"Mesenchymal and neural stem cell systems: fate determination by bi-directional activities"

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Francisco Javier Rivera Gomez-Barris

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Thesis Supervisor: Dr. Jose Minguell

Thesis Co-Supervisor: Dr. Ludwig Aigner

Thesis Co-Supervisor: Dr. Walter Sierralta

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Francisco Javier Rivera Gomez-Barris

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Directores de Tesis:

Dr. Jose Minguell   ....

Dr. Ludwig Aigner   ....

Dr. Walter Sierralta   ....

Comisión de Evaluación de la Tesis:

Dr. Juan Bacigalupo

Dr. Christian Gonzalez

Dra. Jimena Sierralta

Dr. Manuel Santos   ....

DEDICATION

To my supervisor and great friend Ludwig, alias Luchito, for giving me all the chances to grow not only as a scientist but also as a person.

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LIST OF ABBREVIATIONS

α-MEM	Alpha Modified Eagles Medium
bHLH	basic Helix-Loop-Helix domain
BM	Bone Marrow
BME	Betamercaptoethanol
bp	Base pair
BSA	Bovine Serum Albumin
BrdU	Bromo-deoxyuridine
cDNA	Complementary DNA
CNS	Central nervous system
°C	Centigrade
Da	Dalton
DAPI	4',6'-diamidino-2-phenylindol-dihydrochlodire-hydrate
DCX	Doublecortin
DPBS	Dulbeccor's phosphate buffered saline
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleotide acid
dNTP	Desoxyribonucleotide-triphosphate
E	Embryonal day
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EtOH	Ethanol
FGF-2	Basic fibroblast growth factor
g	Gram
GAPDH	Glycerinaldehyde-3-Phosphate-Dehydrogenase
GalC	Galactocerebroside
GFAP	Glial Acidic Fibrilary Protein
HBSS	Hanks Balanced Salt Solution
HC	Hippocampus
HEPE	N-(2-Hydroxyethyl)piperazin-N'-(2-Ethansulfonacid)
hNSC	Hippocampal neural stem cells
HSC	Hematopoietic stem cell
IGF-1	Insuline-like growth factor
IGFBPs	IGF binding proteins
IL-6	Interleukin 6
l	liter
MAP2ab	Microtubule-associated Protein 2a + 2b
MBP	Myelin Basic Protein
MEM	Modified Eagles Medium
mg	milligram
ml	milliliter
mRNA	messenger RNA

MSC	Mesenchymal stem cell
μ	micro (10-6)
NF	Neurofilament
NSC	Neural stem cell
NT-3	Neurotrophin-3
NeuN	Neuronal nuclear antigen
PBS	Phosphate buffered NaCl-solution
PCR	Polymerase chain reaction
pH	Negative Logarithm of the H+-Ion concentration
PFA	Paraform aldehyde
PI	Propidium Iodide
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
Shh	Sonic Hedgehog
SVZ	Subventricular zone
TBE	Tris-Borate-EDTA-Elektrophoresis Buffer
TBS	Tris-Borate buffered saline
TE	Tris –EDTA-buffer
TH	Tyrosine hydroxylase
Tris	Tris-(hydroxymethyl)-aminomethane

ABSTRACT

Adult stem cells reside in different tissues and organs of the adult organism and play a crucial role in cellular homeostasis. Among these cells are mesenchymal stem cells (MSCs) that reside in the adult bone marrow and neural stem cells (NSCs) that are located in the adult central nervous system (CNS). MSCs and NSCs share the typical stem cell characteristics, i.e. proliferation, self-renewal and multipotency. MSCs differentiate into a variety of mesenchymal lineages such as osteocytes, chondrocytes, tenocytes, adipocytes and myocytes, whereas NSCs give rise to neurons, astrocytes and oligodendrocytes. Cell fate of MSCs and NSCs is specified by intrinsic determinants and by extracellular cues present in the respective cellular microenvorinment, the stem cell niche. Among these are soluble factors that regulate proliferation, self-renewal, determination, differentiation and survival in autocrine and/or paracrine modes of action.

The differentiation fate of MSCs and NSCs is not restricted to the mesenchymal or, respectively, neural lineage, but the transdifferentiation phenomena have been demonstrated. For example, when placed in a neurogenic environment such as the hippocampus, MSCs generate cells of the neural lineage and they express a neural-like phenotype. NSCs, in contrast, repopulate the hematopoetic system after experimentally induced depletion. Apparently, the effects on the two stem cell systems are bi-directional, since MSCs affect also the neural microenvironment. For example, MSCs promote axonal re-growth and/or differentiation of NSCs that reside in the tissue. To

date, nothing is known on the molecular signals that mediate the bi-directional effects of the two stem cell systems, but soluble factors are likely to be involved.

The present work proposes that interactions between the MSC and NSC niche, mediated by soluble factors, influence the cell fate and the final phenotype of the stem/progenitor cells. Hippocampus-derived soluble factors promoted proliferation and survival of MSCs and induced a neuronal-like phenotype in MSC derived cells. Vice versa, MSC derived soluble factors did not affect neuronal differentiation of hippocampal NSCs, but promoted astrocyte proliferation and induced oligodendrogenic differentiation of progenitor cells. The MSC derived soluble factors enhanced the expression of oligodendrogenic transcription factors such as the Olig 1, Olig 2, Nkx2.2 and repressed the expression of the oligodendrogenic inhibitor factor Id2. The effect on oligodendroglial differentiation was not mediated by the insulin-like growth factor 1, which was previously described as a potent oligodendrogenic factor. In summary, the work demonstrates that soluble factors play a relevant role in the interaction between the MSC and NSC niche. They induce a neuronal-like phenotype in MSCs and an oligodendrogenic program in NSCs. The present work has a major impact on the understanding of the molecular and cellular relation between the MSC and NSC niche. This is crucial for the development of future therapeutic strategies such as transplantation or stimulation of endogenous stem cell programs for the treatment of degenerative disease, especially in the CNS.

RESUMEN

Las células troncales adultas residen en diferentes tejidos y órganos del organismo y juegan un rol crucial en la homeostasis celular. Dentro de éstas están las células troncales mesenquimáticas (CTM) que residen en la médula ósea y las células troncales neurales (CTN) que se localizan en el sistema nervioso central (SNC). Ambas células comparten características típicas de células troncales, por ejemplo son indiferenciadas, capaces de dar origen a distintos tipos celulares maduros (multipotencia), tienen un alto potencial proliferativo y pueden autorrenovarse. Las CTMs son capaces de diferenciarse a una variedad de linajes mesenquimáticos como osteocitos, condrocitos, tenocitos, adipocitos y miocitos; mientras que las CTNs dan origen a neuronas, astrocitos y oligodendrocitos. El destino celular de las CTMs y de las CTNs esta especificado por factores intrínsecos y por señales externas presentes en el respectivo microambiente celular, conocido como "el nicho celular troncal". Dentro de estas señales están los factores solubles que regulan, en forma autocrina y/o paracrina la proliferación, la autorrenovación, la diferenciación y la sobrevivencia de las células troncales. El destino celular de las CTMs y de las CTNs no esta restringido a linajes mesenquimáticos y neurales, respectivamente, ya que el fenómeno de trans-diferenciación ha sido descrito. Por ejemplo, las CTMs en contacto directo con un microambiente neurogénico, como el hipocampo, son capaces de dar origen a células del linaje neural, expresando un fenotipo "neural-like". Por otra parte las CTNs pueden repoblar el sistema hematopoyético luego de que éste haya sido eliminado.

Aparentemente, los efectos de estos dos sistemas celulares troncales son bidireccionales, ya que las CTMs también afectan al microambiente neural. Por ejemplo, las CTMs promueven el re-crecimiento axonal y/o la diferenciación de las CTNs residentes en el tejido. Hasta el momento, poco se conoce acerca de las señales moleculares que median los efectos bi-direccionales entre estos dos sistemas celulares troncales, sin embargo resulta evidente que los factores solubles liberados por una u otra célula están involucrados.

En este trabajo se propone estudiar cómo las interacciones, mediadas por factores solubles, entre las CTMs y el nicho de la CTN influyen en el destino celular y el fenotipo final de ambas células troncales. Factores solubles derivados del hipocampo inducen, en la CTM, la adquisición del fenotipo neural-like y promueven la proliferación y la sobrevivencia de éstas. Por otra parte, factores solubles liberados por las CTMs no afectan la diferenciación neuronal de las CTNs del hipocampo, sin embargo promueven la proliferación de astrocitos e inducen la diferenciación oligodendrogénica de los progenitores hipocampales. Los factores solubles liberados por las CTMs aumentan la expresión de factores de transcripción oligodendrogénicos como Olig1, Olig2 y Nkx2.2, mientras que reprimen la expresión del factor inhibidor oligodendrogénico Id2. El efecto oligodendrogénico observado no esta mediado por el factor de crecimiento similar a insulina (IGF-1), el cual había sido descrito como un potente inductor oligodendrogénico. En resumen, este trabajo demuestra que factores solubles tienen un papel muy relevante en la interacción entre las CTMs y el nicho de las CTNs, induciendo el fenotipo neural-like en las CTMs y un programa oligodendrogénico en las

CTNs. Este trabajo tiene un alto impacto en el estudio de la interacción molecular y celular entre CTMs y el microambiente neural. La comprensión de estos fenómenos puede ser crucial para el desarrollo de estrategias terapéuticas como transplantes o estimulación de los programas de células troncales endógenas y por ende manipulación del destino celular de éstos, para el tratamiento de enfermedades degenerativas, especialmente en el SNC.

INTRODUCTION

I. - Stem cells

Stem cells are characterized by their unique capacity to proliferate, to renew themselves and to differentiate into mature specialized cell types. Thus, stem cells are responsible for the development and regeneration of tissue and organ systems (Morrison et al., 1997; Weissman, 2000). Stem cells have important characteristics that distinguish them from other cell typess. First, they are non-specialized cells that renew themselves for long periods through cell division. The second is that under certain physiological or experimental conditions, they may be induced to become cells with special functions such as cardiac myocytes of heart, the Langerhands cells of pancreas or mature neurons of the brain. Therefore, stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage cell differentiation (Till and Mc, 1961; Morrison et al., 1997; Weissman, 2000). Stem cells give rise to a multiple lineage of progenitors with limited self-renewal potential, which in turn give rise to progeny that are more restricted in their differentiating potential and finally to functionally mature cells.

II. –Embryonic and fetal stem cells

There are different kinds of stem cells depending on i) the source from where they are obtained and ii) the differentiation potential that they express. Embryonic stem

cells are the earliest in ontogeny, extending from the zygote to the inner cell mass of the blastocyst. They are totipotent, since they can originate all the different mature cell types of the organism. These characteristics make embryonic stem cells a potential therapeutic tool for the treatment of degenerative diseases, like Parkinson's disease, Alzheimer's disease, multiple sclerosis, osteoporosis, cirrhosis, etc. However, the use of embryonic stem cells is limited due to legal and ethical concerns. Moreover, embryonic stem cells have been shown to be tumorigenic after transplantation (Arnhold et al., 2004) and an additional limitation is derived from the necessity of immune suppression of the host.

Recent interest in stem cell biology and its therapeutic potential has led to the search for fetal stem cells in organs obtained from terminated fetuses, which would otherwise be discarded (O'Donoghue and Fisk, 2004). In the past, fetal neural tissue has been investigated and used therapeutically in Parkinson's disease with some evidence of clinical improvement (Lindvall, 2003). However, the use of fetal stem cells or fetal tissue has its limitations due to inconclusive results in human trials, the need of immune supression, and ethical concerns. Ethical issues and immunological problems can be avoided if fetal stem cells are obtained from umbilical cord blood, cryopreserved and, if necessary, used for future autologous transplantation. Stem cells obtained from umbilical cord blood can give rise to hematopoietic and mesenchymal cellular lineages, supporting the potential therapeutic use of these cells (Erices et al., 2000). However, additional experimental data are required for the assessment of the potential and the risk of the use of umbilical cord blood-derived stem cells.

III. - Adult stem cells

Adult stem cells are undifferentiated cells of adult tissues or organs; they are either quiescent or slowly proliferating. Adult stem cells are characterized by their potential to proliferate, to self-renew, and to differentiate into cell types that fulfill specific functions of the tissue or organ they are located in. The primary role of adult stem cells in a living organism is to maintain and to repair the tissue in which they are integrated. Based on their organ-specific location and their differentiation potential, different types of adult stem cells are defined, such as mesenchymal stem cells (MSCs), neural stem cells (NSCs), hematopoietic stem cells (HSCs), skeletal muscle stem cells (like satellite cells), endothelial progenitors, mammary gland stem cells, and others.

III. 1 Self-renewal, fate commitment and differentiation of adult stem cells

During adulthood, stem cells face the problem of maintaining the pool of uncommitted progenitors while generating precursors and mature cell types sufficient for tissue homeostasis. Therefore, proliferation, determination and differentiation have to be tightly regulated. Two modes of proliferation of adult stem cells can be distinguished. Symmetric cell division produces two identical daughter cells, each with stem cell characteristics. Asymmetric cell division generates two different daughter cells; one remains a stem cell (self-renewal) and the other one becomes a committed progenitor for a particular lineage (fate commitment) (Fig. 1). Therefore, to maintain the stem cell pool, self-renewal and fate commitment have to be tightly regulated. On the

route to a fully differentiated cell, stem cells progress through an intermediate cell type or types, called precursor cells (Fig. 1). Precursor cells are usually regarded as "committed", meaning that their fate is already decided along a particular cellular development pathway, although this characteristic may not be as definitive as once thought (Robey, 2000). As was mentioned before, commitment or cell fate determination is tightly linked to asymmetric cell division, since it generates a cell, restricted in its differentiating potential and diminished in its capacity of self-renewal. Cell fate determination and differentiation are regulated by extracellular cues and involve the sequential activation of transcription factors. Therefore, signaling pathways and transcription factors that regulate cell fate are crucial in the development of the cell's final phenotype. Previous studies showed that Wnt, Notch, Shh, Bmi-1 signaling pathways regulate the expression of determinated transcriptional factors (Pringle et al., 1996; Orentas et al., 1999; Tomita et al., 1999; Tanigaki et al., 2001; Tekki-Kessaris et al., 2001; Gaiano and Fishell, 2002; Ohishi et al., 2002; Molofsky et al., 2003; Park et al., 2003; Reya et al., 2003; Kageyama et al., 2005; Molofsky et al., 2005) that participate on self-renewal, cell fate determination and differentiation of adult hematopoietic and neural stem cells (HSC and NSC, respectevely). Importantly, de-regulation of these pathways might be a key event in carcinogenesis (Liu et al., 2005).

III.2 Stem cell plasticity: de- and trans-differentiation

A dogma in cellular biology has considered that under normal circumstances a differentiated cell cannot return to an undifferentiated cell. However, this concept has
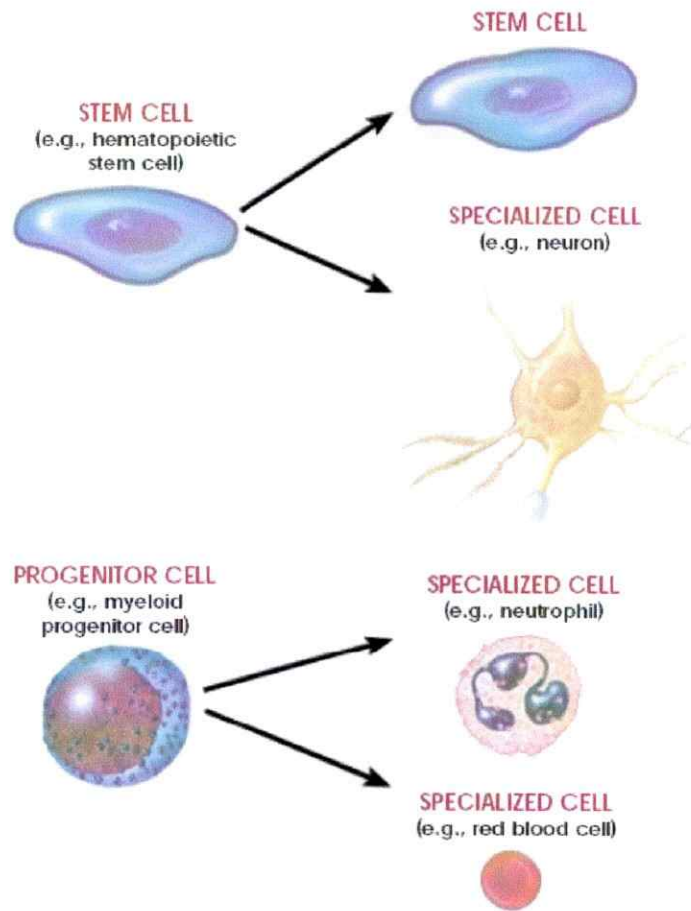
Figure 1.- Distinguishing features of progenitor/precursor cells and stem cells.
A stem cell is an immature cell that has the potential to self renew and to develop into specialized cell types. One product of a stem cell undergoing division is an additional stem cell that has the same capabilities as the progenitor cell (self-renewal). In addition, one daughter cell might develop into a mature, fully-differentiated cell, for example a neuron. A precursor cell is still capable to proliferate, however with limited self-renewal potential. Shown here is an example of a myeloid precursor undergoing cell division to yield two specialized cells (a neutrophil and a red blood cell). (Figure extracted from NIH Resources. www.ncbi.nih.gov)

been challenged within the last few years. De-differentiation describes the process by which a differentiated, organ-specific cell looses its differentiated phenotype and acquires characteristics of a somatic stem cell from the same organ. For example, retinal pigmented epithelium cells, which are fully differentiated, switch on a neural stem cell program after being exposed to in vitro conditions (Engelhardt et al., 2005). The traditional view of adult stem cells being restricted in their fate to a specific organ or tissue has also been challenged in the past few years. In different experimental setups, it has been demonstrated that stem cells pass the boundaries of organ and even germ layer specificity. This process was termed trans-differentiation, since adult stem can differentiate into mature cells that have a different germ layer origin (Fig. 2). For example, *in vivo* studies suggest that bone marrow cells, which usually differentiate into blood cells, migrate and differentiate into neurons and astrocytes (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000; Nakano et al., 2001; Priller et al., 2001), hepatocytes (Petersen et al., 1999) and skeletal and cardiac myocytes (Ferrari et al., 1998; Bittner et al., 1999; Orlic et al., 2001) after transplantation. Furthermore, a recent study suggested that neural stem cells, which usually differentiate into the neural cells, can give rise to blood cells after transplantation (Bjornson et al., 1999). Most of the studies that suggest trans-differentiation processes were performed using bone marrow-derived cells (mesenchymal and hematopoietic stem/progenitor cells) which were transfected with green fluorescent protein (GFP) or LacZ transgene as a tracer for subsequent detection after transplantation into a host (Ferrari et al., 1998; Brazelton et al., 2000; Nakano et al., 2001; Orlic et al., 2001; Priller et al., 2001). In another approach, male donor cells were transplanted into a female recipient and then subjected

to in situ hybridization for the Y chromosome to detect transplanted cells (Eglitis and Mezey, 1997; Bittner et al., 1999; Petersen et al., 1999; Mezey et al., 2000; Orlic et al., 2001). Donor cells were visualized in different host tissues ("new niches", such as liver, brain, skeletal muscle and heart) and their phenotypes were analyzed. The results demonstrated that donor cells migrated and expressed host-tissue specific proteins and markers, having also a cell morphology specific of host tissue cells (Eglitis and Mezey, 1997; Bittner et al., 1999; Eglitis et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Nakano et al., 2001; Orlic et al., 2001; Priller et al., 2001). As an example of these studies, bone marrow cells from male mouse donors were intravenously systemically implanted in female mice. At different timepoints after transplantation (between 2 weeks and 3 months), mice were sacrificed and neural tissues were analyzed. Transplanted cells were monitored using the Y chromosome as a tracer, and immunostaining for different neural markers was performed to verify neural differentiation. Transplanted cells expressing nuclear specific antigen (NeuN) and neuron specific enolase (NSE) were found in cortex, hypothalamus and hippocampus (Mezey et al., 2000). In addition, transplanted cells expressing glial acidic fibrilary protein (GFAP) and F4/F80 antigen (a microglial marker) were found in cortex, hippocampus, thalamus and cerebellum (Eglitis and Mezey, 1997). These results suggest that bone marrow cells, under certain conditions, can migrate to neural regions and trans-differentiate into neurons and astrocytes. In addition it was suggested that tissue damage can induce adult stem cell trans-differentiation. In order to evaluate if tissue damage or injury promotes a better stem cell trans-differentiation, stem cells were transplanted into unlesioned or previously
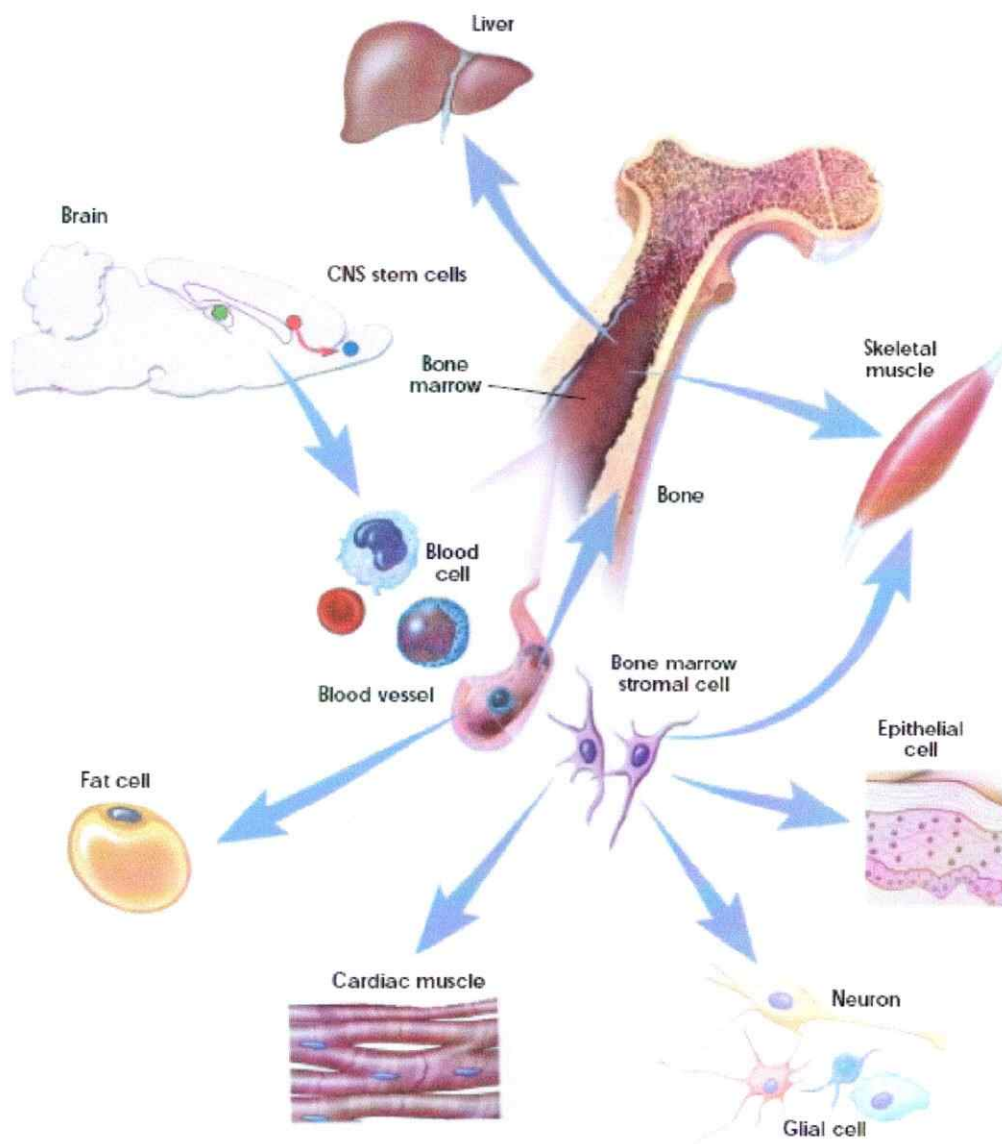
Figure 2. - Adult stem cell plasticity.
The figure illustrates the possibility that different types of adult stem cells can differentiate into a lineage that differs from its tissue of origin. Therefore, adult stem cells can trans-differentiate into lineages that have a different germ layer origin. (Figure extracted from NIH Resources. www.ncbi.nih.gov)

damaged foreign tissues (Ferrari et al., 1998; Bittner et al., 1999; Eglitis et al., 1999; Petersen et al., 1999; Orlic et al., 2001). The results demonstrated that transplanted cells promote functional recovery of previously damaged tissues, suggesting the regenerative potential of adult stem cells (Bittner et al., 1999; Orlic et al., 2001). As an example for these studies, a middle cerebral artery occlusion mouse model (MCAO) was used to induce a stroke in the brain of experimental animals. Bone marrow cells from a male donor were transplanted into female MCAO lesioned mice. After a defined time period, mice were sacrificed and neural tissues were analyzed. Transplanted cells were monitored using the Y chromosome as a tracer and immunostained for different neural markers to verify neural differentiation. The results showed that in the ipsilateral side, GFAP-expressing transplanted cells migrate to the ischemic cortex, whereas in the contralateral non-ischemic hemisphere, no migrating transplanted cells are detected in the cortex. This finding suggested that lesioned brain tissue promotes attraction and trans-differentiation of bone marrow cells (Eglitis et al., 1999). In summary, all these studies together suggested that transplanted adult stem cell trans-differentiate into cell types specific for the host tissue. The present thesis is aimed to analyze in more detail the cellular phenomenon of trans-differentiation, in particular the mesenchymal stem cell trans-differentiation into neural lineage.

The host environment instructs or induces the transplanted stem cell to acquire a specific phenotype. For example, neural stem cells transplanted into hippocampus give rise mostly to neurons, whereas if they are transplanted into spinal cord, they give rise mostly to glial cells (Palmer et al., 1999). Therefore, the external signals that control

adult stem cell fate and homeostasis collectively make up the "stem cell micro-environment" or "niche" (Fig.3) (Morrison et al., 1997; Potten et al., 1997; Watt and Hogan, 2000). This niche affects not only stem cells, but also their daughter cells (the progenitors). Different kinds of stimuli are now known, that affect the self-renewal, cell fate, differentiation, maturation, proliferation, survival and migration of adult stem cells, like soluble or secreted factors (e.g. cytokine), extracelular matrix (e.g. integrins) and cell-cell interactions (e.g. Notch) (Watt and Hogan, 2000). These are the main three components of the stem cell niche (Fig. 3).

Since the present thesis focuses on the neural fate determination and differentiation of adult mesenchymal stem cells (MSCs) and neural stem cells (NSCs), both systems will be described in the following chapters.

IV. Adult mesengenesis and adult mesenchymal stem cell biology

IV.1 Mesenchymal stem cells (MSCs)

In vivo and *in vitro* studies have identified the bone marrow stroma as a source of multipotent stem cells that give rise to a variety of committed progenitors that later differentiate into different mesenchymal mature cells like bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), tendon cells (tenocytes), muscle
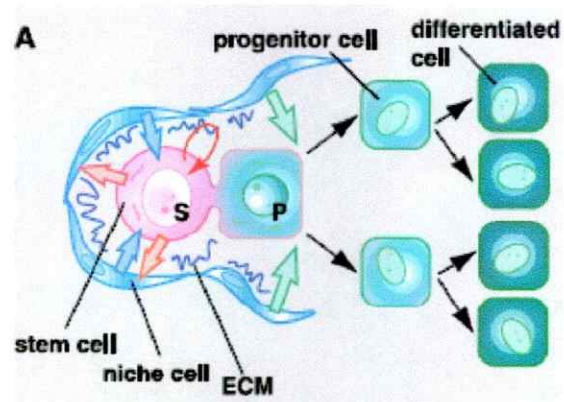
Figure 3.- Stem cell niche.
The figure illustrates the different niche components that interact with stem cells: soluble factors, extracelular matrix (ECM) and niche cell interactions. These factors regulate self-renewal, proliferation, fate determination, differentiation and maturation of stem/progenitor cells. (Watt and Hogan, 2000)

cells (myocytes) and hematopoietic supporting stoma cells (Caplan, 1994; Majumdar et al., 1998; Dennis et al., 1999; Minguell et al., 2001) (Fig. 4). Since their original description, these bone marrow stroma multipotent progenitor cells were referred to, among other names, as mesenchymal stem cells (MSCs). *In vitro*, the adherent capacity of MSCs is used to separate them from the hematopoietic system that is also present in the bone marrow. In practice, the cell population obtained by the enrichment with this differential adherence method is not homogeneous; it consists of mesenchymal stem cells and committed progenitors. Thus, the denomination MSC refers to a heterogeneous cell population (Minguell et al., 2001). Recently, investigators have tried to isolate the "true" mesenchymal stem cell subpopulation by taking advantage of the uncommitted and relatively quiescent properties, which distinguish the stem cells from the mesenchymal committed progenitors. Proliferative mesenchymal progenitors are forced to metabolic death by exposure to 5-flourouracil, which integrates in DNA during S-Phase and kills proliferating cells (Conget et al., 2001). The remaining subpopulation (5 to 20%) contains quiescent, uncommitted and undifferentiated mesenchymal stem cells, which are denominated MSC Go cells, due to their proliferative resting stage. Upon stimulation, the isolated cells can self-renew and give rise to committed precursors to osteogenic and adipogenic lineage (Conget et al., 2001).

IV. 2 Mesenchymal fate determination and differentiation

The differentiation of mesenchymal stem cells is ascertained by studying the expression of lineage-specific functional and molecular properties. These properties
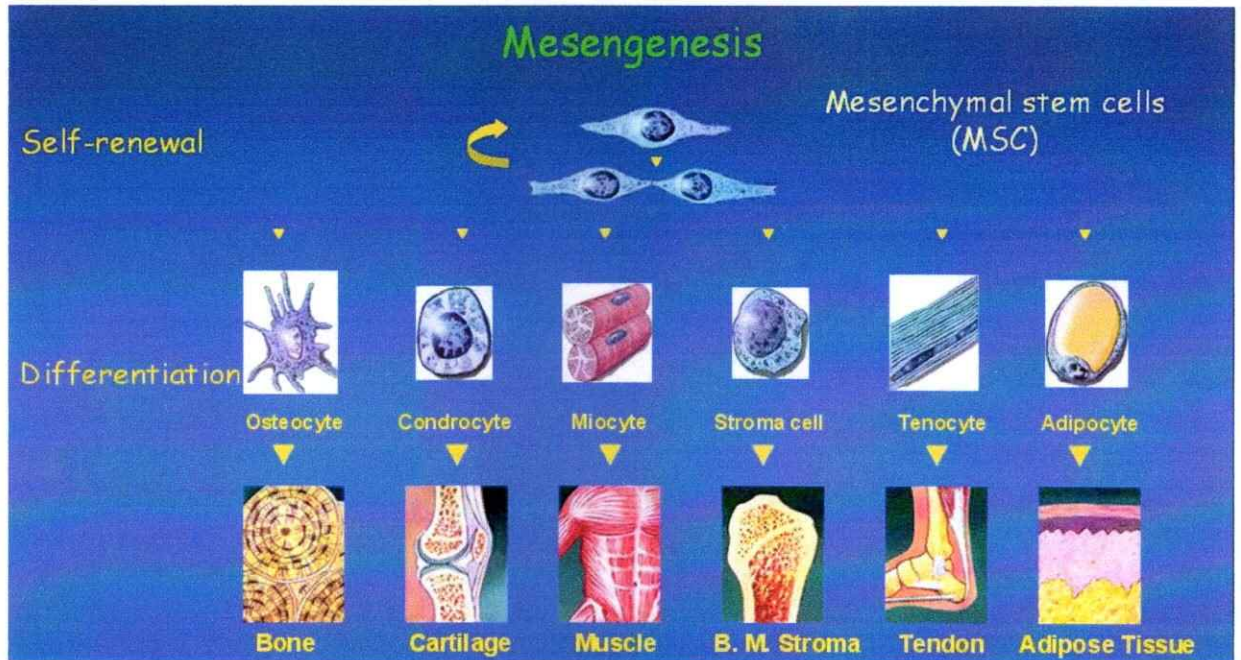
Figure 4.- Mesenchymal stem cells and their differentiation potential.
Mesenchymal stem cells can self-renew and are multipotent. They can generate lineage-restricted precursor cells, which further differentiate into the different mesenchymal cell: osteocyte (Bone), crondrocyte (Cartilage), myocyte (Muscle), stroma cells (Bone marrow stoma), tenocyte (Tendon) and adipocyte (Adipose tissue).

include extracellular matrix (ECM) mineralization (osteoblast), accumulation of intracellular lipid droplets (adipocytes), fiber contractions (myocytes), expression of specific structural proteins (osteoblast, chondrocytes, myocytes, tenocytes), and expression of lineage-specific transcription factors (Minguell et al., 2001). At the molecular level, cell fate determination of MSCs and differentiation is orchestrated by intracellular signal transduction pathways that activate specific transcription factor programs and ultimately induce expression of specific genetic programs. The expression of the transcription factors core-binding factor alpha1 (Cbfa1)/Runx2, peroxisome proliferator–activated receptor γ (PPARγ), Sox9, and myocyte-specific enhancer factor 2 (MEF2) play important roles in the commitment of multipotent stem cells into specific lineages and in the maintenance of differentiated phenotypes (Olsen et al., 2000; Harada and Rodan, 2003; Waddington et al., 2003; McBeath et al., 2004). For instance, two key transcription factors, Runx2 and PPARγ, induce MSCs to differentiate into osteoblasts and adipocytes, respectively. Moreover, transcription factors also participate in the differentiation and maturation progression of mesenchymal progenitors cells into a certain lineage, i.e. the osteo-lineage (Madras et al., 2002; Harada and Rodan, 2003; Song and Tuan, 2004). In summary, transcriptional factors play a crucial role at different stages during mesenchymal stem cell differentiation.

IV.3 Mesenchymal stem cell niche

Bone marrow stroma is the tissue source most commonly utilized in growing mesenchymal progenitors. It is well described that distinct niches exist within the bone

marrow that support HSC survival and growth by providing specific factors and adhesive properties to maintain their viability, while facilitating an appropriate balanced output of progeny for the lifetime of an organism (Janowska-Wieczorek et al., 2001). It has also been determined that these niches are formed by stromal precursor cells, specifically osteoblasts (Calvi et al., 2003). Therefore, bone marrow contains the co-existing hematopoietic and mesenchymal stem cell systems (Minguell et al., 2001) (Fig. 5). This suggests that mesenchymal progenitors in bone marrow contribute to the formation and function of a stromal microenvironment, which produces inductive/regulatory survival, proliferation and differentiation signals not only for themselves but also for the development of hematopoietic progenitors and non-mesenchymal stromal cells present in the bone marrow (Klein, 1995; Majumdar et al., 1998; Cheng et al., 2000; Minguell et al., 2001). It is within this dynamic cellular microenvironment where MSCs are presumed to exist (Fig. 5). However, it is not known whether hematopoietic and mesenchymal systems reside in the same or different niches within the bone marrow. It may be argued that these two cell compartments (hematopoietic and mesenchymal cells in the bone marrow) occupy the same niche, given the close physical proximity to one another. However, the extracellular and/or intracellular signals that are required to maintain both the hematopoietic and mesenchymal stem cell developmental program in the bone marrow microenvironment are likely to be different. A complete characterization of the cellular, biochemical, and molecular interactions of MSCs within their niche is needed in order to understand how these cells are regulated *in vivo*.
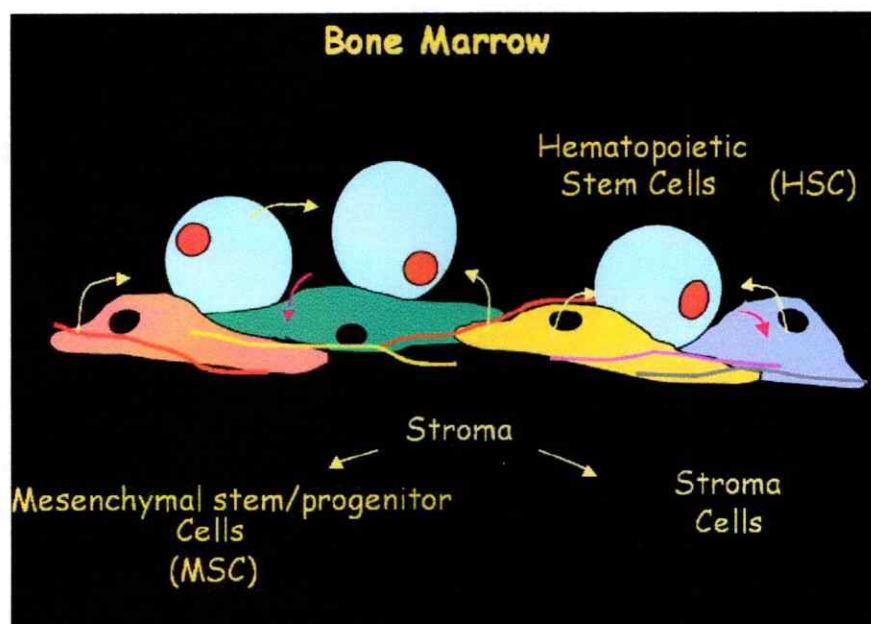
Figure 5. - Mesenchymal stem cell niche.
Mesenchymal stem cells in the bone marrow. The figure illustrates the co-existence with hematopoietic stem cell system (round cells). The interactions between them are mediated by soluble factors (red and yellow curve arrows), extracellular matrix (lines) and cell-cell interactions. Mesenchymal stem/progenitor cells are present in the bone marrow stroma supporting the hematopoietic system.

Despite the fact that bone marrow is considered a well-accepted source of MSCs, these cells have been also isolated from other tissue sources, including trabecular bone (Noth et al., 2002), adipose tissue, synovium, skeletal muscle, lung, deciduous teeth (Tuan et al., 2003), and human umbilical cord perivascular cells derived from the Wharton's Jelly (Sarugaser et al., 2005) suggesting that the MSC niche may not be restricted to bone marrow. These findings reveal that MSCs are widely distributed in vivo, and as a result may occupy an ubiquitous stem cell niche.

IV.4 Mesenchymal stem cell plasticity: Neural-like trans-differentiation

MSCs are not restricted to their mesenchymal differentiation fate but may also differentiate into cells of ectodermal lineage such as neural cell types. *In vitro* studies have shown that neural differentiation of MSCs can be induced when these cells are exposed to a number of different non-physiological compounds. Murine or human bone marrow, as well as cord blood-derived MSCs may be induced to express a neural-like phenotype by compounds such as dibutyryl cyclic AMP, adenylate cyclase activators (forskolin, isobutyl-methylxanthine), dimethyl sulfoxide, inductors of gamma-aminobutyric acid synthesis (valproic acid) and/or antioxidants (D609, Beta-mercaptoethanol, butylated hydroxyanisole) (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Deng et al., 2001; Woodbury et al., 2002; Munoz-Elias et al., 2003; Padovan et al., 2003; Rismanchi et al., 2003).The criteria to assess the neural differentiation properties induced by these compounds were based on the appearance of cells exhibiting a typical neural-like morphology and/or the expression (by immunhistochemistry,

Western blot and RT-PCR) of distinctive neural-specific genes such as neuron specific enolase (NSE), medium neurofilament (NF-M), neuron-specific nuclear protein (NeuN), glial fibrilary acidic protein (GFAP), tau, TUC-4, microtubule-associated protein 1B (MAP1B), neuron-specific tubulin (TuJ-1), sinaptophysyn, syntaxin, etc. V. Adult neurogenesis and adult neural stem cell biology.

V. Adult neurogenesis and adult neural stem cell biology

V.1 Adult neural stem cells (NSCs)

For many years it was believed that the fully developed central nervous system (CNS) is devoid of any mitotic activity and thus cannot generate new cells. However, in the mid 1960s, Altman and colleagues proposed a model of persistent neurogenesis in the adult rodent brain (Altman and Das, 1965). However, only when Kaplan and Hinds showed convincing data on the fate of newly generated neurons in regions of the hippocampus in 1977, the dogma of the "static brain" became outdated (Kaplan and Hinds, 1977). Today, it is widely accepted that neurogenesis is a common phenomenon in specialized regions of the adult brain.

Much research has been done focusing in the cellular and molecular basis of adult neurogenesis. The main question has been how new neurons and other neural cell types can be originated in the adult brain. It is unlikely that these cells come from mature neural cells such as neurons or oligodendrocytes, since these cells do not have a potent

mitotic activity. Thus, a more feasible hypothesis was that mature neural cells could be generated from uncommitted cells (neural progenitors) that are present in the adult brain. In this context, numerous studies revealed that some brain regions are rich in neural stem/progenitor cells (NSCs) which proliferate, self-renew and differentiate into mature neural cell types.

The NSCs have been shown to proliferate both *in vivo* and *in vitro,* and to give rise to the three major cell types of the CNS: nerve cells (neurons) and two categories of non-neuronal (glial) cells (astrocytes and oligodendrocytes) (Fig. 6) (Gage, 2000). NSCs are originated during development and reside in the CNS during adulthood. They have been defined as slow dividing and uncommitted progenitor cells, generating fast-dividing, lineage-restricted progenitor cells (precursor cells) that are able to undergo a limited number of cell cycles (Davis and Temple, 1994; Lillien, 1998).

Several aspects influence the final fate of neural stem/progenitor cells. Transcription factors can function as intracellular determinant signals linking the extracellular signals (such as growth factors and neurotrophins) via second messenger pathways and other intracellular responses.
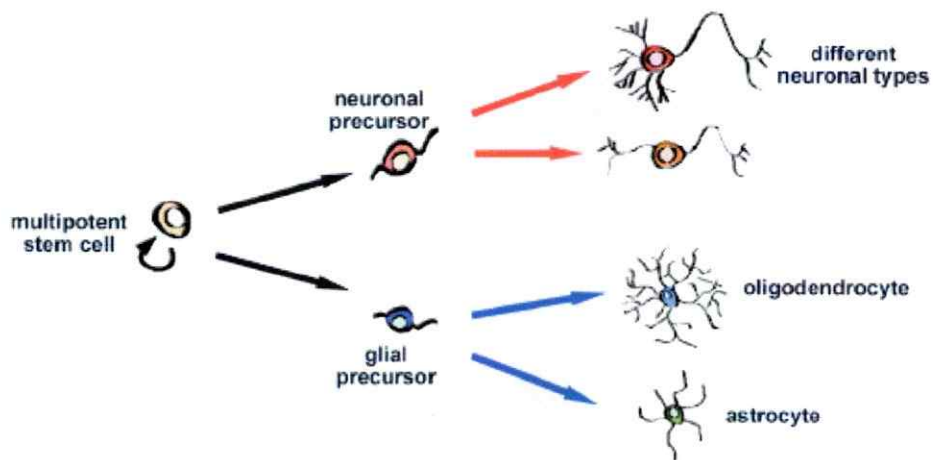
Figure 6.- Neural stem cells and their progeny.
Neural stem cells can self-renew and are multipotent. They can generate lineage-restricted precursor cells, which further differentiate into the three major cell types of the CNS: neurons, astroglia and oligodendroglia.

V.2 In vitro culture of adult neural stem cells

The starting material for the isolation of adult NSCs is typically a region of the adult brain that has been shown to contain dividing cells, for example the subventricular zone (SVZ) or the hippocampus (HC) (Reynolds and Weiss, 1992; Gage et al., 1995). However, adult NSCs can be also derived from non-neurogenic regions like spinal cord, striatum and neo-cortex (Palmer et al., 1995; Palmer et al., 1999). Usually, the tissue is disaggregated and the dissociated cells are exposed to 10 or 20 ng/ml of mitogens such as fibroblast growth factor-2 (FGF-2) (Richards et al., 1992) or epidermal growth factor (EGF) (Reynolds and Weiss, 1992; Gritti et al., 1996) in a defined medium. Under non-adherent conditions, i.e. in the absence of an adequate substrate such as the extracellular matrix molecule laminin, cells are expanded as floating aggregates, called "neurospheres" (Reynolds and Weiss, 1992; Gritti et al., 1996). These neurospheres consist of proliferating neural stem and progenitor cells. The proliferating cells express neural stem cell markers, such as nestin, an intermediate filament found in neuroepithelial stem cells. NSCs are induced to differentiate by withdrawal of mitogens. Targeted differentiation into one or another neural cell type is induced by specific factors: retinoic acid (RA), brain derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) for neuronal differentiation (Takahashi et al., 1999); leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP-2) for astrocyte differentiation (Nakashima et al., 1999); insulin-like growth factor-1 (IGF-1) for oligodendroglial differentiation (Hsieh et al., 2004). Typically, the differentiation fate is analyzed by staining with antibodies directed against antigens specific for neurons (for example β-III

tubulin, Map2ab); for astrocytes (for example GFAP, S-100) and for oligodendrocytes (for example RIP, GalC, MBP) (Fig. 6).

V.3 Self-renewal, cell fate determination and differentiation of NSCs

Neural differentiation is a tightly regulated process that involves control of neural stem cell number and regulation of proliferation, symmetric and/or asymmetric cell division, determination, migration, differentiation and cell death. Moreover, it is associated with the sequential changes in cell identity from a slowly proliferating neural stem cell with unlimited self-renewal capacity, to the mature neural cell. Intermediate stages are fast cycling progenitors and dividing precursors with limited self-renewal and limited differentiation potential. The initial steps are crucial, since the regulation of self-renewal and cell fate determination assures the maintenance of the neural stem cell pool and the final phenotype of one daughter cell after the asymmetric cell division. Experiments using animals with targeted deletion of the cell cycle regulator p21 demonstrated that it is required for maintenance of the stem cell pool (Kippin et al., 2005). In these experiments the increased proliferation rates of adult neural stem cells derived from p21-/- mice were associated with a reduction in neural stem cell number relative to p21+/+ mice (Kippin et al., 2005). In addition to this, *in vitro* experiments concluded that the polycomb family transcriptional repressor B lymphoma Mo-MLV insertion region 1 (Bmi-1) promotes NSCs self-renewal (Molofsky et al., 2003; Molofsky et al., 2005). The reduced self-renewal of Bmi-1-deficient neural stem cells (from Bmi-1 knockout mice) leads to their postnatal depletion (Molofsky et al., 2003).

Moreover, in the absence of Bmi-1, the cyclin-dependent kinase inhibitor gene p16Ink4a is up-regulated in neural stem cells, reducing the rate of proliferation. However, restricted neural progenitors from the forebrain proliferate normally in the absence of Bmi-1. Thus, Bmi-1 dependence distinguishes stem cell self-renewal from restricted progenitor proliferation (Molofsky et al., 2003). In addition to this, activation of the Notch signaling up-regulates the expression of basic Helix-Loop-Helix (bHLH) transcription factor hairy-enhancer-of-split-1 (Hes1) and -5 (Hes5) and maintains the cells in an undifferentiated stage (Gaiano and Fishell, 2002; Kageyama et al., 2005). In contrast, it was described that Notch signaling promotes astrocyte differentiation in adult rat hippocampus-derived multipotent neural progenitors (Tanigaki et al., 2001). However, recent data indicate that subpopulations of astrocytes act as stem cells in the adult making sense on the first two findings (Gotz et al., 2002; Heins et al., 2002). Therefore there are several molecular cues that participate on NSCs self-renewal/cell fate determination.

As was mentioned before, NSCs can diferentiate into the three mayor neural lineages: neurons, astrocytes and oligodendrocytes. Neuronal differentiation can be triggered by stimulation with retinoic acid and neurotrophic factors like BDNF, NT-3 (Takahashi et al., 1999). In addition, activation of the cyclic-AMP (cAMP) signaling cascade induces neuronal differentiation (Takahashi et al., 1999). In the intrinsic signal level, bHLH transcriptional factor family members (such as Neurogenin 1 and 2, Mash-1, NeuroD, etc) play a crucial role in neuronal cell fate determination and differentiation of NSC and progenitors (Ross et al., 2003; Kageyama et al., 2005). Another class of

neuronal cell fate determinants are transcriptional factors from the homeodomain family, such as Pax6 (Osumi, 2001; Heins et al., 2002). For instance, *in vivo* studies showed that just half of the neurons have been developed in Pax6 mutant mice (Heins et al., 2002). Moreover, transfection of Pax6 in radial glial cells but also in astrocytes isolated from cortex promotes neurogenesis (Heins et al., 2002). These results demonstrate an important role of Pax6 as an intrinsic neuronal fate determinant (Heins et al., 2002).

In comparison to neuronal determination and differentiation, less is known on the molecular control of astrogenesis. However, recent work has revealed a role for Notch and Hes factors in gliogenesis. For instance, in cultured adult hippocampal progenitors, Notch promotes astrocyte differentiation (Tanigaki et al., 2001) indicating that hippocampal neural progenitors are directed towards an astrocyte fate by Hes activity induced by Notch signaling. In the retina, forced expression of Hes1 or Hes5 promotes the conversion of postnatal retinal progenitors into Müller glia, the only astroglial cell type present in the adult retina (Furukawa et al., 2000; Hojo et al., 2000). Similarly, in neural progenitors derived from the spinal cord, over-expression of Hes1 causes differentiation into astrocytes (Wu et al., 2003). Collectively, these findings suggest that regulation of astrocyte formation by Notch and Hes factors is a mechanism present throughout the CNS. Other pathways and molecules trigger astrocyte differentiation at the expense of oligodendrocyte fate determination. For example, BMP signaling activates the expression of the bHLH proteins inhibitor of differentiation 2 (Id2) and 4 (Id4). These ID proteins sequester OLIG proteins and/or their E proteins (E12 and E47), thereby preventing the Olig-E complex formation and its entry into the nucleus.

Therefore its oligodendrogenic activity is inhibited, thereby promoting the astrocytic differentiation of neural progenitors cells (Fig. 7) (Wang et al., 2001; Samanta and Kessler, 2004).

During development, astrocytes are produced by a second wave of cell differentiation. Formation of oligodendrocytes occurs even later during the early postnatal period, when neurons are surrounded by the supportive glia and synaptic connections have been established. Several soluble factors such as bFGF, IGF-1, BDNF, platelet-derived growth factor (PDGF), thyroid hormone (TH), ciliary neurotrophic factor (CNTF), LIF, BMP-2, Shh and the Notch receptor have been shown to influence glial fate decision in the developing telencephalon in NSCs and more mature progenitors *in vitro* (Furukawa et al., 2000; Gaiano et al., 2000; Morrison et al., 2000; Jiang et al., 2001; Morrow et al., 2001; Tanigaki et al., 2001; Billon et al., 2002; Miller, 2002). Oligodendrocyte specification is controlled by bHLH transcriptional factors. For example, the two bHLH transcriptional factors Olig1 and Olig2 trigger an oligodendrogenic fate in neural progenitors (Zhou et al., 2000; Wegner, 2001; Ross et al., 2003). Recent data indicate that the specific cell fate of a NSC is determined by a combination of different factors, rather than a single transcription factor. For instance, when Neurogenin2 (Ngn2) is co-expressed with Olig2 in neural precursors, this combination promotes motor neuron development (Mizuguchi et al., 2001; Novitch et al., 2001). Thus, Olig2 regulates development of both neurons and oligodendrocytes in a context-dependent manner (whether or not Ngn2 is co-expressed). This evidence indicates that different combinations of bHLH genes can specify for different types of
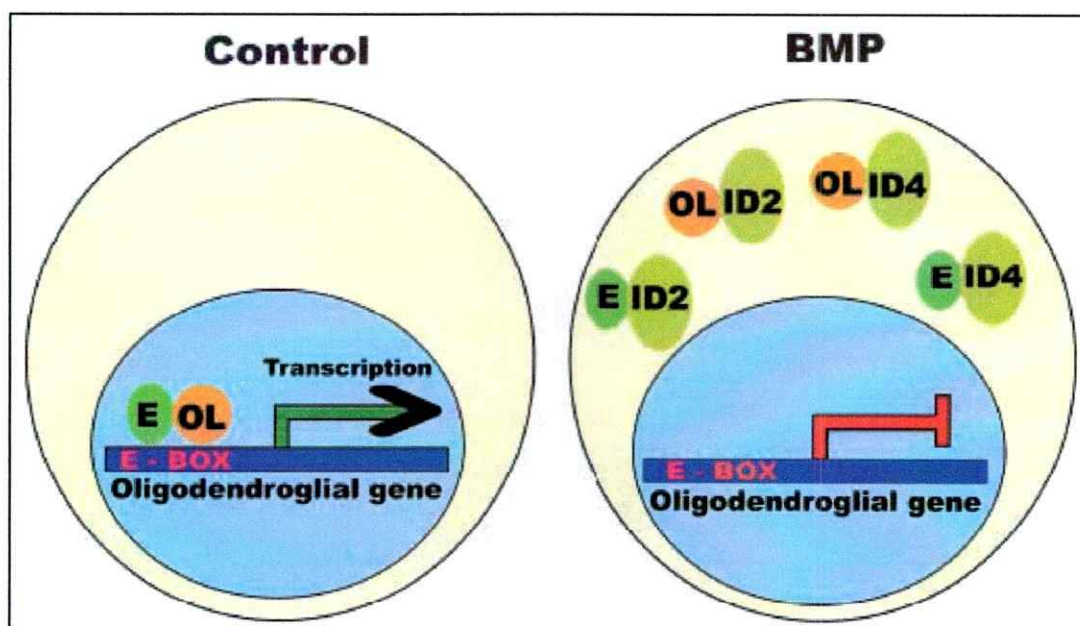
Figure 7.- Model of the role of ID proteins in mediating effects of BMP signaling. OLIG proteins bind to promoter regions after heterodimerizing with the E2A proteins, resulting in transcription of their downstream targets. In the presence of BMP4, all four ID proteins are induced, resulting in sequestration of OLIG 1/2 by ID2 and ID4 and of E2A proteins by all the IDs in the cytoplasm of progenitor cells. This inhibits OLIG binding to promoters of target genes, resulting in inhibition of oligodendrocyte development and enhancement of commitment to the astrocytic fate. (Samanta and Kessler, 2004)

cells. Moreover, as was mentioned before, astrocyte/oligodendrocyte cell fate determination can be regulated the balance of olig genes and Id proteins (Fig. 7), two proteins that belong to the bHLH family (Wang et al., 2001; Samanta and Kessler, 2004). This suggested that oligodendrocyte differentiation is controlled by the balance of bHLH factors (Fig. 7). In addition, oligodendroglial fate determination is controlled by non-bHLH transcription factors. Here, Nkx2.2 acts together in collaboration with olig proteins to promote oligodendrocyte fate on neural progenitor cells during development and adulthood (Zhou et al., 2001; Zhang et al., 2005b). In chick embryos' spinal cord progenitors, the co-expression of Olig2 with Nkx2.2 promotes ectopic and precocious oligodendrocyte differentiation (Zhou et al., 2001). In human neural progenitor cells that have been derived from adult olfactory neuroepithelium targeted Olig2 or Nkx2.2 expression alone exhibits no phenotypic lineage restriction. In contrast, simultaneous transfection of Olig2 and Nkx2.2 cDNA produced characteristic oligodendrocyte morphology and antigenicity including expression of myelin basic protein (MBP) (Zhang et al., 2005b).

In adult neural stem cells, very little is known about the oligodendrocyte cell fate regulation. As was previously mentioned in olfactory neuroepithelium progenitors, Olig2 and Nkx2.2 act together in the oligodendrocyte specification (Zhang et al., 2005b). In addition, experiments using adult hippocampus-derived NSCs have demonstrated that adult NSCs are induced to differentiate into oligodendrocytes by IGF-1 (Hsieh et al., 2004).

V.4 Neural stem cell niche

Neurogenesis has been documented in two regions of the adult central nervous system (CNS) throughout the mammalian phylogeny. In the SVZ of the lateral ventricle wall, neural stem cells divide and give rise to proliferating neuronal-determined precursors (Fig. 8) (Gates et al., 1995; Kuhn et al., 1997). In rodents, these precursors depart from the SVZ leaving the ventricle wall and migrating along the rostral migratory stream towards the olfactory bulb where they functionally integrate (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Winner et al., 2002; Carleton et al., 2003) (Fig. 8). In the subgranular lining of the hippocampal dentate gyrus, cells also divide along the border of the hilus and continuously generate a pool of differentiating neuronal precursors that functionally integrate into the overlaying hippocampal granule cell layer (Altman and Das, 1965; Kaplan and Hinds, 1977; Kuhn et al., 1996; van Praag et al., 2002) (Fig. 8). In addition, it has been suggested that there is a close interaction between the neural stem system and the vascular system (Palmer et al., 2000). Neurogenesis is intimately associated with the process of active vascular recruitment and remodeling, suggesting that adult neurogenesis occurs within an angiogenic niche. Neural stem and progenitor cells are embedded in a cellular and extracellular microenvironment, the stem cell niche (Palmer et al., 2000; Doetsch, 2003). Alterations in the stem cell niche change the rate of neurogenesis with functional consequences for regeneration (Nakatomi et al., 2002).
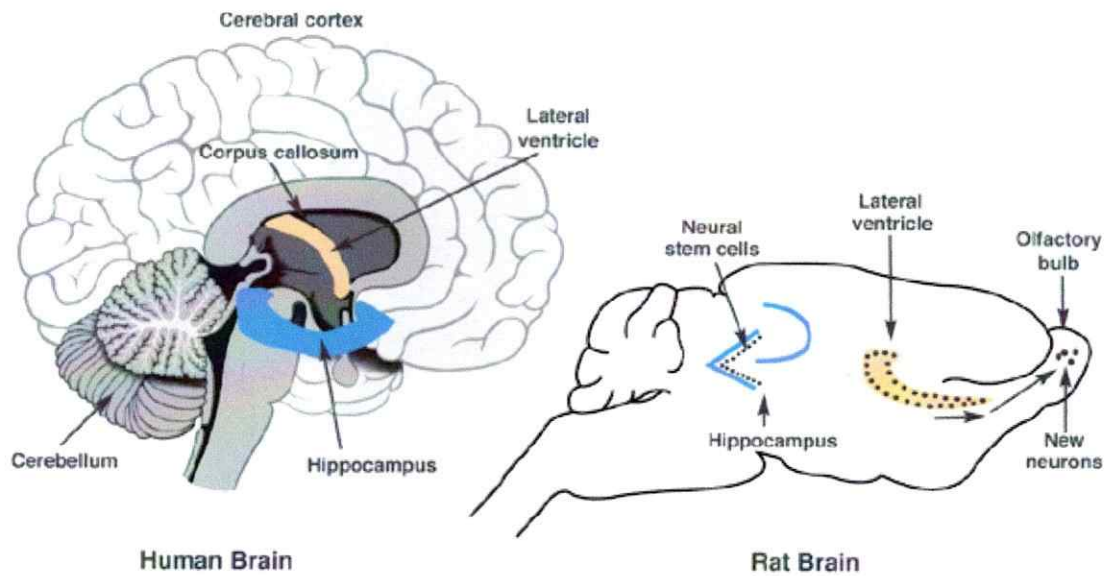
Figure 8.- Neural stem cell niche in the adult human and rat brain.
Dentate girus of hippocampus, subventricular zone and olfactory bulb are the known natural niche for neural stem cells. In rat brain neuroprogenitors migrate from lateral ventricule on the subventricular zone along rostral migratory stream up to the olfactory bulb where they differentiate and integrate. The interactions with extracelular matrix, niche cells and soluble factors present make this phenomenon possible.

VI. Interactions between MSC and the NSC's niche

Recent transplantation studies suggest that MSCs and NSCs or the associated neural niches influence each other. For example, the interaction between MSCs and neural tissues *in-vivo* is neuroprotective and promotes regeneration in the lesioned CNS after transplantation in murine model (Dezawa et al., 2001; Hofstetter et al., 2002; Zhang et al., 2004a; Lu et al., 2005; Neuhuber et al., 2005). The regenerative effect of MSCs in the damaged CNS is mediated by so far unknown mechanisms, but it is intriguing to speculate on different possibilities: i) trans-differentiation of MSCs into neuronal phenotypes and functional integration of the cells; ii) by-stander effects of MSCs on the survival of damaged neurons (neuroprotection); iii) by-stander effects of MSCs on the fate, and differentiation of NSCs present at the lesion site. In either way, it is important to understand the interaction of MSCs and their neural microenvironment.

The possibility that MSCs-neural microenvironment interactions affect the fate of MSCs is attractive, since some studies suggested a neurogenic potential of MSC *in-vivo* and *in-vitro* (Azizi et al., 1998; Kopen et al., 1999; Li et al., 2000; Woodbury et al., 2000; Li et al., 2001; Mahmood et al., 2001; Woodbury et al., 2002; Hermann et al., 2004). *In-vivo*, MSCs were transplanted directly in the striatum, and after 2 or 3 months cells migrated to striatum and cortex (Azizi et al., 1998). In another study, MSCs that were placed into the mouse lateral ventricle were later detected in cerebellum, hippocampus molecular layer and olfactory bulb, where they expressed markers specific for astrocytes and neuronal lineage (Kopen et al., 1999). In addition, after MSCs were

transplanted into a head trauma, stroke or Parkinson model mouse, transplanted cells express mature astrocyte- or neuronal-specific markers (Li et al., 2000; Li et al., 2001; Mahmood et al., 2001). The identity of physiologically-relevant factors present in neural tissues that induce or maintain a neural phenotype of MSCs is not known. Initial supporting evidence for the existence of such activities was provided recently by co-culture systems with hippocampal tissue. Since the adult hippocampus is a region of persistent neurogenesis, it presumably contains factors that favor neuronal differentiation. In co-cultures of MSCs with hippocampal slices, MSCs acquire a neuron-like morphology and express mature neuron markers such as NeuN (Abouelfetouh et al., 2004).

The possibility that MSCs-neural microenvironment interactions depend on activities that promote neuroprotection and/or influence the fate and differentiation of endogenous NSCs is fascinating. MSCs release a variety of cytokines and growth factors that promote proliferation, differentiation and survival of other cells in the bone marrow microenvironment (Minguell et al., 2001). When transplanted into lesioned neural tissues (injured spinal cord and MCAO) MSCs promote axonal re-growth of damaged neurons and enhance proliferation and differentiation of endogenous neural progenitor cells (Hofstetter et al., 2002; Zhang et al., 2004a; Lu et al., 2005; Neuhuber et al., 2005). The latter is very interesting, since it suggests the possibility of the interaction between different stem cell or progenitor populations.

The present studies investigate the interaction between the MSC and the neural microenvironment, specifically the adult hippocampus, which contains a NSC niche. Bi-directional effects were initially studied in co-culture systems. Since the emphasis of the present work is in soluble factors derived from the different stem cell microenvironments, conditioned medium derived from the different sources was further investigated for stem cell relevant activities. Among these were effects on proliferation, determination, differentiation and survival.

HYPOTHESIS

The interactions between the mesenchymal and the neural stem cell niche induce neural differentiation, mediated by soluble factors, on both mesenchymal and neural progenitor cells.

OBJECTIVES

General objective 1

Analysis of the effects of soluble factors released from neural stem cell niche on mesenchymal stem/progenitor cells.

a) Specific objective 1: To verify the potential of mesenchymal progenitor cells to differentiate into a neural-like lineage, based on morphology changes and immunophenotype.

b) Specific objective 2: To study if soluble factors released from adult rat hippocampus (neural stem cell niche) affect the proliferation, survival and cell fate of mesenchymal stem/progenitor cells.

General objective 2

Analysis of the interactions between bone marrow mesenchymal and hippocampal neural progenitor cells.

a) Specific objective 1: To determine the role of direct interactions between mesenchymal cells and hippocampal neural progenitor cells on neural differentiation.

b) Specific objective 2: To determine the ability of soluble factors released from mesenchymal progenitor cells to induce differentiation on neural progenitor cells.

c) Specific objective 3: To study, on a cellular and molecular base, the effects of MSC-soluble factors on NSCs differentiation.

d) Specific objective 4: To determine the biological nature of the oligodendrogenic soluble factors released by MSC and evaluate the participation of a candidate cytokine.

MATERIALS AND METHODS

I. MSC culture

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the local institutional guidelines on animal care and ethics (INTA, Universidad de Chile). Female Fisher-344 rats (alternatively Wistar rats were also used) were killed by cervical dislocation. Femurs and tibias were obtained from two legs after getting rid of the muscular tissue. Bone marrow plugs harvested from 2-4 femurs and tibias were resuspended in approximately 10 ml of α-minimun essential medium (α–MEM) (Gibco Invitrogen, Pasley, UK), desegregated, homogenized and centrifuged (800 x g). The resulting cell pellet was resuspended in α–MEM containing 10% Fetal Bovine Serum (FBS, PANbiotech) (α–MEM-10%FBS) and cell number was determined by Trypan blue exclusion. Cells were seeded ($1x10^6$ nucleated cells/cm^2) into 56 cm^2 culture dishes and incubated in a humidified atmosphere 37°C, 5%CO2. Three days later, non-adherent cells were washed off and adherent cells were further incubated in fresh α–MEM-10%FBS until they reached confluency. Cells were tripsinized (0.25% Trypsin-EDTA (Gibco-Merck), resuspended in complete medium and seeded (8,000 cells/cm^2). After 3-5 days the resulting monolayer of cells, hereafter named rat bone marrow-derived mesenchymal stem cells (MSC), was tripsinized and aliquots were either frozen and stored in liquid nitrogen or further cultivated (Santa Maria et al., 2004).

To confirm the multipotentiality of MSCs used in this study cultures (passage 2-4) were submitted to osteogenic and adipogenic differentiation conditions. Osteogenic differentiation was induced by incubation of cells in α–MEM-10%FBS supplemented with 0.1μM dexamethasone (Sigma, Saint Louis Missouri), 10mM β-glycerophosphate (Sigma, Saint Louis Missouri), and 50μM L-ascorbic acid-2-phosphate (Sigma, Saint Louis Missouri). Osteogenic differentiation was analyzed after 4 weeks by deposit of mineralized hydroxyapatite in the extracellular matrix and detected by microscopy after staining with 40mM Alizarin Red S (Sigma, Saint Louis Missouri) (Peter et al., 1998). Adipogenic differentiation was induced by incubation in α–MEM-10%FBS supplemented with 1μM dexamethasone (Sigma), 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (Calbiochem) and 100 μM indomethacin (Sigma, Saint Louis Missouri). Adipogenic differentiation was detected by staining for cytoplasmatic lipid droplets with saturated Oil Red O (Merck) (Jones et al., 2002).

II. Adult neural stem and precursor (NSC) cultures

Neural stem and progenitor cell cultures from adult rat hippocampus and lateral ventricle wall were prepared as described (Wachs et al., 2003). Briefly, adult female Fischer-344 rats (2-4 months; Charles River, Germany) were killed, brains were removed and placed in 4°C Dulbeccor`s phosphate buffered saline (DPBS) (PAN, Germany) with 4.5g/L glucose (Merck, Germany) (DPBS/glu). Hippocampus was aseptically removed and the dissected tissue was mechanically dissociated using a

scalpel. Then, the cell suspension was then washed in DPBS/glu and resuspended in PPD-solution containing 0.01% Papain (Worthington Biochemicals, England), 0.1% dispase II (Boehringer, Germany), 0.01% DNase I (Worthington Biochemicals, England) and 12,4mM MgSO4 in HBSS (PAN, Germany) without Mg^{2+}/Ca^{2+} (PAA, Germany) and digested for 30 to 40 min at room temperature. The cell suspension was triturated every 10 min. Dissociated cells were collected and resuspended in serum-free Neurobasal (NB) medium (Gibco BRL, Germany) containing 2mM L-glutamine and 100 U/ml penicillin / 0.1g/L streptomycin and washed three times with accurate trituration with a pipette tip. Finally the single cell suspension was resuspended in NB medium supplemented with B27 (Gibco BRL, Germany) (NB/B27), 2mM L-glutamine (PAN, Germany), 100 U/ml penicillin / 0.1g/L streptomycin (PAN, Germany), 2µg/ml heparin (Sigma, Germany), 20ng/ml bFGF-2 (R&D Systems, Germany) and 20ng/ml EGF (R&D Systems, Germany). Cells were seeded in T-25 culture flasks and cultures were maintained at 37°C in an incubator with 95% air, 5% CO2. Single cells began to form spheres within 5 to 7 days of suspension culture and continued to grow in mass and number over the next weeks. Medium was changed every 7 days. For passaging, the culture medium containing floating neurospheres was collected in a 15ml centrifuge tube and centrifuged at 120 x g. The pellet was resuspended in 200µl of Accutase™ (Innovative Cell Technologies Inc., USA, distributed by PAA, Germany) and triturated using a pipette tip. Then, the cell suspension was incubated at 37°C for 10 min. 10^5 cells were plated in T75 culture flasks for long term in NB/B27 medium. NSCs used in this study were derived from cultures of passage number 4 to 6.

To analyze the differentiation potential of rat hippocampal-derived neural stem cells (hNSC), single cell suspensions were plated on poly-ornithine (250 µg/ml)-and laminin (5µg/ml)-coated, HCl pre-treated, glass coverslips in 12 well plates at a density of 10^4 cells/cm^2 and they were incubated with NB/B27-5% FBS or α–MEM-10%FBS for 7 days. Cells were fixed with PBS 4% pre-warmed paraformaldehyde (37°C, pH 7.4) (4% w/v paraformaldehyde, 100mM NaH$_2$PO$_4$, 0.4mM CaCl$_2$, 50mM sucrose) for 30 minutes and processed for inmunofluorescence.

To characterize hNSC culture, neurospheres from passage 4 were dissociated, seeded and cultured on a poly-ornithine/laminin matrix incubated in α-MEM 10%FBS, which is the habitual MSC growth media. After 12-24 hours, cells were fixed for immunofluorescence analysis.

III. Other primary cultures and cell lines

The following cell lines were used as positive and negative controls for the expression of neural markers and as sources of different conditioned media:

1) Cell line Neuro2a (ATCC U.S.A. # CCL-131): N2a cell line is derived from albino mouse brain neuroblastoma. It comprises cells that can be differentiated into neurons using 5mM dibutyryl-cAMP. Neuro-2a cells produce large quantities of microtubular protein which is believed to play a role in a contractile system which is responsible for

axoplasmic flow in nerve cells (Olmsted et al., 1970). Cells were cultured according to the supplier's instructions. Briefly, 10,000cells/cm^2 were seeded and incubated in T-25 flasks in DMEM (Gibco) 10% FBS with antibiotics (100 U/ml penicillin / 0.1g/L streptomycin) at 37°C and 5% CO2. For cell passage, the cells were treated with 0.25% - 0.53mM Trypsin-EDTA (Gibco-Merck), centrifuged for 5 minutes at 800 x g, resuspended in DMEM 10% FBS and seeded.

2) Cell line SH-SY5Y (ATCC U.S.A. # CRL-2266): SH-SY5Y cell line is derived from human brain neuroblastoma with bone marrow metastasis. It comprises cells that can be differentiated into neurons using 10 μM retinoic acid. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity (Biedler et al., 1978). Cells were cultured according to the supplier's instructions. Briefly, 30,000 cells/cm^2 were seeded and incubated in T-25 flasks in MEM/F12 medium (Gibco) with 15mM Hepes, 1x non-essential amino acids 10% FBS with antibiotics (penicillin/streptomycin) at 37°C and 5% CO2. For cell passage, the cells were treated with 0.25% - 0.53mM Trypsin-EDTA (Gibco-Merck), centrifuged for 5 minutes at 800 x g, resuspended in 1:3 conditioned medium: fresh medium and seeded in a new plate.

3) Mouse embryonic forebrain cells (MEF): Primary mouse embryonic forebrain (MEF) cultures. MEF has neural progenitors that after 1 week under differentiating conditions differentiate to mature neural cells. MEF cultures were prepared as follows: 10.5–12.5 day pregnant C57Bl/6Ncrl female mice (Charles River Laboratories, Sulzfeld,

Germany) were killed and the uteri promptly removed and immersed in ice-cold Dulbecco's phosphate-buffered saline solution. Embryonic day 10.5–12.5 embryos were released from the uteri and forebrains dissected and separated from surrounding tissues. Cell dissociation was performed as described previously (Karl et al., 2005). Dissociated cells were collected by 5 min centrifugation at 120 g. The pellet was resuspended in Neurobasal medium (Invitrogen-Gibco, Karlsruhe, Germany) and washed three times. Finally, the cell preparation was resuspended in MEF differentiation medium (Neurobasal medium supplemented with B27 (Invitrogen-Gibco), 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5% fetal calf serum), seeded on coverslips coated with poly-ornithin (250 lg/mL) and laminin (15 lg/mL) at a density of 2.5 x 105 cells/cm2 and maintained for one week at 37°C in a 5% CO2-containing humidified atmosphere before immunofluorescence analysis.

4) Cell line MCF-7 (ATCC U.S.A. # HTB-22): MCF-7 cell line is derived from human mammary gland epithelial adenocarcinoma with pleural metastasis (Soule et al., 1973). The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to respond to estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Cells were cultured according to the supplier's instructions. Briefly, 10,000cells/cm^2 were seeded and incubated in T-25 flasks in α-MEM 10% FBS suplemented with 200mM L-glutamin, and 100mM sodium pyruvate with antibiotics (100U/ml penicillin / 0.1g/L streptomycin) at 37°C and 5% CO2. For cell passage, the confluent plate was treated with 0.25% - 0.53mM Trypsin-EDTA

(Gibco-Merck), resuspended in complete medium and 1/20 of this cell suspension was seeded.

5) Human umbilical venous endothelial cells (HUVEC): Primary culture of endothelial cells derived by human umbilical cord veins (Clark et al., 1986). Human umbilical cord was washed with PBS and incubated with colagenase (0.02%) (Sigma) for 15 minutes at 37°C. The soluble fraction was collected in M199 medium (Gibco 3110-035) and centrifuged for 10 minutes at 800 x g, and the pellet was resuspended in M199 medium. Cells were seeded on a 6 well plate previously coated with 2% gelatin (Sigma) and incubated in M199 medium containing EGF (Sigma E-4127), heparin and 20% FBS with antibiotics. For cell passage, after the formation of growing colonies, cells were treated with trypsin (0.025%) with EDTA (0.01%) and seeded.

6) Cell line COS-7 (ATCC U.S.A., # CRL-1651): The Cos-7 cell line is derived from African green monkey kidney fibroblast-like cells transformed with SV40 virus (Gluzman, 1981). Cells were cultured according to the supplier's instructions. Briefly, 10,000 cells/cm^2 were seeded and incubated in T-25 flasks in DMEM (PANbiotec, Germany) 10% FBS suplemented with 4mM glutamin (PAN Biotec), 1,5 g/L sodium bicarbonate, 4,5 g/L glucose and 1mM sodium pyruvate with antibiotics (100 U/ml penicillin / 0.1g/L streptomycin) at 37°C and 5% CO2. For cell passage, the confluent plate was treated with 0.25% - 0.53mM Trypsin-EDTA (Gibco-Merck) and 1/20 of this cell suspension was seeded.

7) Cell line HeLa (ATCC U.S.A. # CCL-2): The HeLa cell line is derived from human cervix epithelial adenocarcinoma. HeLa cells express keratin and are susceptible to poliovirus propagation (Scherer et al., 1953). Cells were cultured according to the supplier's instructions. Briefly, 10,000 cells/cm^2 were seeded and incubated in T-25 flask in MEM 10% FBS suplemented with 200mM L-glutamin, 1x non-essential amino acids (PAN Biotec, Gmbh, Germany) and 100mM sodium pyruvate with antibiotics (100 U/ml penicillin / 0.1g/L streptomycin) at 37°C and 5% CO2. For cell passage, the confluent plate was treated with 0.25% - 0.53mM Trypsin-EDTA (Gibco-Merck), centrifugated for 5 minutes at 120 x g, resuspended in complete medium and 1/20 of this cell suspension was seeded.

8) Cell line CTX TNA2 (ATCC U.S.A. # CRL-2006): The CTX TNA2 cell line was established from primary cultures of type 1 astrocytes from brain frontal cortex tissue of 1 day-old rats. The cultures were transfected 3 days after initial plating with a DNA construct containing the oncogenic early region of SV40 under the transcriptional control of the human GFAP promoter (pGFA-SV-Tt) (Radany et al., 1992). Cells were cultured according to the supplier's instructions. Briefly, 5,000cells/cm^2 were seeded and incubated in T-25 flasks in DMEM (PANbiotec, Germany) 10% FBS, suplemented with 200mM glutamin, 100mM sodium glutamate with antibiotics (100 U/ml penicillin / 0.1g/L streptomycin) at 37°C and 5% CO2. For cell passage, the confluent plate was treated with 0.25% - 0.53mM Trypsin-EDTA (Gibco-Merck), centrifugated for 5 minutes at 200 x g, resuspended in complete medium and 1/10 of this cell suspension was seeded.

IV. MSC's neural-like induction with beta-mercaptoethanol (BME)

For neural-like induction of MSC, cells were plated on glass coverslips ($2,500$cells/cm^2) and incubated in presence of beta-mercaptoethanol (BME) as previously described (Woodbury et al., 2000). Briefly, the pre-induction step was performed incubating MSC in α-MEM 10%FBS containing 1mM beta-mercaptoethanol (BME) for 24h. Later, in the inductive step, serum was withdrawn and MSC was incubated in α-MEM containing 10mM BME up to 5 hours. Finally, cells were washed 3 times with TBS and immediately fixed.

V. Preparation of Tissue-Conditioned Media.

Adult rats (Wistar, 200-400 g) were used following the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Institutional Animal Care and Use Committee of INTA, Universidad de Chile. Animals were killed by cervical dislocation and the intact hippocampus, cerebellum, olfactory bulb and cortex were removed under sterile conditions. In addition, the tibialis anterior muscles were excised. Intact tissues were weighed and placed into a 15 ml flat bottom culture tube to which 2 ml fresh α–MEM medium was added. After incubating with gentle stirring (to avoid tissue damage) (2 h, 4°C), the soluble fraction was collected (19,000 x g, 30 min, 4°C) and filtered through a 0.2 μm filter. The different tissue soluble factions were analyzed for protein content (Bradford) and stored at -80°C. Cells

were incubated with tissue-conditioned media, which consists of 200 µg/ml of protein from the tissue soluble fraction plus 0.5% FBS.

VI. MSC proliferation studies

For proliferation studies, MSC suspended in α–MEM-10%FBS were plated in 24- or 4 multiwell plates (2,500 cells/cm^2), which were previously coated with poly-L-lysine (Ferri and Levitt, 1995). Cells were incubated for 24 h and then switched to the control (α–MEM-0.5%FBS) or hippocampus-conditioned media (HCM). At various time intervals, adherent cells were washed (2x) with phosphate buffered saline (PBS), trypsinized and the number of viable cells was determined (Trypan blue).

VII. Western blot analysis

For total protein extraction, cells were treated with RIPA buffer (0.5% sodium deoxycholate, 1%Triton-X100, 1% SDS, 1%NP-40, 150mM NaCl, 50mM Tris, pH 8, Sigma) containing the protease inhibitors 1mM PMSF, 2 µg/ml aprotinin, 2 µg/ml pepstatin and were lysed using rubber policeman. The solution was centrifugated at 19,000 x g for 15 minutes at 4°C, and the supernatant was stored at -20°C. For protein determination, BioRad method and a bovine serum albumin (BSA) standard curve were used. For electrophoresis, 20-25 µg of total protein was loaded for each sample in 8% and 12% acrylamide-bisacrylamide (29:1) gel and was performed at 120 V for 2 hours.

Next, gel protein content was transferred to a nitro-cellulose membrane (Millipore Corporation, Bedford, MA, USA) overnight at 50mA. The membrane was blocked using 5% skim milk powder in TBS-0.05% Tween20. After blocking, membranes were incubated with mouse anti-NF-H antibody (1:2500) (Sigma, Saint Louis Missouri) (8% gel) or mouse anti-NeuN antibody (1:200) (Chemicon) (12% gel) for 2 hr at room temperature. Later, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000) for 1 hr at room temperature. Finally, the membrane was developed by a chemiluminescence assay (ECL, Amersham Pharmacia Biotech, England).

VIII. Co-culture MSC-hNSC

MSC (passage 3-4) from wild-type Fisher-344 rats were prepared as described above and plated on poly-ornithine (250µg/ml)-and laminin (5µg/ml)-coated glass coverslips in 12-well plates at a density of 2,000-4,000 cells/cm^2. After 12 hrs, NSCs (passage 3-4), isolated from adult hippocampus of transgenic rats ubiquitously expressing GFP (Lois et al., 2002), were plated over the MSC layer at a density of 10,000 cells/cm^2 in MEM-10% FBS and co-cultured for 7 days. Media was refreshed on the third day. Cells were fixed for 30 minutes with 4% prewarmed paraformaldehyde as previously described and then processed for immunofluorescence. In some experiments, the GFP signal was enhanced using a goat anti-GFP antibody (Rockland).

IX. Preparation of conditioned media and testing

MSC were plated (12,000 cells/cm^2) and incubated in α-MEM 10% SFB. After three days, the supernatant was collected and filtered using a 0.22 μm-pore filter. The filtrate was termed MSC conditioned media (MSC-CM). In some experiments, heat-sensitive factors present in the MSC-CM media were inactivated by heating the MSC-CM in boiling water for 10 minutes.

Hela and CTX-TNA2 cells (see above) were plated (10,000 cells/cm^2) and incubated for three days, either in their habitual media and α-MEM 10% FBS. Conditioned media from Hela (Hela conditioned), CTX-TNA2 (astrocyte conditioned) was filtered using a 0.22 μm-pore filter and was used to incubate hNSC.

To determine the effects of the different conditioned media on adult neural progenitors, hNSC were plated on poly-ornithine (250μg/ml)-and laminin (5μg/ml)-coated glass coverslips at a density of 8,000-10,000 cells/cm^2 in α-MEM 10% FBS for 12-24 hours. Then, medium was changed and hNSC were incubated in CM for up to 7 days. Finally, cells were fixed for 30 minutes with 4% prewarmed paraformaldehyde as described and then processed for immunofluorescence.

X. Application of bromo-deoxyuridine (BrdU) and propidium iodide (PI)

For analysis of proliferation and cell death, bromo-deoxyuridine (BrdU) and propidium iodide (PI) was used. hNSCs were prepared as described above, plated at a density of 8,000-10,000 cells/cm^2 and incubated in α-MEM 10% FBS or MSC-CM up to 7 days. To detect proliferating cells, 10 µM BrdU (Sigma) was added to the cells after 2 or 6 days. After 24 hours, cells were washed and immediately fixed or they were washed and incubated in the respective media for further cultivation. To detect dying cells, 50 µg/ml propidium iodide (PI) was added after 3 or 7 days. After 10 minutes at 37° C, cells were washed 3 times with TBS and immediately fixed.

XI. Immunofluorescence stainings and analysis

Immunofluorescence stainings and analysis was done according to the different experiments.

1) For the analysis of the neurogenic potential of MSC, the cells were grown on uncoated glass coverslips or poly-L-lysine coated glass coverslips and fixed with 4% p-formaldehyde in PBS for 20 minutes. Cells were treated with methanol for 10 min at –20°C and with 0.1 % Triton X-100/PBS for 10 min at room temperature for permeabilization. After washing twice, cells were treated with PBS-5% FBS (blocking solution) for 1h at room temperature. Cells were then incubated for 2h at room temperature in antibody-containing blocking solution. The following primary antibodies

were used: mouse NF-H 1:500 (Sigma, Saint Louis Missouri); mouse GFAP 1:400 (Sigma, Saint Louis Missouri); goat GAP-43 1:20 (Sta. Cruz Biotechnology, California); NeuroD 1:100 (Sta. Cruz Biotechnology, California); mouse NeuN 1:50 (Chemicon International); mouse Map2ab 1:250 (Sigma, Saint Louis Missouri); mouse-ß-III Tubulin 1:100 (Chemicon, California). After 4 washing steps, cells were incubated with secondary antibody diluted in blocking solution for 2h at room temperature. Secondary antibodies were rabbit anti-Goat FITC and goat anti-Mouse FITC (1/250 and 1/150) (Sta. Cruz Biotechnology, California). Nuclear counterstaining was done with Hoechst 33240 (1µg/ml bisbenzimide in PBS-3% FBS, Sigma). After the final wash, samples were mounted on slides using Fluoromount-G (Southern Biotechnology Associates Inc., USA). For quantification, the number of immunoreactive cells was determined by counting specifically stained cells in at least 10 random fields on the coverslip using an Epifluorescent microscope Axiophot (Zeiss, D-37030, RFA). For quantitative analysis, between 200 and 300 cells (MSC) per experiment in random fields were counted. Experiments were done in triplicate.

2) For the analysis of the effects of MSC and MSC-CM on hNSC, cells were fixed for 30 minutes and thoroughly washed in TBS, then blocked for 1 hour at room temperature with a fish gelatin buffer containing 0.1% Triton-X100 (Sigma), 1% BSA (Biomol) and 0.2% fish skin gelatin (Sigma, Germany) in Tris-buffered saline (TBS). The same solution was used for the incubation with antibodies. Primary antibodies were applied overnight at 4°C. The following antibodies and final dilutions were used. The primary antibodies were: rabbit anti-GFAP 1:1000 (Dako, Denmark); rabbit anti-GalC 1:200

(Chemicon International); rabbit anti-NG2 Chondroitin Sulfate Proteoglycan 1:200 (Chemicon International); IgM mouse anti-A2B5 1:200 (Chemicon International) mouse anti-rat nestin 1:500 (Pharmingen International,USA); mouse anti-Map 2a+2b 1:250 (Sigma); mouse anti-βIII-tubulin 1:500 (clone 5G8; Promega, Madison, Winsconsin); mouse anti-βIII-tubulin 1:500 (clone Tuj-1, Babco, USA); mouse anti-Myelin Basic Protein (MBP) 1:750 (SMI-92, Sternberger Monoclonals Incorporated); goat anti-GFP 1:1000 (Rockland). The secondary antibodies used were: donkey anti-goat, mouse, rabbit or rat conjugated with fluorescein (FITC), rhodamine X (RHOX) 1:500 (Jackson Immuno Research, West Grove, PA, USA); goat anti-mouse, rabbit or rat conjugated with fluorescein (FITC), rhodamine X (RHOX) 1:500 (Jackson Immuno Research, West Grove, PA, USA). In cases in which antigens were sensitive to detergents (GalC, A2B5 and NG2), Triton-X100 was omitted from fish skin gelatin buffer. When one of these antigens was co-stained with an antigen that needed permeabilization with Triton-X100, some considerations were taken as follows: first, coverslips were incubated under non-permeabilizating conditions and the respective antibody; second, after washing and blocking, coverlips were incubated under permeabilizating conditions and the respective antibody; third, a post-fixation step with 4% paraformaldehyde was added between the incubation of both primary antibodies; and finally, the incubation with secondary antibodies was performed as described above in presence of Triton-X100. Nuclear counterstaining was performed with 4´, 6´-diamidino-2-phenylindole dihydrochloride hydrate at 0.25μg/μl (DAPI; Sigma). Fixed cells were placed on Superfrost Plus slides (Menzel-Gläser, Germany) and mounted in Prolong Antifade kit (Molecular Probes,

Eugene, USA). Epifluorescence observation and photo-documentation were realized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, Sterling Heights, USA). For the quantitative analysis, between 50-100 cells/field, approximately 500-1,000 cells (hNSC) were counted per condition in each experiment and the expression of different markers was determined. Each analysis considered three independent experiments. For nuclei size measurements, cell nuclei diameters were measured after DAPI staining using a standard pixel/μm scale. Nuclei were analyzed in the different conditions.

3) For analysis of cell proliferation, BrdU-treated cells were processed as follows: fixed hNSCs were incubated with primary antibodies against A2B5, Map2ab, GalC and GFAP as previously described. Coverlips were then post-fixed for 30 minutes with 4% paraformaldehyde. This post-fixation step is to make the antigen-antibody interaction stronger and less sensitive to future treatments. Next, coverslips were incubated with 2N HCl for 30 minutes at 37°C, washed with borate buffer 0.5M pH 8.5 for 10 minutes at room temperature, then washed 4 times with TBS buffer and blocked with fish gelatin buffer. HCl acts on DNA histone proteins to allow room for antibody binding. Finally, cells were incubated with a rat anti-BrdU antibody (Oxford Biotechnology, Oxford, UK) dilution 1:500, overnight, and revealed using secondary antibodies as previously described. The fraction of BrdU positive cells, were determined in the total cells and

only those positive for each marker were considered. The staining counting was analyzed as described above.

4) For evaluation of cell death, PI treated cells were processed as follows:

Fixed hNSCs were treated always under dark conditions to avoid PI fluorescence loss. Fixed cells were stained against A2B5, Map2ab, GalC and GFAP as described above except that secondary antibodies had to be conjugated to FitC, since PI has red fluorescence. The fraction of PI-positive cells was determined in the total cell population and in each neural marker-expressing subpopulation. The staining counting was analyzed as described above.

XII. MSC RNA extraction and RT-PCR

MSCs from passage 2 were grown in α-MEM 10% FBS for 2 days. After incubation, RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the RETRO Script kit (Ambion, Cambridgeshire, UK) with random primers. For specific amplification of an IGF-1 fragment, PCR using the following primers was performed: forward 5´CAA AAT GAG CGC ACC TCC AAT A 3´and reverse 5´TTG AGG GAA ATG CCC ATC TCT G 3´. The size of the expected fragment is 555 bp. The following temperature profile was used: activation of polymerase 95°C, 15 minutes; 45 cycles of denaturing 94°C, 20 sec, annealing 58°C, 30

sec, elongation 72°C, 60 sec. Total RNA from rat pancreas and liver were used as positive controls for IGF-1 expression.

XIII. hNSC RNA extraction and quantitative real time RT-PCR

hNSCs were plated on poly-ornithine (250 µg/ml)-and laminin (5µg/ml)-coated glass coverslips at a density of 8,000-10,000 cells/cm^2 in MEM 10% SFB for 12-24 hours. Then hNSC were incubated in MEM 10% SFB or MSC-CM. RNA was extracted at day 0, 3 or 7 with the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the RETRO Script kit (Ambion, Cambridgeshire, UK) with random primers. The expression analysis was performed by real-time quantitative PCR with the Rotor Gene 2000 (Corbett Research, Sydney, Australia) with the QuantiTect SYBR Green PCR kit (Qiagen) using the specific primers listed on Table 1. The following temperature profile was used for all the genes analyzed: activation of polymerase 95°C, 15 minutes; 45 cycles of denaturing 94°C, 20 secs, annealing 56°C, 30 secs, elongation 72°C, 60 secs. Different product sizes are given on Table 1. The quality of the products was controlled by a Melt curve. An example of RNA quantification will be given on results section.

XIV. IGF-1 and IGFBP-1 studies

hNSC were plated on poly-ornithine (250 µg/ml)-and laminin (5µg/ml)-coated glass coverslips at a density of 8,000-10,000 cells/cm^2 in α-MEM 10% FBS for 12-24

Table 1.- Product size and primer pair sequence for the different genes analyzed[1]

Gene	Primer forward 5'-3'	Primer reverse 5'-3'	Size
Olig1	GCCCCACCAAGTACCTGTCTC	GGGACCAGATGCGGGAAC	109bp
Olig2	CACAGGAGGGACTGTGTCCT	GGTGCTGGAGGAAGATGACT	144bp
Nkx2.2	CACGCAGGTCAAGATCTG	TGCCCGCCTGGAAGGTGGCG	188bp
Id2	TTTCCTCCTACGAGCAGCAT	CCAGTTCCTTGAGCTTGGAG	160bp
Hes1	TACCCCAGCCAGTGTCAACA	TTCATTTATTCTTGCCCGGC	140bp
Mash-1	GATGAGCAAGGTGGAGACGC	CGGAGAACCCGCCATAGAGT	165bp
NeuroD1	CATGAAGCGCTGCGTTTAAC	CTTCAGCTCCCTCTCCCTCA	140bp
Pax6	CTCCGTACATGCAAACACAC	GTCAGGTTCACTTCCGGGAA	140bp

[1]Product size and 5'-3'sequence of the forward and reverse primers are given for Olig1,

Olig2, Nkx2.2, Id2, Hes1, Mash-1, NeuroD1 and Pax6.

hours. Then hNSC were incubated for up to 7 days in α-MEM 10% FBS or MSC-CM

containing: 20 ng/ml human recombinant insulin-like growth factor (IGF-1) (R&D

Systems, USA) or 600 ng/ml human recombinant insulin-like growth factor binding

protein (IGFBP-1) (R&D Systems, USA), or both. At the end of the experiment, cells

were fixed for 30 minutes with 4% prewarmed paraformaldehyde, as described, and then

processed for immunofluorescence.

XV. Statistical Analysis

Experiments were performed in triplicate or more. All error bars represent

Standards Deviations (SDs). Data from the different experiments were analyzed as

follows: 1) data from co-culture effect on hNSCs, nuclei size measurements and

BrdU/PI incorporation: Student's t test; 2) data from tissue-conditioned medium effect

on MSCs, MSC-CM and As-CM effect on hNSCs, MSC-CM heat-sensitive and IGF-1

effect on hNSCs by 1-way ANOVA followed by Tukey's post-hoc test; and 3) data from

HCM effect on MSC survival/proliferation, quantitative RT-PCR and temporal

expression of neural markers on hNSCs, by 2-way ANOVA. Values were considered

statistically significant at $p<0.05$. Statistical analysis was performed using SYSTAT 11.

RESULTS

I. Mesenchymal differentiation of MSCs

A characteristic feature of MSCs is their differentiation potential into adipocytes and osteocytes. To confirm these properties in the cells used in the present study, cells isolated from bone marrow stroma from adult rats were incubated in adipogenic and osteogenic differentiation media. Osteogenic differentiation was induced by incubating the cells in α–MEM-10%FBS supplemented with 0.1μM dexamethasone, 10mM β-glycerophosphate and 50μM L-ascorbic acid-2-phosphate. For adipogenic differentiation, cells were incubated in α–MEM-10%FBS supplemented with dexamethasone, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 100 μM indomethacin.

Under proliferation conditions, MSCs were adherent with a fibroblast-like morphology (Fig. 9 a). After 3 weeks of incubation in osteogenic medium, MSCs formed a confluent cell layer and synthesized hydroxyapatite, a typical sign for extracellular matrix mineralization and development of osteoblastic cells (Fig. 9 b and c). For adipogenic differentiation, MSCs were exposed to adipogenic medium for 1 week. Cells accumulated intracellular neutral lipid droplets indicating an adipocyte phenotype (Fig 9 d and e). These results demonstrate the bipotent nature of MSCs, i.e., that they were capable of differentiating into two mesenchymal lineages.
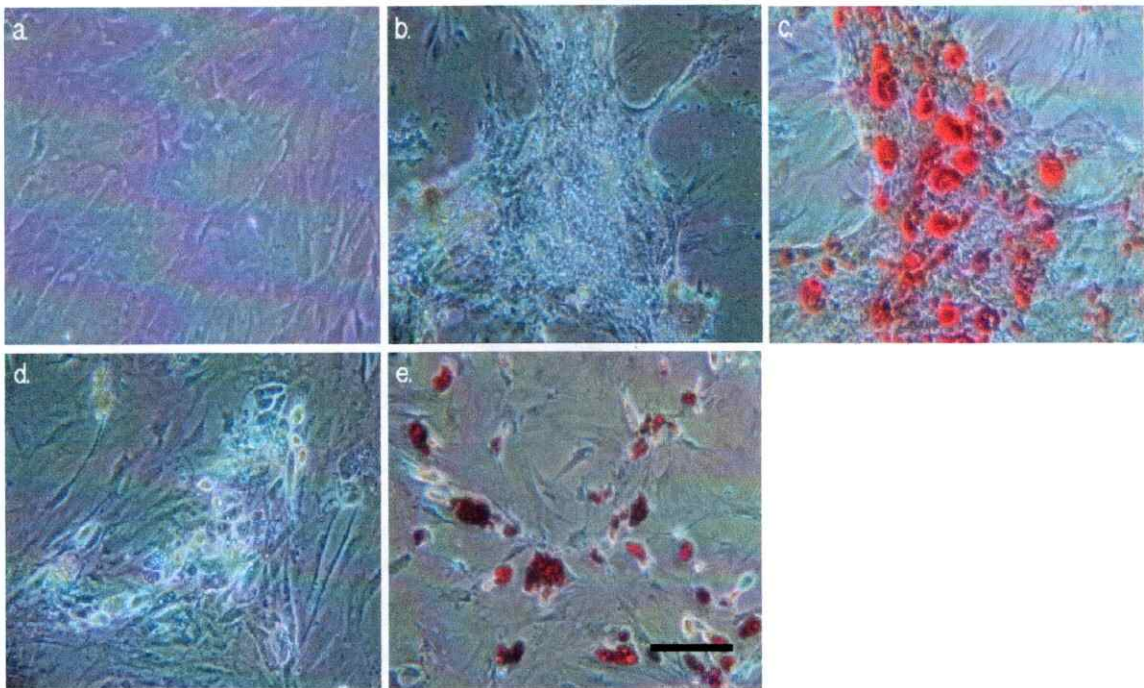
Figure 9. MSCs differentiate into cells of the mesenchymal lineage
MSCs were incubated in control medium (MEM-10% FBS) (a), osteogenic medium (b, c) or adipogenic medium (d, e) and analyzed for their differentiation potential. (a, b, d) phase contrast images. (c) MSCs in osteogenic medium and staining with Alizarin red. (e) MSCs in adipogenic medium and staining with Oil Red O. Scale bar = 125 μm. These results show that the MSCs used are at least bipotent cells, since they are able to differentiate to osteoblasts and adipocytes.

II. Mesenchymal progenitor cells can differentiate into neural-like cells

In addition to their mesenchymal differentiation fate, MSCs demonstrate characteristics of neural stem cells. For example, they express the neuroectodermal stem cell marker Nestin (Vogel et al., 2003) and upon stimulation with substances known to promote neuronal differentiation, such as antioxidants, they express neuronal genes including NeuN, Tau, synoptophysin, NSE (Woodbury et al., 2000; Woodbury et al., 2002).

In order to test whether the MSCs used can be induced to differentiate into cells of the neural lineage, we used a two-step protocol described by Woodbury et al. (Woodbury et al., 2000; Woodbury et al., 2002). This protocol consists of i) a pre-induction step, where MSCs are incubated in α-MEM 10%FBS containing 1mM beta-mercaptoethanol (BME) for 24h and ii) an induction step, where MSCs are incubated in α-MEM containing 10mM BME in the absence of serum. According to Woodbury (Woodbury et al., 2000; Woodbury et al., 2002), morphological changes that include process formation can be detected within 5 hours of the induction step. The MSC morphology changes from a fibroblastoid to a neuronal-like bipolar or multipolar one. In addition, cells start to express neural specific markers such as the neuron-specific nuclear antigen (NeuN), neurofilament medium chain (NF-M), neuron enolase (NSE) and tau (Woodbury et al., 2000).

In the present studies, MSCs were sequentially incubated with pre-inductive and indutive media. Pre-inductive media without BME (α-MEM 10%FBS) was used as a control. Cell differentiation was analyzed by immunocytochemical detection of neural-specific markers such as NeuN, NF-M, neurofilament heavy chain (NF-H) and the microtubule-associated protein (MAP-2ab). Differentiated mouse embryonic forebrain cells (MEF), the mouse brain neuroblastoma neuro2a cells and the human brain neuroblastoma SH-SY5Y cells served as positive control for the expression of these markers (Glass et al., 2002), whereas the endothelial HUVEC cells and mammary gland epithelial MCF-7 cells were used as a negative control.

Treatment of MSCs with BME using the pre-incubation and incubation procedures resulted in morphological changes from an elongated fibroblastoid shape with a large surface, to a considerably smaller, elongated, bipolar or multipolar (neuron-like) shape with long processes (Fig 10 a and b). The morphological changes were accompanied by induction of neuronal specific marker expression. Almost all MSCs expressed NF-M (Fig 10 c and d) and NF-H (Table 2) after treatment with BME. Under control conditions, cells did not express NF-M and NF-H. NeuN was expressed in presence or absence of BME, but NeuN-positive cells were present more frequently after stimulation with BME (Table 2). Expression of MAP2ab was not detected in any of the conditions evaluated (Table 2). In conclusion, these observations confirm the existing data from the literature (Woodbury et al., 2000; Woodbury et al., 2002) and verify the

a.

b.

c.

d.
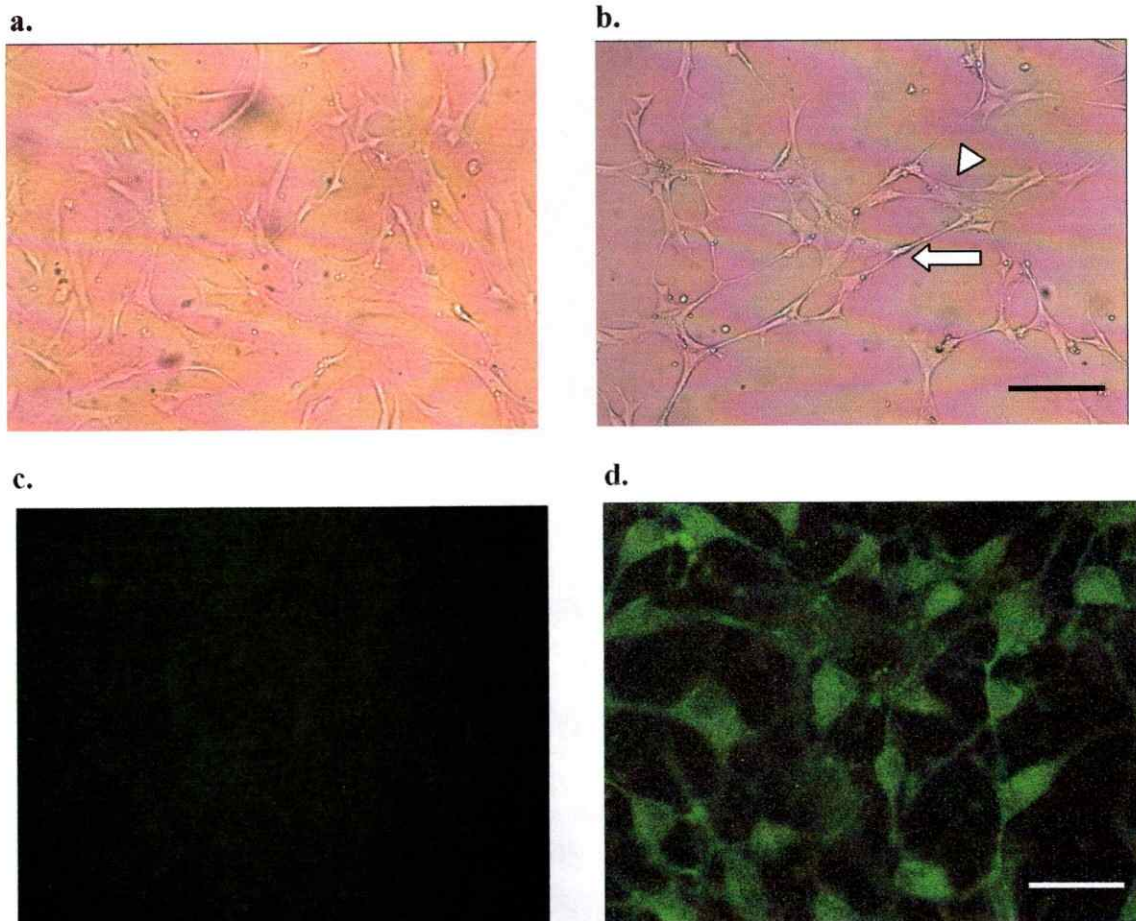
Figure 10.- BME induces a neuronal-like morphology in MSCs.
a) MSC grown in control medium display the typical elongated fibroblastoid shape. b) MSC incubated in the presence of BME; bipolar (arrow) and multipolar (arrowhead) processes reminiscent of neuronal processes can be observed. Scale bar = 125 μm. Immunofluorescence analysis of the expression of NF-M. c) MSC grown in control conditions do not express neurofilament proteins. d) MSCs stimulated with BME are immunoreactive for NF-M. Scale bar 62, 5 μm.

Table 2. Expression of neuronal specific markers in MSCs[1]

Cells and Culture Conditions	NeuN	NF-M	NF-H	Map2ab
MSC under Control Conditions	+/-	-	-	-
MSC under BME Conditions	+	+	+	-

[1]MSCs were incubated in control medium or stimulated with BME first for 24 hours (pre-induction) and then for 5 hours at a higher concentration of BME (induction). Expression of neuronal specific markers NeuN, NF-M, NF-H and Map2ab was analyzed by immunocytochemistry. Under control conditions, only few MSCs express NeuN. Expression of NF-M and NF-H was induced under BME treatment. 300 cells were counted in different fields. In neither condition Map2ab-positive cells were observed. -: no expression; +/-: less than 10%; +: more than 10%.

potential of MSCs to change their morphology and express markers associated with a neurogenic process.

In summary, the results demonstrate the potential of MSCs to i) differentiate into cells of mesenchymal lineages such as adipocytes and osteocytes, and ii) express neuronal morphology and neuronal-specific genes.

III. Hippocampus-conditioned medium induces a neuronal-like phenotype in MSCs

In addition to their mesenchymal differentiation fate, MSCs have the potential for showing characteristics of neural stem cells. For example, they express the neuroectodermal stem cell marker Nestin (Vogel et al., 2003) upon stimulation with substances known to promote neuronal differentiation (Woodbury et al., 2000; Woodbury et al., 2002). Moreover, in co-cultures with early postnatal hippocampal tissue, MSCs are induced to express the neuron-specific marker NeuN and a neuron-like morpholgy (Abouelfetouh et al., 2004). The present work aimed to determine if adult brain tissue contains soluble factors that induce the expression of a neuronal-like phenotype in MSCs. For that purpose, MSCs were incubated for three days in conditioned medium (CM) derived from adult hippocampus (HCM), cortex (CoCM) or cerebellum (CeCM). CM derived from muscle (MCM) and medium alone served as controls. The effects were assessed by analyzing the appearance of cells with a neuron-like morphology and by analyzing the expression of neural lineage markers.

Conditioned media from the different brain regions promoted a morphological change in MSCs, with a switch from a fibroblast-like morphology into cells with bipolar and multipolar processes (Fig 11). In addition, MSCs stimulated with CMs from the different brain regions induced expression of the neuronal gene NF-H (Fig. 11). CM derived from muscle tissue (used as a control), had no effects on morphology or on NF-H expression of MSCs (ANOVA, Fig. 11 and 13).

Media conditioned by different neural tissues significantly increased the proportion of NF-H positive cells (ANOVA, $p<0.001$, experiment done in triplicate). The strongest effect in induction of neuronal-like morphology and in expression of NF-H was displayed by HCM (ANOVA-Tukey Post Hoc, $p<0.005$) (Fig. 13), since 41.1 +/- 3% of MSCs expressed NF-H after stimulation with HCM, while only 11.4 +/- 2.1% and 17.6 +/- 2.8% of MSCs expressed NF-H after stimulation with CeCM or CoCM, respectively (Fig. 13). The CeCM and CoCM effects on NF-H expression (11,4% and 17,6%) were not statistically different. The expression of NF-H by MSCs incubated with HCM (but not with control media), was further demonstrated by Western blot analysis for NF-H, which detected a single band of the expected size of molecular weight 200 kDa (Fig 12).

Since the hippocampus is known to be a region of ongoing neurogenesis in the adult, and the HCM effects on induction of neuronal morphology and marker expression were most pronounced, the effects of hippocampus derived CM were analyzed in more
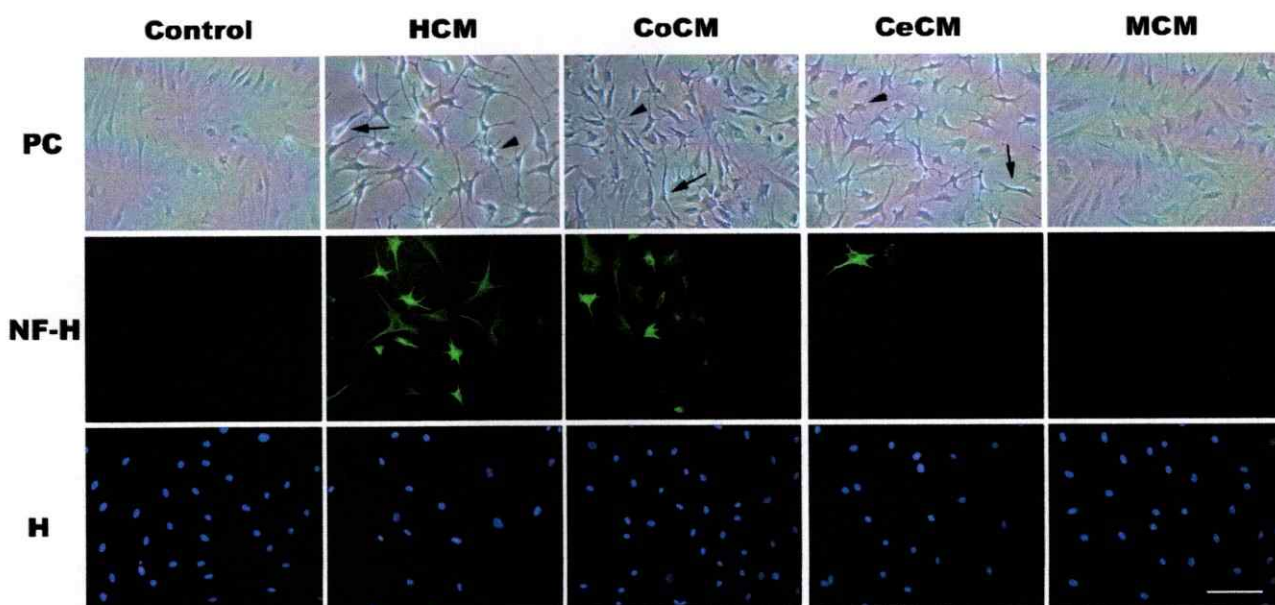
Figure 11. Soluble factors from different brain regions induce the expression of NF-H in MSCs.

MSCs were incubated for 3 days in control medium (MEM-0,5% FBS), HCM, CoCM, CeCM or MCM and analyzed for NF-H expression by immunefluorescence staining. Phase contrast images showing the morphological differences (upper panels). Cells with a bipolar (arrows) and with multipolar (arrowheads) morphology are present only in HCM, CoCM and CeCM. Fluorescence images for NF-H (green, middle panels) and Hoechst (blue, lower panels) are shown. NF-H-positive cells are present only in HCM, CoCM and CeCM. Scale bar = 125 µm.
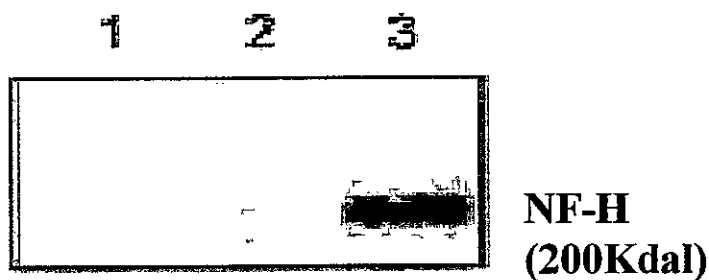
Figure 12.- Hippocampal soluble factors induce the expression of NF-H in MSCs.
Western blot analysis of NF-H (200 kDa), in cells incubated for 3 days in α -MEM
containing 10 % FBS (lane 1), α-MEM containing 0.5 % FBS (lane 2) or HCM (lane 3).
Equal amounts of protein (25ug) were loaded in each lane. Note that expression of NF-H
was only observed under HCM conditions.
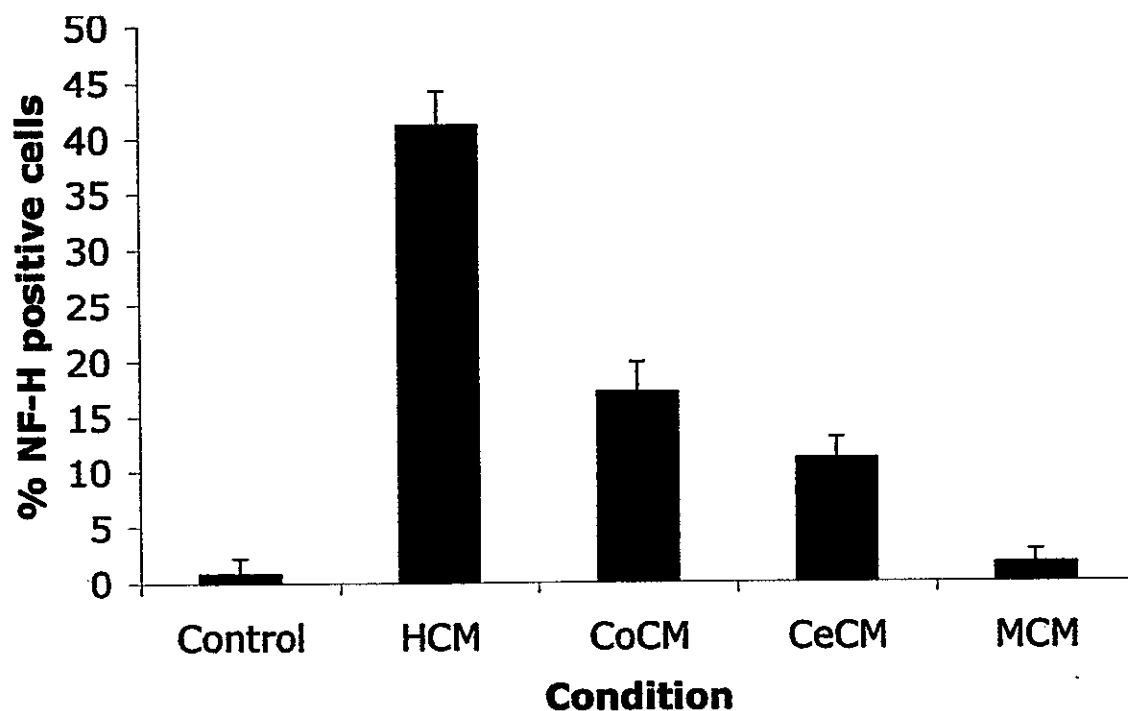
Figure 13. Quantitative analysis of NF-H expression in MSCs .
Experiments were performed as described in Figure 11. The percentage of NF-H positive cells was determined. The highest percentage of cells expressing NF-H was found in HCM. 300 cells were counted in different fields in each experiment. Experiments were done in triplicate. Data represent means ± SDs. For statistical analysis, 1-way ANOVA and Tukey Post Hoc analyses were used.

detail. This was done by immunocytochemistry for the neuronal markers NeuroD, β-III tubulin, NeuN, NF-H, GAP-43, for the glial marker GFAP, and by quantitative analysis of the number of cells expressing these markers. Under either control or HCM conditions, more than 70% of MSCs expressed the neuronal determination marker NeuroD and the young neuronal marker β -III tubulin (Table 3). Very few cells (< 1%) expressed NeuN under these conditions (Table 3). When stimulated for three days with HCM and evaluated for the expression of different neural markers, a dramatic difference in the percentage of cells expressing NF-H (<1% in control, 41.1 % in HCM) (Fig. 14 and Table 3) and GAP-43 was observed (Table 3). In contrast, the astrocytic marker GFAP was not expressed by MSCs regardless of the conditions used, indicating that hippocampal-derived CM did not induce an astrocyte-like phenotype in MSCs (Table 3). Taken together, these results suggest that hippocampal soluble factors induce a neuronal-like phenotype on MSCs.

IV. Hippocampus-conditioned medium promotes expansion and survival on MSCs

The generation of mesenchymal mature cells through MSCs is a tightly regulated process that involves control of cell proliferation, symmetric- and asymmetric cell division, cell determination, differentiation and apoptosis (Minguell et al., 2001). Proliferation of MSCs is regulated by different cytokines and soluble factors. Moreover, since MSCs constitutes a heterogeneous culture of different mesenchymal progenitors, different soluble factors promote proliferation of a specific subpopulation of progenitors,

Table 3. Expression of different neural markers in MSCs[1]

Marker	Control	HCM
NeuroD	+++	+++
ß-III Tubulin	+++	+++
NeuN	+	+
NF-H	-	++
GAP-43	-	+
GFAP	-	-

[1]MSC were incubated for 3 days in control medium or in HCM, fixed and processed for immunocytochemistry using the markers listed. Under control conditions, MSC express the early neuronal markers NeuroD and βIII-tubulin, and only few cells express NeuN. Expression of NF-H and GAP-43 was induced by HCM. 300 cells were counted in different fields en each experiment. In neither condition GFAP-positive cells were observed. -: no expression; +: less than 10%; ++: between 10 and 70%; +++: more than 70%.

which differentiate towards a specific mesenchymal lineage. For instance, Wnt signaling can control osteoprogenitor proliferation and block the adipogenic differentiation, increasesing MSC osteocyte differentiation (De Boer et al., 2004). In addition, insulin-like growth factor-I (IGF-I) specifically increases cell proliferation and lipid accumulation, playing a critical role on MSCs adipocyte differentiation (Scavo et al., 2004). Therefore, differentiation and proliferation are coupled toward MSCs lineage-restricted differentiation, thus differentiation studies must be accompanied with proliferation experiments. However, *in vivo* studies in which MSCs were transplanted into different brain regions (Azizi et al., 1998; Kopen et al., 1999; Hofstetter et al., 2002) MSCs proliferation has not been investigated sufficiently. Due to these reasons and to the potential application of MSC transplantation in neurological / neurodegenerative diseases, the influence of neural tissue (hippocampus) and in particular of its soluble factors, on MSC proliferation was studied.

In a first set of experiments, which aimed to define optimal baseline conditions for MSC proliferation, the effect of serum (FBS) concentration on the proliferative potential of MSCs was tested. MSCs were grown in α-MEM containing different concentrations of FBS and the number of total and of living cells was determined over a period of nine days. MSCs proliferated significantly more, over time, under high serum concentrations compared to low serum conditions, (ANOVA, $p < 0.001$, experiments were done in triplicate) (Fig 14 a). In addition to this, MSC's also have a better survival

a.
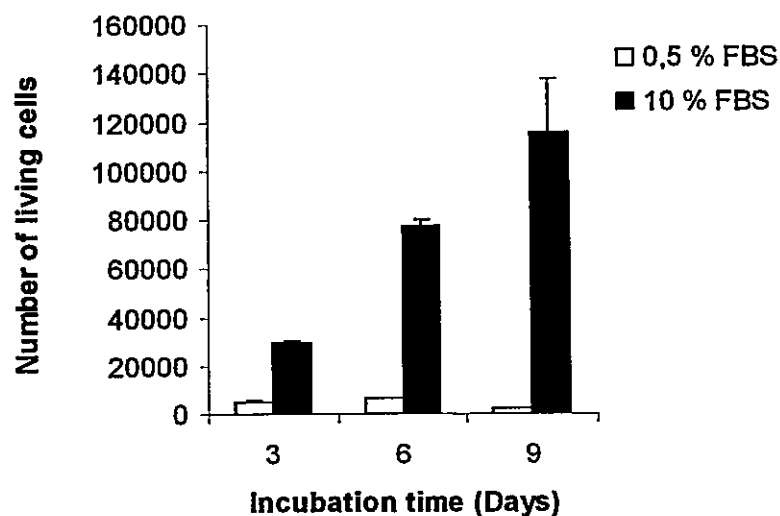
b.
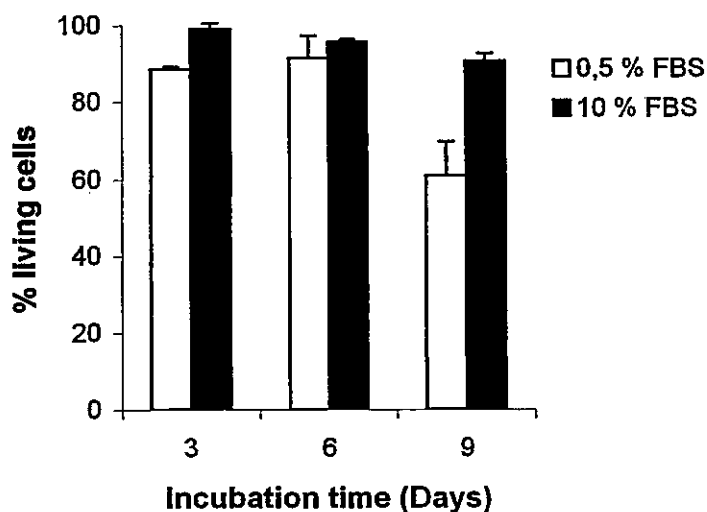
Figure 14. MSC proliferation and survival is serum dependent.
MSC were seeded (4,000 cells) and incubated up to 9 days in low serum media (α-MEM 0,5% FBS) and high serum concentrations (α-MEM 10% SFB). Total number (a) and percentage (b) of living cells were determined at day 3, day 6 and day 9. A clear proliferative and survival effect was observed on the highest serum concentration condition. Experiment was done in triplicate. Error bars represent SDs and 2-way ANOVA-Tukey Post-Hoc was used for statistical analysis.

(% living cells among the total amount of cells), over time, under high serum concentration conditions, (p<0.05, experiments were done in triplicate) (Fig 14 b). Specifically, survival in low serum decreased between days 6 and 9, since there was approximately 60% of living cells (respect to the total cells), whereas in high serum a survival rate of 90% was maintained. In conclusion, MSCs proliferation and survival are serum dependent, being the best condition the highest concentration tested (10% SFB).

The next experiments targeted the question of whether soluble factors derived from hippocampus tissue influenced MSC proliferation and survival. Therefore, HCM was prepared as described in the methods section, and used to stimulate MSCs. Cell proliferation and survival were determined by counting the total number of cells and the percentage of live cells over a period of nine days. HCM promoted a significant increase on the proliferation, over time, of MSC cultures compared to the control condition (ANOVA, p<0.001, experiment done in triplicate). Specifically, the number of cells after three days in HCM was about 80% higher compared to the control (Fig. 15 a). After that, the cell number was maintained under HCM conditions. In addition to this, MSC's also survive better, over time, under HCM conditions respect to control (ANOVA, p<0.05, experiments were done in triplicate). It was noticed that during the period between days 6 and 9, HCM prevented MSCs from substrate detachment and cell death that otherwise occurred when cells were grown under control conditions (Fig. 15 b). Accordingly, viability was 91+/- 7% and 61+/- 8% for cells incubated in the presence

a.

b.
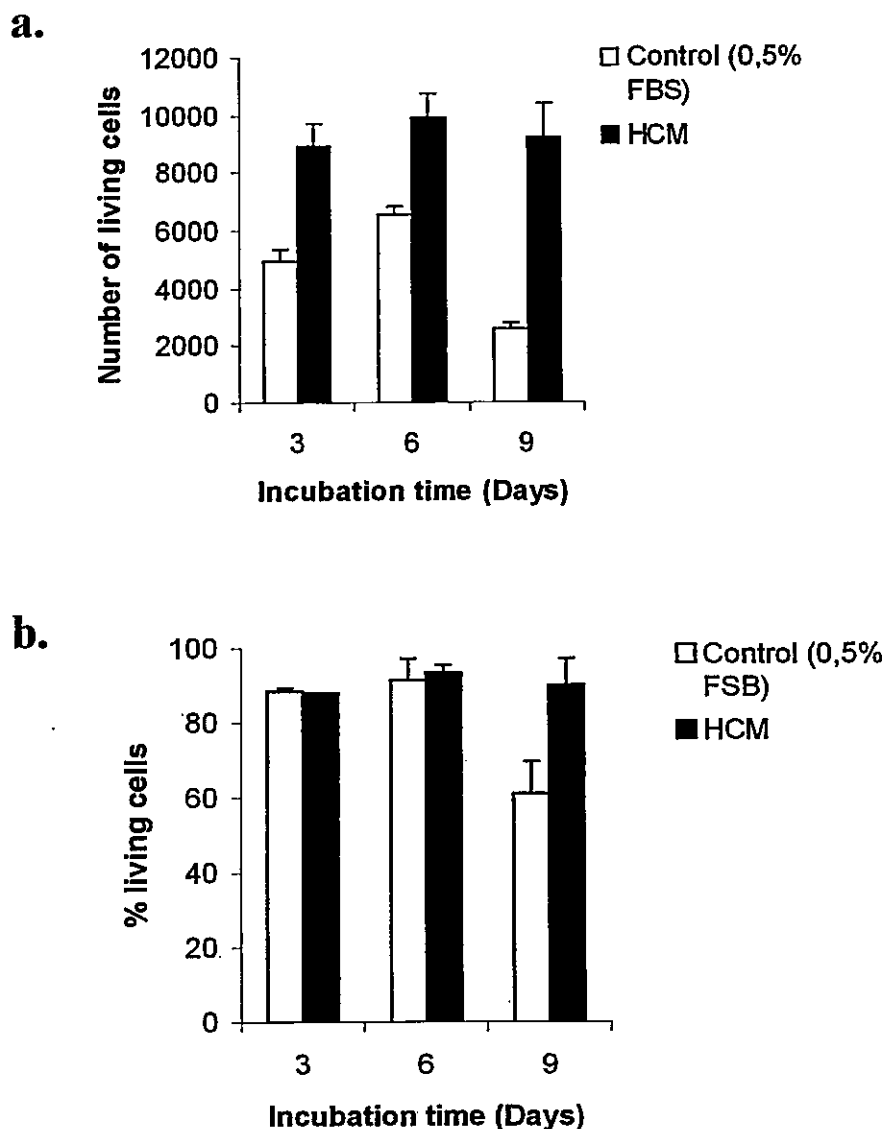
Figure 15. Hippocampal soluble factors promote expansion and survival of MSCs
MSC were seeded (4,000 cells) and incubated up to 9 days in control media (α-MEM 0,5% FBS) and in HCM. Total number (a) and percentage (b) of living cells were determined at day 3, day 6 and day 9. Note the early and significant increase and the protracted maintenance in cell number in HCM respect to their control. Experiment was done in triplicate. Error bars represent SDs and 2-way ANOVA-Tukey Post-Hoc was used for statistical analysis.

and absence of HCM, respectively. In summary, the hippocampus-derived CM promoted MSC expansion and survival.

Taken together, soluble factors derived from adult brain tissue, in particular from hippocampus, promote proliferation and survival of MSCs and induce a neuronal-like phenotype in these cells.

The overall aim of this thesis was to identify and characterize bidirectional effects between mesenchymal and neural stem and progenitor cells. The previous sections of this thesis were dedicated to mesenchymal stem cells and to the question of i) whether these cells have the ability to induce neuronal differentiation programs in addition to their mesenchymal differentiation potential and ii) whether proliferation, differentiation and survival of these cells is influenced by adult neural tissues, in particular by soluble factors derived from the hippocampus. The goal of the sections that follow was to identify and characterize the effects of mesenchymal stem cells on neurogenic processes, in particular on the cell fate of neural stem cells.

In consistence with the first part of this thesis, where the effects of hippocampus-derived soluble factors of MSCs were analyzed, neural stem and progenitor cells derived from adult rat hippocampus (hNSC) were used in the sections that follow. These cells hold the potential to proliferate, to self-renew and to differentiate into the three neural

lineages, i.e neurons, astrocytes and oligodendrocytes (Wachs et al., 2003). The characterization of the hNSCs used is presented below.

V. hNSCs: proliferation and differentiation potential

To analyze the effects of MSCs on neural stem cell biology, NSC cultures derived from adult rat hippocampus (hNSC) were generated as described (Wachs et al., 2003). These cultures grow under proliferation conditions in so-called neurospheres (Neurobasal medium containing B27 supplement, FGF-2, EGF and heparin), that consist of undifferentiated neural stem and progenitor cells and, to a lesser extent, of already differentiated neuronal and glial cells (Wachs et al., 2003) (Fig. 16a). Under conditions that promote differentiation such as growth factor removal and presence of serum, the cells adhere and differentiate into neurons and glia.

Here, the identity of the cells and their differentiation fate under proliferation- and differentiation-promoting conditions were analyzed by immunocytology for the expression of the following neural stem, progenitor and neural differentiation markers:

1) **Nestin**: Intermediate filament, is usually used to identify multipotent neural stem cells (Dahlstrand et al., 1992).

2) **A2B5**: An epitope of a glycoprotein, a marker originally thought to be specific for bipotent glial progenitors cells (Galiana et al., 1993). More recent data

demonstrated that A2B5-immunopurified cells can also generate neurons indicating their multipotent nature (Nunes et al., 2003).

3) **NG2**: Chondroitin sulfate proteoglycan, is highly expressed in bipotent glial-restricted progenitors (O2A) and oligodendrocyte precursors cells (OPC) (Nishiyama et al., 1996b, 1996a; Fidler et al., 1999; Dawson et al., 2000; Dawson et al., 2003).

4) **Map2ab**: Microtubule associated protein 2a + 2b, usually expressed in mature neurons, specifically in dendrites (Ferreira et al., 1987).

5) **GFAP**: Glial acidic fibrillary protein, usually expressed in astrocytes (Cohen et al., 1979).

6) **GalC**: Galactocerebroside, highly expressed in oligodendrocytes (Monge et al., 1986).

7) **MBP**: Myelin basic protein, highly expressed in mature oligodendrocytes (Monge et al., 1986).

hNSC neurospheres from passage 4 were dissociated, seeded and cultured on a poly-ornithine/laminin matrix incubated in α-MEM 10%FBS. After 12-24 hours, cells were fixed for immunofluorescence analysis. Under these conditions hNSCs expressed high levels of the neural stem cell and progenitor markers nestin (Fig. 16 b) and A2B5 (Fig. 16 c). The quantitative analysis revealed that approximately 94% of hNSCs were immunopositive for A2B5 (table 4). Additionally, approximately 40% of the neural progenitors in our cultures were positive for NG2 (table 4), Nestin was expressed in
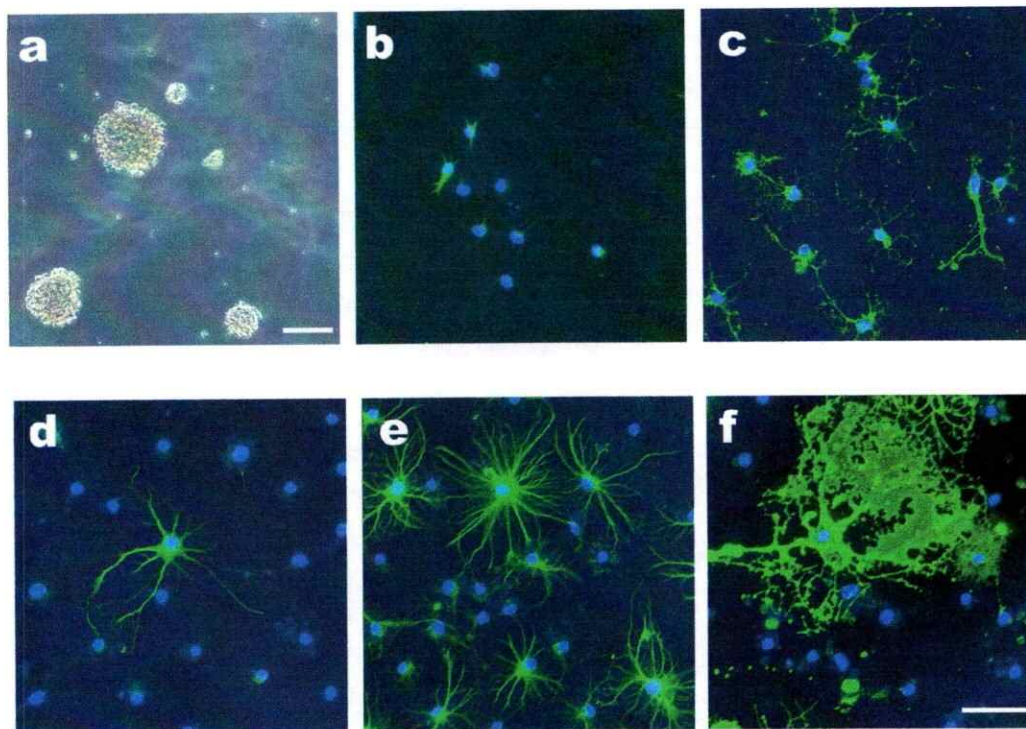
Figure 16.- Hippocampal neural stem/progenitor cells (hNSCs) cultured under proliferation and differentiation conditions.

hNSC incubated under proliferative conditions (NB/B27 + FGF-2 + EGF+ heparin), were dissociated and plated on polyornithin/laminin coating coverslips for cell attachment, incubated for 24 hours in α-MEM 10%SFB and fixed for immunofluorescence. Alternatively, cells were incubated for 7 days in α-MEM 10%SFB (differentiating conditions) and fixed for immunofluorescence. Phase contrast image of hNSC under proliferation conditions showed the formation of floating neurospheres (a). Scale bar =100 μm. Fluorecence images of previously dissociated hNSCs neurospheres, plated and incubated for 24 hours, expressing Nestin (b) and A2B5 (c) stem/progenitors markers. Fluorescence images of previously dissociated hNSCs neurospheres, plated and incubated for 7 days under differentiating conditions, expressing Map2ab (d), GFAP (e) and Gal C (f) mature neural lineage markers. Scale bar =50 μm.

Table 4.- Markers expression in hNSC under expansive conditions[1]

Markers	% positive cells
Nestin	18,5 +/- 0,28
A2B5	93,4 +/- 0,99
NG2	39,9 +/- 1,56
Map2ab	1,5 +/- 0,33
GFAP	6,1 +/- 0,07
GalC	0,8 +/- 0,21

[1]hNSC floating neurospheres from passage 4 under proliferative conditions were dissociated and single cells were cultured under differentiating medium. After 24 hours, cells were fixed and immunostained using antibodies against Nestin, A2B5, NG2, Map2ab, GFAP and GalC. Note the high percentage of cells expressing the glial-restricted markers A2B5 and NG2. On the other hand, there is a low percentage of cells expressing any of the mature neural markers analyzed (Map2ab, GFAP and GalC). 250-400 cells were counted in different fields in each experiment. Experiments were done in triplicate. Results are presented as means +/- SD.

approximately 20% of the hNSCs (table 4) (Kuo et al., 2003). Importantly, less than 10% of the neural progenitors expressed mature neural cells markers (i.e. GFAP, GalC and Map2ab) (table 4). Taken together, these results suggest that hNSC culture is rich in glial-restricted progenitors and poor in mature neural cells.

After 1 week of incubation in differentiating medium (α-MEM 10%FBS), hNSCs expressed the neuronal marker Map2ab (Fig. 16 d), the astroglial marker GFAP (Fig. 16 e) or the oligodendroglial marker GalC (Fig. 17f). The expression occurrence of the various markers was further quantified. 56.5 +/- 6.6% of the cells were GFAP-positive, 5.3 +/- 3.9 % were GalC-positive cells and approximately 2.0 +/- 0.2% were Map2ab-positive.

In summary, the results confirmed that the hNSCs used in the present study were multipotent and generated all three neural cell lineages: neurons, astroglia and oligodendroglia.

VI. MSCs influence the differentiation fate of hNSCs in co-cultures

Considering the described effect of hippocampal soluble factors in the MSC neural-like fate acquisition, it is reasonable to ask whether the neural stem/progenitors cells (NSCs) present in the hippocampus release soluble factors that induce this phenotype on MSC. Conversely, and considering the stromal nature of MSC, it is logical

to wonder if MSC can affect the neural fate of NSCs present in the hippocampus. To investigate potential reciprocal effects between hNSCs and MSCs, co-cultures of the two cell types were established. Cell identity and differentiation fate was analyzed using cell type-specific markers (see above). As a control experiment, hNSC and MSC were cultured separately (Fig. 17 a and b, respectively). In order to distinguish the hNSCs from the MSCs in co-cultures, the hNSCs were derived from a transgenic rat line that expresses the GFP reporter gene under a ubiquitous promoter (Fig. 17 c and d) (Lois et al., 2002). Moreover, MSCs could be identified by their bigger nuclei in comparison to hNSCs, as revealed by Dapi staining. Quantitative analysis of nuclear size showed that MSC nuclei had a diameter of 26.07 +/- 3.71 µm, whereas hNSC had a diameter of 15.84 +/- 2.37 µm, which is significantly different (Student t test, p<0.0001).

The habitual MSC medium (α-MEM 10%SFB) promotes differentiation on hNSC as was before described (differentiating condition), so hNSCs (from GFP transgenic rat) were single cultured or co-cultured with MSC in α-MEM 10%SFB. After 7 days in culture, expression of neural markers was analyzed. In hNSCs, single or co-cultured with MSCs, expression of markers for the three mature neural lineages could be detected: Map2ab for neurons (Fig. 17 e-h), GFAP for astrocytes (Fig. 17 i-l) and GalC for oligodendrocytes (Fig. 17 m-p). Co-cultures of hNSCs with MSCs significantly increased the proportion of hNSCs expressing GalC compared to the hNSC single-cultures (74 +/- 7.8% vs. 4 +/- 5% respectively, Student t test p<0.01, experiment done
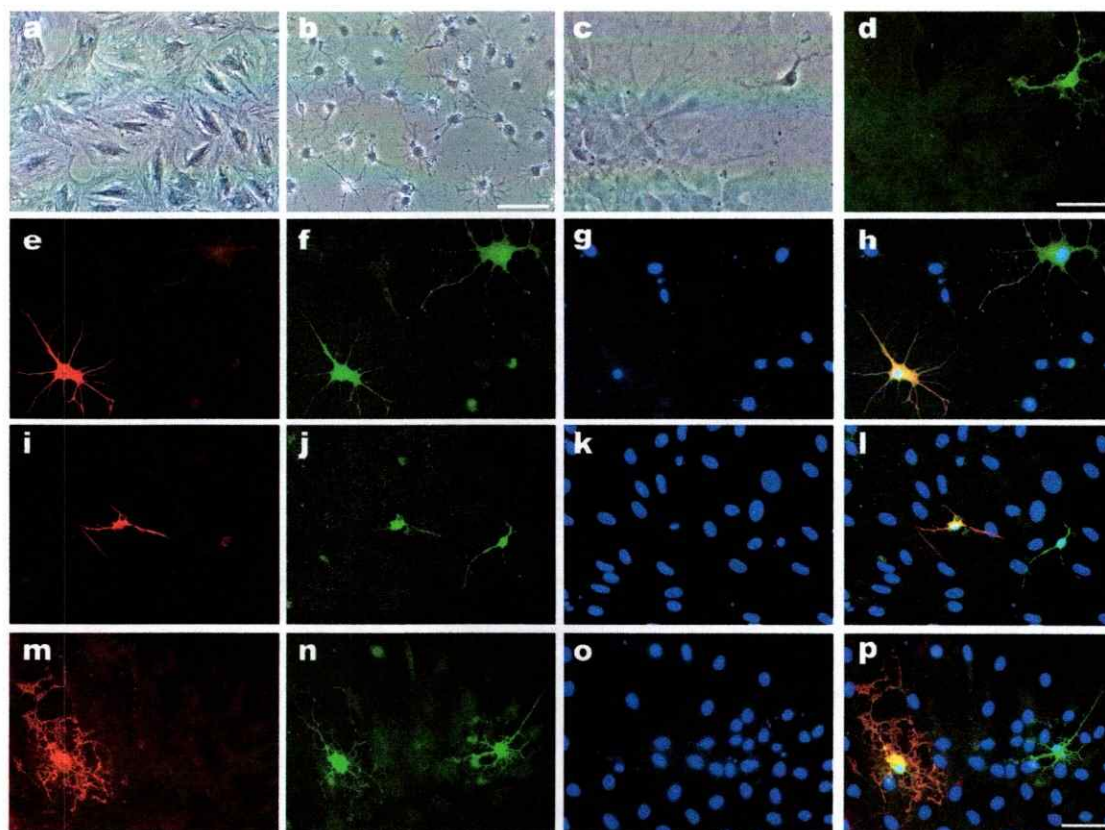
Figure 17.- Co-cultures of MSC-hNSC: effects on differentiation fate.

MSCs and hNSCs were cultured in single and co-culture conditions. To distinguish each cell type, hNSC were isolated from a GFP transgenic rat (hNSC(GFP)). Cells were incubated in a-MEM 10%SFB for 7 days, fixed and immunofluorescence was performed. Phase contrast images for MSC culture alone (a), hNSC culture alone (b), scale bar = 100 μm. Phase contrast image (c) and flourescence image (d) for MSC-hNSC(GFP) co-culture, scale bar = 50 μm. Flourescence images for co-culture: Map2ab expression (red) (e); GFAP expression (red) (i); GalC expression (m); GFP expression (green) (f, j, n); DAPI (blue) (g, k, o); merge e,f,g (h); merge i, j, k (l) and merge m, n, o (p). Scale bar = 50 μm.

in triplicate) (Fig. 18). To strengthen the finding on the increased percentage of GalC expressing, and therefore oligodendroglial cells, an additional marker for oligodendrocytes, Rip (Reynolds and Hardy, 1997), was evaluated. Confirming the above-mentioned results, a higher percentage of cells expressing the Rip antigen was detected in the co-culture condition (data not shown). A lower percentage of hNSCs expressed GFAP in co-cultures with respect to single-culture hNSC (19 +/- 9.1% vs. 53 +/- 9.4%, respectively, p<0.01) (Fig. 18) .In contrast, the percentage of Map2ab-expressing cells was the same in the two culture conditions (Fig. 18). None of the mature neural specific markers (GalC, Rip, GFAP or Map2ab) was expressed in MSCs for any of the culture conditions tested.

In summary, these results suggest that there was no effect of hNSCs on the differentiation fate of MSC. Conversely, there is a MSC-induced oligodendrogenic effect on the hNSCs.

VII. The oligodendrogenic effect of MSCs on hNSCs is mediated by soluble factor(s)

The previous chapter described that in co-culture systems, MSCs influence the differentiation fate of hNSCs towards an oligodendroglial specification. To investigate if soluble factors secreted by MSCs in contrast to cell contact-mediated signals were responsible for this effect, hNSCs were incubated in MSC-conditioned medium (MSC-
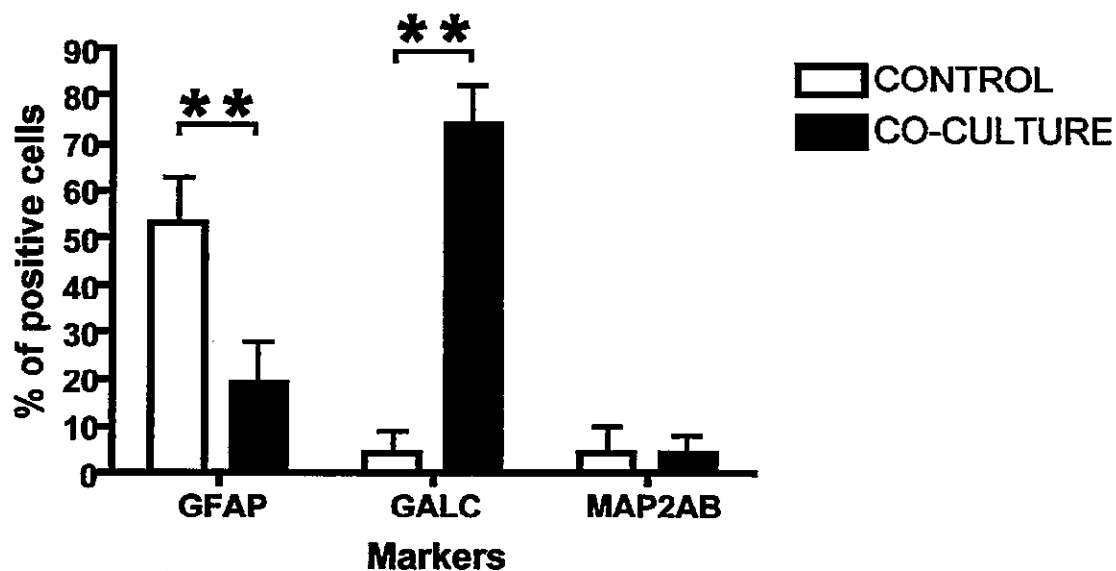
Figure 18.- Quantitative analysis of the expression of neural differentiation markers in hNSCs co-cultured with MSCs.

hNSCs were cultured for 7 days in single and co-culture with MSCs. To distinguish each cell type, hNSC were isolated from a GFP transgenic rat (hNSC(GFP)). 500-1,000 hNSC (green cells) were counted in different fields for both conditions in each experiment. Among them, different mature neural markers expressing cells (red) were counted. Thus, the percentage of expressing hNSCs (yellow cells) was determined for each marker. In co-cultures a higher percentage of hNSC-derived cells expressed the oligodendroglial marker GalC, a lower percentage expressed the astroglial marker GFAP. No difference in the percentage of cells expressing Map2ab was detected. Experiments were done in triplicate. Data represent means +/- SD. **p<0.01, Student's *t* test.

CM). hNSCs treated with MSC-CM for 7 days displayed a multipolar morphology with extensive arborization and secondary processes and small cell bodies (Fig 19 b). In contrast, hNSCs exposed to unconditioned (control) MSC medium displayed fewer processes and had bigger cell bodies (Fig. 19 a). Moreover, hNSCs incubated with MSC-CM for 7 days had smaller nuclei than hNSC incubated under control conditions (unconditioned MSC medium) as revealed by Dapi staining. Quantitative analysis of nuclear size showed that hNSC nuclei, incubated in MSC-CM, had a diameter of 11.74 +/- 2.77 μm, whereas hNSC nuclei, incubated in control conditions, had a diameter of 15.84 +/- 2.37 μm (Student t test, p<0.0001).

Immunofluorescence analysis revealed the presence of an important proportion of A2B5/GFAP and A2B5/GalC double positive cells in both cultures (data not shown), suggesting that GFAP and GalC positive cells can be originated from A2B5-expressing neural progenitors cells.

The quantitative analysis of marker expression for mature neural cell types in hNSCs revealed a significantly higher proportion of GalC-positive cells among cultures containing MSC-CM as compared to those in control conditions (50 +/- 6% conditioned vs. 5 +/- 4% control, ANOVA and Tukey post-hoc, p<0.001, experiment done in triplicate) (Fig. 19 c, d and Fig. 20). Myelin basic protein (MBP) is a marker usually expressed in mature oligodendrocytes, since it is one of the mayor constituents of the myelin sheath (Monge et al., 1986), which develops during the last stages in
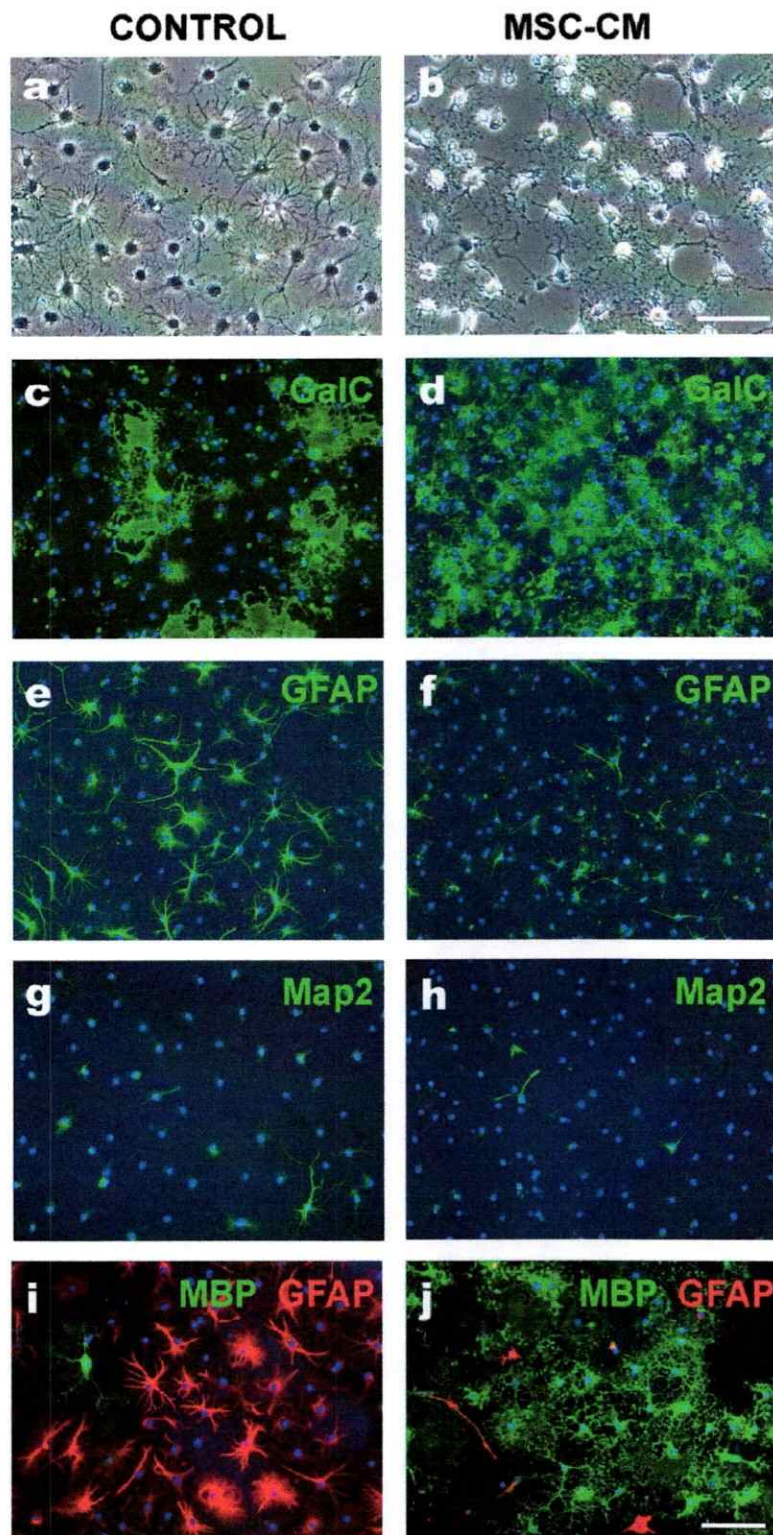
CONTROL   **MSC-CM**

Figure 19.- MSC soluble factors induce the expression of oligodendrocyte mature markers in adult hippocampal progenitor cells (hNSCs).
hNSC were incubated for 7 days in control media (α-MEM 10% SFB) and in MSC-CM and processed for immunocytology. Phase contrast images for neural progenitors incubated in the different conditions described (a, b). Scale bar = 100 μm. Fluorescence images for the different neural markers (c-j). Scale bar = 100 μm.

oligodendrogensis (Monge et al., 1986; Reynolds and Hardy, 1997). This marker is expressed at a later stage than GalC and RIP in oligodendrocyte ontogenesis (Monge et al., 1986; Daston and Ratner, 1994; Reynolds and Hardy, 1997), which makes it is a good marker for oligodendrocyte maturation. Similarly to GalC expression, a significantly higher percentage of cells expressing MBP was found in cultures exposed to conditioned medium compared to control medium (36.6 +/- 7.3% conditioned vs. 0.5 +/- 0.3% control, ANOVA-Tukey post-hoc, p = 0.001, experiment done in triplicate) (Fig. 19 i, j and Fig. 20). On the other hand, there was a lower frequency of GFAP-expressing cells in cultures that were incubated in MSC-CM (30 31 +/- 8% conditioned vs. 56 +/- 7% control conditions, p<0.05) (Fig. 19 e, i, f, j and Fig. 20). Finally, the occurrence of Map2ab expression, which was very low in our cultures, was not influenced by the presence of conditioned medium (Fig. 19 g, h and Fig. 20).

Taken together, these results indicate that soluble factors released from MSC could promote the generation of the mature oligodendrocytes in neural progenitor cultures at the cost of the astrocytic lineage.

To determine whether the oligodendrogenic activity present in MSC-CM is specifically produced by MSCs, the oligodendrogenic potential of another cell type was also evaluated. For that purpose, conditioned medium from the non-mesenchymal rat astrocyte cell line CTX TNA2 was tested for the presence of oligodendrogenic activity(ies) using the hNSC cultures. hNSCs were incubated for 7 days in astrocyte-
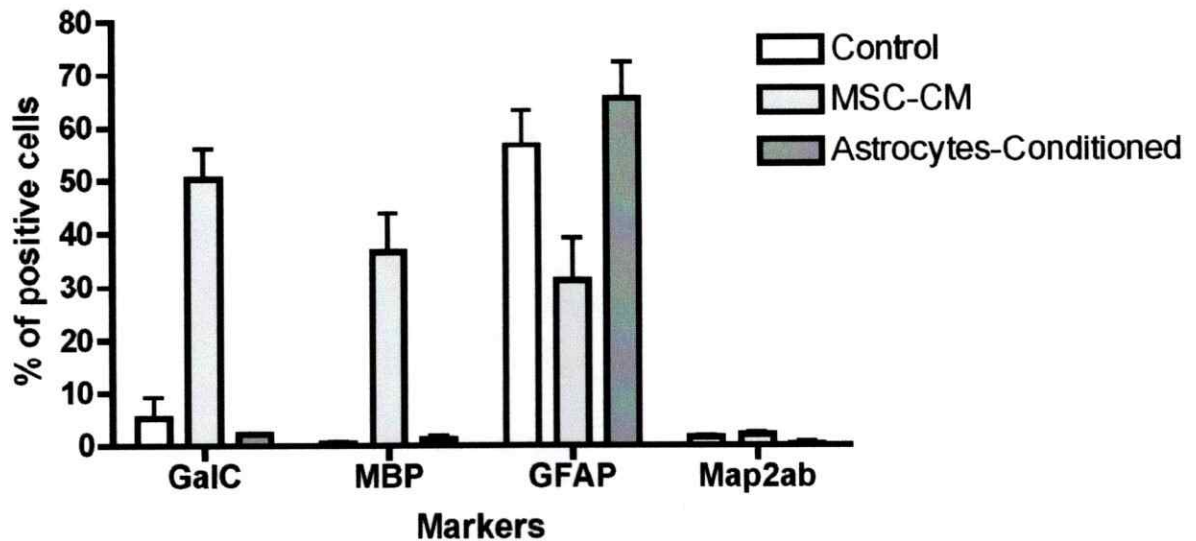
Figure 20.- Quantitative analysis of the effects of MSC-CM on the expression of neural differentiation markers in hNSCs.

hNSC were incubated for 7 days in control media (α-MEM 10% SFB), in MSC-CM and in rat astrocyte cell line conditioned media (As-CM) and processed for immunocytology. 500-1,000 hNSCs were counted in each experiment and the percentage of GalC, MBP, GFAP and Map2ab-expressing cells was determined. Compared to, control and As-CM, MSC-CM showed a significantly higher proportion of GalC and MBP positive cells. However, under MSC-CM condition, a significantly lower proportion of GFAP positive cells was found when compared with control and As-CM. The percentage of Map2ab expressing cells did not differ in the three conditions tested. Experiments were done in triplicate. Results show means +/- SDs. 1-way ANOVA and Tukey post-hoc test were used for statistical analysis.

conditioned media (As-CM), and cells were fixed and analyzed for the expression of neural lineage markers by immunocytochemistry. The results showed that 2.1 +/- 0.1% of the cells expressed GalC, whereas almost no MBP positive cells were found, indicating no significant difference compared to control conditions (ANOVA-Tukey Post Hoc, experiment done in triplicate) (Fig. 20). These proportions were lower that those found under MSC-CM conditions (p<0.005) (Fig. 20). Therefore, no oligodendrogenic effect was detected on hNSC upon incubation with As-CM. In addition to this, a significant higher proportion of GFAP positive cells (65.3 +/- 7%) were found under As-CM with respect to MSC-CM conditions (p<0.01). Therefore As-CM has an astrogenic effect on hNSC. Finally, no significant differences were found in the proportion of Map2ab positive cells (0.3%) between the different conditions tested. Moreover, no difference in GalC-expressing cells was found when hNSCs were incubated in Hela conditioned media (data not shown). In summary, soluble factors released from other cell types (non-mesenchymal) cannot induce oligodendrogenesis in adult neural progenitor cells as MSC `s soluble factors did.

VIII. MSC soluble factors instruct neural progenitors to the oligodendrogenic fate in hNSC cultures and promote astrocyte proliferation

In order to determine whether soluble factors released by MSCs have an instructive and/or a selective activity on neural progenitor cells, we i) monitored the effects of MSC-CM on hNSC cell morphology and marker expression over time and ii)

analyzed proliferation and death of cells in hNSC cultures that were exposed to MSC-CM. At day 0 (cells were seeded the day before, day -1), hNSCs displayed mostly bipolar morphologies with few processes and phase-dark cell bodies (Fig. 21 a, d). Three days later, cells displayed multipolar morphologies with secondary processes, an effect more pronounced in MSC-CM (Fig. 21 b, e). After 7 days, most of the cells in MSC-CM had secondary processes, reminiscent of oligodendrocyte morphology, and phase-bright somata (Fig. 21 f). On the other hand, hNSCs incubated in control medium were multipolar with few secondary branches and flat cell soma reminiscent of astrocytic morphology (Fig. 21 c).

In summary, these observations suggest that under MSC-CM conditions, hNSC display an oligodendrocyte morphology that over time becomes more complex, with more secondary process, and the number of cells displaying this morphology increases over time, however, under control conditions cells display an astrocyte morphology.

To support the time-dependent changes in morphology described above, a time course monitoring of the percentage of A2B5-, GFAP-, GalC- and Map2ab-expressing cells was performed. The kinetics of the expression of different cell type specific markers was analyzed in hNSC cultures in the presence or absence of MSC-CM. For A2B5 expression, a significant time-dependent decrease in the percentage of positive cells was observed in both culture conditions (ANOVA, $p < 0.001$). The presence of MSC-CM did not influence the kinetics of the A2B5 expression's decrease ($p > 0.05$)
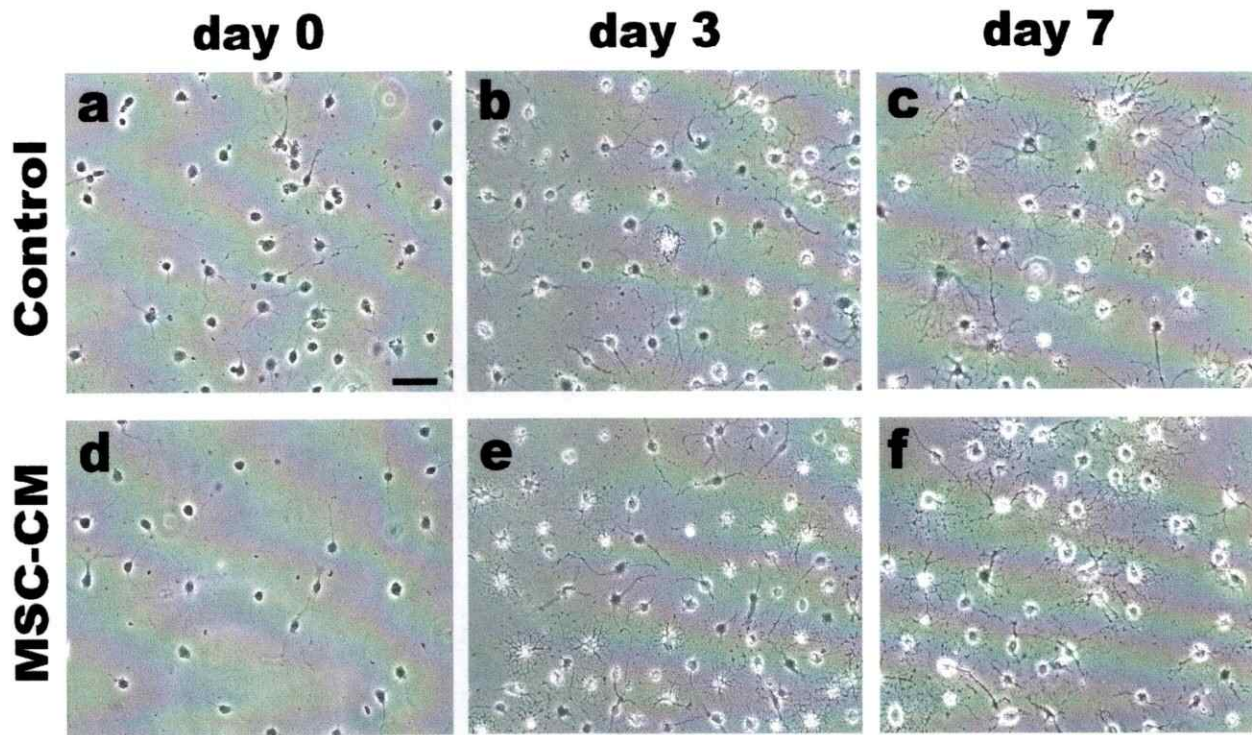
Figure 21.- Morphological changes of hNSCs induced by MSC-CM.

hNSCs were incubated for up to 7 days in control medium (a,b and c) and in MSC-CM (d, e and f). Cell morphology was analyzed at days 0, 3 and 7 on phase contrast microscope and images are shown (a-f). Scale bar = 100 μm. Note the increase, over time, of the amount of cells having an oligodendrogenic morphology, in hNSCs grown under MSC-CM condition.

(Fig. 22). The percentage of GalC expressing cells significantly increased over time, under MSC-CM conditions as compared to cultures maintained in the control medium (p = 0.001) (Fig. 22). On the other hand, the proportion of GFAP-positive cells increased in both conditions. However, compared to control medium, a lower percentage of cells expressed GFAP over time in MSC-CM condition (p<0.05) (Fig. 22). Finally, Map2ab expression pattern remained the same over time and there was no difference between the two culture conditions evaluated (p>0.05) (Fig. 22).

Two mechanisms may be responsible for the increased number of oligodendrocytes present in hNSC cultures after exposure to MSC-CM. First, soluble factors released by MSCs could selectively increase the proliferation or the survival of oligodendrocytes (precursors and/or mature cells). Second, soluble factors released by MSCs could instruct neural progenitors to differentiate into oligodendrocytes. These possibilities are, however, not exclusive.

The next experiments were performed to test whether the oligodendrogenic effect of the MSC-CM is due to a selection or/and an instructive process. Therefore, we analyzed proliferation and death of cells in hNSC cultures that were exposed to MSC-CM. Proliferation and cell death rates were measured by bromo-deoxyuridine (BrdU) and propidium iodide (PI) incorporation, respectively, at days 3 and 7 of MSC-CM treatment. PI is used to stain DNA and allows the identification of dying cells due to the appearance of pyknotic nuclei. The percentage of proliferating and dying cells was
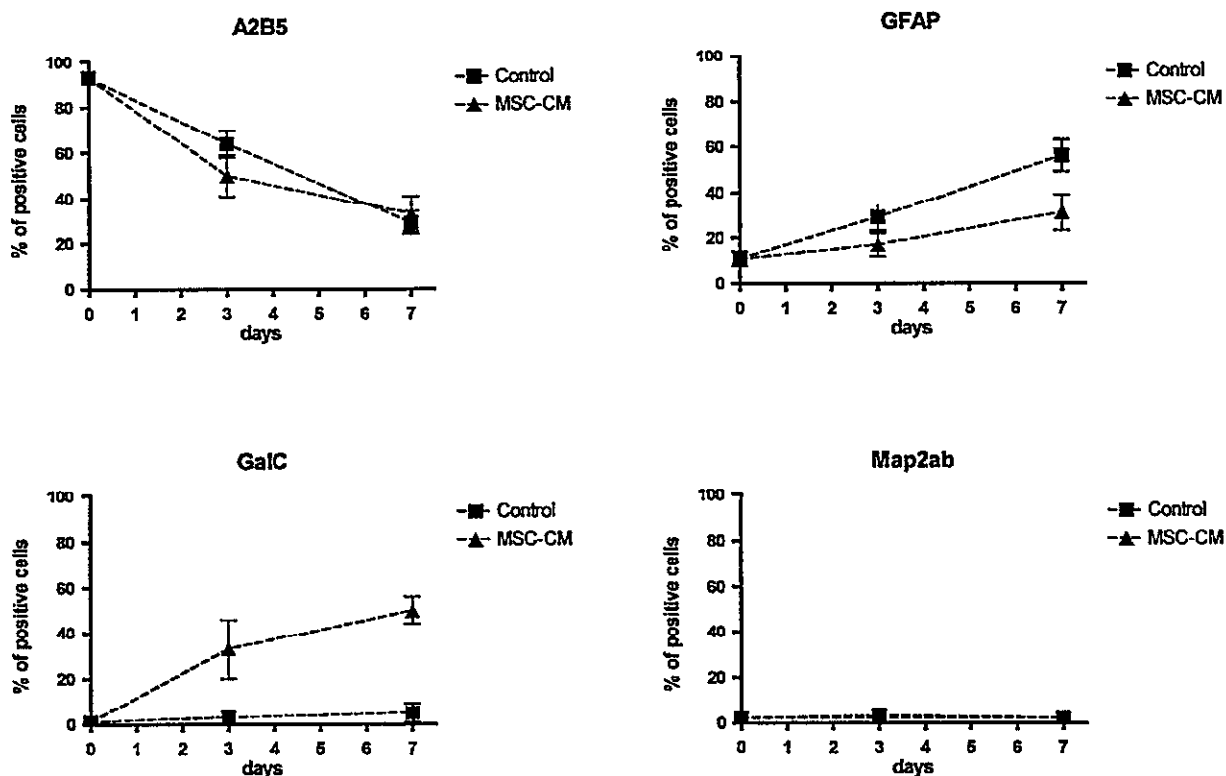
Figure 22.- Time course of expression of cell type specific markers in hNSCs treated with MSC-CM.

hNSCs were incubated in control media and MSC-CM for 7 days. Cells were fixed and stained for the different neural markers at day 0, 3 and 7. The percentage of cells expressing the different neural markers (A2B5, GFAP, GalC, and Map2ab) in hNSC cultures over time is shown. The percentage of A2B5-positive cells decreases with time in control and MSC-CM conditions, however the percentage of GalC-positive cells increases only in MSC-CM condition. The increase in the GFAP-positive cell fraction is lower in MSC-CM than in the control condition. A small percentage of hNSCs expresses the neuronal marker Map2ab, independent of the culture conditions used. Results are shown as means +/- SDs. Experiments were done in triplicate. For statistical analysis 2-way ANOVA was used.

determined for the total cell population as well as for the different cell types present in the cultures. An example of BrdU/A2B5, BrdU/GalC, BrdU/GFAP and PI/GFAP double staining is given in figure 23. The proliferation rates of most cell types were not modified by the presence of MSC-CM, as compared to control medium. This was evident for the total cell population, as well as for the A2B5-, the GalC-, and the Map2ab-positive cell subpopulation cultured for 3 or 7 days (Fig. 24 a, b). In contrast, GFAP-positive cells cultured in the presence of MSC-CM for 3 days displayed a significantly higher proliferation rate compared to the control (Student t test, $p<0.05$) (Fig. 24a). However, no significantly difference was found in the percentage of GFAP-positive cells that co-label for BrdU at day 7 (Fig. 24 b). In addition no differences in cell death rates were detected between hNSC cultures treated with control media or MSC-CM. Hence, differences were neither observed in the total cell population, nor when specific subpopulations were scrutinized (Fig. 24 c, d). In summary, the only difference observed in these experiments was the early increase in the proliferation rate of astrocytes induced by MSC-CM. Taken together, the results indicate that neither a selective proliferation nor selective death of one particular cell type could explain the oligodendrogenic effect of MSC-CM on hNSC cultures, and therefore suggest an instructive (inductive) mechanism.

To confirm the hypothesis that a proliferating progenitor population gives rise to oligodendrocytes under MSC-CM by an instructive signal, a BrdU pulse-labeling
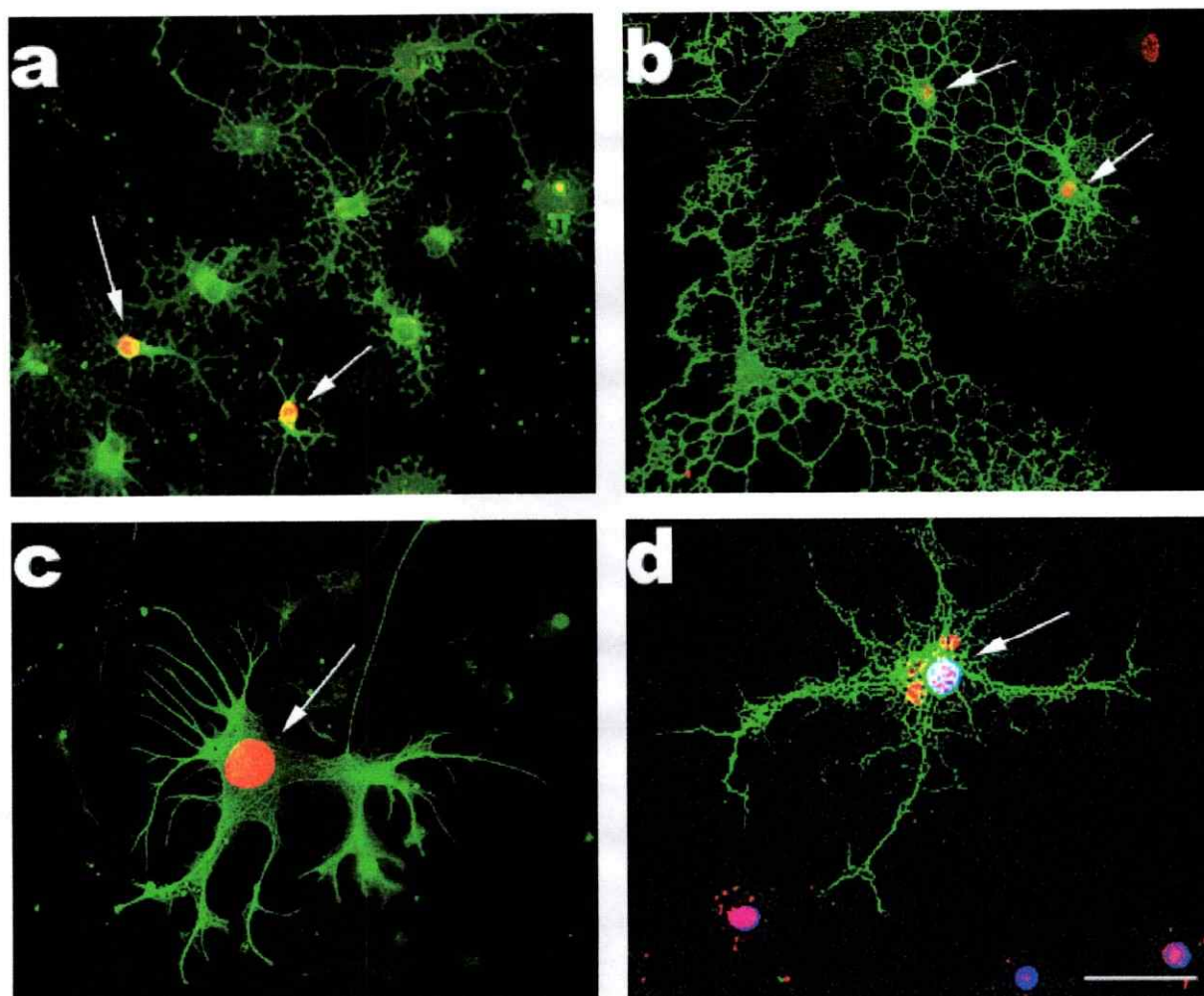
Figure 23.- Cell type specificity of proliferating and dying hNSC-derived cells after MSC-CM stimulation

The figure illustrates BrdU and PI double stained with different neural markers. hNSC were incubated under MSC-CM for up to 7 days. 10 µM BrdU was added after 2 or 6 days of incubation for nuclear incorporation. After 24 hours in contact with BrdU, cells were fixed and treated for immunoflourescence. Photomicrographs show cells co-labelled for BrdU (red) and the different neural markers (green): A2B5 (a), GalC (b) and GFAP (c). Arrows indicate BrdU incorporation in differentiating hNSCs. 50 µg/ml of PI was added after 3 or 7 days of incubation. After 10 minutes at 37°C, cells were fixed and immunostained. Dapi stained was included. Photomicrograph shows a PI (red)/GFAP (green)/Dapi (blue) triple stained cell (d). Scale bar = 50 µm.
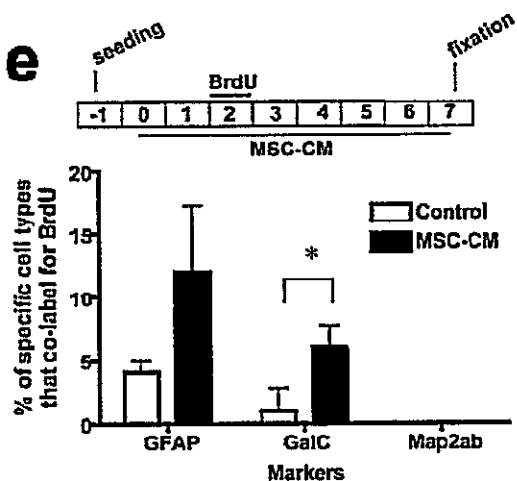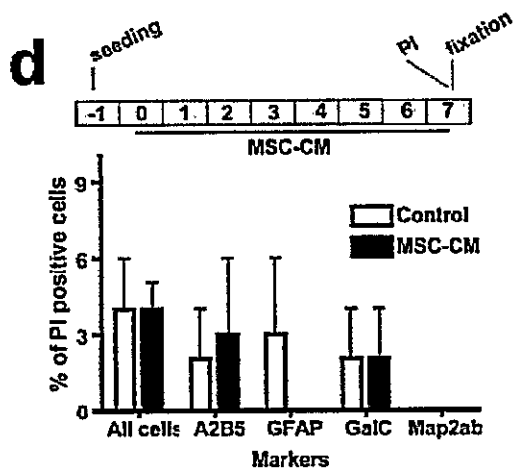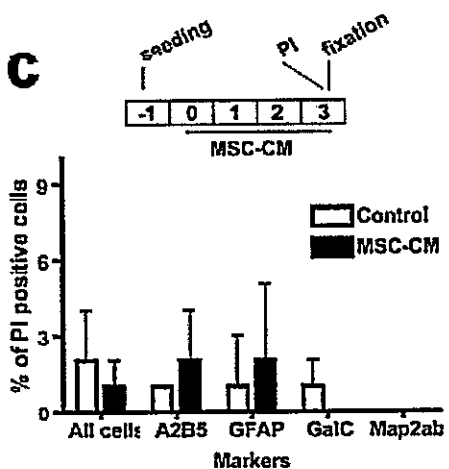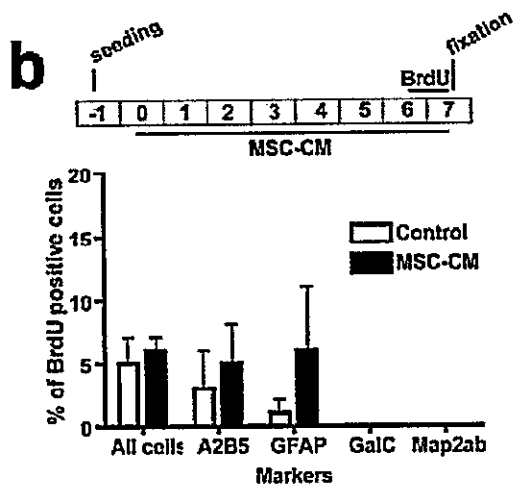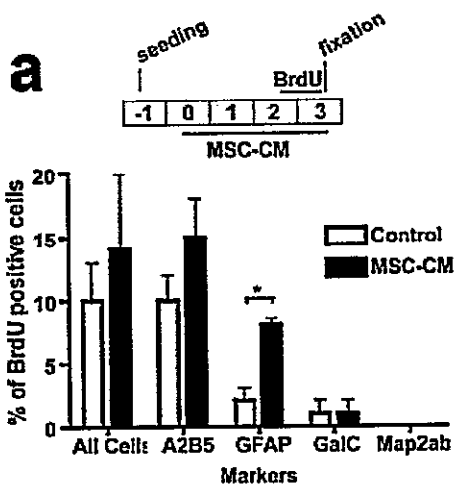
Figure 24. Effects of MSC-CM on proliferation and survival of hNSCs and on specific cell types in hNSC-cultures.

(a and b): cell proliferation: hNSCs were incubated in control medium or in MSC-CM for up to 7 days. 10 μM BrdU was added at day 2 or 6, and 24 later cells were washed and prepared for immunoflourescence analysis for BrdU and different neural markers (A2B5, GFAP, GalC and Map2ab). The percentages of BrdU-labeled cells among the total cell population, the A2B5-, the GFAP-, the GalC- and the Map2ab-expressing cells were determined. BrdU incorporation between days 2 and 3 (a) and between days 6 and 7 (b) was determined for each cell population in both experimental conditions. Note that only for GFAP-positive cells the BrdU incorporation rate day 2-3 was statisitically different between MSC-CM and control condition. (c-d) cell death: cultures were as above, but 50 μg/ml of PI was added at day 3 or 7 for 10 minutes. Cells were fixed and immunostained for different neural markers (A2B5, GFAP, GalC and Map2ab). The percentages of PI labeled cells were determined among the total cell population, the A2B5-, the GFAP-, the GalC- and the Map2ab-expressing cells. Thus, the PI incorporation at day 3 (c) and 7 (d) was determined for each cell population in both conditions. The PI incorporation rate was not different for any cell type tested. For a-d: 300-500 cells were counted for each experiment. Experiments were done in triplicate. Error bars represent SDs, statistical analysis was performed by Student's t test, * = p<0.05. (e) quantitative cell fate analysis: NSCs were incubated for 7 days under control or MSC-CM conditions. During the second day of culture a 24-hour pulse of BrdU was given. Then, cells were washed and further incubated until day 7. Cells were fixed and co-immunostained for BrdU and GFAP, GalC or Map2ab. The percentages of BrdU positive cells among GFAP or GalC or Map2ab expressing cells were determined. Only in cells that expressed GalC at day 7, the percentage that incorporated BrdU was significantly higher in MSC-CM compared to control. Experiments were done in tetraplicate. Results are shown as mean +/- SD. For statistical analysis Student's t test was performed, * = p<0.05.

experiment was performed. hNSCs were incubated for 7 days in control medium or in MSC-CM, a pulse of BrdU was given between days 2 and 3 for 24 hours, and on day 7,cells were fixed and analyzed for the presence of BrdU in the different cell populations (Fig. 24 e). The percentage of BrdU-positive cells among the total population of a specific cell type was determined. In this analysis, the total number of a specific cell type co-labeled for BrdU should represent the sum of the number of cells expressing a specific marker that proliferated between days 2 and 3, plus proliferating progenitors (mostly A2B5 expressing cells) that by day 7 expressed a cell-type specific mature marker. The percentage of GFAP-expressing cells that integrated BrdU in MSC-CM was not significantly different compared to control medium (Student t test) (Fig. 24 e). Therefore, this result, together with the fact that the proliferating rate of neural progenitors (mostly A2B5 expressing cells) was similar under both conditions tested, and that MSC-CM promotes the specific proliferation of GFAP-expressing cells (Fig 22 and 24 a), indicate that MSC-CM hinders the astrocyte instructive signal fate of hNSC (negative astrocytic cell fate instruction). Moreover, these data together suggest that MSC-CM promotes an increment over time of GFAP expressing cells (Fig. 22), mostly by a proliferative effect. In contrast, a significantly higher percentage of GalC-expressing cells co-labeled with BrdU was found in the MSC-CM-treated cultures compared to control conditions (Student t test $p<0.05$) (Fig. 24 e). Thus, MSC-CM promotes oligodendrogenic differentiation on proliferating neural progenitor cells. Therefore, since the proliferating rate of neural progenitors (mostly A2B5-expressing cells) was similar under both conditions tested (Fig. 24 a), and almost no GalC proliferating cells were detected at any time point or at any condition analyzed (Fig. 24

a, b), it is suggested that the higher percentage of cells that adopted the GalC phenotype in cultures exposed to MSC-CM (Fig. 20 and 22) is due to an induction on hNSC into an oligodendrocyte differentiation (oligodendrocytic fate instruction) triggered by MSC´s soluble factors. Finally no Map2ab-expressing cells co-labeled with BrdU were found (Fig. 24 a, b, e), suggesting that Map2ab-positive cells did not proliferate and that they were not originated from proliferating A2B5 positive cells. However, due to the very low abundance of Map2ab-expressing cells found in the culture conditions used here, no final conclusion can be drawn regarding the impact of MSC secreted factors on this cell type.

In summary, the results indicate that MSC-CM induces oligodendrogenic differentiation of A2B5-positive progenitors and promotes proliferation of GFAP-positive astrocytes.

IX. MSC-derived soluble factors induce oligodendrocyte fate decision in hNSCs by stimulating the expression of oligodendrogenic transcriptional factors and repressing an oligodendrogenic inhibitor factor

The experiments reported above demonstrated that MSC-CM induces the oligodendrogenic differentiation program in hNSCs. Fate decision of cells is controlled by extrinsic and by intrinsic determinants that result in the sequential acquisition of cell identity. Oligodendroglial determination is governed by different growth factors like;

Shh, IGF-1, IL-6 etc (Lu et al., 2000; Hsieh et al., 2004; Zhang et al., 2004b), and by different transcriptional factors like; Olig1, Olig2, Nkx2.2, etc (Zhou et al., 2000; Wegner, 2001; Zhou et al., 2001; Ross et al., 2003; Zhang et al., 2005b). The following experiments were aimed to identify the molecular pathways that mediate or participate in the MSC-CM inductive effect of hNSC oligodendrogenic determination/differentiation. Therefore, MSC-CM-treated hNSCs were analyzed for the temporal expression pattern of candidate transcription factors by quantitative RT-PCR. These included oligodendrogenic, astrogenic and neuronogenic transcription factors.

IX.1 Establishment of quantitative RT-PCR for delvelopment genes

hNSC were incubated in control or MSC-CM conditions for up to 7 days. Cells were lysed and RNA was obtained at days 0, 3 and 7. Quantitative real time RT-PCR was performed as described in the Methods section. For final quantification, a standard curve was established by amplification of the different mRNAs tested (Olig1, Olig2, Nkx2.2, Id2, Hes1, NeuroD1, Mash-1 and Pax6) using serial dilutions (1/1 to 1/64) of brain cDNA obtained from rat embryos on day 14 (E14) and postnatal on day 9 (P9). A mixed solution of both kinds of cDNA was made to ensure that it contained a reasonable amount of all the transcriptional factors tested. As an internal reference, the rRNA primer pair QuantumRNA™ Universal 18S Internal Standard (Ambion, Cambridgeshire, UK) was used. An example of mRNA quantification is illustrated in figure 25 using olig1 gene. First, a fluorescence threshold was established using the standard curve

mRNA Quantification Method (olig1)

a.
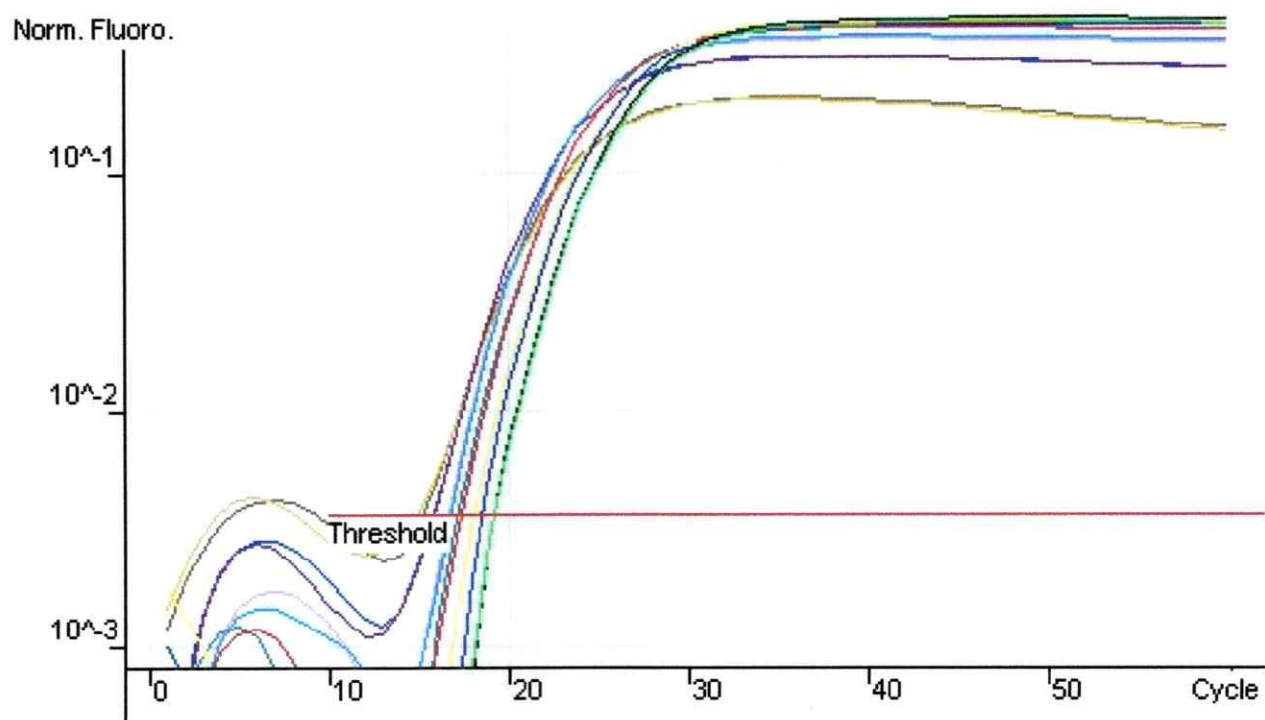
b.
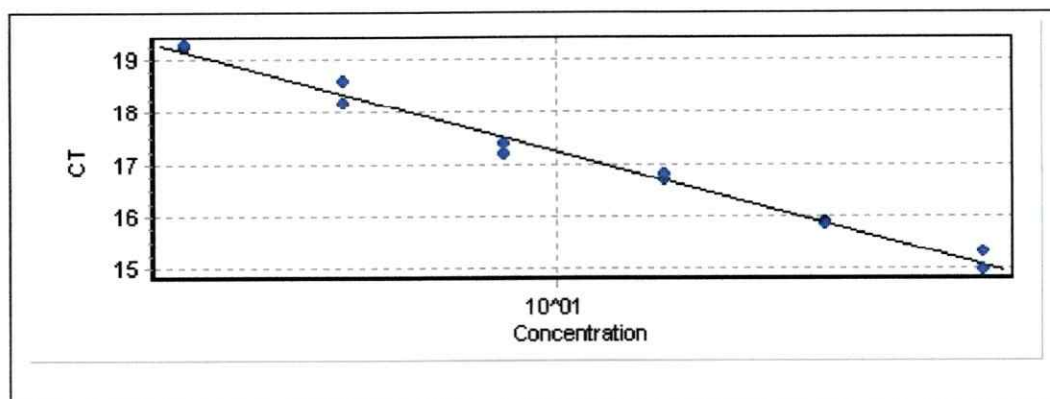
Threshold	0,0036
R Value	0,99323
Standard Curve (2)	CT = -2,720*log(conc) + 19,982

c.

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
1		Standard Olig1 64	Standard	15,33	64,000	51,367	19,7%
2		Standard Olig1 64	Standard	14,97	64,000	69,674	8,9%
3		Standard Olig1 32	Standard	15,88	32,000	32,099	0,3%
4		Standard Olig1 32	Standard	15,85	32,000	33,110	3,5%
5		Standard Olig1 16	Standard	16,69	16,000	16,229	1,4%
6		Standard Olig1 16	Standard	16,80	16,000	14,750	7,8%
7		Standard Olig1 8	Standard	17,19	8,000	10,597	32,5%
8		Standard Olig1 8	Standard	17,40	8,000	8,867	10,8%
9		Standard Olig1 4	Standard	18,16	4,000	4,660	16,5%
10		Standard Olig1 4	Standard	18,57	4,000	3,297	17,6%
11		Standard Olig1 2	Standard	19,25	2,000	1,853	7,3%
12		Standard Olig1 2	Standard	19,28	2,000	1,806	9,7%

d.

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)
1		d0a	Unknown	17,91		5,783
2		d0a	Unknown	18,07		5,039
3		d3ca	Unknown	17,71		6,836
4		d3ca	Unknown	17,94		5,614
5		d3mca	Unknown	18,08		4,986
6		d3mca	Unknown	17,83		6,201
7		d7ca	Unknown	18,74		2,856
8		d7ca	Unknown	18,87		2,558
9		d7mca	Unknown	16,98		12,744
10		d7mca	Unknown	17,00		12,477
11		NTC	NTC	31,20		,000

e.

Name	Average Conc	Olig1 * 30	rib18s	olig1/rib
d0	5,40	161,94	7,69	21,054
d0				
d3c	6,20	185,85	10,84	17,138
d3c				
d3mc	5,56	166,80	5,15	32,375
d3mc				
d7c	2,70	81,09	9,06	8,952
d7c				
d7mc	12,61	378,30	3,58	105,661
d7mc				

f.
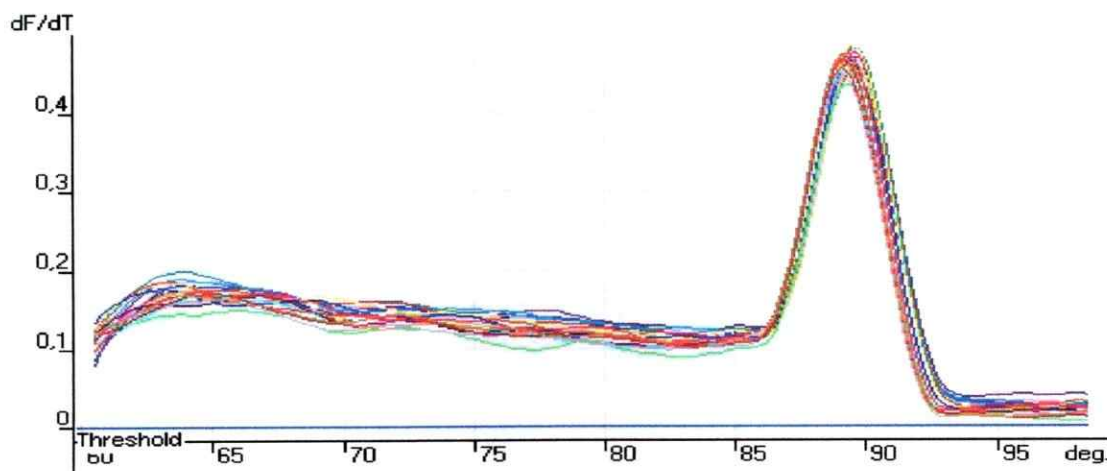
Figure 25.- Establishment of quantitative RT-PCR for development genes (Olig1 as an example).
hNSCs were cultured as described, mRNA was purified and quantitative RT-PCR for Olig1 was performed as described. A standard curve was performed by a serial dilution from 1/2 up to 1/64 with an E14 + P9 total RNA. a) Fluorescence intensity plotted against number of cycles for standards. Fluorescence increased within the time course of PCR (cycle progression) until reaching a plateau. Note that the threshold fluorescence is determined by the best linear regression. b) Linear regression plot between Logarithms of relative concentration v/s Ct. Ct is the cycle number in which the different standards have the same previously fixed threshold fluorescence, thus each standard has one Ct value. The linear equation, r and threshold parameters are given. c) Calculated relative concentration values derived from the linear regression, for the different standards. d) Ct and the calculated relative concentration values for the different samples obtained by interpolation from the standard curve. The samples are named as follows: day 0 (d0); day 3 control (d3c); day 3 MSC-CM (d3mc); day 7 control (d7c); day 7 MSC-CM (d7mc). e) Final calculation of olig1 mRNA relative concentration (considering a previous 1/30 dilution) and Olig1/rib18s ratio determination for each sample. g) Melting curve for each sample, note that there is just one peak, meaning that there is just one PCR product, confirming the absence of unspecific products.

considering the best linear regression in the plot between the logarithms of relative concentration and Ct (cycle number in which each standard has the same fluorescence threshold). Next, the samples were analyzed using the same procedure and threshold, so the concentration of each sample was determined by interpolation on the standard curve. Finally, the ratio in the same sample between the relative concentration of Olig 1 and rRNA 18s (in arbitrary units) was used for quantities and time course analysis. This was performed for all the genes analyzed.

IX.2 MSC-CM up-regulates expression of oligodendrogenic transcription factors

A list of transcriptional factors that can promote oligodendrocyte fate determination in neural stem/progenitors cells have been described, such as: Olig1, Olig2, Sox4, Sox10, Oct6, Brn-1, Brn-2, Gtx, etc (Wegner, 2001). However it has been reported that the expression of bHLH transcriptional factors Olig1 and Olig2 genes together with the homeodomain transcriptional factors Nkx2.2 play a crucial role for an oligodendrocyte cell fate (Zhou et al., 2000; Zhou et al., 2001; Fu et al., 2002; Ross et al., 2003; Zhang et al., 2005b). hNSCs were incubated under control or MSC-CM conditions for up to 7 days. At days 0, 3 and 7, the expression levels of the oligodendrogenic transcriptional factors Olig1, Olig2 and Nkx2.2 were quantified. MSC-CM induced an increase, over time, in the relative amount of mRNA for Olig1 and Olig2 compared to control conditions (ANOVA, $p<0.001$ for Olig1 and $p = 0.001$ for Olig2) (Fig. 26 a, b). After 7 days of incubation, MSC-CM induced an approximately

12-fold increase in the Olig1 mRNA (Fig. 26 a) and almost 7-fold increase in Olig2 mRNA (Fig. 26 b), as compared to the control medium. In addition, there was a significant increase in the level of Nkx2.2 mRNA in MSC-CM conditions with respect to the control ($p<0.05$) (Fig. 26 c). Taken together, these results indicate that MSC-derived soluble factors up-regulate the expression of key transcriptional factors involved in oligodendrogenic differentiation programs in hNSCs.

IX.3 MSC-CM down-regulates the oligodendrogenic inhibitor ID2

Cell fate determination is controlled by the composite arrangement of promoting and inhibitory factors. Therefore, up-regulation of specific oligodendrogenic transcriptional factors may not be sufficient for cell fate commitment into oligodendrocyte. Indeed, the presence of an inhibitory system constituted of proteins inhibiting the activity of Olig1 and Olig2 genes has been described (Wang et al., 2001; Samanta and Kessler, 2004). The presence of this system promotes astrocytic differentiation of neural progenitor cells. It was recently described that the inhibitor of differentiation 2 (Id2) could sequester Olig proteins, thereby preventing them from entering the nucleus and thus inhibiting their activity (Samanta and Kessler, 2004). Therefore, the expression level of Id2 in MSC-CM treated hNSCs was analyzed in the present study. The highest levels of Id2 mRNA were detected on day 0 of MSC-CM or control treatment (Fig. 26 d). In addtition, MSC-CM induces a higher decrease, over time, in the levels of Id2 mRNA compared with control conditions (ANOVA, $p<0.005$)
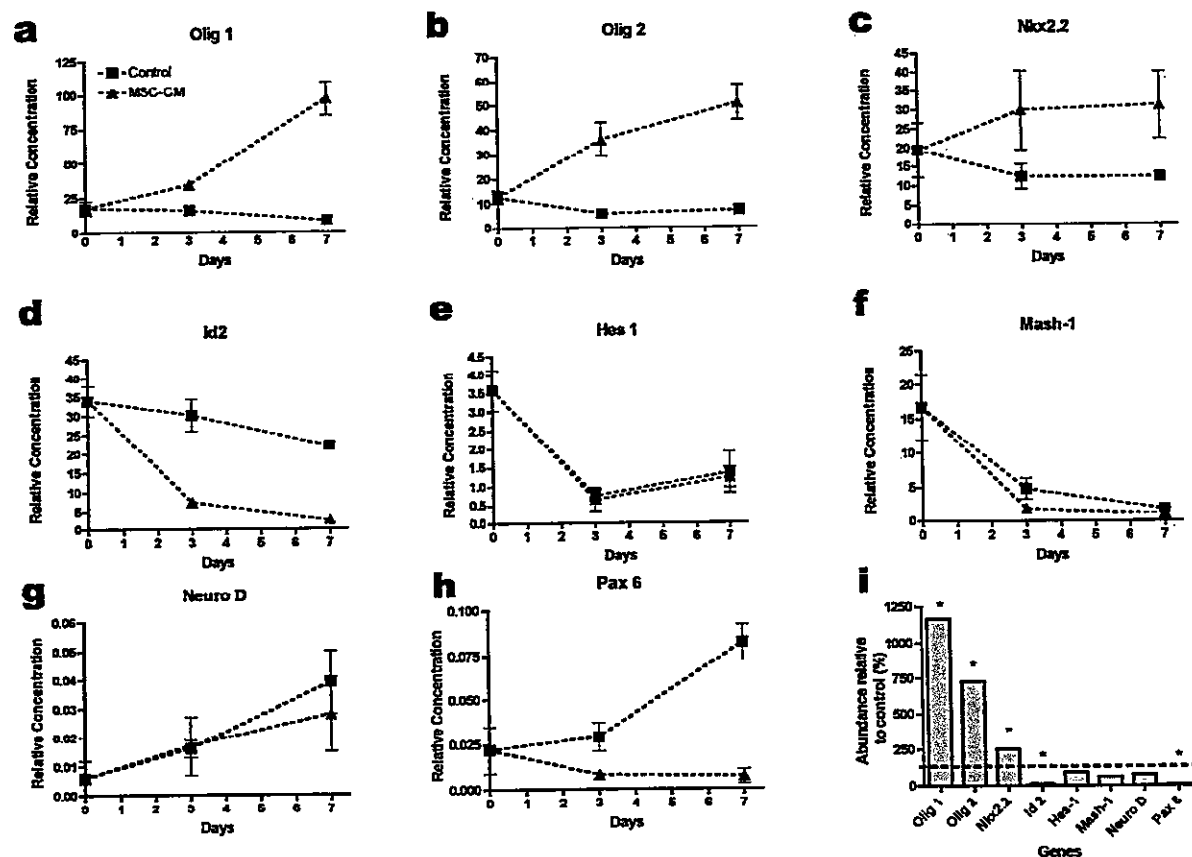
Figure 26.- Temporal expression pattern of neural cell fate determinants in hNSCs after stimulation with MSC-CM.

hNSCs were incubated in control medium or in MSC-CM for 0, 3 and 7 days. mRNA was prepared and quantitative RT-PCR was performed. Data are expressed as relative mRNA concentrations with respect to a standard dilution curve versus time for Olig1 (a), Olig2 (b), Nkx2.2 (c), Id2 (d), Hes1 (e), Mash1 (f), NeuroD1 (g) and Pax6 (h). Note the increase in the relative amount of mRNA for Olig1, Olig2 over time. The levels of Nkx2.2 mRNA were significantly higher in MSC-CM with respect to the control condition. The levels of Id2 mRNA were significantly decreased in MSC-CM with respect to control conditions over time. Levels of Hes1 and Mash-1 mRNA significantly decreased over time in both conditions with no differences between conditions. NeuroD1 levels increased significantly over time in both conditions similarly. Lower levels of Pax6 mRNA in MSC-CM compared to control medium were found over time. Experiments were performed in triplicate. Data represent means +/- SD. 2-way ANOVA was used for statistical analysis. (i) The relative amount of mRNA of 7 days of exposure to MSC-CM versus control medium is shown for each gene, * = significant increase or decrease compared to control, p<0.05.

(Fig. 26 d). After 7 days of incubation, cultures exposed to MSC-soluble factors contained 10 times less Id2 mRNA as compared to those in control medium (Fig. 26 d). Therefore, the MSC-CM induction in oligodendrogenic determination involved transcriptional down-regulation of the Id2 inhibitor of oligodendrogenic differentiation.

IX.4 MSC-CM induced oligodendrogenic effect is associated with down-regulation of Hes1, but it is not a specific effect

A candidate family of transcription factors, the Hes genes, have been reported to act as inhibitors of differentiation into the neuronal lineage. The hes gene family encodes for transcriptional factors able to block neurogenesis and promote astrocytic differentiation (Ross et al., 2003). One of these genes, Hes1, was reported to participate in inhibition of neuronal differentiation of neural progenitor cells and of pheochromocytoma PC12 cells (Ishibashi et al., 1994; Strom et al., 1997; Nakamura et al., 2000; Ross et al., 2003; Ishibashi, 2004). Similarly to the Id2 mRNA, the highest levels of Hes1 mRNA were detected on day 0 (Fig. 26 e). Thereafter, the levels of Hes1 mRNA significantly decreased over the next 7 days (ANOVA, $p < 0.001$) (Fig. 26 e). However, no significant differences could be detected between the Hes1 mRNA levels measured in the hNSC cultures maintained in control and those in MSC-CM (Fig. 26 e). Therefore, the decrease in Hes1 expression was associated with the oligodendrogenic effect induced by MSC-CM, but is not specific for MSC-derived soluble factors.

IX.5 MSC-CM- induced oligodendrogenic fate is associated with changes in

expression of neurogenic transcription factors

To monitor the neuronal inductive transcriptional factors, we analyzed the kinetics

of Mash-1, NeuroD1 and Pax-6 mRNA expression. These factors are involved at

different levels of the neuronal determination and differentiation program (Lee et al.,

1995; Miyata et al., 1999; Nieto et al., 2001; Osumi, 2001; Heins et al., 2002; Ross et

al., 2003). The highest level of Mash-1 mRNA was detected on day 0 (Fig. 26 f).

Although the levels of Mash-1 mRNA significantly decreased over time (ANOVA, p =

0.001) (Fig. 26 f), no difference in Mash-1 mRNA levels could be detected after 7 days

of culture in MSC-CM, as compared to control medium (Fig. 26 f). With respect to the

bHLH transcriptional factor, NeuroD1, only very low levels of mRNA could be detected

(Fig. 26 g). Nevertheless, the amount of NeuroD1 mRNA increased significantly over 7

days in culture (p<0.05) (Fig. 26 g), but no differences were observed between the two

culture conditions (Fig. 26 g). Similarly, very low levels of pax6 mRNA could be

detected (Fig. 26 h). hNSCs exposed to MSC-secreted soluble factors had significantly

lower pax6 mRNA levels over time, as compared to the levels measured under control

conditions (p<0.005) (Fig. 26 h). Therefore, soluble factors present in the MSC-CM did

not specifically increase the expression of neuronal fate-related transcription factors in

hNSC, suggesting that they are not participating specifically instructing the hNSC

towards a neuronal cell fate.

In summary, MSC-CM-induced oligodendrocyte differentiation in hNSCs is associated with changes in the expression pattern of a number of cell fate transcription factors. MSC-CM stimulates the expression of the oligodendrogenic transcriptional factors Olig1, Olig2 and Nkx2.2 and represses the expression of the Id2 oligodendrogenic inhibitor factor (Fig. 28 i). This oligodendrogenic tendency is associated to a repression in the expression of the astrogenic transcriptional factor Hes1. Finally, the changes in the expression of the neurogenic transcriptional factors NeuroD1, Mash1 and Pax6, are suggestive of a lack of participation of MSC-derived soluble factors specifically instructing hNSC into a neuronal fate (Fig. 28 i).

X. MSC-CM Characterization: Analysis of the activity

With the aim to analyze the activity of MSC-CM and to characterize the putative oligodendrogenic factors that are released by MSC, two different approaches were performed: 1) a biochemical approach; 2) an oligodendrogenic candidate approach.

X. 1 Biochemical approach: The oligodendrogenic activity present in MSC-CM is partially heat-sensitive

In order to characterize the nature of the oligodendrogenic activity present in MSC-CM, the activity was tested for its heat-sensitivity. Therefore, MSC-CM was treated for 10 minutes in boiling water and tested for its ologidendrogenic activity on

hNSCs. After hNSC incubation for 7 days in MSC-CM and heated-inactivated MSC-CM, the expression of different markers was analyzed by immunocytochemistry. A lower percentage of GalC positive cells was detected under heat-inactivated MSC-CM (20.4 +/- 6.9%) in comparison to untreated MSC-CM (50 +/- 6%) (ANOVA-Tukey Post Hoc, $p < 0.001$, experiment done in triplicate) (Fig. 27). In addition to this, a lower percentage of MBP-positive cells was found when hNSCs were incubated in heat-inactivated MSC-CM (6.2 +/- 0.9%), when compared to untreated MSC-CM (37+/- 7.3%) ($p < 0.01$) (Fig. 27). However, in comparison to control conditions, where only 5 +/- 4% of cells expressed GalC, the percentage of GalC-expressing cells was still higher when cells were stimulated with heat–inactivated MSC-CM ($p < 0.005$) (Fig. 27) indicating that the heat-treatment did not completely destroy the activities. There was no difference in the percentage of MBP-positive cells between heat-inactivated MSC-CM and control conditions (Fig. 27).

With respect to other neural lineage markers, there were no significant differences between heat-inactivated and untreated MSC-CM, since the percentages of GFAP and of Map2ab-positive cells were comparable under both conditions (Fig. 27).

In summary, these experiments demonstrate that the oligodendrogenic activity(ies) can be partially inactivated by heat-treatment, indicating the partial heat-sensitivity. It would be of interest to test if a more robust heat-treatment would further
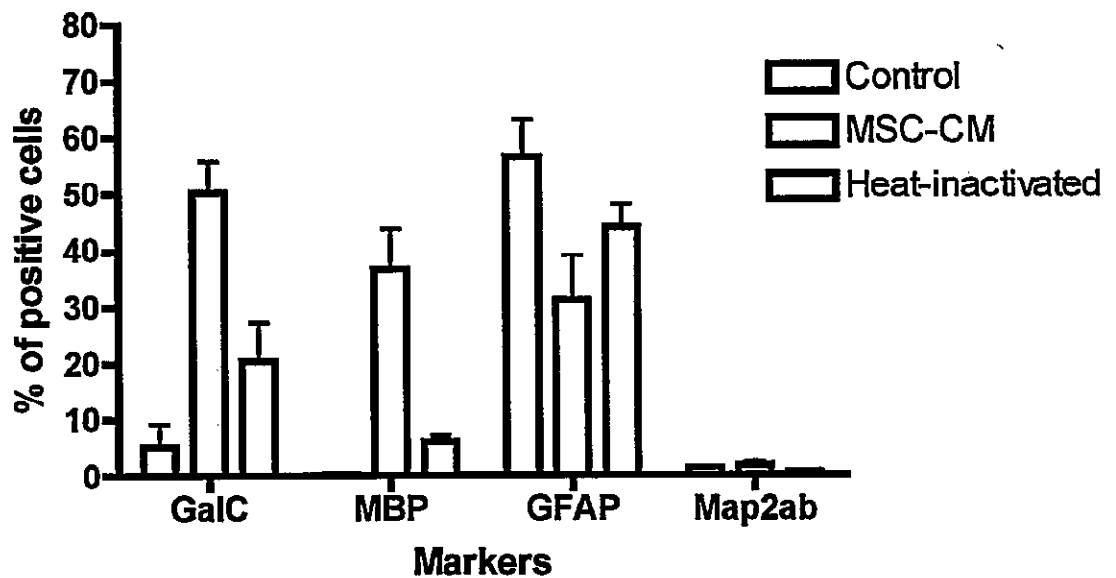
Figure 27.- Effect of heat inactivation on MSC-CM .
hNSC were incubated up to 7 days under control medium, untreated MSC-CM and heat-inactivated MSC-CM. Heat-inactivation was achieved by placing the MSC-CM for 10 minutes in boiling water. After incubation, cells were fixed and immunostained against GFAP, GalC, MBP and Map2ab. Quantification was performed as described in Methods section. Heat-inactivated MSC-CM-treated cells show a lower proportion of GalC and MBP positive cells in comparison with untreated MSC-CM. The experiment was performed in triplicate. Error bars represent SDs and 1-way ANOVA-Tukey Post Hoc was used for statistical analysis.

inactivate the factor(s). Moreover, it remains to be tested if the oligodendrogenic activity

is derived from a single factor, or if more than one factor contribute to the activity.

X.2 Oligodendrogenic candidate approach: IGF-1 is not sufficient to explain the MSC-soluble factors' oligodendrogenic effect observed in the neural precursors

MSCs express and secrete a number of different cytokines. These include

interleukins: 1α, 6, 7, 8, 11, 12, 14 and 15; leukaemia inhibitory factor (LIF); stem cell

factor (SCF); etc (Minguell et al., 2001). During development, a list of growth factors

act in differentiation, maturation and migration of oligodedrocyte precursors, such as

neurogulin-1 (NRG), platelet-derived growth factor (PDGF), FGF-2, ciliary

neurotrophic factor (CNTF), Shh, IL-6 (Lu et al., 2000; Miller, 2002; Liu and Rao,

2004; Zhang et al., 2004b). Recently, insulin-like growth factor-1 (IGF-1) was reported

to induce adult neural progenitor cells to differentiate into oligodendrocytes (Hsieh et

al., 2004). IGF-1 is to date the only potent oligodendrogenic growth factor described for

adult hippocampal neural progenitor cells. Similarly, IGF-1 was also described as a

potent oligodendrogenic factor for O2A bipotent precursors, whereas IGF binding

proteins 1, 2 and 6 (IGFBP-1, 2, 6) could inhibit this activity (Kuhl et al., 2002; Kuhl et

al., 2003; Hsieh et al., 2004). Finally, it was described that MSC can produce and secrete

IGF-1 (Abboud et al., 1991). These data make IGF-1 a good candidate to explain the

MSC-CM oligodendrogenic effect observed on hNSC.

To test whether the oligodenrogenic effect of MSC-CM on hNSCs is mediated by IGF-1, i) MSCs were tested for expression of IGF-1 mRNA by RT-PCR and ii) specific inhibitors of IGF-I (IGF-binding proteins) were employed in the experiments. MSCs expressed IGF-1 mRNA as demonstrated by RT-PCR (Fig. 28), supporting that IGF-1 might be a candidate factor for the oligodendrogenic activity present in MSC-CM. Next, IGFBP-1 was used in hNSC cultures that were treated with MSC-CM. Briefly, hNSC were incubated for 7 days in control and MSC-CM conditions in the presence or absence of either IGF-1, IGFBP-1, or both. Expression of the neural lineage markers (GFAP, Map2ab, GalC and MBP) was then assessed by immunocytochemistry. In cultures grown under control conditions, no significant differences in the expression pattern of neural lineage markers were detected with or without IGF-1, IGFBP-1 or both (Fig. 29 a, ANOVA, experiments were done in triplicate). Similarly, the presence of IGF-1, IGFBP-1 or both did not influence the expression pattern of neural markers MSC-CM treated hNSCs (Fig. 29 b, ANOVA). These observations clearly suggest that IGF-1 is neither sufficient nor necessary to explain the oligodendrogenic effect present in the MSC-CM. In contrast, these results support the hypothesis that there might be one or more yet undescribed oligodendrogenic activity(ies) derived from MSCs. However there are more candidates to test from the oligodendrogenic factors before described, like IL-6, which is an oligodendrogenic factor in embryonic stem cells that can be produced and secreted by MSC (Minguell et al., 2001).
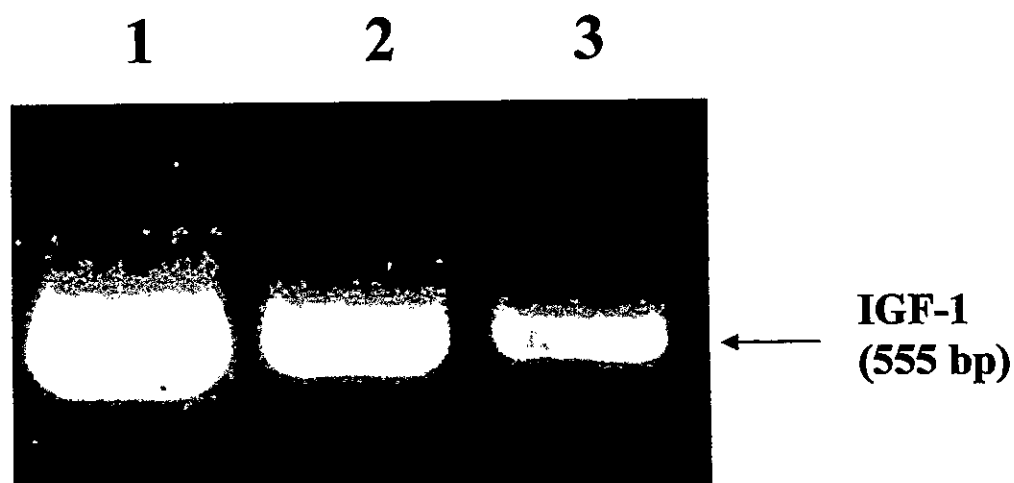
Figure 28.- IGF-1 mRNA expression in MSC.
RT-PCR analysis of IGF-1 mRNA (555 bp) expression in liver (lane 1), pancreas (lane 2), both as positive controls, and MSC incubated in α -MEM containing 10 % FBS (lane 3). MSCs express IGF-1 mRNA.

Control Medium
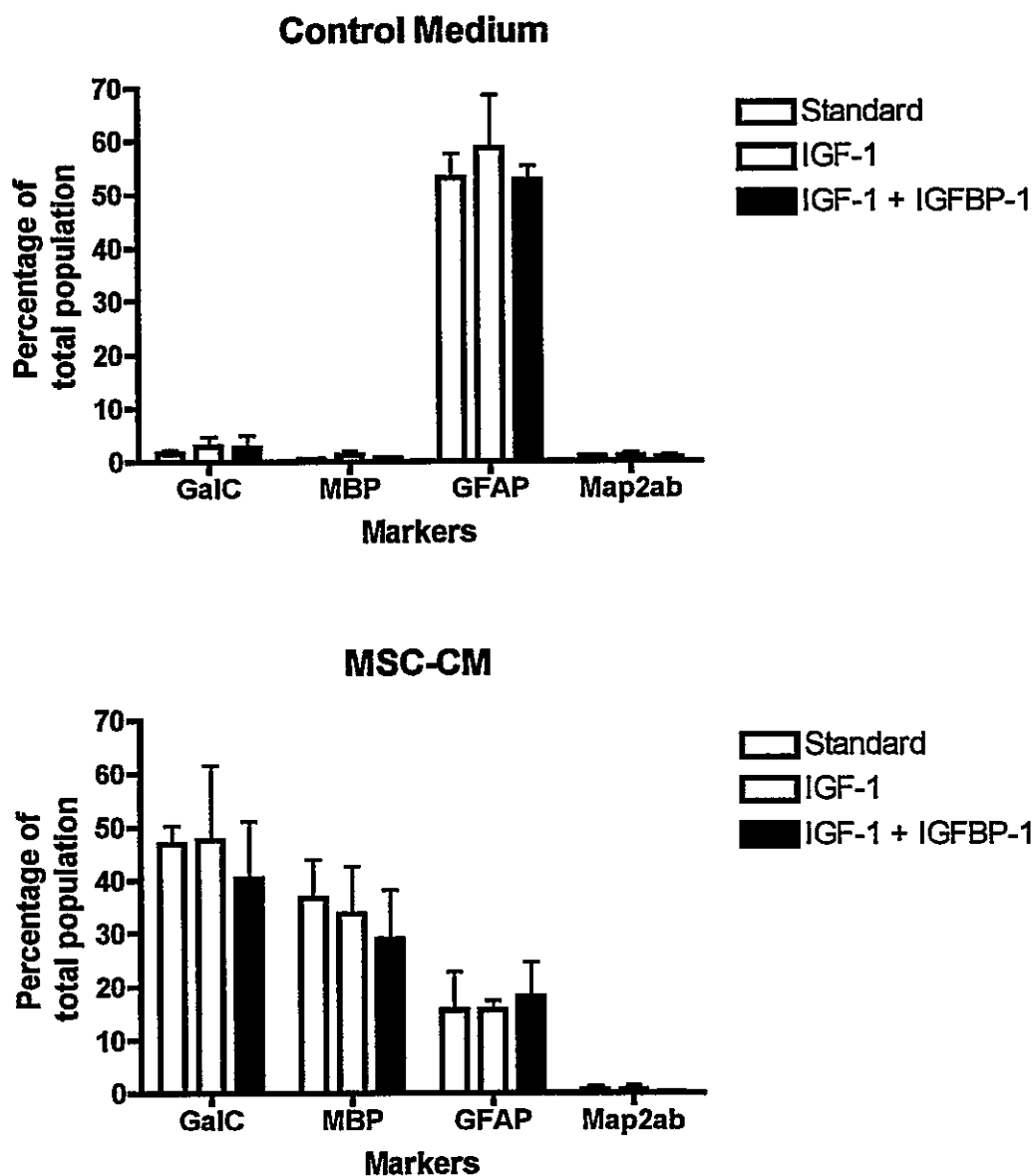
MSC-CM

Figure 29.- IGF-1 effects on neural progenitor differentiation.
Percentage of cells expressing different markers after incubation for 7 days with or without IGF-1 and/or IGFBP-1 in control media (a) and MSC-CM condition (b). Note that the presence of IGF-1, IGFBP-1 and IGF-1/IGFBP-1 did not affect the cell type composition of hNSC cultures, independently of control or MSC-CM conditions. 300-500 cells were counted in different fields for each experiment. Experiments were done in triplicate. Results are shown as means +/- SD. 1-way ANOVA was used for statistical analysis.

XI. Attached results

XI. 1 Characterization of hNSC from earlier passages

hNSC neurospheres from passage 1 were dissociated, seeded and cultured on a poly-ornithine/laminin matrix incubated in α-MEM 10%FBS. After 12-24 hours, cells were fixed for immunofluorescence analysis. Results were compared with those obtained from hNSC passage 4. Under these conditions, cultures from passage 1 have more cells expressing mature markers like: GalC (21% passage 1 v/s 6% passage 4) and GFAP (12% passage 1 v/s 1% passage 4). In addition to this, cultures from passage 1 have a lower proportion of A2B5-positive cells (44, 4% passage 1 v/s 94% passage 4) while the proportion of nestin-positive cells remain almost the same (approximately 20% in both passages). Therefore, this indicates that older cultures are richer in neural progenitors and have less mature neural cells.

XI. 2 In vitro, β–III tubulin is not a high fidelity marker for neuronal precursors

In the MSC-CM conditions, we observed a significantly higher percentage of β-III tubulin-expressing cells (30,8 %) as compared to control conditions (6,0%) (Student *t* test, $p<0.01$). However, there were 43.8% of β-III tubulin-expressing cells that concomitantly expressed GalC (Fig. 30 and Table 5). In addition to this, under control

conditions there were 35.7% of β-III tubulin-expressing cells that also expressed GFAP (Table 5). This result suggests that β-III tubulin is not a high fidelity neuronal-commited marker. To corroborate this hypothesis, the expression of the same marker protein using a different monoclonal antibody (Tuj1) was analyzed. Under MSC-CM conditions, we found 49.8% of Tuj1-expressing cells that concomitantly expressed GalC (Fig. 30 and Table 5) and under control conditions, 53% of Tuj1-expressing cells expressed GFAP as well (Table 5). Therefore, the results indicate that in the presently used in vitro culture condition, β-III tubulin was not a specific marker for neuronal precursors and neuronal commitment.
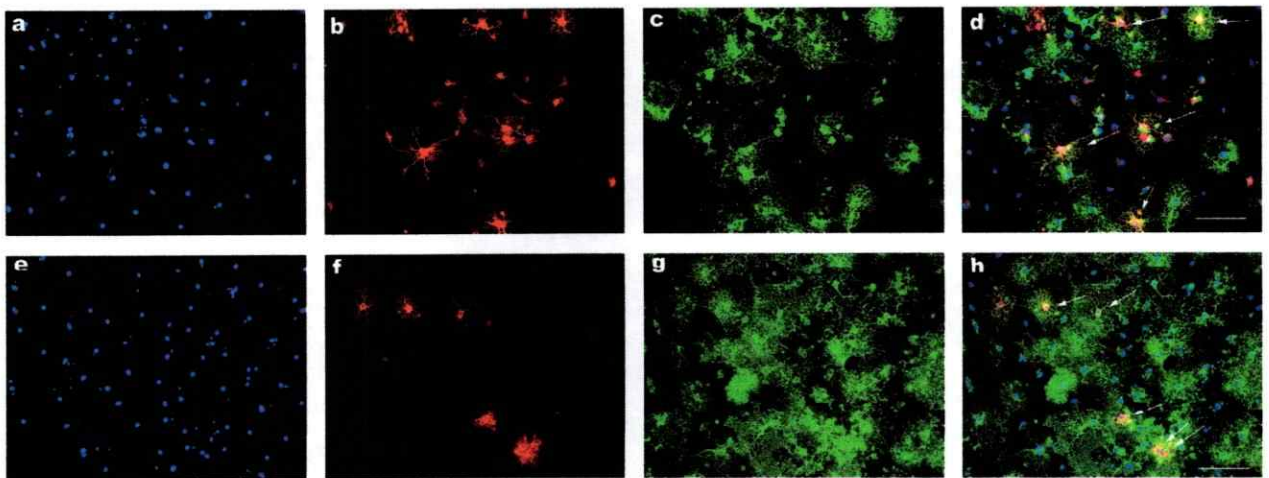
Figure 30.- Co-expression of GalC and β–IIItubulin in hNSCs.

hNSC were incubated under MSC-CM conditions for 7 days. After incubation, cells were fixed and double immunostained against β–III tubulin/GalC and Tuj1/GalC. Fluorescence images of: Dapi (a, e); β–III tubulin (b); Tuj1 (f); GalC (c, g) and merge (d, h). The figure illustrates the presence of GalC/β–III tubulin and GalC/Tuj1 double positive cells (arrows). Scale bar = 100 μm.

Table 5.- Quantitative analysis for neural markers co-expression in hNSCs under different conditions[1]

Type of double positive cells	Conditions	Percentage
βIIItubulin-GalC / βIIItubulin	MSC-CM	43.8 +/- 3.9
Tuj1-GalC/ Tuj1	MSC-CM	49.8 +/- 0.3
βIIItubulin-GFAP / βIIItubulin	Control	35.7 +/- 6.2
Tuj1-GFAP/ Tuj1	Control	53 +/- 4.2

[1]hNSCs were grown and treated as described in figure 32. The table shows the percentage of: β–III tubulin-positive cells that co-label for GalC, β–III tubulin-positive cells that co-label for GFAP, Tuj1 positive cells that co-label for GalC and Tuj1 positive cells that co-label for GFAP. Experiments were done in triplicate. Results are given as means +/- SDs.

DISCUSSION

This thesis focused on bidirectional effects between adult bone marrow-derived mesenchymal stem/progenitor cells (MSCs) and the adult hippocampus as a foreign micro-environment (neural stem cell niche), mediated by the soluble factors. These studies demonstrate that soluble factors derived from hippocampus induce a neuronal-like morphology and the expression of neuronal markers in adult MSCs, while soluble factors produced by MSCs were found to strongly induce hippocampal neural progenitor cells (hNSCs) to adopt an oligodendrocyte phenotype (Fig. 32). Therefore, there are bidirectional activities between one population of stem/progenitor cells and a foreign niche influencing the biology of each other, namely the paracrine activity of the bone marrow-derived mesenchymal progenitor cells and the neural progenitors isolated from the hippocampus.

Over the recent years, several studies have focused on the analysis of the transdifferentiation potential of stem/progenitor cells from different adult organs or tissues (Abboud et al., 1991; Eglitis and Mezey, 1997; Bjornson et al., 1999; Kopen et al., 1999; Brazelton et al., 2000; Cheng et al., 2000; Mezey et al., 2000; Woodbury et al., 2000; Deng et al., 2001; Nakano et al., 2001; Woodbury et al., 2002; Munoz-Elias et al., 2003; Munoz-Elias et al., 2004). *In vivo* studies have demonstrated that after adult stem/progenitor cells transplantation into a foreign micro-environment, trans-differentiation occurs (Eglitis and Mezey, 1997; Bjornson et al., 1999; Kopen et al.,

1999; Brazelton et al., 2000; Mezey et al., 2000; Nakano et al., 2001; Munoz-Elias et al., 2004). Therefore, the interaction with a foreign micro-environment or niche exerts an influence on the stem/progenitor cell fate determination. In addition, *in vivo* studies suggest that transplanted stem/progenitor cells promote and induce endogenous regeneration of previously damaged foreign micro-environment (Hofstetter et al., 2002; Zhang et al., 2004a; Lu et al., 2005; Neuhuber et al., 2005; Zhang et al., 2005a). However, the influence of the transplanted stem/progenitor cells onto the foreign niche had not been studied in depth. In addition to this, since soluble factors play a central role in cellular signaling, modulating for example proliferation, cell fate, differentiation, survival, it is relevant to study the interactions between stem/progenitor cell and a foreign niche mediated by soluble factors.

I. Trans-differentiation of MSCs: wishful thinking?

Although there are numerous studies supporting *in vivo* the trans-differentiation phenomenon (Eglitis and Mezey, 1997; Ferrari et al., 1998; Bittner et al., 1999; Petersen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Nakano et al., 2001; Orlic et al., 2001; Priller et al., 2001), other *in vivo* studies reveal that after bone marrow transplantation, cell fusion between transplanted stem/progenitors cells with tissue mature differentiated cells can occur, and thus the evidence has been misinterpreted as a trans-differentiation phenomenon (Terada et al., 2002; Alvarez-Dolado et al., 2003). For

this reason, it is necessary to check the diploid condition of differentiated stem cells after transplantation.

The present work demonstrates that MSCs isolated from adult bone marrow stroma, can adopt a neuronal-like phenotype when stimulated with beta-mercaptoethanol or when exposed to soluble factors released by the neural environment (hippocampus). A crucial question is whether the results indicate a neuronal trans-differentiation phenomenon or not. Moreover, in addition to trans-differentiation, the results can be also explained by a de-differentiation phenomenon. De-differentiation describes the process by which a differentiated, organ-specific cell looses its differentiation markers and acquires characteristics of a somatic stem cell from the same organ. Therefore, a differentiated cell can de-differentiate into an undifferentiated stem cell, able to originate different mature cells. The possibility that a differentiated cell can, first, de-differentiate and later trans-differentiate into a mature cell from a different lineage, cannot be excluded.

Both trans-differentiation and de-differentiation, have some requirements to be demonstrated *in vitro*. Different research groups have focused on searching for physiological inductors for neural differentiation on MSC. With that aim, the studies found culture conditions that allow neurosphere formation from bone marrow MSC and the subsequent differentiation into the neural lineages (Wislet-Gendebien et al., 2003; Hermann et al., 2004; Wislet-Gendebien et al., 2005). The investigators interpreted the

results as a MSC *in vitro* trans-differentiation process. However, they did not do a clonal analysis for mesenchymal and neural lineages; therefore they certainly cannot rule out that they are isolating neural progenitors from the bone marrow as opposed as actually witnessing a trans-differentiation phenomenon. The latter is supported by a study that described that a minor nestin-positive subpopulation in MSC culture can form nerospheres, while nestin-negative cells cannot (Wislet-Gendebien et al., 2003; Wislet-Gendebien et al., 2005). These neurospheres, when co-cultured with neurons or neuroprogenitors, can be induced to form mature neural cells, such as neurons and astrocytes (Wislet-Gendebien et al., 2003; Wislet-Gendebien et al., 2005). In this thesis it cannot be concluded that MSC suffered a trans-differentiation and/or de-differentiation into neural lineages because experiments such as clonal analysis were not performed. Moreover, the expression of early neuronal markers was observed under control conditions (without differentiation-inducing stimuli), indicating the existence of subpopulations of MSC able to express neuronal proteins. These observations suggest the possibility that the neuronal-like induction mediated by hippocampal soluble factors on MSC, could be acting on a subpopulation of neuronal committed progenitors that already exist in the bone marrow. Therefore, the possibility of a neural induction on a selective bone marrow subpopulation of neural progenitors can not be excluded.

Regardless of the underlying mechanism (whether it is trans-differentiation, de-differentiation, selective neuronal induction or not), hippocampal soluble factors promote a neuronal-like phenotype on MSC culture. But the question is whether the

neuronal-like phenotype meets the criteria to be considered a "real" neuron or not. For "true" neuronal trans-differentiation, one would ask the cells to fulfill certain requirements: i) cells should express genes and proteins specific for neurons, ii) cells should demonstrate morphological characteristics of neurons, iii) cells should have sub-cellular structures characteristic for neurons including an axon, dendrites and synapses, and iv) cells should demonstrate functional neuronal characteristics of neurons, in particular in electrophysiological experiments.

In these studies, it was shown that both hippocampal soluble factors and BME promote the expression of genes and proteins specific for neurons, displaying a bipolar or multipolar morphology, thus suggesting the appearance of the neuronal-like phenotype on MSCs. First, BME promotes a rapid acquisition of neuronal-like phenotype on MSC. In previous studies it was demonstrated that the induction of neural genes in MSC could be achieved through stimuli with non-physiological substances (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Deng et al., 2001; Woodbury et al., 2002; Munoz-Elias et al., 2003; Padovan et al., 2003; Rismanchi et al., 2003). The criteria to assess the neural differentiation 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, recent studies have recommended caution in the interpretation of results assessing the neural differentiation properties induced by non-physiological compounds, since a disruption of the actin cytoskeleton may facilitate the outcome of neurite-resembling processes (Neuhuber et al., 2004). When cells were

treated with cytocalasin-D or latrunculin-A (to disrupt F-actin network), a neuronal-like morphology was acquired, similar to that obtained with neuronal induction media. In addition to this, some of the non-physiological substances used to induce neuronal differentiation in MSCs have negative side-effects. For example, valproic acid has been demonstrated to induce changes in the chromatin structure, and butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have cytotoxic and apoptotic effects (Saito et al., 2003; Marchion et al., 2005). Moreover, in a study by Lu and coworkers, it was 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 regulated steps in a complicated cellular differentiation process (Lu et al., 2004). Furthermore, by comparing a pool of different lineage gene expression profiles (21,000 genes) it was demonstrated that gene transcription was not affected when MSCs were exposed to these non-physiological compounds (Bertani et al., 2005). In addition, the gene expression profile of MSC and an unrelated non-neural organ, such as liver, treated with these compounds was compared. The treatment was no more effective in redirecting MSC toward a neural phenotype than toward an endodermal hepatic pathway (Bertani et al., 2005). In addition, almost no studies have reported electrophysiological properties on MSC neuronal–like phenotype. Finally, to avoid misleading effects of non-physiological compounds, studies should focus in the research of physiological inductors for MSCs neural differentiation, which was an important aim in the present work. In conclusion, since BME is a non-physiological compound, the

induction of the neural-like phenotype on MSCs by BME, observed in this study, cannot be conclusively considered a trans-differentiation phenomenon.

When comparing non-physiological compounds with hippocampal soluble factors on neuronal-like induction on MSC, there are important differences: 1) Upon exposure to hippocampal soluble factors, MSC adopted the neuronal-like phenotype in 2 to 3 days, taking more time than using non-physiological compounds (5 hours). Thus, the hippocampal soluble factors' induction is more likely a physiological process than an aleatory toxic effect, as was shown for non-physiological compounds induction. 2) The survival and proliferation was enhanced under hippocampal soluble factors condition, while using non-physiologycal compounds did not have this effect. These observations suggest that hippocampal soluble factors neuronal-like induction on MSCs is not an artifact as was suggested for non-physiological compounds induction. However, although hippocampal soluble factors induce the expression of NF-H and GAP-43, the mature neuronal marker Map2ab was not observed. Nevertheless, *in vivo* studies have shown that the expression of Map2ab can be observed after MSC transplantation in adult injured and fetal brain (Li et al., 2000; Munoz-Elias et al., 2004), suggesting that the interaction with extracellular matrix and/or the direct interaction with neural cells could be necessary for the expression of mature neural markers and is not impossible for MSCs. Finally, 3) under hippocampal soluble factors, MSCs displayed no identifiable axon or dendrites on their bipolar or multipolar morphology. In conclusion, the induction of the neural-like phenotype on MSCs by hippocampal soluble factors needs

more studies to confirm whether it is a trans-differentiation phenomenon or not. MSCs can be induced to express a neuronal-like phenotype; however trans-differentiation or de-differentiation are not fully demonstrated.

II. The adult hippocampus: a region with signals promoting neuronal differentiation

This thesis demonstrates that soluble factors derived from different brain tissues (physiological compounds) induce a neuronal-like morphology and the expression of neuronal markers in adult MSCs. In comparison with cerebellum and cortex, hippocampus-derived conditioned medium was most effective in promoting the neuronal-like phenotype in MSCs. The adult hippocampus, contrary to cerebellum and cortex, is a brain region with ongoing neurogenesis even in the adult (Kuhn et al., 1996). For example, hippocampal astrocytes release soluble factors that induce neuronal differentiation of adult neural progenitor cells (Song et al., 2002). Regardless of the underlying mechanism (whether it is trans-differentiation or not), the hippocampus provides an environment that promotes the expression of neural genes, being a rich source of signals required for adult stem/progenitor cell differentiation.

Several groups have demonstrated that after transplantation into brain tissue MSCs migrate in several neural tissues, including hippocampus, and differentiate into cells expressing neural markers including NeuN, NF, tyrosine hydroxylase, MAP-2 and GFAP (Azizi et al., 1998; Kopen et al., 1999; Li et al., 2000; Li et al., 2001; Mahmood

et al., 2001; Priller et al., 2001; Hofstetter et al., 2002; Zhao et al., 2002). The underlying mechanism was investigated using a co-culture system with postnatal hippocampal tissue and MSCs, suggesting that direct contact between MSCs and neural cells is required for the induction of a neuronal phenotype of MSCs (Abouelfetouh et al., 2004). In co-cultures without direct cell-cell contact, MSCs did not express the late neuronal marker NeuN (Abouelfetouh et al., 2004). In contrast to the present work, where an adult hippocampus was used as a source of soluble factors, the study by Abouelfetouh and co-workers used a postnatal hippocampus, therefore the soluble factors delivered by these tissues may not be strictly the same. In addition to this, in the present work a panel of early and also late neural markers were used, as opposed to the study by Abouelfetouh and co-workers, where they used solely the late neuronal marker NeuN to assess neuronal differentiation (Abouelfetouh et al., 2004). In accordance with that study, we did not find a higher number of NeuN positive MSCs exposed to brain tissue conditioned medium versus the control. However, the expression of other neuronal markers such as NF-H and GAP-43 was induced in MSCs after stimulation with adult hippocampus-conditioned medium, suggesting that hippocampus-derived soluble factors are sufficient to induce a neuronal-like phenotype in MSCs. The neuronal-like induction activity observed in this study seems to be restricted to neural tissues, since muscle derived soluble factors did not induce morphological changes in MSCs and did not induce the expression of neural markers characteristic of a neural-like phenotype.

Fetal neural tissues also promote neural-like differentiation on transplanted MSC (Munoz-Elias et al., 2004). This observation together with the results of this thesis, suggest that neurogenic tissues produce differentiating signals, being a good neural niche that promotes a neural-like phenotype on mesenchymal stem/progenitors cells. However, micro-environments with an intrinsic potent capability of generating new mature cells, like hippocampus or fetal tissues, are not the only rich source of differentiating signals for adult stem/progenitor cells. Previous studies have shown that injured brains can also induce a neuronal-like or glial-phenotype on transplanted MSC (Morrison et al., 1997; Eglitis et al., 1999; Li et al., 2000; Li et al., 2001). Moreover, soluble factors released from damaged skeletal muscle but not undamaged tissue can induce myogenic differentiation on MSC (Santa Maria et al., 2004). Therefore, tissue damage is also an issue to consider, since soluble factors from injured tissues could have a stronger differentiating effect than those released by undamaged tissue.

Apparently, and similar to the co-culture experiments by Abouelfetouh and co-workers, the activities present in the hippocampus-conditioned media do not induce MSC astroglial differentiation, thereby excluding gliogenic activities, since no GFAP-expressing cell was found in this study. As was mentioned before, hippocampal astrocytes release soluble factors that induce more neuronal than astrocyte differentiation on adult neural progenitors cells (Song et al., 2002). Moreover, when GFP-expressing bone marrow-derived cells (BMC) were co-cultured with organotypic hippocampal slices, BMC differentiated into ramified microglia, but not into GFAP-

expressing astrocytes (Wehner et al., 2003). Together, the aforementioned studies suggest that *in vitro*, hippocampal soluble factors have a strong differentiating activity in different adult stem/progenitor cells, preferably towards the neuronal direction only. This is in contrast to the MSCs – brain transplantation experiments (Azizi et al., 1998; Kopen et al., 1999; Li et al., 2000; Li et al., 2001; Mahmood et al., 2001; Hofstetter et al., 2002; Zhao et al., 2002), where both neuronal and astroglial differentiation was observed. This suggests that compared to the *in vitro* situation, MSCs, when transplanted in the brain, are exposed to a versatile micro-environment consisting of glio- and neurogenic activities, contrary to when they are exposed only to the hippocampal soluble factors.

In addition to the effect on neuronal-like differentiation, hippocampal conditioned medium (HCM) affected cell expansion and survival. The increase in the proportion of NF-H-positive cells in HCM might have been due to an inductive effect and/or a selective proliferation of NF-H-positive cells. A pure selective proliferation effect can be excluded having in mind that the percentage of NF-H-positive cells in control conditions is less than 1% and in HCM almost 40%. Such proliferation would require more than six cell cycles in three days, that is, less than 12 hours per cycle, which is rather unlikely. This might suggest that the increase in the proportion of NF-H-positive cells in HCM is due to an inductive or instructive effect more than and selective proliferative one. In addition, enhanced survival might also contribute to the increase in the proportion of NF-H positive cells.

Finally, an important issue to investigate in the future is the nature of the activities present in the hippocampus-derived conditioned media that mediate the effect of neuronal-like differentiation of MSCs. Growth factors such as fibroblast growth factor (FGF-2), brain-derived neurotrophic factor (BDNF) and other members of the neurotrophin family (NT) might be candidates, since they induce neuronal differentiation of MSCs *in vitro* (Sanchez-Ramos et al., 2000; Kim et al., 2002). Moreover, it has been described that, under certain conditions, the hippocampus produces and can secrete neurogenic soluble factors like neurotrophins (Katoh-Semba et al., 1999). A first approach was used in this study in order to evaluate which hippocampal cells could be responsible for the induction of the neuronal-like phenotype observed on MSCs. After co-culturing MSC with hippocampal neural stem cells, no morphology changes or neural marker expression was observed on MSC, indicating that hNSC are not responsible for the neuronal-like induction on MSCs. However, another study described that secreted factors from hippocampal astrocytes can promote neuronal differentiation on NSC (Song et al., 2002). Therefore, it is recommended to search for other hippocampal cell sources, such as astrocytes or pyramidal neurons, responsible for releasing soluble factors that induce the neuronal-like phenotype on MSC.

III. Oligodendrocytic fate determination in hNSCs: an increment in the balance of Olig/Id2 determinant transcriptional factors triggered by MSCs' soluble signals.

One of the most surprising and crucial results of the present thesis is the finding that MSCs induce oligodendrogenic determination and differentiation in hNSCs.

The oligodendrogenic fate was reached at the cost of the astrocytic differentiation, whereas very low neurogenic differentiation was observed in any of the conditions studied. The oligodendrogenic activity originating from the MSCs was found to be present in the medium conditioned by these cells and thus may be attributed to one or multiple MSC secreted factors. The percentage of hNSCs that differentiated into oligodendrocytes was however significantly lower (approx. 50%) when hNSCs were only exposed to MSC conditioned medium as compared to co-culture conditions (approx.70%, Student t test, $p<0.05$). Lower concentrations of active secreted factors present in the conditioned medium, probably as a result of short factor half-life, could explain the reduction in oligodendrocyte differentiation as compared to the co-culture conditions. Alternatively, in addition to soluble factors derived from the MSCs, direct cell-cell contact or matrix bound molecules might contribute to the oligodendrogenic activity.

MSC-derived soluble factors did not only induce the differentiation of hNSCs into oligodendrocytes, but further supported their maturation. Hence, the expression of the myelin basic protein was detected in a large proportion (approx. 70%) of the GalC-

expressing cells after 7 days of incubation in MSC-conditioned media. The expression of the myelin basic protein is activated in the mature oligodendrocyte before the myelination process can be induced (Baumann and Pham-Dinh, 2001). Therefore, MSC-secreted factors did not simply induce aberrant expression of a specific marker, but contributed to the establishment of a *bona fide* genetic program.

This work shows that soluble factors released by MSCs instruct the A2B5 expressing progenitors into an oligodendrocyte fate (GalC-expressing cells), without affecting their proliferation and survival rates (Fig.31). Therefore, the increment of oligodendrocyte was not due to a proliferation/survival selective signal. Interestingly, the active MSC-secreted factors increased significantly the proliferation rate of the GFAP-expressing cells; however they had a weak astrocyte-instructive signal on A2B5-expressing progenitors (Fig.31). The oligodendrogenic program was induced rapidly by MSC-secreted factors, since after three days of culture more than 30% of the hNSCs were found to be differentiated enough into GalC-expressing cells. This induction progressed without affecting the differentiation rate of A2B5-expressing cell, since almost 30% of the hNSC expressed GFAP under control conditions. This observation further supports that the activity of MSC-secreted factors was primarily on the progenitor differentiation fate decision and not on their capacity to undergo differentiation *per se* (Fig. 31).

MSC-secreted factors induce oligodendrocyte fate decision by rapidly and strongly up-regulating the expression of transcriptional factors responsible for the
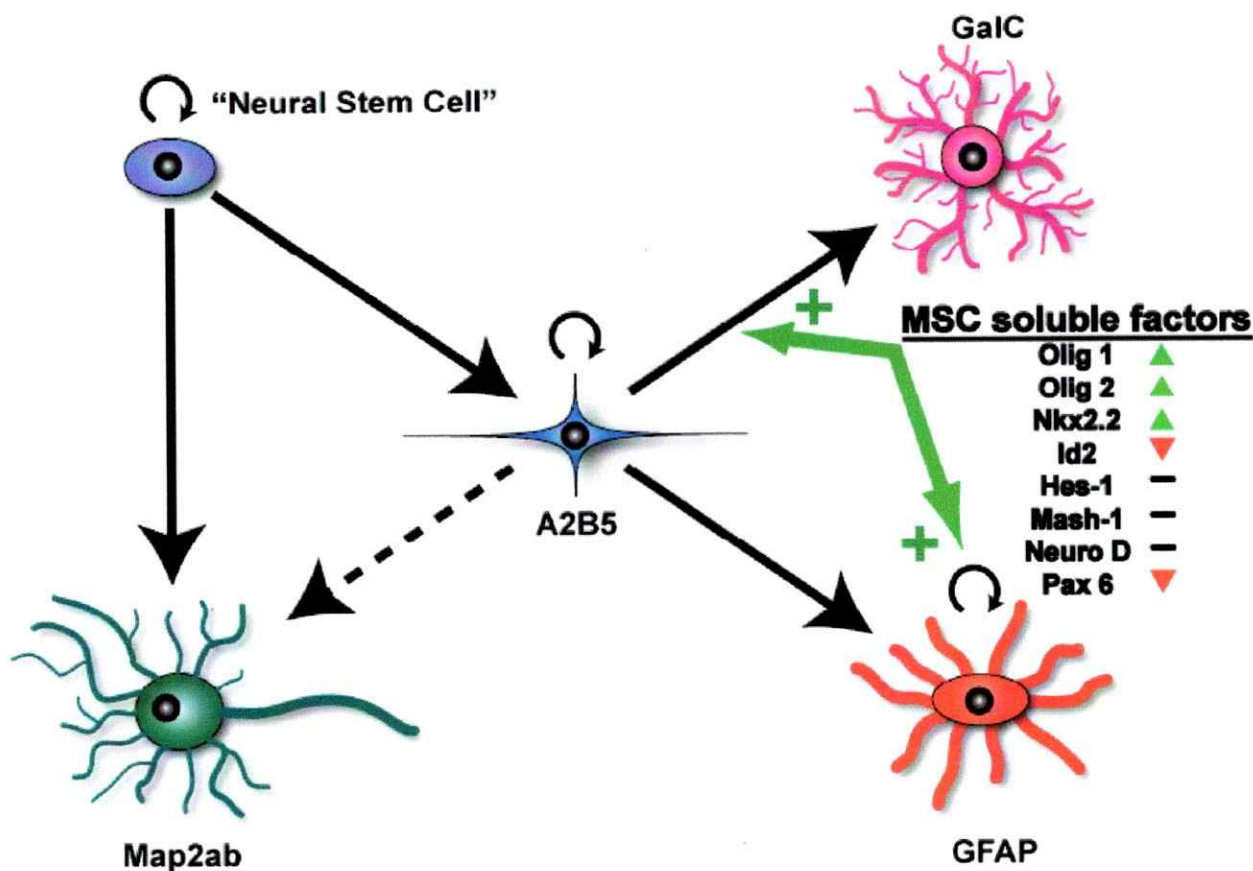
Figure 31.- Representative scheme for the oligodendrogenic effect of MSC-derived soluble factors on adult NSCs.
Self-renewing and proliferative NSC (violet cell with upper curve arrow) can give rise to the three major neural lineages: neurons (Map2ab, green cell), astocytes (proliferating GFAP red cell, with upper curve arrow) and oligodendrocytes (GalC, pink cell). NSCs give rise to proliferative glial-restricted A2B5 expressing cells (multipolar blue cell with upper curve arrow). A2B5-positive cells differentiate, frequently, to oligodendrocytes and astrocytes (black arrows) but some of them, under certain conditions, rarely can differentiate to neurons (black segmented arrow). Under MSC soluble factors, A2B5-expressing cells were induced to the oligodendrocyte (GalC) lineage (green arrows and plus sign), while the astrocytic differentiation pathway was partially inhibited. MSC soluble factors promote astrocyte proliferation (green arrows and plus sign), however the total amount of astrocytes never reach the total amount of oligodendrocytes under this condition. On the left side, the expression of different cell fate determining transcription factors is shown. Every oligodendrogenic transcription factor is up-regulated (Olig1, Olig2 and Nkx2.2) (green up arrowhead), the oligodendrogenic inhibitor factor Id2 and the neurogenic factor Pax6 are down-regulated (red down arrowhead), whereas Hes 1, NeuroD and Mash-1 are not specifically downregulated as compared with control conditions (black minus sign).

induction of oligodendrocytic differentiation, such as Olig1, Olig2 and Nkx2.2 and by down-regulation of the expression of a factor inhibiting the oligodendrocytic differentiation, namely the inhibitor of differentiation 2 (Id2) (Fig. 31). It was recently described that Id2 could sequester olig proteins and prevent their translocation into the nucleus, thereby inhibiting their activity (Samanta and Kessler, 2004). Moreover, the expression of Id2 promotes astrocyte differentiation (Samanta and Kessler, 2004). Hence, differentiation of the progenitor cells was promoted concomitantly by the induction of pro-oligodendrogenesis transcriptional factors and the release of oligodendrogenesis- inhibition. Some studies have suggested that during development oligodendrocyte differentiation may be triggered when the balance of bHLH factors favors oligs over Ids (Wang et al., 2001; Samanta and Kessler, 2004)(Fig. 7). This is the first time that olig and Id mRNA were measured for adult neural stem/progenitors cells under oligodendrogenic conditions. The findings of this thesis suggest that the Olig/Id balance is also crucial for oligodendrocyte fate during adulthood.

IV. Possible candidate molecules and pathways for the oligodendrogenic activity on adult NSCs.

In order to approach a characterization of oligodendrogenic factors released by MSC, heat sensitivity was analyzed. Although under heat-inactivated MSC-CM conditions there were 2 to 3 times less GalC-expressing cells compared to non-heated MSC-CM, the heated medium still exerted some oligodendrogenic activity. This could have two main explanations. First, it is possible that the heating inactivation was not

sufficient, leaving a low remaining activity. Alternatively, the activity could be heat-insensitive. It has been described that some eukaryotic cytokines, peptides and proteins are thermostable (Brauer et al., 1982; Rey et al., 1984; Szmigielski, 1985; Fantozzi et al., 1992; Nishihira et al., 1995). Moreover, since the hNSC culture is not homogenous, there could be a subpopulation of cells that react to a heat -insensitive oligodendrogenic factor that is released from MSC. Taken together, more detailed analysis, like more vigorous heat inactivation and a clonal analysis, is required to exclude or confirm one or another hypothesis.

IV. 1 Insulin-like growth factor (IGF-1)

To date, the only potent oligodendrogenic factor described in adult hippocampal neural/progenitor cells is the insulin-like growth factor 1 (IGF-1) (Hsieh et al., 2004). Moreover, IGF-1 was also described as a potent oligodendrogenic factor for O2A bipotent precursors, whereas IGF binding proteins 1, 2 and 6 (IGFBP-1, 2, 6) could inhibit this activity (Kuhl et al., 2002; Kuhl et al., 2003; Hsieh et al., 2004). Futhermore, it has been shown that murine MSC can produce and secrete IGF-1 (Abboud et al., 1991) and in the present study, the expression of IGF-1 mRNA was verified. These reasons made IGF-1 a good candidate for the oligodendrogenic phenomenon observed in this study. However, the present work excluded IGF–1 to be the factor, because: 1) IGF-1 alone did not induce oligodendrogenesis in our cultures and, 2) IGFBP-1 did not affect the oligodendrogenic effect induced by MSC secreted factors on hNSC. These results, however, seem to contradict those reported by Hsieh and coworkers (Hsieh et al., 2004),

which describe an oligodendrogenic effect on adult hippocampal neuroprogenitors induced by IGF-1. A difference in the experimental conditions that may account for that, for example, is the passage number of cells used. As demonstrated in the present work, the cell type composition of the hNSC cultures depends on the passage number. Thus, cultures from passages 2 to 4 have a 10% of cells expressing mature neural markers under undifferentiated conditions, while cultures of earlier passages have more abundant mature neural cells (33 % GFAP- and GalC-expressing cells). The work by Hsieh and co-workers used cells of relatively late passages (between 10 and 20) and no characterization of the undifferentiated culture was done in terms of different neural progenitor and mature markers expression. In the present study, cells of relatively early passages (between 4 and 6) were used. Therefore, different passages have a different composition of neural cells and they can behave differently when exposed to the same stimulus.

IV.2 Sonic hedgehog (Shh)

As already mentioned, signal transduction pathways that involve the regulation of the Oligs and Id2 expression are crucial for understanding the molecular mechanism by which MSC-secreted factors induce oligodendrocytic fate on hNSCs. Sonic-hedgehog (Shh) signaling participates in cell commitment, regulating oligodendrocyte cell fate during development of precursors in spinal cord and telencephalon (Pringle et al., 1996; Orentas et al., 1999; Tekki-Kessaris et al., 2001). Experiments were done in a heterozygous Danforth's short tail (Sd/+) mutant mice, which lack a notochord and floor

plate in caudal regions of the neural tube. In this animal model, no oligodendrocyte precursors were found at the ventricular surface (Pringle et al., 1996). In addition, no oligodendrocyte development was found in explant cultures of Sd/+ spinal cord in the absence of a floor plate. Moreover, oligodendrocytes developed in explants of intermediate neural tube only when they were co-cultured with fragments of notochord or in the presence of purified Sonic hedgehog (Shh) protein. Therefore, signals from the notochord/floor plate, possibly involving Shh, are necessary and sufficient to induce the development of ventrally derived oligodendroglia (Pringle et al., 1996). This initial commitment would be established by Shh through the induction of oligodendrocyte-specific Olig genes (bHLH type transcriptional factor), as the expression of these genes is lost in Shh null mice (Lu et al., 2000; Zhou et al., 2000). Olig genes are expressed at the telencephalon-diencephalon border and adjacent to the floor plate, a source of the secreted signaling molecule Shh. This signal pathway is both necessary and sufficient for Olig gene expression *in vivo*, as was demonstrated by gain- and loss-of-function analyses in transgenic mice (Lu et al., 2000). In addition to this, embryonic cortical precursors were cultured in presence of recombinant Shh. It was observed that after cell adherence, oligodendrocyte progenitors emerged and continued to increase in number, while the ratio of neuronal cells decreased compared to control (Murray et al., 2002). All this data support the hypothesis that the activation of Shh pathway is required for increasing the expression of Olig genes when adult hippocampal neural stem/progenitors cells are exposed to MSC-soluble factors. Therefore, Shh is a good candidate to evaluate its participation in the oligodendrogenic effect observed on adult hippocampal neural progenitors.

IV.3 Bone morphogenetic protein-4 (BMP-4)

The oligodendrogenic effect mediated by MSC secreted factors not only consists on the up-regulation of oligs genes, but also the down-regulation of Id2 expression, which is an oligodendrogenesis inhibitor. It was shown that BMP-4 signaling up-regulate Id2 expression (Samanta and Kessler, 2004). OLPs isolated from embryonic lateral ganglionic eminence, BMP signalling, activates the expression of the bHLH proteins inhibitor of differentiation 2 (Id2) and 4 (Id4), inihibiting oligodendrogensis and promoting astrocytic differentiation of neural progenitor cells (Wang et al., 2001; Samanta and Kessler, 2004). Therefore the inhibition of the BMP signaling could be necessary for the down-regulation of Id2 expression. However it was described that MSC secrete BMP-4, but only a small subpopulation called "nestin-positive" secrete the active form of BMP-4, while the "nestin-negative" fraction produce only the inactive form of BMP-4 (Wislet-Gendebien et al., 2004). Therefore most of MSC do not produce and secrete active BMP-4. In addition, soluble factors released by "nestin-positive" fraction induce astrocyte differentiation on embryonic neural progenitor cells (Wislet-Gendebien et al., 2004). This result is not inconsistent with the results of this thesis, since here were used the total population of MSCs and the effect was measured on adult neural progenitor cells. Therefore, one hypothesis is that most of the MSCs could inhibit BMP signaling on hNSC, secreting soluble factors antagonist to BMP that blocks the union to its receptor, like chordin (Bachiller et al., 2000).

IV. 4 Neurotrophin-3 (NT-3)

Recently is has been described that neurotrophin-3 (NT-3) induces oligodendrocyte differentiation in hippocampal post-natal neural stem cells via the Erk1/2 pathway (Hu et al., 2004). NT-3 induced a series of intracellular responses including enhancement of phosphorylation of Erk1/2 or Akt and an increase of the expression of Olig-1. Application of a specific inhibitor (U0126) of MEK1/2 (which is upstream to Erk1/2) blocked the phosphorylation of Erk1/2, suppressed the expression of Olig1 preventing NSC differentiation into oligodendrocyte precursors (OLPs) in response to NT-3 stimulation. Blockade of TrkC, the NT-3 receptor, also inhibited the oligodendrocyte differentiation of NSCs induced by NT-3 (Hu et al., 2004). Therefore, NT-3 could be also a good candidate for testing the oligodendrocyte instructive effect of MSC secreted factors.

IV.5 Interleukin-6 (IL-6)

Since, IGF-1 is not sufficient to explain the oligodendrogenic activity, it is imperative to search for different cytokine candidates that could participate on this phenomenon. There are two possible candidates. First, it is already known that MSC can produce and release interleukin-6 (IL-6) (Minguell et al., 2001). IL-6, among other functions, participates on hematopoietic stem/progenitor cells (HSCs) fate determination. In MSC-HSC co-culturing experiments, it was demonstrated that IL-6 secreted by MSC participates on the osteoclast fate determination of HSCs (Mbalaviele

et al., 1999). In addition to this, it has been described that IL-6 can induce an oligodendrogenic stimulus on embryonic stem cells (Zhang et al., 2004b). Moreover, activated microglia-associated interleukine (IL)-6 impairs neuronal and favors glial differentiation during adult neurogenesis (Monje et al., 2003). Therefore, these observations make IL-6 a good candidate for future studies.

IV. 6 Other candidates

In order to continue searching for more candidates, a good approach is to focus on different neural progenitors from development. Astrocytes and oligodendrocytes proceed through a sequence of progenitors that become progressively more restricted. A recently-identified cell population that arises from totipotent NSCs, called tripotential glial-restricted precursor (GRP), can generate oligodendrocytes and two types of astrocytes (Rao et al., 1998; Herrera et al., 2001; Gregori et al., 2002). This population in turn differentiates into the more restricted oligodendrocyte-type 2 astrocyte progenitor cells (O-2A) that can generate oligodendrocytes but only one type of astrocyte (Raff et al., 1983; Skoff and Knapp, 1991). It has been described that O2A glial-restricted progenitor can differentiate into oligodendrocytes under the influence of thyroid hormone (TH), retinoic acid and CNTF (Liu and Rao, 2004). Thus, these molecules are good candidates for the oligodendrogenic effect observed in this study; however, the expression and secretion of these soluble factors by MSC are not well documented.

Taken together, these results indicate that soluble factors secreted by MSC induce specific astrocyte proliferation and an oligodendrocytic fate on adult neural progenitors, affecting the neural stem cell niche. Therefore, this study demonstrates the bivalent nature of adult bone marrow stromal cells, since they can behave as stem cells differentiating under particular conditions and as stromal cells, producing different kinds of signals affecting the biology of adult neural stem/progenitor cells.

V. Do MSC derived soluble factors affect neural stem cells, progenitor cells or precursor cells?

This work shows that soluble factors secreted from the MSC did not significantly promote or repress the neuronal determination of hNSC cultures (Fig. 33). But, why was neuronal differentiation not observed under these conditions? It is possible that MSC do not secrete neurogenic factors, but it is also possible that most of the neuroprogenitors isolated from the adult hippocampus, are unable to differentiate into a neuronal lineage, since they were already committed into glial-restricted lineage. Moreover, it is possible that the same stimulus induces different cellular process depending whether they are acting on neural stem cells or committed neuroprogenitors.

In the hNSC cultures used in the present study, although almost 94% of the cells expressed A2B5, suggesting some homogeneity at the level of marker expression, the cells did not have uniform response to the MSC derived soluble factors. This supports the heterogeneity of the adult hippocampus neural stem/progenitor cell culture,

suggesting that it is composed of different neural progenitors having distinct degree of commitment and differentiation. This is not a novel observation, since it was described that adult neocortex and subcortical white matter have different kinds of cycling glial-restricted progenitors, supporting the heterogeneity hypothesis (Gensert and Goldman, 2001).

The hNSCs used in the present study were shown to be pluripotent, demonstrated by their capacity to differentiate upon appropriate stimuli into neurons, astrocytes and oligodendrocytes. This culture system likely contained stem cells, but was largely composed of proliferating progenitors in an undifferentiated state. It is noteworthy that in the present study, a large proportion (approx. 95%) of the neural progenitors expressed the marker A2B5. The latter was reported to be expressed by glial-restricted progenitors (GRP), although some neuronal differentiation potential of A2B5-expressing cells has also been reported (Galiana et al., 1993; Nunes et al., 2003). For instance, white matter progenitor cells (WMPC) have been identified from adult brains, they are A2B5 immunoreactive, small bipolar cells, capable of proliferation and differentiation into neurons, astrocytes and oligodendrocytes (Roy et al., 1999; Nunes et al., 2003) (Fig. 31). The large amount of A2B5-expressing cells could be the result of a faster proliferation of this subpopulation, leading to its progressive enrichment in the culture, or alternatively could result from a selective process via our culture techniques and medium. Both hypotheses could explain the different composition of early and late passage cultures. In comparison to early passage NSCs, later passages were richer in the A2B5-positive progenitor population.

MSC-derived soluble factors may specifically act on the A2B5-positive subpopulation of progenitor cells and not on others. It is not clear from the results that MSC soluble factors induce an oligodendrogenic fate on "true" neural stem cells and is also not clear if they can induce or repress a neuronal fate (Fig.31). In other words, can MSC soluble factors induce an oligodendrogenic fate on "true" tripotent neural stem cells? Do they have no neurogenic effect? To approach this problem, an experimental procedure is suggested. First, we need to isolate neural stem cells and different committed progenitor cells (glial-restricted and neuronal-restricted), obtaining a culture that is as homogeneous as posible. To select each population, cells should be isolated from hippocampus of different transgenic mice and selected by cell sorting. These transgenic mice should express the green fluorescent protein (GFP) driven by regulatory elements of the different genes (markers): nestin gene, (neural stem cells) (Gleiberman et al., 2005); A2B5 gene (glial-restricted progenitors) and doublecortin gene (DCX) (neuronal-restricted progenitors) (Couillard-Despres et al., 2005). Therefore, after cell sorting, there should be three cell populations "enriched" in 1) neural stem cells, 2) glial-restricted progenitor cells and 3) neuronal-restricted progenitor cells. Then, a clonal analysis is recommended to evaluate their potential, using the stimulus known for each neural lineage differentiation. Finally, the three different populations should be incubated with MSC soluble factors and the final phenotype should be evaluated in each culture. If a significant amount of oligodendrocytes are obtained from the nestin-enriched population and the A2B5-enriched population, that would mean that soluble factors secreted by MSC can indeed promote oligodendrogenesis on a homogeneous tripotent neural stem cell population. If no significant amount of neurons can be

obtained from the DCX-enriched population, it would mean that MSC-derived soluble factors have no neurogenic inductive signal.

This kind of analysis would allow us to work with purified cellular systems, and giving a much clearer and more reliable information about adult stem cell biology.

VI. MSC-NSC interactions: is there an in vivo relevance?

VI.1 Relevance of interactions of endogenous stem cell populations and niches

Considering the dense network of capillaries present in the brain, any position in the brain parenchyma is located within 50 μm from the next capillary. Since MSCs are found in the blood circulation (Kuznetsov et al., 2001; Roufosse et al., 2004), the spacing between NSCs and MSCs might be minimal. Moreover, the location of NSCs is tightly associated with the vascular system (Palmer et al., 2000)

Interactions between the CNS and the vascular system are strictly controlled through the blood-brain barrier. Hence, factors locally secreted by the circulating MSCs may not be available for the NSCs in normal conditions. However, following CNS lesions, the blood-brain barrier becomes leaky (Lanens et al., 1993) and MSCs as well as their secreted factors can penetrate the nervous tissue and act locally. It was described that after brain lesion, like an ischemic one, different cytokines and chemokines are expressed, like macrophage inflammatory protein-1alpha (MIP-1 alpha); monocyte

chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) (Wang et al., 2002a; Wang et al., 2002b). These soluble factors promote and enhance the MSC attraction and migration. Through their secreted factors at the lesion site, MSCs could locally induce resting progenitors to differentiate into oligodendrocytes and hence participate in a regenerative effort. For example, in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE), lesions and inflammation were shown to induce the differentiation of neural progenitors into oligodendrocytes (Blakemore and Keirstead, 1999; Keirstead and Blakemore, 1999).

VI.2 Relevance of interactions of stem cell populations after transplantation

Several studies have focused on MSC transplantation on neurological disease models in mice or rats. After transplantation, MSC phenotypes, survival and mouse/rat recovery was analyzed. For instance, MSCs were transplanted on a mouse model of Parkinson's disease (PD) (Li et al., 2001). MSCs were harvested from donor adult mice, and then cultured and prelabeled with bromodeoxyuridine (BrdU). PD mice with MSC intrastriatal transplantation exhibited significant improvement on the performance test. Immunohistochemistry showed that BrdU reactive cells survive in the transplanted areas at the damaged striatum, expressing tyrosine hydroxylase (TH). The findings suggested that MSCs injected intrastriatally survived, expressed dopaminergic protein, and promoted functional recovery (Li et al., 2001). In another study, MSCs were transplanted into injured spinal cord. After transplantation, cells integrated well into the injured side and promoted a strong axonal growth (Neuhuber et al., 2005). In another

study, MSCs were transplanted intravenously into a rat previously damaged by a middle cerebral artery occlusion (MCAO) to produce brain ischemia. Treatment of MCAO with MSCs significantly improved functional recovery. In addition, MSC treatment significantly increased BrdU(+), DCX(+), endogenous cells. The authors concluded that MSCs may contribute to improved functional recovery and increased neurogenesis after stroke (Zhang et al., 2004a). The mechanism of how MSCs contribute to functional recovery was not analyzed. The present thesis opens the possibility that MSC triggered tissue endogenous NSCs to differentiate into oligodendrocytes. Recently, it was shown that transplantation of MSC in EAE animal model, enhances functional recovery, possibly via reducing inflammatory infiltrates and demyelination areas and stimulating oligodendrogenesis (Zhang et al., 2005a).

In conclusion, the interaction of adult mesenchymal stem/progenitors cells and the adult hippocampus tissue, as a foreign niche, has two main effects. First, hippocampus-secreted soluble factors induce a neural-like phenotype on MSC (Fig. 32). Second, MSC-derived soluble factors modulate the differentiation fate of hippocampal neural progenitors (Fig. 32). The oligodendrogenic activity(ies) derived from MSCs are IGF-1 independent (Fig. 32). Based on the possibility that MSCs and NSCs might be in close proximity under physiological or pathophysiological conditions, as well as after transplantation, the present work helps to understand and to promote regenerative processes in the adult CNS.
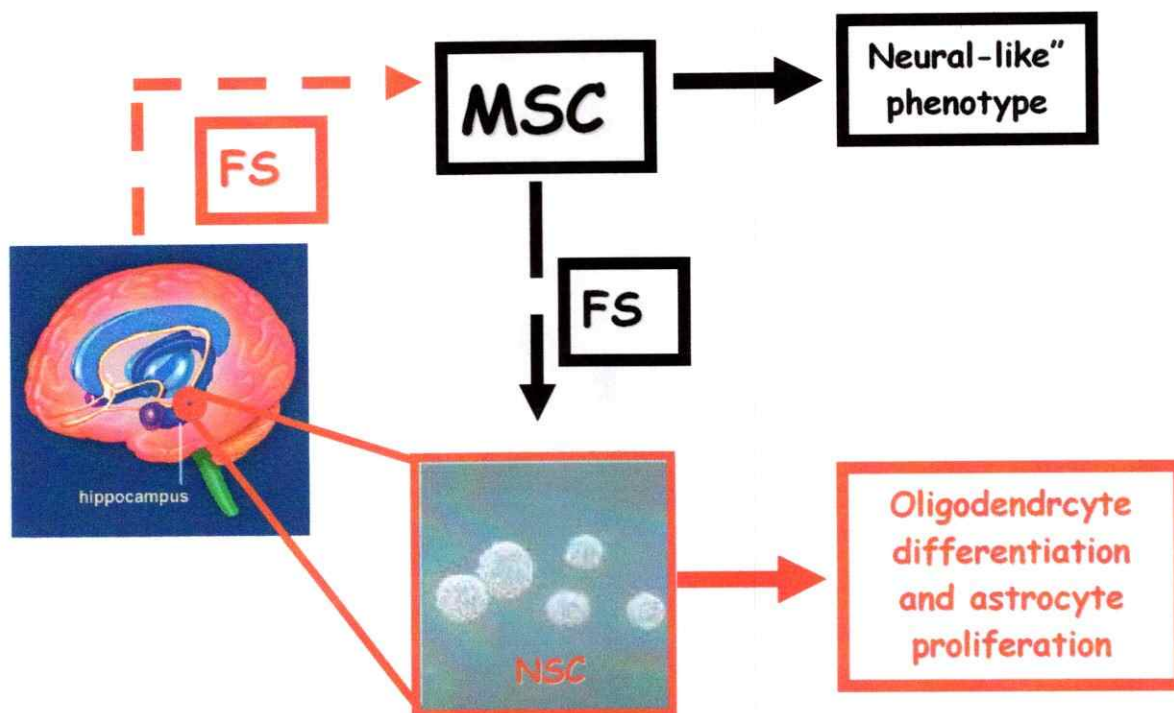
Figure 32.- Representative scheme for interactions between adult hippocampus, NSCs and MSCs.
The hippocampus compartment is represented in red, the bone marrow mesenchymal stem/progenitor cells (MSC) compartment is represented in black. Soluble factors (FS) released by hippocampus tissue (red segmented arrow) induce MSCs to adopt a neural-like phenotype (upper black squares), while soluble factors (FS) released by MSCs (black segmented arrow) induce an oligodendrocyte fate determination and differentiation, together with an astrocyte proliferation in NSCs (bottom red squares).

ADDITIONAL METHOLOGICAL CONSIDERATION

I. The use of the β-tubulin isotype III as a neuronal marker?

As mentioned above, neuronal differentiation was assessed using the expression of Map2ab as a marker. The later was more specific than the frequently used detection of the β-tubulin isotype III, which was expressed in up to 50% of the GalC expressing-cells as well as in an important percentage of the GFAP-expressing cells under our culture conditions. This was verified using another monoclonal antibody against the same antigen, Tuj1, having almost the same result. In addition to this, the analysis of the expression of transcriptional factors revealed that there is no neuronal induction genetic program triggered by MSC on adult neuronal progenitors. In conclusion, under our *in vitro* conditions, the marker β-tubulin isotype III is not specific for neuronal commitment. In addition to this, β-tubulin isotype III was used to study neuronal trans-differentiation in adult mammalian retina (Engelhardt et al., 2005). It was observed that β-tubulin isotype III turned out to be a misleading marker for neuronal transdifferentiation, since β-tubulin isotype III was expressed in almost all pigment epithelial derived cells in vitro, however only a subpopulation co-expressed the neuronal precursor molecule doublecortin. Moreover, only the doublecortin-expressing subpopulation displayed a neuronal morphology, whereas most of the β-tubulin isotype III-expressing cells had a flat fibroblastic morphology (Engelhardt et al., 2005). This suggests that it is recommendable, before any conclusion about the final phenotype of

differentiating cells, to analyze not only the morphology changes and the expression of the markers frequently used for the detection of certain neural lineages, but also to analyze the co-expression of these markers and transcriptional factors that participate in the fate determination of stem/progenitor cells.

SUMMARY

1) MSC isolated from adult bone marrow stroma, can adopt a neuronal-like phenotype when they are in contact with beta-mercaptoethanol.

2) Soluble factors derived from hippocampus induce a neuronal-like phenotype, provoking morphology changes and the expression of neuronal markers, also promoting proliferation and survival in MSCs.

3) Soluble factors released by MSCs induce, in a time-dependent manner, oligodendrogenic differentiation, provoking morphological changes and the expression of oligodendrocyte markers in NSC.

4) NSC oligodendrogenesis induced by MSC`s soluble factors, is not caused by a proliferative or survival selective signal but is caused by an inductive or instructive signal.

4) Soluble factors delivered by MSC promote astocytic proliferation in hNSC.

5) Soluble factors released by MSC, induce oligodendrocytic fate in hNSC, enhancing the expression of oligodendrogenic transcriptional factors and inhibiting the expression of Id2, a specific inhibitor of oligodendrogenesis.

6) The oligodendrogenic effect of soluble factors secreted by MSC on hNSC is IGF-1 independent.

7) Soluble factors released by MSCs have no effect on neuronal fate determination in hNSC.

8) Bidirectional interactions between soluble factors released by adult hippocampus and MSC determine the fate of adult mesenchymal and neural stem/progenitor cells.

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