ground substance, composed of glycosaminoglycans and proteoglycans, rich in hyaluronic acid, fills the space between cells and fibers (Kanitakis, 2002; Prost-Squarcioni, 2006).

4.1 Embryonic origins of the dermis

The dermis has multiple embryonic origins (Driskell et al.). Fibroblasts are differentiated based on their position along three anatomical divisions: anterior-posterior, proximal-distal and dermal-nondermal (Rinn et al., 2006). Head and facial fibroblasts derive from the neural crest, while dorsal and ventral trunk fibroblasts derive from somitic and lateral plate dermomyotomes, respectively (Driskell et al.; Rinn et al., 2008). It has been shown that primary adult fibroblasts retain several features of the embryonic pattern of expression of *HOX* genes, homeodomain transcription factors which act to specify position identity during development, homeostasis and regeneration (Rinn et al., 2008).

Cells derived from the Follicular Dermal Papilla (FDP) and the Dermal Sheath (DS) differ from other dermal fibroblasts by their *in vitro* biological properties; support for epidermal cell growth, aggregative behavior in culture depending on Versican (Feng et al.) and upregulation of specific biomarkers

(alkaline phosphatase, alpha smooth muscle actin, epimorphin and protease-activated receptor-1 (reviewed in (Ohyama et al., 2010)). After formation of the hair follicle precursor, the placode (E14.5), an aggregate of mesenchymal cells, the dermal condensate, is recruited below the placode at the base of the follicle (Driskell et al.; Ohyama et al.). Signaling between the condensate and the placode leads to the downward growth of the follicle into the dermis and encapsulation of the dermal condensate by epithelial cells, forming the mature FDP (Driskell et al.; Millar, 2002). The FDP cell number does not increase during follicular downward growth; its size is correlated with the hair fiber dimensions (Ohyama et al.). The mature FDP induces the surrounding epithelial matrix cells to proliferate, migrate and differentiate (Driskell et al.; Millar, 2002; Schneider et al., 2009).

4.2 Dermis - niche and signaling pathways

Platelet-derived growth factor A (PDGF-A) is expressed in developing hair follicle epithelium, and its receptor (PDGF-Rα) is expressed in the dermal condensate. Knockout mice for *PDGF-A* develop thinner dermis, misshapen hair follicles, smaller dermal papillae, abnormal dermal sheaths, thinner hair and reduced cell proliferation in the dermis and dermal sheaths compared to wild type mice, suggesting that PDGF-A plays a role in FDP, dermal sheath and dermal fibroblast establishment (Karlsson et al., 1999). Wnt signaling has a key role in recruitment of the dermal condensate, which is unable to develop in the absence of epithelial β-catenin, a downstream effector of the WNT signaling pathway (Schneider et al., 2009; Zhang et al., 2009). Sonic hedgehog (Shh) knockout mouse embryos show disrupted formation of the FDP (Karlsson et al., 1999). Shh signaling controls the expression of a subset of FDP-specific signature genes, being critical for subsequent signaling modulating proliferation and further downward growth of the follicular epithelium, in addition to development of the FDP (Schneider et al., 2009; Woo et al.). Wnt5a is expressed in the developing dermal condensate in wild type but not in Shh-null mice embryos, indicating that Wnt5a is a target of Shh in hair follicle morphogenesis (Reddy et al., 2001). Shh-null skin analysis showed that Shh is not a component of the first epithelial signal (Schneider et al., 2009). Dermal Smoothened (*smo*) loss of function results in loss of the dermal condensate and overexpression of Shh-dependent Noggin. This phenotype is partially rescued by the knockdown of *noggin* in the hair follicle by increasing the expression of epithelial *shh* (Woo et al.).

Laminin-511 mutants show developmental defects by E16.5, with decreased length and structure of primary cilia *in vitro* and *in vivo*. Inhibition of the laminin-511 receptor β1 integrin disrupted FDP primary cilia formation and hair development. Laminin-511 triggers noggin expression through a mechanism that is dependent both on Shh and PDGF (Gao et al., 2008), showing the importance of laminin-511 for FDP maintenance.

The peribulbar DS, which covers the outside of the hair follicle, contains mesenchymal cells that contribute to the maintenance and regeneration of the FDP (Jahoda and Reynolds, 2001; Driskell et al., 2011). Upon amputation of the lower vibrissae follicle or surgical removal of the FDP alone in adult mice a new FDP was formed, suggesting that DS cells contribute to reconstitution of the new FDP (reviewed in (Ohyama et al., 2010)).

4.3 Specification and differentiation of the dermal papillae and its role in hair follicle development

The FDP gene expression pattern is heterogeneous and depends on the hair follicle type. It has been shown that Sox2 is expressed in all dermal papillae at E16.5, but from 18.5E onwards its expression is confined to the FDP of guard/awl/auchene follicles, whereas CD133 is expressed in FDP associated with all hair follicle types (Driskell et al., 2009). Sox2 distinct subpopulations express different sets of genes in addition to the FDP gene signature (Driskell et al., 2009; Driskell et al.). FDP cells are not believed to divide, but during anagen the number of dermal cells in the dermal papillae increases, probably due to migration of cells from the DS (Chi et al.). Inhibition of β-catenin signaling in FDP cells resulted in reduced proliferation of epithelial cells inducing catagen and preventing anagen induction, possibly through inhibition of the FGF pathway (Driskell et al., 2011; Enshell-Seijffers et al., 2010). During anagen, FDP induces downward growth of the stem cells in the secondary hair germ. A quantitative analysis using the rodent vibrissa model indicated that the hair inductivity capacity of FDP cells is altered between early anagen and mid-anagen, being higher in the former, a phenomenon probably linked to the proliferative activity (Iida et al., 2007). Specific FDP markers which are upregulated during anagen in the adult mouse skin are the Corin serine protease and Sox-2 (Driskell et al., 2011), highlighting the crucial role of FDP signaling for hair follicle development. The FDP is also a reservoir of multipotent stem cells (Biernaskie et al., 2009; Hoogduijn et al., 2006; Lorenz et al., 2008; Wong et al.). At least three different subpopulations of progenitor cells may be identified: (i) Sox-2 positive cells, which are associated with Wnt, BMP, and FGF signaling; (ii) Sox-2 negative cells, associated with Shh, Insulin Growth Factor (IGF), Notch, and Integrin pathways; and (iii) skin-derived precursors (SKPs), which may differentiate into adipocytes, smooth myocytes and neurons *in vitro*; they are believed to originate from Sox-2 positive cells, and in part from the neural crest (Biernaskie et al., 2009; Lorenz et al., 2008; Wong et al.). The fact that cells isolated from different anatomical locations (including the back skin derived from the dermomyotome) display multipotent stem cell characteristics suggests that the hair follicle environment, rather than the embryonic origin, induces the generation of cells with the characteristics of neural crest derivatives (Driskell et al., 2011). A comparative analysis between the mesenchymal stem cells (MSCs) isolated from FDP and DS and those isolated from bone marrow showed that these MCs have similar morphology and population doubling time and express the same cell surface biomarkers (Hoogduijn et al., 2006). Also, both cell populations had the capacity to differentiate into the same mesenchymal lineages (osteoblasts, adipocytes, chondrocytes and myocytes) at similar rates and extent of differentiation (Hoogduijn et al., 2006). Progenitor cells derived from the FDP have also been explored as a source for generation of iPS cells (Tsai et al., 2010; Tsai et al., 2011). As a non-invasive source of progenitor cells, the dermal papilla and dermal sheath are viable and promising candidates for use in the clinic.

5. STRUCTURE AND MORPHOLOGY OF THE SEBACEOUS GLAND

Sebaceous glands are important in the maintenance of the hair, since absence of these glands was associated with scarring

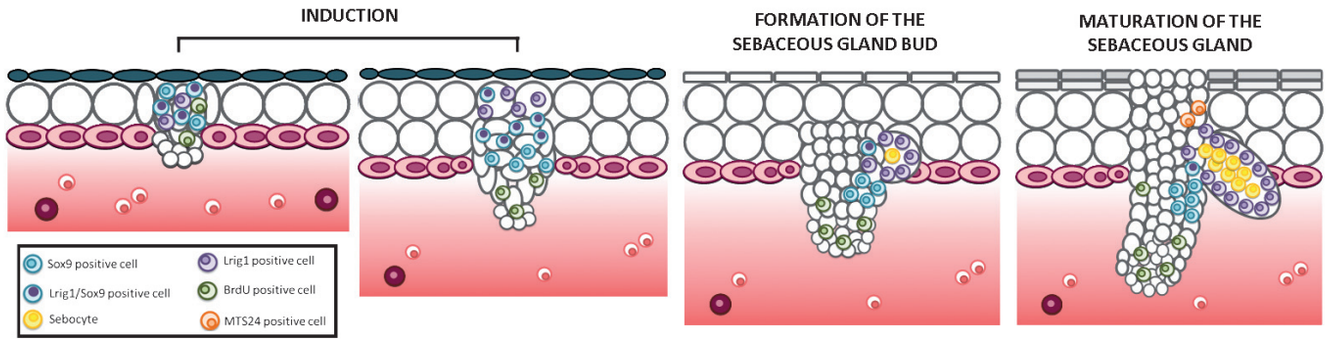

Figure 3: Stem cell involvement during Sebaceous Gland embryonic development

alopecia and doxorubicin-induced hair loss (Al-Zaid et al., 2011; Selleri et al., 2006). The sebaceous glands are composed mainly of sebocytes, which are highly specialized epithelial cells that release their sebum content through a process that culminates in the rupture of the cell membrane and cellular extravasation known as holocrine secretion (Frances and Niemann, 2012).

The majority of sebaceous glands are an integral part of a pilosebaceous unit, although this structure can appear as an independent structure in mutant mice lacking hair follicles (Mecklenburg et al., 2001; Nakamura et al., 2001; Schneider and Paus, 2010).

5.1 Embryonic origins of the of the sebaceous gland

The Sebaceous Gland (SG), of ectodermal origin, develops late in embryogenesis in the upper portion of the HF, from the same lineage as that of keratinocytes. During the differentiation process Sox9 and Lrig1 are initially co-expressed by epidermal progenitor cells, but SG is driven by the asymmetric cell fate decision of Lrig1- positive stem cells but not of MTS24/Plet1-positive precursor cells (Frances and Niemann, 2012). Nevertheless, Sox-9 ablation in the embryo led to failed SG formation, even though Sox-9 is not expressed in the SG lineage or in its resident precursors (Nowak et al., 2008), indicating a more complex role for Sox-9 in SG morphogenesis. During homeostasis of adult mouse skin, Lrig1-positive cells contribute to the infundibulum and the sebaceous glands (Jensen et al., 2009), as illustrated in Fig. 3.

5.2 Sebaceous gland stem cell -- niche and signaling pathways

Blimp1, a transcriptional repressor, was shown to be a marker of the early stage SG-residing progenitor cell linage. Loss of Blimp1 leads to elevated c-*myc* expression, augmented cell proliferation and SG hyperplasia, resulting in enhanced bulge stem cell activity (Horsley et al., 2006), suggesting that Blimp1 is important for maintenance of SG progenitor cells. In addition, SG fails to develop in gamma-secretase null mice, in a mechanism dependent on Notch proteolysis (Pan et al., 2004). Inhibition of the Shh pathway selectively suppresses sebocyte development, whereas its activation leads to an increase in the size and number of SG (Allen et al., 2003). Knockout mice for CD109, a glycosylphosphatidylinositol glycoprotein which negatively regulates TGF-β signaling, also display SB hyperplasia (Mii et al.). Activation of the Protein Kinase C system by phorbol 12-myristate 13-acetate (PMA) in immortalized sebocytes stimulated lipid synthesis (a marker of sebocyte differentiation) with translocation and downregulation of the cPKCα and nPKCδ isoforms (Geczy et al.). On the other hand, Wnt pathway inhibition appears to be crucial for SG development, since Smad7 transgenic induction perturbed hair follicle morphogenesis and differentiation and accelerated SG morphogenesis (Han et al., 2006). Smad7 binds to β-catenin, inducing its degradation and thereby inhibiting the Wnt/β-catenin signaling pathway (Han et al., 2006). The Wnt pathway is also overexpressed in sebaceous gland carcinoma (Erovic et al.). Taken together, these findings show that SG development and maintenance involves a unique gene signature that interplays with the HF and FDP signaling pathways.

6. CONCLUDING REMARKS

The skin constitutes a reservoir for adult stem cells of different embryonic origins. Skin stem cell populations reside in the adult hair follicle, sebaceous gland, dermis and epidermis; however, the origin of most of these stem cell populations is still unknown. In this review we attempted to clarify the emergence, structure, markers and embryonic development of diverse populations of stem cells from the epidermis, dermis and related appendages such as the sebaceous gland and hair follicles. Further studies on skin stem cell specification and commitment are crucial for development of the knowledge of the dynamics of this tissue and for effective cell therapy protocols.

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Sonic Hedgehog in cancer stem cells: a novel link with autophagy

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ABSTRACT

The Sonic Hegdehog/GLI (SHH/GLI) pathway has been extensively studied for its role in developmental and cancer biology. During early embryonic development the SHH pathway is involved mainly in pattern formation, while in latter stages its function in stem cell and progenitor proliferation becomes increasingly relevant. During postnatal development and in adult tissues, SHH/GLI promotes cell homeostasis by actively regulating gene transcription, recapitulating the function observed during normal tissue growth. In this review, we will briefly discuss the fundamental importance of SHH/GLI in tumor growth and cancer evolution and we will then provide insights into a possible novel mechanism of SHH action in cancer through autophagy modulation in cancer stem cells. Autophagy is a homeostatic mechanism that when disrupted can promote and accelerate tumor progression in both cancer cells and the stroma that harbors tumorigenesis. Understanding possible new targets for SHH signaling and its contribution to cancer through modulation of autophagy might provide better strategies in order to design combined treatments and perform clinical trials.

Key words: Cancer Stem Cells, Sonic Hedgehog, Cell Survival, Autophagy, neuroblastoma, cancer therapy

There are several developmentally expressed signaling molecules that have relevance in tumorigenesis and cancer. One of them, the hedgehog (HH) pathway, plays a key role in the regulation of embryonic development and governs processes such as cell differentiation, cell proliferation and tissue patterning. In the adult, Sonic Hedgehog, the most studied member of the vertebrate HH family, functions in tissue repair and regeneration, along with maintenance of stem cells. In adult tissues, SHH can recapitulate the gene expression that is achieved during embryogenesis through the selective activation of transcription factors. In recent years the number of identified genes that are directly regulated by the SHH pathway has increased, and can be related to oncogenic processes since several of the new targets are implicated in cancer biology (Table 1). Here, we will shortly summarize the multifaceted potential of SHH in tumor growth and maintenance according to the current literature. We will then focus on a previously unreported process controlled by SHH, autophagy, and propose an intriguing connection between a pivotal growth factor and a key cellular response as an emerging therapy that could be targeted to induce tumor cell death.

THE SHH/GLI PATHWAY AT A GLANCE

SHH is a secreted glycoprotein that activates signaling in target cells by binding to its 12-pass transmembrane receptor Patched 1 (Ptc/Ptch), which unleashes Smoothened (Smo), a seven-pass transmembrane protein G-coupled co-receptor to trigger downstream activation of the GLI family transcription factors. In mammals, canonical SHH signaling promotes localization of Smo to the primary cilium, a microtubule-based specialized cell surface protrusion, considered today a critical organizer for molecules involved in SHH signaling in vertebrates (Goetz et al., 2009, Ezratty et al., 2011). SHH ultimately exerts its effects by influencing the balance between GLI activator and repressor forms. Smo activation leads to the stabilization of GLI3 transcription factor, that, together with GLI2, act as the canonical effectors activating *gli1* and other target genes. In the absence of SHH, GLI3, and, to a lesser extent, GLI2 truncated forms can mediate in transcription acting as repressors (Aza-Blanc et al., 2000).

SHH/GLI PATHWAY IN CANCER

Altered SHH pathway activation, as revealed by up-regulation of *gli1* or *patched1* expression, has been involved in different types of solid and non-solid cancers, including glioma, medulloblastoma, neuroblastoma, leukemia, gastric cancer, and other tumors (Katoh and Katoh, 2009). Indeed, aberrant activity of SHH has been extensively connected to different aspects of cancer development, from tumorigenesis to metastasis. Accordingly, recent clinical trials with Hh pathway antagonists have validated this pathway as a promising anticancer target. Next, we will review the role of SHH in cancer pathogenesis, in particular how SHH impacts to promote and maintain malignancy from normal tissue to tumors focusing on diverse aspects of gene regulation ranging from tumor initiation, invasiveness promotion to nutrient recycling by autophagy.

INITIATION

There has been a long debate to describe the initiation cells that give rise to cancer. Even though the classification of cancers is relatively direct, the understanding of the ontogenetic origin of cancer remains elusive. One reason is that every cancer is different in terms of originating tissue, formation, and gene expression (Ezratty et al., 2011). It has been proposed that cancer initiates with a small number of "stem cells", that have the capacity to replenish the tumor in its entirety (Hill and Wu, 2009). Cancer stem-like cells (CSCs) have been identified

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TABLE 1

SHH targets associated to cancer. State-of-the-art listing of SHH-GLI controlled genes related to tumorigenesis and tumor progression

Target gene	Function in cancer	Regulation	Reference
ABCG2	ABC transporter (drug resistance, cell survival)	Direct	Singh et al., 2011(Singh et al., 2011)
Ascl1	Neurogenic bHLH transcription factor, gene regulation	Direct	(Voronova et al., 2011)
Bcl-2	Antiapoptotic protein, cell survival	Direct	(Bigelow et al., 2004)
Bmi1	Stem cell marker	Direct	(Wang et al., 2012)
BMP-2	Patterning, endochondral ossification	Direct	(Zhao et al., 2006)
Cathepsin B	Lysosomal protease	Non determined	(Hwang et al., 2009)
Cyclin D1	Cell cycle	Direct	(Hu et al., 2006)
Cyclin D2	Cell cycle	Direct	(Yoon et al., 2002; Yoon et al., 2009)
cyr61	Pro-angiogenic factor	Direct	(Harris et al., 2011)
DNMT1	Epigenetic gene regulation	Direct	(He et al., 2011)
DNMTa	Epigenetic gene regulation	Direct	(He et al., 2011)
DR4	TRAIL induced death receptor	Direct	(Kurita et al., 2010)
Fbn2	Microfibrills component	Direct	(Yu et al., 2009)
Fgf15	CNS development	Direct	(Komada et al., 2008)
FOXA2 (HNF3β)	Transcriptional activator in liver	Direct	(Sasaki et al., 1997)
FoxF1	Mesenchymal transcription factor and potential tumor suppressor	Direct	(Madison et al., 2009)
FoxL1	Mesenchymal transcription factor	Direct	(Madison et al., 2009)
Hes1	Transcriptional repressor, Notch target	Direct	(Wall et al., 2009)
Hhip	Hedgehog interacting protein	Direct	(Vokes et al., 2007)
Hrt3	Notch target	Indirect	(Morrow et al., 2009)
IGFBP3	Igf binding protein	Direct	(Yu et al., 2009)
IGFBP6	Igf binding protein	Direct	(Yu et al., 2009)
K17	Epithelia development, EMT	Direct	(Bianchi et al., 2005)
KLK2	Serine protease	Non determined	(Chen et al., 2010)
KLK3	Serine protease	Non determined	(Chen et al., 2010)
Krox-20	Associated with desmoplastic medulloblastoma	Direct	(Yoon et al., 2009)
MEF2C	Myogenesis and angiogenesis	Direct	(Voronova et al., 2011)
Mycn	Oncogene	Direct	(Hu et al., 2006)
Myf5	Muscle differentiation	Direct	(Gustafsson et al., 2002)
Nkx2.1	Potential oncogene	Direct	(Vokes et al., 2007)
Nkx2.2	Potential oncogene	Direct	(Vokes et al., 2007)
Osteopontin	EMT regulation	Direct	(Yoon et al., 2002; Das et al., 2009)
Pax2	Tumor suppressor target gene	Direct	(Hu et al., 2006)
Plakoglobin	Catenin-cadherin complex, EMT	Direct	(Yoon et al., 2002)
Ptch1	Catenin-cadherin complex, EMT	Direct	(Yoon et al., 2002)
	Negative regulator of Hh	Direct	(Alexandre et al., 1996)
Ptch2	Patched homolog	Direct	(Vokes et al., 2007)
RegIV	Multiple functions in cancer	Direct	(Wang et al., 2011)
RGS4	G protein regulator	Direct	(Yu et al., 2009)
Sall1	Transcription factor involved in tumorigenesis	Direct	(Hu et al., 2006)
Sox9	Transcription factor involved in development and oncogenesis	Direct	(Bien-Willner et al., 2007)
Stathmin 1	Microtubule dynamic-regulating oncoprotein	Non determined	(Chung et al., 2010)

in solid tumors of the breast, colon, brain and other sites. They can differentiate into all the cell phenotypes of the parental tumor. This developmental scheme has been demonstrated for many cancer types, including neural (Hemmati et al., 2003) and non-neural tumors (Richardson et al., 2004, O'Brien et al., 2007). The origin of CSCs is not fully understood, but data suggest that they originate from normal stem or progenitor cells, or possibly other cancer cells. These cells are capable to self-renewal, to give rise the different tumor cell types, and to maintain tumor growth. Other key features include activation of pluripotency genes (Oct4, Sox2, Nanog), formation of tumor spheres in low-adherence cultures, and multi-drug resistance. CSCs can be identified by distinct markers, including the cell surface marker CD133 (also known as prominin 1), BMI1 and CD44 (Neuzil et al., 2007) [Figure 1 and Table 1]. Noteworthy, these cells have been shown as resistant to cancer therapies and CSCs have therefore been proposed to be the cells of origin for tumor relapse. Thus, while the transcriptome of CSCs may not fully match that of the cognate stem cells, pluripotent tumor cells with stem cell phenotype and capacity probably contribute significantly to the phenotypic heterogeneity seen in cancers.

CSCs use a variety of signaling pathways to undergo self-renewal and differentiation, including Wnt, Notch, and HH (Barker and Clevers, 2006; Wang et al., 2012a). There is increasing evidence that connects the SHH/GLI pathway and tumor initiation specific markers as targets (Katoh and Katoh, 2009). In neuroblastoma, for instance, a SHH pathway pharmacological loss of function reduces a CD133/CD15 positive compartment (Schiapparelli et al., 2011). In medulloblastoma, stem cell markers have been involved in SHH tumor propagation (Read et al., 2009), and are also directly controlled by the pathway (Wang et al., 2012). Thus, the SHH/GLI pathway may have different roles during cancer initiation, activating in cells with tumorigenic potential, and up-regulating genes that are involved in cell "stemness" maintenance.

GROWTH

The SHH pathway has been classically involved in growth during embryonic development, controlling key genes that modulate cell proliferation such as *cyclins* and *n-myc* (Figure 1). Abnormal SHH signaling activity during cancer has similarities to normal development and organ growth; tumor cells recapitulate development, but aberrantly. Gain of function mutations in key components of the SHH pathway, such as Patched 1 or Smo, are sufficient to generate tumors in different tissues such as skin, cerebellum and prostate (Athar et al., 2006,Yang et al., 2008, Sanchez et al., 2004). SHH controls genes (Mill et al., 2005), both directly and indirectly, that amplify a secondary response signal, since many of these target genes control their own multiple targets (Eilers and Eisenman, 2008).

MAINTENANCE

During embryonic development and in normal tissue homeostasis, SHH is involved in progenitor cell maintenance and has also been shown to act as a survival factor in different

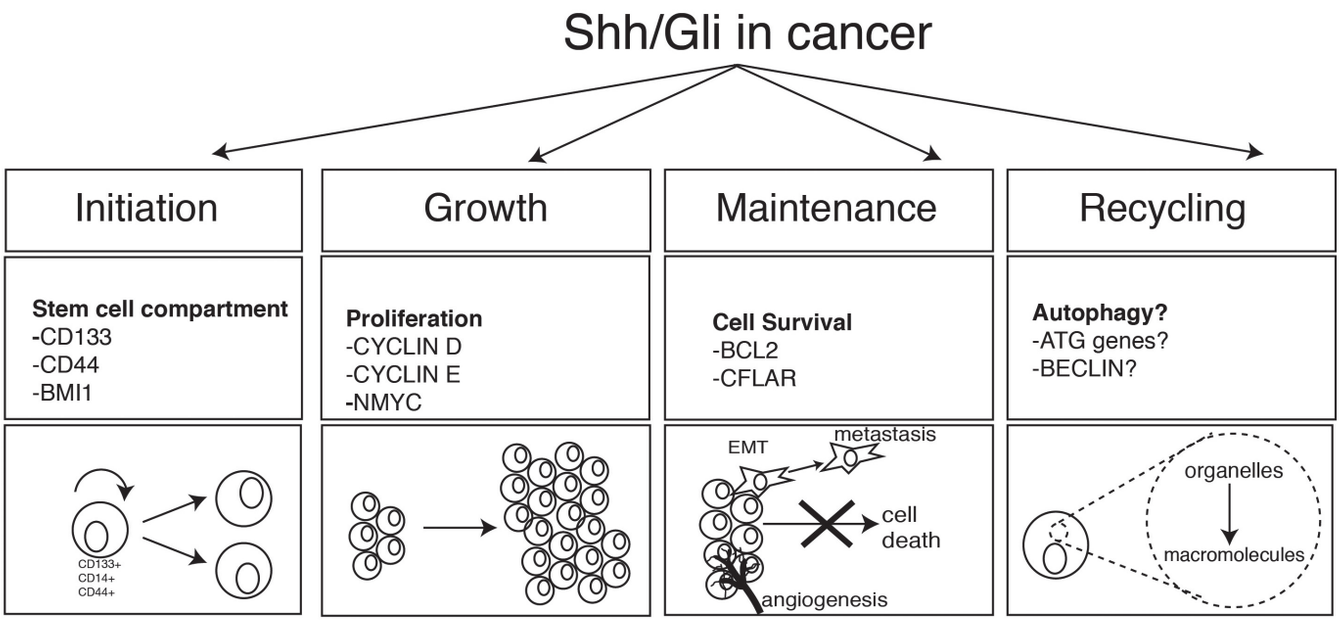

Figure 1: SHH functions in cancer. The canonical SHH pathway has been related to many aspects of tumorigenesis and tumor progression. During cancer initiation, there is a SHH/GLI dependent up-regulation of the CSC marker BMI1, sharing expression with CD15 or CD133. SHH is also involved in maintaining the stem cell niche responsible for tumor initiation. Later on, during tumor growth SHH/GLI controls transcription of genes implicated in cell proliferation and cell cycle progression. In order to maintain tumor size and support pro-invasive processes, SHH pathway activates cell survival, EMT transition, angiogenesis and metastatic mechanisms through extensive direct gene regulation. SHH is also acting indirectly related to other signaling pathways. We propose that SHH could be involved in the autophagic process as a way to recycle nutrients, promoting cell survival and resistance to adverse environments. All these functions highlight the importance of the SHH pathway in cancer biology and justify its use as a strategic target for combined pharmacologic treatments.

tissues (Krüger et al., 2001; Machold et al., 2003). In cancer progression, both processes are deregulated, implicating SHH dysfunction. In order to maintain the number of cells growing in a tumor, SHH can control cell death avoidance/survival. Canonical SHH signaling positively controls *bcl-2* (Bigelow et al., 2004), an important regulator of apoptosis and proto-oncogene that has been related to cell survival in multiple solid and non-solid cancers. SHH also modulates apoptosis induced by the Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through repression by GLI3, binding the cognate death receptor-4 (DR4) promoter (Kurita et al., 2010).

ANGIOGENESIS

The term angiogenesis literally means "new blood vessel formation", and performs a critical role for cancer progression that facilitates tumor growth and survival. Angiogenesis enables tumor expansion, local expansion and dissemination (Bergers and Benjamin, 2003). It is a well-regulated process, driven by specific pro-angiogenic factors and extracellular proteins expressed by endothelial cells (Schmidt et al., 2007). Pro-angiogenic factors such as cyr61, VEGF, neuropilin-1 and CD24 have recently been shown as regulated by the canonical SHH pathway. SHH appears to play a critical part in the biology of the perivascular niche and has been implicated in vascular formation and function within the tumor (Harris et al., 2011; Cao et al., 2012, Geng et al., 2007). Of note, the angiogenic process is closely related to other massive changes in cancer cells, in order to promote colonization of new niches.

EPITHELIAL MESENCHYMAL TRANSITION (EMT) AND METASTASIS

EMT refers to a cellular reorganization process that is key to embryonic development. It results in down-regulation of cell adhesive mechanisms, loss of cell polarity, and gaining of invasive and migratory mesenchymal properties. The EMT series of events also occur during tumorigenesis, allowing tumor-initiating cells to metastasize. During EMT, cells down-regulate E-cadherin, a membrane-bound glycoprotein involved in the adherence of adjacent cells. The loss of E-cadherin in primary tumor tissue has been linked to tumor metastasis and poor prognosis. SHH is involved in EMT along with Notch and BMP signaling pathways and other niche factors, such as members of the transforming growth factor-β (TGF-β) superfamily of cytokines (Bailey et al., 2007; Yoo et al., 2011). These different pathways are also connected, since SHH for instance, directly upregulates Jagged2 to activate the Notch pathway response (Kasper et al., 2006). SHH also regulates the expression of other proteins involved in EMT and metastasis as Snail (Wanshura et al., 2011) and the down-regulation of E-cadherin, contributing to the EMT and metastatic phenotype (Maitah et al., 2011).

AUTOPHAGY AS A MECHANISM FOR SELECTIVE TUMOR GROWTH

Tumor cells often acquire the ability to evade death by inactivating survival pathways that normally function to eliminate damaged and harmful cells. A strictly regulated mechanism that achieves this removal and reutilization is autophagy. Autophagy is generally thought to play a pro-survival role and can be up-regulated in response to both external and intracellular factors, including amino acid starvation, growth factor withdrawal, low cellular energy levels, endoplasmic reticulum (ER) stress, hypoxia, oxidative stress, pathogen infection, and organelle damage (Janku et al., 2011). It is therefore considered a self-defense mechanism, where macromolecules and complete organelles are engulfed in perinuclear double membrane vesicles and degraded in lysosomes (Mizushima, 2007;Chen and White, 2011).

The role of autophagy in cancer is complex, depending mainly on the tumor stage. It has been proposed that autophagy may have a dual role, as a tumor suppressor in normal cells by degrading oncoproteins and, later, allowing cancer cells to survive during metabolic stress (Janku et al., 2011). In this sense, although autophagy is a mechanism of tumor suppression, it also confers stress tolerance that enables tumor cells to survive under adverse conditions by recycling of nutrients for metabolic needs, a fundamental aspect of tumor progression. It has been observed that extensive autophagy is generated by tumor hypoxia and anaerobic glycolysis, whereas angiogenesis maintains low autophagic activity. In fact, autophagy localizes to hypoxic regions of tumors most distal to blood vessels where is supports tumor cell survival (Sivridis et al., 2011).

Autophagy inhibition may result an interesting strategy for pharmacological studies in order to limit tumor nutrient availability and energy demands. The successful development and application of autophagy regulators is important. Signaling pathways that promote autophagy are therefore potential candidates for inhibitor development.

A NEW PARADIGM FOR SHH/GLI THERAPEUTIC ACTION: INHIBITION OF AUTOPHAGY WITH HH INHIBITORS

To date, a relationship between the SHH and autophagy pathways has not been reported, although it has been shown that there are cancers that are sensitive to both pathways, as for instance neuroblastoma (Mao et al., 2009; Mohan et al., 2011; Xu et al., 2012).

In order to shed light on a possible link between SHH and autophagy we tested the effect of a pharmacological loss of function for the SHH pathway in the number of LC3 [microtubule-associated protein light chain 3] positive vesicles, using the neuroblastoma cell line SHSY5Y (Biedler et al., 1978). To date, the detection of processed LC3 by western blot or fluorescence studies, together with electron microscopy for autophagosome formation, have been the mainstays for autophagy detection (Lazova et al., 2010). Cyclopamine (cyc), an alkaloid that functions as SHH antagonist, with several derivatives under clinical trials, was used under a nutrient starvation protocol consisting of serum starvation (Allison, 2012). Two hours of cyc pretreatment of SHSY5Y cultures prevented LC3 vesicles formation (Figure 2A-B). This reduction in LC3 autophagosome positive cells was accompanied with increased Caspase 3 positive cells, suggesting that these cells underwent apoptosis, probably also related to genes controlled by SHH/GLI (Figure 2D-H). To evaluate if the SHH pathway controls essential autophagic genes such as *atg5* or *beclin1 (bcn1)*, we used a SHH-sensitive cell line, C3H10T1/2, treated with SHH-N conditioned media or control (Δ64-SHH) and evaluated *atg5* and *bcn1* levels. Pathway activation was monitored by *ptc1*. Of note, *atg5* decreased in

Δ64-SHH treated cells, whereas *bcn1* did not change under these conditions. This suggests that autophagy regulation by SHH could be driven by a transcripcional control of specific key autophagic genes. Importantly, using a yeast-reverse one-hybrid system (Milla et al., 2012) and bioinformatics we were able to detect non -consensus GLI binding sites (GBS) in the first and seventh intron of the mouse *atg5*. We also found multiple GBS in the human ATG5 promoter (unpublished

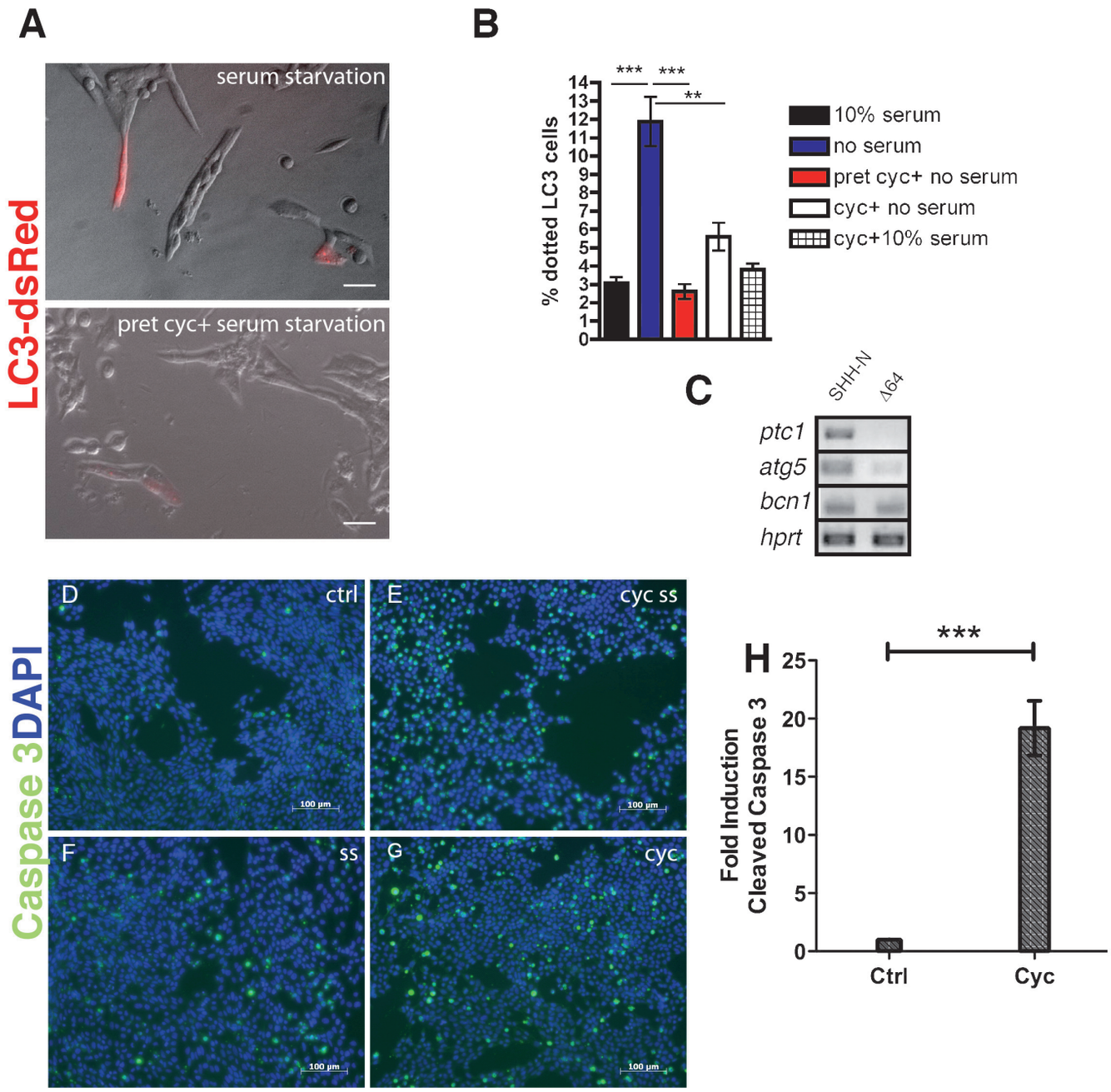

Figure 2: SHH signaling regulates autophagy in the neuroblastoma cell line SHSY5Y. 6-hour serum-starved cells increase dotted LC3-DsRed positive cells in comparison to 10% fetal bovine serum cultured controls, suggesting increased autophagy (A-B). Treatment with 10μM cyc significantly decreases the number of LC3-dotted cells compared to the control serum-starved condition. Strikingly, the decrease in LC3 positive cells after a 2-hour cyc pre-treatment is even more pronounced (B). Representative images of replicates of 4 experiments are shown in upper and lower panel. Bar=50 μm. (C) The SHH pathway reporter cell line C3H10T1/2 were treated for 24 hours with conditioned media obtained following 48-hour transfection of C3H10T1/2 cells with either SHH-N or Δ64-SHH, a mutant form of Shh, which is unable to signal. Pathway activation was monitored by *ptc1*. Note the *atg5* decrease in Δ64-SHH treated cells. The *bcn1* autophagic gene does not change under these conditions. (D-G), Cyc treatment drives cells to apoptosis as evidenced by an increased number of cleaved caspase 3-positive cells $p<0.0006$. Quantitation in (H). Each bar in the graph represents the average of separate triplicate determinations showing the standard deviation of the mean. P value $p<0.001$.

results). These data suggest the attractive possibility that SHH pathway might control the autophagic series of events in cancer cells. From a therapeutic point of view, it would be interesting to evaluate the effect of combined anticancer pharmacological antiautophagic agents and Hh pathway inhibition on tumor cell survival. SHH pathway inhibition could act as a novel sensitizer to increase efficiency of conventional chemotherapeutic agents in cancer by inducing apoptosis. Consistent with our findings, previous reports have suggested that several cytotoxic chemotherapeutic agents induce autophagy, and inhibition of autophagy enhanced their efficiency *in vitro* (Guo et al., 2012). Combinations of other anticancer drugs with autophagy inhibitors have also shown success in preclinical models (Amaravadi et al., 2007, Carew et al., 2010).

Autophagy could also act in the cells that fuel the tumor, especially in the early stages, modulating the tumor stroma or the "stem cell niche". Tumor cells exploit the surrounding stromal environment through the recruitment of these nonmalignant cells that provide physiological resources to facilitate tumor progression. It has been proposed that the stromal cells increase autophagy in order to speed their metabolism and generate anaerobic extra-mitochondrial glycolisis, allowing them to boost their energy production and oxidative stress, accelerating a random mutagenesis in cancer cells (Lisanti et al., 2010). We only analyzed the changes in neuroblastoma cells produced by the SHH pathway, it is important, however, to conceive these changes as cooperative and strongly related to the tumor microenvironment, occurring in parallel during each tumor stage. Experiments conducted using co-cultures, as a model, would help to understand this relationship and shed light on the *in vivo* situation. Even though autophagy plays a similar role in tumor cells as it does in normal cells, tumor cells face more stress and the dependence on autophagy may be more substantial. This differential response between normal and tumor cells in autophagy dependence may be useful for exploiting autophagy modulation in cancer therapy. In tumor initiation, for example, autophagy should be studied in the tumor-initiating cells and in the tumor niche, the stroma, in an independent manner.

Cross talk between independent yet intertwined signaling pathways of metabolism and cancer is currently a topic of intense research. Of note, SHH has recently been proposed as a general positive metabolic regulator in cancer (Bhatia et al., 2012). This observation, combined with our data indicating a SHH mediated autophagic activation, could influence cancer cells to survive and proliferate. More elaborate analysis is needed to determine if the SHH and autophagic pathways activation are coupled in different cancer types.

CONCLUDING REMARKS

Induction of cell death and inhibition of growth are the main targets of cancer therapy. In this short paper, we summarized new insights into molecular mechanisms of SHH action in cancer with special focus on autophagy. Understanding the role of autophagy in cancer treatment is critical since many anticancer therapies activate autophagy, possibly limiting their therapeutic efficacy. Here we propose that autophagy could be connected with the HH signaling pathway. The nature of this relation is of interest for the design of anticancer combined therapies, with HH and autophagy antagonists. Elucidating the interplay between autophagy, tumor cell metabolism and SHH/GLI will provide unique opportunities to identify new therapeutic targets and develop synthetically lethal treatment strategies that preferentially target cancer cells, while sparing normal tissues.

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A cell junction pathology of neural stem cells leads to abnormal neurogenesis and hydrocephalus*

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ABSTRACT

Most cells of the developing mammalian brain derive from the ventricular (VZ) and the subventricular (SVZ) zones. The VZ is formed by the multipotent radial glia/neural stem cells (NSCs) while the SVZ harbors the rapidly proliferative neural precursor cells (NPCs). Evidence from human and animal models indicates that the common history of hydrocephalus and brain maldevelopment starts early in embryonic life with disruption of the VZ and SVZ. We propose that a "cell junction pathology" involving adherent and gap junctions is a final common outcome of a wide range of gene mutations resulting in proteins abnormally expressed by the VZ cells undergoing disruption. Disruption of the VZ during fetal development implies the loss of NSCs whereas VZ disruption during the perinatal period implies the loss of ependyma. The process of disruption occurs in specific regions of the ventricular system and at specific stages of brain development. This explains why only certain brain structures have an abnormal development, which in turn results in a specific neurological impairment of the newborn. Disruption of the VZ of the Sylvian aqueduct (SA) leads to aqueductal stenosis and hydrocephalus, while disruption of the VZ of telencephalon impairs neurogenesis. We are currently investigating whether grafting of NSCs/neurospheres from normal rats into the CSF of hydrocephalic mutants helps to diminish/repair the outcomes of VZ disruption.

Key words: hydrocephalus, abnormal neurogenesis, neural stem cells, stem cell transplantation

INTRODUCTION

It is now understood that hydrocephalus is not only a disorder of CSF dynamics, but also a brain disorder, and that derivative surgery does not resolve most aspects of the disease (Jones and Klinge, 2008). Indeed, 80-90% of the neurological impairment of neonates with fetal onset hydrocephalus is not reversed by derivative surgery. How can we explain the inborn and, so far, irreparable neurological impairment of children born with hydrocephalus? In 2001, Miyan and his co-workers asked a key question: "Humanity lost: the cost of cortical maldevelopment in hydrocephalus. Is there light ahead?" We think that there is some light. There is evidence that the common history of congenital hydrocephalus and brain maldevelopment starts early in the embryonic life with the disruption of the ventricular (VZ) and subventricular (SVZ) zones. However, the nature, mechanisms and extent of the brain impairment linked to hydrocephalus are far from been fully unfolded. We agree with Del Bigio (2001) and Williams et al., (2007) that better treatment of hydrocephalus and the associated neurological impairment will come from a better understanding of the biological basis of the brain abnormalities in hydrocephalus. We think that this view may represent one of the 'lost highways' in hydrocephalus research, as described by Jones and Klinge (2008).

Virtually all cells of the developing mammalian brain are produced in two germinal zones that form the ventricular walls, the VZ and the SVZ (Fig. 1A) (Jacobsen, 1991; Brazel et al., 2003; Gotz and Huttner, 2005; Merkle and Alvarez-Buylla 2006; Malatesta et al., 2008). The VZ is a pseudostratified neuroepithelium that contains multipotent radial glia/stem cells, hereafter called neural stem cells (NSCs). NSCs line the ventricular lumen and through a long basal process reach the pial surface. A landmark of NSCs is their primary cilia that project to the ventricle and are bathed by the fetal cerebrospinal fluid (CSF, Fig. 1E) (Mori et al., 2005; Kazanis et al., 2008). During a fixed period of brain development, NSCs divide asymmetrically, with one daughter cell remaining as a NSC and the other becoming a neural progenitor cell (NPC). Late in development a population of NSCs differentiates into immature ependyma, which during the first postnatal week mature into ependyma (mouse) (Fig. 1A). In the human, ependymal cell differentiation starts at about the fourth week of gestation and is completed around the 22nd gestational week (Sarnat, 1992). The SVZ is located underneath the VZ along the lateral walls of the lateral ventricles of the embryonic

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brain; it contains the neural precursors, which lose contact with the ventricular surface, proliferate extensively and then differentiate into migratory neuroblasts (Fig. 1A) (Brazel et al., 2003; Bonfanti and Peretto, 2007).

Over the years, based on our own and other investigators' evidence, we have progressively come to the view that a *disruption of the VZ and SVZ*, in most cases due to genetic defects, triggers onset of congenital hydrocephalus *and* abnormal neurogenesis (Fig. 1B). We discuss this evidence in the present review.

CELLULAR AND MOLECULAR MECHANISMS OF VENTRICULAR ZONE DISRUPTION

For clarification purposes we shall define the terms used in the present paper to refer to the VZ. At stages of development when the VZ is mostly formed by radial glia/NSCs, the acronym "VZ" will be used. When the VZ is mostly or exclusively formed by multiciliated ependymal cells, the term "ependyma" will be used. The terms "denudation", "disruption" or "loss" will be alternatively used to refer to the disassembling, disorganization, or loss of the VZ cells.

The disruption of the VZ follows a program that has temporal and spatial patterns, progressing as a "tsunami" wave running from the caudal to rostral regions of the developing ventricular system, leaving behind a severe damage. Radial glial/neural stem cells, immature ependyma and mature ependyma all have distinct phenotypes and certainly play quite different roles (Kazanis et al., 2008). What do they have in common so that the denudation wave will hit them all? Junctional proteins appear to be the key to understanding this devastating phenomenon. Up to embryonic day 12 (E12), neuroepithelial cells lining the neural tube are joined together by gap, adherens and tight junctions (Fig. 1A) (Mori et al., 2005; Kazanis et al., 2008). From E12 onward, tight junctions are missing and cell-to-cell adhesion relies on gap and adherens junctions. It is exactly at this time, E12, when disruption of the VZ starts in the mutant hyh mouse (Jiménez et al., 2001) (Fig. 1A). What do all the different mutant mouse strains undergoing VZ denudation have in common? Overall, a series of findings indicates that disruption of the VZ arises from a final common pathway involving alterations of vesicle trafficking, abnormal cell junctions and loss of VZ integrity (Ferland et al., 2009).

Cadherins play a key role during neural tube formation (Ivanov et al., 2001) and represent the major calcium-dependent cell junction molecule in the VZ and later in the ependyma (Fig. 1A, 2A-E) (Hatta et al., 1987; Chenn et al., 1998). Antibodies against chicken N-cadherin injected into the fetal CSF disrupt the VZ and lead to denudation of the SVZ and formation of periventricular rosettes (Ganzler-Odenthal et al., 1998). In the hyh mouse the mutated gene encodes for αSnap (Chae et al., 2004), a key protein in intracellular trafficking. This mutation results in abnormal transport of N-cadherin to the plasma membrane of NSCs (Chae et al., 2004). As discussed below, these mice undergo a massive disruption of the VZ. Disruption of the VZ occurs in mice in which adherens junction formation has been impaired by removal of regulatory molecules such as Lgl1 (Klezovitch et al., 2004), atypical protein kinase C-lambda (aPKCλ) (Imai et al., 2006), and non-muscle myosin II-B (NMII-b) (Ma et al., 2007). Various other animal models with a defect in cell-cell junctions undergo VZ disruption, abnormal translocation of cells into the ventricle and hydrocephalus (Kamiguchi et al., 1998; Tullio et al., 2001; Bátiz et al., 2009).

Gap junctions are now regarded not only as channels between neighboring cells, but also as signaling complexes that regulate cell function (Saez et al., 2003; Dbouk et al., 2009). Connexins also form functional hemichannels that provide a pathway linking the intra and extra-cellular milieu (Saez et al., 2005; Dbouk et al., 2009; Orellana et al., 2009). Gap junctions play an important role in cell-cell coupling to maintain synchronized ependymal ciliary beating (Goodenough et al., 1996; Perez Velazquez et al., 2000; Rouach et al., 2002) and CSF flow (Banizs et al., 2005). A series of studies indicates that the formation of gap and adherens junctions are interrelated phenomena (Jongen et al., 1991; Meyer et al., 1992; Fujimoto et al., 1997; Wei et al., 2005; Oka et al., 2006; Laird 2006; Derangeon et el., 2009). This may explain how in the SA of Spina Bifida Aperta (SBA) patients the same VZ cells display abnormalities in both N-cadherin and connexin 43 (Fig. 2E-H) (Sival et al., 2011).

Recently we have demonstrated that the VZ/ependymal cells of human fetuses are joined together by N-cadherin-based adherens junctions and gap junctions (Guerra et al., 2010; Sival et al., 2011). In SBA patients, areas of SA about to become denuded display disorganized VZ/ependyma cells with an abnormal subcellular location of N-cadherin and connexin 43 (Fig. 2D-H). This mirrors what is seen in animal models with a defect in cell-cell junctions. The increased amount of both junction proteins in the cytoplasm of the abnormal VZ cells might reflect abnormalities in their transport to the plasma membrane or, less likely, in their internalization and degradation (see Laird 2006).

In brief, abnormal cell junctions of the VZ cells appear as a final common pathway in the alteration of a series of molecules directly or indirectly involved in the assembly of adherens and gap junctions (Chae et al., 2004; Ferland et al., 2009; Sival et al., 2010; Bátiz et al., 2006; Klezovitch et al., 2004; Imai et al., 2006; Ma et al., 2007; Rasin et al., 2007; Nechiporuk et al., 2007). This may explain how a series of *different* genetic defects affecting the VZ/ependyma finally leads to its disruption, hydrocephalus and abnormal neurogenesis.

A recent finding has shown that non-genetic mechanisms can also lead to VZ disruption (Fig. 1B). Lysophosphatidic acid, a blood-borne factor found in intracranial haemorrhages, binds to receptors expressed by the VZ cells and triggers VZ disruption and hydrocephalus (Yung et al., 2011).

DISRUPTION OF THE VENTRICULAR ZONE OF THE SYLVIAN AQUEDUCT LEADS TO HYDROCEPHALUS

We have extensively studied the mutant mouse hyh (hydrocephalus with hop gait) that develops fetal onset hydrocephalus. This mutant displays certain characteristics that make it an appropriate animal model of congenital hydrocephalus. Phenotypical characteristics, such as time of onset, type of abnormal CSF dynamics, clinical evolution, and survival/death rate (Jiménez et al., 2001; Wagner et al., 2003; Bátiz et al., 2005; Páez et al., 2007), are similar to those found in several types of human congenital hydrocephalus.

In the hyh mouse, a programmed disruption of the VZ of the ventral wall of the aqueduct (SA) starts early in fetal life (E12.5) (Fig. 1A) and *precedes* the onset of a moderate

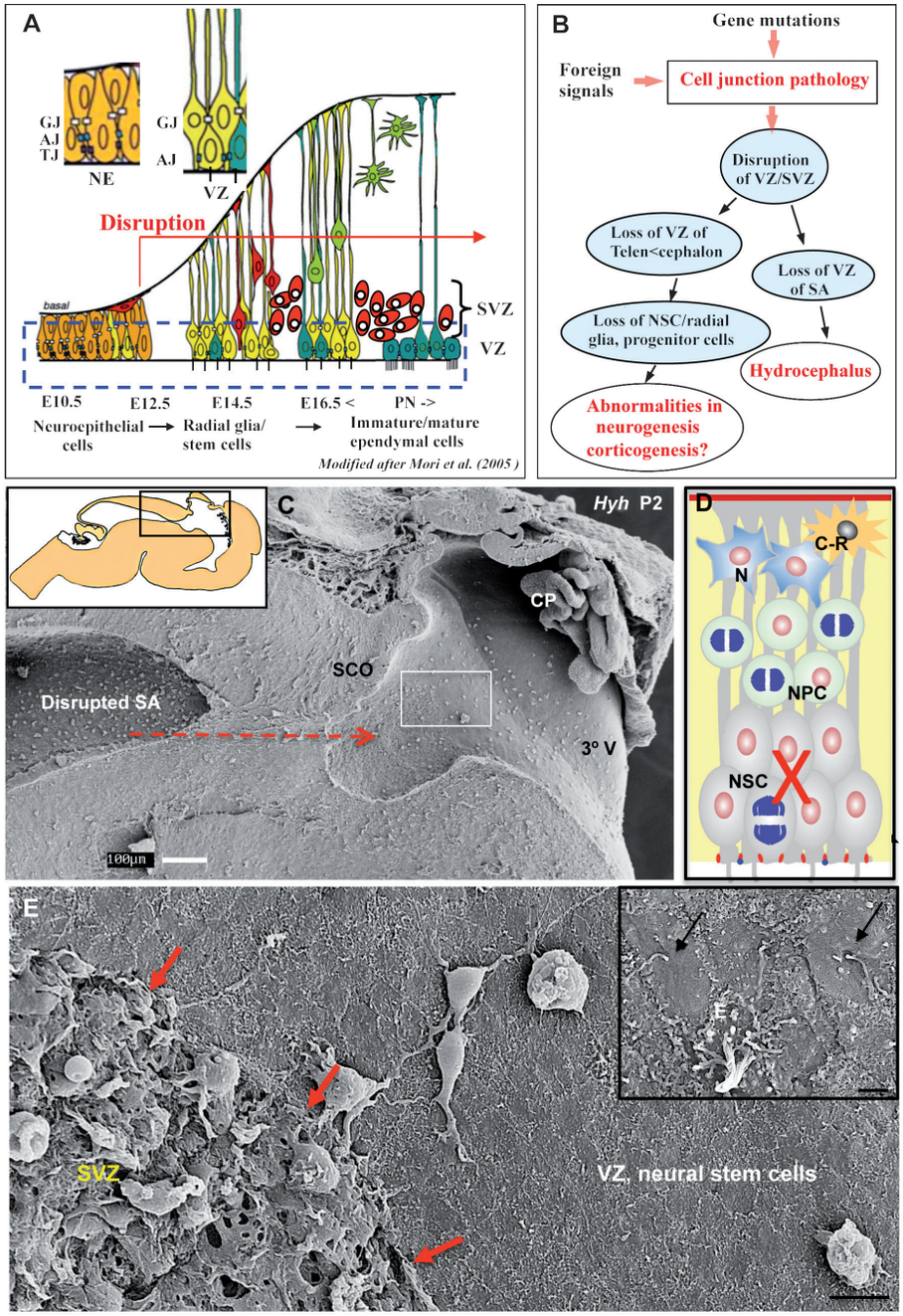

Figure 1. Ventricular zone development and its disruption in hydrocephalic mutant mice. A, Drawing depicting development of the mouse neural tube. Up to E12, the tube is lined by neuroepithelial cells (orange) joined together by gap (GJ), adherens (AJ) and tight (TJ) junctions. After E12, neuroepithelial cells start to differentiate into radial glial/stem cells (yellow). These cells form the ventricular zone (VZ), lining the lumen of the neural tube. At about E14, the stem cells begin to divide asymmetrically, with one of the daughter cells becoming neural precursor cells (red) that form the subventricular zone (SVZ). Late in embryonic life and early in postnatal life, radial glia/stem cells differentiate into ependymal cells that line the ventricular walls of the adult brain. In the mutant mouse hyh, disruption of the ventricular zone starts at E12. **B**, Flow chart representing the hypothesis that cell junction pathology of the VZ cells leads to both hydrocephalus and abnormal neurogenesis. **C**, Scanning electron microscopy of a newborn hyh hydrocephalic mutant mouse. Disruption of ventricular zone along the Sylvian aqueduct and ventricle progresses as a wave expanding caudal-rostrally (broken red arrow). The rectangle frames the disruption front shown in E. **Inset**: Drawing of the hyh mouse brain. The area framed is similar to that shown in C. CP, choroid plexus; 3º V, third ventricle; SCO, subcommissural organ. **D**, Drawing of the cells forming the ventricular zone (NSC, neural stem cells) and the subventricular zones (NPC, neural precursor cells) of the developing mouse brain. N, Neuron; C-R, Cajal Retzius cell. Disruption of the VZ (large red X) implies the loss of NSC. **E**, Detailed view of area framed in figure C. Red arrows point to the disruption front, leaving behind the denuded subventricular zone (SVZ). **Inset**: High magnification of two stem cells (arrows) displaying a primary cilium and one multiciliated ependymal cell (E). Bars: C, 100 µm; E, 6 µm; inset in E, 1 µm.
Figs. C and E: from Wagner et al. 2003

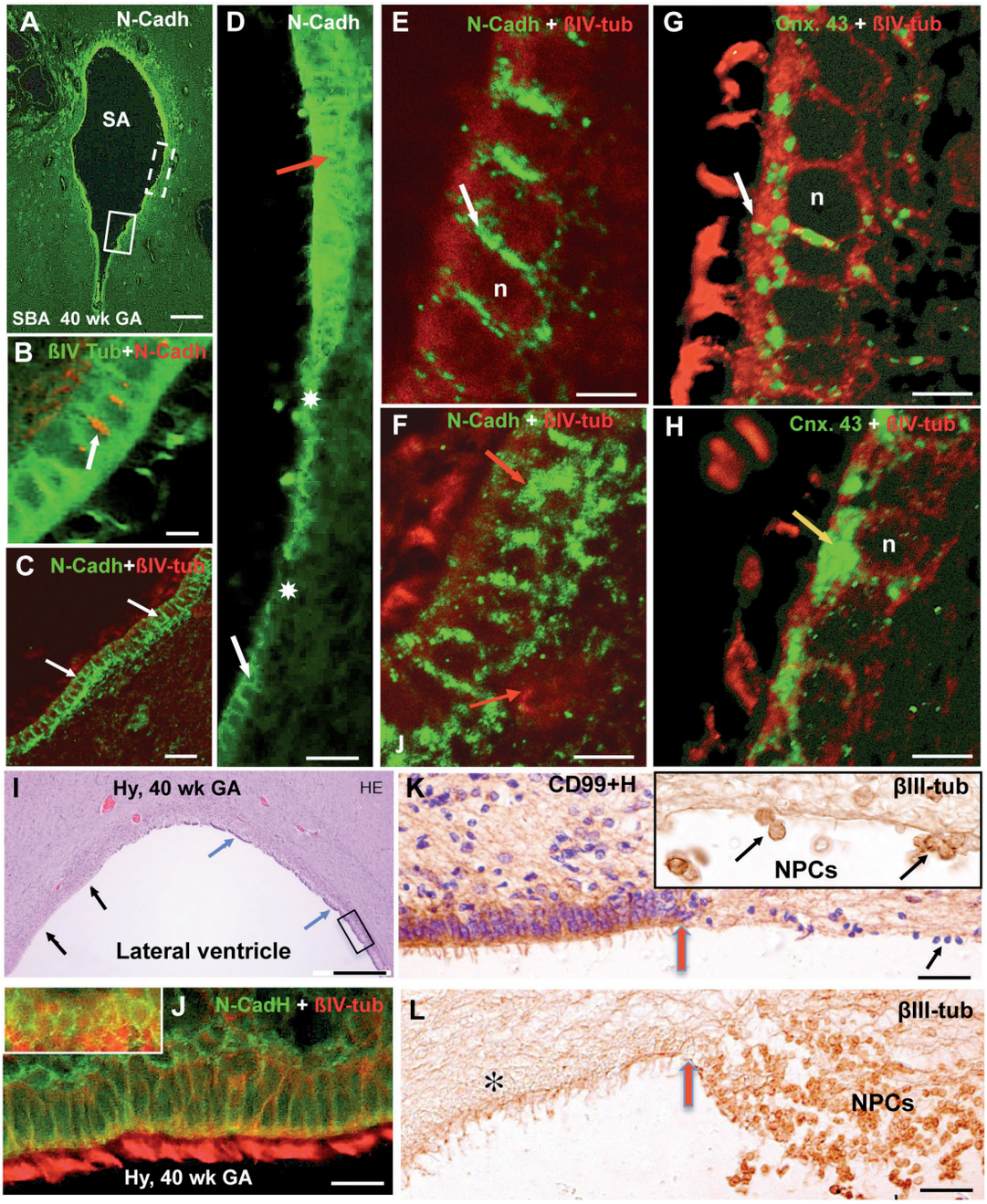

Figure 2. **The ventricular zone of the Sylvian aqueduct and telencephalon of human hydrocephalic fetuses undergo disruption**. **A-H**, Cross section of the Sylvian aqueduct of a fetus with spina bifida aperta (SBA), 40 week gestational age. **A**, Immunofluorescence for N-cadherin. In the wall of the SA, regions with a normal appearance (solid line rectangle) coexist with others undergoing disruption (broken line rectangle). **B**, **C**, Detailed magnification of area shown in solid line rectangle of Fig. A. Normal expression of N-cadherin in ependymal cells not yet affected. The protein is mostly located at the lateral plasma membrane, forming adherens junctions (arrows). **D**, Micrograph of area similar to that framed by the broken line rectangle in Figure A. There are areas with normal location of N-cadherin (full white arrow), others with little or no N-cadherin (asterisk) and still with cells loaded with this protein (red arrow). **E**, **F**, Confocal microscopy of ependyma with normal (E, white arrow) and abnormal (F, red arrow) expression of N-cadherin. n, cell nucleus. **G**, **H**, Confocal microscopy of ependyma with normal (E, white arrow) and abnormal (F, orange arrow) expression of connexin 43. n, cell nucleus. **I-L**, Telencephalon of a hydrocephalic human fetus, 40 weeks gestational age. **I**, In the lateral ventricle there are large areas devoid of ependyma (black arrows) and others lined by ependyma (blue arrows). **J**, Area of normal ependyma similar to that framed in Fig. I, double immunostained for N-cadherin (green) and bIV-tubulin (red). N-cadherin is arranged in the lateral plasma membrane as a complete belt (see insert). **K**, Border region between normal and denuded ependyma (red arrow). Immunostaining with anti-CD99. In the denuded area, βIII-tubulin+ neural precursors (NPCs) (see insert) reach the ventricular lumen. **L**, A large mass of βIII-tubulin+ neural precursor cells (NPCs) are translocated to the ventricle. Arrow, border between intact (asterisk) and disrupted ependyma. Bars: A, 350 µm; B, 9 µm; C, 36 µm; D, 21 µm; E-F, 7 µm; G, 8 µm; H, 5 µm; I, 250 µm; J, 11 µm; K-L, 25 µm.
Figs. A-H: from Sival et al. 2011. Figs. I, K, L: from Domínguez-Pinos et al. 2005.

communicating hydrocephalus. The loss of the ependyma of the dorsal wall of the SA occurring shortly after birth leads to fusion of the denuded ventral and dorsal walls of SA, resulting in aqueductal obliteration (Fig. 3E) and severe hydrocephalus (Fig. 3F) (Jiménez et al., 2001; Wagner et al., 2003; Páez et al., 2007). The phenomenon of VZ denudation associated with the onset of hydrocephalus has also been found in several mutant mice (Klezovitch et al., 2004; Imai et al., 2006; Ma et al., 2007; Rasin et al., 2007; Nechiporuk et al., 2007). Moreover, in human hydrocephalic fetuses, VZ/ependymal denudation precedes, and probably triggers the onset of hydrocephalus (Domínguez-Pinos et al., 2005; Sival et al., 2011). It can be postulated, on solid grounds, that a primary alteration of the VZ due to various genetic defects triggers the onset of congenital hydrocephalus (Fig. 3). The loss of the VZ may trigger hydrocephalus through two different mechanisms. First, the abnormal expression of connexin 43 and the loss of the ependyma in the SA imply the abnormal function or the absence of multiciliated cells; consequently the flow of CSF through the aqueduct would be curtailed. Later, the complete loss of ependyma (perinatal period) leads to fusion of the denuded neuropiles and stenosis/obliteration of the SA. Collectively, these alterations cause severe hydrocephalus (Fig. 3A, E, F).

DISRUPTION OF THE VENTRICULAR ZONE OF THE TELENCEPHALON LEADS TO ABNORMAL NEUROGENESIS

In hyh mutant mice, the disruption of the VZ follows a program that has temporal and spatial patterns. The program is turned on at E12 (Fig. 1A) and turned off by the end of the second postnatal week. After the third postnatal week, and throughout the life span, the denuded areas remain devoid of ependyma (Fig. 3F). Spatially, the loss of the VZ progresses as a "tsunami" wave running from caudal to rostral regions of the developing ventricular system, leaving behind a severe damage (Fig. 1C, E, 3D) (Jiménez et al., 2001; Wagner et al., 2003; Páez et al., 2007). Disruption of the VZ after birth implies the loss of ependyma; however, the disruption of the VZ during fetal life results in the loss of NSCs (Fig. 1D, E) and a disorganization of the SVZ, *indicating that abnormal neurogenesis and hydrocephalus are linked at the etiological level* (Fig. 1B, 3A).

In the pathophysiologic program of VZ disruption, the loss of VZ/ependyma occurs in *specific regions* of the SA and ventricular walls, and at *specific stages* of brain development. This explains why only certain brain structures have an abnormal development, which in turn results in a specific neurological impairment (Jimenez et al., 2001; Wagner et al., 2003; Páez et al., 2007).

HOW AND TO WHAT EXTENT ARE THE PROLIFERATION, MIGRATION AND DIFFERENTIATION OF NEURAL PROGENITOR CELLS OF THE SVZ AFFECTED BY THE LOSS OF THE ADJACENT VZ?

Abnormal proliferation of neural progenitors. NPCs divide to generate either two proliferative daughter cells or one or two postmitotic neuronal daughter cells; the former predominates early, with neuron-generating divisions predominating later. In the SVZ of hyh mice, devoid of a VZ, there is an early overproduction of neurons at the expense of progenitor cells; this would explain the progressive loss of progenitor cells in hyh mutants from E12.5 on (Takahashi et al., 1994, 1995; Caviness and Takahashi, 1995; Caviness et al., 1995; Chae et al., 2004). The loss of the radial glia/neural stem cells forming the VZ is also expected to contribute to a reduced number of SVZ progenitors. There is apparently no information on the proliferative activity of SVZ neural precursors of human fetuses with disruption of the VZ.

Abnormal migration of neuroblasts. In human hydrocephalic fetuses, an abnormal expression of N-cadherin, as seen in SA, has also been found in the VZ/ependyma of the telencephalon (Guerra et al., 2010). Many of these cases displayed extensive areas of the lateral ventricles with a disruption of the VZ/ependyma and displacement of NPCs into the ventricle (Fig. 2I-L) (Domínguez-Pinos et al., 2005; de Wit et al., 2008; Guerra et al., 2010).

Impairment of neuronal migration gives rise to several genetic malformations of the developing cortex: lissencephaly (smooth brain), subcortical band heterotopia (heterotopic neurons arrested under the normal cerebral cortex) and periventricular heterotopia (PH) (Barkovich et al.,1991; Ricci et al., 1992; Kamuro and Tenokuchi 1993; Dobyns et al., 1996; Ferland et al., 2009). PHs are clusters of neuroblast/neurons ectopically positioned along the lateral ventricles (Fig. 3C) (Ferland et al., 2009). Humans with disruption in the VZ of the telencephalon carry PH primarily composed of later-born neurons (Ferland et al., 2009). Mutations in either of two human genes, filamin A (FLNA) or ADPribosylation factor guanine exchange factor 2 (ARFGEF2), cause PH (Sheen et al., 2001, 2003). In the mouse, the loss of FlnA function affects cell adhesion, disrupts the VZ and impairs neuronal migration. In the hyh mouse (carrying a mutation in αSnap), the progressive denudation of the VZ also leads to PH formation (Chae et al., 2004; Ferland et al., 2009). These findings have led to the proposal that PH formation arises from a disruption in the VZ resulting from alterations of vesicle trafficking and cell-cell adhesion (Ferland et al., 2009). Furthermore, disruption of the VZ implies the loss of radial glia. Therefore, neuronal migration would be expected to be impaired at the sites of VZ disruption, leading to the formation of PH (Fig. 3C) (de Wit et al., 2008; Ferland et al., 2009). Nevertheless, little is known about how much the disruption of the VZ in human fetuses affects corticogenesis. This key issue is under current investigation in our laboratories.

Abnormal neuron differentiation. In the hyh mutant, the brain cortex is markedly smaller as compared with wild-type embryos (Chae et al., 2004; Páez et al., 2007) and it has excessive early-born neurons (thicker layer VI) and few late-born neurons (thinner layers II-IV). However, alteration of the cortex is not a widespread phenomenon. The loss of the VZ in specific regions of the ventricular walls and at specific stages of brain development explains why only certain brain structures have an abnormal development. Thus, in hyh mice and other animal models, the brain cortex is not affected homogeneously, with the cingular and frontal cortices being the most altered regions (Jones et al., 1987, 1991; Bruni et al., 1988; Páez et al., 2007).

How does disruption of the VZ affect the two main populations of cortical neurons, gabaergic and glutamatergic, considering that they arise from VZ located at different anatomical sites? In the mouse, most gabaergic neurons

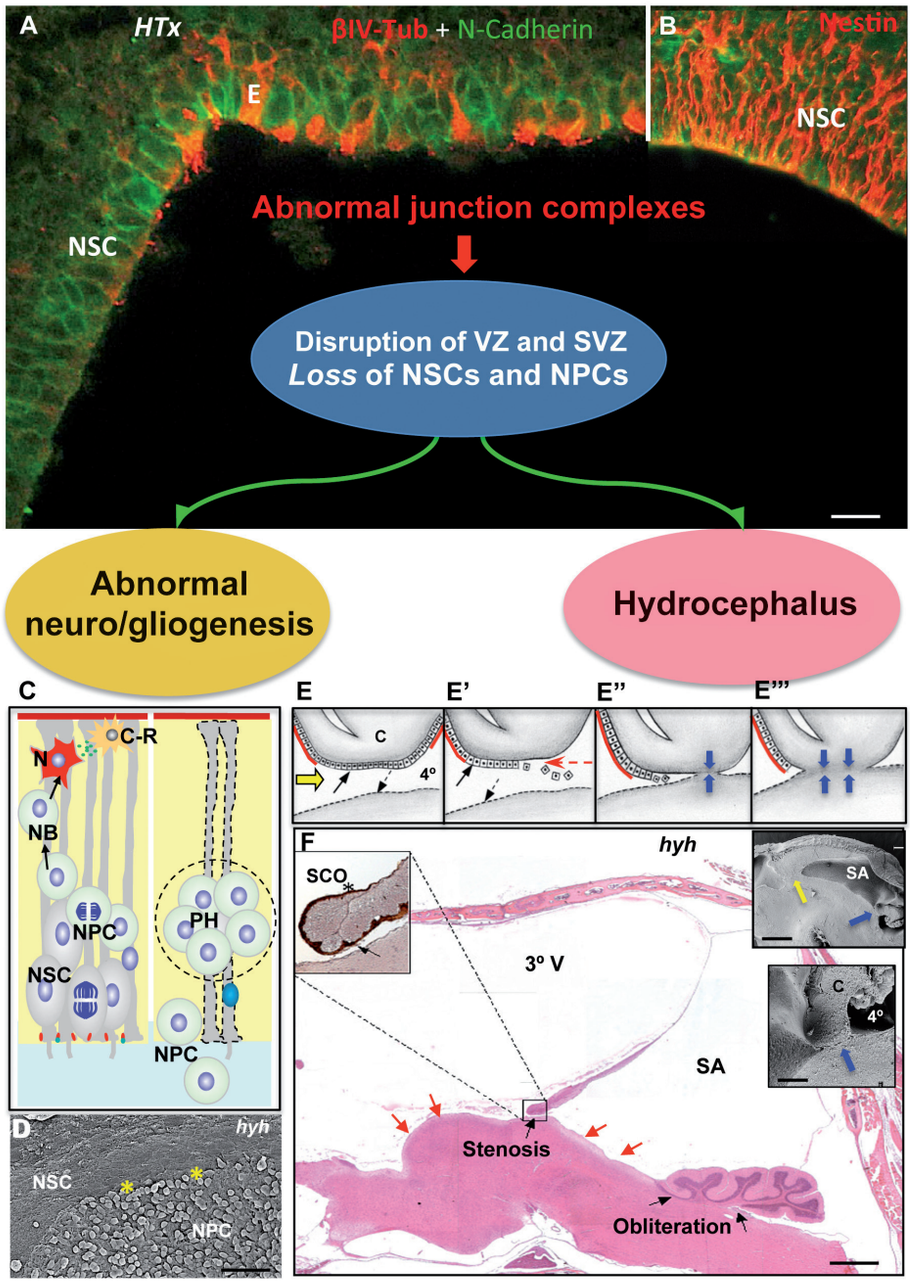

Figure 3. **Abnormal junction complexes of cells forming the ventricular zone may lead to disruption of the ventricular and subventricular zones, hydrocephalus and abnormal neurogenesis. A**, The lateral ventricle of PN1 HTx rats is lined by numerous neural stem cells (NSC, green) and a few ependymal cells (E, red), both cell types expressing N-cadherin. **B**, Area similar to that in Fig. A. Immunostaining for nestin shows that most cells of the ventricular zone are neural stem cells (NSC). **C**, Line drawing depicting the normal neurogenic process, from neural stem cells (NSC) to neural precursor cells (NPC), to migratory neuroblasts (NB) and finally to neurons (N). The loss of NSCs (right side of drawing) would lead to arrested migration of neuroblasts, thereby forming paraventricular heterotopias (PH) and translocation of NPCs into the ventricle. C-R, Cajal Retzius cell. **D**, In the hyh mouse, disruption of the ventricular zone (NSC, asterisks) exposes the NPCs of the subventricular zone to the ventricular cerebrospinal fluid. **E-E'''**, In hyh mice, disruption of the VZ lining the ventral wall of the aqueduct occurs during early fetal life (E, broken line). Disruption of the dorsal wall of aqueduct occurs shortly after birth (E'', red arrow). Then the ventral and dorsal denuded walls fuse, leading to aqueduct obliteration (E'', E''', blue arrows) and hydrocephalus. **F**, Sagittal section of the brain of a hyh mouse with severe hydrocephalus. A marked stenosis of the rostral end of the aqueduct at the site of the subcommissural organ interferes with CSF circulation between the third ventricle (3° V) and the Sylvian aqueduct (SA) and the obliteration of the caudal end of the SA blocks CSF circulation towards the fourth ventricle. Red arrows, denuded ventral walls. **Top left inset**, Detail of the stenosed region of SA. SCO, subcommisural organ. **Right top inset**, Scanning electron microscopy of a PN2 hydrocephalic hyh mouse, showing the zones of stenosis (yellow arrow), obliteration (blue arrow) and the expansion of the SA. **Right bottom inset**, Detail of obliterated region of SA (Blue arrow), C, cerebellum; 3°, fourth ventricle. Bars: A, 25 μm; D, 20 μm; F, 250 μm; right top inset, 400 μm; right bottom inset, 100 μm.
Fig. E: from Wagner et al. 2003. F: from Batiz et al. 2006.

originate from the ganglionic eminences (Anderson et al., 1997, 2002; Corbin et al., 2001; Marín and Rubenstein, 2001, 2003; Wichterle et al., 2001; Nadarajah and Parnavelas, 2002). In hyh mice, the disruption of the VZ of the ganglionic eminences occurs from E14 on (Jiménez et al., 2001; Ferland et al., 2009), thus severely impairing neurogenesis of gabaergic neurons (Vío et al., 2010). By contrast, disruption of the VZ of the pallium occurs during the late period of development. Here, late glutamatergic neurons and gliogenesis would be expected to be impaired.

There is virtually no information on brain cortex alterations of human cases with disruption of the telencephalic VZ. One study reported a widespread loss and disorganization of the VZ in the brain of children with lissencephaly and other disorders of neuroblast migration (Sarnat et al., 1993).

AT SITES OF VZ DISRUPTION, NEURAL PROGENITORS OF THE SVZ ARE ABNORMALLY DISPLACED INTO THE VENTRICLE. WHAT IS THEIR FATE?

All mutant mice carrying a disruption of the VZ show neural progenitors reaching the ventricle. A puzzling question is the fate of neural progenitors reaching the ventricle. Do they undergo cell death? Do they get free in the CSF and move to distant locations? Do they continue proliferating in the CSF? The presence of apparently healthy NPCs on the denuded ventricular surface of hydrocephalic human fetuses (Fig. 2K, L) (Domínguez-Pinos et al., 2005) and their collection from the CSF of hydrocephalic fetuses (Krueger et al., 2006) support the possibility that in these human and animal mutant fetuses undergoing VZ disruption, those NPCs migrating through the denuded ventricular surface finally get free in CSF. The interesting question of the fate of the NSCs and NPCs reaching the CSF is fully open. Ongoing experiments in our laboratory indicate that these cells can be collected from the CSF and, under specific culture conditions, develop into neurospheres.

ALTERATIONS OF THE MICROENVIRONMENT OF THE NEUROGENIC NICHE AFTER DISRUPTION OF THE VZ

The microenvironment of the VZ and SVZ regulates the behavior of neuronal progenitors through diffusible signals (Kazanis et al., 2008). Many such signals have been found in fetal CSF (fCSF), indicating that fCSF is part of the neurogenic niche (Owen-Lynch et al., 2003; Gato et al., 2005; Miyan et al., 2006; Johanson et al., 2008, 2011; Gato and Desmond, 2009). Two proteomic analyses of fetal CSF of rats and humans have revealed numerous compounds that likely are cues for different phases of neurogenesis (Parada et al., 2005; Zappaterra et al., 2007). Although the origin of most of these fCSF compounds is unknown, some are secreted by specialized regions of the VZ such as the choroid plexus (Zappaterra et al., 2007; Johanson et al., 2008) and subcommissural organ (Rodríguez et al., 1998; Montecinos et al., 2005, Vío et al., 2007). Considering fCSF as part of the microenvironment of the VZ and SVZ, an abnormal hydrocephalic fCSF would be expected to affect the VZ and SVZ. Indeed, *in vivo* and *in vitro* studies of HTx rats that develop inherited hydrocephalus revealed that changes in fCSF composition may lead to abnormal corticogenesis (Mashayekhi et al., 2002).

IS THERE AN OPPORTUNITY FOR DIMINISHING/REPAIRING THE DISRUPTION OF THE VZ AND SVZ AND ITS CONSEQUENCES: ONSET OF HYDROCEPHALUS AND ABNORMAL NEUROGENESIS?

A distinction must be made between (i) brain *maldevelopment* due a primary pathology of the VZ that precedes or accompanies the onset of hydrocephalus, and (ii) neurological impairment due to brain *damage* caused by hydrocephalus. The former occurs during development and consequently hydrocephalic neonates are born with a neurological deficit. Brain *damage* is a postnatal acquired defect essentially caused by ventricular hypertension and abnormal CSF.

Brain damage is associated with regional ischemia, disruption of white matter pathways and alteration of the microenvironment of neural cells (Del Biggio, 2001, 2010). Derivative surgery, the almost exclusive treatment of hydrocephalus today, is aimed to prevent or diminish brain *damage*. It is clear that hydrocephalic patients improve clinically after shunting or ventriculostomy. The improvement is due to reduced intracranial pressure, which likely increases white matter blood flow (Del Bigio., 2001), and probably to resumption of the clearance role of CSF. However, derivative surgery does not reverse the inborn brain defects. This has led a study group on hydrocephalus to conclude that "Fifty years after the introduction of shunts for the treatment of this previously untreatable disorder, we must acknowledge that the shunt is not a cure for hydrocephalus; it is only a patch" (Bergsneider et al., 2006). One of the conclusions of the National Institutes of Health–sponsored workshop on "Priorities for hydrocephalus research" held in 2007 was**:** **"**The most forward-looking research priorities are for future treatments. These include not only the development of novel medical devices or surgical techniques, which represent a continuation of the first 50 years of hydrocephalus therapeutic research, but also the development of novel therapies that should emerge from improved understanding of the basic biology of hydrocephalus and its impact on the brain…There is a need to determine the potential role of stem cell therapy, for example, to supply trophic agents" (Williams et al., 2007).

Based on the evidence that the common history of fetal onset hydrocephalus and abnormal neurogenesis begins with disruption of the VZ and SVZ, we have begun to explore strategies for diminishing/repairing such disruptions.

Grafting of stem cells or neurospheres into the CSF. There is growing evidence that NSC transplantation represents a great opportunity for the treatment of many neurological diseases (Sievertzon et al., 2005; Buddensiek et al., 2010, Gage, 2000; Armstrong and Svendson, 2000; Weissman, 2000; Neuhuber et al., 2008). Stem cells used for transplantation into the CNS include mesenchymal stem cells (hMSCs) (Satake et al., 2004), NSCs (Bai et al., 2003; Buddensiek et al., 2010) and NPCs (Kim et al., 2004; Ohta et al., 2004, Wu et al., 2002). NSCs can be obtained from fetal brain (Gage, 2000) and from regions of the adult brain such as the hippocampal subgranular zone/dentate gyrus and the subventricular zone (Rietze et al., 2001). Under certain culture medium conditions, NSCs grow to form "neurospheres" (Park et al., 2008). Neurospheres are able to generate neurons, astroglia and oligodendroglia (Fig. 4A-J).

Worth noticing is that fetal and adult CSF is an important component of the microenvironment of NSCs and NPCs during

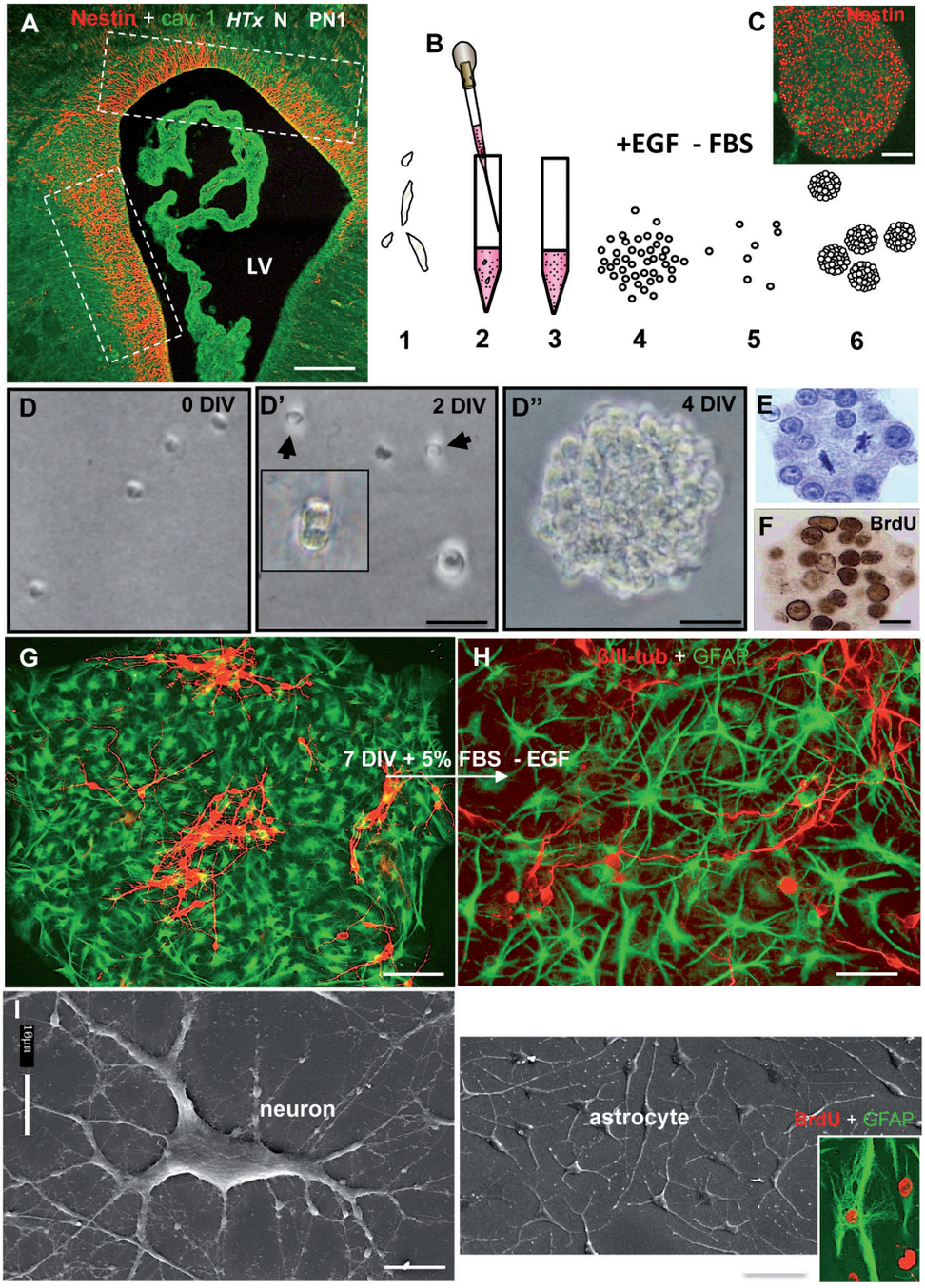

Figure 4. A single neural stem cell forms a neurosphere that after a few days *in vitro* can differentiate into neurons and glia. **A**, Brain of a HTx rat, at PN1, processed for double immunofluorescence to reveal nestin (red) and caveolin 1 (green). At this age, the dorsal and lateral walls of the lateral ventricles (LV) are lined by neural stem cells (red). These areas can be dissected out and used for NSC cloning. **B**, The neurosphere assay is a classical neural stem cell culture technique. After an initial dissection of the lateral ventricular wall (step 1), the tissue is transferred to a plastic falcon tube and mechanically disaggregated to obtain a single cell suspension (steps 2, 3). The cells are then cultured in serum-free media in the presence of EGF as a mitogen (step 4). In the course of the first two days *in vitro* (DIV), the vast majority of differentiated cells die (step 5). The surviving ones undergo cell division and form spherical clones called "neurospheres" (3-4 DIV, step 6). **D**, **D'**, **D''**, Phase-contrast micrographs of NSC developing into neurospheres after 4 DIV. **Inset:** dividing NSC. **E**, A neurosphere fixed, embedded in paraffin, sectioned and stained with toluidine blue. Cells undergoing mitosis are seen. **F**, A neurosphere cultured in the presence of BrdU, showing that all its cells are proliferative. **G**, A neurosphere cultured in a medium containing 5% fetal bovine serum (FBS) and devoid of EGF starts to differentiate into neurons (βIII-tubulin, red) and astrocytes (GFAP, green). **H**, After a few days *in vitro* in this medium most cells have differentiated into neurons (red) and astrocytes (green). **I, J,** Cells similar to those seen in Fig. H, but viewed under scanning electron microscopy. **Inset**: Double immunofluorescence for BrdU and GFAP to show that the differentiated astrocytes originate from the proliferative cells of neurospheres. Bars: D, D', 55 μm; D'', 20 μm; E-F, 10 μm; G, 100 μm; H, 40 μm; I-J, 10 μm;

pre- and post-natal neurogenesis (Johanson et al., 2008, 2011). Therefore, CSF is expected to be a beneficial medium for NSC survival. Indeed, NSCs cultured in a medium containing CSF show increased survival and proliferation as compared to standard culture media (Gato et al., 2005). Adult human leptomeningeal (intrathecal) CSF is also an appropriate environment for the *in vitro* survival and differentiation of adult human NSCs (Buddensiek et al., 2010). Successful NSCs transplantation into the ventricular or subarachnoidal CSF also supports the view that CSF is a beneficial environment for NSCs to survive, growth and migrate (see below).

Current investigations in our laboratories indicate that neurospheres developed from NSCs collected from the SVZ of normal rats (see Fig. 4), when cultured in the presence of CSF from normal or hydrocephalic HTx rats, differentiate into neurons and glia. It seems likely that NSCs obtained from normal HTx rats, when grafted into a lateral ventricle of hydrocephalic HTx rats, would generate normal neuronal and glial lineages. We have just started exploring this possibility using neurospheres transfected with green fluorescence protein. Our goal is to promote incorporation of the grafted NSCs into the disrupted VZ and SVZ, in order to improve brain functions compromised by hydrocephalus

Delivery of NSCs, NPC or MSCs into CSF. CSF transplantation or explantation of stem/progenitor cells is emerging as an alternative to intraparenchymal grafts of therapeutic cells near injured neural tissue. The multifocal nature of multiple sclerosis makes intraparenchymal cellular therapy difficult. However, a hopeful strategy is to use the CSF administration pathway, which has widespread flow capabilities. Mice with experimental autoimmune encephalomyelitis (EAE) were grafted with neurospheres placed into ventricular CSF; the NPCs entered into demyelinating areas, differentiated into mature brain cells and promoted multifocal remyelination and functional recovery (Pluchino et al., 2003). In mice with induced stroke, NPCs have been administered into the parenchyma near the infarcts and into a lateral ventricle; CSF administration was more efficient in distributing NPCs to the lesion area (Kim et al., 2004). Hippocampus-derived neurosphere cells, isolated from a transgenic rat expressing green fluorescent protein, were transplanted into the fourth ventricle or cisterna magna of rats with spinal cord injury. The injected cells followed the flow of subarachnoidal CSF and survived as clusters on the pial surface of the spinal cord. Notably, a large number of them migrated into the lesion site, integrated into the injured spinal cord and survived within the host spinal cord for as long as 8 months without any tumorigenic changes (Wu et al., 2002, 2004; Bai et al., 2003). NSCs transplanted into the 4th ventricle of animals with injured dorsal roots reached the lesion root and associated with axons in the same manner as Schwann cells (Ohta et al., 2004). These two experiments indicate that grafted NSCs differentiate in a site-dependent manner (see also Rosser et al., 2000). Another approach has been the delivery of NSCs by lumbar puncture to rats with spine injury; cells travelled through the CSF to the site of injury in the spinal cord, differentiated into nestin-positive, immature neurons or glial cells (Satake et al., 2004) and supported neuroprotection and partial recovery of function (Neuhuber et al., 2008).

Immune cells and immune molecules have been shown to support neurogenesis from NSCs and NPCs in the adult brain (González-Pérez et al., 2010; Molina-Holgado and Molina-Holgado, 2010). On the other hand, NPCs transplanted into mice after spinal cord injury contributed to functional recovery mostly by inducing microglia/macrophages to express their repairing phenotype secreting factors such as BDNF and noggin (Ziv et al., 2006; Ziv and Schwartz 2008). The onset of disruption of the VZ is associated with the arrival of microglia/macrophages. Thus, the possibility of a fascinating cross talk between NSCs undergoing disruption and immune cells is being explored, since it could become a therapeutic target.

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Typical and atypical stem cells in the brain, vitamin C effect and neuropathology

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ABSTRACT

Stem cells are considered a valuable cellular resource for tissue replacement therapies in most brain disorders. Stem cells have the ability to self-replicate and differentiate into numerous cell types, including neurons, oligodendrocytes and astrocytes. As a result, stem cells have been considered the "holy grail" of modern medical neuroscience. Despite their tremendous therapeutic potential, little is known about the mechanisms that regulate their differentiation. In this review, we analyze stem cells in embryonic and adult brains, and illustrate the differentiation pathways that give origin to most brain cells. We also evaluate the emergent role of the well known anti-oxidant, vitamin C, in stem cell differentiation. We believe that a complete understanding of all molecular players, including vitamin C, in stem cell differentiation will positively impact on the use of stem cell transplantation for neurodegenerative diseases.

Key words: Stem cells, radial glia, vitamin C, SVCT2, brain development, tanycytes, Bergmann glia, neurogenic niche, type B cells, neuroblast, Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis.

1. STEM CELLS DURING BRAIN DEVELOPMENT AND ADULT LIFE

Early during embryogenesis, a layer of neuroepithelial cell folds and fuses to form the neural tube, a well characterized anatomical structure that gives rise to most brain regions (Geelen and Langman, 1979; Ray and Niswander, 2012). Thus, neuroepithelial cells are the primordial neural stem cells of the embryonic brain. These cells have three distinguishable features: i) highly polarized, they face both the ventricular zone (VZ) and the pial surface (Huttner and Brand, 1997); ii) expression of the intermediate filament, nestin (Lendahl et al., 1990); and iii) ability to undergo inter-kinetic nuclear migration, a phenomenon involved in the determination of neural progenitors (Del Bene et al., 2008; Taverna and Huttner, 2010).

In the neural tube, neuroepithelial cells are capable of proliferating actively and symmetrically to increase their population (Egger et al., 2011; Tawk et al., 2007). As development continues, these cells begin to experience asymmetrical divisions, which lead to the generation of the first neuroblasts and basal progenitors, initiating the embryonic neurogenesis process (Haubensak et al., 2004; Kriegstein and Alvarez-Buylla, 2009). Following the onset of neurogenesis, at embryonic days 9-10 in mice (E09-E10), neuroepithelial cells experience several molecular and morphological changes that transform them into a new type of cell, the radial glia. These new progenitor cells uniformly extend a long radial process from the cell body, located at the VZ, all the way to the most external portion of the cortex, the pial surface (Gotz and Huttner, 2005; Morrens et al., 2012; Yamasaki et al., 2001). Such morphological changes coincide with the expression of different markers, including GLutamate ASpartate Transporter (GLAST), Brain Lipid-Binding Protein (BLBP), Tenascin-C and Glial Fibrillary Acidic Protein (GFAP), which is absent in rodents (Anthony et al., 2004; Hartfuss et al., 2001; Malatesta et al., 2003). Some proteins are highly enriched in radial glial cells. For example, Radial Glia 1 and 2 (RC1 and RC2) are two proteins specific to these cells alone (Edwards et al., 1990). The protein 3-phosphoglycerate dehydrogenase (3-PGDH), a key enzyme for L-serine biosynthesis, is also characteristically expressed in radial glia (Yamasaki et al., 2001). Using immunohistochemical methods, we show the presence of 3-PGDH, in radial glial cells of both E15 embryonic mouse cortex and human cortex at 9 weeks gestation (Figure 1A,B).

Through continuous asymmetrical divisions, radial glial cells give rise to intermediate precursors and neuroblasts (Malatesta et al., 2000) that will then generate most neuronal classes (e.g., pyramidal, stellate, etc). Soon thereafter, these radial glia derived-newborn neurons will use the same radial glial processes as scaffolds to migrate to the appropriate cortical layers (Noctor et al., 2002) (Figure 1C).

Close to the perinatal period, radial glial cells stop producing neurons and differentiate into astrocytes (Culican et al., 1990) . In the presence of appropriate trophic factors, radial glial cells in culture differentiate into neuronal and glial cells (Liu and Lauder, 1992; Yamasaki et al., 2001). Interestingly, the number of each differentiated cell type (neuron or glia) depends on the time at which radial glial cells are isolated for the in vitro studies. For example, neuronal formation is favored by radial glia obtained between embryonic days 13-

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16 (E13-E16) while glia generation is promoted by radial glial taken at later stages (embryonic day 18 to postnatal days) (Malatesta et al., 2000; Temple, 2001).

Between postnatal days 1-15 (PN1-PN15), most radial glial cells disappear from almost all brain regions (Tramontin et al., 2003). Radial glia-like cells are however maintained in specific regions of the brain. For instance, the Bergmann glia is a radial glia-like cell that is predominant in cerebellar cortex. These cells like radial glia present high levels of GFAP and S100 protein expression (Figure 2A-D), generate neuronal and glial progenitors and participate in the migration of newborn granule cells. Such neurogenic activity disappears at later stages of postnatal development. Tanycytes represent another radial glial cell in the hypothalamus (Figure 2E-G). Unlike most radial glial cells, tanycytes maintain their neurogenic activity even in the adult brain (Cifuentes et al., 2011; Lee et

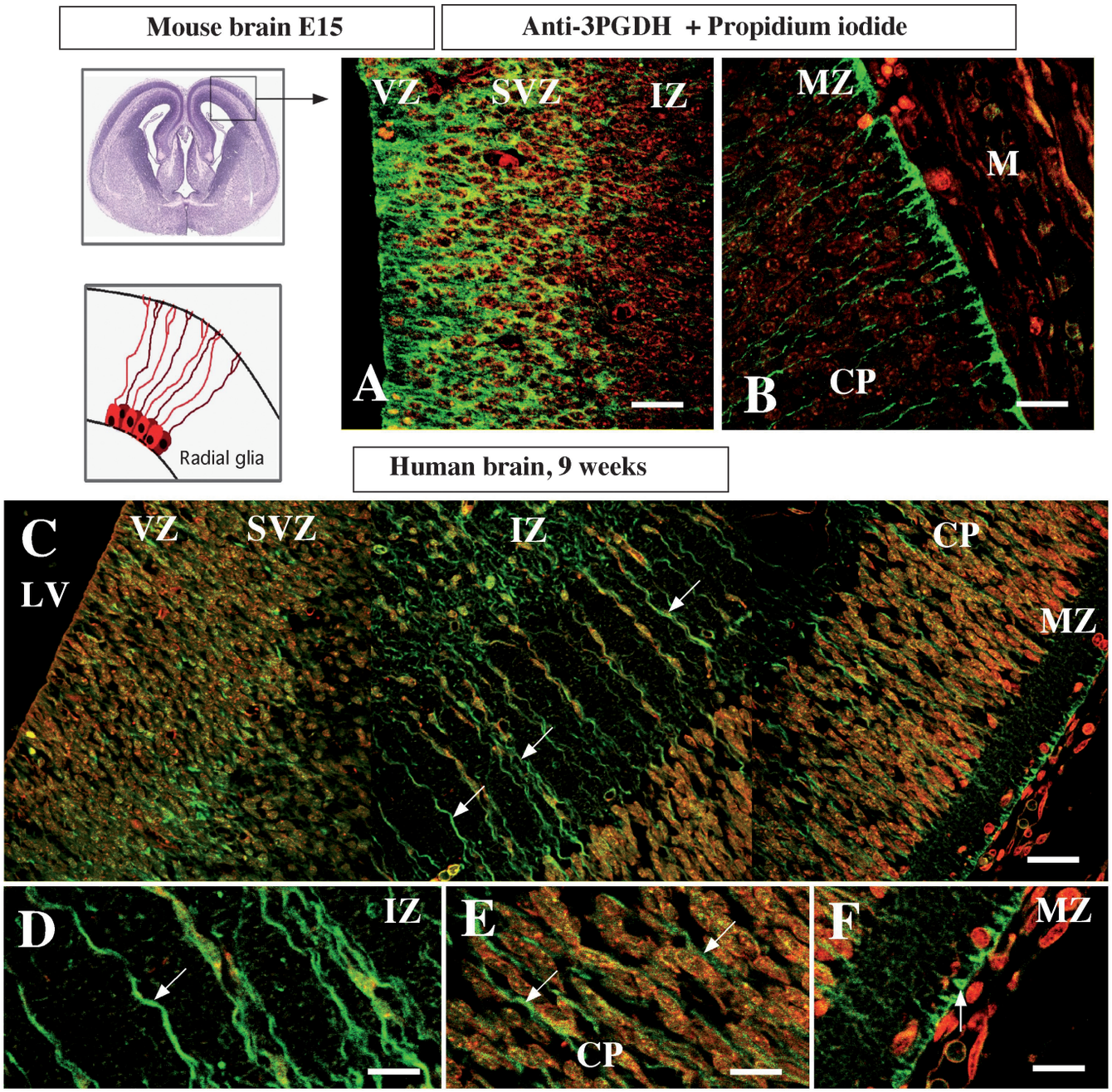

Figure 1. Typical stem cells during embryonic development, Radial glia cells and 3-PGDH expression in mouse and human embryonic brain: (A-B) Embryonic mouse frontal sections immunostained using a specific antibody anti-3-PGDH and a Cy2-labeled secondary antibody. Propidium iodide was used for nuclear staining. (C-D) Similar analyses were performed in sagittal sections of human embryonic brain at 9-weeks of development. A positive reaction in radial glia cells present in the ventricular zone (VZ) and subventricular zone (ZVS) of the mouse and human embryonic cortex was observed. The radial glial processes and end-terminal on meninges (M) were also positive (B and D-F, arrows). CP, cortical plate. IZ, intermediate zone. LV, lateral ventricle. MZ, marginal zone. Scale bars: 15 μm.

al., 2012), the reason by which they are considered atypical stem cells. Tanycytes play a major role in the formation of the median eminence-cerebrospinal fluid barrier (Figure 2E). More recently, these cells have been found to be crucial for the brain glucose sensing mechanism (Cortes-Campos et al., 2011; Orellana et al., 2012).

Perhaps the most active neurogenic site present in the mature brain is concentrated in the ventricular wall of lateral ventricle. These radial glial-like stem cells or Type B cells show intense expression of GFAP (Figure 3A-D), maintaining long basal processes in contact with blood vessels (Figure 3E) like radial glial cells. Type B cells are close to ependymal cells, which produce factors that regulate neuronal production; they generate Type C cells, which then proliferate to form Type A cells or neuroblasts (Alvarez-Buylla et al., 1998; Doetsch et al., 1997; Garcia-Verdugo et al., 1998) (Figure 3A). Different studies have shown that the structure of these neurogenic sites is variable across species. For example, in guinea pig brain, we observed an ependymal cell layer positive for Isolectin B4 expression within the subventricular zone (Figure 3F, arrows). Additionally, in the subventricular zone it is possible to find a ribbon of Type B cells (Figure 3F). A similar cellular

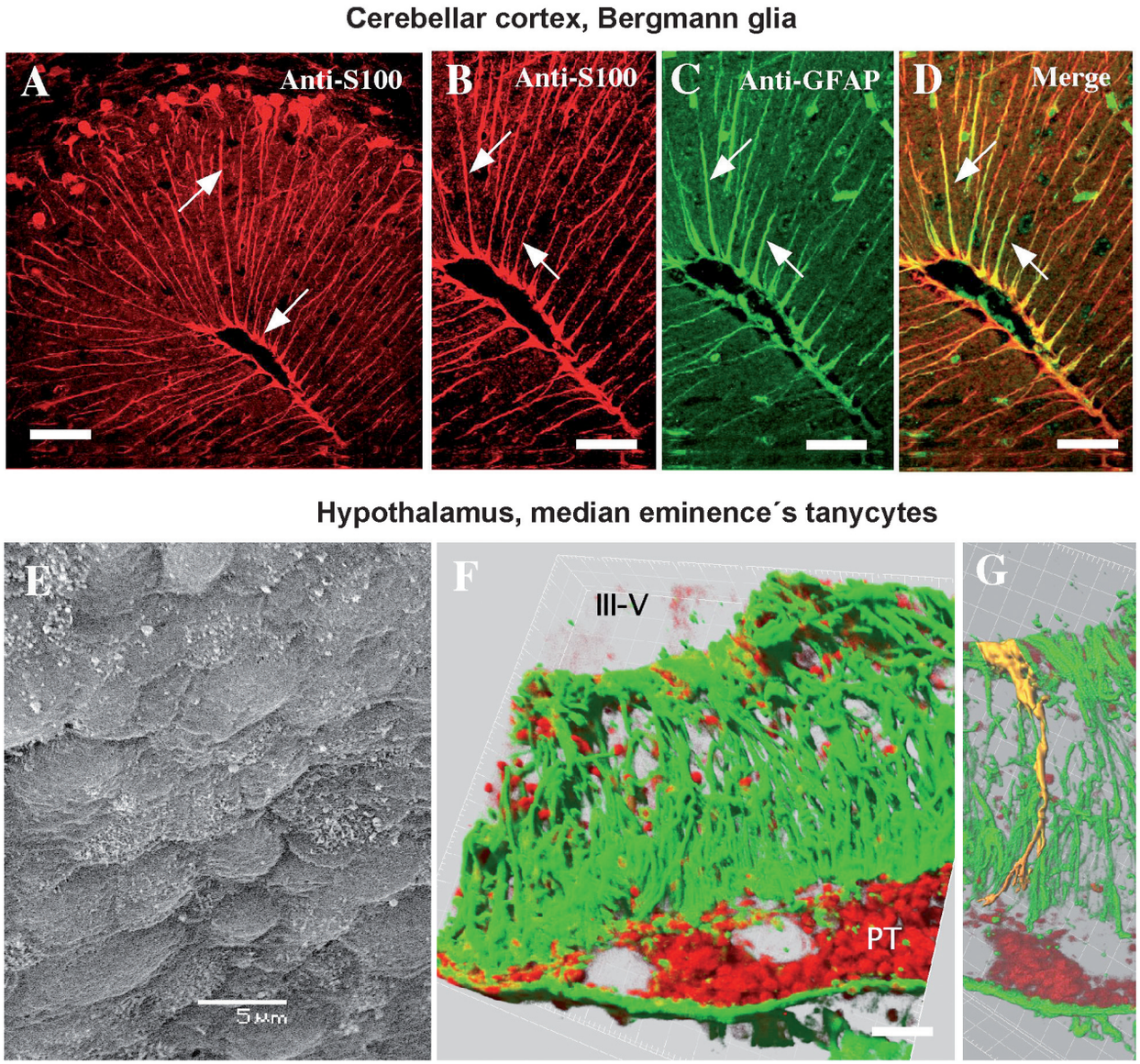

Figure 2. Atypical stem cells during post-natal and adult life, cerebellar Bergmann glia and hypothalamic tanycytes: (A-D) Post-natal cerebellar frontal section of mouse brain immunostained using specific antibodies anti-GFAP or anti-S100, and two different secondary antibodies labeled with Cy2 or Cy3, respectively. The Bergmann glial process and the end-terminal on meninges (A, arrow) were positive (B-D, arrows). (E-G) Tanycytes from the median eminence analyzed using scanning electron microscopy on the ventricular surface, showing the cerebrospinal fluid-median eminence barrier. The processes of these cells are detected using anti-vimentin and a Cy2-labeled secondary antibody. Propidium iodide was used for nuclear staining. The image was processed using the software Imaris. One tanycyte is depicted in yellow in G. III-V, third ventricle. PT, pars tuberalis. Scale bars: 20μm.

organization is observed in the subventricular zone of the human brain (Sanai et al., 2004). In these niches the neuroblasts migrate between Type B and ependymal cells (Figure 3H-I, arrows). Therefore, neurogenic niches seem to present different structures depending on the species studied (Chojnacki et al., 2009).

2. STEM CELL DIFFERENTIATION: CELLULAR SIGNALING

How do radial glial cells regulate their fate to first generate neurons and then astrocytes? Experimental evidence suggests that both internal and external signals trigger neurogenesis and gliogenesis (Behar et al., 1994; Colombo et al., 1993; Jiang et al., 1998; Liu and Lauder, 1992; Yamasaki et al., 2001). Once these signals become available in quantities above the activation threshold, radial glial cells respond by differentiating towards a defined cell type. Along with these signals, different genetic programs are activated, allowing radial glial cells to express receptors and proteins necessary to respond specifically to the external stimuli (Ciccolini, 2001; Okano and Temple, 2009; Qian et al., 2000). Of the signals that promote neuronal differentiation, the helix-loop-helix type transcription factors, neurogenin 1 and 2 (Ngn1 and Ngn2); (Guillemot et al., 1993; Ma et al., 1996; Ma et al., 1998; Sommer et al., 1996), Mash1 (Guillemot et al., 1993; Nieto et al., 2001), Math (Gradwohl et al., 1996; Kageyama et al., 2005) and NeuroD (von Bohlen und Halbach, 2011), are among the most well-studied. Ngn1 is a factor primarily expressed during the neurogenic period

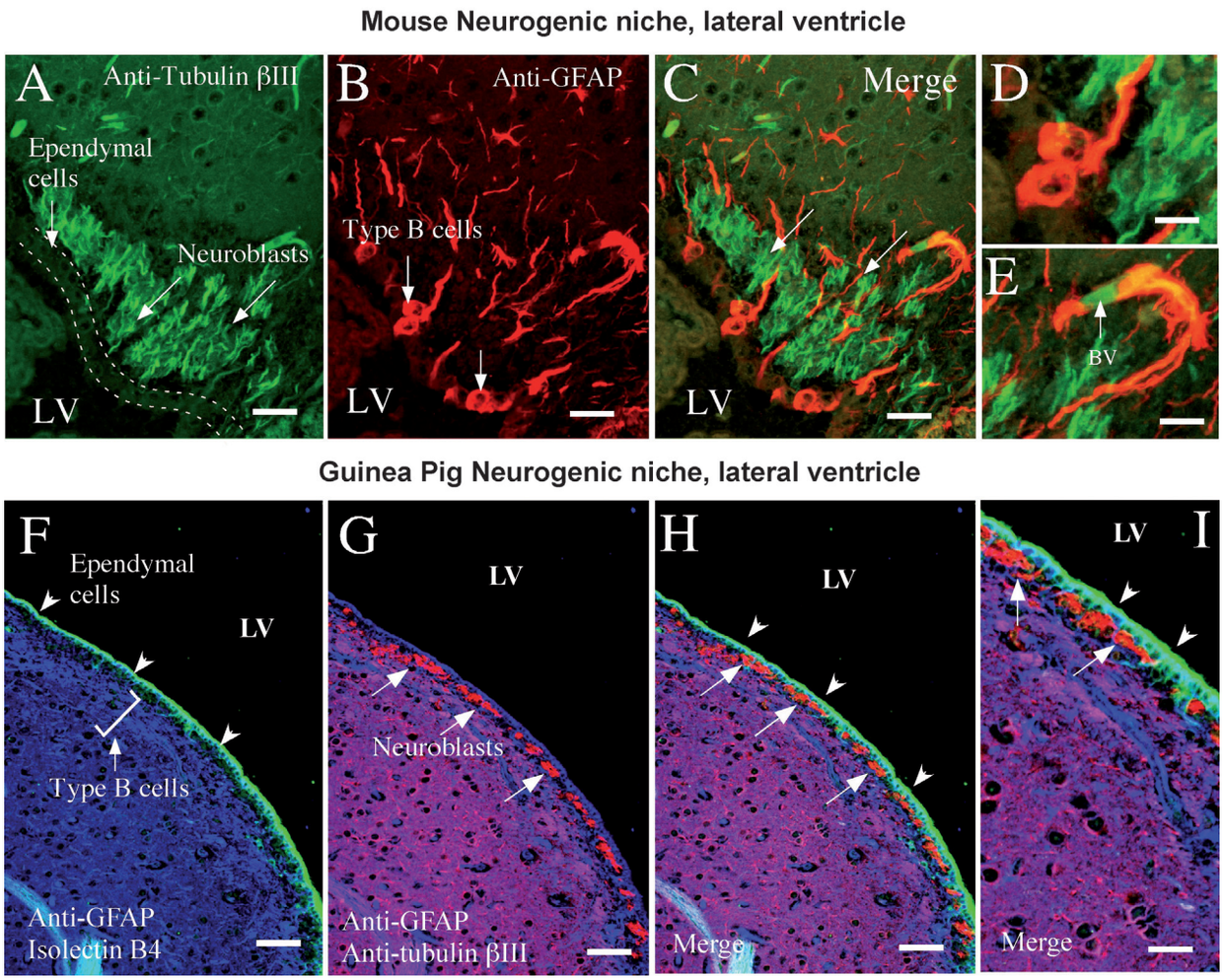

Figure 3. Comparative cellular distribution of typical stem cells present in the adult neurogenic niche of mouse and guinea pig brain: (A-E) Adult mouse frontal brain section of the lateral ventricle neurogenic area immunostained using antibodies specific for anti-tubulin βIII (neuroblasts marker) or anti-GFAP (type B cells marker), and two different secondary antibodies labeled with Cy2 or Cy3, respectively. The neuroblasts are observed inside the subventricular zone near to the ependymal cells, type E cells (A). Type B cells are detected (GFAP-positive) in the ventricular and subventricular zone close to ependymal cells (B and D). The processes were in contact with the blood vessel of the subventricular zone (E). (F-I) Adult guinea pig frontal brain section of the lateral ventricle neurogenic area immunostained using specific antibodies anti-tubulin βIII (red) or anti-GFAP (blue), and two different secondary antibodies labeled with Cy3 or Cy5, respectively. The ependymal cells were observed by using Isolectin B4-FITC labeled (green, arrowheads). The astrocytes or Type B cells form a ribbon in the subventricular zone (F). The neuroblasts are detected near the ependymal cells (G-H, arrows). The neurogenic niche structure observed in guinea pig (I) is similar to the neurogenic niche previously described in adult human brain. Bv, blood vessels; LV, lateral ventricle. Scale bars: 20μm.

in the cortex (Fode et al., 2000). Because it induces neuronal differentiation but also inhibits glial differentiation by interfering with the glial signaling pathway JAK/STAT (Sun et al., 2001), it is recognized as a factor capable of modifying neural stem cell fate. Ngn2 is primarily a neurogenic factor expressed in a small population of neural stem cells, and cells that lack Ngn2 requires the presence of Mash1 to generate neurons (Nieto et al., 2001).

Little is known about the extracellular factors that regulate neurogenesis. Recent work indicates that sonic hedgehog (Shh) regulates neurogenesis by directly modulating Ngn1 expression (Ota and Ito, 2003). Neurotrophin 3 (NT3), a factor expressed in the ventricular zone, has also been implicated in neurogenesis (Ohtsuka et al., 2009). Once NT3 is released to the extracellular medium, it activates the ERK1/2 signaling pathway, which ultimately leads to the activation of transcription factors that control neuronal differentiation (Bartkowska et al., 2010; Ohtsuka et al., 2009).

Several factors are known to induce gliogenesis. For example, Notch1 is a membrane receptor expressed in most neural stem cells that upon activation by its Jagged or Delta ligands (Zhou et al., 2010) leads to a cascade of proteolytic events, which culminates with the translocation of its internal domain to the nucleus. In the nucleus, Notch1 acts as a transcriptional transactivator (Morrison et al., 2000; Zhou et al., 2010), inducing demethylation of the binding sites for STAT proteins, such as the promoter of the GFAP and S100B gene. This allows radial glial cells to respond to the JAK/STAT signaling cascade, a well established gliogenic pathway (Freeman, 2010). Factors, such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and cardiotrophin 1 (CT1), also regulate gliogenesis. After binding to their respective receptors, each factor activates the JAK/STAT signaling cascade that leads to GFAP expression, a well known marker for glia (Barnabe-Heider et al., 2005; Bhattacharya et al., 2008; Bonni et al., 1997).

Growth factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), are widely used to maintain stem cells in culture; they also participate in stem cell differentiation (Chojnacki et al., 2009). At the onset of neural development, receptors for bFGF are highly enriched in radial glial like stem cells. Interestingly, a large fraction of these receptors are associated with a variety of proteoglycans present in the extracellular matrix (Thisse and Thisse, 2005). Mechanistically, the receptors may uptake the bFGF from the extracellular matrix (Aigner et al., 2002), and upon activation, collaborate in the determination of astrocytic lineage. At present, it is necessary to understand the dynamical relationships between extracellular signals and transcriptional factors. For example, in high concentrations of Ngn1, BMP2 promotes neuronal differentiation. On the contrary, if Ngn1 levels fall, BMP2 promotes gliogenesis (Nakashima et al., 2001). Therefore, studying the different combinations that occur over time might provide insights into the patterns of cellular differentiation.

3. ROLE OF VITAMIN C IN NEURAL STEM CELL DIFFERENTIATION

Over the years, we have biochemically characterized vitamin C function in brain tissue. Known as a critical antioxidant, recent results suggest that vitamin C plays an important role in stem cell physiology. To gain a deeper understanding into this novel role, we have critically revised the role of vitamin C in stem cell generation, proliferation and differentiation. Although the mechanisms by which vitamin C modulates stem cell biology are unclear, the revision presented here suggests a unique role for vitamin C during stem cell transplantation in a number of brain diseases.

3.1. Vitamin C in the central nervous system

Most mammals are able to synthesize vitamin C from glucose. Humans and primates however are deficient in the enzyme that catalyzes vitamin C biosynthesis (L-.gulonolactone oxidase); therefore, they must obtain it from the diet (Nishikimi and Yagi, 1991). Lack of vitamin C during development and/or adulthood can produce severe physiological disorders that could lead to death, implying that as micronutrient, vitamin C is essential for the normal functioning of the organism (Harrison and May, 2009; Rice, 2000; Tveden-Nyborg et al., 2009). Its best known role is that of an antioxidant agent, taking away highly reactive free radicals that are constantly generated by the cellular machinery (Arrigoni and De Tullio, 2002; Harrison and May, 2009; Nualart et al., 2003; Wayner et al., 1986). This role is highly relevant for both the maintenance of cerebral functions and protection of brain structures (Rice, 2000). Given that the brain is among the organs with the highest rate of metabolism, it is subjected to elevated levels of oxidative stress, and thus high levels of vitamin C are required.

In addition to its protective role, vitamin C acts as a cofactor in several enzymatic reactions. It participates in the synthesis of catecholamines, carnitine (Rebouche, 1991), cholesterol, amino acids, and several other hormonal peptides (Glembotski, 1987). It also facilitates the hydroxylation of proline and lysine residues in collagen, which are required for its correct folding (Murad et al., 1981; Peterkofsky, 1972, 1991) as well as the hydroxylation of hypoxia-inducible factor-1 alpha (HIF1a), which is degraded by the proteosome under normal oxygen conditions (Harrison and May, 2009). Recent investigation indicates that vitamin C modulates dopaminergic, glutamatergic and cholinergic neurotransmission (Rebec and Pierce, 1994; Sandstrom and Rebec, 2007).

Vitamin C can also promote myelin formation (Eldridge et al., 1987). Using adult salamander dorsal root ganglia, Olsen and Bunge (1986) observed myelination *in vitro* and described vitamin C as a critical factor for Schwann cell myelination. Similarly, Eldridge et al. (1987) described that chick embryo extracts and L-AA led to the formation of large quantities of myelin and to the assembly of basal laminae. They strongly suggest that AA promotes Schwann cell myelin formation. Furthermore, in vitro and animal studies showed that AA improves the clinical and pathological phenotype of a mouse model of Chercot-Marie-tooth disease 1A (CMT1A) (Kaya et al., 2007; Passage et al., 2004), which led to various clinical trials examining AA administration in CMT1A. However, none of these trials showed a significant benefit of AA in the treatment of CMT1A patients (Burns et al., 2009; Micallef et al., 2009; Verhamme et al., 2009). The lack of effectiveness in the treatments was difficult to interpret because there were no studies assessing SVCT2 expression in Schwann cells and peripheral nerves, data that was recently obtained by (Gess et al., 2010). Additionally, they used the heterozygous SVCT2+/-

mice to elucidate the functions of SVCT2 and AA in the murine peripheral nervous system. SVCT2 deficiency causes hypomyelination and extracellular matrix defects in peripheral nervous system (Gess et al., 2011). Also, they demonstrated that expression of various collagen types was reduced in the sciatic nerves of SVCT2$^{+/-}$ mice, and hydroxyproline levels were surprisingly normal. These results indicate that collagen formation was regulated on the transcriptional and not the posttranslational level (Gess et al., 2011).

3.2. Vitamin C transporters in the brain

Two types of proteins are required for the uptake and maintenance of vitamin C levels in the brain: the sodium-ascorbate co-transporters (SVCTs) and the hexose transporters (GLUT1, GLUT3, GLUT4), the latter incorporating only the oxidized form of vitamin C (Nualart et al., 2003; Rumsey et al., 2000; Rumsey et al., 1997; Vera et al., 1993). Therefore, SVCTs are the major homeostatic regulators of vitamin C levels in the central nervous system (CNS; Rice, 2000), actively uptaking its reduced form, L-ascorbate (Caprile et al., 2009; Castro et al., 2001; Garcia et al., 2005). L-ascorbate is transported against its concentration gradient (Daruwala et al., 1999; Tsukaguchi et al., 1999), and thus the energy required for the transport is supplied by sodium exchange through the Na^+/K^+-ATPase pump (Castro et al., 2001). The relationship between sodium and ascorbate transport was found to be 2:1, and the order of union to the substrate was sodium-ascorbate-sodium (Godoy et al., 2007). At the molecular level, two SVCTs (SVCT1 and SVCT2) encoded by different genes have been described

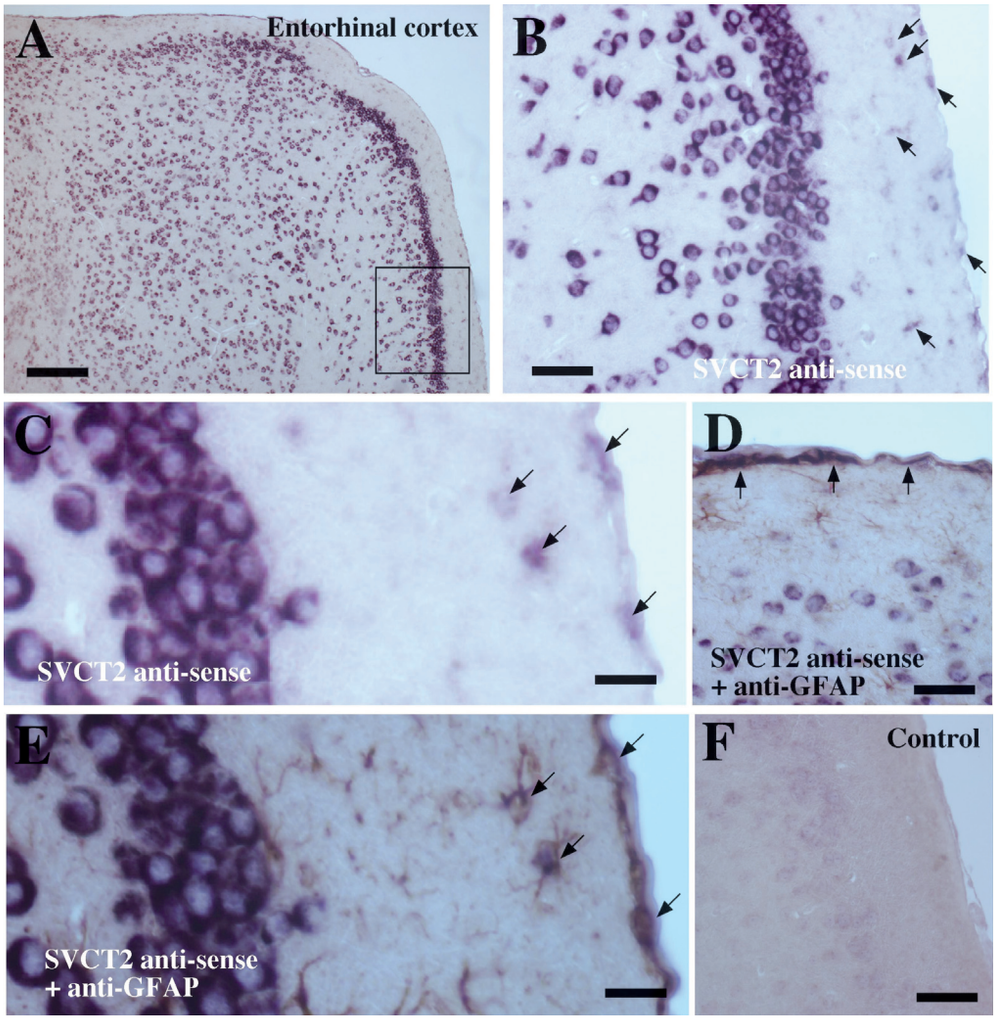

Figure 4. SVCT2 mRNA detection by *in situ* hybridization in adult brain: SVCT2 expression at the mRNA level by *in situ* hybridization using digoxigenin-labeled cRNA probes specific for SVCT2. An intense hybridization signal was observed in different neurons of the mouse brain, the most intense signal was detected in hippocampus, thalamic nuclei, hypothalamus and the entorhinal cortex (A, B, C). No labeling was detected in endothelial cells of the blood–brain barrier and astrocytes. To confirm the presence of negatively-labeled astrocytes in the tissue sections, we carried out *in situ* hybridization for SVCT2 in combination with immunohistochemical analysis using antibodies against GFAP and a peroxidase-labeled secondary antibody (D-E, arrows). Most astrocytes were negative for SVCT2 in the different areas of the brain (B-C); however, the astrocytes detected in the external area of the entorhinal cortex and marginal zone of the brain showed SVCT2 expression (C, D and E, arrows). Experiments using an antisense for SVCT2 and anti-GFAP in the same section confirmed SVCT2 expression in GFAP-positive cells (C and E, arrows). F, Control experiments using a sense riboprobe. Scale bars: 20μm.

(Faaland et al., 1998; Tsukaguchi et al., 1999; Wang et al., 1999). Each transporter has a distinct distribution. For example, *in situ* hybridization and immunohistochemical experiments have shown that SVCT2 is expressed mostly in CNS within choroid plexus cells (Garcia et al., 2005; Tsukaguchi et al., 1999), embryonic neurons (Castro et al., 2001), tanycytes (Garcia et al., 2005) and adult cortical and hippocampal neurons (Astuya et al., 2005) (Figure 4A-B). Although most astrocytes do not express SVCT2, our results indicate that astrocytes of the external area of the entorhinal cortex express SVCT2 (Figure 4C,E). Additionally, glia limitans, the thin barrier of astrocytes associated with the parenchymal basal lamina surrounding the brain, also showed double labeling for SVCT2 and GFAP (Figure 4C-E, arrows). In contrast, SVCT1 is typically found in peripheral organs, such as kidney, intestine, or liver (Castro et al., 2008; Tsukaguchi et al., 1999). Cloned for the first time by Tsukaguchi et al. (1999), both transporters share an amino acid identity sequence of 60-65%, and according to predictions of hydrophobicity, each has 12 transmembrane domains with the N- and C-terminal domains facing the cytosolic side (Savini et al., 2008). SVCT1 and SVCT2 have "non-functional" variants generated by alternative splicing. In the case of SVCT2, Lutsenko et al. (2004) described a short isoform that lacks the transmembrane segment of domains 4, 5 and 6 (Lutsenko et al., 2004). This isoform acts as a dominant negative when inhibiting the transport of ascorbate mediated by the complete isoform of SVCT2. The expression of both SVCTs is regulated at different levels: transcriptionally by the presence of two promoting sequences with distinct efficiencies in SVCT2, and post-transductionally by the presence of phosphorylation and glycosylation sites in SVCT1 and SVCT2. Recently, these cellular modifications were described as determinant of the functionality and subcellular localization of these transporters (Subramanian et al., 2008).

Different research groups have shown the physiological importance of both transporters. Sotiriou et al. (2002) generated knockout mice for the gene encoding SVCT2, and observed that animals developed respiratory problems and cerebral hemorrhages dying within minutes immediately after birth. Biochemical analysis showed that vitamin C levels in brain and other areas were undetectable, indicating that SVCT2 is critical for the maintenance of vitamin C levels. The cerebral hemorrhages observed in these animals suggest poor formation of collagen, which requires vitamin C as cofactor for its synthesis (Sotiriou et al., 2002). Histological analysis showed no morphological alterations at the cerebral level, suggesting that SVCT2 is not required at least in part for brain development. The above leaves open the possibility that other isoforms of the transporter not yet identified contribute to the incorporation of vitamin C to trigger the differentiation of cerebral cells.

3.3. Vitamin C and stem cell differentiation

Recently, Pei and colleagues (2010) found that vitamin C favored the generation of induced pluripotent stem cells (iPS) (Esteban et al., 2010). Cells grown *in vitro* in the presence of vitamin C expressed two histone demethylases, Jhdm1a and Jhdm1b (Wang et al., 2011), which are required for iPS cells production. Vitamin C also helped to maintain iPS cells *in vitro*, which favored their proliferation (Esteban et al., 2010). The increase in the number of iPS was found to be mediated at least in part by a reduction in the levels of p53, the tumor suppressor protein that elicits cell death (Esteban et al., 2010). Together, these results suggest that vitamin C is able to positively regulate stem cell generation and proliferation.

Neural tissue has been shown to attain ascorbic acid (AA) concentrations that rank among the highest of mammalian tissues (Horning et al., 1975; Kratzing et al., 1982; Milby et al., 1982). In fact, vitamin C levels are particularly high in fetal rat brain, doubling from the 15th to the 20th day of gestation and then dropping significantly by the time of birth (Kratzing et al., 1985). A similar pattern was observed in chicken embryos in which the AA concentration increased to 5.6 nmol/mg by D10 in ovo, and gradually declined to 32% before birth (Wilson, 1990). Surprisingly, normal brain differentiation has been observed in SVCT-null mice, suggesting that embryonic cells may use different mechanisms or transporters to take up and maintain high levels of vitamin C (Sotiriou et al., 2002). Recently, Yan and collaborators (Yang et al., 2001) reported high expression of SVCT2 in embryonic mesencephalic neurons, and suggested an important role for vitamin C in dopaminergic differentiation. They observed that the stem cells grown with AA gave rise to a 10-fold increase in the number of dopaminergic neurons compared to the untreated cultures (Yang et al., 2001).

To characterize the localization of the vitamin C transporter, SVCT2, in early brain development, the expression of SVCT2 was examined through RT-PCR in fetal mouse brain. High levels of SVCT2 expression were observed in the cerebellum, hippocampus, and hypothalamus (Castro et al., 2001). Kinetic analysis of AA uptake in primary neuronal cultures showed two affinity constants, 8 and 103 uM. The first kinetic constant is in accordance with previous reports for SVCT2 (Tsukaguchi et al., 1999). The second kinetic constant suggests an alternative path for the transport of vitamin C in mouse embryonic neurons. Because the sole presence of a given mRNA does not always correlate with protein expression and functional activity, it is, therefore, necessary to study both mRNA expression and protein distribution to obtain a more definitive answer regarding the expression and localization of SVCT2 in fetal rat brain. By a variety of methods, Caprile and collaborators demonstrated that SVCT2 is highly expressed in the ventricular and subventricular areas of fetal rat brain. Further functional analysis carried out in immature neurons isolated from either embryonic brain cortex or cerebellum showed that SVCT2 is localized in the cellular membrane and is involved in vitamin C uptake in these cells (Caprile et al., 2009). Similar results have been obtained in Neuro2a and HN33.11 cell lines, demonstrating that SVCT2 is critical for vitamin C transport (Caprile et al., 2009). In line with our hypothesis, SVCT2 has also been implicated in neuronal maturation possibly by regulating the differentiation of embryonic cortical precursors into neurons and astrocytes. AA promoted *in vitro* differentiation of CNS precursor cells into neurons and astrocytes in a cell density-dependent manner (Lee et al., 2003). Also, AA seems to affect the functional maturation of post-mitotic neurons. For example, the addition of AA to these neurons enhanced the frequency and amplitude of synaptic potentials, an effect that was dose-dependent and highly specific (Lee et al., 2003). An AA-dependent increase in the expression of genes, which could potentially compound the effects of AA on cell differentiation, was also observed (Qiu et al., 2007), suggesting that AA may stimulate the brain in the

generation of CNS neurons and glia. It has also been observed that in cultured hippocampal neurons, SVCT2 was required for normal maturation of glutamatergic function (Qiu et al., 2007). Hippocampal neurons from days 16-18 *in utero* Slc23a2-/- and wild-type embryos were isolated from mice and functionally compared. It was consistently seen that the lack of SVCT2 resulted in smaller amplitude of synaptic potentials, and these mice also exhibited less complexity in terms of number of primary dendrites as well as total dendrite length (Qiu et al., 2007). Hippocampal neurons from Slc23a2-/- embryos lack AA while the intracellular concentration in wild-type neurons was within the range of 0.1-0.2mM (Qiu et al., 2007).

How does vitamin C promote stem cell differentiation? How do neural stem cells, or any other pluripotent cell, uptake vitamin C? What set of transporters are present in these cells? The answers to these questions are necessary to understand the role of vitamin C in neural stem cell differentiation.

4. ROLE OF VITAMIN C IN BRAIN STEM CELL TRANSPLANTATION

4.1. Stem cell transplantation in the CNS

Stem cell transplantation has emerged as a *unique* therapeutic strategy for most neurological disorders (i.e., Parkinson's Disease (PD), Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS), Spinal Muscular Atrophy (SMA), stroke, etc.). The goal of this strategy is the partial or complete replacement of damaged or dead neural cells by ***newer*** and ***healthier*** ones, which in theory should lead to functional restoration of the afflicted tissue. Depending on the type of cells used (e.g., induced pluripotent, embryonic or adult stem cells), their physiological and metabolic state and the disease to be targeted, stem cell transplantation therapy has obtained promising results (see below). The low number of cells transplanted, their limited survival, decreased differentiation efficiency, poor cellular integration, in addition to tissue immune rejection, are all factors that lead to inconsistencies in the results. In spite of these problems, the scientific optimism for cellular transplantation keeps growing, with the National Institute of Health (USA) devoting additional resources to it.

We describe below some of the neurodegenerative diseases being considered for *"treatment"* with stem cell transplantation, their results and problems, and the future directions of the approach. In this context, we speculate that the treatment with vitamin C acts in favor of cellular replacement in all disorders listed.

4.2. Stem cell transplantation in neurodegenerative disorders

4.2.1. Parkinson's disease

Parkinson's disease (PD) is a progressive neurological disorder that affects ~1% of the population over 55 years of age. It is caused by the progressive loss of dopaminergic neurons from the ***Substantia Nigra*** and ***Corpus Striatum***, which provokes severe motor dysfunctions, including uncontrollable shaking, rigidity, walking and coordination problems, and at later stages, cognitive and behavioral deficits. Although the cause(s) of dopaminergic neuronal loss is (*are*) unknown, a distinctive feature of PD is the accumulation of filamentous inclusions known as ***Lewy bodies,*** which consist of protein aggregates formed mainly by α-synuclein, ubiquitin and neurofilaments (Trojanowski and Lee, 1998). The current view is however that ***Lewy bodies*** are a consequence and not a cause of the disease. A more recent hypothesis suggests that metabolic alterations in dopaminergic neurons are the primary cause of PD. To support this view, a number of labs have shown that the enzymes involved in the metabolism of dopamine produce elevated quantities of H_2O_2 (Przedborski et al., 2000). In the presence of oxygen, these peroxide species form reactive oxygen species (ROS), which at augmented levels alter the health of the neurons. Additionally, dopaminergic cells are highly dependent on adenosine triphosphate, and inhibitors of mitochondrial function (i.e., MPP) accumulate specifically in dopaminergic neurons, affecting their ATP production and promoting the generation of ROS (Winklhofer and Haass, 2010). More recently, several genes have been linked to mitochondrial dysfunction and have been consequently implicated in PD (Blandini and Armentero, 2012; Winklhofer and Haass, 2010). Although the mechanism through which these genes alter free radical generation and mitochondrial function *specifically* in dopaminergic neurons is not yet clear, the excessive production of free radicals coupled with perturbed mitochondrial function strongly correlates with dopaminergic neuronal death (Betarbet et al., 2002a; Betarbet et al., 2002b; Winklhofer and Haass, 2010).

Levodopa is currently the only pharmacological option with a recognized effect on stopping the progression of PD; it allows the recovery of dopamine levels in the afflicted areas, which in turn alleviates the motor defects observed in PD patients (Dawson et al., 2010). Unfortunately, the effectiveness of the treatment decreases with time (Dawson et al., 2010). Due to this, and the lack of pharmacological alternatives, stem cell transplantation has emerged as a compelling strategy to restore dopaminergic neurons in PD patients. In theory, stem cells have the potential to generate an unlimited number of dopaminergic neurons. In practice, it has been tremendously difficult to produce them (Cao et al., 2001; Li et al., 2007). By over-expressing genes that encode transcription factors present primarily in dopaminergic neurons, embryonic stem cells have been derived into dopaminergic neurons *in vitro* (Jacobs et al., 2009; Perrier et al., 2004). These pioneer results immediately raised the possibility of transplanting dopaminergic neurons in PD animal models (Kriks et al., 2011; Tabar et al., 2008) and patients. Kriks and colleagues (2011) were the first to introduce stem cell-derived dopaminergic neurons into PD mice. Transplanted neurons survived, integrated into the mature neuronal parenchyma and established neural connections with the existent neurons (Kriks et al., 2011). More importantly, these cellular transplants were able to significantly improve motor function in PD mice (Kriks et al., 2011). Several labs are now trying to inject stem cell-derived dopaminergic neurons into human PD patients.

Although the results in animal models are quite encouraging, stem cell transplantations have not been exempt of problems. A large number of the transplanted cells often die after the procedure, reducing the possibilities of replacing the damaged cells. In other cases, the newly differentiated neurons become pluripotent cells again, and no longer express the identifying dopaminergic factors. A major problem is that the exogenous transplants may generate aggressive tumors, making the treatment strategy quite dangerous. It is therefore fundamental to solve these and other problems before stem cell

therapy could be used safely and efficiently in human patients. It is also of major priority to understand the factors that might regulate stem cell transplantation in PD patients. In this context, experimental evidence suggests that vitamin C might positively modulate stem cell transplantation. First, as an anti-oxidant, vitamin C can drastically reduce the accumulation of free radicals produced by both the endogenous tissue, and the stem cell-derived neurons *exogenously* incorporated into the tissue. The scavenging effect of vitamin C has an important consequence; it dramatically reduces cell death. Such improvement in survival should have a direct impact on the number of stem cell-derived neurons available for insertion into the afflicted tissue. Thus, vitamin C indirectly modulates neuronal integration. A second role of vitamin C is to *directly* favor stem cell production and differentiation into dopaminergic neurons. The idea is supported by preliminary results in which mouse or human somatic cells incubated with vitamin C significantly increased the induction of stem cells (Esteban et al., 2010; Yan et al., 2001), a mechanism mediated at least in part by regulating the activity of histone demethylases, enzymes required for the expression of Nanog (a master transcription factor for stem cell production). In other experiments, vitamin C has been directly implicated in cellular differentiation. Stem cells incubated with vitamin C increased the number of cells that acquired a cardiac phenotype (i.e., contractile properties and GATA4, a and b-myosin heavy chain expression). Similarly, it is possible that under appropriate conditions, vitamin C can promote specific neuronal differentiation (Figure 5). In general, we believe vitamin C is a promising co-adjuvant to achieve successful stem cell transplantation in PD.

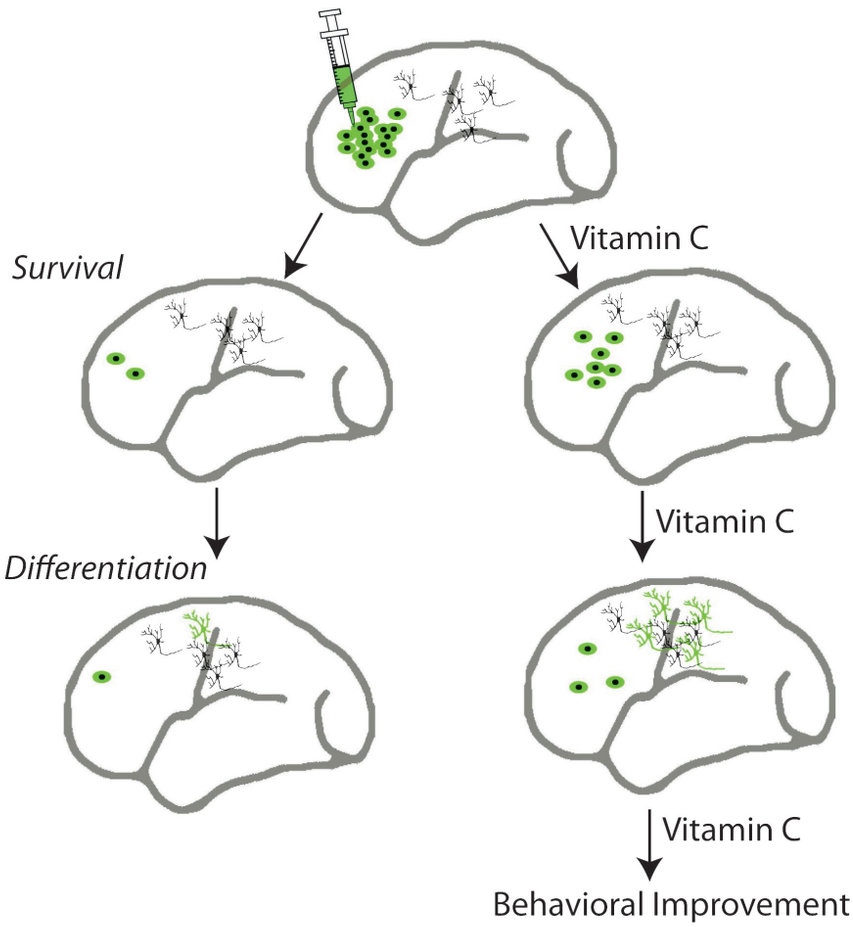

Figure 5. Vitamin C promotes survival, proliferation and differentiation of pluripotent stem cells upon transplantation in neurodegenerative disorders: In all neurodegenerative diseases, patients' lives are dramatically altered by the massive loss of neurons associated with the disease (black cells, panel). Transplantation of stem cells aims to replace the damaged tissue (injection of cells, needle, top) and correct most of the functional defects. To date, replacement therapies have generally been unsuccessful because of both the low level of stem cell survival (left, middle panel), and limited and unspecific differentiation (left, bottom panel). Vitamin C may positively regulate the outcome of stem cell therapy by regulating such cellular events (right). In presence of vitamin C, transplanted stem cells (green) become healthier (less free radical agents) favoring cellular survival and thus proliferation. This step increases the number of cells available for tissue replacement (right, middle panel). In a second step, vitamin C modulates stem cell differentiation (right, middle panel). Acting via currently unknown pathways, vitamin C induces the formation of specific cells types (in this case neurons, green), which upon proper integration, recreates the previously defective neuronal network (green and black neurons). Thus, vitamin C might ultimately lead to an improvement of behavioral alterations.

4.2.2. Alzheimer's disease

Alzheimer's disease (AD) is a devastating neurodegenerative disorder. It is characterized by the progressive loss of memory and cognitive function. Although the exact mechanistic cause(s) of AD is (are) unknown, experimental evidence has guided our attention to the accumulation of beta-amyloid peptide (Aβ-plaques) and hyperphosphorylated tau proteins (neurofibrillary tangles, NFTs) (Lambert et al., 1998; Lee et al., 2005). Animal models of AD in which Aβ plaques or NFT are expressed, have become the standard tools to study the cellular and molecular alterations present in neurons undergoing AD (Morgan et al., 2000; Mucke et al., 2000; Woodruff-Pak, 2008). Although our understanding has grown significantly in recent years, there is limited treatment for the disease (Morgan et al., 2000). The main complication for any therapeutical approach is that almost all neurons (i.e., inhibitory and excitatory neurons as well as local or projection neurons) are equally affected in AD (Woodruff-Pak, 2008). Thus, AD has no unique cellular target. A further complication is that AD affects not just one brain region but several, including cortex, hippocampus and brain stem neurons.

Stem cell transplantation is considered the restorative solution for Alzheimer's patients. However, transplantation in AD patients is complicated by the factors previously indicated (i.e., variety of cells undergoing degeneration and brain regions afflicted). Despite this fundamental problem, researchers have injected stem cells or stem cell-derived neurons into various brain regions in AD mouse models with the hope of restoring brain function. While in some cases the transplanted animals showed no improvement, in others stem cell transplantation produced a substantial improvement in cognitive function (Blurton-Jones et al., 2009; Marutle et al., 2007). The mechanisms by which these stem cell transplants achieved behavioral improvements are however controversial; optimistic researchers have suggested that the improvement is due to the replacement of damaged neurons (Marutle et al., 2007). However, more conservative investigators indicate that the injected stem cells did not replace damaged neurons but instead released trophic factors (e.g., BDNF, brain derived neurotrophic factor; NT-3, neurotrophin 3) that support cell survival and neuronal plasticity (Blurton-Jones et al., 2009). Further experiments are necessary to clarify such controversy. In any case, the results indicate that stem cell transplantation is a potentially valuable alternative for AD patients. As we indicated previously, stem cell transplantation can be enhanced by treating the patients with vitamin C because it quenches free radicals and promotes survival of the transplanted stem cell-derived neurons (Figure 5). Such improvement in neuronal survival should favor the insertion of the newly transplanted neurons into the afflicted tissue (Figure 5). In addition, vitamin C should also enhance the proliferation and differentiation of stem cells (described above). Future experiments are necessary to support our view.

4.2.3. Amyotrophic lateral sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a fatal and progressive disease in which spinal cord motor neurons die. As motor neurons and their neuromuscular connections degenerate, muscle strength decays, leading to paralysis and respiratory failure (Donkervoort and Siddique, 1993). A number of comprehensive studies have determined that approximately 10% of the ALS cases are caused by inherited genetic defects, while the rest of the cases have no obvious familial background (Blauw et al., 2012). Of the genes implicated in ALS, some (*SOD1*) encode proteins present in the mitochondria (e.g., copper-zinc superoxide dismutase type 1, SOD1) while others encode proteins that bind to DNA/RNA sequences (e.g., fused in sarcoma/translocation in liposarcoma, FUS/TLS; and 43kDa-TAR-DNA binding protein; TDP43). Mutations in any of these genes cause ALS. For example, *SOD1* mutations (G93A) alone in motor neurons and glial cells or motor neurons can cause their loss (Renton et al., 2011; Traub et al., 2011). Aberrant mitochondrial function, endoplasmic reticulum stress, axonal transport defects, and the excessive production of free radicals species have been suggested as the main causes of motor neuron degeneration (Papadeas et al., 2011; Philips and Robberecht, 2011; Van Den Bosch et al., 2006; Yamanaka et al., 2008). How the mutations in TDP-43 and FUS/TLS produce motor neuron death remains less clear. Alterations in the regulation of gene expression, RNA splicing, RNA transport and translation, as well as microRNA processing have all been implicated as causes of the disease (Buratti and Baralle, 2008). More recently, a new genomic factor was found to be responsible for ALS; the presence of hexanucleotide repeats in a gene known as *C9ORF72*, which has no known function (DeJesus-Hernandez et al., 2011; Renton et al., 2011), directly correlates with many ALS cases. Together, these findings have the potential to lead us to significant advances into understanding how the disease develops. Clinically, however there are no options; no drug has been able to stop or even delay ALS progression. For this reason, stem cell transplantation has emerged as a distinctive strategy to replace ALS afflicted motor neurons and glial cells (Kim et al., 2010; Lepore et al., 2008) . Indeed, it has been possible to differentiate stem cells into either motor neurons or glial cells *in vitro* (Dimos et al., 2008; Karumbayaram et al., 2009; Song et al., 2011). In a transgenic mouse model of ALS, stem cell transplantation delayed the disease progression, reduced motor neuron loss and improved motor function (Lepore et al., 2008). More recently, clinical trials of human ALS patients treated with either autologous or xenogenous neuronal stem cells are underway.

As with PD and AD, we believe vitamin C treatment could positively favor stem cell transplantation in ALS patients by first reducing the generation of free radicals, which are particularly toxic for motor neurons that contain low amount of molecular scavengers. Therefore, vitamin C should promote the survival of both differentiated and transplanted stem cell-derived motor neurons (Figure 5). Vitamin C should also help to promote stem cell proliferation, and differentiation by the mechanisms previously described (see above). Validation of the predicted adjuvant role of vitamin C in ALS remains to be demonstrated.

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Adult mesenchymal stem cell therapy for myelin repair in Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a demyelinating immune-mediated disease of the central nervous system (CNS). It is the most frequent neurological disease in young adults and affects over 2 million people worldwide. Current treatments reduce the relapse rate and the formation of inflammatory lesions in the CNS, but with only temporary and limited success. Despite the presence of endogenous oligodendroglial progenitors (OPCs) and of spontaneous remyelination, at least in early MS its levels and its qualities are apparently insufficient for a sustained endogenous functional repair. Therefore, novel MS therapies should consider not only immunemodulatory but also myelin repair activities. Mesenchymal stem cells (MSCs) represent an attractive alternative to develop a cell-based therapy for MS. MSCs display stromal features and exert bystander immunemodulatory and neuroprotective activities. Importantly, MSCs induce oligodendrocyte fate decision and differentiation/maturation of adult neural progenitors, suggesting the existence of MSC-derived remyelination activity. Moreover, transplanted MSCs promote functional recovery and myelin repair in different MS animal models. Here, we summarize the current knowledge on endogenous mechanisms for remyelination and proposed autologous MSC therapy as a promising strategy for MS treatment.

Key words: multiple sclerosis, remyelination, oligodendrocyte progenitor cells, mesenchymal stem cell therapy and functional recovery.

1. MULTIPLE SCLEROSIS: ETIOLOGY AND CLASSIFICATION

Oligodendrocytes are the myelin-producing cells of the central nervous system (CNS) and are responsible for electrical insulation and protection of axons. Electrical insulation is required for a salutatory conduction along the axons. Demyelination in the CNS as a consequence of a number of different pathologies leads to a variety of dysfunctions that cause a wide range of neurological symptoms resulting in physical and cognitive disabilities.

Multiple Sclerosis (MS) represents the most frequent demyelinating disease, and in young adults it is the major cause of neurological disabilities. There are approximately 2.5 million MS patients worldwide. MS primarily affects the Caucasian population from the Northern Hemisphere with a higher frequency in central and northern compared to southern Europe (Ebers and Sadovnick, 1993; Noseworthy et al., 2000). MS has a gender bias, since it appears more frequently in females than in males (Alonso and Hernan, 2008; Orton et al., 2006; Ramagopalan et al., 2010). The reason for this is unclear, but the higher MS incidence in females might be more related to specific female physiology (i.e. hormones) rather than to an MS-associated X-linked gene (Whitacre, 2001). MS patients suffer several neurological symptoms such as weakness, changes in sensation, spasticity, visual problems, fatigue and depression, acute/chronic pain, and paralysis.

Although the MS etiology is still under debate, it certainly involves an autoimmune response in which T and B cells react against CNS myelin. This causes inflammatory lesions in the CNS and culminates in the loss of oligodendroglia and in axonal degeneration (Kornek and Lassmann, 2003; Lassmann, 1998; Lassmann, 1999; Lassmann et al., 2007; Noseworthy et al., 2000; Siffrin et al.; Sospedra and Martin, 2005). The current concepts on MS etiology include i) dysregulation of the immune system and induction of an autoimmune response, ii) viral infections as the initial trigger, and iii) genetic and environmental risk factors (Ebers and Sadovnick, 1994; Noseworthy et al., 2000; Rodriguez, 2007; Sadovnick and Ebers, 1993; Sadovnick et al., 1996). For example, specific alleles of genes related to the immune response such as antigen-presentation (HLA, specifically DR genes), cell-adhesion (CD58), and cytokine receptors (IL7RA, IL2RA) have been described as genetic risk factors for MS (De Jager et al., 2009; Fugger et al., 2009; Svejgaard, 2008). In addition, smoking, lack of sunlight and vitamin D deficiency have been identified as environmental MS predisposing factors (Ascherio et al.; Hedstrom et al., 2009).

Based on the mode of progression, MS is classified in three major clinical forms: primary progressive (PP), secondary progressive (SP), and relapsing-remitting (RR) MS (Lublin and Reingold, 1996). The RR is the most frequent type, which is characterized by acute episodes of neurological dysfunction named relapses, followed by variable recovery and periods of clinical stability (remission). While RR-MS and SP-MS are most likely distinct phases of the same disease, PP-MS may imply completely different processes. More than 50% of the RR-MS patients eventually develop progressive neurological symptoms and sustained deterioration without a clear remission period. This form is called the SP variety of MS (Lublin and Reingold, 1996). Finally, between 10 and 15% of MS patients suffer form the PP type, which is characterized by the absence of remission periods (Lublin and Reingold, 1996).

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Patients with PP-MS worsen at similar speeds, while those with the RR-MS may have very different clinical courses.

2. REMYELINATION: THE PHYSIOLOGICAL RESPONSE TO MYELIN DAMAGE

The CNS is generally referred to as an organ with a limited capacity for regeneration. As a consequence, traumatic injuries, demyelinating or degenerative diseases generally result in irreversible deficits. However, endogenous repair activities exist and can be activated in order to regain structure and function. The first evocative suggestion that remyelination exists in the CNS was made by Joseph Babinski at the end of the nineteenth century. In his studies on MS pathology he illustrated demyelinated axons that displayed short areas with thin myelin sheaths, which he interpreted as remyelination. Today, it is evident that myelin sheaths are re-established along demyelinated axons, restoring structure and function (Franklin and Ffrench-Constant, 2008; Lassmann et al., 1997; Smith et al., 1979; Woodruff and Franklin, 1999).

Research on animal models has provided a substantial contribution to our knowledge of the cellular and molecular mechanisms of remyelination. Animal models to study remyelination use mainly drugs that specifically induce demyelination. Cuprizone (bis-cyclohexanone-oxaldihydrazone) is one of the toxins widely used in preclinical research. It is easily administrated orally through food pellets, and once in the organism it chelates copper, resulting in a systemic copper deficiency. For still unknown reasons, the cuprizone-induced copper deficiency affects in particular oligodendrocytes and induces a synchronous and rapid demyelination in various brain regions such as the corpus callosum (CC), superior cerebellar peduncles, cortex, olfactory bulb, hippocampus, optic chiasm, brainstem, etc (Blakemore, 1972; Blakemore, 1973; Kesterson and Carlton, 1971; Komoly et al., 1987; Ludwin, 1978; Matsushima and Morell, 2001; Silvestroff et al.; Skripuletz et al., 2008). Remyelination is quite evident one to two weeks after cuprizone removal and largely complete after four weeks (Matsushima and Morell, 2001; Silvestroff et al.). Other toxic agents that are used to investigate de- and remyelination are lysophosphatidylcholine (lysolecithin) and ethidium bromide (EtBr). These are, in contrast to the systemic application of cuprizone, injected locally into the desired site of demyelination. Lysolecithin is a membrane-dissolving agent which acts mainly on myelin-producing cells, while EtBr is a DNA intercalating agent that damages not only oligodendrocytes but also astrocytes (Woodruff and Franklin, 1999). The substances are generally injected into white matter CNS regions such as caudal cerebellar peduncle, spinal cord or CC, provoking a rapid local demyelination (Jablonska et al., 2010; Woodruff and Franklin, 1999; Zawadzka et al., 2010).

Myelin repair represents a crucial therapeutic goal for the treatment of MS. Therefore it is critical to understand how this reparative phenomenon occurs in adult CNS. Which are the cells responsible for remyelination? What is the molecular and cellular mechanism that underlies this process? One would expect that adult remyelination might recapitulate the full program of developmental myelination. However, for still unknown reasons, remyelinated sheaths end up thinner than the myelin sheaths produced during development (Blakemore, 1974; Ludwin and Maitland, 1984). Two hypotheses which might explain this observation are currently under discussion: i) since myelination depends on the coordinated interaction between oligodendrocytes and axons, the thin myelin sheath formation might be a consequence of differences in the axonal properties (Franklin and Hinks, 1999); ii) alternatively, the intrinsic remyelination capability of adult oligodendroglial progenitors might be weaker compared to those of developmental progenitors. The different proliferation rates and migratory capacities of developmental versus adult progenitors might contribute to this possibility (Wolswijk and Noble, 1989). Although neither of these hypotheses has yet been confirmed, it is more likely that myelination and remyelination display relatively different cellular and molecular mechanisms.

At least two different cellular sources for newly generated myelinating oligodendrocytes have been identified: i) oligodendroglial precursor/progenitor cells and ii) subventricular zone-derived neural stem/progenitor cells. In the early 1980s, Martin Raff and co-workers identified for the first time oligodendroglial precursor/progenitor cells (OPCs). Optic nerve-derived OPCs are proliferating cells capable of differentiating into oligodendrocytes and type 2 astrocytes (also termed O-2A progenitors) (Raff et al., 1983; Raff et al., 1984). OPCs are widely spread throughout the CNS in the white and grey matter, representing 5 to 8% of total glial cells (Levine et al., 2001). OPCs can be identified through the expression of specific markers such as ganglioside antigens recognized by the A2B5 antibody (Wolswijk and Noble, 1989), chondroitin sulfate NG2 (Dawson et al., 2000; Keirstead et al., 1998), platelet-derived growth factor receptor-α (PDGFRα) (Redwine and Armstrong, 1998) and the transcription factor olig1 (Arnett et al., 2004). There is substantial evidence that OPCs are the major source of new myelinating oligodendrocytes in adult CNS (Franklin and Kotter, 2008). First, *lacZ*-encoding retroviral tracing studies demonstrated focal lysolecithin-induced demyelination in the white matter labeled proliferating cells that give rise to remyelinating oligodendrocytes (Gensert and Goldman, 1997). Second, transplanting adult OPCs into a myelin-deficient (md) rat was shown to remyelinate nude axons (Zhang et al., 1999). Third, after focal demyelination OPC repopulation was observed before new mature oligodendrocytes appeared (Levine and Reynolds, 1999; Sim et al., 2002; Watanabe et al., 2002). Finally, the existence of cells with a transitional expression of markers for OPCs and mature oligodendrocytes argues for OPCs being the source of newly generated myelin in the adult CNS (Fancy et al., 2004; Zawadzka et al., 2010). Although Schwann cells have been thought to remyelinate solely axons of the peripheral nervous system, they can also be a source for CNS myelin (Dusart et al., 1992; Felts et al., 2005). Conversely, a recent report using a genetic fate mapping strategy demonstrated that CNS-resident PDGFRα/NG2-expressing cells (OPCs) give rise to remyelinating oligodendrocytes and to Schwann cells after chemical-induced demyelination (Zawadzka et al., 2010).

The process of OPC-derived remyelination may be divided into three steps: OPC activation, recruitment and differentiation (Bruce et al.; Franklin and Kotter, 2008). Each individual step is tightly regulated by extrinsic and intrinsic factors that act as either remyelination inhibitors or activators (Rivera et al., 2010). Upon demyelination, OPCs become mitotically active and induce the expression of oligodendrogenic genes such as Olig2 and Nkx2.2 (Fancy et al., 2004; Levine and Reynolds,

1999; Reynolds et al., 2002). The proliferation stimulus is mediated via astrocytes and microglia, which are activated upon demyelination to release mitogens that act on OPCs (Olah et al., 2012; Redwine and Armstrong, 1998; Schonrock et al., 1998; Wilson et al., 2006). OPC recruitment is intrinsically modulated by the cell cycle regulatory protein p27Kip1 (Crockett et al., 2005) and promoted by platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Murtie et al., 2005; Woodruff et al., 2004; Zhou et al., 2006). Also, the coordinated interaction between cell surface molecules and extracellular matrix (ECM) is crucial for OPC recruitment (Larsen et al., 2003). Oligodendroglial differentiation and maturation is further subdivided into the following steps: first, OPCs establish contact with bare axons, then OPCs activate myelin genes and generate the myelin membrane that finally wraps compactly around the axons forming the myelin sheath (Franklin and Kotter, 2008).

Apparently, OPCs are not the only immature cells within the adult CNS which can generate new oligodendrocytes. The original findings of newly generated neurons in the adult brain by Altman and Das (Altman, 1969; Altman and Das, 1965; Altman and Das, 1964) evoked the hypothesis of neural stem cells that are the source of new neurons in the adult brain. Neural stem/progenitor cells (NPCs) reside in a particular cellular and extracellular microenvironment called the stem cell niche, in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) of the wall of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch and Scharff, 2001; Gage, 2000). In the SVZ NPCs divide and differentiate into neuronal precursors, migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they functionally integrate and differentiate into granule and periglomerular neurons (Carleton et al., 2003; Doetsch and Scharff, 2001; Lois et al., 1996). SVZ-derived NPCs generate not only neurons but also oligodendrocytes. Retroviral tracing has demonstrated that NPCs give rise to a small subpopulation of Olig2-expressing transit-amplifying precursor cells that in turn generate PSA-NCAM/PDGFRα-positive cells (Menn et al., 2006). These cells migrate towards the corpus callosum (CC), the striatum and to the fimbria fornix, where they differentiate into oligodendrocytes (Menn et al., 2006). SVZ-derived NPCs also respond to demyelinating lesions that enhance basal oligodendrogenesis. For example, the number of SVZ-derived newly generated oligodendrocytes was significantly increased after lysolecithin-induced demyelination (Menn et al., 2006). Moreover, SVZ-derived EGF-responsive NPCs migrate to the lesion area and differentiate into remyelinating oligodendrocytes (Gonzalez-Perez et al., 2009). Upon lysolecithin-induced demyelination of the CC, SVZ-derived PSA-NCAM-expressing progenitors in the RMS increased their proliferation rate, migrated towards the injured CC and differentiated into oligodendrocytes and astrocytes (Nait-Oumesmar et al., 1999). The shift towards the oligodendroglial fate apparently involves the BMP antagonist chordin, since chordin is upregulated in the SVZ after demyelination, and elevated levels of chordin inhibit the generation of GAD65-positive and DCX-positive cells and redirect the newly generated cells towards the CC, where they differentiate into oligodendrocytes (Jablonska et al., 2010). SVZ-derived progenitors also respond to chronic demyelination as it is presented in a MS animal model. Here, SVZ-derived remyelinating oligodendrocytes were found in CC, fimbria and striatum. In white matter areas remote from SVZ such as the cerebellum, however, no SVZ-derived newly generated oligodendrocytes were found (Picard-Riera et al., 2002), suggesting that unlike OPCs, SVZ-derived oligodendrogenesis is restricted to the SVZ near regions. In conclusion, the SVZ stem cell niche constitutes a source for new oligodendrocytes. A number of crucial questions are yet to be answered: i) how similar are SVZ-derived remyelinating cells and OPCs? ii) what are the molecular mechanisms of the neuronal-oligodendroglial fate switch?

Remyelination is a reparative response to myelin damage; however during MS this phenomenon largely fails. Although some MS patients generate autoantibodies against OPC epitopes such as NG2 and might destroy OPCs (Niehaus et al., 2000), the majority of data supports a failure of OPC differentiation and maturation in MS. OPCs are typically found in demyelinated areas, but they fail to differentiate and to remyelinate (Chang et al., 2002; Kuhlmann et al., 2008; Reynolds et al., 2002; Wolswijk, 1998). Also, the proliferation of glial progenitors in the SVZ and in demyelinated lesions in MS brains is 2 to 3-fold higher than in controls (Nait-Oumesmar et al., 2007) indicating that the number of OPCs is not a limiting factor for remyelination in MS. In the acute situation, the large number of immune cells and inflammatory cytokines facilitate and promote remyelination. However, chronic MS brains show microenvironmental changes that limit remyelination. These changes include a lower level of inflammatory responses, which are required for successful remyelination (Franklin, 2002). Therefore, an impaired OPC differentiation ability and variations in the CNS inflammatory status restrict remyelination capability during MS.

3. THE OLIGODENDROGENIC PROGRAM: MOLECULAR MODULATION OF OLIGODENDROGENESIS AND MYELIN REPAIR

The generation of new myelinating oligodendrocytes (oligodendrogenesis) is a process composed of several hierarchically structured events (de Castro and Bribian, 2005; Franklin and Kotter, 2008; Liu and Rao, 2004; Miller, 2002). During oligodendrogenesis, each step is tightly regulated by context-dependent stimulatory as well as inhibitory signals that are orchestrated in an oligodendrogenic program (Rivera et al., 2010).

Several molecular signals involved in the activation of oligodendrogenesis such as PDGF and thyroid hormone (TH) have been identified. The role of PDGF in oligodendrocyte development is well established. PDGF stimulates the proliferation of OPCs, and in the absence of PDGF, OPCs exit the cell cycle and differentiate pre-maturely (Barres et al., 1994; Raff et al., 1988). PDGF might also play an important role in the process of myelination. Excess PDGF increases the number of OPCs within demyelinating lesions (Woodruff et al., 2004), and accelerates remyelination (Allamargot et al., 2001). However, PDGF infusion also induces SVZ type B cell proliferation and tumor initiation (Jackson et al., 2006). In summary, although PDGF is an attractive candidate to enhance remyelination in MS, it might have detrimental side effects such as tumor formation. Another molecule that stimulates endogenous myelin repair is TH. This hormone induces proliferation of OPCs, promotes their differentiation and finally enhances morphological and functional maturation of post-mitotic oligodendrocytes (Ahlgren et al., 1997; Billon et al., 2001;

Rodríguez-Peña, 1999). Indeed, inhibition of this hormone leads to a decrease in oligodendrocyte numbers (Ahlgren et al., 1997; Koper et al., 1986). Consistent with these findings, TH also affects myelination and remyelination. Thus it has been shown *in vivo* in different demyelination and MS animal models that TH enhances and accelerates remyelination by promoting neural progenitor differentiation into OPCs and oligodendrocytes (Calza et al., 2002; Calzà et al., 2005; Franco et al., 2008). In conclusion, TH induces OPC differentiation, maturation and enhances remyelination.

In addition to oligodendrogenesis-promoting factors, a number of molecules and transduction pathways that inhibit oligodendrogenesis and remyelination have been identified. For instance, bone morphogenetic proteins (BMPs) are rapidly up-regulated after CNS injuries and are involved in astrogliosis and glial scar formation (Fuller et al., 2007; Setoguchi et al., 2001). Upon BMP stimulation, OPCs upregulate Id2 and Id4, which sequester the pro-oligodendrogenic Olig factors and prevent them from translocating into the nucleus and thereby block their activity (Samanta and Kessler, 2004). Besides BMPs, Notch signaling has been implicated in inhibition of oligodendrogenesis. The Notch downstream targets Hes1 and Hes5 (Jarriault et al., 1998; Wang et al., 1998) inhibits neuronal and oligodendroglial differentiation and promotes astrocyte fate decision in neural progenitor cells (Artavanis-Tsakonas et al., 1999; Kageyama and Ohtsuka, 1999; Kageyama et al., 2005; Ohtsuka et al., 2001; Tanigaki et al., 2001; Wang et al., 1998; Wu et al., 2003). Notch also inhibits the expression of genes relevant for oligodendroglial maturation and myelination (Givogri et al., 2002; Jessen and Mirsky, 2008; Woodhoo et al., 2009). Therefore, Notch signaling inhibits oligodendrocyte fate decision as well as oligodendroglial differentiation, maturation and myelination. A further interesting target for remyelinating therapies is the cyclin-dependent kinase inhibitor p57kip2. In addition to cell cycle control, p57kip2 is involved in oligodendrogenesis. It has been demonstrated that in Schwann cells suppression of endogenous p57kip2 levels uncouples cellular differentiation from axonal contact (Heinen et al., 2008). A similar role for p57kip2 has now been revealed during the oligodendroglial differentiation process. Although oligodendrocytes –in contrast to Schwann cells– showed spontaneous differentiation in culture, long-term p57kip2 suppression accelerated morphological maturation as well as myelin protein expression. Moreover, p57kip2 is dynamically regulated during MS and inhibits oligodendroglial maturation (Kremer et al., 2009). Furthermore, p57kip2 regulates glial fate choice in adult NPCs, since after p57kip2 suppression a significant increase in oligodendrogenesis at the expense of astrogenesis has been noticed (Jadasz et al., 2012). Therefore, p57kip2 blocks oligodendrocyte fate decision, differentiation and maturation. Finally, Wnt signaling was shown to interfere with oligodendrogenesis during development as well as during adult CNS remyelination (Fancy et al., 2009; Shimizu et al., 2005). It has been shown that stabilization of Axin2, a negative regulator of Wnt signaling, accelerated oligodendrocyte differentiation and remyelination (Fancy et al., 2011). In summary, Wnt is an inhibitory signal for oligodendrogenesis, affecting differentiation and maturation as well as remyelination.

There are several other molecular key regulators of oligodendrogenesis and remyelination, however, a review of all factors would be beyond the scope of this review. Nevertheless, therapeutic strategies aiming to enhance pro-oligodendrogenic activities and/or to suppress anti-oligodendrogenic signals might represent an attractive possibility for the treatment of demyelinating diseases such as MS.

4. INNOVATIVE THERAPIES FOR REMYELINATION AND MS TREATMENT

Current MS treatments use disease-modifying drugs, which have proven to have only limited efficacy, primarily in the RR type of MS. These include the immune-suppressive cytokines interferon beta-1a and interferon beta-1b, the immune-modulating drug glatiramer acetate and the immune-suppressant mitoxantrone. A novel and frequently used drug is the monoclonal anti alpha4-integrin antibody natalizumab, which reduces the ability of immune cells to cross the blood-brain-barrier (BBB). All these MS treatments have major side effects, have only minor effects in the progressive forms of MS, and most likely have no repair-promoting activity.

Preclinical development of novel MS therapies widely uses the experimental autoimmune encephalomyelitis (EAE) animal model (Lassmann, 2007a). This was first described in monkeys (Rivers et al., 1933), but now mainly rodent species are used. EAE is induced by active immunization with myelin-derived antigens such as myelin oligodendrocyte protein (MOG), myelin basic protein (MBP), myelin proteolipid protein (PLP), or with immunodominant peptides from these antigens such as MOG_{35-55}. Alternatively, EAE can also be evoked through the adoptive transfer of myelin-reactive T lymphocytes (Kabat et al., 1951; Kuchroo et al., 2002). A typical susceptible rodent will debut with the first clinical symptoms around 2 weeks after immunization and develop a RR EAE. Besides the clinical symptoms, the EAE models also resemble most, if not all the pathological characteristics of MS such as demyelination, inflammation and neurodegeneration, which makes this model particularly attractive for the development of new MS therapies.

A molecular therapy might not be sufficient to target all different aspects of MS pathogenesis. MS is a multi-factorial disease with inflammatory, myelin- and axon-degenerative components. Moreover, this disease is progressive, initiating with acute episodes characterized by T and B cell infiltration and subsequent inflammatory reactions that ultimately lead to a chronic situation encompassing an anti-regenerative microenvironment (Franklin, 2002; Lassmann, 2007b). Therefore the diseased microenvironment as well as the pathogenic parameters change during the course of MS, thus it is unlikely that a single molecular therapy would be able to cover the entire range of MS pathogenesis and provide sufficient structural and functional repair. Ideally, a MS therapy should: i) target the autoimmune-inflammatory component and exert an immunemodulatory activity, ii) target the neurodegenerative component and be neuroprotective, and iii) promote structural and functional repair mechanisms such as remyelination. In this respect, a cell therapy strategy that provides all these activities might represent an attractive therapy for MS treatment.

5. MESENCHYMAL STEM CELLS TRANSPLANTATION: AN ATTRACTIVE AND PROMISING THERAPY FOR MS TREATMENT

Adult mesenchymal stem cells (MSCs) reside in the bone marrow and in most connective tissues within the body (da

Silva Meirelles et al., 2006; Minguell et al., 2001). MSCs are characterized by their capability to differentiate into cells and tissue of the mesenchymal lineage such as bone, adipose tissue, cartilage, tendons, and muscle (Minguell et al., 2001). In bone marrow MSCs also display stromal cell properties, since they regulate the activity and fate of hematopoietic stem cells (HSCs), presumably through paracrine mechanisms (Minguell et al., 2001). Therefore the dual nature of MSCs as stem and stromal cells represents an advantage for these cells to "adapt" to neural microenvironments that arise from pathological conditions such as MS. In contrast to NPCs and OPCs that are embedded in the adult CNS and thus require invasive techniques to obtain them, MSCs are highly accessible. Altogether, MSCs are multipotent, stromal and accessible cells that might represent an attractive alternative to develop an autologous cell therapy for MS treatment.

During the last decades, several research groups have evaluated the effect of MSC transplantation into the diseased CNS. MSC transplantation promotes neuroprotection and regeneration in the lesioned areas of different animal experimental models (Dezawa et al., 2001; Gerdoni et al., 2007; Hofstetter et al., 2002; Lu et al., 2005; Neuhuber et al., 2005; Zhang et al., 2005; Zhang et al., 2004). Importantly, in the case of MS, several studies have demonstrated that transplanted MSCs reduce demyelination, increase neuroprotection, modulate inflammation and enhance functional recovery in the EAE animal model (Bai et al., 2009; Barhum et al., 2010; Gerdoni et al., 2007; Gordon et al., 2008; Gordon et al., 2010; Kassis et al., 2008; Kemp et al., 2010; Lanza et al., 2009; Lu et al., 2009; Rafei et al., 2009a; Rafei et al., 2009b; Zappia et al., 2005; Zhang et al., 2009; Zhang et al., 2005; Zhang et al., 2006). A list of these studies with the most relevant findings is summarized in Table I. It seems that systemic transplantation represents the best MSC administration route compared to others. For instance, while no negative side effects have been reported when MSCs were intravenously administrated into an EAE model, MSC-derived ectopic connective tissue has been detected within the CNS of EAE mice after intracerebroventricular transplantation (Grigoriadis et al., 2011). Clinical trials are currently ongoing with MSC autologous systemic transplantation into RR-, SP- and PP-MS patients (Freedman et al., 2010; Martino et al., 2010). Moreover, in a recent preliminary study in which autologous MSC transplantation was performed in SP-MS patients, a significant functional, structural and physiological visual improvement has been described (Connick et al., 2012). Although pre-clinical and clinical trials suggest MSC transplantation as a promising therapy for MS, more studies and long-term clinical trials are necessary to provide final conclusions.

The underlying mechanisms of the therapeutic effects of MSCs are still unknown, but they may involve one or more of the following possibilities: i) transdifferentiation of MSCs into functional integrated mature neurons and/or oligodendrocytes (MSC plasticity); ii) immunoregulatory effect of transplanted MSCs on host-derived immunoreactive cells (immunemodulation); iii) bystander effects of MSCs on the survival of damaged neurons and/or oligodendroglia (neuroprotection); iv) bystander effects of MSCs on the fate and differentiation of endogenous NPCs or OPCs present at the lesion site (remyelination).

5.1 MSC neural transdifferentiation: a fact or wishful thinking?

Several studies have considered and tested the hypothesis that transplanted adult MSCs might transdifferentiate into mature neurons or glial cells, which would integrate into the

TABLE I

Summary of the main findings (X) in studies where functional recovery has been reported after MSC transplantation into EAE mice

MSCs Source	Administration Route	IM	NP	RM	References
Human bone marrow	Intravenous	**X**	**X**		(Zhang et al., 2005)
Mouse bone marrow	Intravenous	**X**	**X**		(Gerdoni et al., 2007)
Human bone marrow	Intravenous	**X**		**X**	(Bai et al., 2009)
Human bone marrow (neurotrophic factor-producing MSCs)	Intracerebroventricular	**X**	**X**		(Barhum et al., 2010)
Human bone marrow	Intraperitoneal				(Gordon et al., 2008)
Human bone marrow	Intravenous		X		(Gordon et al., 2010)
Mouse bone marrow	Intravenous, Intraventricular	**X**	**X**	**X**	(Kassis et al., 2008)
Human (CNTF-overexpressing MSCs)	Intravenous	**X**	**X**	**X**	(Lu et al., 2009)
Mouse bone marrow	Intraperitoneal				(Rafei et al., 2009a)
Mouse bone marrow	Intravenous	**X**	**X**		(Zappia et al., 2005)
Mouse bone marrow	Intravenous		X		(Zhang et al., 2009)
Human bone marrow	Intravenous		X		(Zhang et al., 2006)
Mouse bone marrow	Intraperitoneal	**X**			(Rafei et al., 2009b)

Abbreviations: Immunemodulation (IM), Neuroprotection (NP), Remyelination (RM)

damaged CNS and promote functional recovery. For instance, MSCs that were injected into the mouse lateral ventricle were later detected in the cerebellum, hippocampus molecular layer and olfactory bulb. Surprisingly, transplanted MSCs were found to express markers specific for astrocytes and neuronal lineage. Moreover, after MSCs were placed into a CNS trauma, stroke or Parkinson mouse model, transplanted cells were found to express mature astrocyte- or neuronal-specific markers (Kopen et al., 1999; Li et al., 2001; Li et al., 2000; Mahmood et al., 2001). Together this *in vivo* evidence supported a MSC transdifferentiation mechanism; however, follow-up studies revealed the possibility of fusion events between transplanted stem/progenitors cells with endogenous differentiated cells (Álvarez-Dolado et al., 2003; Kemp et al., 2010; Terada et al., 2002). This observation clearly indicates caution when interpreting results and claiming conclusions. MSC transdifferentiation has been tested under both *in vivo* and *in vitro* conditions. A number of studies have shown that the induction of neural genes in MSCs could be achieved through stimulation with non-physiological substances such as betamercaptoethanol, dimethylsulfoxide, hydroxyanisole and butylated hydroxytoluene, etc (Deng et al., 2001; Munoz-Elias et al., 2003; Padovan et al., 2003; Rismanchi et al., 2003; Sánchez-Ramos et al., 2000; Woodbury et al., 2002; Woodbury et al., 2000). The criteria to assess MSC neural transdifferentiation properties of these compounds were based on the appearance of cells exhibiting a typical neural-like morphology and/or the expression of distinctive neural-specific genes. However, it has been observed that these non-physiological compounds induce a disruption of the actin cytoskeleton and may facilitate the outcome of neurite-resembling processes (Neuhuber et al., 2004). Moreover a study by Lu and coworkers demonstrated that morphological changes and increases in immunolabeling for certain neural markers upon "neural chemical induction" of MSCs are likely the result of cellular toxicity, cell shrinkage, and changes in the cytoskeleton and do not represent a true neural transdifferentiation phenomenon (Lu et al., 2004). Consequently, caution is recommended in the interpretation of results assessing the MSC neural transdifferentiation induced by non-physiological compounds. Therefore, to avoid misleading effects *in vitro* studies should focus on the investigation of physiological inductors for MSC neural differentiation. In this respect, we have shown that soluble

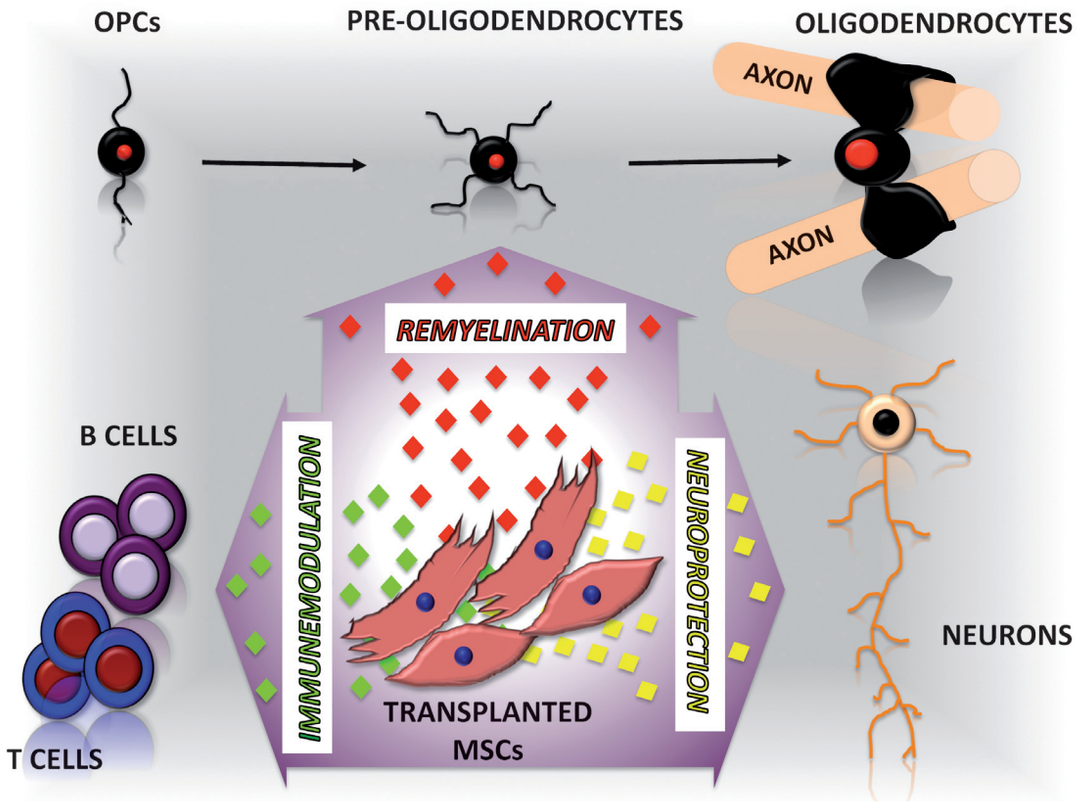

Figure 1. Therapeutic activities of transplanted MSCs in MS. Bone marrow-derived MSCs are accessible stromal multipotent cells that after transplantation display bystander therapeutic activities for MS treatment. It seems that mainly soluble factors (cytokines, growth factors, neurotrophins, etc) mediate the MSC-induced recovery in MS. Transplanted MSCs can home to and infiltrate the diseased CNS and lymph nodes. After transplantation, MSCs can modulate the immune system and inhibit encephalitogenic T and B cell activation (immunemodulatory activity (♦) in green). In addition, transplanted MSCs protect neurons and oligodendrocytes from cell death (neuroprotection activity (♦) in yellow). Finally, transplanted MSCs induce oligodendrocyte, stimulate endogenous OPCs differentiation and maturation that might enhance remyelination *in vivo* (remyelination activity (♦) in red). Therefore, MSC transplantation represents an attractive alternative to develop a novel therapeutic strategy for the treatment of MS. Abbreviations: Oligodendrocyte Progenitor Cells (OPCs), Mesenchymal Stem Cells (MSCs).

factors derived from adult hippocampus induce a neuronal-like phenotype in MSCs (Rivera et al., 2006a). However, differentiated MSCs did not display mature neuronal features. In conclusion, although some *in vivo* and *in vitro* studies indicate that MSCs might transdifferentiate into cells from the neural lineage, there is no convincing evidence and more studies are required to claim this conclusion.

Considering that MS is a CNS disease that mainly affects oligodendroglia, there is no substantial evidence showing that MSCs can transdifferentiate *in vivo* into mature remyelinating oligodendrocytes. In two different studies mouse and human-derived MSCs were systemically administrated into EAE mice. Although green fluorescent protein (GFP) labeled-MSCs were found in CNS demyelinating areas after intravenous infusion, no significant sign of MSC oligodendroglial transdifferentiation was observed (Gerdoni et al., 2007; Gordon et al., 2010). In summary, it is unlikely that neural transdifferentiation might be part of the transplanted MSC-derived repair mechanism in MS. Alternative mechanisms by which MSCs might enhance functional recovery in MS are mediated through bystander effects on the host immune system and CNS cells (Zhang et al., 2005). These activities involve CNS-homing, immunemodulation, neuroprotection and remyelination (Karussis et al., 2008).

5.2 Transplanted MSCs home into demyelinated CNS and exert immunemodulatory activity during MS

Interestingly, after systemic or intraperitoneal administration of GFP labeled MSCs, transplanted cells home and infiltrate into CNS demyelinated regions as well as into lymph nodes of EAE mice and promote functional recovery (Table I) (Gerdoni et al., 2007; Gordon et al., 2008; Gordon et al., 2010; Kassis et al., 2008; Zappia et al., 2005). These observations indicate that MSCs display CNS homing and immunemodulatory properties. The immunemodulatory effects of MSCs involve impairment of the maturation and function of dendritic cells (DCs) through the inhibition of molecules associated with antigen presentation and IL-12 release (Aggarwal and Pittenger, 2005). Additionally, MSCs inhibit the differentiation of monocytes into immature antigen-presenting myeloid DCs and modulate macrophage activity (Nemeth et al., 2009). The immunemodulatory effect of MSCs is not restricted to DCs and monocytes/macrophages, since they also influence B and T lymphocytes during MS. MSCs affect B cell proliferation and differentiation, promote T cell anergy and stimulate the production of regulatory T cells (Selmani et al., 2008; Uccelli et al., 2007; Uccelli et al., 2006). MSC-injected EAE mice display a reduction of CNS inflammatory infiltrates and a decrease in encephalitogenic T cell proliferation in lymph nodes with a subsequent reduction in demyelination (Kassis et al., 2008; Zappia et al., 2005). The MSC-derived inhibitory effect on encephalitogenic T cells has been also confirmed *in vitro* (Kassis et al., 2008; Zappia et al., 2005). Moreover, transplanted MSCs regulate the T cell phenotype and modulate the immune response in the EAE animal model (Bai et al., 2009; Liu et al., 2009; Rafei et al., 2009a; Rafei et al., 2009b). Intravenously injected MSCs inhibit Th1 and Th17 production with a concomitant increase in Th2 and anti-inflammatory cytokines, promoting functional recovery in EAE mice (Bai et al., 2009). Further studies have shown that transplanted MSCs inhibit the production of CD4 Th17 cells in a CCL-2-dependent manner (Rafei et al., 2009a; Rafei et al., 2009b). Interestingly, it seems that the immunemodulatory effect of MSCs during MS is mainly mediated through their secretome properties. In a recent study where conditioned medium derived from MSCs (MSC-CM) was infused into EAE mice, a significant decrease in pro-inflammatory cytokine (IFN-γ, IL-17, TNF-α, IL-2) expression together with a consequent increase in anti-inflammatory cytokine (IL-10, IL-14) expression has been observed (Bai et al., 2012). In this study, authors showed that at least partially, hepatocyte growth factor (HGF) signaling mediates these MSC-CM-induced changes in cytokine expression in EAE (Bai et al., 2012). Moreover, inhibition of HGF signaling decreased MSC-derived functional recovery in EAE. In summary, transplanted MSCs exert immunemodulatory activities by diverse mechanisms that might be involved in the functional recovery in MS (Figure 1).

5.3 Transplanted MSCs induce neuroprotection and enhance CNS remyelination during MS

The MSC-derived immunemodulatory activity is probably not sufficient to explain the functional recovery observed in EAE mice after MSCs transplantation. In a recent report, MSCs were transplanted into experimental autoimmune neuritis (EAN) mice, a non-MS autoimmune neuropathy. Contrary to EAE, although MSCs inhibited CD4+ T cell proliferation, transplanted cells failed to promote functional recovery in EAN (Sajic et al., 2012). This result suggests that the MSC-derived immunemodulatory activity is not sufficient to promote functional recovery in all autoimmune-based neuropathies, and therefore MSCs may exert other activities that provide better success in demyelinated diseases such as MS. Besides the immunemodulatory effects of MSCs, transplanted MSCs protect axons and oligodendrocytes from cell death. For instance, it has been shown that transplanted MSCs reduce axonal loss in EAE mice (Zhang et al., 2006). Moreover, transplanted MSCs decrease the cellular expression of proNGF and p75, reducing oligodendrocyte apoptosis and enhancing functional recovery in EAE model (Zhang et al., 2009). In addition to this, the neuroprotective effect of transplanted MSCs is endowed with a strong antioxidant effect in EAE (Lanza et al., 2009). In summary, transplanted MSCs enhance neuronal and oligodendroglial survival (Figure 1).

In addition to immunemodulatory and neuroprotective activities, MSCs display neuroreparative properties by affecting the fate of CNS endogenous progenitor cells. For example, it has been shown that transplanted MSCs enhanced endogenous oligodendrocyte differentiation and remyelination in EAE mice (Bai et al., 2009; Kassis et al., 2008; Lu et al., 2009). However, with this experimental setup (EAE model) is still not clear whether the MSC-derived regenerative effect is an indirect consequence of the MSC-mediated immunemodulation or whether MSCs could directly exert a bystander activity on endogenous progenitors. A recent study partially addressed this question by co-transplanting MSCs together with OPCs into the myelin deficient *shiverer* mouse strain (Cristofanilli et al., 2011). This study showed that MSCs enhanced OPC migration and maturation into oligodendrocytes promoting myelination in the corpus callosum. Consistent with this study, we have shown that *ex vivo* co-transplantation of MSCs together with NPCs onto hippocampal slice cultures (free of immune derived cells) induces NPCs to acquire an

oligodendrocytic phenotype, while NPCs transplanted alone generated mostly astrocytes (Rivera et al., 2009). Regarding the underlying mechanisms, we have studied the effects of MSCs on NPCs *in vitro* and demonstrated that soluble factors present in MSC-CM strongly activate oligodendrogenesis in postmitotic NPCs (Rivera et al., 2006b). First, we observed a strong increase in the number of GalC- and MBP-expressing cells in the MSC-CM treated cultures, indicating that MSC-CM promotes oligodendroglial differentiation and maturation. This was apparently at the expense of astrogenesis, since the number of GFAP-expressing cells was dramatically reduced. Moreover, we observed that MSC-CM augmented the expression of the pro-oligodendrogenic determinants Olig1/2, while it diminished the expression of Id2, a specific inhibitor of oligodendrogenesis. Therefore we suggested that MSC-CM not only promoted oligodendrocyte differentiation and maturation, but also induced oligodendrocyte fate decision, most likely through modulating the relative expression levels of Oligs/Ids (Rivera et al., 2006b). In agreement with this conclusion, we have recently published that MSC-CM primed or predisposed proliferating NPCs towards the oligodendrocyte lineage (Steffenhagen et al., 2011). Therefore, soluble factors derived from MSCs can activate NPC oligodendrogenesis at different progression stages. In the light of our findings, a recent report has shown in EAE mice that the MSCs derived oligodendrogenic and myelin repair activity or activities reside in soluble factors secreted by these cells (Bai et al., 2012). Thus, MSC-CM infusion into EAE mice enhanced oligodendrocyte development and remyelination. Hence the identification of the oligodendrogenic activity derived from MSCs becomes a priority to develop new remyelination therapies for MS treatment.

We performed a candidate approach in order to identify the MSC-derived oligodendrogenic activity. We have excluded a number of growth factors, cytokines and hormones as candidates for this activity: Insulin-like growth factor-1 (IGF-1), thyroid hormone (TH), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), transforming growth factor beta-1 (TGFbeta-1), neurotrophin-3 (NT-3), sonic hegdehog (Shh), PDGF-AA, UDP-glucose and noggin (Rivera et al., 2006b; Rivera et al., 2008). Even though ciliary neurotrophic factor (CNTF) is expressed by MSCs and promotes oligodendrocyte differentiation of adult NPCs, it did not decrease astroglial differentiation (Rivera et al., 2008). Therefore it seems that, in contrast to MSC-CM, CNTF does not induce a change in the fate of NPCs from astrocytes towards oligodendrocytes. In experiments using neutralizing antibodies we demonstrated that CNTF, although expressed by MSCs, is not involved in the pro-oligodendrogenic effect triggered by MSCs (Rivera et al., 2008). A recent study concluded that HGF mediates MSC-induced recovery in MS (Bai et al., 2012). In addition, these authors showed that HGF accelerated remyelination of lysolecithin-induced demyelinated spinal cord. Although HGF seems to represent an attractive candidate for the MSC-CM derived oligodendrogenic activity, it does not induce oligodendrogenesis in NPCs and no decrease in the proportion of oligodendrocytes generated has been observed after blocking HGF in MSC-CM (unpublished observations). The nature of the MSC-CM-derived oligodendrogenic activity remains unclear at present, but molecules other than proteins might be considered. For example, recent studies demonstrated that MSCs secrete vesicles that contain miRNAs, which could exert effects on neighboring cells (Chen et al., 2010). In summary, MSCs might activate oligodendrogenesis and contribute to remyelination in MS, but this hypothesis requires further investigation (Figure 1).

6. CONCLUSION AND FINAL REMARKS

The CNS remyelination capacity is impaired during chronic MS, since neural progenitors are insufficiently recruited into the lesion site and fail to differentiate. Current MS treatments reduce the formation of inflammatory lesions within the CNS but do not enhance endogenous myelin repair. Therefore, in addition to immunemodulation, boosting endogenous oligodendrogenesis and remyelination through cell therapies is a highly attractive alternative, since it may cover several target mechanisms in one shot. Here, MSCs represent an attractive source to develop a cell therapy for MS. First, MSCs are accessible cells, easy to obtain and thus invasive techniques can be avoided. Second MSCs can be used in an autologous transplantation mode. Third, MSCs home into the demyelinated CNS and therefore systemic transplantation rather than invasive cell administration techniques could be used. Finally, MSCs exert stromal bystander immunemodulatory, neuroprotective and eventually remyelinating activities in the damaged CNS. Therefore, autologous MSC transplantation might be considered for developing novel therapeutic approaches for MS treatment (Figure 1).

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Mesenchymal Stem Cell treatment for autoimmune diseases: a critical review

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ABSTRACT

Mesenchymal stem cells (MSCs) are now known to display not only stem cell multipotency, but also robust antiinflammatory and regenerative properties. After widespread *in-vitro* and *in-vivo* preclinical testing, autologous and allogeneic MSCs have been applied in a range of immune mediated conditions, including graft versus host disease, Crohn´s disease, multiple sclerosis, refractory systemic lupus erythematosus and systemic sclerosis. Current data suggests that MSCs may not only replace diseased tissues, but also exert several trophic, regenerative and antiinflammatory effects. While the clinical outcome in case reports and phase I-II trials seems occasionally striking, these limited results point to the need to perform controlled multicenter trials. Future advances from stem cell science can be expected to pinpoint significant MSC subpopulations and/or stem cell markers for improved regenerative or immunoregulatory properties.

Key words: Mesenchymal stem cells, autoimmune diseases, cellular therapy.

1. INTRODUCTION

Mesenchymal Stromal Cells, originally described in the 1960s as bone forming cells in the bone marrow (Friedenstein et al., 1996), are more accurately called Multipotent Mesenchymal Stromal Cells, though they are often named Mesenchymal Stem Cells (MSCs) since they display adult stem cell multipotency. Although they differentiate to bone, cartilage and other connective tissues at the single cell level *in vitro* (Pittenger et al., 1999), debate persists regarding their true multipotential capacity *in vivo*. Unlike hematopoietic stem cells originating from bone marrow, MSCs can be isolated from a variety of other sources including placenta, umbilical cord, adipose tissue, teeth and menstrual fluid (Hass et al., 2011). Their ability to differentiate into classical mesodermal tissues led to an early emphasis on the regenerative potential of MSCs; however, the findings of Bartholomew and colleagues in 2002 (Bartholomew et al., 2002) pointed to new features of these progenitor cells, the consequences of which are presently being elucidated in several areas of medicine; MSCs were found to escape T-cell recognition, suppress T cell response to mitogens and also to prolong skin graft survival in baboons. In spite of this array of effects that were proven subsequently to affect T and B lymphocytes, natural killer (NK) cells and also antigen/presenting cells (Uccelli et al., 2006; Tyndall et al., 2007), MSCs remain immune privileged. Since they exhibit low levels of major histocompatibility (MHC) class I molecules, rarely express cell surface MHC class II and do not express co-stimulatory molecules (CD40, CD40L, CD80, CD86), they escape T cell recognition (Chamberlain et al., 2007). Furthermore, their effects on immunocompetent cells are not MHC restricted, allowing allogeneic MSCs to be used with no need to match with host human leukocyte antigens (HLAs). These properties have provided the basis for the development of "off the shelf" cellular therapy when needed. In this review we analyze data on MSC treatment in immune-mediated diseases with emphasis on systemic lupus erythematosus (SLE), we discuss possible mechanisms of action of MSCs and address some areas of concern regarding stem cell treatment with MSCs.

2. USE OF MSCS IN AUTOIMMUNE AND INFLAMMATORY DISEASES

Given their vast proliferative potential, immunosuppressive properties and the ease of access to available tissue sources, therapies with autologous or allogeneic MSCs have been tested in a variety of immune-mediated disease models, including experimental allergic encephalomyelitis (Rafei et al., 2009; Bai et al., 2009) –a model of multiple sclerosis–, diabetic NOD/scid mice (Lee et al., 2006), collagen induced arthritis (Augello et al., 2007; González et al., 2009), and several lupus murine models (Zhou et al., 2008; Sun et al., 2009; Gu et al., 2010; Yamaza et al., 2010; Youd et al., 2010; Chang et al., 2011; Schena et al., 2010). Results have been mainly encouraging, but not altogether consistent, particularly in the case of arthritis (Schurgers et al., 2010), and lupus mice (Youd et al., 2010; Chang et al, 2011).

At the time of this review, 228 MSC registered human trials were found at the **National Institutes of Health** (NIH) website, including 19 for graft-versus-host disease (GVHD), 18 for diabetes, 11 for Crohn´s disease or ulcerative colitis, 7 for multiple sclerosis, 3 for amyotrophic lateral sclerosis, one each for Sjögren syndrome, rheumatoid arthritis and systemic sclerosis and 3 for SLE (http://clinicaltrials.gov/). Some of these trials point to non immune-mediated conditions associated with tissue injury such as hepatic cirrhosis, myocardial infarction or congestive heart failure. In several of these instances it has become apparent that MSCs are not necessarily replacing diseased tissues or differentiating into separate cell lineages but rather exert a complex pattern of

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trophic, regenerative and antiinflammatory effects, as we discuss later (Block et al., 2009; Chen et al., 2008).

3. CLINICAL TRIALS IN HUMAN DISEASE

Autoimmune disease is one of the top 10 leading causes of death in women up to 64 years of age (Walsh and Rau, 2000) and the second leading cause of chronic illness in the United States (Faustman, 2010). Commonly used immunosuppressant treatments lead to devastating long-term side effects; thus the NIH has recently recognized the need for "*Translation of… knowledge into new, broadly applicable strategies for treatment and prevention of [such] diseases*" (Biennial Report of the Director, NIH). Cell therapy indeed appears to be one of these broadly applicable translational strategies for autoimmune diseases.

A. Graft versus Host Disease (GVHD)

In humans, the most studied application for MSCs is GVHD, a complication of hematopoietic stem cell transplantation in which donor T cells attack an immunocompromised and genetically disparate recipient (English et al., 2010). In 2004, Le Blanc *et al*, treated a 9-year-old boy with severe treatment-resistant acute GVHD of the gut and liver with third party haploidentical mother-derived MSCs (Le Blanc et al., 2004). The clinical response was striking, with improvement of liver and intestinal function. A later phase II clinical study from the same group involved 55 steroid-resistant patients (25 children and 30 adults) with severe acute disease. Treatment with HLA-identical and haploidentical sibling donor bone marrow or third-party mismatched bone marrow MSCs induced a 70% initial response rate that was not related to age or HLA match. None of the patients had side effects either during or immediately after the MSC infusion (Le Blanc et al., 2008). The most recent placebo controlled trials have confirmed the significant improvement in liver and gastrointestinal GVHD, but did not reach significance for durable complete responses or other primary endpoints. (http://investor.osiris.com/releasedetail.cfm?releaseID=407404)

B. Crohn´s Disease (CD)

CD is a disorder of uncertain etiology that can involve the entire gastrointestinal tract with persistent transmural inflammation and fistulization. The first report of a phase I clinical trial of cell therapy using autologous adipose-derived MSCs was published in 2005. Local injection led to healing of fistulas (6/8) with no adverse effects (Garcia-Olmo et al., 2005). These results were confirmed by the same group in 2009 in a phase II multicenter in a randomized controlled trial including 49 patients with complex perianal fistulas (Garcia-Olmo et al., 2009). However, the intravenous infusion of MSCs in CD patients has produced mixed results. Onken et al. reported a clinical response (≥100 point reduction in the Crohn Disease Activity Index) in 3/9 (33.3%) patients, while Duijvestein et al. observed no efficacy in a small group of patients treated with allogeneic Bone Marrow Derived MSCs (BM-MSC) (Onken et al., 2006; Duijvestein et al., 2010). Currently, there is a phase III, multicenter, placebo-controlled, randomized and blind study to evaluate the safety and efficacy of allogeneic BM-MSCs, conducted by Osiris Therapeutics. (http://www.clinicaltrials.gov/ct2/show/NCT00482092).

C. Multiple Sclerosis (MS)

MS is the most common autoimmune inflammatory demyelinating disease of the central nervous system, often resulting in major disability. In the first report of a pilot study injecting autologous MSCs intrathecally, no significant clinical response or adverse events were observed in 10 patients with non-responsive disease, indicating the feasibility of autologous MSC for treatment of MS (Mohyeddin Bonab et al., 2007). Further phase I/II studies involving 10-15 patients each (Yamout et al., 2010; Karussis et al., 2010) confirmed the absence of adverse effects during follow-up (6-28 months). An increase in the proportion of CD4+CD25+ regulatory T cells with decreased proliferative responses of lymphocytes and activation markers on dendritic cells was detected hours after MSC transplantation (Karussis et al., 2010). Connick et al. recently reported a proof-of-concept study including 10 patients with MS treated with an intravenous infusion of autologous MSCs (Connick et al., 2012). Patients improved on measures of visual function, without evidence of significant adverse events. Progression of general disability was also reduced after treatment. The reproducibility and clinical significance of these findings remains to be confirmed. An international MSC Transplant Study Group (MSCT) has recently derived guidelines on the utilization of MSCs in MS, along with protocols for the culture of the cells and the treatment of patients (Freedmann et al., 2010)

D. Systemic Lupus Erythematosus (SLE)

Perhaps the most remarkable results of human MSC therapy emerge now from clinical trials aimed at severe, treatment-refractory SLE (Sun et al., 2010; Liang et al., 2010). While these are still uncontrolled surveys, the recent report of successful MSC treatment for other renal conditions akin to the SLE spectrum (Tögel and Westenfelder et al., 2010, Lee et al., 2010) lend support to these notoriously favorable outcomes. Taken together, these results highlight the need to advance the clinical science of stem cell therapy, identifying specific mechanisms of action and also promoting the development of safe but accessible controlled clinical protocols (Singer and Caplan, 2011).

Prompted by the positive results in the Fas-deficient MRL/lpr mice treated with human MSCs from healthy individuals (Zhou et al., 2008), Sun et al. treated four patients with active disease and lupus nephritis that was unresponsive to monthly i.v. cyclophosphamide and oral prednisone (≥ 20 mg/day) (Sun et al., 2009). The Disease Activity Index (SLEDAI) improved significantly at one, six and twelve months follow-up, as did urinary protein. CD4+ Foxp3 (T regulatory) cells increased at 3 months follow-up, and treatments were tapered and even suspended in two patients. None had complications after 12-18 months follow-up. These encouraging results led to a larger phase I trial in 15 patients also with refractory disease, including the first 4 cases reported. In this trial one third of the patients had previously failed oral mycophenolate mophetil (1-2 gr/day x 3 months) (Liang et al., 2010). Non-renal manifestations were prominent, including arthritis, severe skin disease, serositis and non-responsive cytopenias. Patients received one infusion of allogeneic BM-MSCs from passage 3-5 from non HLA matched healthy family members. Clinical and serological changes were quite dramatic for

those patients with truly severe disease as gauged by a high baseline SLEDAI, in spite of treatment with glucocorticoids and immunesuppressants. Follow-up reached 17.2 (3-36) months, with no adverse effects, deaths or ensuing GVHD. Quite surprisingly, 24 h proteinuria decreased significantly as early as one week after MSC therapy, even preceding changes in anti-dsDNA antibodies, which decreased significantly at one month and three months post transplant. T regulatory (Treg) cells, found to be quantitatively and qualitatively deficient in active SLE, (La Cava, 2008; Valencia et al., 2007), were restored at week one as judged by the percentage of CD4+ Foxp3+ cells among peripheral blood mononuclear cells.

A second trial from this group in Nanjing, China followed, reporting the use of umbilical cord-derived MSCs (UC-MSCs) also in severe lupus patients (n=16) (Sun et al., 2010). This time 5 of 15 renal cases had histological confirmation of proliferative nephritis, and 11 were preconditioned with cyclophosphamide prior to MSC infusion. Cords for MSC culture were derived from normal deliveries, minced and cultured with 10% bovine serum through passages 2-5 before use. Follow-up was only 8.25 months, but significant improvement was verified for SLEDAI score, serum albumin, 24 h urinary protein, serum creatinine, serum complement and anti-dsDNA antibodies. A decrease in serum IL-4 (with a nonsignificant increase of IFN-γ) was interpreted by the authors as a hint of improvement of pathogenic Th2 imbalance, though animal lupus models have shown rather the opposite cytokine change (Chang et al., 2011). These trials, although with shorter follow-up (8-17 months) seem to compare favorably with hematopoietic stem cell transplants in SLE that still exhibit 4-12% mortality (Burt et al.,2006; Jayne et al., 2004). Undoubtedly MSC therapy must be further explored in SLE. The EULAR Stromal Cell Group is now conducting a prospective, double-blind, comparative, multicenter trial of renal lupus treated with allogeneic MSCs (Tyndall, 2011).

More than 50 years ago Dr. Paul Klemperer suggested that the histopathological connective tissue changes found in SLE were common to *"connective tissue or collagen diseases"* (Klemperer, 1962). Little did he know that a cure for such diseases might be found within connective tissues!

E. Systemic Sclerosis (SS)

SS is an immune mediated disease with a prominent vascular and microvascular component often leading to ischemic complications (Guiducci et al., 2007) Since MSCs can differentiate to endothelial cells *in vitro* and also participate in blood vessel formation in adult tissues (Martens et al., 2006), therapy both with autologous and haploidentical third party donor MSCs has been reported, leading to striking improvement in two separate case reports (Christopheit et al., 2008; Guiducci et al., 2010). In a most interesting investigation by Akiyama, transplantation of allogeneic MSCs in 5 patients with SS triggered the induction of T cell apoptosis, lymphopenia and Treg induction, leading to skin ulcer healing in one case, and significant improvement in the Skin Score, Health Assessment Questionnaire and autoantibody titer in the whole group. In this report MSCs from SS patients were found to be deficient in the expression of FAS and FAS-L, the main molecules mediating the immunoregulatory effects described by the authors (Akiyama et. al., 2012).

4. MECHANISMS OF THE THERAPEUTIC EFFECT OF MSC TREATMENT

Despite the *in vitro* and *in vivo* evidence for therapeutic effects of MSCs, the mechanisms by which MSCs exert their immunomodulatory and reparative effects are still incompletely understood, but most likely involve multiple pathways (Figure 1).

4.1. Proinflammatory "licensing" of MSCs

In contrast to therapies that cause global immune suppression, MSCs have been dubbed as ''smart" immune modulators since their suppressive effects require a previous *licensing* step that occurs in the presence of an inflammatory environment and is mediated by the secretion of specific cytokines (Jones et al., 2007, Jorgensen, 2010). Thus, IFN-γ, alone or together with tumor necrosis factor (TNF)-α, IL-1α or IL-1β, are required to trigger the expression by MSCs of high levels of soluble factors involved in immunosuppression such as IDO, HGF, TGF-β, and NO (Aggarwal and Pittenger, 2005; Ryan et al., 2007; Krampera et al., 2006, Ren et al., 2008). The need for this activation step has been confirmed in a model of GVHD, since recipients of IFNγ−/− T cells did not respond to MSC treatment, evolving into fatal GVHD (Polchert et al., 2008). Others have attributed the immunomodulatory function of MSCs mainly to IL-6-dependent secretion of prostaglandin E2 (PGE2) (Boufii et al., 2010).

4.2. Tipping of the Th1/Th2 balance

Although still controversial, an imbalance in IFN-γ and IL-4 cytokine levels suggestive of a pathogenic T helper 2 (Th2) response has been reported in SLE. Accordingly, experimental data suggests that MSC therapy might ameliorate disease by promoting the conversion from a Th2 humoral response to a Th1 cellular immune response through modulation of IL-4 and IFN-γ levels in effector T cells. Zhou et al. showed that intraperitoneal infusion of human BM-MSCs in MRL/lpr mice decreased production of IL-4 and increased IFN-γ in peripheral blood T cells (Zhou et al., 2008). Sun et al. reported similar findings with UC-MSCs transplantation in patients with refractory SLE 3 months after treatment, also suggesting a polarization toward the Th1 phenotype, that was associated with clinical improvement (Sun et al., 2010). In gastrointestinal models of disease, pretreatment of MSCs with IFN-γ markedly inhibited dextran sodium sulfate (DSS) induced colitis in mice, leading to improvement of body weight, colitis scores and better survival rates compared to untreated mice (Duijvestein et al., 2011). Recent studies have suggested that Toll Like Receptor (TLR) signaling regulates the proliferation, differentiation and immune function of MSCs. Waterman et al. provided evidence that human MSCs polarize into distinct phenotypes following specific TLR-activation. TLR3-priming would favor an immunosuppressive (MSC2) phenotype, expressing CCL10, CCL5 and to a lesser degree IL-4 and IL-10, while TLR4-priming triggered a pro-inflammatory phenotype (MSC1), secreting IL-6 and IL-8 and even reversing MSC-established suppressive effects (Waterman et al., 2010). The *in vivo* effects of these polarized MSCs in autoimmune or proinflammatory diseases remain to be clarified.

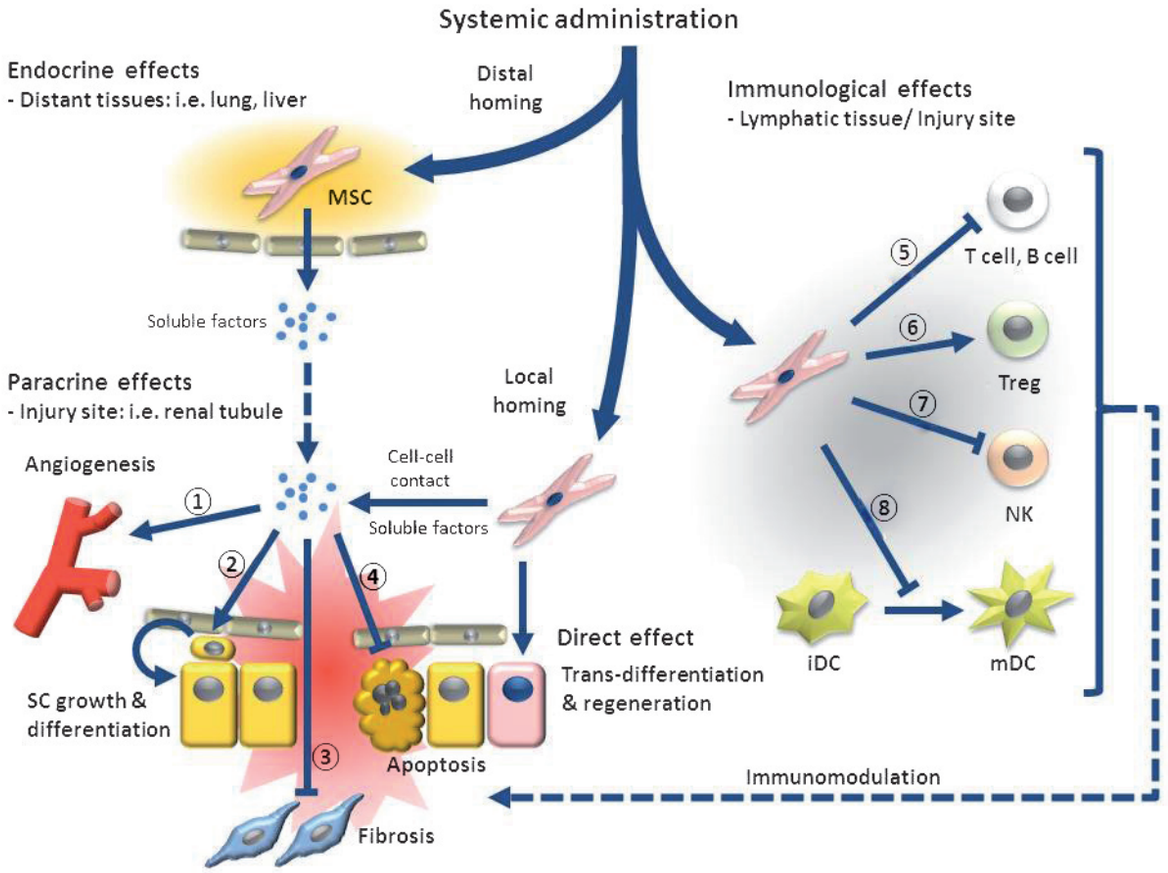

Figure 1. Systemic administration of mesenchymal stem cells can: trigger distal (endocrine) or local (paracrine) effects that include cellmediated actions. 1) Promotion of angiogenesis: vascular endothelial growth factor (VEGF), insulin like growth factor 1 (IGF-1), monocyte chemoatractant protein 1 (MCP1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL6). **2)** Stem cell growth and differentiation: stem cell factor (SCF), leukemiainhibitory factor (LIF), macrophage colonystimulating factor (MCSF), stromal derived factor 1 (SDF1), angiopoietin1 and activin A. **3)** Inhibition of fibrosis: hepatocyte growth factor (HGF), bFGF, adrenomedullin (ADM). **4)** Inhibition of apoptosis: VEGF, HGF, IGF1, transforming growth factor (TGF)β, bFGF, granulocyte macrophage colonystimulating factor (GMCSF), activin A and thrombospondin1. Immune mediated effects include the following (5 to 8). **5)** Suppression of T and B cells: human leukocyte antigen G5 (HLAG5), HGF, inducible nitric oxide synthase (iNOS), indoleamine2,3dioxygenase (IDO), prostaglandin E2 (PGE2), bFGF and TGFβ. **6)** Induction of regulatory T cells (Treg) differentiation and expansion by TGFβ expression. **7)** Inhibition of natural killer (NK) cells by secretion of IDO, PGE2 and TGFβ. 8) Inhibition of dendritic cell (DC) maturation by secretion of PGE2. Figure reproduced from Carrión and Figueroa, Stem Cell Res Ther 2011 May 11;2(3):23.

4.3. Effects on CD4 + T cell populations: Treg/Th17 ratio

Several studies have reported a quantitative and/or qualitative defect of Treg cells in human SLE, as well as increased production of Th17 proinflammatory cells (La Cava, 2008; Valencia et al., 2007; Crispin and Tsokos, 2010). MSCs have also been shown to induce the generation of functional Tregs both *in vitro* and *in vivo* (González et al., 2009; Prevosto et al., 2007; Gonzalez-Rey et al., 2010). In MLR/lpr mice, transplantation of MSCs from many sources (bone marrow, umbilical cord or exfoliated deciduous teeth), can restore Treg cells and induce a significant reduction in Th17 levels, consequently up-regulating the ratio of Treg/Th17 cells (Sun et al., 2009; Gu et al., 2010; Yamaza wet al., 2010). Recently we have shown that MSCs also generate functional active CD4+CD25+Foxp3+ T-regulatory cells during the *in vitro* differentiation phase of Th1 and Th17 cells (Luz-Crawford 2012). In human SLE the transplantation of either allogeneic or autologous MSC derived from bone marrow or UC has also increased Treg cells, suggesting that this may be one of the mechanisms of the MSC-mediated improvement of disease (Sun et al., 2009; Sun et al., 2010; Liang et al., 2010). However in two patients with active, but not highly inflammatory SLE, we reported that the infusion of autologous MSCs induced no amelioration, in spite of generating a marked increase in Treg cells (Carrión et al., 2010). In experimental autoimmune encephalomyelitis (EAE), considered a model of human MS, the administration of *ex vivo* culture-expanded MSCs has been shown to reverse neuroinflammation (Rafei et al., 2009). The effect seems to be dependent on the MSC-driven proteolytic processing of CC Chemokine Ligand 2 (CCL2) to an antagonistic derivative that interferes with CD4 Th17 cell function, thus suggesting that the therapeutic effects of MSCs in EAE occur via the paracrine conversion of CCL2 from agonist to antagonist of inflammatory cell recruitment (Rafei et al., 2009).

4.4. MSC homing and survival

One of the most relevant ongoing debates in the field of cell therapy is whether the engraftment of MSCs at the target site of injured tissues is mandatory, or can be replaced by systemic or paracrine effects. Local delivery and homing of cells toward the injury site is beneficial due to the cell-to-cell interaction with host tissues, accompanied by an increased concentration of the secreted trophic factors. However, in some preclinical models of disease, cell homing to the damaged tissue (i.e. infarcted heart or kidney) following systemic infusion remained largely inefficient. This is mainly due to the limited homing capacity of MSCs, and their entrapment in the microvasculature and other organs such as the liver and lungs (Karp and Leng Teo, 2009). Moreover, the migration of the cells was shown to be negatively affected following their *ex vivo* expansion, probably because of a lower expression of migratory and adhesion ligands such as CXCR4 and CCR1; a genetic modification of MSCs to overexpress CXCR4 was necessary to re-establish their homing properties (Cheng et al., 2008).

Long-term engraftment is another hallmark for showing the beneficial effect of stem cell-based therapies. The long-term persistence of autologous or allogeneic MSCs after a single intravenous infusion has been described in baboons, with levels of tissue engraftment ranging from 0.1% to 2.7% (Devine et al., 2003). Long-term engraftment of MSCs that differentiated to form myogenic cells in dogs with Duchenne muscular dystrophy has been recently reported (Nitahara-Kasahara et al., 2012). In NZB/W F1 lupus mice treated with $1x10^6$ human UC-MSCs via the tail vein, Chang and colleagues (Chang et al., 2011) found evidence of MSCs in kidney tissues at week 2 of infusion, but no long-term engraftment. Even if MSCs protect and improve recovery from several models of acute and chronic myocardial and renal injury (Humphreys and Bonventre, 2008; Choi et al., 2009), paracrine and endocrine effects seem most important, since conditioned medium from MSCs has been able to mimic the beneficial effects of stem cell therapy (Bi et al., 2007).

4.5. MSC paracrine factors in the repair mechanism

Cellular regeneration of an ischemic tissue necessitates massive cell supply, on the order of a billion for an infarcted heart, for example (Laflamme and Murry, 2005). Experimental studies and clinical trials have revealed that MSC-mediated therapeutic benefit might largely rely on the contribution of the secreted amounts of growth factors and cytokines rather than on their potential for differentiation into cardiomyocytes, vascular or renal cells (Bi et al., 2007). The panel of regulatory and trophic factors secreted by MSCs include a large number of growth factors, cytokines and chemokines. This MSC secretome was shown to be responsive to stress, including physiological changes (hypoxia or anoxia), small molecule stimulation and cytokine treatments (Kamota et al., 2009). Despite the absence of *in vivo* profiling of the MSC secretome and its response to disease, current MSC-based therapies have shown results largely related to a paracrine effect. Nguyen et al. showed that the injection of MSC-derived factors (MDFs) achieved protection by paracrine effects rather than direct cardiac regeneration in a swine model of myocardial infarction (Nguyen et al., 2010). The array of potential therapeutic mechanisms offered by MDFs includes antiapoptotic (Shabbir et al., 2009), anti-inflammatory (Bartosh et al., 2010), antifibrosis (Mirotsou et al., 2011), angiogenic (Kinnaird et al, 2004) and also regenerative effects. The intricacy of such factors *in vivo* has been also illustrated by Lee et al. in a model in which the reduced size of myocardial infarction in response to the infusion of human MSCs was due to the secretion of the anti-inflammatory protein (TSG-6), triggered by the entrapment of MSCs in the lung (Lee et al., 2009).

MDFs also appear to contribute to improved function and renal repair in response to MSCs (Tögel and Westenfelder, 2010). In an effort to address the mechanisms involved in the amelioration of renal disease induced by MSCs, we recently evaluated several functional and molecular markers of kidney damage and regeneration in rats subjected to 5/6 nephrectomy (NPX) (Villanueva et al., 2011). In this well known model of chronic kidney disease, a single intravenous infusion of 0.5×10^6 MSCs was associated with significant reduction of serum creatinine and inflammatory markers including macrophage infiltration and interstitial α-SMA (α-smooth muscle actin). Treated rats exhibited a significant induction in epitheliogenic molecules [Pax-2, bFGF (basic fibroblast growth factor) and BMP-7 (bone morphogenetic protein-7)], and increased expression of transcription factors Tie-2 and VEGF –involved in angiogenesis–, with respect to sham operated animals or NPX animals treated only with culture medium (Figure 2). These results are in agreement with *in vitro* studies (Tögel and Westenfelder, 2010), suggesting that there is a pathway related to vascular protection induced by MSC.

Finally, the importance of epigenetic regulatory factors in the control of biological processes and in the immune response has also been stressed. Common miRNA patterns of expression have been found in three different murine models of SLE (Dai et al., 2010), suggesting these might be targeted therapeutically. Since MSCs have been shown to secrete microparticles enrichened in miRNAs (Chen et al., 2010), several authors have suggested that microvesicle-mediated transfer of mRNA from MSC to target tissues might also participate in some of the processes involved in immunoregulation or in the recovery from kidney injury in response to stem cell treatment (Camussi et al., 2010).

5. CONCLUSIONS

A wealth of information has now accumulated linking the biology of MSCs to beneficial effects in a number of animal and human diseases. Even if the *in vivo* role of endogenous MSCs remains speculative, harnessing the therapeutic effects of *ex vivo* expanded MSCs for tissue repair and regeneration seems to have a significant clinical potential. Initial hypotheses centered on the role of the differentiation of MSCs into healthy cells and tissues have led to a wider view of MSC mediated effects, including immune modulation, paracrine and endocrine effects and even genetic regulatory mechanisms. In the midst of these scientific developments, the results of the first clinical trials with MSC therapy are undoubtedly encouraging in some cases. However, the heterogeneity of MSCs as defined today and the intricate circuitry of cellular, humoral and regenerative factors that mediate their presently known effects still point to many issues to be resolved. Long term safety concerns remain an issue, given the description of *in-vitro* malignant MSC transformation (Miura et al., 2006)

and the unknown interaction of regular immunosupressants with single or repeated MSC therapy (Spaeth et al., 2009). Regulatory and technical conditions must be defined, allowing the development of better clinical surveys. Along with the need for larger randomized controlled clinical trials, future advances from stem cell science can be expected to pinpoint significant MSC subpopulations and/or stem cell markers for regenerative or immunoregulatory properties, as well as new mechanisms of action (Psaltis et al., 2010). Thus assays for *in vitro* or *in vivo* MSC potency could be developed, leading to the use of more potent stimulated or primed pretreated MSCs. This is an exciting era for the development of safe and effective regenerative therapies, but special efforts will be required to build both the basic and clinical foundation for stem cell applications.

LIST OF ABBREVIATIONS

bFGF, basic fibroblast growth factor; BM-MSCs, bone marrow-derived mesenchymal stem cells; BMP-7, Bone morphogenetic protein 7; CD40L, CD40 ligand; dsDNA, double-stranded DNA; EGF, epidermal growth factor; GVHD, graft versus host disease; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; HLA-G, human leukocyte antigen G; HRCT, high resolution computed tomography; i.v., intravenous; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; IL-1RA, Interleukin 1 receptor antagonist; MHC, major histocompatibility complex; MSC, mesenchymal stem cell; NK, natural killer cells; NO, nitric oxide; NPX, nephrectomized; Pax-2, paired box protein Pax-2; PGE-2, prostaglandin E2; SDF-1, stromal cell-derived factor-1; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus disease activity index; Th2, T helper 2; Tie-2, angiopoietin-1 receptor; TNF, tumor necrosis factor; Treg, T regulatory cells; UC-MSCs, umbilical cord-derived mesenchymal stem cells; VEGF, vascular endothelial growth factor.

COMPETING INTEREST

The authors declare that they have no competing interests. MK holds a consulting position with "Cells for Cells" S.A.

AUTHORS' CONTRIBUTIONS

All authors contributed to the writing of the manuscript and read and approved the final version.

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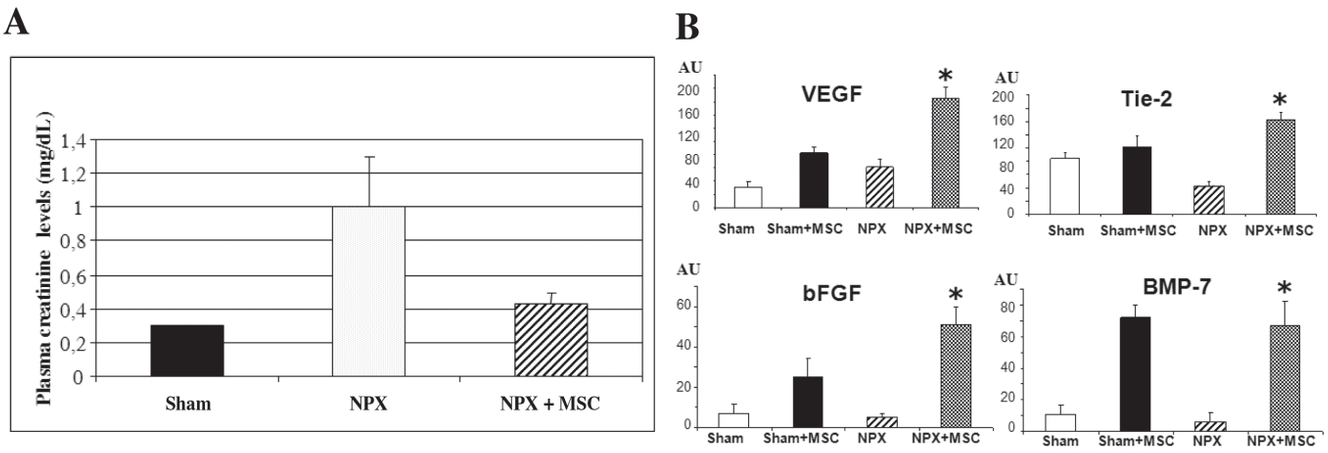

Figure 2. Functional renal damage: A. Renal function was assessed by plasma creatinine levels in sham animals (Sham) infused with fresh XVivo medium, 5/6 nephrectomized (NPX) rats infused with fresh XVivo medium and NPX injected with MSC (NPX+MSC). Sham rats injected with XVivo had normal plasma creatinine levels (0.3 mg/dL). In NPX animals, creatinine levels increased to 1.0±0.3 mg/dL which was significantly higher than both Sham groups ($p<0.05$). In rats submitted to NPX+MSC, creatinine level was reduced to 0.4±0.1 mg/dL, ($p<0.05$ *vs*. NPX group). Furthermore, creatinine values of the NPX+MSC were not significantly different from those in Sham animals.
B. Five weeks after nephrectomy, the presence of transcriptional factors involved in angiogenesis, VEGF, Tie2, and epithelial markers bFGF, BMP7 were analyzed. VEGF and Tie2 levels were minimal in Sham (31±7, 84±9 Arbitrary Units (AU) respectively) and NPX animals (61±12, 43±6 AU respectively) measured 35 days after damage. However, Sham+MSC had augmented angiogenic proteins (VEGF: 83±8, Tie2: 102±16 AU). All markers were elevated in NPX+MSC animals (VEGF: 165±16, Tie2: 142±11 AU). The differences between NPX and NPX+MSC were significant ($p<0.05$). On the other hand, a significant difference was found in epithelial markers among controls and MSC kidneys treated. The expression of bFGF and BMP7 in Sham kidneys was scarce, whereas in NPX animals the expression level was even lower (8±4 and 9±3 AU respectively). However, in Sham+MSC an increased expression of epitheliogenic markers was observed (bFGF: 25±3, BMP7: 68±13 AU) that was higher in NPX+MSC rats (bFGF: 52±7, BMP7: 67±15 AU). These differences were significant ($p<0.05$). The expression of alpha tubulin was used to correct for variation in sample loading.

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In Osteoporosis, differentiation of mesenchymal stem cells (MSCs) improves bone marrow adipogenesis

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ABSTRACT

The formation, maintenance, and repair of bone tissue involve close interlinks between two stem cell types housed in the bone marrow: the hematologic stem cell originating osteoclasts and mesenchymal stromal cells (MSCs) generating osteoblasts. In this review, we consider malfunctioning of MSCs as essential for osteoporosis. In osteoporosis, increased bone fragility and susceptibility to fractures result from increased osteoclastogenesis and insufficient osteoblastogenesis.
MSCs are the common precursors for both osteoblasts and adipocytes, among other cell types. MSCs´ commitment towards either the osteoblast or adipocyte lineages depends on suitable regulatory factors activating lineage-specific transcriptional regulators. In osteoporosis, the reciprocal balance between the two differentiation pathways is altered, facilitating adipose accretion in bone marrow at the expense of osteoblast formation; suggesting that under this condition MSCs activity and their microenvironment may be disturbed. We summarize research on the properties of MSCs isolated from the bone marrow of control and osteoporotic post-menopausal women. Our observations indicate that intrinsic properties of MSCs are disturbed in osteoporosis. Moreover, we found that the regulatory conditions in the bone marrow fluid of control and osteoporotic patients are significantly different. These conclusions should be relevant for the use of MSCs in therapeutic applications.

Key words: MSCs, osteoporosis, adipogenesis, bone marrow microenvironment

BACKGROUND

The formation, maintenance, and repair of bone tissue depend on fine-tuned interlinks in the activities of cells derived from two stem cell types housed in the bone marrow interstice. A hematologic stem cell originates osteoclasts, whereas osteoblasts derive from mesenchymal stem cells (MSCs). Bone tissue is engaged in an unceasing process of remodelling through the turnover and replacement of the matrix: while osteoblasts deposit new bone matrix, osteoclasts degrade the old one.

Bone marrow provides an environment for maintaining bone homeostasis. The functional relationship among the different cells found in bone marrow generates a distinctive microenvironment via locally produced soluble factors, the extracellular matrix components, and systemic factors (Raisz, 2005; Sambrook and Cooper, 2006), allowing for autocrine, paracrine and endocrine activities. If only the main cellular components of the marrow stroma are considered, the activity of adipocytes, macrophages, fibroblasts, hematopoietic, endothelial and mesenchymal stem cells and their progeny bring about a complex range of signals.

Osteoporosis is a bone disease characterized by both decreased bone quality and mineral density. In postmenopausal osteoporosis, increased bone fragility and susceptibility to fractures result from increased osteoclastogenesis, inadequate osteoblastogenesis and altered bone microarchitecture.

The pathogenesis of the disease is hitherto unknown, hence the interest in basic and clinical research on the mechanisms involved (Raisz, 2005; Sambrook and Cooper, 2006). Cell studies on the origin of postmenopausal osteoporosis initially focused on osteoclastic activity and bone resorption processes; then on osteoblastogenesis, and more recently on the differentiation potential of mesenchymal stem cells (MSCs) (Shoback, 2007). Moreover, distinctive environmental bone marrow conditions appear to provide support for the development and maintenance of unbalanced bone formation and resorption (Nuttall and Gimble, 2004; Tontonoz et al., 1994). In this review, we consider the participation of the differentiation potential of MSCs, the activity of bone marrow adipocytes and the generation of a distinctive bone marrow microenvironment.

MESENCHYMAL STEM CELLS (MSCs)

Bone marrow contains stem-like cells that are precursors of nonhematopoietic tissues. These cells were initially referred to as plastic-adherent cells or colony forming-unit fibroblasts and subsequently as either mesenchymal stem cells or marrow stromal cells (MSCs) (Minguell et al., 2001; Lindnera et al., 2010; Kolf et al., 2007). There is much interest in these cells because of their ability to serve as a feeder layer for the growth of hematopoietic stem cells, their multipotentiality for differentiation, and their possible use for both cell and gene therapy (Minguell et al., 2001; Kolf et al., 2007). Friedenstein et al. (1970) initially isolated MSCs by their adherence to tissue culture surfaces, and essentially the same protocol has been used by other investigators. The isolated cells were shown to be multipotential in their ability to differentiate in culture or after implantation *in vivo*, giving rise to osteoblasts, chondrocytes, adipocytes, and/or myocytes.

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MSCs populations in the bone marrow or those that are isolated and maintained in culture are not homogenous, but rather consist of a mixture of uncommitted, partially committed and committed progenitors exhibiting divergent stemness (Baksh et al., 2004). These heterogeneous precursor cells are morphologically similar to the multipotent mesenchymal stem cells, but differ in their gene transcription range (Baksh et al., 2004). It has been proposed that in such populations, cell proliferation, differentiation and maturation are in principle independent; stem cells divide without maturation, while cells close to functional competence may mature, but do not divide (Song et al., 2006).

Several molecular markers identify committed progenitors and the end-stage phenotypes, but at present there are no reliable cell markers to identify the uncommitted mesenchymal stem cells. Given the difficulty to identify a single marker to evaluate the population of stem cells, various combinations of these markers may be used (Seo et al., 2004; Lin et al., 2008; Xu et al., 2009). Therefore, MSCs are mainly defined in terms of their functional capabilities: self-renewal, multipotential differentiation and transdifferentiation (Baksh et al., 2004).

Hypothetically, the fate of MSCs appears to be determined during very early stages of cell differentiation ("commitment"). During this mostly unknown period, both intrinsic (genetic) and environmental (local and/or systemic) conditions interplay to outline the cell's fate towards one of the possible lineages. Based on microarray assays comparing gene expression at the stem state and throughout differentiation, it has been proposed that MSCs multilineage differentiation involves a selective mode of gene expression (Baksh et al., 2004; Song et al., 2006). It appears that "stemness" is characterized by promiscuous gene expression, where pluripotential differentiation results from the maintenance of thousands of genes at their intermediate expression levels. Upon commitment to one fate, only the few genes that are needed for differentiation towards the target tissue are selected for continuous expression, while the rest are downregulated (Zipori, 2005; Zipori, 2006).

The gene expression profile of undifferentiated human MSCs (h-MSCs) show high expression of several genes (Song et al., 2006; Tremain et al., 2001), but the contribution of such genes in preserving h-MSC properties, such as self-renewal and multilineage differentiation potential, or in regulating essential signalling pathways is largely unknown (Song et al., 2006). Several factors like age (Zhou et al., 2008), culture condition (Kultere et al., 2007), microenvironment (Kuhn and Tuan, 2010), mechanical strain (McBride et al., 2008) and some pathologies (Seebach et al., 2007; Hofer et al., 2010) appear to affect MSCs´ intrinsic activity.

MSCs´ commitment towards either the osteoblast or adipocyte lineage is determined by a combination of regulatory factors in the cells´ microenvironment. The adequate combination leads to the activation of lineage-specific transcriptional regulators, including Runx2, Dlx5, and osterix for osteoblasts, and PPARγ2 and a family of CAAT enhancer binding proteins for adipocytes (Murunganandan et al., 2009). Although the appropriate collection of regulatory factors required for suitable differentiation of MSCs is largely unknown, the TGF/BMPs, Wnt and IGF-I signals are briefly considered.

Several components of the BMP family are secreted in the MSCs´ microenvironment (Lou et al., 1999, Gori et al., 1999; Gimble et al., 1995); BMP-2/4/6/7 have been identified as mediators for MSCs differentiation into osteoblasts or adipocytes (Muruganadan et al., 2009). The intracellular effects of BMPs are mediated by an interaction with cell surface BMP receptors (BMPRs type I and type II) (Gimble et al., 1995). It seems that differentiation into adipocytes or osteoblasts is highly dependent on the type of receptor I expressed by the cells, so that adipogenic differentiation requires signaling through BMPR IA, while osteogenic differentiation is dependent on BMPR IB activation (Gimble et al., 1995). The active receptors trigger the activation of Smad proteins, which induce specific genes. Under osteogenic differentiation, BMP action promotes osterix formation through Runx2-dependent and Runx2-independent pathways, thereby triggering osteogenic differentiation (Gori et al., 1999; Shapiro, 1999).

In addition to the role of BMPs in bone formation, BMPs also positively mediate the adipogenic differentiation pathways (Haiyan et al., 2009). It has been demonstrated that there is a binding site for Smad proteins in the promoter region of PPARγ2 (Lecka-Czernik et al., 1999), and over-expression of Smad2 protein suppresses the expression of Runx2 (Li et al., 1998). These observations suggest that adequate content of osteoblasts and adipocytes in the bone marrow is dependent on balanced signaling through this pathway. Moreover, considering the distinct role assigned to BMPRIA and BMPRIB, the temporal gain or loss of a subtype of BMP receptors by MSCs could be critical for commitment and subsequent differentiation (Gimble et al., 1995144).

Wnt signaling in MSCs is also decisive for the reciprocal relationship among the osteo/adipogenic pathways. Activation of the Wnt/β-catenin pathway directs MSCs differentiation towards osteoblasts instead of adipocytes (Bennett et al., 2005; Ross et al., 2000; Moldes et al., 2003). Animal studies have shown that activation of the Wnt signaling pathway increases bone mass, preventing both hormone-dependent and age-induced bone loss (Bennett et al., 2005). Furthermore, Wnt activation may control cell commitment towards osteoblasts by blocking adipogenesis through the inhibition of the expression of both C/EBP and PPARγ adipogenic transcription factors, as demonstrated in vivo in humans (Qiu et al., 2007), in transgenic mice expressing Wnt 10b (Bennett et al., 2005) and *in vitro* (Rawadi et al., 2003). MSCs´ self-renewing and maintenance of the undifferentiated state appear to be dependent on appropriate canonical Wnt signaling, promoting increased proliferation and decreased apoptosis (Boland et al., 2004; Cho et al., 2006). The overexpression of LRP5, an essential co-receptor specifically involved in canonical Wnt signaling, has been reported to increase proliferation of MSCs (Krishnan et al., 2006). In addition, disruption *in vivo* or *in vitro* of β–catenin signaling promoted spontaneous conversion of various cell types into adipocytes (Bennett et al., 2002). Moreover, the importance of this pathway for bone mineral density has been highlighted by the observation that genetic variations at either the LRP5 or Wnt10b gene locus are associated with osteoporosis (Brixen et al., 2007; Usui et al., 2007).

Also, insulin-like growth factor-I (IGF-I) signalling is clearly an important factor in skeletal development. The IGF regulatory system consists of IGFs (IGF-I and IGF-II), Type I and Type II IGF receptors, and regulatory proteins including IGF-binding proteins (IGFBP-1-6) and the acid-labile subunit (ALS) (Rosen et al., 1994). The ligands in this system (i.e. IGFs) are potent mitogens, and in some circumstances differentiation factors, that are bound in the circulation and interstitial fluid

as binary (to IGFBPs) or ternary complexes (IGF-ALS-IGFBP-3 or -5) with little free IGF-I or -II. IGF bio-availability is regulated by the interaction of these molecules at the receptor level; hence changes in any component of the system will have profound effects on the biologic activity of the ligand. The IGFBPs have a particularly important role in regulating IGF-I access to its receptor, since their binding affinity exceeds that of the IGF receptors. The IGF system is unique because the IGFBPs are regulated in a cell-specific manner at the pericellular microenvironment, such that small changes in their concentrations could strongly influence the mitogenic activity of IGF-I (Jones and Clemmons, 1995; Hwa and Rosenfeld, 1999; Firth and Baxter, 2002). IGFs are expressed virtually by all tissues, and circulate in high concentrations. Although nearly 80% of the circulating IGF-I comes from hepatic sources, both bone and fat synthesize IGF-I and these tissues contribute to the total circulating pool. Locally produced IGF-I predominates over circulating IGF-I in maintaining skeletal integrity (Rosen et al., 1994; Kawai and Rosen, 2010), and both ALS and IGFBP-3 participate in regulating bone function. However, the possible autocrine/paracrine roles of IGF-I and IGFBPs in marrow (Liu et al., 1993; Peng et al., 2003) or in osteoblast (Zhao et al., 2000; Zhang et al., 2002; Wang et al., 2007) are practically unknown.

RELATIONSHIP BETWEEN THE OSTEO- / ADIPOGENESIS PROCESSES - THE FAT THEORY FOR OSTEOPOROSIS

Since in the bone marrow MSCs are the common precursor cells for osteoblast and adipocytes, adequate osteoblast formation requires diminished adipogenesis. As pointed out above, MSCs commitment and differentiation into a specific phenotype depends on hormonal and local factors (paracrine/autocrine) regulating the expression and/or activity of master differentiation genes (Nuttall and Gimble, 2004; Muruganadan et al., 2009) (Figure 1). A reciprocal relationship has been postulated to exist between the two differentiation pathways whose alteration would facilitate adipose accretion in the bone marrow, at the expense of osteoblast formation, thus decreasing bone mass (Reviewed in Rosen et, al 2009; Rodríguez et al.. 2008; Rosen and Bouxtein, 2006). Such unbalanced conditions prevail in the bone marrow of osteoporosis patients, upsetting MSC activity and the microenvironment (Nuttall and Gimble, 2004; Moerman et al., 2004; Rosen and Bouxtein, 2006). This proposition is known as the fat theory for osteoporosis. Moreover, this alteration of osteo-/adipogenic processes is also observed in other conditions characterized by bone loss, such as aging, immobilization, microgravity, ovariectomy, diabetes, and

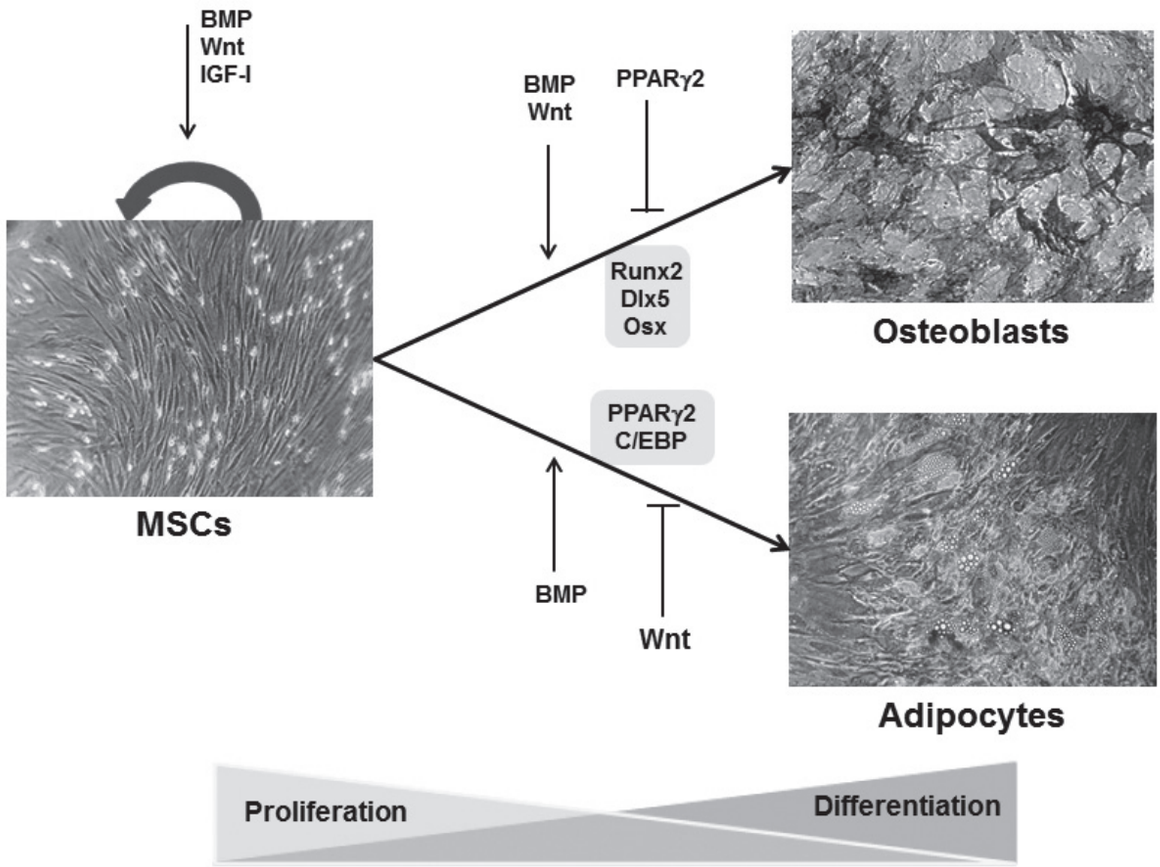

Figure 1: Schematic representation of mesenchymal stem cells (MSCs) differentiating into osteoblasts or adipocytes. Cell differentiation depends on specific hormonal and local factors regulating the expression and/or activity of master differentiation genes (enclosed in grey box). Abbreviations: MSCs: Mesenchymal stem cells, BMP: Bone Morphogenetic Protein, Wnt: IGF-1: insulin-like growth factor-1, Runx2: Runt-related transcription factor 2, Dlx5: Distal-Less Homeobox 5, Osx: Osterix, PPARγ2: Peroxisome proliferator-activated receptor gamma 2, C/EBP: CCAAT/enhancer-binding protein

glucocorticoid or tiazolidindione treatments, highlighting the harmful consequence of marrow adipogenesis in osteogenic disorders (Wronski et al., 1986; Moerman et al., 2004; Zayzafon et al., 2004; Forsen et al., 1999).

Cell studies comparing the differentiation potential of MSCs derived from osteoporotic patients (o-MSCs) with that of control MSCs (c-MSCs) have shown unbalanced osteogenic/adipogenic processes, including increased adipose cell formation, counterbalanced by reduced production of osteogenic cells (Nuttall and Gimble, 2004; Rodríguez et al., 2008; Rosen and Bouxtein, 2006). Further research on MSC differentiation has shown that activation of PPARγ2, a master transcription factor of adipogenic differentiation, positively regulates adipocyte differentiation while acting as a dominant negative regulator of osteogenic differentiation (Lecka-Czernik et al., 1999; Jeon et al., 2003; Khan and Abu-Amer, 2003). In contrast, an increase in bone mass density was observed in a PPARγ deficient mice model; even the heterozygous deficient animals showed high bone mass and increased osteoblastogenesis (Cock et al., 2004). On the other hand, Runx2 expression by MSCs inhibits their differentiation into adipocytes, as may be concluded from experiments in Runx2-/- calvarial cells, which spontaneously differentiate into adipocytes (Kobayashi et al., 2000).

In vivo observations further support the fat theory. Early studies observed that osteoporosis was strongly associated with bone marrow adipogenesis. Iliac crest biopsies showed that bone marrow from osteoporotic patients had a considerable accumulation of adipocytes in relation to that of healthy elderly women (Moerman et al., 2004; Meunier et al., 1971). More recently, increased bone marrow adiposity measured by in vivo proton magnetic resonance (^{1}H-MRS) has been associated with decreased bone mineral density in patients with low bone density (Griffith et al., 2005; Yeung et al., 2005; Blake et al., 2008).

In newborn mammals there is no marrow fat; however the number of adipocytes increases with age such that in humans over 30 years of age, most of the femoral cavity is occupied by adipose tissue (Moore and Dawson, 1990). The function of marrow fat is largely unknown; in humans it was first considered to be 'filler' for the void left by trabecular bone during aging or after radiation. Later, these cells have been proposed to have a role as an energy source, or as modulators of adjacent tissue by the production of paracrine, and autocrine factors (reviewed in Rosen et al., 2009). In fact, adipokines, steroids, and cytokines (Lee et al., 2002; Pino et al., 2010; Rosen et al., 2009;) can exert profound effects on neighboring marrow cells, sustaining or suppressing hematopoietic and osteogenic processes (Omatsu et al., 2010; Krings et al., 2012; Rosen et al., 2009; Rodríguez et al., 2008). Thus, the function of bone marrow adipose tissue may be similar to that of extra medullary fat. As such, it has been well established that unbalanced production of signaling products from subcutaneous or visceral fat modulates several human conditions including obesity, lipodystrophy, atherogenesis, diabetes and inflammation. Recent studies in mice, suggest a complex fat phenotype in the bone marrow, presenting mixed brown and white adipose properties (Lecka-Czernik, 2012). Further work is needed to find out whether differences in the quality or quantity of marrow fat, take part in deregulated bone remodelling in some bone diseases.

STUDIES ON THE ACTIVITY OF OSTEOPOROTIC MSCs

Because of their ability to self-renew, human MSCs can be expanded and differentiated in vitro, offering many perspectives for tissue engineering and regenerative medicine approaches. However, there is scarce information on whether specific diseases affect the properties of MSCs, because of the difficult accessibility to human bone marrow in health and disease (Cipriani et al., 2011; Corey et al., 2007).

Our research has focused on the properties of MSCs isolated from bone marrow of control and osteoporotic post-menopausal women. We grouped our observations on functional characteristics of o-MSCs and c- MSCs in three categories, which are summarized in Table I, as follows:

a) General activities: h-MSCs isolated from osteoporotic and control donors have similar CFU-F, but different proliferation rates. O-MSCs showed significantly diminished proliferation rate and decreased mitogenic response to IGF-I. The pERK/ERK ratio is increased in o-MSCs, compared with control c-MSCs. In other cell types, activation of the MEK/ERK signalling pathway enhances the activity of adipogenic transcription factors (Prusty et al., 2002). We also observed decreased TGF-β production by o-MSCs, as well as decreased capacity to generate and maintain a type I collagen-rich extracellular matrix, both conditions supporting cell differentiation into the adipocyte phenotype. Then, considering that the lineage fate of MSCs is dependent on early activation by specific BMPs, PPARγ and Wnt signaling (Ross et al., 2000; Rawadi et al., 2003; Westendorf et al., 2004; Baron and Rawadi, 2007), we compared the expression level of some genes related to these pathways in c- and o- MSCs. Results obtained by RT-PCR showed that in c- and o-MSCs the expression level of mRNA for β-catenin, Dkk-1, and BMPRIB was similar; while the level of mRNA for Wnt 3a was undetectable in both types of samples. The expression level of mRNA for GSK-3β, LRP6 and Osx was lower in o-MSCs than in c-MSCs, while the mRNA level for Ror2, Wnt 5a, BMPRIA showed doubtful. To further quantify the expression level of GSK-3β, LRP6, Osx, Ror2, Wnt 5a, BMPRIA real time RT-PCR was performed. As shown in Table I, statistically significant decreased mRNA levels for GSK-3β, LRP6 and Osx (0.64, 0.26 and 0.18 fold, respectively) were observed in o-MSCs, as compared to c-MSCs. In addition, mRNA levels for Ror2, Wnt 5a, and BMPRIA were similar in both types of cell samples.
   These data suggest impaired regulation by the BMPs and Wnt pathways in o-MSCs, representing some intrinsic deviation from control cells that might underlie the impaired self-renewal, and adipogenic/osteogenic differentiation potential observed in o-MSCs. mRNA levels for Ror2, Wnt 5a, and BMPRIA were similar in both types of cell samples.

b) Differentiation potential of cells: under osteogenic differentiation conditions, cells derived from osteoporotic donors had diminished alkaline phosphatase activity and less calcium deposition, compared with cells from control donors, in agreement with their reduced ability to form mature bone cells. On the other hand, the increased

adipogenic potential of o-MSCs was tested by incubating cells in adipogenic medium; under this condition o-MSCs showed favoured adipogenesis compared with c-MSCs. In conjunction, these observations sustain the notion that in the bone marrow of osteoporotic women, fat overload occurs at the expense of osteogenesis (Meunier et al., 1971).

c) Adipocyte characteristics: Adipocytes derived from both MSCs types were similar in cell size and granularity (unpublished observations); however, the fluorescence index in adipocytes originated from c-MSCs was significantly higher than those from o-MSCs (Table I), suggesting that c- and o-adipocytes differ in the quality of their lipid content. As far as we know, this is the first observation on qualitative differences in the lipid content among c- and o-adipocytes, matching some observations in the quality of lipids in the bone marrow fluid (Li et al., 2012).

STUDIES ON THE ACTIVITY OF BONE MARROW FLUID OF POST-MENOPAUSAL WOMEN

Distinctive environmental bone marrow conditions appear to support the development and maintenance of the balance between bone resorption and bone formation. Knowledge is

TABLE I

Functional characteristics of osteoporotic- and control- MSCs

	Condition	Incubation Time (days)	c-MSCs	o-MSCs	Reference
General Activities:					
Total Colonies Number (CFU-F)	Basal	14	12.7±5.6	14.1±2.6	Unpublished observations
Proliferation rate	Basal		High	Low	Rodríguez et al. 1999
IGF-1 mitogenic response (0 – 50 ng/ml)	Basal	4	Yes	No	Rodríguez et al. 1999
p-ERK/ERK	Basal	3	0.55±0.05	1.3±0.25	Rodríguez et al. 2004
TGF-β Synthesis (units/10^6 cells)	Osteogenic	14	16	7	Rodríguez et al. 2000
Collagen Type I Synthesis (μg/10^6 cells)	Basal	1	10.2±1.9	5.1±2.7	Rodríguez et al. 2000
GSK-3β mRNA level (relative to c-MSCs)	Basal	-	1.06±0.21	0.56±0.05*	Unpublished observations
LRP6 mRNA level (relative to c-MSCs)	Basal	-	1.00±0.30	0.197±0.05*	Unpublished observations
Osx mRNA level (relative to c-MSCs)	Basal	-	1.023±0.48	0.098±0.04*	Unpublished observations
Differentiation potential:					
Alkaline Phosphatase Activity (μmol PNP/min/10^6 cells)	Osteogenic	12	19.4±1.16	7.8±0.28	Rodríguez et al. 1999
Calcium Deposition (μg/plate)	Osteogenic	16	34±0.5	14.5±1.1	Hess et al, 2005
Adipocytes (%)	Adipogenic	14	11.5±3.3	22.3±6.5	Hess et al, 2005
Adipocytes characteristics:					
Granularity	Adipogenic		326±147	493±152	Unpublished observations
Size	Adipogenic		87.5±23.8	95.2±3.7	Unpublished observations
Fluorescence Index	Adipogenic	14	3.64±0.43	2.13±0.15*	Unpublished observations

Basal: Non differentiation condition; OS: Osteogenic differentiation condition; AD: Adipogenic differentiation condition; * $p<0.05$.
GSK-3β: Glycogen Synthase Kinase-3
LRP6: low-density lipoprotein-related receptor protein-6
TGF-β: Transforming growth factor beta
Osx: Osterix
IGF-1: Insulin-like growth factor 1
pERK: Phospho-Extra-cellular regulated kinase
ERK: Extra-cellular regulated kinase

scarce about the intramedullar concentration of compounds with recognized regulatory effects on bone formation or resorption and is limited to some pathologic conditions or estimated from measurements in plasma (Wiig et al., 2004; Iversen and Wiig, 2005; Lee et al., 2002; Khosla et al., 1994). Measurement of soluble molecules found in human bone marrow has been particularly difficult, not only because of tissue seclusion, but also because of the complicated anatomy and blood perfusion of bone. Since it may be expected that concentrations measured in the bone marrow fluid (BMF) more reliably reflect the physiologically relevant levels in the interstitial compartment surrounding the bone cells than values found in blood, we isolated the extracellular bone marrow fluid by directly spinning bone marrow samples for 20 min at 900xg. Considering the complex organization in such a regulatory milieu, we opted for evaluating some molecules recognized as markers of adipocyte, proinflammatory or osteoclastic/osteoblastic activity (Pino et al., 2010).

The concentrations of cytokines or receptors measured in the bone marrow extracellular fluid from control and osteoporotic human donors are indicated in Table II. In addition, the concentrations of IGF-I and its IGFBPs were analyzed, as well as the C-terminal telopeptide cross-links of type I collagen (CTX). Results summarized in Table II indicate significantly different concentrations of regulatory molecules in the extracellular fluid of control versus osteoporotic women; this last group was characterized by higher content of proinflammatory and adipogenic cytokines. Also, osteoporotic samples showed decreased leptin bioavailability, suggesting that insufficient leptin action may characterize the osteoporotic bone marrow (Pino et al., 2010). In addition, bioavailability of IGF-I appears diminished in o-BMF, as shown by the increased IGFBP3/IGF-I ratio.

Taken together our results and those of other researchers identify significant differences between functional properties of control and osteoporotic MSCs, displayed *in vitro*, in cells under basal or differentiating conditions. Moreover, it can be concluded that such divergence prevails also *in vivo*, because the bone marrow fluid of osteoporotic patients characterizes by unfavourable content of several regulatory molecules. Therefore, the properties of both MSCs and bone marrow microenvironment are significantly impaired in osteoporotic patients, negatively affecting bone formation.

CONCLUSIONS

In the pathogenesis of osteoporosis, impairment of both MSCs functionality and microenvironment add to the known detrimental effect of increased osteoclast activity, resulting in decreased bone formation.

O-MSCs are characterized by intrinsic functional alteration leading to poor osteogenic capability and increased adipogenesis. Osteoporotic bone marrow microenvironment differs from the control microenvironment by increased concentration of pro-adipogenic and pro-inflammatory regulatory factors.

The content and/or quality of adipocytes in the bone marrow appear critical to delineate impairing of MSCs; in this

TABLE II

Regulatory activity in bone marrow fluid of post-menopausal women

Regulating Factor concentration	Control BMF	Osteoporotic BMF	Reference
Interleukin-6 (pg/mL)	4.8±2.5	6.2±2.5*	Pino et al. 2010
Soluble interleukin-6 receptor (ng/mL)	33.7±13.1	47.0±13.7*	Pino et al. 2010
TNF-α (pg/mL)	72.3±55.0	148.9±82.0*	Pino et al. 2010
Adiponectin (μg/mL)	9.5±2.4	5.7±2.7*	Pino et al. 2010
Soluble RANKL (pmol/L)	0.27±0.16	0.14±0.05*	Pino et al. 2010
Osteoprotegerin (pmol/L)	2.9±0.9	4.4±1.8*	Pino et al. 2010
Leptin (ng/mL)	14.5±11.3	7.0±4.4*	Pino et al. 2010
Soluble leptin receptor (ng/mL)	44.6±14.7	48.9±17.8	Pino et al. 2010
Leptin bioavailability	0.33±0.22	0.15±0.16*	Pino et al. 2010
IGF-1 (ng/ml)	76,1±25,4	48,2±18,5*	Xian et al. 2012
IGFBP-3 (ng/ml)	24,52±5,98	27,88±8,52	Unpublished observations
IGF-1/IGFBP-3	3.1	1.72	Unpublished observations

BMF= Bone marrow fluid. *$p<0.05$.
TNF-α: Tumor necrosis factor alpha
RANKL: receptor activator of Nuclear Factor κ Beta ligand
IGFBP: Insulin-like growth factor binding protein

sense osteoporosis could be homologated to other age-related diseases such as obesity, atherogenesis and diabetes, which are characterized by extramedullar unbalanced adipocyte formation and signaling.

Currently it is not known how damaged o-MSCs emerge, further work is needed to ascertain the role of the microenvironment, and genetic and epigenetic factors, as proposed for other stem cells-related pathologies.

The conclusion that intrinsic properties of MSCs are altered in osteoporosis should be relevant for the therapeutic use of MSCs, which represent an interesting promise for regenerative medicine for several severe human diseases.

The possibility of reversing o-MSCs impairment opens new perspectives for osteoporosis therapy.

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MSC transplantation: a promising therapeutic strategy to manage the onset and progression of diabetic nephropathy

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ABSTRACT

Currently, one of the main threats to public health is diabetes mellitus. Its most detrimental complication is diabetic nephropathy (DN), a clinical syndrome associated with kidney damage and an increased risk of cardiovascular disease. Irrespective of the type of diabetes, DN follows a well-known temporal course. The earliest detectable signs are microalbuminuria and histopathological changes including extracellular matrix deposition, glomerular basement membrane thickening, glomerular and mesangial expansion. Later on macroalbuminuria appears, followed by a progressive decline in glomerular filtration rate and the loss of glomerular podocytes, tubulointerstitial fibrosis, glomerulosclerosis and arteriolar hyalinosis. Tight glycemic and hypertension controls remain the key factors for preventing or arresting the progression of DN. Nevertheless, despite considerable educational effort to control the disease, a significant number of patients not only develop DN, but also progress to chronic kidney disease. Therefore, the availability of a strategy aimed to prevent, delay or revert DN would be highly desirable.

In this article, we review the pathophysiological features of DN and the therapeutic mechanisms of multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs). The perfect match between them, together with encouraging pre-clinical data available, allow us to support the notion that MSC transplantation is a promising therapeutic strategy to manage DN onset and progression, not only because of the safety of this procedure, but mainly because of the renoprotective potential of MSCs.

Key words: Regenerative medicine. Diabetes mellitus. Diabetic nephropathy. Multipotent mesenchymal stromal cells. Mesenchymal stem cells.

According to the World Health Organization, the total number of people with diabetes mellitus (DM) is projected to rise from 285 million in 2010 to 439 million in 2030 (Shaw et al., 2010). Regarding its etiology, DM is classified as type 1 (T1DM) or type 2 (T2DM). While T1DM is due to autoimmune destruction of pancreatic beta cells leading to insulin deficiency (Cnop et al., 2005), T2DM is a metabolic disorder due to insulin resistance along with impaired insulin secretion (Cnop et al., 2005). Over the past decades, medical advances have substantially improved the management of patients with DM, thereby prolonging their survival (Penfornis et al., 2011). Nevertheless, available treatments do not guarantee a tight glycemic control, since patients do not often adhere well to medical indications. Thus even under treatment patients develop chronic macro- and microvascular diseases including stroke, neuropathy, retinopathy and nephropathy (Stolar, 2010; Maric and Hall, 2011). Among these, diabetic nephropathy (DN) is the most detrimental consequence with regard to both premature morbimortality and medical expenses (Blazquez-Medela et al., 2010; Stolar, 2010). Furthermore, DN represents a major concern for public health worldwide, since 25% to 40% of the patients with DM develop it, and also because as DN progresses to end-stage chronic kidney disease, patients require hemodialysis and even kidney transplant (McCrary, 2008; Reutens and Atkins, 2011).

PATHOPHYSIOLOGICAL FEATURES OF DIABETIC NEPHROPATHY

DN is a clinical syndrome consisting of kidney damage and increased risk of cardiovascular diseases. Its main risk factors are gender, genetic factors, renal hemodynamics and age of DM onset (Blazquez-Medela et al., 2010). Although the time of clinical debut of DN varies between patients with T1DM and T2DM, clinical and histological progressions in both conditions are quite similar (Najafian et al., 2011). Changes in the filtration unit begin soon after DM onset, and take place "silently" for a long time before the appearance of the first clinical signs of the disease. In susceptible patients DN follows a well-known physiopathological course (Figure 1). Microalbuminuria is the earliest clinically detectable sign of kidney damage. It is associated with histological changes that include extracellular matrix deposition, glomerular basement membrane thickening and glomerular mesangial expansion. In later stages patients develop macroalbuminuria, followed by a progressive decline in the glomerular filtration rate. At this stage, histological changes include glomerulosclerosis, tubulointerstitial fibrosis and arteriolar hyalinosis (Najafian et al., 2011).

Although hyperglycemia itself is not sufficient to provoke development of DN, the main promoting factors are the following metabolic and hemodynamic alterations (Figure 1):

– **augmented oxidative stress.** High glucose flux increases the production of superoxide anions in the mitochondrial electron transport chain (Rolo and Palmeira, 2006). Excessive production of superoxide anions results in the formation of more superoxide anion and secondary reactive oxygen species (ROS) including peroxynitrite and hydroxyl radicals, which modify DNA, proteins and lipids (Brownlee, 2001). Along with a deregulation of anti-oxidant enzymes, increased oxidative stress leads to endothelial damage (Evans et al., 2002). Furthermore, ROS up-regulate

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the expression of TGF-beta1, PAI-1 and extracellular matrix (ECM) proteins in glomerular mesangial cells, triggering mesangial expansion (Fujita et al., 2009).

– **accumulation of advanced glycation end products and fibrosis**. High glucose concentration results in non-enzymatic glycation of proteins, lipids and nucleic acids (Yamagishi and Matsui, 2010). These advanced glycation end products (AGEs) interact with membrane receptors that induce crosslinking of ECM proteins and slow down their turnover. Thus the normal interactions among ECM proteins are disrupted in a way that compromises their function and leads to fibrosis. Furthermore, aberrant cell-ECM interactions lead to the alteration of cell adhesion, proliferation and epithelial phenotype maintenance, stimulating epithelial-to-mesenchymal transition (Simonson, 2007; Yamagishi and Matsui, 2010). In addition, AGEs induce ROS synthesis and ROS accelerate AGE formation (Singh et al., 2011). This positive feedback between AGEs and ROS worsens renal tissue damage.

– **chronic inflammation**. DN was considered to be a non-immune disease. Nevertheless, it has been shown recently that inflammation is crucial for the development of microvascular complications of DM, including nephropathy (Mora and Navarro, 2006; Ortiz-Munoz et al., 2010). Accordingly, lymphocytes, monocytes and macrophages have been involved in DN progression (Galkina and Ley, 2006; Ninichuk et al., 2007). Moreover, it has been proved that IL-1beta, IL-6 and TNF-alpha are relevant for the development of DN (Mocan et al., 2006; Mora and Navarro, 2006). IL-1beta and IL-6 increase vascular endothelial permeability and alter ECM dynamics at both the mesangial and podocyte levels, contributing to interstitial infiltrates, glomerular basement membrane thickening, mesangial expansion, and tubular atrophy (Pecoits-Filho et al., 2002; Dalla et al., 2005). TNF-alpha is cytotoxic to renal cells and contributes to sodium retention and renal hypertrophy, alterations that are observed during the earlier stages of DN (DiPetrillo et al., 2004). Also, the exposure of tubular epithelial cells to TNF-alpha results in a significant increase in the synthesis and secretion of lymphocyte- and neutrophil-chemoattractant factors, and in the cell surface expression of ICAM-1 (Ishikura et al., 1991). Accordingly, the up-regulation of MCP-1 and ICAM-1 in the kidney has been associated with macrophage and lymphocyte recruitment, urinary albumin excretion, tubulointerstitial injury and DN progression (Matsui et al., 1996; Chow et al., 2006). In addition, TNF-alpha directly promotes the local generation of ROS, resulting in the alteration of the function of glomerular capillary barrier wall, which allows the permeation of albumin (McCarthy et al., 1998).

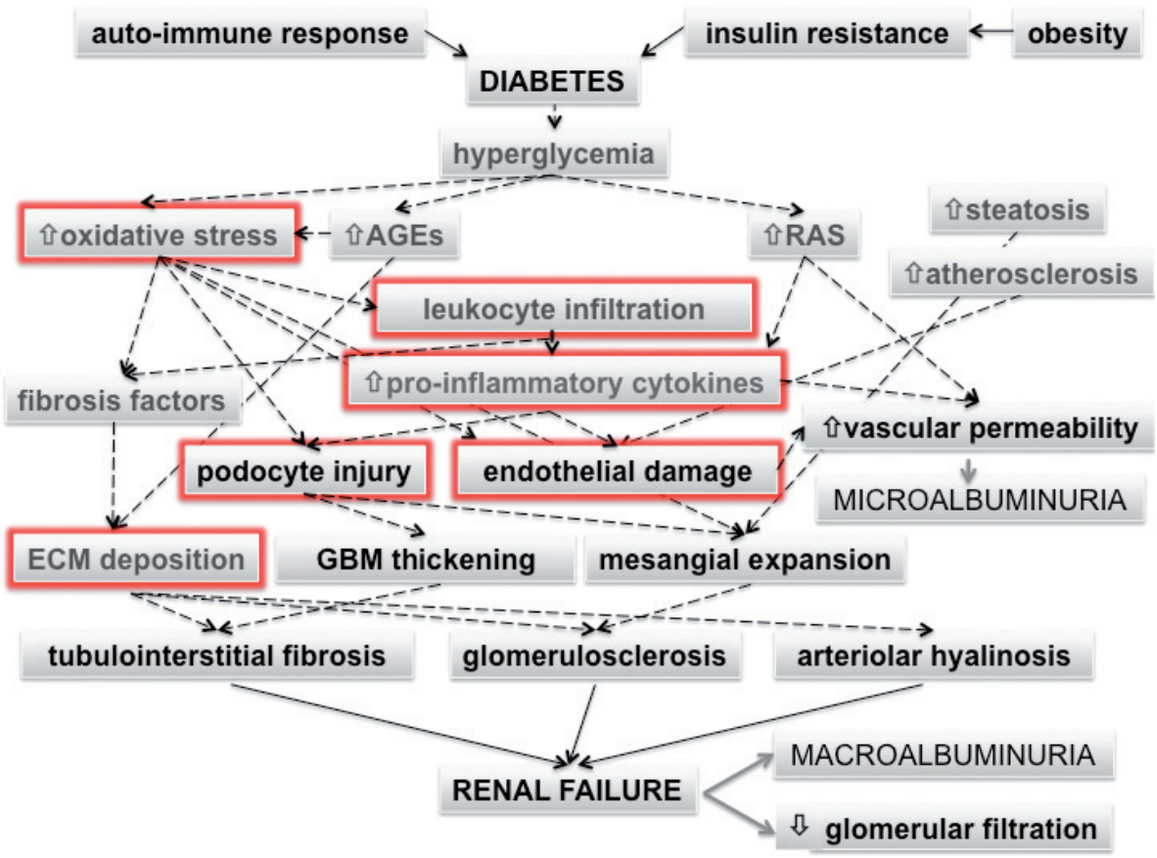

Figure 1. Pathophysiological features triggered at the onset and required for the maintenance of DN: The putative therapeutic targets of donor MSC that might contribute to the prevention, delay or reversion of DN are highlighted in red.
AGEs: advanced glycation end products, RAS: renin-angiotensin system, ECM: extracellular matrix, GBM: glomerular basement membrane.

– **altered renin-angiotensin system**. Angiotensin II shows increased activity during DN, and causes hypertrophy of mesangial and tubular epithelial cells (Chawla et al., 2010). Also, it has pressor effects on arteriolar smooth muscle, increasing vascular pressure. Furthermore, Angiotensin II induces inflammation, apoptosis and promotes the production of TGF-beta and MCP-1, two prosclerotic cytokines that have been identified as responsible for glomerular sclerosis.

– **steatosis and atherosclerosis.** Patients with T2DM present additional factors that aggravate renal damage, i.e. obesity, dyslipidemia, atherosclerosis that results in renal ischemia and hypertension (Maric and Hall, 2011; Packham et al., 2011). Lipid deposition in the kidneys produces direct glomerular injury, and may also result in glomerular mesangial cell activation and proliferation (Wang et al., 2005). Activation of these cells leads to chemokine production, which promotes the recruitment of monocytes and their maturation into macrophages in the mesangium. Furthermore, it has been shown that renal mesangial and tubular cells grown in culture and incubated with LDL or VLDL up-regulate the expression of TGF-beta and PAI-1 and accumulate ECM proteins (Vaziri and Norris, 2011), thus demonstrating that lipids have a direct role in the activation of glomerulosclerosis mediators.

AVAILABLE STRATEGIES TO MANAGE PATIENTS WITH DIABETIC NEPHROPATHY

There is currently no cure for patients with DN. Palliative therapeutic strategies include the use of drugs to control hyperglycemia, blood pressure and proteinuria (Yamagishi et al., 2007; Choudhury et al., 2010). In advanced stages patients receive renal replacement therapy, which consists of hemodialysis and, if possible, kidney transplantation (Reutens and Atkins, 2011). Unfortunately, the latter is only useful when the kidney is co-transplanted with pancreatic beta-islets; if this is not done, renal failure reappears (Fioretto and Mauer, 2012). Therefore, the need for therapeutic strategies to prevent, delay or revert DN is compelling.

STEM CELL-BASED STRATEGIES TO MANAGE PATIENTS WITH DIABETIC NEPHROPATHY

Pharmacological interventions often target only a single pathophysiological feature of the disease, e.g. the inhibitors of the renin-angiotensin system used for the management of patients with DN suppress urinary albumin excretion in a relatively short term but do not prevent renal function decline and the progression to end-stage chronic kidney disease (Jerums et al., 2008). Conversely, stem cell-based intervention is known to act through multiple mechanisms, a clear advantage when facing diseases with highly complex pathophysiology, as is the case of DN. Choosing the adequate stem cell for this purpose should take into account the following notions:

– **embryonic stem cells** are obtained from the inner cell mass of a blastocyst and are pluripotent; i.e., they can give rise to endo-, meso- and ectodermal cells. Their teratogenicity raises a major concern regarding biosafety. Bioethical, religious and political issues have limited the studies aimed to evaluate their putative massive therapeutic use (Mertes and Pennings, 2009).

– **induced pluripotent stem cells** are generated after somatic cell reprogramming (Takahashi et al., 2007). These cells are pluripotent and teratogenic, thus they share the biosafety concerns with embryonic stem cells. To avoid this problem, researchers have proposed that pluripotent stem cells should be first differentiated *in vitro* and then transplanted. This imposes at least two major technical problems to solve; the definition of proper and efficient differentiation conditions, and the development of procedures for the delivery of differentiated cells into damaged tissues.

– **adult stem cells** are found in all non-embryonic tissues; hence they may be isolated from fetus, newborn, child and adult individuals. They contribute to both maintenance of cellular homeostasis and regeneration of damaged organs. Adult stem cells are multipotent, and due to their limited self-renewal potential, not teratogenic. Some of them also have plasticity, i.e., they can differentiate into cells from lineages different from their origin (Phinney and Prockop, 2007).

Since adult stem cells pose less bioethical and technical concerns, the first candidate for a stem cell-based strategy to treat DN was bone marrow-derived stem cells. These cells have shown to contribute to the regeneration of damaged kidneys (Kale et al., 2003; Poulsom et al., 2003). Accordingly, bone marrow-derived stem cells have been shown to differentiate or transdifferentiate into mesangial cells (Ito et al., 2001; Imasawa et al., 2001), tubular epithelial cells (Poulsom et al., 2001), endothelial cells (Rookmaaker et al., 2003), and podocytes (Prodromidi et al., 2006).

Bone marrow harbors at least two distinct adult stem cells; the hematopoietic stem cells that give rise to blood and endothelial cells (Wagers and Weissman, 2004) and the multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), that give rise to adipocytes, chondrocytes, osteocytes and myocytes (Minguell et al., 2001; Dominici et al., 2006). Additionally, it has been suggested that MSCs might cross the germ line barrier and generate cells from the endo- and ectodermal lineages (Phinney and Prockop, 2007).

The main advantage of MSCs over hematopoietic stem cells for clinical use is their hypoimmunogenicity, since histocompatibility between donor and receptor is not required. Also, recipients do not need to be conditioned before MSC transplantation, as is the case in total bone marrow or hematopoietic stem cell transplantation (Uccelli et al., 2008). Furthermore, it is currently agreed that MSCs contribute to tissue regeneration not only because of their differentiation potential, but also because of the following therapeutic mechanisms (Figure 2):

– **scavenging of oxidative stress.** MSCs are highly resistant to *ex vivo* culture and ionizing radiation, which are two conditions that generate strong oxidative stress. Recently we demonstrated that the low susceptibility of MSCs to the deleterious effect of ROS and reactive nitrogen species correlates with the ability of these cells to effectively scavenge peroxide and peroxynitrite, due to the constitutive expression of SOD1, SOD2, CAT and

GPX1 enzymes and high levels of glutathione (Valle-Prieto and Conget, 2010). Moreover, MSCs possess the main enzymatic mechanisms to detoxify reactive species and to prevent oxidative damage of the proteome and genome (Salmon et al., 2009). Thus, MSCs are endowed with the main molecular mechanisms to manage oxidative stress efficiently.

– **anti-fibrosis.** The role of MSCs in fibrosis is still a matter of controversy. Some studies have indicated that MSCs have no effect, others show an increase and others show a decrease (Carvalho et al., 2008; di Bonzo et al., 2008; Ezquer et al., 2011). These differences may be related, at least in part, to the characteristics of target tissues, fibrosis etiology, the stage of disease at the moment of MSC administration and follow up time. The anti-fibrosis effect of MSCs could be direct, i.e. through the regulation of ECM protein synthesis and degradation, or indirect, i.e. due to the preclusion of leukocyte infiltration and/or the inhibition of pro-fibrotic cytokine secretion (Higashiyama et al., 2007; Semedo et al., 2009a).

– **immunomodulation.** MSCs express constitutively major histocompatibility complex class I, and after induction major histocompatibility complex class II (Rasmusson, 2006). However, they do not present in the cell surface co-stimulatory molecules such as B7-1, B7-2, CD40 y CD40L. Hence, they activate neither allogeneic lymphocytes nor a proliferative response in helper CD4+ T lymphocytes, and they are not targeted by CD8+ cytotoxic T lymphocytes (Tse et al., 2003). Furthermore, MSCs are capable of inhibiting the differentiation of monocyte precursors into activating dendritic cells, and of altering the function of mature dendritic cells (Jiang et al., 2005). Thus MSCs indirectly limit the cytotoxic expansion and activity of NK cells and T lymphocytes. And last but not least, MSCs promote the appearance of regulatory T lymphocytes, inducing antigen-specific tolerance (Maccario et al., 2005). MSCs reduce the serum levels of IL-5, IL-12(p40) and TNF-alpha, resulting in a reduction of leukocyte infiltration into damaged tissues (Togel et al., 2005; Semedo et al., 2009b). In addition, MSC administration leads to modulation of the inflammation through down-regulation of the Th1 cytokines (IL-1beta, IL-6, IL-12, TNF-alpha and INF-gamma), and up-regulation of Th2 cytokines (IL-4 and IL-10) (Semedo et al., 2009b).

– **secretion of trophic factors.** It is known that MSCs have the ability to secrete *in vivo* and *in vitro* a wide range of trophic factors, including VEGF, bFGF, PDGF, IGF-1, HGF and EGF (Caplan and Dennis, 2006). The biological effect of these factors can be both direct, i.e. triggering intracellular signalling in the target cell, or indirect, i.e. inducing neighbor cells to secrete bioactive factors. Therefore it has been proposed that MSCs have a catalytic role in tissue regeneration, since once in the damaged tissue they are able to modify the microenvironment by secreting factors that would: (i) prevent parenchymal cells from dying; e.g. anti-apoptotic factors such as HGF and IGF, in models of myocardial infarction and acute renal failure (Nigam and Lieberthal, 2000; Kinnaird et al., 2004b); (ii) induce the proliferation and differentiation of endogenous progenitors; e.g. neurogenic factors such as NGF and BDNF, in models of neuronal damage (Neuhuber et al., 2005); (iii) promote neovascularization; e.g. angiogenic and vasculogenic factors such as VEGF and bFGF, in models of acute myocardial infarction and ischemic acute renal failure (Kinnaird et al,. 2004a; Togel et al., 2007).

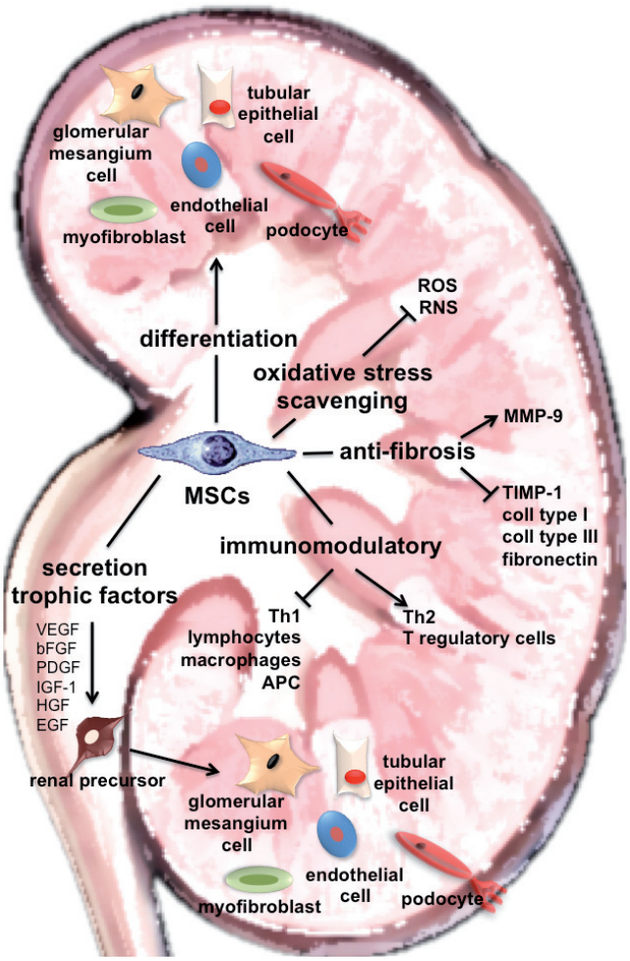

Figure 2. Predictable cellular and molecular mechanisms underlying MSC renoprotection: Once administered into an individual with DN, MSCs will circulate into the bloodstream. In damaged kidney, they will cross the endothelium, home into the parenchyma and migrate to the injured areas. MSCs will contribute to renal tissue regeneration through, at least, one of the mechanisms shown in the figure.
ROS: reactive oxygen species, RNS: reactive nitrogen species, APC: antigen presenting cell.

MSC TRANSPLANTATION: A PROMISING STRATEGY TO MANAGE PATIENTS WITH DIABETIC NEPHROPATHY

General support

Although MSCs are scarce (less than 0.01% in the bone marrow), they appear as ideal candidates to prevent, delay or revert DN, since (i) they can be obtained from donors without major complications; (ii) they can be expanded *ex vivo*; (iii) they are hypoimmunogenic; (iv) once administered intravenously, they are able to home into damaged organs where they may protect the parenchyma from noxa, organize endogenous

regenerative mechanisms and/or differentiate into tissue-specific cells (Figure 2). Furthermore, MSC transplantation has been successfully performed in human patients to treat diverse pathologies such as graft-versus-host disease (Le Blanc et al., 2004), cerebral stroke (Bang et al., 2005), myocardial infarction (Gnecchi et al., 2005; Ripa et al., 2007), metachromatic leukodystrophy (Koc et al., 2002), idiopathic aplastic anemia (Fouillard et al., 2003), osteogenesis imperfecta (Le Blanc et al., 2005) and dystrophic epidermolysis bullosa (Conget et al., 2010). So far MSCs have been administered to more than 1,000 human patients with no evidence of adverse effects or tumor formation.

Indirect support

Indirect support for the putative contribution of donor MSC to the management of individuals with DN includes: (i) recipient MSCs play a key role in normal turnover and remodeling of renal structures including renal vessels, interstitial myofibroblast cells, glomerular mesangium, podocytes and tubular epithelium (Cornacchia et al., 2001; Grimm et al., 2001; Poulsom et al. 2001; Gupta et al., 2002); (ii) in mice models of Alport syndrome and glomerulonephropathy, MSC administration results in clinical improvements (Sugimoto et al., 2006; Wong et al., 2008); (iii) in rodent models of acute tubular epithelial injury and experimental glomerulonephritis, donor MSCs contribute to the functional and structural recovery of both glomerular and tubular compartments (Morigi et al., 2004; Krause and Cantley, 2005; Qian et al., 2008); (iv) in rat remnant kidney models, MSC transplantation attenuates renal fibrosis and produces a reduced glomerulosclerosis index (Semedo et al., 2009a). This was correlated with a reduction in the expression of pro-fibrotic molecules such as collagen type I, collagen type III, fibronectin, vimentin, ASMA, FSP-1 and TGF-beta. It was also associated with a change of the ratio between MMP-9 and TIMP-1, indicative of recovery in the balance between synthesis and degradation of ECM components; (v) in animal models of acute kidney injury MSC transplantation was beneficial (Lange et al., 2005). It was also observed that IGF-1 produced by MSCs reduces apoptosis and increases cell proliferation of the proximal tubular epithelium, whereas HGF secreted by MSCs enhances the remodeling of fibrotic renal tissue (Imberti et al., 2007); (vi) ongoing clinical trials are assessing the safety and efficacy of MSCs to treat cisplastin-induced acute renal failure and lupus nephritis (Giordano et al., 2007; www.clinicaltrials.gov).

Direct support

To our knowledge, there are only four published reports showing, at the pre-clinical level, that MSC-based therapy could be useful for the prevention or the reversion of renal failure in diabetic individuals. In an immunodeficient non-obese diabetic mouse model, it has been shown that after the intracardiac injection of a large number of human MSCs ($\approx 250x10^6$/kg body weight), few donor cells were found in the kidneys (Lee et al., 2006). Unfortunately, it is not known whether this had any functional consequence, as animals did not present renal failure before the intervention or during the follow-up period. In mice with T1DM induced by the administration of five low doses of streptozotocin, we showed that the intravenous administration of syngeneic MSCs ($\approx 20x10^6$/kg body weight) results in the reduction of microalbuminuria and the preservation of normal renal histology (Ezquer et al., 2008). By contrast, untreated diabetic mice remained albuminuric and presented glomerular hyalinosis and mesangial expansion. In rats with diabetes induced by the administration of a single high dose of streptozotocin, the intracardiac infusion of allogeneic MSCs ($\approx 10x10^6$/kg body weight) along with cyclosporine resulted in a transient amelioration of renal function and structure (Zhou et al., 2009). In the latter two reports, after MSC administration an improvement in the diabetes condition was also observed. To determine whether the renoprotective effect of MSCs is indirect, i.e. due to hyperglycemia correction, or direct, i.e. due to protection/regeneration of renal tissue, we administered syngeneic MSCs in a mouse model that develops severe diabetes after the infusion of a single high dose of streptozotocin (Ezquer et al., 2009). Despite not sharing the etiology of either T1DM or T2DM, these animals showed a rapid progression of renal failure and developed most of the pathognomonic signs of DN. In these diabetic mice, MSC administration did not result in hyperglycemia correction; however, renal failure did not progress. In contrast, in untreated diabetic mice microalbuminuria gradually increased and renal histopathological alterations were evident at the end of the study period. Interestingly, at least up to three months after MSC administration donor cells were found in the kidney of severe diabetic mice. None of the published reports explored the mechanisms behind renoprotection. But, due to the scarce numbers of donor cells found in recipient kidneys, it is expected that mechanisms different than cell differentiation will be relevant.

Potential limitations to clinical translation

Data supporting the contribution of donor MSCs to the management of renal failure in diabetic individuals have been generated in the available animal models of DN. Unfortunately, those models only reproduce the earlier stages of human DN (Breyer et al., 2005; Inada et al., 2005; Alpers and Hudkins, 2011). Non-obese and streptozotocin-induced diabetic mice progress to proteinuria and hyperfiltration. They also present variable degrees of mesangial matrix expansion and glomerular capillary basement membrane thickening, but infrequently develop nodular glomerulosclerosis, a pathognomonic sign of advanced human DN. Hence the impact of MSC transplantation in individuals with advanced DN remains unproved.

No dose-response studies have been performed, since the optimal dose of MSCs is unknown. Also, the cellular and molecular mechanisms behind MSC renoprotection in a diabetic environment are still unidentified. Thus more pre-clinical and clinical trials should be designed and performed in order to assess the safety and efficacy of MSC transplantation in individuals with DN.

CONCLUSION

The perfect match between the pathophysiological features of DN and the therapeutic mechanisms of MSCs, together with the encouraging pre-clinical data available supports the notion that MSC transplantation is a promising therapeutic strategy

to manage DN onset and progression, not only because of the safety of this procedure, but mainly because of the renoprotective potential of MSCs.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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Molecular signature of cancer stem cells isolated from prostate carcinoma and expression of stem markers in different Gleason grades and metastasis

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ABSTRACT

Prostate cancer (PCa) is the most frequently diagnosed malignancy in men worldwide. Chemotherapy response is very poor and resistance to hormone-based treatments is frequent in advances stages. Recently, tumor-initiating cells or cancer stem cells (CSCs) have been identified in several cancers, including PCa. These cells are thought to be responsible for therapy resistance, relapse and metastasis. In the present work, enriched populations of CSCs were obtained using a mixed procedure that included differential clone-forming ability, sphere growing induction (prostatospheres) and magnetic-associated cell sorting (MACS). Also, stem marker expression was determined in PCa biopsies of different histological grades and metastasis samples. The signature for stem markers of the isolated CSCs was CD133+/CD44+/ABCG2+/CD24-. Expression of stem markers (CD133, CD44, and ABCG2) was higher in medium Gleason biopsies than in lower and higher grades, and lymph-node and bone metastasis samples. These results suggest that the CSCs in PCa reach an important number in medium Gleason grades, when the tumor is still confined into the gland. At this stage, the surgical treatment is usually with curative intention. However, an important percentage of patients relapse after treatment. Number and signature of CSCs may be a prognosis factor for PCa recurrence.

Key words: prostate cancer, cancer stem cell, spheres cultures

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of male malignancy death throughout the world (Dunn and Kazer 2011). In Chile, this disease reached that level very rapidly and currently over 1,700 patients die annually due to this cancer, representing an observed rate exceeding 20 per 100,000 men (Minsal 2011). This number may be even underestimated considering that PCa occurs mainly in men over 60 years, where other associated medical conditions may be contributing to the cause of death. If localized, PCa can be cured by surgical treatment. However, more than 30% of patients may relapse after surgery (Kotb and Elabbady 2011). Once disseminated, PCa can be controlled by hormonal treatment (androgen deprivation) and mortality is primarily associated with metastatic disease when patients become resistant to treatments (hormone therapy, radiotherapy and chemotherapy).

Recent studies on cancer pathogenesis have identified the presence of tumor stem-like cells that could influence the key processes of tumor progression (Clarke et al. 2006; Shipitsin and Polyak 2008). These cells have tumor-initiating and self-renewing abilities, divide asymmetrically, and express several pluripotency genes (La Porta 2012). For these reasons, many authors have called them cancer stem cells (CSCs). In recent years, CSCs were identified in several cancers, including PCa, and have been proposed to explain the metastatic capacity, recurrence, and resistance to hormone, radio and chemotherapy (Gao 2008; Ishii et al. 2008; Li et al. 2007). In established cell lines from PCa origin, particularly from metastasis, CSCs have been identified and isolated using different approaches, such as flow cytometry, magnetic-associated cell sorting (MACS), the ability of differential cloning and sphere growing under non-adherent culture conditions (Collins and Maitland 2006; Collins et al. 2005; Miki and Rhim 2008; Miki et al. 2007; Tang et al. 2007). Most of these studies have used cell lines as PC3, DU145, and LNCaP, and animal models. These reports have identified several molecular markers for CSCs such as CD44, CD133, CD24, CD40 and $\alpha 2\beta 1$ integrin, among others (Patrawala et al. 2007; Pfeiffer and Schalken 2010). In addition, the ability to exclude Hoechst 33342 staining has been widely used for identifying and separating stem cells (side population) (Patrawala et al. 2005; Zhou et al. 2001). The exclusion of this dye is caused by the ABCG2 transporter that is over-expressed in CSCs. The presence of a side population has been also reported in PCa cells (Pascal et al. 2007). Interestingly, ABC transporters are thought to be responsible for drug resistance in most cancers (Sharom 2008; Stavrovskaya and Stromskaya 2008), including PCa (Sánchez et al. 2009; Sánchez et al. 2011).

In our laboratory we developed cell culture systems from PCa explants to study several aspects of this disease, such as hormone sensitivity, drug resistance and the effect of various compounds with therapeutic potential (Castellón et al. 2005; Castellón et al. 2006; Clementi et al. 2009; Mendoza et al. 2009; Sánchez et al. 2005). The aim of this work was to isolate and characterize CSCs from our tumor explant-derived cultures in order to determine specific molecular stem signatures and to evaluate these markers in relation to Gleason grades

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and metastasis. This analysis may represent an important prognostic tool for metastasis potential, relapse timing and resistance development. In addition, genetic and functional characterization of these CSCs is being carried out in order to identify potential therapeutic targets for the selective elimination of these cells.

MATERIAL AND METHODS

Prostate cancer tissue

Tumor samples were derived from radical prostatectomies of patients with localized PCa (mostly patient Gleason score 5-6, histological sample Gleason grade 3) from our institutional hospital. Tissue pieces were received in sterile culture medium with RNase inhibitor and then taken to the laboratory in a period no longer than 1 hour after collection. Once in the laboratory, when necessary, soft healthy or hyperplasic tissue was separated from harder cancerous tissue. For control purpose, small pieces of all samples used for cell isolation and cultures were processed for histological diagnosis to confirm Gleason grade. Successful cultures from six surgical pieces were obtained for this work. All protocols of this study were approved by Ethics Committees of the Faculty of Medicine and the clinic hospital of our university.

Isolation and culture of epithelial cells from prostate cancer

Small pieces (1 mm3) of PCa tissue underwent enzymatic digestion with collagenase (2 mg/ml), hyaluronidase and deoxyribonuclease (0.02 mg/ml). Digestion was carried out at 37°C for 10-12 hours in a shaking water bath. The process was checked every hour in order to separate dispersed stromal cells. After enzymatic digestion, aggregates of epithelial cells were washed with culture medium and plated. These cell aggregates were cultured in Dulbecco MEM/HAM F-12 (1:1) medium supplemented with 10% FBS in culture bottles. After 2 or 3 days, once cell aggregates had adhered and spread, the medium was changed by a chemically defined medium according to the methods previously described (Castellón et al. 2005; Castellón et al. 2006).

Isolation of prostate cancer stem cells

Prostate CSCs were identified and isolated from tumor explant-derived cultures of epithelial cell (2-4 passages), using a combination of methods previously described.

Differential cloning ability: Detached and washed epithelial cells from explant-derived cultures were counted and diluted. 100 cells were taken and suspended in 10 ml of culture medium. Cells were plated by transferring 100 ul of cell dilution in each well of a of 96-well culture plate. The cells were monitored during the first week to observe and characterize the obtained clones and allowed to grow for another week to evaluate and characterize the different clones, namely, holoclones, meroclones and paraclones (Barrandon and Green 1985). To evaluate the morphology of these clones, cells were washed, fixed with cold methanol 100% and stained with 0.5% violet crystal / 25% methanol for 10 minutes.

Sphere growing induction: Cells obtained from the differential cloning cultures were trypsinized and centrifuged at 2000 rpm for 5 minutes. The pellet was suspended and plated, at a density of $4x10^4$ cells/ml, in DMEM medium without FBS and supplemented with the following factors: 5 ug/ml of human transferrin, 5 ug/ml of insulin, 20 ng/ml of EGF, 10 ng/ml of FGF-2, 200 ng/ml of retinol, 200 ng/ml of vitamin E, 10 nM of hydrocortisone, 2 ng/ml of sodium selenite, and 0.4% of human serum albumin-free globulins. This culture medium (non-adherent medium) allowed the formation of prostate spheroids (prostatospheres) enriched in CSCs from day 7 of culture (Fan et al. 2010). For control purposes, parallel cultures in adherent conditions were used.

Magnetic-associated cell sorting (MACS): Cell populations enriched in CSCs were dispersed, washed and incubated with antibody-coupled magnetic microbeads for 1 hour. Then, cells were mounted into proper columns and settled in a magnetic support (Pascal et al. 2007). For positive separation, negative cells passed throughout the columns were used as control. Positive retained cells were washed and plated in proper CSCs medium. For negative separation (in case of using a non-stem marker antibody), negative cells were processed as the enriched stem cell population. The selected antibodies coupled to the microbeads for these separations included anti-CD133, anti-CD44, anti-CD24 and anti-ABCG2 (Miltenyi Biotec).

Immunocytochemistry of prostatospheres

Prostatospheres were washed and fixed with paraformaldehyde 10% in phosphate buffer and then resuspended in HistoGel reagent (Thermo Scientific, HG-4000-012). These structures formed a three-dimensional matrix to preserve the integrity of prostatospheres. Then, the matrix was included in paraffin and sections of 5 um were obtained. Afterwards, samples were deparaffinized with xylene and rehydrated with decreasing ethanol concentrations. Later, sections were incubated for 2 hours with specific antibodies targeting extracellular epitopes of surface proteins CD133 (Miltenyi Biotech.), CD44 (Santa Cruz Biotechnology Inc.), CD24 (Santa Cruz Biotechnology Inc.), ABCG2 (Thermo Scientific) and ALDH2 (Thermo Scientific). After washing to remove non-specifically bound antibody, samples were incubated for 1 hour with secondary biotinylated antibodies followed by peroxidase-labeled streptavidin. Diaminobenzidine was used as chromogen. For immunofluorescence, secondary antibody was conjugated to FITC (Thermo Scientific) and to observe nuclei, DAPI for 5 minutes was used.

Prostate cancer biopsies and metastasis samples

Samples of PCa of different Gleason grades and lymph-node and bone metastases were obtained from our institutional archive of biopsies with the corresponding authorizations. The following samples were used considering the histological grade (Gleason 1 to 5) of the biopsies:

Low Gleason (grade 2): 11 samples. Medium Gleason (grade 3): 14 samples. High Gleason (4-5): 9 samples. Lymph-nodes metastasis: 7 samples. Bone metastasis: 5 samples.

Immunohistochemistry of prostate cancer biopsies and metastasis samples

Expressions of all markers studied were evaluated by quantitative immunohistochemistry. Only specimens fixed

and included in optimal conditions for immunohistochemical studies were selected. The samples were deparaffinized and rehydrated. Hydrogen peroxide was used to inactivate endogenous peroxidase activity and antigen retrieval was performed with target retrieval solution. To avoid unspecific binding, samples were incubated with PBS-BSA 1% for 1 hour at room temperature. Samples were incubated with first antibodies directed against proteins CD133 (Santa Cruz Biotechnology Inc.), CD44 (Santa Cruz Biotechnology Inc.) and ABCG2 (Thermo Scientific) for 1 hour at 37ºC. The signal was amplified using a second biotinylated antibody followed by peroxidase-labeled streptavidin. Diaminobenzidine was used as chromogen. The samples were counterstained with hematoxylin (Ross et al. 2003). All samples were analyzed using Image ProPlus 6.2 software (Media Cybernetics, Bethesda USA), to quantify the immunoreactive area (IA) in um^2 and the integrated optical density (IOD), which allows a quantitative densitometric analysis of the specific areas (Ruifrok and Johnston 2001).

Statistic analysis

Statistic evaluation of data was performed using ANOVA analysis and non-parametric test of Kruskal-Wallis followed by Dunnett's post-test. Statistic significance was considered for $P< 0.05$. Results were expressed as mean $\pm$ SD.

RESULTS

Enrichment of CSCs by differential cloning ability

We obtained different clone populations using limiting dilution assays from PCa explant-derived cultures, knowing that CSCs have the ability to form compact colonies. Holoclones (compact colonies), meroclones (loose colonies), and paraclones (dispersed colonies) were obtained from explant-derives cultures (Fig. 1A). From some cultures, more than 35% of holoclones (Fig. 1B) were yielded. However, it was not possible to obtain compact colonies, with the same expansive characteristics, from all explant-derived cultures (data not shown). These differences could be due to the effect of the number of passages that underwent each culture. It was observed that the greater the number of previous passages of original culture, the higher the holoclones yield. Holoclones obtained by this method showed higher expression of the stem markers than mero- and paraclones. A representative marker (CD44) in shown in Figure 1A and 1C. The successful holoclones maintained their morphological and clonogenic characteristics for at least 3 successive passages (Fig. 2A). However, it should be noted that these clones showed some degree of plasticity, as well as producing primarily holoclones (> 90%), the presence of few mero- and paraclones in the cultures was observed (data not shown).

Enrichment of CSCs by sphere growing induction

Cells obtained from holoclones were plated under non-adherent condition, knowing that CSCs can survive and aggregate under this condition and generate spheres that grow without differentiation. Successful spheres cultures (prostatospheres) were obtained after a week of culture (Fig. 2A and 2B). Some prostatospheres were fixed and processed for immnocytochemistry, showing a molecular signature that included high expression of CD133, CD44, and ABCG2 and no expression of CD24 (Fig. 2B). A few cells of the spheres expressed also ALDH2, another marker described for CSCs. The prostatospheres can be maintained in culture for several weeks without expression of differentiation makers (data not shown).

Enrichment of CSCs by magnetic-associated cell sorting (MACS)

Some prostatospheres were disaggregates and resulting cells washed and processed for MACS separation (see materials and methods). Using the stem markers CD133, CD44 and CD24, further enriched CSCs populations were obtained. In Figure 3, a representative separation protocol using CD44 is shown. Cells retained in the MACS column (expressing CD44) were washed and plated in proper medium for 48 and 72 hrs (Fig. 3A). Resulting cultures were enriched over 95% in CD44-expresing cells (Fig. 3B).

Expression of CSCs markers in PCa biopsies of different histological grades and metastasis samples

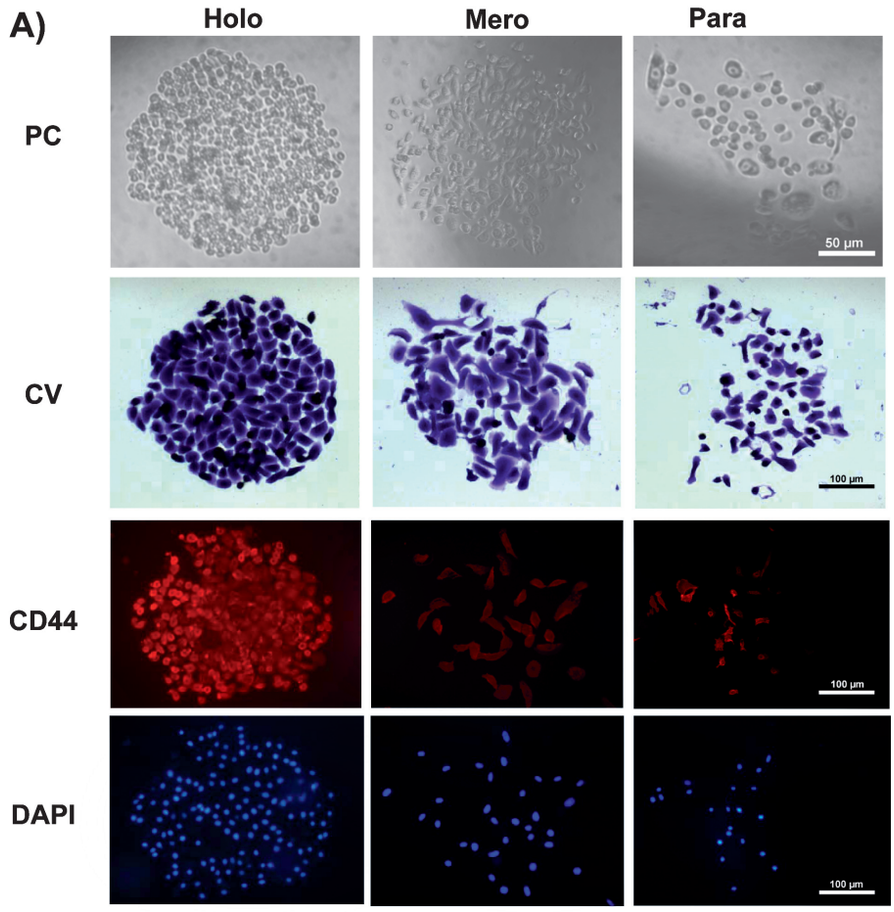
In order to evaluate the expression of the stem markers described for CSCs isolated from explant-derived cultures in tumor progression and metastasis, we analysed a collection of biopsies of low, medium and high Gleason grade and samples from lymph-node and bone metastasis by immunohistochemistry. Expression of stem markers CD133, CD44 (Fig. 4A), and ABCG2 (Fig. 5A) was observed in all samples. For the three markers studied, medium Gleason biopsies showed the highest expression (Figs. 4B and 5B). Interestingly, lymph-node and bone metastasis showed the lowest expression of CSCs proteins. Tissue for benign prostatic hyperplasia (BPH) was used as non-malignant control. As expected, low expression of stem markers were found in BPH (Figs. 5 and 6). It is known that in normal prostate CD44 is restricted to basal cells and ABCG2 to endothelial cells.

DISCUSSION

Stem cells are defined upon their self-renewal capacity and the ability to give rise to complete tissues, organs or even individuals (i.e. embryonic stem cells) (Glinsky 2008; Reya et al. 2001; Visvader and Lindeman 2008). Nearly a decade ago, evidence about the existence of CSCs came from haematopoietic cancers (Bonnet and Dick 1997; Lapidot et al. 1994). In the same way that normal stem cells can give rise to normal tissue or organs, CSCs can give rise to malignant tissue or tumors. Considering that the mechanisms underlying cancer initiation and development remain unclear, the presence of CSCs in most cancers has focussed attention on the role of these cells in carcinogenesis (Cabanillas and Llorente 2009). The identification of CSCs has challenged the hypothesis of "clonal evolution" for cancer development and may implicate new approaches for cancer therapy (Filip 2008; Gil 2008; Lewis 2008). It is still not clear whether CSCs originate from malignant transformation of normal stem cells, or if malignant cells acquire stem characteristics through the epithelial-mesenchymal transition (EMT) (Lobo et al. 2007; Ward and Dirks 2007). Most evidence suggests that cancer originate from progenitor cells rather than stem cells (Pardal et al. 2003; Wicha et al. 2006). There is still debate regarding

the existence of an actual stem cell in cancer. For these reasons, some authors prefer to refer to these cells as tumor-initiating cells (TICs). Probably, as part of EMT, a small cell population re-expresses some pluripotency genes. These cell populations, with acquired "stemness" features, can be considered as cancer stem-like cells or CSCs.

It is believed that malignant cells undergoing EMT become aggressive due to invading and migrating abilities. They could invade the surrounding stroma, enter lymph and blood circulation and produce local and distant metastases. It is assumed that solid tumors are a rather homogeneous group of cells with high proliferative and invasive capacity and, in a stochastic way, some of these cells leave the tumor and produce metastasis. This explains that most treatments are focussed on eliminating the bulk of these cells (Le Tourneau et al. 2008). However, the presence of a small number of CSCs in many cancers makes it necessary to re-consider that view. Current evidence suggests that CSCs are really responsible for metastasis, recurrence and drug resistance (Borst et al. 2007; Chiang and Massagué 2008; Croker and Allan 2008). Prostate CSCs has been also identified. There have been successful efforts to isolate and characterize PCa CSCs from established cell lines (mainly PC3, LNCaP and DU145) (Celià-Terrassa et al. 2012; Duhagon et al. 2010; Li et al 2008; Setoguchi et al. 2004; Tang et al. 2007). This is a very interesting finding since cell lines have been believed to be very homogeneous



Type of clone	Nº of clones	Percentage
Holo	13	36
Mero	17	47
Para	6	17
Total	46	100

Figure 1. Prostate cancer cell clones obtained by limiting dilution assays. A) Representative images of clones with different morphology. Holo: holoclone, Mero: meroclone, Para: paraclone observed by phase-contrast light microscopy (PC) and stained with crystal violet (CV). A set of clones showing immunofluorescence (red) staining for a representative stem marker (CD44). DAPI (blue) was used for nuclei staining. B) Number and percentage of clones obtained by limiting dilution assay. C) Quantitative analysis of immunofluorescence. Data are expressed as percentage of positive area (PA). Different superscripts represent statistical significance ($p<0.05$).

populations. Apparently, they are not, distinguishing at least two very different cell types, one of them CSCs (Celià-Terrassa et al. 2012). Many authors report molecular signatures for these CSCs isolated from cell lines. Most of them are coincident with high expression of the markers CD133, CD44, CD40 and $\alpha 2\beta 1$ integrin, among others (Yu et al. 2012). Interestingly, some of these markers are also common with CSCs from other solid tumors (La Porta 2012). However, the remaining CSC populations in cancer cell lines may not represent actual CSCs from tumors. Phenotypic, functional and gene expression characteristics may differ substantially. Indeed, there is still uncertainty about the source of CSCs in PCa. Evidence supports both basal and luminal cell compartments as potential prostatic niche for CSCs (Yu et al, 2012). Considering that PCa is a heterogeneous and multi-focal disease, malignant cells may come from different stem sub-populations. In order to address this problem, we have isolated and partially characterized a CSCs population from explant-derived cell cultures from PCa tumors, using combined methods. We were able to obtain enriched cultures of CSCs expressing a consistent molecular signature displaying a CD133+/CD44+/ABCG2+/CD24- pattern, from these explant-derived cell cultures. Interestingly, this stem signature is similar to those obtained from PCa cell lines (Yu, et al 2012), suggesting that prostate CSCs are very conservative.

Keeping in mind that prostate CSCs could represent a potential therapeutic target (Lang et al. 2009), we evaluated the stem signature described in enriched population of CSCs from tumors, in representative biopsies of the different histological grades and lymph-node and bone metastasis samples. We found, that all stem markers studied were present in low, medium and high Gleason grades and metastasis. However, the highest expression of CSCs proteins was observed in biopsies of medium grades. These samples correspond to patients with medium Gleason score (grades 5 to 6). At this stage, the tumor is usually confined to the prostate gland and most patients are subjected to radical surgery. However, many of them (around 30%) relapse months after surgery (Kotb and Elabbady 2011). This is a very interesting point, considering that metastasis is a very inefficient process, due to less than 1% of the neoplastic cells reaching blood circulation can actually colonize distant organs, and even a smaller percentage produce metastases (Chiang and Massagué 2008). It is well known that primary tumors secrete signals (altered proteins) that affect, in a selective way, specific stromal tissue favouring CSCs niche (Chiang and Massagué 2008; Witz 2008). Once CSCs colonize these conditioned stromas, they may remain for long periods of time in a dormant state before activating and producing metastases (Kaplan et al. 2006; Wels et al. 2008). This may explain the recurrence after apparently curative surgery in many cancers. In PCa, when a tumor is diagnosed as localized and without clinically evident metastases (medium Gleason grades), it is believed that radical surgery is curative. However, at this stage many cancer cells may have reached blood circulation (according to our results, some of them presumably CSCs), colonized lymph nodes or other tissues and entered

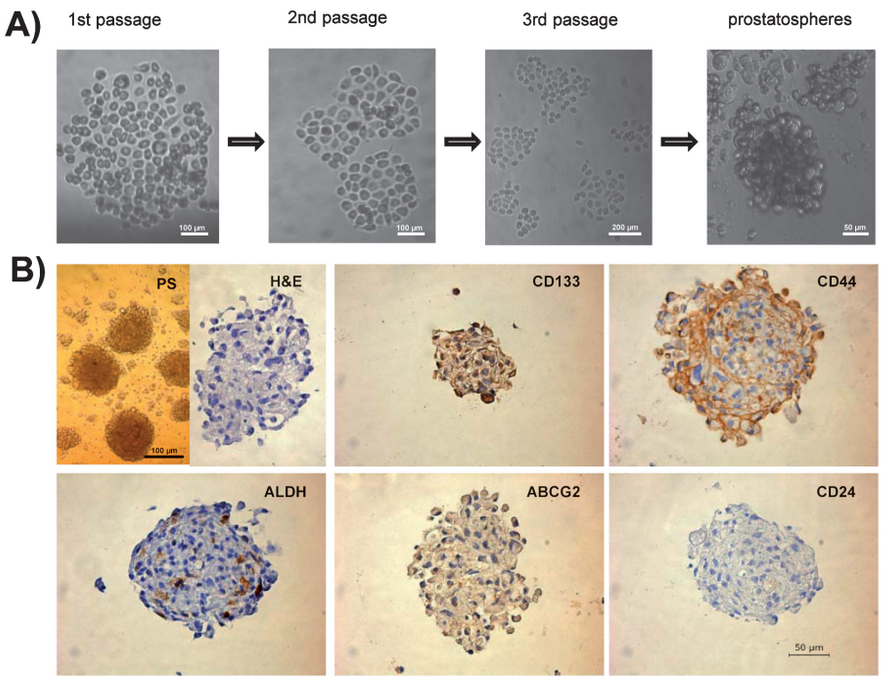


Figure 2. Holoclones sub-cultures and characterization of holoclone-derived spheres. A) Sequence of 3 successive passages of a representative holoclone and representative cell spheroids (prostatospheres) generated from the 3rd passage of holoclones. During the period of time taken by the sequence of passages (approximately 3 weeks) no changes in characteristic morphology was observed. B) Immunocytochemistry analysis of prostate cancer spheres enriched in cancer stem cells. Spheres were fixed, embedded in HistoGel (see materials and methods) and included in paraffin. H&E: Control Hematoxylin/Eosin staining. 5 um sections were immunocytochemically analyzed for CD133, CD44, ALDH2, ABCG2 and CD24. The main molecular signature observed was CD133+/CD44+/ABCG2+/CD24-. A few cells within prostatospheres were positive for ALDH2. Insert: Representative prostatospheres (PS) obtained by culturing cells in non-adherent conditions after 15 days of cultures.

a dormant state. These CSCs may be responsible for later relapse in those patients. Interestingly, lymph-node and bone metastasis showed the lowest expression of CSCs markers. This may be explained because in growing metastasis, progenitors and malignant differentiated cells (originated from CSCs) are predominant.

Generally, prostate CSCs have been involved in EMT (Hollier et al. 2009), pre-metastatic niche preparation, relapse and metastasis (Drewa and Styczynski 2008; Kelly and Yin 2008; Takao and Tsujimura 2008). For these reasons, our results may represent an important step in the efforts to further characterize CSCs from prostate tumors in order to identify potential therapeutic targets for selective elimination. Taking into account that prostate CSCs have been studied mainly in established cell lines, we consider that our PCa explant-derived culture system is more suitable as a model for the study of these CSCs. Nevertheless, the main feature of these cells is to induce tumor growth resembling the original cancer in a recipient organism. For this purpose, animal models have been widely used, but most of them are suitable to study tumor growth and local dissemination. To evaluate metastasis, orthotopic models have been developed and for PCa, human cancer lines have been implanted in the ventral prostate lobe of immuno- compromised mice, resulting in rapid tumor growth and spreading to distant organs (Celià-Terrassa et al. 2012). The main disadvantages of this model are the size of ventral lobe and the high grade of local dissemination of malignant cells. At present, we have developed a modified orthotopic model injecting PCa cells into anterior prostate lobe of NOD/SCID mice. This model allows the surgical resection of the initial tumor and evaluating relapse and further metastasis progression. The isolated CSCs from tumor samples are been currently evaluated in this model.

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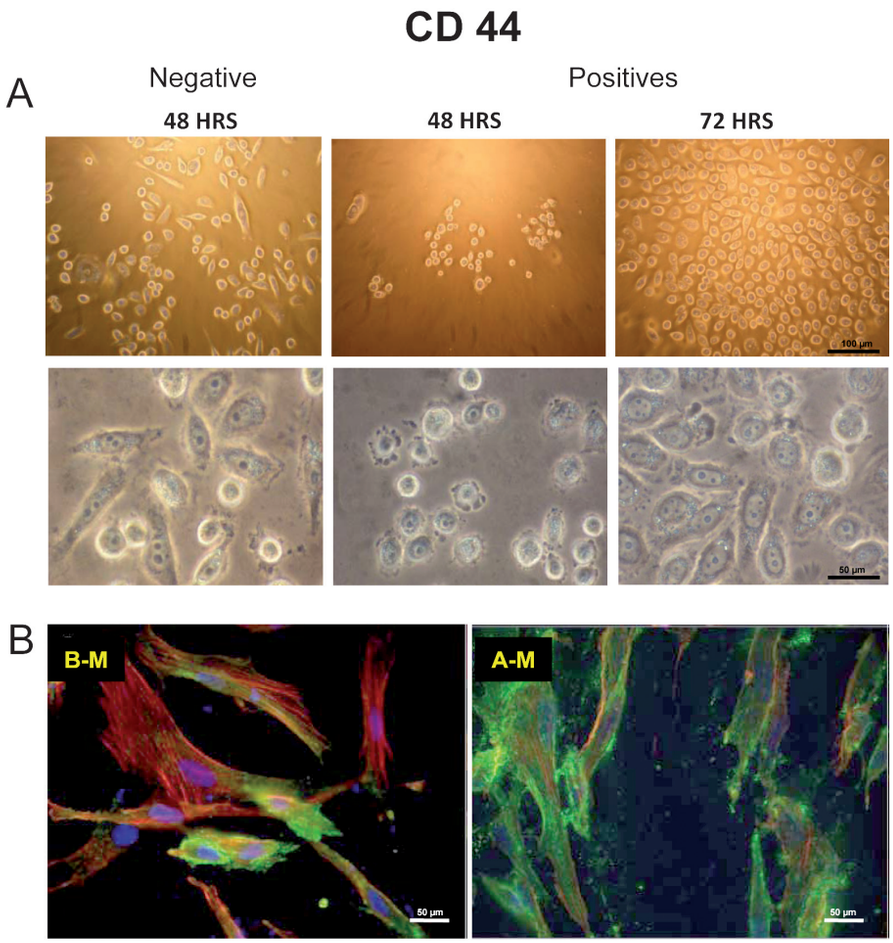

Figure 3. Representative separation of prostate cancer cells by magnetic-associated cell sorting (MACS). Enriched population of prostate cancer stem cells were processed for MACS separation using several stem marker antibody-coupled microbeads. A representative positive separation using microbeads coupled to anti-CD44 is shown. A) CD44-negative and CD44-positive cell populations 48 and 72 hrs after separation observed under the phase-contrast microscope at different magnification (see size-bars) B) Immunofluorescence analysis for CD44 (green) before MACS (B-M) and after MACS (A-M) separation. Phalloidin (red) for cytoskeleton and DAPI (blue) for nuclei staining were used.

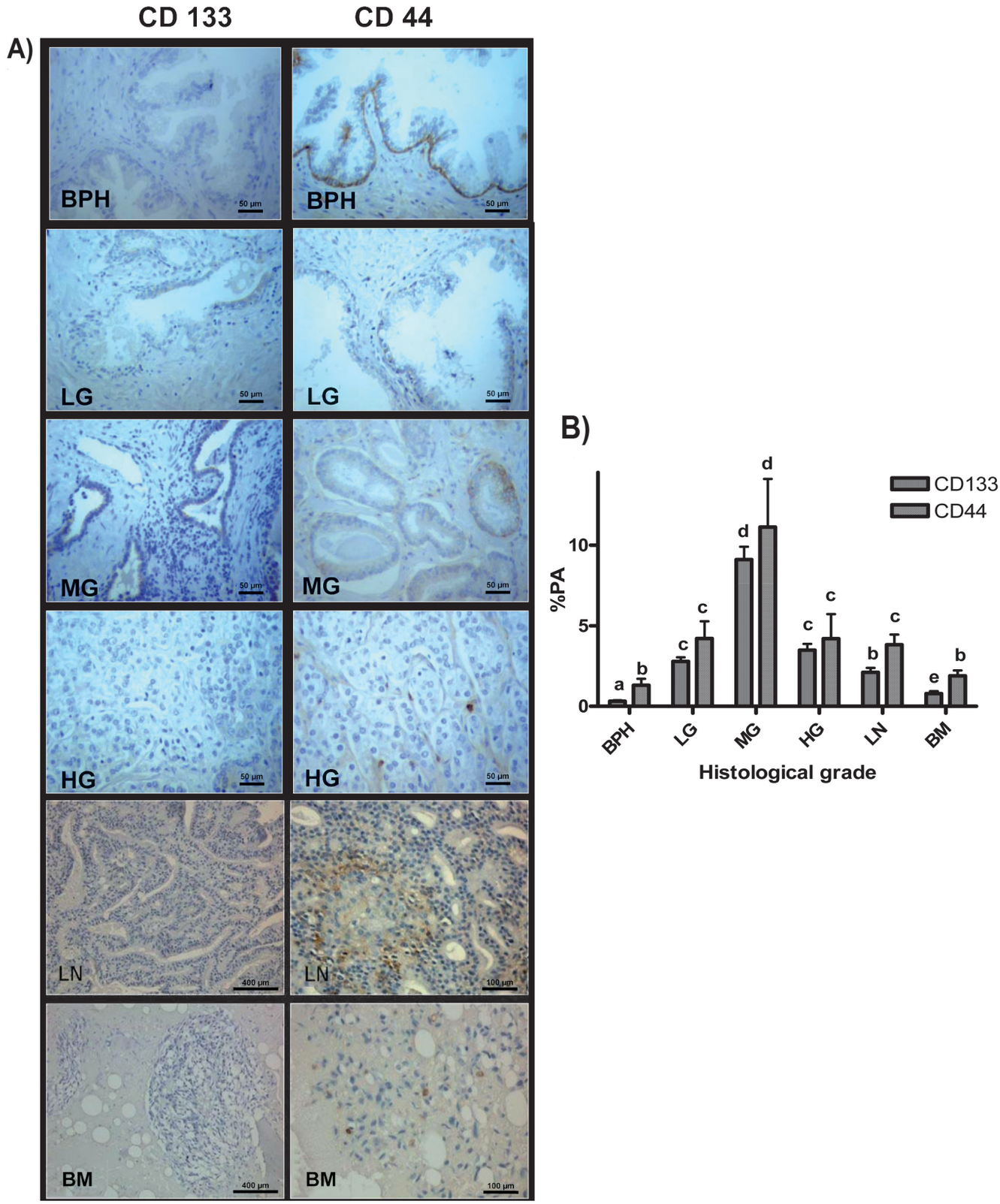

Figure 4. Immunohistochemistry analysis of cancer stem cell markers CD133 and CD44 in prostate cancer biopsies of different Gleason grades and metastasis samples. A) CD133 (left) and CD44 (right) immunostaining. BPH: Benign prostatic hyperplasia. LG: low histological Gleason grade (grade 2). MG: Medium histological Gleason grade (grade 3). HG: high histological Gleason grade (grade 4-5) LN: lymph-node metastasis. BM: Bone metastasis. See size-bars for magnification. B) Quantitative analysis of immunostaining using Image ProPlus 6.2 software. Data are expressed as percentage of positive area (PA). Different superscripts represent statistical significance ($p<0.05$).

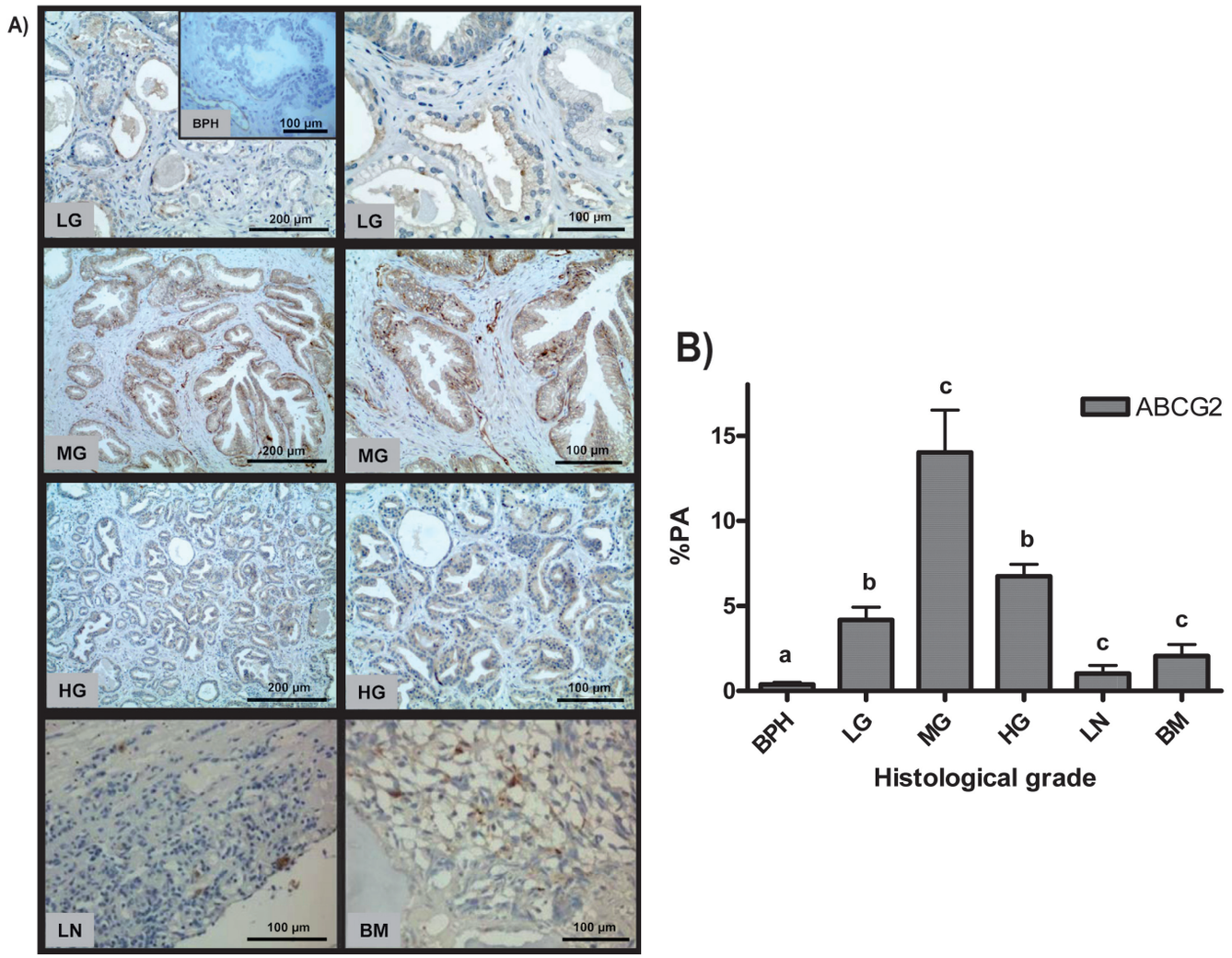

Figure 5. Immunohistochemistry analysis of cancer stem cell marker ABCG2 in prostate cancer biopsies of different Gleason grades and metastasis samples. A) ABCG2 immunostaining. BPH: Benign prostatic hyperplasia. LG: low histological Gleason grade (grade 2). MG: Medium histological Gleason grade (grade 3). HG: high histological Gleason grade (grade 4-5) LN: lymph-node metastasis. BM: Bone metastasis. See size-bars for magnification. B) Quantitative analysis of immunostaining using Image ProPlus 6.2 software. Data are expressed as percentage of positive area (PA). Different superscripts represent statistical significance ($p<0.05$).

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Hematopoietic stem cell transplantation: clinical use and perspectives

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ABSTRACT

Hematopoietic stem cell transplantation is the accepted therapy of choice for a variety of malignant and non-malignant diseases in children and adults. Initially developed as rescue therapy for a patient with cancer after high doses of chemotherapy and radiation as well as the correction of severe deficiencies in the hematopoietic system, it has evolved into an adoptive immune therapy for malignancies and autoimmune disorders. The procedure has helped to obtain key information about the bone marrow environment, the biology of hematopoietic stem cells and histocompatibility. The development of this new discipline has allowed numerous groups working around the world to cure patients of diseases previously considered lethal. Together with the ever growing list of volunteer donors and umbilical cord blood banks, this has resulted in life saving therapy for thousands of patients yearly. We present an overview of the procedure from its cradle to the most novel applications, as well as the results of the HSC transplant program developed at our institution since 1989.

Key words: hematopoietic, stem cells, transplantation, allogeneic

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) remains until now the only proven clinical use of stem cells. Since the discovery of this procedure in the early 1960s as a cure for hematologic cancer and both congenital and acquired diseases of the hematopoietic system (Fig. 1) (Pasquini & Wang, 2011; Thomas et al., 1975), significant progress has been made to make HSCTs safe and available to all patients with a clinical indication of the procedure.

HEMATOPOIETIC STEM CELLS (HSC) AND THE NICHE

HSC sustain blood cell production over the entire life of an individual. Initially during embryonic life HSCs develop in different anatomical places, beginning in the mesodermal germ layer which gives rise to the hemangioblasts (Huber et al., 2004). Subsequently a second short wave of erythroid progenitors originates from the yolk sac and later the liver becomes the third structure that gives rise to definitive erythroid differentiation (Gekas et al., 2005). HSCs that originate complete hematopoiesis are found initially in the aorta-gonad-mesonephros (AGM) region for a short period in a low number of cells. Subsequently the main place of HSC accumulation coming from the yolk sac and the AGM region corresponds to the fetal liver (Ema & Nakauchi, 2000). Finally, the hematopoiesis transfers to the bone marrow where it will contribute to the definitive adult hematopoietic system through the entire life of the individual.

The first demonstration of the existence of HSCs was reported in 1961 by Till and McCulloch (1961). Later the concept of the hematopoietic stem cell niche emerged, a highly specialized place where stem cells localized in the bone marrow (Schofield, 1978).

Currently the adult HSC niche is known to involve several other cell types that support HSCs including osteoblasts, osteoclasts, mesenchymal stem cells, adipocytes and neuronal cells, among others (Doan & Chute, 2012). Also, a network of extracellular matrix proteins and surface molecules are pivotal for HSC survival, self-renewal, proliferation, differentiation and trafficking (Rettig et al., 2012).

At least 2 different niches have been described within the niche concept; the osteoblastic niche and the vascular niche, with different localizations and functions (Till & McCulloch, 1961). The former corresponds to the area where HSCs are in direct contact with the endosteal surface and the osteoblasts; evidence has shown that stem cell fate is regulated by the direct contact between HSCs and osteoblasts through Notch activation (Calvi et al., 2003; Yin & Li 2006). The vascular niche in the adult individual has been shown to be an area of HSC regulation. It has been demonstrated that *in vitro*, endothelial cells are able to expand significantly HSCs, probably through via soluble factors (Chute et al., 2002; Zhang et al., 2008). *In vivo* several studies have supported the existence of an anatomical vascular niche (Kiel et al., 2005; Lo Celso et al., 2009; Kopp et al., 2005).

The HSC-niche interactions subsequently induce the production of more differentiated precursors and finally mature blood cells which will be the components of the hematological and immune systems.

CLINICAL HSC TRANSPLANTATION

HSC transplantation (HSCT) is a procedure in which the entire hematopoieses and immune system are replaced by the donor's cells (Copelan, 2006). HSCT can be classified according to its purpose, HSC origin and HSC donor type (Tables 1, 2 and 3). Matching in allogeneic transplantation is done by comparing alleles of the human histocompatibility locus (HLA) located in chromosome 6. HLA antigens are classified in class I (A, B, C) and class II (DRB1, DQB1, DPB1), and matching between donor and patient can be done at low, intermediate and high resolution (Petersdorf, 2008). Indications for HSC

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transplantation are malignant and non-malignant diseases (Fig. 1). Most HSCT procedures are performed with autologous HSC for different forms of cancer, with multiple myeloma being the most common indication. Nevertheless, the development of equally effective and less toxic procedures is resulting in a decreasing use of this procedure as therapy for these patients. Most allogeneic transplants are performed for patients with hematologic malignancies, mainly acute leukemias. Chronic myelogenous leukemia was the most common indication until tyrosine kinase-based therapy became available and proved to

TABLE 1

Purpose of HSCT

1. Rescue a patient with cancer from the toxic effects of high dose chemotherapy +/- radiation therapy (autologous or allogeneic)
2. Correct a congenital or acquired severe blood disorder by replacing the patient´s with the donor´s hematopoietic system (allogeneic)
3. Increase the control of a malignant disease by alloimmune effector mechanisms of the graft versus host reaction (allogeneic)
4. Reset the immune system to abolish autoimmunity (autologous or allogeneic)

TABLE 2

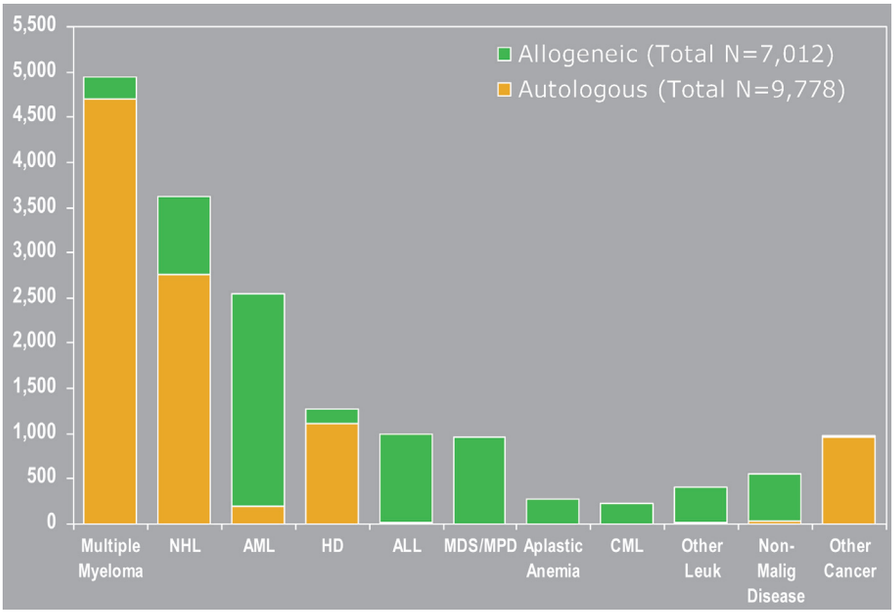
Donor sources in HSCT

1. Bone marrow, harvested by multiple punctures and aspiration of posterior iliac crests
2. Mobilized peripheral stem cells, obtained by leucopheresis after administration of granulocyte stimulating factor
3. Placental blood collected through the umbilical cord at the time of delivery

TABLE 3

Donor types in HSCT

1. Autologous: HSC are obtained from the patient
2. Allogenic: HSCs are obtained form a donor
   a. Matched related, sibling.
   b. Mismatched related: partially matched family member. This graft usually requires graft manipulation either negative T cell selection or positive CD34 selection (haploidentical)
   c. Matched unrelated: HSC from an unrelated donor that satisfies match criteria similar to a sibling donor
   d. Mismatch unrelated: HSC from an unrelated donor with major o minor mismatches in HLA type



NHL: Non Hodgkin´s lymphoma, HD: Hodgkin´s disease, AML: acute lymphoblastic leukemia ALL: acute lymphoblastic leukemia, MDS/MPD: Myelodisplastic syndromes, CML: chronic myelogenous leukemia.

Figure 1. Indications for Hematopoietic Stem Cell Transplants in the United States, 2009. Center for International Bone Marrow transplant Research (www.cibmtr.org).

be a better alternative, particularly in older patients in the early phases of the disease. Transplantation in this group is now reserved for younger patients and when leukemia becomes resistant to tyrosine kinase inhibitors.

To engraft allogeneic HSCs successfully, a patient has to receive some form of immune ablative therapy or conditioning regimen before transplantation. In patients with hematological malignancies this is usually accomplished by the use of chemotherapy and total body radiation, which also function as anticancer therapy. Conditioning regimens are termed myeloablative when high doses of both radiation and chemotherapy are used and reduced intensity when lower doses are used. The intensity of the conditioning regimen depends on the underlyimg diagnosis, age of the patients and co-morbidities. Reduced intensity regimens have allowed extension of the procedure to older and sicker patients (Kassim et al., 2005).

The most limiting complication for allogeneic HSCT is graft versus host disease (GVHD), an immune rejection to host tissues mediated by donor lymphocytes which results in a skin rash, diarrhea and liver disease (Ferrara et al., 2009). This condition can become chronic and produce a systemic sclerosis-like illness which can produce scarring of the skin, gut and eyes (Horowitz & Sullivan, 2006). The most important factor that determines the incidence and severity of GVHD is HLA matching.

Transplantation failure is due to either disease relapse in patients transplanted for malignancies or to mortality related to the procedure, almost always due to infections and sometimes as a result of conditioning-induced organ damage. Infections by common and opportunistic microorganisms is a consequence of the profound immune suppression of patients and the prolonged recovery of innate and adaptive immunity (Table 4). GVHD is a contributing factor to infectious complications because of the further delay in immune reconstitution it provokes.

HISTORICAL EVOLUTION: HOW HURDLES WERE OVERCOME

Bone marrow transplantation was performed successfully as a result of the studies done by Donnall Thomas and the group at the Fred Hutchinson Cancer Center during the 1960s. Early studies demonstrated the effect of high radiation therapy doses and chemotherapy in the bone marrow as well as the capacity to regenerate the individual's hematopoietic function by reinfusion of stored bone marrow cells from the individual or a donor (Lorenz et al., 1951; Mannick et al., 1960; Santos & Owens 1969).

To control GVHD two issues had to be dealt with: histocompatibility and post-transplant immune suppression. The first was accomplished with the continuous improvement of HLA matching from antigen to allelic typing, as well as the discovery of an ever growing list of class I and II alleles. The second was successfully dealt with by the introduction of calcineurin inhibitors, mainly cyclosporine, a potent immune suppressor developed for solid organ transplantation (Choi et al., 2010).

Infection control has improved significantly with the introduction of wide spectrum antibiotics, effective antifungals and an ever growing list of antivirals developed to curb the activation of latent or acquired disease. The use of controlled hospital environments to control the spread of airborne fungi goes in this direction (Tomblyn et al, 2009).

The second source of HSCs to enter the clinic were peripheral SC obtained form a healthy donor after mobilization with granulocyte stimulating factor and collected via leukapheresis. This avoids a painful surgical, and results in faster engraftment of granulocytes and platelets as well as improved immune reconstitution compared to bone marrow grafts (Storek et al, 2001).

The recognition of umbilical cord blood as a source of HSCs that can repopulate the marrow of a patient was reported in 1988 in a Fanconi anemia patient (Gluckman, 2009), based on previous work by Broxmeyer (2009) and Knudtzon (1974). The successful results of transplantation in children using cord blood grafts stimulated its use in adult patients. The main limitation was and remains the low cell number, with slow hematologic and immune reconstitution and initially high transplant-related mortality (Gluckman et al., 1997; Rubinstein et al., 1998). Several strategies are under evaluation to improve the results of umbilical cord blood transplantation (UCBT) in

TABLE 4

Prevalence of opportunistic infections after allogenic HSCT in the different post transplantation periods.

Phases of allogenic HSCT	Hematologic reconstitution (day +30)	Early immunologic reconstitution (day +100)	Late immune reconstitution (day +365)
Host factors	Neutropenia, lymphopenia, mucositis,	Deficiency in humoral and cellular immunity, acute GvHD	Deficiency in humoral and cellular immunity, chronic GvHD
Infectious agents	Gram negative bacteria		
	Coagulase negative Staphylococcus		
	Enterococci		Encapsulated bacteria
	Candida spp		
	Aspergillus spp		
	Respiratory and enteric viruses (RSV, influenza, parainfluenza, rotavirus)		
			Adenovirus
			Cytomegalovirus
	Pneumocystis jeroveci		

adults and large children. The most successful corresponds to double UCBT. Considering that most of the adult patients do not qualify for UCBT due to their weight and their minimum nucleated cell requirements, this is achieved with 2 UCB units. Several groups have published their results (Barker et al., 2003; Gutman et al., 2009; Brunstein et al., 2010). Overall, these studies have shown that myeloablative conditioning followed by double UCB graft infusion is associated with acceptable engraftment rate and long term overall survival, similar to adult graft sources. Interestingly, these studies have demonstrated the predominance of one of the 2 units shortly after transplant. The mechanisms are not fully understood but most likely are immunological in nature (Gutman et al., 2010; Ramírez et al., 2012). Large retrospective studies in Europe and the US comparing results between unrelated cord blood and adult volunteer donors have shown similar overall survival (Rocha et al., 2004; Laughlin et al., 2004). Important differences between the HSC sources are depicted in Table 5.

UNRELATED DONOR TRANSPLANTATION

Once transplantation from fully matched siblings and well matched relatives was done safely, the problem of patients without such a donor became the first priority. To this effect volunteer donor registries were started first in Europe (Anthony Nolan, UK, 1974) and later in the US (National Donor Marrow Program, 1986). These registries were quick to collaborate and large databases of donors from many registries were united (Bone Marrow Donors Worldwide). These efforts have resulted in more than 20 million donors recruited to date in all continents, providing thousands of HSC products for transplantation (WMDA) (Foeken et al., 2010). Despite this effort only 50% of the patients searching for an unrelated donor find one, and this average is widely variable among different ethnic backgrounds. The finding that umbilical cord blood was an alternative source of HSCs spurred the creation of public access cord blood banks which have meant a

TABLE 5

Comparison of the main features of UCBT and BMT (adapted from SCHOEMANS et al.)

	UCB	BM/PBSC
Number of available donors as 07 07/2012	563,6926 units	19,585,600 donors
Major limiting factor	Fixed unit cell content	HLA match and donor attrition
Minimum number of total nucleated cells for transplant	Aprox 2,5x107/kg	Aprox 2.0 x 108/kg
Second graft or DLI	Impossible	Possible
Median speed of donor availability	1 day	3-4 months
Donor morbidity	None	Fatigue, local pain, lower back pain
EBV/CMV transmission to recipient	Negligible	Possible
Risk for transmission of congenital diseases	Theoretically possible	None
Standard HLA match requirements	Minimum 4/6	Mostly 8/8

UCB: umbilical cord blood; BM/PBSC: bone marrow/peripheral blood stem cells; DLI: donor lymphocyte infusion; EBV/CMV: Epstein Barr virus/Cytomegalovirus

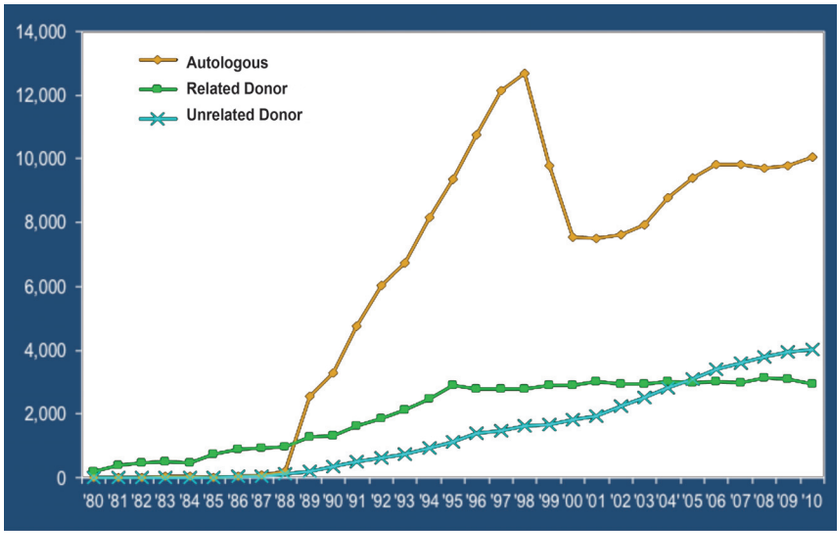


Figure 2. Transplant activity in the US 1980-2010. Center for International Bone Marrow transplant Research (www.cibmtr.org)

significant increase of the unrelated donor pool, especially for the pediatric age population. By 2012 an estimated 500,000 cord blood units have been stored globally (Bone Marrow Donors Worldwide). These banks obtain cord blood from donations; the blood is collected on delivery and white blood cells are purified, mixed with DMSO and frozen in liquid nitrogen. The units are then characterized (HLA, infectious diseases) and offered for clinical use through the multiple donor registries.

DONOR AVAILABILITY FOR UNRELATED DONOR TRANSPLANTATION

As more transplant teams are established and more donors are available worldwide, the number of allogeneic procedures has increased by nearly 30% in the past decade both in the US (Fig. 2) (Pasquini & Wang, 2011) and in Europe (Fig. 3) (Passweg et al., 2012). Mobilized peripheral stem cells have become the fastest growing stem cell source in adult transplantation and umbilical cord blood in children (Foeken et al., 2010) (Fig. 4). The growth of adult volunteer donor registries and the establishment of large cord blood banks have increased the available donor pool for unrelated transplantation. The present number of registered donors in Bone Marrow Donors Worldwide exceeds 20,000,000, including over 500,000 stored umbilical cord blood units which represent a nearly 12-fold increase in 20 years (Figure 5). Despite all this progress, transplant activity differs widely between developed and developing countries. The World Marrow Donor Association

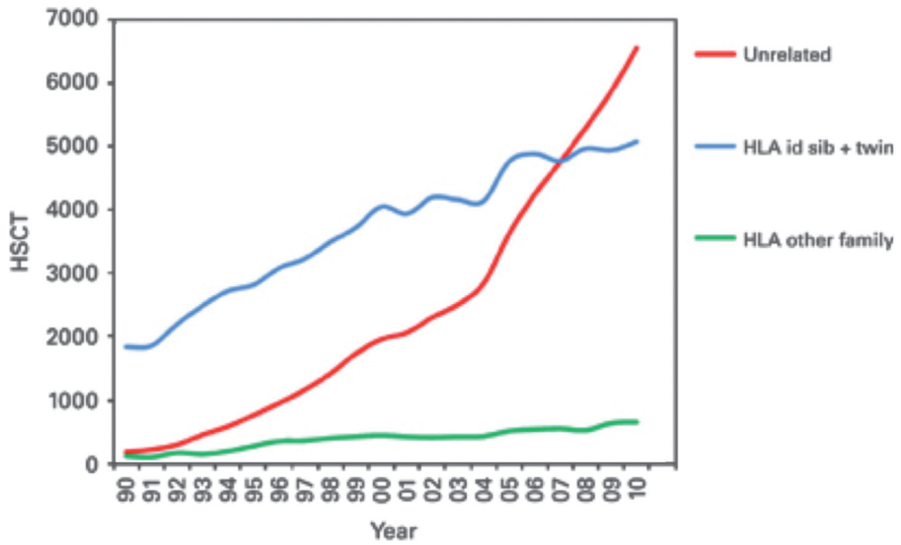


Figure 3. Allogenic Transplant activity in Europe (EBMT) 1990 to 2010 (Passweg et al., 2012).

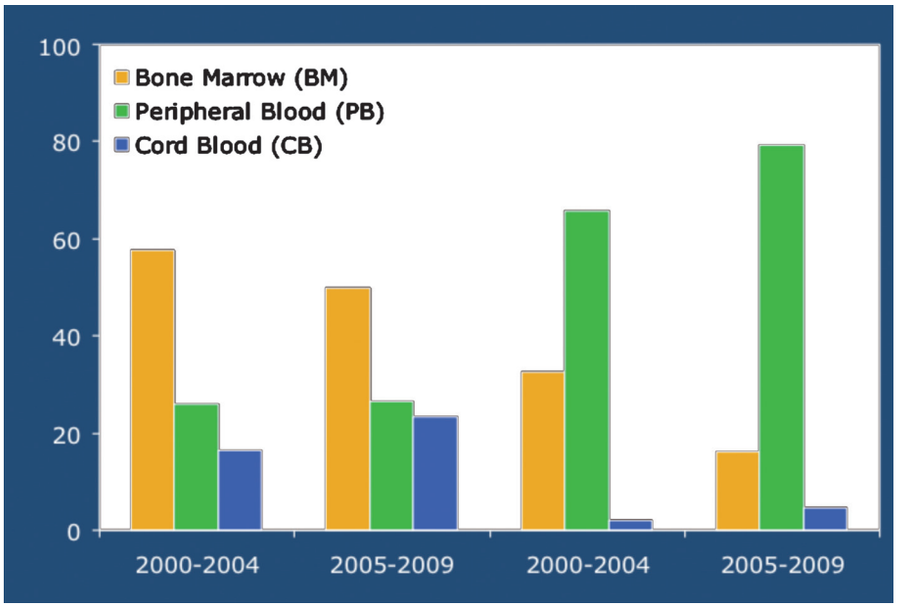


Figure 4. Allogeneic Stem Cell Sources by Recipient Age 2000-2009 (Pasquini ans Wang et al., 2011).

reported in 2010 the rate of unrelated donor transplants. Procedures per 10 million habitants in developed countries ranged from 82 (Spain) to 224 (Germany) while the reported activity in South America in that same period was 6 to 14 transplants per 10 million. This reflects the high level of medical infrastructure and human resources needed to implement this procedure successfully.

The use of T lymphocyte-depleted HSC grafts from haploidentical family donors has also gained acceptance in many groups trying to overcome donor shortage. This procedure is directed towards eliminating T alloreactive cells in the graft and avoiding GVHD; many centers have improved its efficacy and safety, resulting in a slow gain of acceptance.

ALLOGENIC TRANSPLANTATION FOR HEMATOLOGICAL MALIGNANCIES

Acute and chronic leukemia constitute the most common indications for allogeneic HSCT. The mechanisms by which HSCT can cure leukemia are twofold: the high doses of chemotherapy and radiation the patients receives before infusion of the HSC graft (conditioning or preparatory regimen); and the immune-mediated graft *versus* leukemia reaction which mirrors GvHD. The relative importance of each is not well defined, and as the graft *versus* leukemia GVL reaction is better understood, more patients are being prepared with less intense doses of chemotherapy and radiation, favoring engraftment but reducing toxicity and transplant-related mortality.

Long term results in malignant diseases have classically depended on patient age, donor source (matched versus mismatched, related versus unrelated) and stage of disease. Patients in early clinical remission fare much better than patients who have experienced multiple relapses or are no longer responsive to chemotherapy. Because of this many centers will omit transplanting a patient with active disease outside a clinical trial.

ALLOGENIC TRANSPLANTATION FOR NON-MALIGNANT DISEASES: EXPANDING INDICATIONS

Allogeneic HSCT not only plays an important role in the treatment of children and adolescents with malignant diseases but it is also an effective therapy for a wide range of non-malignant diseases including hemoglobinopathies (thalassemia, sickle cell disease), congenital or acquired bone marrow failure syndromes, primary immunodeficiencies and inherited metabolic disorders. In each of these diseases, donor-derived cells have the ability to correct the underlying defect, either by direct repopulation of the hematopoietic and immune systems or by indirect delivery of the missing enzymes or other critical building blocks across the cell membranes. Contrary to what is emerging in HSC transplants for malignant diseases, the best results are still obtained with the use of identical sibling donors, which only applies to 20% of these patients. Thus the number of transplants using unrelated donors (cord blood and adult peripheral blood or bone marrow) has increased in recent years, with variable results that depend on the donor, number of cells, degree of mismatches, co-morbidity of the patient and time of transplantation. The European Group for Blood and Marrow Transplantation published the recommendation for the use of HSCT in non-malignant diseases; in some entities the transplant is considered the standard of care and in other cases this option of treatment must be considered in relation to the condition of the patient and the decision of the team and the family (Ljungman et al., 2010).

Primary immunodeficiencies are inherited disorders characterized by impairment of innate or adoptive immunity, commonly leading to lethal complications. Allogeneic HSCT can cure most of the lethal forms of immunodeficiencies, including severe combined immunodeficiency (SCID), several T-cell immunodeficiencies, Wiskott-Aldrich syndrome, phagocyte disorders such as leukocyte adhesion deficiency and chronic granulomatous diseases, haemophagocytic syndromes such as familial lymphohistiocytosis, Chediak-Higashi

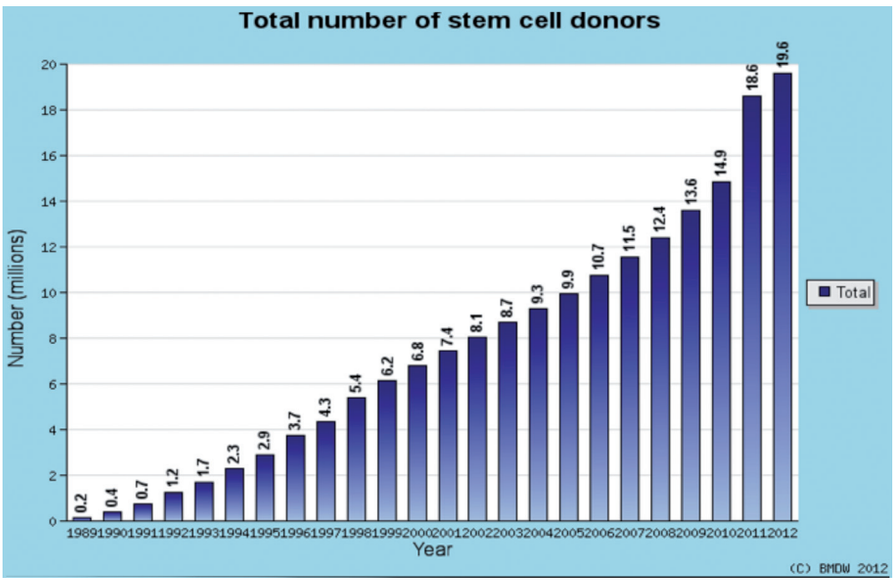


Figure 5: Unrelated stem cell donors registered with Bone Marrow Donors Worldwide 1989 to 2012 (www.bmdw.org).

syndrome, Griscelli's disease and X-linked lymphoproliferative syndrome. Treatment by HSCT is increasingly successful and is indicated from both HLA-identical and alternative donors. Patients with severe congenital immunodeficiencies need to be grafted as soon as possible. An allogeneic HSCT results in a survival rate of more than 90% when carried out shortly after birth. Prognostic factors are age, type of SCID, clinical state at the time of diagnosis, in particular the presence of a lung infection and degree of HLA histocompatibility (Wachowiak et al., 2008; Slatter & Cant, 2012). Allogeneic HSCT with an HLA-identical family donor is the treatment of choice for children with acquired severe aplastic anemia (Muñoz Villa et al., 2008). The use of alternative donors is restricted to patients who have failed other forms of therapy, and in these patients transplant-related mortality remains a significant problem mainly due to late or non engraftment (Maury et al., 2007). Children with Blackfan–Diamond anemia having a matched sibling should be transplanted if they do not respond to steroids or if they become dependent on them (Vlachos & Muir, 2010). Children with Fanconi anemia should be transplanted when they develop severe hematological disease if they have an HLA-identical sibling donor or a well-matched unrelated donor.

Allogeneic HSCT is the only currently available clinical treatment for many inherited metabolic diseases that lack effective enzyme replacement therapy, such as lysosomal and peroxisomal storage disorders (adrenoleukodystrophy, certain mucopolysacharidoses and sphyngolipidoses). Allogeneic HSC can correct these disorders in part by differentiation into monocyte macrophage cells such as microglia in the brain, Kupffer cells in the liver and alveolar macrophages in the lungs. These can induce long-term metabolic correction and ameliorate neurocognitive and functional problems (Krivit, 2004). Moreover, donor-derived cells induce 'cross-correction', a phenomenon by which the close proximity of normal cells can correct the biochemical consequences of enzymatic deficiency within the neighboring cells. Evidence of extensive distribution of donor cells in the blood vessels, peri-ventricular tissues, cerebral white matter, cerebellum, choroid plexus and forebrain parenchyma have been described (Krivit, 2004). Recent reports of large series have underlined the importance of transplantation in the early stages of the disease before significant organ and tissue damage occur (Prasad et al., 2008).

Hemoglobinopathies, such as thalassemia, sickle cell disease (SCD) and other complex defects, can cause major morbidity, poor quality of life and early death from the combined effects of anemia, hemolysis, iron overload and ineffective erythropoiesis. Early transplantation from a suitable donor prevents and reverses many of these problems. Because of different natural histories, the specific questions regarding the time of transplantation, criteria for patient selection and supportive care guidelines differ for patients with thalassemia and SCD. However, limitations of donor availability and risks of potentially serious toxicities have prevented HSCT from becoming the standard of care. To help define the patient selection criteria in thalassemia and an individual patient's risks from transplantation, Lucarelli et al. developed a scoring system on the basis of chelation therapy (regular or irregular), hepatomegaly and liver fibrosis, which predicted the risks of transplantation in patients with beta thalassemia (Lucarelli et al., 1993). This score has helped in the selection of both patients and donors for transplantation. Developments of conventional therapy have improved both the quality and the duration of life for patients with sickle cell disease (SCD). For this reason, HSCT from an HLA-identical sibling is offered only to a subset of patients at high, life-threatening risk or to patients who cannot receive adequate support (Shenoy, 2011).

THE HSC TRANSPLANT PROGRAM AT THE PONTIFICAL CATHOLIC UNIVERSITY OF CHILE

Our institution commenced this program in late 1989 and its evolution has paralleled the challenges and advances in the field. 475 transplant procedures have been performed in children and adults since its inception. Initially, autologous and matched sibling transplants were performed in standard risk patients with malignant and non-malignant diseases. Allogeneic transplantation quickly became the center of our effort as indications widened during the 90´s. Having safely established the sibling donor program we began to perform unrelated cord blood transplants in 1996, shortly after the first reports of its use were published (Rubinstein, 1998). During that period adult volunteer registries were restricted to centers outside the US and Europe, making this donor source unavailable. Unrelated cord blood, despite being the last source to be developed, bridged the gap that allowed us to solve the problem of patients lacking a family donor. Ten years later we started to procure grafts from adult donor registries after the National Marrow Donor Program launched an initiative to widen the use of unrelated transplantation worldwide. Nowadays our search process is initiated through Bone Marrow Donors Worldwide and registries with potential donors are contacted after the initial search. From 1989 through 2011, 268 patients have received an allogeneic transplant. Figure 6 depicts our program´s allogeneic transplant activity in 4-year periods since 1989, showing that in the last period the number of unrelated donor transplants surpassed those of siblings, in accordance with the global tendency.

Indications for allogeneic transplants in our program have mirrored those reported by CIBMTR (Table 6). Most patients are transplanted for hematological malignancies, mainly acute leukemia (68%), and most of them have received myeloablative conditioning including total body irradiation. 150 patients (71%) transplanted for malignancy had early disease (acute leukemia in first or second complete remission, chronic myelogenous leukemia in chronic phase, untreated myelodysplasia). 58 patients have been transplanted for non-malignant disorders, 45 of them children (77%) and 26 (40%) with the diagnosis of severe aplastic anemia have been almost all children. 170 patients received a graft from a sibling or well matched relative (63%), and 98 from an unrelated donor (86% cord blood).

5-year overall survival for patients with malignancies was 48%. We analyzed risk factors for overall survival during the period 1997-2007, when the unrelated donor program started. The only predictive factor for survival was disease stage (early 58%, advanced 12%, $p<0.001$, Fig. 7). For patients with early disease no differences were seen comparing patient age (<18 years, 43%; 18 or more 51%), donor source (related 62%; unrelated 59%), nor study period (1997 to 2005, 57%; 2006 to 2011 63%).

5-year overall survival for patients with non-malignant disorders in the pediatric age group was 62%. Due to the wide diversity of diseases in this group we sought to identify prognostic factors that could apply to all of them. Three such

TABLE 6

Patient and donor characteristics of the HSCT program at the Catholic University of Chile

Indications	Children < 18	Adults
Malignant		
Acute lymphoblastic leukemia	44	34
Acute myelogenous leukemia	23	35
Chronic Leukemia, myelodysplasia	17	34
Lymphoma	3	7
Other tumors	4	1
Early disease	66	84
Late disease	24	24
Non malignant		
Severe aplastic anemia	13	10
Hystiocytosis	4	
Congenital		
Marrow failure syndromes	9	1
Immunodeficiency	11	
Hemoglobinopathy		1
Metabolic disorders	8	
Sibling or other related donors	76	91
Unrelated donors	66	32
Cord blood	60	26
Adult volunteer	6	6

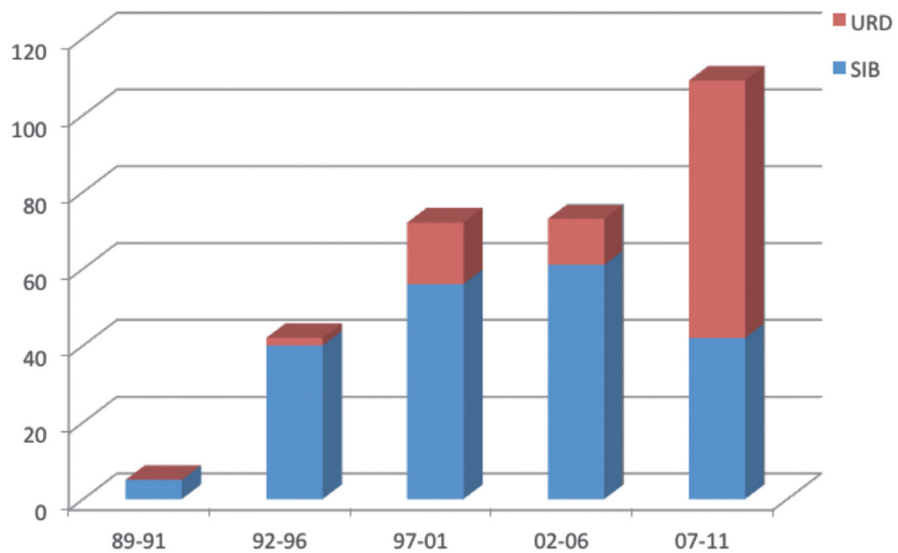


Figure 6. Evolution of donor types in the HSCT program at the Catholic University of Chile. Number of patients transplanted in different age periods. SIB: sibling or other related. URD: unrelated donor.

factors emerged; donor source (sibling versus unrelated), the immune status of the patient (lymphopenic versus non-lymphopenic) and the presence of significant co-morbidities which were disease-related (active infections, severe iron overload and poor performance status). A worse prognosis was associated with the use of unrelated donors, with patients that had normal lymphocyte counts at transplantation and the presence of co-morbidities. When all three factors were scored together a powerful prognostic index was identified which allowed us to identify a group with a high risk of failure (5 year overall survival of 87% versus 26%. $p<0.001$, Fig. 8).

CONCLUSIONS

HSC transplantation has developed into a mature field of medicine, reaching an ever growing number of patients worldwide thanks to improvements in transplantation techniques, center experience and donor availability. Our experience as well as that of many other centers has shown that patients with malignant diseases in need of transplantation should be evaluated and performed as soon as a donor is identified to avoid progression of their illness. Careful patient and donor selection will optimize results in patients with non-malignant disorders. Finally, continued improvements in the transplant process, in the understanding of the biology of hematopoietic stem cells and the ability to manipulate the immune system to develop a safer transplant procedure will help to benefit more patients without other realistic hope for a cure.

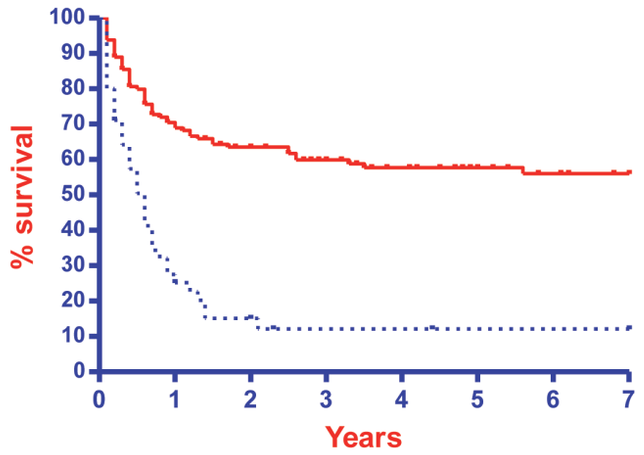


Figure 7. Allogenic HSCT results for patient with malignant diseases

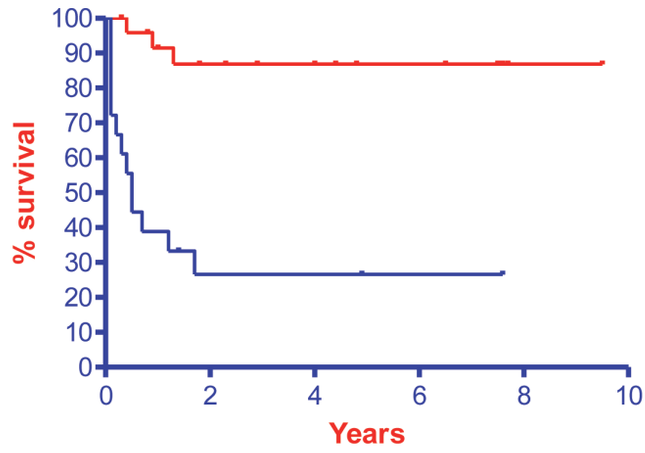


Figure 8. Allogeneic HSCT results in children with non malignant diseases. Prognostic score (PS): related donor: 0; unrelated donor: 1; lymphopenic patient: 0; nonlymphopenic: 1; without comorbidities:0; with comorbidities

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Bioethical aspects of basic research and medical applications of human stem cells**

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ABSTRACT

The new discoveries, the extraordinary dynamism in human stem cell (SC) research, and the great expectations of the benefits in clinical treatment of many diseases are on the edge of unparalleled advances in both: 1) the understanding of basic mechanisms of cell differentiation and development and 2) the translation from basic research to new clinical therapies. Human stem cells are obtained from different sources, such as embryo, fetal, and adult tissues, *in vitro* induction (iPS cells) or transdifferentiation. The evidence that these cells are pluripotent (or multipotent), meaning they have the ability to differentiate into all body tissues or tissues of the same lineage, raises the possibility that they could regenerate diseased or damaged tissue in diseases that until now have had no effective treatments. Human stem cell research and therapy raise important bioethical considerations because of the human nature of these cells and their peculiar characteristics. Here we discuss the bioethical aspects of basic human SC research and the conditions necessary for the translation of basic preclinical research into clinical use of SC.

Key words: Human Stem cells, Basic research, Bioethics; Clinical trials, translational research

INTRODUCTION

The production and use of human stem cells (SC), especially embryonic stem cells (ES) has attracted major scientific and public interest for two reasons: 1) From the scientific perspective, the evidence that these cells are pluripotent, meaning they have the ability to differentiate into all body tissues raises the possibility that they could regenerate diseased or damaged tissue in diseases that until now had no effective treatments (Andersson and Lendahl, 2009; Lindvall and Hyun, 2009; Riazi et al., 2009). This has resulted in high expectations in the media and public about treatments and eventual cures for a wide range of diseases, some of which have no effective treatment and mean painful situations for patients and their families, such as Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis. Taking into consideration the rapid development of human SC research, there is a vast amount of information offered to patients, researchers, clinicians and members of the public. This information may be in many aspects questionable and presented in the media uncritically (Peddie et al., 2009). Sometimes scientists themselves, in interviews in the media contribute to expectations that are not consistent with the current state of SC use in human medical treatments (Choumerianou et al., 2008; Devereaux and Loring, 2010). 2) The other aspect discussed in the scientific literature and media is the ethical issues involved in how SC are obtained (for example from human embryos), how they are stored and how are they used in Medicine (Gómez-Lobo, 2004; Knoepffler, 2004).

AIM OF THIS PAPER

We discuss the bioethical aspects involved in basic SC research and the conditions necessary for the translation of preclinical basic research to the clinical use of SC. Furthermore, we deal with the sometimes thin line between legitimate medical innovation and offers of treatments that do not fulfill ethical and scientific standards but nevertheless excite anxious patients who find these treatments outside of regular medicine in what has been called "SC tourism"

Definition and types of SC

There are several types of human SC: embryonic stem cells (ESC), adult stem cells (ASC), induced pluripotent stem cells (iPS) and reprogrammed adult differentiated cells obtained by transdifferentiation.

All SC have the following characteristics: they are undifferentiated cells, i.e. they are not specialized or specific like the cells of different tissues; they can replicate themselves and they maintain their differentiation potential and may eventually regenerate various tissues (Álvarez et al., 2012).

The different types of SC differ in their origin, potential to generate various tissue and adverse effects when used in living organisms. Until recently only two types and ways to obtain stem cells were available. However, in 2006 Yamanaka and his group produced stem cells with embryonic characteristics by reprogramming differentiated cells from the skin. These stem cells were called induced pluripotent (iPS) cells (Takahashi and Yamanaka, 2006). More recently, direct transdifferentiation was obtained between two types of fully differentiated adult cells, thus bypassing the pluripotential state (Vierbuchen, 2010).

Embryonic stem cells (ESC)

In 1981, Evans and Martin successfully isolated and cultured *in vitro* the inner cell mass cells of mouse blastocyst. Since these

Abbreviations: SC: Stem Cells; ESC: Embryonic Stem Cells; ASC: Adult Stem Cells, FSC: Fetal Stem Cells; iPS: induced Pluripotent Stem cells

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cells can differentiate into various tissues of the body, they are called pluripotent. These *in vitro* cultured cells are called embryonic stem (Evans, 2011). This was a landmark advance in the understanding of development, tissue homeostasis and progress of Regenerative Medicine. Then, in 1998 inner mass cells from a human blastocyst were successfully cultured (Conaghan J, et al. 1998). The main feature of ESC cells is the ability to multiply indefinitely *in vitro* while maintaining their pluripotent nature and the ability to be biologically manipulated to differentiate into different cells of various tissues

Adult Stem Cells (ASC)

Adult stem cells are found in various tissues of an organism already formed and are able to form and regenerate cell lines from a particular tissue or tissues. This feature is called multipotency. ASC may be cultured in vitro but this is more difficult compared with multiplication of ESC (Schuijers and Clevers, 2012). The most studied and clinically used ASC are the hematopoietic and mesenchymal cells in bone marrow. It is also possible to obtain this type of cells from the cord blood (Rao et al., 2012) and from the umbilical cord (Hayward et al., 2012).

Fetal Stem Cells (FSC)

Pluripotent stem cells are also found in fetal tissues, called Fetal Stem Cells (Ryan et al., 2011). FSC have also been found recently in amniotic fluid (Antonucci et al., 2012).

Induced pluripotent stem (iPS) cells

Being able to reprogram somatic cells to the pluripotent state through the introduction of specific genes was considered almost impossible. However this was experimentally done and at present is an important way to obtain pluripotent stem cells. Research in the production and use of iPS cells has followed a rapid advance (Takashi and Yamanaka, 2006; Pietronave and Prat, 2012).

Transdifferentiated cells

Finally, we should mention that transdiferenciation of ASCs have been achieved (Liu and Chang, 2006) and more recently direct reprogramming of somatic cells in cardiomyocytes and nerve cells has been achieved. This is called direct transdifferentiation which bypasses the pluripotent state with the advantage of reducing the chances of producing tumors (Vierbuchen, 2010; Yang, 2011). Nevertheless total risks of this type of cells have not yet been resolved (Pournasr et al., 2012)

BIOETHICAL ISSUES INVOLVED IN HUMAN STEM CELL BASIC RESEARCH

Embryonic SC

The main ethical issue in obtaining human ESC is the use and destruction of human embryos. Decades of biology has shown that a new human organism begins at fertilization (Haeckel, 1912; Gilbert, 2006). By basic conceptual logic, a human organism is a human being. This is the today focus of discussion in terms of the anthropological ethics dealing with whether the human being at this stage of development is morally relevant and has the right to the inviolability of life at this period (Valenzuela, 2007; Zarcseczny and Caulfield, 2009; Santos and Ventura-Juncá, 2009; Shoemaker, 2010; Ventura-Juncá and Santos, 2011). For some people, the personhood of a human embryo is reached at a later phase of development (for example at the 14th day of development), therefore the destruction of this so-called pre-embryo to obtain ESC form blastocysts (5-7th day) is morally acceptable (Wells, 1984).

Source of human embryos to obtain ESC

1. Embryos regularly obtained by IVF ("surplus" embryos): These are embryos obtained by IVF that are not used for implantation and are stored frozen at -180°C (Brett S et al., 2009) . Informed consent of the parents is required to use these embryos. Parents should understand that their embryos will be destroyed and parts will be used (inner cell mass) to obtain stem cells for research or in clinical applications. In some countries, such as the USA, a waiver of consent is permitted, thus allowing the use of embryos for research.

2. Production of new human embryos

2.1. Production of new human embryos from gamete donors: Human embryos may be created with oocytes and sperm from donors. If this is the case, obtaining Informed consent from donors should specify the terms of human embryo research. (National Academy of Sciences Guidelines for Human Embryonic Stem Cell Research, 2005, 2010; http://www.nap.edu/catalog.php?record_id=12923#description/ access July 26, 2012). These guidelines imply that embryos are not people and not subject to respect as research subjects.

2.2. Production of new human embryos by Somatic Cell Nuclear Transfer: French et al (2008) have produced human embryos with somatic cell nuclear transfer. The authors published the development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. Claims in Science in 2004 and 2005 by Hwang et al. to have cloned human embryos from healthy women and diseased patients,respectively, were proven to be fraudulent (Editorial Science, 2006). The production of human embryos by cloning represents a major ethical issue in that the production of cloned humans undermines human dignity by making a person into a product.

3. ESC produced by "parthenogenetic embryos": Parthenogenesis is a form of reproduction in which an unfertilized egg develops into a new individual. The first pESC lines derived from human parthenotes were obtained by Revazova et al. (2007) and Revazova et al. (2008). The main ethical question arising is whether a parthenote blastocyst is an altered human embryo or just a ball of cells without organization. This is a major anthropological question, with significant ethical consequences. The discussion continues and at the center is the question of the ontological status of parthenotes, which needs to be established before activated oocytes can be considered as an ethical source for pluripotent stem cells (Rao et al., 2008).

4. Production of ESC without destroying human embryos: The ethical objections in obtaining human ESC have encouraged efforts to obtain them with techniques that do not involve

the destruction of human embryos. Several attempts have been proposed to circumvent to produce entities that are not considered human embryos but that could be capable of producing ESC. They have been complicated and most of them have not reached satisfactory results (White paper, 2005; Condic, 2008; Ventura-Juncá et al., 2009).

a) **Altered Nuclear Transfer and Oocyte Assisted Reprogramming:** Altered Nuclear Transfer is a modification of the nuclear transfer technique, with preemptive genetic and epigenetic modification of the nucleus of the somatic cell to be transferred (White Paper, 2005). When there is also an alteration of the oocyte cytoplasm before transfer is done, the technique is termed Altered Nuclear Transfer with Oocyte Assisted Reprogramming. The ethical hypothesis of those who defend this line of research is that the technique will result in a biological entity without the characteristics of an organism. The philosophical debate has centered on what are the necessary characteristics of a new organism and how can we differentiate a biological entity from an organism. Scientists and philosophers with the same anthropology regarding the human embryo, differ in their evaluation of this technique (Colombo, 2004; Austriaco, 2006; Hurlbut et al.,2006).

   This strategy has been abandoned due to the complexity of the technique and doubts about its results from a scientific perspective, as well as ethical objections. As we stated in our previous paper in 2009 (Ventura-Juncá et al., 2009): "There is one published paper in which ESC lines were obtained with this strategy in mice. The technique consisted of silencing the Cdx2 gene from the nucleus of the somatic cell transferred to the oocyte (Meissner and Jaenisch, 2006). The cloned blastocysts were morphologically abnormal with no expression of the CDx2 gene. They lacked a functional trophoblast and failed to implant in foster mothers. Nonetheless, ESC lines were derived from these blastocysts. It is relevant that Meissner and Jaenisch, authors of this article, recognize that the ethical dilemma may not be resolved since the Cdx2-deficient embryo appears to be normal in the first stages of development before the Cdx2 gene is expressed. In theory, the so-called biological entity by its defenders could be transformed into an embryo during the development to blastocyst by one single gene. It seems unlikely that the ontological condition of a biological organism could depend only on the simple silencing and activating of a gene. Thus, we could have new human beings that appear and disappears with this action alone. This demonstrates that the ethical aspects have not been solved and that the facts show that what are being produced are disabled embryos and not biological entities".

b) Human-animal chimeras: A chimera is a hybrid creature that is part human and part animal. Chinese scientists at the Shanghai Second Medical University in 2003 successfully fused human cells with rabbit eggs. The embryos were reportedly the first human-animal chimeras successfully created. They were allowed to develop for several days in a laboratory dish before the scientists destroyed the embryos to harvest their stem cells (reported by Mott, Maryann on January 25, 2005). "Animal-Human Hybrids Spark Controversy". National Geographic News. http://news.nationalgeographic.com/news/2005/01/0125_050125_chimeras.html. (access July 26, 2012). But creating human-animal chimeras (named after a monster in Greek mythology that had a lion's head, a goat's body, and a serpent's tail) has raised ethical questions: What new sub-human combination could be produced and for what purpose? At what point would it be considered human? And what rights, if any, should it have? . Creating chimeras diminishes human dignity (Beca JP, 2007). In the UK, production of human-animal (cow-sheep, etc) chimeras is allowed to obtain ESC, provided the chimeric embryos are destroyed by day 14th. (http://www.hfea.gov.uk/docs/Hybrids_Chimera_review.pdf/ (access July 26, 2012)

Fetal SC

In countries where abortion is allowed, fetal stem cells are obtained from aborted fetuses. This is highly controversial, concerning mainly the ethical issue of abortion, i.e the termination of a human life (Lo and Parham, 2009).

Adult SC

Adult stem cells can be isolated from cord blood and umbilical cord, adult blood, adult bone marrow, or other adult tissues. Although their use does not pose the ethical issues raised by the use of human embryos, there are particular ethical issues common to all types of stem cells, especially related to the fact that after transplantation they remain in the host and may behave unpredictably in the human body (i.e. possible development of tumors). In addition, there are other relevant ethical issues such as the use of children (American Academy of Pediatrics, 2010), intellectual property and patents (Bahadur and Morrison, 2010), economic conflicts of interest (Caulfield, 2010) and how the information about the basic research and clinical applications are delivered to the public through the media (Lo et al., 2008; Sugerman, 2008, Lo and Parham, 2009). In relation to these specific matters, an adequate process of informed consent is a key feature. In terms of public and private cord SC banks, there is a debate over the type of information publicized in the media. Specifically, several private banks of umbilical stem cells can raise parents' expectations in ways not consistent with the findings of the scientific literature (Samuel et al., 2008). Specific regulations for public and private stem cell banks are needed, as well as clear information of their possible benefits (Smith, 2011).

CLINICAL MEDICAL APPLICATION OF HUMAN STEM CELLS: TRANSLATIONAL MEDICINE

Embryonic Stem Cells

The regenerative capacity of ESC has been tested in preclinical studies *in vitro* and in animals. The move to human clinical studies is challenged by unresolved ethical objections related to obtaining ESC from human embryos and the unresolved scientific problem concerning the production of tumors. In January 2009, the FDA approved the first Phase I clinical trial with differentiated cells obtained from ESC for treating paraplegics and in April 2011 for the treating Stargardt's

Macular Dystrophy http://www.clinicaltrials.gov (access July 26, 2012). Updated results have recently been published (Schwartz et al., 2012). On the other hand, the emblematic Geron embryonic stem cell clinical trial for spinal cord injury has been shut down (http://www.nytimes.com/2011/11/15/business/geron-is-shutting-down-its-stem-cell-clinical-trial.html). The production of ESC cell lines has aroused great expectations in both the scientific community and the public with the possibility that these cells could regenerate diseased or damaged tissues in several diseases that represent difficult and painful situations for patients and their families. Patients have unfounded expectations that SC therapy will improve their functioning (Daley, 2010). The major source of information on stem cells is the media rather than physicians. To reduce patients' exposure to inappropriate messages, doctors should make more effort to educate patients using mass media with accurate information (Kim et al., 2012)

Adult Stem Cells

The first marrow transplantation with multipotent stem cells from bone marrow, conducted in 1950 by Edward Thomas for treatment of leukemia opened a new way to regenerate tissues (Thomas, 2005). The progress of this procedure allowed its use in the treatment of other malignant diseases of the blood and is being studied for the use of other ASCs in various pathologies such as Multiple Sclerosis, Alzheimer's disease, Amyotrophic Lateral Sclerosis, heart tissue regeneration and Diabetes. The possibility to cure any sort of diseases by Regenerative Medicine (Andersson and Lendall, 2009) using ASC has also raised high expectations among the public and the media. More than hundreds worldwide clinical trials are being conducted or in progress with ASCs (http://clinicaltrials.gov (access July26, 2012). Apparently, ASCs produce less frequency of tumors and from the ethical point of view, human embryos are not destroyed to obtain them.

In Chile, treatment since 1995 has mainly involved proven ASC, especially those related to bone marrow transplantation for malignant blood diseases (Barriga F et al., 1995, Barriga et al., this issue). They are performed primarily in academic centers and approved by Ethical Research Committees. There are some experimental studies for the treatment of Dystrophic Epidermolysis Bullosa (Conget et al., 2010); Osteoarticular pediatric diseases (Norambuena et al., 2012) and Amyotrophic Lateral Sclerosis with small groups of patients (Soler et al., 2011).

Fetal Stem Cells

One phase I clinical trial is underway using neural stem cells derived from aborted fetuses for the treatment of Batten's disease (Lo and Perham, 2009). As mentioned above, from the ethical point of view this is highly controversial, due to the issue of abortion, i.e the termination of a human life. Several clinical trials are currently going on such as the use Human Fetal Liver Cell Transplantation in Chronic Liver Failure http://www.clinicaltrials.gov/ct2/show/NCT01013194?term=fetal+stem+cells&rank=1 (access July 26, 2012) or and the use of human neural stem cells transplantation for the treatment of Amyotrophic Lateral Sclerosis (http://www.clinicaltrials.gov/ct2/show/NCT01640067?term=fetal+stem+cells&rank=4) (access July 26, 2012)

iPS cells

In the case of iPS, it should be noted that there are complex issues to resolve, such as ensuring complete multicellular reprogramming and stability, before iPS cells can be used in regular clinical treatment. If the iPS cells and transdifferentiated cells finally replace ESCs, broad ethical and scientific consensus could be reached among researchers with different anthropological and ethical positions regarding the respect due to the human embryo. For all of those who recognize a human being in the human embryo, with the dignity and rights of a member of the human family, the iPS cells and transdifferentiated cells is a major line of research that merits significant support and work. But one should be cautious in creating too much expectation about the potential benefits of the use of these cells (Pietronave and Prat, 2012).

BIOETHICAL ISSUES IN THE MEDICAL USE OF HUMAN STEM CELLS

The anthropological debate on the status of the human embryo is and has been central to the case of ESC. But there are other ethical issues common to all SC, which are related to translating biotechnological progress to clinical use. These are becoming increasingly important. (Lo et al., 2008; Lo and Parham L, 2009; Hyun, 2010a,b). These issues, though common to all clinical research, have specific implications and consequences in the case of SC that require special attention. These include, among others, informed consent, (Lo et al., 2008); the use of children as donors (American Academy of Pediatrics Committee on Bioethics, 2010); intellectual property and the need for regulation of any patents derived from research with SC (Simon et al., 2010; Bahadur and Morrison; 2010), financial conflicts of interest arising out of the market potential of SC, which can influence the direction of research, public information and equitable access to treatment (Lanoszka, 2003; Caulfield, 2010). Developing countries in this regard are particularly vulnerable (Pratt and Loff, 2010). Unlike a new drug, SC transplantation remains in the organism and its behavior is still in many respects unpredictable. In addition to these issues, there is the area of ethical and scientific implications of storing cord blood in private banks (family) and advertising and the information given to parents about their potential benefits (Onisto et al., 2011; cryopreservation in Chile: http://www.cryo-cell.cl/ access July, 2012; http://www.vidacel.cl/ access July, 2012). One particular bioethical issue common to the use of any SC is how to inform the public about the real contribution of SC to treat diseases, so the public may make better and more informed decisions. This poses a major responsibility for doctors and researchers to provide such information (Peddie et al., 2009).

Reasonable expectations of Regenerative Medicine with SC: The danger of transforming hope into hype

Regenerative Medicine based on SC therapy can dramatically change Medicine (Andersson and Lendhall, 2009). This hope has solid foundations in the substantial preclinical research in the laboratory and in animals with various types of SC, as well as results of some clinical ASC treatments. However, there are still serious unresolved scientific problems to overcome before the move to clinical use. These are mainly related to the basic

safety needed for any new treatment (Hyun et al., 2008). The problem of tumor production is one of the most relevant. The International Society for Stem Cell Research (ISSCR) (http://www.isscr.org (access July 26, 2012) has stated that so far, the only therapy with SC incorporated as a standard medical treatment is the use of ASC for a few number of diseases with such as hematopoietic ASC transplantation for leukemia and other blood diseases, epithelial stem cells to treat burns and some problems with the cornea (Rama et al., 2010). Bone marrow transplantation has certainly brought a qualitative improvement of great importance in medicine in recent decades (Perry and Linch, 1996). The number of worldwide ongoing clinical trials with ASC exceeds hundreds. In the case of human ESC, only one clinical trail is currently underway (Schwartz et al., 2012). In the case of iPS cells, there are currently more than 10 ongoing clinical trials but no treatment has been approved for regular clinical use. It is highly likely that the dynamics of research in this field can reach clinically validated treatments in several other areas of medicine with different types of SC.

However, public perception, the media and many patients have expectations beyond the current development of Regenerative Medicine with SC. The anguish of patients and sometimes the enthusiasm of doctors and researchers can obscure the fact that there is a long road ahead for preclinical research before safe clinical application. Scientists have noted that it is important that the great expectations raised for years with gene therapy and the very limited results may not be repeated with SC therapy (Verma, 1994; Couzin and Kaiser, 2005; Rosenecker, 2010).

In summary, the translation from basic research to clinical practice in the case of SC raises particular ethical and scientific issues that currently concern both scientists and bioethicists.

Clinical trials, and innovation in Medicine

The clinical trial path. There is consensus among scientists and bioethicists that generally the safest and most responsible route for approval of new treatments is the process of approved clinical trial protocols. The experience of decades has shown the importance of this route, especially in the case of new drugs (Meadows, 2001), which includes first preclinical studies and then a sequence of clinical phases intended to test the safety and benefits of the new treatment known as phase I-II-III and IV. The rigorousness of this process includes scientific and ethical aspects The protocols must be approved by competent scientific and bioethical authorities. This has meant a solid advance in medicine and patient protection, becoming the paradigm of clinical research.

The path of innovation in Medicine. Beyond clinical trials. It is evident that not all medical progress has been made through the clinical trial model. This is a discussion that covers various areas of Medicine that has particular ethical dimensions (Agich, 2001). Progress has also been achieved by medical innovation. The classic example is Surgery. There are numerous surgical procedures that were developed outside the clinical trail scheme for reasons of their own development. Examples are laparoscopy and cardiac transplantation, among others (Cosgrove, 2008). Something similar has happened in other areas of Medicine, especially in Pediatrics and Neonatology This route is not without risks (James and Lanman, 1976; Duc, 1995). There are treatments that have been incorporated into regular use in the clinic, however when they have undergone clinical trials they have lost their validity, due to the contribution of evidence-based Medicine. Thus, medical doctors and patients should be aware of the uncertainties and risks involved in this course of action (Mcculloch et al., 2009). The issue of medical innovation out of the process of clinical research regularly, is of special interest in the case of SC and highly controversial (Hyun, 2010; Martell et al., 2010).

Ethical research policy and ethics of caring for the sick. The purpose of clinical trials is to produce a general knowledge for the use of new treatments with proven effectiveness and safety. The good of an individual patient is not the primary objective. In the case of innovative treatments that have not been tested by clinical research protocols, the perspective is different. They are covered by the field of ethics of the patient care aimed at the welfare of the individual patient. The ethics of clinical research does not have the same parameters as the ethics of the individual patient care. While there is always a risk versus benefit assessment, it requires a particular informed consent because the purpose is different.

In an innovative treatment the goal is the good of the patient without knowing the outcome for certain. This requires a particular form of informed consent to guarantee the security of the patients involved to be consistent with the intended purpose. The experimental treatment plan should be reviewed by qualified peers on scientific and ethical grounds. The results should be reported in scientific journals. Fulfilling these conditions, as expressed by Lindvall and Hyun in 2009, innovative treatment outside the framework of clinical trials may be acceptable from a scientific and ethical perspective. These treatments should be reserved for severely ill patients who have no good treatment options. These patients are usually anxious and more interested in getting better and surviving than in expanding medical knowledge. They and their physicians should be clearly informed, given that SC will remain in the body and behave unpredictably. In order to warrant safety in innovative SC treatment more ethical considerations should be taken care of, such as clarifying the types of patients who qualify for them, maintaining adequate monitoring, taking into account the lack of evaluation of the placebo effect and giving consideration to the route for clinical trials if the gains are positive for a small group of patients (Cohen and Cohen, 2010b). Although medical innovation in the treatment of patients has made significant progress, major disasters have also occurred in Medicine and Surgery in the past (James and Lanman, 1976; Duc, 1995; Mello et al., 2001).

Stem Cell Tourism

The emergence of clinics worldwide that offer treatment with SC: The high expectations created by the potential therapeutic benefits of SC have had two interrelated effects. On the one hand, patients with severe or untreated diseases are eager to access SC treatment of diseases for which conventional treatment are ineffective (Jawad et al., 2012). At the same time, there is an increasing number of unregulated clinics worldwide that offer SC treatment for various diseases (Kiatpongsan and Sipp, 2009). As a result, patients suffering from untreatable or incurable diseases are the subject to unrealistic promises, unpredictable risks, misinformation, and eventual economic

exploitation (Taylor et al., 2010). This is called "stem cell tourism"(Carrera and Bridges, 2006; Chandler, 2010; Cohen, 2010; Crozier and Thomsen, 2010; Lunt and Carrera, 2010; Murdoch and Scott, 2010; Master and Resnick, 2011; Jawad et al., 2012). The stem cell tourism differs from the situation in which patients with serious illnesses, travel to qualified centers in other countries to get a better treatment or more experience. We agree with Mainil et al (2012) that a better alternative terminology for this latter procedure is "'transnational health care", understood as a 'context-controlled and coordinated network of health services", which is rather different from the concept of stem cell tourism.

The emergence of the stem cell tourism clinics has raised concerns among scientists and bioethicists who raise serious scientific and ethical issues underlying this situation about the prestige of SC research and patient safety (Nelson, 2008; Gunter et al., 2010). In 2006 an article in Science reported there were nine research institutions around the world offering SC treatment for a range of very different diseases, mainly neurological disorders (Parkinson's, Amyotrophic Lateral Sclerosis, Spinal Cord injuries, Autism, Depression), but the range included Myocardial Infarction, Diabetes, AIDS, Cancer and even infertility (Enserink, 2006). The SC used were mainly ASC from the patient's own cord blood or from abortions (fetal). The cost of these treatments was about U.S. $20,000, excluding travel expenses and accommodations. The number of patients being treated adds up to many thousands and with results that were, in some cases, dramatic, according to the clinics involved. In 2008, Lau et al. studied 19 clinical treatment offers, the way that the treatments are presented to the public and the clinical evidence for the treatments offered. The results showed that treatment offerings are varied and optimistically presented. However, there was no precise information on the types of SC used, their origin and how they were administered and there was no indication if these treatments had been evaluated by experts. The information given to patients over the Internet was incomplete and could have raised public expectations without solid foundations. All clinics reported an improvement in treated patients, but without statistical support to evaluate the results. There was no clear mention of the risks. Only a few clinics mentioned problems associated with the procedure. The authors warned that the overall results do not imply the clinical assessment of each individual, because there was no access to personal information received by patients or adverse or beneficial results.

The number of these clinics is increasing (Dolgin, 2010; Ryan et al., 2010). They are found in Russia, Santo Domingo, Barbados, China and India, where there are fewer regulations to control such treatments (Pepper, 2010). But there are also clinics in countries with greater safeguards, like Holland and Germany. In the mainstream print media, offers are presented optimistically, influencing the perceptions of patients (Zarzeczny et al., 2010). China is the subject of special attention because of the high number of clinics and patients recruited. It is estimated that there are about 100 laboratories dealing with SC techniques and at least three clinics that offer treatment. By 2009, it was It was estimated that about 6500 patients would receive treatment in two of the largest clinics in China, many of them from other countries (Mcmahon and Thorsteinsdóttir, 2010a). The lack of control over the quality of the treatments offered led the government to implement some regulations, which have been considered inadequate and poorly enforced (Cyranoski, 2009; Nature Editorial, 2010). Cohen and Cohen (2010a) analyzed the situation in Russia and India, where there are similar situations. The way to reach a large number of patients is primarily through the Internet. This avoids regulations in some countries that affect advertising media such as television and brochures. Reports of problems with unregulated SC treatment corroborate the risks of these offers to patients and challenge the prestige of SC research and clinical use. For example, there is a report of a child in Moscow, Russia, with a brain tumor after being injected with fetal neural stem cells in the cerebrospinal fluid (Amariglio, 2009; Macready, 2009). In Thailand a child developed a special form of tumor after a clinic injected autologous ASC to the kidney (Thirabanjasak et al., 2010; Cyranoski, 2010a). In Korea, two patients died after SC treatment (Cyranoski, 2010b) and in Germany a child with Cerebral Palsy died after SC was injected in the brain (Tuffs, 2010).

The urgency to establish regulations

The situation described above has prompted the scientific community and bioethicists to seek regulatory criteria and requirements for information to protect patients and their relatives (Cohen and Cohen, 2010b). For this to be effective in an increasingly globalized and interconnected world, it is necessary that there be international agreements among nations (Mason and Manzotti, 2010, Schalev, 2010).

Recommendations for clinical trials: In 2008 a group of experts at the request of the president of the International Society for Stem Cell Research (ISSCR) published the guidelines for the translation from preclinical research to clinical application (International Society for Stem Cell Research: http://www.isscr.org/ access July 26, 2012). Hyun et al. (2008), commenting on these guidelines, state that SC clinical research involves specific aspects that need to be treated carefully. In agreement with other researchers (Lo and Parham, 2009; Lo et al., 2008), they emphasize the following: the review of treatment protocols by SC experts; the need to clearly define the origin, quality and handling of the SC used; the informed consent process requires additional aspects compared to the one generally used in scientific research, such as information regarding scientific and ethical aspects of particular relevance, especially when they involved the destruction of embryos, the risks involved with different types of SC and the possibility of adverse effects, and the lack of knowledge about long-term effects, etc., regular monitoring of the participants in research to ensure their welfare,transparency in communicating the positive and negative outcomes, and adverse effects and social justice in access to treatment.

Recommendations for treatments of medical innovation beyond clinical trials: The ISSCR guidelines assume that if certain requirements are met treatments beyond clinical trials can be ethically and scientifically valid for application to a small group of patients with severe and intractable diseases http://www.isscr.org/GuidelinesforClinicalTranslation/2480.htm (access July 26, 2012). Given the novelty of the SC, they can have unpredictable behavior that must be considered by doctors and informed patients. The summary of these requirements is: There must be a written protocol of the

procedure (including scientific consistency for use with a particular patient or small group; justify its use as opposed to other possible treatments, characterization of the SCs to be used: origin, type, *in vitro* manipulation, specify method of administration; follow-up plan and monitoring to assess effects and effectiveness); written approval by competent reviewers not involved in the investigation; medical institution and the doctor performing the treatment should take responsibility for this; the institution must have competent personnel and adequate facilities; informed consent must be complete and transparent (It should be determined if the patient has understood that it is an unproven intervention and the possible risks and benefits); regular medical checkups and plan for possible adverse effects; the purpose of the research in contributing to SC research and the generalization of the results should be made explicit by those responsible for the research (this includes: systematic evaluation of the result, communication of results, plan to move to clinical trials in adequate time).

Increased offers of SC treatments in clinics in several countries around the world moved the ISSCR in 2010 to publish the Patient Handbook on Stem Cell Therapies, which is available in several languages /http://www.isscr.org/The_Patient_Handbook.htm; access July 26, 2012).

The debate about regulation and patients' autonomy: This issue is still debated. The International Cellular Medicine Society (ICMS) is an organization formed by scientists, doctors and patient, with the aim of cooperating in informing and educating physicians and patients about the use of and advancement in SCs. The ICMS has a record of the clinics that offer treatment and also provides guidance on quality and safety (http://www.cellmedicinesociety.org/ access July 26, 2012). According to the ICMS, the ISSCR guidelines are more oriented to research and interfere with the autonomy of patients and physicians to use well-regulated SC beyond clinical trials. The debate continues through open letters on the position by both institutions (Audley, 2011; Sipp, 2011).

Finally, we must consider the issue of doctors who use drugs that are experimental or that are used for treatments which they have not been approved for (Okie, 2006; Radley et al., 2006; Lat et al., 2011). This is a relatively common practice. However, it is essential to consider differences between SC and drugs. SC will live for a long time and can affect the patient with unexpected responses and changes depending on environmental signals and intrinsic properties (Ginis and Rao, 2003). The use of SC by some clinics has provoked the intervention of the Federal Drug Administration (FDA) (Cyranoski, 2010). These clinics have defended their position and claim to follow the ISCM guidelines. An article in Nature in 2010 mentioned the lawsuit filed by the FDA against a clinic that was using SC for the orthopedic regeneration. Its medical director C. Centeno defended his position and reported his results in scientific journals (Centeno et al., 2010). Some policies have been established concerning the information provided by SC clinics. According to these policies, there is arguably an ethical duty to provide potential clients of the clinics with the best available information about the risks and benefits of what is essentially an experimental treatment.

Patients affected by untreatable diseases often turn to alternative or natural Medicine, which in many cases does not have a scientific basis. Nobody considers that they do not have the right to do this. But, is this comparable to the case of SC tourism?. We think not. Stem cells remain in the patient's body and can have unexpected harmful effects as in the cases cited above.

CONCLUSIONS

The ethical aspects of research and clinical application of SC are very important. Initially the main ethical debate related to the moral status of the human embryo. While this remains an important issue, new scientific developments, and especially the transfer to clinical application of SC, have raised additional ethical challenges.

The hope of curing various diseases has produced disproportionate excitement in the public that does not correlate with the current status of the clinical use of SC. The existence of unregulated clinics that offer SC treatment, in most cases with serious scientific and ethical shortcomings, has led to hundreds of anxious patients going to these clinics with great expectations and at great cost. Moreover, this jeopardizes the prestige of research in this field.

The scientific community and bioethicists have responded to this situation by providing guidelines for the licit translation from the pre-clinical research to the clinic and providing patients with information on clinics offering SC treatments and how to evaluate the decision to go to them.

We also addressed the issue of innovation and medical progress beyond clinical trials and the scientific and ethical requirements for these exceptional cases. It is not easy to make a clear separation between what is medical tourism and accepted clinical innovation according to precise parameters.

In the future it will be necessary to establish global guidelines and regulations implying an agreement among countries to effectively protect patients and ensure the proper use of the amazing advances expected with SC treatment and research for the sake of science and of all patients considering the requirements of social justice worldwide.

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