Universidade do Minho

Escola de Ciências da Saúde

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Tese de Doutoramento em Ciências da Saúde

Trabalho realizado sob a orientação do
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e co-orientação do
Professor Doutor Nuno Jorge Carvalho Sousa

dezembro de 2014

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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ACKNOWLEDGEMENTS

The development of the present thesis is the result of a strong and wonderful interaction and contribution of several people and institutions. To all of them that in different manners were involved, I want to really express my sincere gratitude.

My first words goes to my parents. Sincerely, there are no words in this world that could express my sincere gratitude to them, for all the support, for believe in me and never questioned my choices of life. If today I am what I am, unquestionably, they were always the reason that made me to believe.

My first scientific acknowledgement goes to my supervisor, Dr. António Salgado, without whom this thesis would not be possible. Sincerely, I would like to thank him for giving me the opportunity to integrate his dynamic research group and for providing me all the tools, and opportunities (even during hard financial periods) to perform all my scientific work. At the same time, I would like to thank him for being always there to hear, talk and discuss not only as a professional, but also, as a friend. For all the support throughout these years, I want to express my sincere gratitude to him.

To Prof. Nuno Sousa, who allowed me to be a part of the NerD family. I want to thank him for the quality of the discussions that we had, for all the questions, comments, suggestions and advices that made me to improve as a scientist.

To Prof. Leo Behie, who gave me the opportunity to integrate his research group in Calgary, Canada. It was for me a pleasure to meet you and be a part of your lab. Thanks for guided all my scientific work in your lab, and for providing me all the tools that I needed. I have learned a lot in the PPRF. At the same time, I would like to thank him for giving me the opportunity to attend to the PPRF Summer Seminars Series, which allowed me to meet wonderful people.

"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth"

Jules Verne

(1828-1905)

Modulation of the Secretome of Mesenchymal Stem cells for Central Nervous System Regenerative Medicine Applications

Abstract

The low regeneration potential of the central nervous system (CNS) represents a challenge for the development of new therapeutic strategies. Different possible solutions (e.g. pharmacological, surgical) have been addressed to tackle these problems. However, so far, the outcomes are still not satisfactory, imposing the need for innovative therapeutical approaches. Therefore, several studies have proposed the use of human adult stem cells as a possible tool for CNS regeneration, and within it, human mesenchymal stem cells (hMSCs) have emerged as a valid therapeutic option. In fact, over the last decade, there has been a substantial effort to assess the impact of hMSCs for CNS repair. From the application standpoint, several studies have shown that most of their therapeutic effects has been attributed to their capacity of secreting a wide panel of neuroregulatory molecules (e.g. secretome) capable of inducing neuroprotective/neurodifferentiative actions in the CNS affected regions. Indeed, although it is hypothesized that the hMSCs-secretome plays a major role in these events, little is known on the mechanisms that regulate all these actions. For that, based in all these concepts, the main goal of the work described in this thesis was to analyse and characterize the secretome of hMSCs collected from different culture conditions (static and bioreactor based), as well as to analyse the success of their application in the survival/differentiation of CNS cells both *in vitro* and *in vivo*, and in the context of Parkinson's disease (PD).

Results have revealed that the secretome of hMSCs (collected under static conditions) was able to increase neuronal cell survival and differentiation of human telencephalon-derived neural progenitors (hNPCs) *in vitro*. *In vivo*, the secretome *per se* revealed to be also able to increase the levels of proliferation and cell neuronal survival in the dentate gyrus (DG) of adult rat hippocampus when compared to hMSCs cell transplantation. However, the use of standard static culture conditions presents some limitations such as heterogeneous culture environment, limited growth surface area per culture, among others. The latter may somewhat limit, or negatively modulate, the secretome of hMSCs. In order to overcome these drawbacks we have proposed the use of dynamic culturing conditions provided by computer-controlled bioreactors. Based on proteomic analysis, it was possible to observe that use of dynamic culturing conditions was able to enhance the secretory profile of hMSCs secretome when compared to the static conditions, enhancing and supporting neuronal cell survival and differentiation both *in vitro* and *in vivo*. In addition to this concept of cell

culture modulation, we have also verified that the oxygen concentration also play a role on the paracrine activity of hMSCs. Indeed, when we compared normoxic (21% O_2) with hypoxic (5% O_2) conditions, proteomic-based results revelead that the use of a hypoxic preconditioning led to an upregulation of several neuroregulatory molecules on the hMSCs secretome. Finally, the possible therapeutic effect of hMSCs secretome, collected from dynamic and static culture conditions, on the recovery of a rat model (6-OHDA) of PD, was also assessed. Results revealed that the injection of hMSCs CM (either from static and dynamic conditions) was able to improve the motor coordination (rotarod test) and the paw reaching motor function of the animals (staircase behavioural test) when compared to 6-OHDA-control group. Additionally, it was also possible to observe that the injection of the hMSCs secretome potentiated the recovery of dopaminergic neurons, thereby supporting the recovery of the parkinsonian rats' on the motor performance.

Thus, both *in vitro* and *in vivo* experiments indicate that the modulation of culture conditions could be one of the ways to improve hMSCs secretome potential, which could open new therapeutic opportunities for its application in the future regarding good manufacturing conditions.

Modulação do Secretoma de Células Estaminais Mesenquimatosas para Aplicação na Medicina Regenerativa do Sistema Nervoso Central

Resumo

A baixa capacidade de regeneração do sistema nervoso central (SNC) representa um dos maiores desafios no desenvolvimento de novas estratégias terapêuticas. Até ao momento, diferentes abordagens (i.e. farmacológicas ou cirúrgicas) têm sido utilizadas no tratamento de lesões do SNC. Contudo, muito dos resultados obtidos não são de todo satisfatórios, reivindicando assim, a necessidade de criar abordagens terapêuticas alternativas. A transplantação de células estaminais adultas tem sido sugerida como uma potencial ferramenta terapêutica para a regeneração do SNC na qual, o uso de células estaminais mesenquimatosas (MSCs) têm assumido um papel de relevo. De fato, estudos têm sido efetuados no sentido de avaliar o impacto destas células na regeneração do SNC. Sob o ponto de vista de aplicação, tem sido demonstrado que a acção terapêutica das MSCs provém da sua capacidade em secretar um vasto painel de moléculas (designado de secretoma), capaz de promover efeitos de neuroprotecção e neuro-diferenciação em lesões do SNC. Assim, tendo por base estes conceitos, o principal objetivo do trabalho descrito nesta tese foi analisar e caraterizar o secretoma das MSCs recolhido de diferentes condições de cultura (estática ou dinâmica), bem como analisar o sucesso da sua consequente aplicação na sobrevivência e diferenciação de células do SNC *in vitro* e *in vivo*, assim como, no âmbito da doença de Parkison (DP).

Os resultados obtidos revelaram que o secretoma das MSCs (recolhidos de uma condição estática) foi capaz de aumentar os níveis de sobrevivência e diferenciação neuronal de células estaminais neurais humanas *in vitro*. Quando aplicado *in vivo*, o secretoma de MSCs revelou por si só, ser igualmente capaz de aumentar os níveis de proliferação e de sobrevivência neuronal no giros denteado (GD) do hipocampo em comparação ao transplante de MSCs. Contudo, tem sido proposto que o uso de condições estáticas de cultura apresenta consideráveis limitações na performance das MSCs tais como: heterogeneidade do ambiente de cultura, limitação da área de crescimento, problemas de difusão de gases, entre outros. Deste modo, no sentido de ultrapassar tais limitações, o uso de condições dinâmicas de cultura através da utilização de bioreactores controlados computorizadamente foi avaliado. Com base numa análise de proteómica, os resultados revelaram que o uso de uma condição de cultura dinâmica aumentou o perfil de secreção das MSCs em comparação à condição estática. Ao mesmo tempo, verificou-se igualmente que o uso do secretoma

das MSCs recolhido da condição dinâmica foi capaz de aumentar e suportar de uma forma significativa os níveis de sobrevivência e diferenciação neuronal *in vitro* e *in vivo* em comparação à condição estática. Todavia, tendo por base este conceito de modulação das condições de cultura, verificou-se também, que o uso de diferentes concentrações de oxigénio (21% O_2 – normóxia; 5% O_2 – hipóxia) era igualmente capaz de modular o perfil do secretoma das MSCs. De fato, após análise de proteómica no secretoma recolhido sob estas condições, foi possível constatar que um pré-condicionamento sob condições de hipóxia levou a um melhor perfil de secreção das MSCs em comparação ao obtido numa condição de normóxia. Finalmente, no âmbito do trabalho descrito nesta tese, o possível efeito terapêutico do secretoma das MSCs (recolhido de condição estática e dinâmica) na recuperação de um modelo da DP foi igualmente avaliado. Os resultados obtidos, revelaram que a injeção do secretoma das MSCs (quer de condição estática ou dinâmica) foi capaz de melhorar a condição motora dos animais com a DP. Adicionalmente, verificamos que a injeção do secretoma de MSCs, foi igualmente capaz de potenciar a sobrevivência dos neurónios dopaminérgicos ao nível da substância nigra, suportando assim as melhorias observadas na componente motora dos animais.

Sendo assim, as experiências efetuadas no trabalho descrito nesta tese (*in vitro* e *in vivo*) revelaram que a modulação das condições de cultura das MSCs poderá ser uma estratégia credível para aumentar o potencial terapêutico do seu secretoma, podendo deste modo levar à criação de novas oportunidades terapêuticas para a sua aplicação no âmbito da medicina regenerativa do SNC.

CHPATER VI

#

6-OHDA – 6-Hydroxidopamine

A

ASCs – Adipose Stem Cells

AM – Amniotic membrane

ACN – Accenture

B

BDNF – Brain-derived neurotrophic factor

BM – Bone marrow

BM-MSCs – Bone marrow mesenchymal stem cells

BMNCs – Bone marrow mononuclear cells

BrdU – Bromodeoxyuridine

bFGF – Beta fibroblast growth factor

bNGF – Beta Nerve growth factor

C

CNS – Central Nervous System

CM – Conditioned media

CMs – Conditioned media static

CMd – Conditioned media dynamic

Ca^{2+} – Calcium

CO_2 – Carbon dioxide

DCX – Doublecortin

Cys C – Cystatin C

CTGF – Connective tissue growth factor

D

DG – Dentate Gyrus

DAPI – 4,6-diamidino-2-phenylindole

DAB – 3,3-Diaminobenzidine

DMEM – Dulbecco's modified Eagle's medium

DA – Dopaminergic neurons

E

ESCs – Embryonic stem cells

EDTA – Ethylenediamine tetraacetic acid

EGF – Epidermal growth factor

EF-2 – Elongation factor 2

ECM – Extracellular matrix

F

FGF-2 – Fibroblast growth factor 2

FBS – Fetal bovine serum

FCS – Fetal calf serum

FA – Formic acid

FDR – False discovery rate

FGF-20 – Fibroblast growth factor 20

G

GDNF – Glial-derived neurotrophic factor

G-CSF – Granulocyte colony-stimulating factor

g - gram

GVHD – Graft-versus-host disease

GMP – Good manufacturing process

g/L – gram per liter

GFP – Green fluorescent protein

GCL – Granular cell layer

GDN – Glia-derived nexin

GFAP – Glial fibrillary acidic protein

H
HGF – Hepatocyte growth factor

HEK – Human embryonic kidney

HNA – Human nuclear antigen

H_2O_2 – Hydrogen peroxide

HCl – Hydrochloric acid

Hsp70 – Heat Shock protein 70

HUCPVCs – Human umbilical cord perivascular cells

HSA – Human serum albumin

I
ISCT – International society of cellular therapy

IGF-1 – Insulin growth factor 1

IGF-2 – Insulin growth factor 2

IL-10 – Interleukin 10

IS – Ischemic stroke

IL-1 – Interleukin 1

IL-8 – Interleukin 8

IL-6 – Interleukin 6

IDA – Information-dependent acquisition

K
KI – Kidney injury

L
L-DOPA – Levodopa

LC – Liquid chromatography

M

MSCs – Mesenchymal stem cells

Mi – Myocardial ischemia

MC – Microcarriers

MCAo – Middle cerebral artery occlusion

MCP-1 – Monocyte chemoattractant protein 1

MAP-2 – Microtubule-associated protein 2

mg/mL – milligram per milliliter

mg/μL – milligram per microliter

mL – milliliter

mg – milligram

min – minutes

mg/Kg – milligram per kilo

M – molar

MMTS – S-methyl thiomethanesulfonate

MS – Mass spectrometry

ms – millisecond

m/z – mass-to-charge ratio

MS/MS – Tandem mass spectrometry

MRP1 – Multidrug resistant protein 1

MIF – Macrophage migration inhibition factor

MFB – Medial forebrain bundle

Mg^{2+} - Magnesium

N
NPCs – Neural progenitors cells

NGF – Nerve growth factor

nm – nanometers

NT-3 – Neurotrophin 3

NSCs – Neural stem cells

NaCl – Sodium Chloride

O

O$_2$ – Oxygen

TEAB – Triethylammonium bicarbonate buffer

P

PD – Parkinson's Disease

PBS – Phosphate buffer saline

PBS – Phosphate buffer saline tween

PPRF-msc6 – Serum-free medium

PMSF – Phenylmethanesulfunyl fluoride

PEDF – Pigment epithelium-derived factor

Prx1 – Peroxideroxin 1

U

μm – micrometer

μL - microliter

μg/μL – microgram per microliter

μL/min – microliter per minute

μg/mL – microgram per milliliter

UCHL1 - Ubiquitin carboxy-terminal hydrolase L1

R

Rpm – Rotations per minute

ROS – Reactive oxygen species

V

VEGF – Vascular endothelial growth factor

S

SCF – Stem cell factor

SDF-1 – Stromal derived factor 1

SCI – Spinal cord injury

SNc – Substantia nigra

s – second

SGZ – Subgranular zone

SEM – Standard error of mean

W

WJ – Wharton Jelly

WJ-MSCs – Wharton Jelly mesenchymal stem cells

T

TGF-β1 – Transforming growth factor beta 1

THP-1 – Human monocytic cell line

TH – Tyrosine hydroxylase

TCA – Trichloroacetic acid

TCEP – Tris(2-carboxyethyl)phosphine hydrochloride

The present thesis is organized in six different chapters. The body of each chapter is presented as research papers (accepted or in preparation).

Section I – Chapter I

In chapter I, it is presented a comprehensive and exhaustive overview of the use of human mesenchymal stem cells (hMSCs) and its secretome as new therapeutic tool for central nervous system (CNS). It starts covering all the topics regarding hMSCs characteristics (different sources, expression markers, multilineage differentiation potential), after which a full overview on hMSCs secretome concept is also presented. At the end, an extensive description of the effects of the hMSCs secretome in some neurodegenerative disorders (e.g. Stroke, Spinal Cord Injury and Parkinson's Disease) is provided, as well as some insights on the use of bioreactors to culture these cells. It is finalized by a section where new research directions are proposed.

Section II – Chapter II to V

All the chapters within this section describe all the experimental work performed on the scope of this thesis.

Chapter II compares the application of hMSCs transplantation and its secretome on the neuronal cell survival and differentiation both *in vitro* and *in vivo.* Results revealed that hMSCs secretome as similar to increased effects in vivo regarding neuronal survival and differentiation in neurogenic niches

Chapter III describes the development of new strategies to improve the hMSCs secretome potential. The use of computer-controlled bioreactors (i.e. dynamic culturing conditions) was found to be a modulator of the hMSCs secretome, enhancing their neurotrophic secretory profile when compared to the conventional static culture flasks,. The effects of these obtained secretomes on the neuronal cell survival and differentiation both *in vitro* and *in vivo* was also addressed. Results

revealed that the dynamically collected secretome induce higher rates of neuronal survival and differentiation

Chapter IV reports that the oxygen concentration is also a modulator of the hMSCs secretome profile. In this chapter, normoxic and hypoxic conditions were used in order to address the impact of different percentages of oxygen in the hMSCs secretome. Proteomic-based analysis was performed in order to address the impact of the above-referred conditions on hMSCs secretome composition. Their impact o the neuronal cell survival and differentiation both *in vitro* was also addressed. Results revealed that in fact hypoxic conditions change the profile of hMSCs secretome. However this did not have an impact on the neuro-regulatory character of the latter.

Chapter V presents the final experimental work within the scope of this thesis. In this chapter the effects of the hMSCs secretome (collected from dynamic and static conditions) in a rat model of Parkinson's Disease were addressed. Animal behavior tests (were performed in order to evaluate the effects of hMSCs secretome injections. At the end, dopaminergic neuronal cell survival was also addressed (TH-immunostaining). Results revealed that hMSCs secretome was able to partially revert the phenotype of the hemi-parkinsonian animals.

Section III – Chapter VI

In this chapter it is presented a general discussion of all the works carried out in the scope of this thesis, in which specific considerations and perspectives were also provided.

SECTION I

Chapter 1

Mesenchymal Stem Cells Secretome: A New Paradigm for Central Nervous System Regeneration?

Mesenchymal Stem Cells Secretome: a new paradigm for Central Nervous System Regeneration?

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Abstract

The low regeneration potential of the central nervous system (CNS) represents a challenge for the development of new therapeutic strategies. Mesenchymal stem cells (MSCs) have been proposed as a possible therapeutic tool for CNS disorders. In addition to their differentiation potential, it is well accepted nowadays that their beneficial actions can be also mediated by their secretome. Indeed, it was already demonstrated, both *in vitro* and *in vivo*, that MSCs are able to secrete a broad range of neuroregulatory factors that promote an increase in the levels of neurogenesis, inhibition of apoptosis/glial scar, immunomodulation, angiogenesis, neuronal and glial cell survival, as well as relevant neuroprotective actions into different physiological contexts. Considering their protective action in lesioned sites, MSCs secretome might also improve the integration of local progenitor cells in neuroregeneration processes, which opens a door for the creation of new treatments for future applications in human clinical trials, based in stem cell technology. Thus, we analyze the current understanding of MSCs secretome as a new paradigm for the treatment of CNS neurodegenerative diseases.

1.   Introduction - Mesenchymal Stem Cells (MSCs)

The use of stem cells as a new strategy for cell-based therapies has shown promising results in a variety of health related problems, including neurodegenerative diseases [1]. In fact, during the last few years there have been a great deal of progress in the development of new protocols and strategies based in stem cells for the treatment of central nervous system (CNS) disorders [2, 3]. Indeed, studies have shown that they display some capability to differentiate into several cells types and also to exert trophic and protective actions [4-6]. Mesenchymal stem cells (MSCs) are a stem cell population that has emerged in the last few years as a promise in regenerative medicine of different tissues [7, 8]. This great potential has been associated with their widespread availability throughout the human body, along with the fact that, when isolated, they display great proliferative potential with minimal senescence through multiple passages [9, 10]. According to the definition introduced by the International Society for Cell Therapy (ISCT), there are some minimal criteria for the identification of MSCs populations, such as the adherence to plastic in standard culture conditions; positive expression for specific markers like CD73, CD90, CD105 and negative expression for hematopoietic markers like CD34, CD45, HLA-DR, CD14 or CD11B, CD79α or CD19; and *in vitro* differentiation into at least osteoblasts, adipocytes and chondroblasts [11]. Friedenstein and colleagues [12] were the first to isolate and describe MSCs as fibroblastoid cells with clonogenic potential and plastic culture adherence in rodent bone marrow. Following these early studies several reports have confirmed that MSCs are not only present within the bone marrow, but also in other tissues like adipose tissue [13, 14], dental pulp [15, 16], placenta [17, 18] and, umbilical cord blood [19] and Wharton Jelly [20, 21], and brain [22]. Although all these populations are within the definition of MSCs, they do present subtle differences, specifically in their membrane antigen markers. Studies have shown that such differences can be the result of different cell culture protocols in their isolation and expansion or, alternatively, be related with the tissue source from where they are isolated [23, 24]. At the same time, besides the membrane antigens proposed by ISCT (CD73, CD90 and CD105) in the characterization of MSCs, CD29, CD44, CD51, CD71, CD106, and Stro-1 are also other membrane antigens that have been associated to the MSCs definition [23, 25, 26]. In addition to these findings, further studies demonstrated that all these MSCs populations could be sub-passaged and differentiated *in vitro* into different cell lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts [26, 27]. Curiously, several reports also showed that MSCs could also differentiate into neuronal and epithelial populations [26, 28-31].

While the differentiation into epithelial cells seems to occur, the differentiation of MSCs into functional neuronal lineages is still matter of intense debate [26, 32].

In this sense, in addition to the necessity into clarify the phenotypic identity of MSCs and the best culture parameters in their handling, it becomes also important to characterize the MSCs secretome in order to understand if in fact, the factors secreted by these cells may be the main effectors of their therapeutic actions when applied in the CNS.

1.1.   MSCs secretome

Recently it is becoming accepted that the regenerative effects promoted by MSCs is mainly associated with the secretion of bioactive molecules, that is, with their secretome [33]. Recently the concept of the secretome has been defined as the proteins which are released by a cell, tissue or organism being afterwards crucial on the regulation of different cell processes [34]. Therefore, today it is believed and accepted that in response to injury, MSCs have the capacity to migrate to the damage site and promote the repair process through the secretion of growth factors, cytokines as well as antioxidants [35, 36]. According to Wagner and colleagues [37], the secretion of all these factors may be dependent according to the type and stage of injury. For these reasons, authors believe that beyond cell-cell interaction, the secretome of MSCs could be the main reason of their immunomodulation and regenerative capacity in the lesion site [38, 39]. Although studies suggest that MSCs transcriptome/secretome can be modulated with different environment conditions, it becomes also important to analyze how far these changes can be relevant according to the normal or pathological conditions in which they are being applied [32, 40]. Therefore, it has been suggested that these protective actions promoted by MSCs secreted molecules may explain their remarkable therapeutic plasticity in the CNS [9, 41].

1.1.1. Protein secreted fraction

In 2007 Caplan and Dennis [42] were the first to designate MSCs as trophic mediators. Indeed, these authors considered that despite their potential to differentiate into different cell lineages, these cells are also able to secrete a panel of growth factors and cytokines with direct effects into a variety of mechanisms such as immune system suppression, inhibition of apoptosis, increase of angiogenesis and stimulation of tissue adjacent cells [42]. Crigler and coworkers [43] were the first to demonstrate that BM-MSCs were able to promote neuronal survival and neuritogenesis through the secretion of neurotrophic factors such as BDNF and beta-NGF *in vitro*. Recently, from a

characterization study of the conditioned media (CM) of BM-MSCs, Nakano and coworkers [44] demonstrated that these cells were able to secrete IGF-1, HGF, VEGF and TGF-β, which were the related to higher levels of neuronal survival and neurite outgrowth *in vitro*. In line with this, further studies also showed that the BM-MSCs CM were also able to promote neuronal and glial survival *in vitro* [45, 46]. In addition to these findings, when applied into animal models, BM-MSCs were also able to release a different trophic factors, such as BDNF, FGF-2, GDNF and IGF-1, fact that could explain not only the increase on neuronal survival after lesion, but also the improvement of animal behavior upon cell transplantation [47, 48].

Similar to what has been reported for BM-MSCs growth factors such VEGF, HGF, bFGF, IGF1, TGF-β1 and others have also been found in the adipose stem cells (ASCs) secretome [49, 50]. *In vitro*, Lu and coworkers [51] revealed that ASCs secretome was able to exert an active protection in a PC12 cell line model against to inducement of excitotoxicity mediated by glutamate. This outcome was partially correlated with the presence of different levels of VEGF, HGF and BDNF [51]. At the same time, another study using the same cell line revealed that ASCs-CM was able to induce neuritogenesis, relating this effect with the presence of secreted NGF [52]. Wei and coworkers [53] demonstrated that after incubation with ASCs-CM, it was observed a significant increase in the neuroprotection of cerebellar granule neurons from apoptosis, through the action of IGF-1 released by ASCs. Recently, our group has also revealed, *in vitro,* that ASCs-CM was able to increase the viability of neuronal and glial populations through the presence of NGF, SCF, HGF and VEGF in their secretory profile [54]. *In vivo*, several reports already demonstrated a trophic benefit promoted by ASCs [55, 56]. For instance, Lopatina T *et al.* [57] showed that ASCs were able to stimulate the regeneration of peripheral nerves through the secretion of BDNF, promoting *de novo* axon growth. Finally, concerning Wharton Jelly stem cells (WJ-MSCs) and human umbilical cord perivascular stem cels (HUCPVCs), studies already showed that they are also able to contain neurotrophic factors in their secretome [54, 58, 59]. Recently, Salgado and coworkers [59] verified that the CM of HUCPVCs was able to increase the proliferation and the survival of primary cultures of hippocampal neurons and glial populations. In line with this, Ribeiro *et al.* [54] also showed similar results, demonstrating that HUCPVCs CM was able to secrete NGF and VEGF. Koh and coworkers [58], performing an objective analysis of WJ-MSCs secretome, revealed that the secretion of G-CSF, VEGF, GDNF and BDNF could be correlated with their neuroprotective effect when transplanted *in vivo*. Similar observations were also found by Ding and colleagues [60], which revealed that after transplantation of WJ-MSCs, they were able to promote functional recovery, reduction of lesion site

as well as to express high levels of SDF-1, BDNF and GDNF. Recently, our group demonstrated that the secretome of HUCPVCs was able to increase the secretion levels of neurotrophic factors such as NFG and FGF-2 in the dentate gyrus of the hippocampus, contributing for the increase of neural proliferation, survival and differentiation. Altogether, these outcomes already published, strongly suggest that the soluble factors secreted by MSCs populations may explain their apparently therapeutic effect both *in vitro* and *in vivo*. Nonetheless, a deep analysis of the factors existing in their secretome into different physiological conditions is still lacking. In fact, despite the inexistence of a full characterization of MSCs secretome, studies already shown that the use of MSCs as well as their trophic action could be a potential therapeutic tool in the regenerative processes of some neurodegenerative disorders such as Parkinson's disease, Stroke and Spinal cord injury [47, 61, 62].

1.1.2. Exosomal/Microvesicle fraction

In addition to the paracrine soluble factors that are released by MSCs to the extracellular space, recent efforts on MSCs secretome research suggest that the presence of a second component composed by secreted vesicles [63, 64]. Indeed MSCs are able to secrete large amounts of vesicles (microvesicles, exosomes), either constitutively or after activation of signals [63]. These vesicles secreted by MSCs have been considered as a vehicle of paracrine or endocrine signaling, able to transport defined signaling molecules to target cells or tissues at distant sites [65-67]. Nevertheless, the biochemical composition, the complex of biogenesis of these vesicles as well as their physiological role has not been fully elucidated [64, 68]. Even though their potential as mediators of cell communication has not gone unnoticed however, these vesicles presented remarkable features such as the ability to transfer proteins and functional genetic material such as miRNAs to other cells [69, 70]. In particular, the release of exosomes has received much attention. The secretion of these nanovesicles was described for the first time by Harding and colleagues [71] during the maturation of sheep reticulocytes. Typically, exosomes are formed by inward budding of late endosomes, having a diameter of 40-10 nm, with a density of 1.13-1.19 g/mL in a sucrose solution [72]. At the same time, the most unique function that has been associated to exosomes is their capacity to interact with targeted cells, which putatively enable cell-to-cell communications, which are implicated in important physiological processes such as antigen presentation, genetic exchange, immune responses and angiogenesis [72].

MSCs were recently considered an efficient mass producer of exosomes [73]. Indeed, it has been tested and suggested that MSCs can produce higher amounts of exosomes than other type of cell lines such as myoblasts, human acute monocytic leukemia cell line (THP-1) and human embryonic kidney cell line (HEK) [73]. MSCs-derived exosomes common display surface markers such as CD9, CD63 and CD81 as well as, markers of adhesion molecules such as CD29, CD44 and CD73, which are also expressed on the membrane of MSCs [72]. Although proteomic studies are still scarse, studies have proposed that exosome concentration within the secretome of MSCs varies according to the batch that is being used. [70, 72]. Indeed, Lai and colleagues [70] demonstrated through liquid chromatography-mass spectrometry/mass spectrometry that MSCs-derived exosomes obtained from three different bacthes presented different proteins in their constitution, among which few of them were common to the three different batches.. From the application standpoint, MSCs-derived exosomes have demonstrated promising results in a wide panel of diseases [74, 78-85]. Lai and colleagues [80] were the first to investigate the effects of MSCs-derived exosomes into an animal model of myocardial ischemia/reperfusion (MI/R) injury, where the results revealed that the application of MSCs-derived exosomes were able to neutralize the progression of MI/R, acting as an effective adjuvant therapeutic agent. Similar outcomes were also presented by Arslan and colleagues [78] that showed that the intact MSCs-derived exosomes were able to restore the bioenergetics levels, reduce the oxidative stress and activate pro-survival signaling, thereby enhancing cardiac function after myocardial MI/R injury. In kidney injury (KI), MSCs-derived exosomes also revealed beneficial effects [83]. For instance, Reis and colleagues [83] demonstrated that the exosomes-like microvesicles extracted from the BM-CM, had miRNA that could be implicated in the treatment of KI. Also into immunological problems, MSCs-derived exosomes revealed to be effective [77]. The latter have also shown to have impact on immunomodulation. For instance, through the co-culture of MSC-CM and splenic mononuclear cells, isolated from an animal model of autoimmune encephalomyelitis, Mokarizadeh and colleagues [86] demonstrated that MSCs-derived exosomes were able to inhibit lymphocyte proliferation as well as to promote the secretion of anti-inflammatory cytokines including interleukin 10 (IL-10) and TGF-β. With these observations, the authors suggested that the application of MSCs-derived exosomes could represent a potential tool able to induce peripheral tolerance and to modulate immune responses [86]. In line with is, the application of MSCs-derived exosomes also revealed promising results when applied into graft-*versus*-host disease (GVHD) [77].

Finally, as it was already described previously, when applied into the CNS, MSCs and its secretome play a potential therapeutic benefit in the treatment of neurological impairments. The action of the exosomes is also implied in this therapeutic action [72]. Indeed, studies have suggested that one of the ways of MSCs to communicate with the CNS cells is through miRNAs that are present in the exosomes [87, 88]. Xin and colleagues [87], a middle cerebral artery occlusion (MCAo) model, revealed that after MSCs transplantation there was an increase in the levels of microRNA 133b (miR-133b) into the ipsilateral hemisphere of the rats. In order to link this result with the treatment of MSCs, *in vitro* they verify that the levels of miR-133b were increased in the exosomes derived from MSCs, demonstrating in this way that MSCs induced their effects via exosome-miR-133b transfer, leading to the enhance of neurite outgrowth and functional recovery [87].

1.2. MSC Secretome as potential neuroprotective tool for neurodegenerative disorders such as Stroke, Spinal Cord Injury and Parkinson's Disease

1.2.1. Stroke

Cerebrovascular diseases, such as stroke, represent a kind of lesion that results from blood vessel occlusion or damage, leading to focal tissue loss and death of endothelial cells and multiple neural populations [2, 89] (Figure 1).
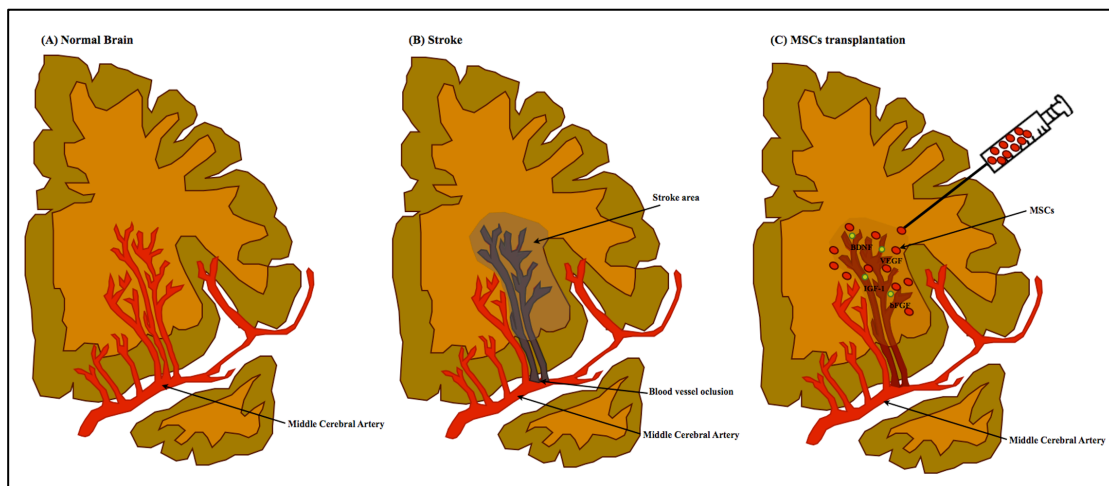
Figure 1. Mesenchymal stem cell-based therapy for stroke. This pathology is caused by occlusion of a cerebral artery, leading to focal tissue loss with death of different neural cells, including neurons and glial cells as well as endothelial cells (B). MSCs transplantation has been shown to have a beneficial role in the reduction of lesion size and in the protection of surviving cells (C). The secretion of growth and trophic factors has been associated with motor and functional recovery, having a key role on neuroprotection and modulation of inflammation.

In addition to their occurrence normally are associated mechanisms like acidosis, caused by anaerobic glucose metabolism, intracellular calcium accumulation and excitotoxicity, which leads to high levels of glutamate release, and excessive production of free radicals and inflammatory mediators [90, 91]. It has been proposed that the transplantation of MSCs could also be a feasible therapeutic option after IS [61, 92]. Indeed, studies have shown that after intravenous administration of BM-MSCs, they have the capacity to migrate to lesion site promoting tissue regeneration and behavioral improvement [93]. Nevertheless, studies have suggested that these cells not only were able to promote the recovery of animal behavior as well as to increase the levels of neurogenesis, providing the survival of neuroblasts and reducing the volume of lesion after IS [94, 95]. In addition to this finding, previous studies also showed that the possible mechanism that could be associated with this phenomenon resides in their capacity to migrate selectively to ischemic lesion through the action of SDF-1, and in their trophic and differentiation capacity into neural/glial cells [96, 97]. Indeed it has been reported in animal models that MSCs are really involved in the production and increase of trophic factors such as IGF-1, VEGF, EGF, BDNF and bFGF, which according Wakabayashi and colleagues [98], seems to be the responsible mechanism in the reduction of the lesion site mainly associated with the capacity of modulation of inflammatory environment and host cells. Regarding ASCs, in a recent report, Leu and colleagues [99] proposed that like BM-MSCs, ASCs therapy also enhances the angiogenesis and neurogenesis processes. In addition to this assumption, these authors' also saw that ASCs applications were able to increase the number of small vessels in the lesion site, considering that the neurological function recover could be explained by the promotion of angiogenesis. Although the exact mechanism of these cells still remains unclear, other studies have suggested that homing properties, cytokines (SDF-1α, IL-1, IL-8) effects, and paracrine mediators (HGF, BDNF, IGF-1, VEGF) could also be processes coupled with ASCs effects, contributing to the tissues regeneration and functional behavior [100-102]. Nonetheless, the mechanisms of action through the secretion of growth factors and cytokines could be an exciting stimulus not only to promote repair through the induction of progenitor cells to differentiate and replace lost tissues but also, in the activation of survival and anti-inflammatory pathways [53]. Wei and colleagues [53] were the first to show that after application of ASC-CM in brain damage, they were able to exert neuroprotection blocking the neuronal damage and tissue loss through the factors present in their composition particularly IGF-1 and BDNF. Regarding WJ-MSCs, Ding and coworkers [60] demonstrated that they may also be beneficial for the treatment of brain ischemia. The high expression of SDF-1, BDNF and GDNF was found after WJ-MSCs implantation,

suggesting that these cells gave the ability to induce the molecular pathways involved in the neuroprotection of neural tissue. In the same line, Koh and colleagues [58] also demonstrated that in fact WJ-MSCs can be seen as a therapeutical alternative to use in stroke, given that they proved that they produce more trophic factors such as G-CSF, VEGF, GDNF and BDNF than BM-MSCs after transplantation. However, despite the fact that WJ-MSCs do not differentiate into functional neurons and remain undifferentiated after transplantation, it was shown that they exhibited an exciting migratory tropism to the lesion site which, related with the production of trophic factors allows the creation of new network between the host neural and transplanted stem cells [58]. Concerning the clinical application of MSCs, few studies have been performed. For instance, in 2005, Bang and colleagues [103] demonstrated that transplantation of MSCs (BM) had no adverse cell response but, and improved the neurological function of the patients. Recently, Lee and colleagues [104] also showed that after long-term application of BM-MSCs, there was a safe improvement in the neurological and in the motor function of the patients. As in the case of SCI patients, the precise mechanism that could explain the recovery of stroke patients remains still unclear; however, some evidences have associated the clinical improvement with the increase of serum levels of SDF-1α as well as with the increase of neurogenesis in the subventricular zone of the lateral ventricle [104]. Although studies defend that the secretion of neurotrophic factors could be the probable reason for the improvement of stroke impairments, more studies are needed in order to clarify the precise action and interaction of MSCs and their factors with the resident cells where they are being implemented [105, 106].

1.2.2. Spinal Cord Injury

Spinal cord injury (SCI) is characterized by long-term functional deficits in ascending and descending motor and sensitive neuronal pathways as a result of accidental injury in most of the cases, leading to a complex cascade of reactions that result in loss of neurons and glial cells, inflammation, demyelination and pain [107, 108] (Figure 2). The occurrence of this kind of lesion creates a non-permissive inflammatory and chemical environment along with abnormal secretion and accumulation of neurotransmitters, which result in a high grade of excitotoxicity that is destructive for neuronal function and regeneration [62, 107]. The application of pharmacological treatments has been, according with the literature the best approach for SCI neuroprotection [109]. However, despite the multiple treatments that were developed and those that are being developed and applied, most of these trials have failed to show a great efficacy in the recovery of sensory–motor

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function, leaving many patients facing significant neurologic dysfunction and disability [109]. Cell-based therapies through the use of MSCs have grown in the last few years as a potential promise for SCI applications [55, 110]. Despite the complexity of SCI lesions, transplantation with BM-MSCs already showed that these cells were able to promote remyelination, axonal sparing and functional recover in different SCI stages [111, 112]. Moreover it has been hypothesized that MSCs have the capacity to migrate to the lesion site, surviving for a long period of time and improving animal behavior [113, 114]. Although studies suggest that MSCs promote functional recovery after transplantation in SCI, the precise mechanism of action still remains unclear [115]. Besides the fact that MSCs are immunosuppressive, studies have shown that they can modify the SCI milieu directly through the release of trophic factors such as BDNF, NGF and VEGF, promoting axonal regeneration, neurite outgrowth and glial scar reduction [43, 116] (Figure 2C).
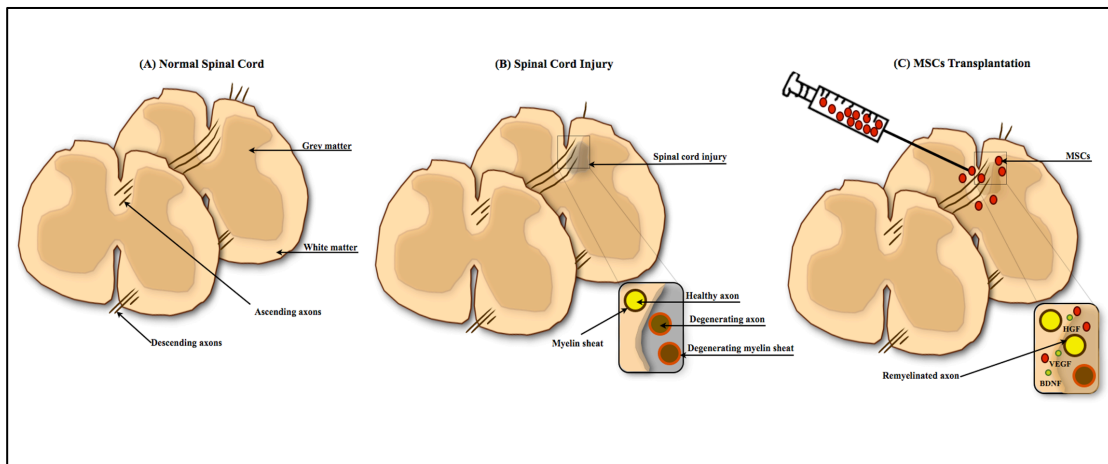
Figure 2. Mesenchymal stem cell-based therapy for SCI. SCI leads to immediate neuronal and glial cell death with interruption of ascending and descending pathways, followed by intense inflammatory reaction and glial scar formation (B). The transplantation of MSCs has been described to contribute for the recruitment of new neural stem cells, neuronal and glial cells, promoted by cell-cell interaction or by the release of cytokines and trophic factors (C). The secretion of this cytokines and trophic factors seems to be the main effector of neuroprotective processes and for reduction of the glial scar, modulation of the inflammation and stimulation of the remyelination (adapted from Lindvall and Kokaia [2]).

Lu and coworkers [117] showed that after transplantation of BM-MSCs, they were able to secrete NGF, NT-3 and high levels of BDNF, contributing to the extent of host axonal growth, and enhancing the growth of host serotonergic, coerulospinal, and dorsal column sensory axons after SCI. Similar findings were also reported by Neuhuber *et al.* [118], which demonstrated that the CM of BM-MSCs were able to promote axon growth and functional recovery due to the presence of BDNF, VEGF, IL-6,

MCP-1, SCF and SDF-1α in their constitution. Recently, Gu *et al.* and Park *et al.* [119, 120] showed that these cells were able to secrete neurotrophic factors such as HGF, VEGF, BDNF and GDNF, suggesting that this secretory activity could be the main reason to promote axonal regeneration of spinal neurons both *in vitro* and *in vivo*. Concerning ASCs, it was also shown that these could be similar to Schwann cells, secreting neurotrophic factors such as BDNF and improving re-myelination [57]. Moreover, predifferentiated ASCs can be yet another promising approach to axonal regeneration that has been associated with their paracrine action [55]. With WJSCs, so far only two studies have examined their use in SCI. Nonetheless, the outcome of these studies indicates that WJSCs transplantation into SCI were able to potentiate repair and recovery due to the release of trophic factors such as NT-3, VEGF, bFGF and BDNF [113, 121]. In addition to these promising results observed on animal models of SCI, clinical trials have already showed promising results for the transplantation of MSCs, namely BM-MSCs, in cases of SCI [122-124]. In a pilot study, Saito and colleagues [125] demonstrated that the autologous transplantation of BM-MSCs by lumbar puncture seems to be safe and relevant for the patients, leading to motor improvement. Similar results were also obtained by Karamouzian and colleagues [123] in subacute SCI stages. In this study, after the transplantation of the BM-MSCs, the authors observed that 45,5% of the patients presented improvements in their neurological and motor function [123]. However, the precise mechanism that could explain this recovery after transplantation is still unclear. As discussed in respect of experiments with animal models, also on clinical trials some authors considered that the transdifferentiation of MSCs into neural lineages or their secretome through the release of growth and trophic factors seems to be the main explanation for the improvement of the condition of the patients [122, 126]. Although these cells continue in projection, nowadays, evidences suggest that MSCs-based therapies could be in fact a new approach for the regeneration of SCI tissue damages, providing neuroprotection and trophic support for the prevention of cell death and axonal degeneration [127, 128].

1.2.3. Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by the progressive degeneration of dopaminergic neurons (DA) in several dopaminergic networks, most intensively in the mesostriatal pathway at the level of the *substantia nigra pars compacta* (SNc) [129, 130] (Figure 3). As a result, patients develop several motor complications including rigidity, bradykinesia, and

postural instability [131]. The application of Levodopa (L-dopa) or DA agonists has been considered the *gold standard* treatment for PD as well as for the reduction of its major symptoms [132]. However, even if it improves the behavior performance, most of these treatments have shown some limitations such as undesirable side effects, non-total recovery of PD symptomatology, long-term inefficiency, as well as, inability to recover lost DA neurons or to protect the remaining ones [133-135].
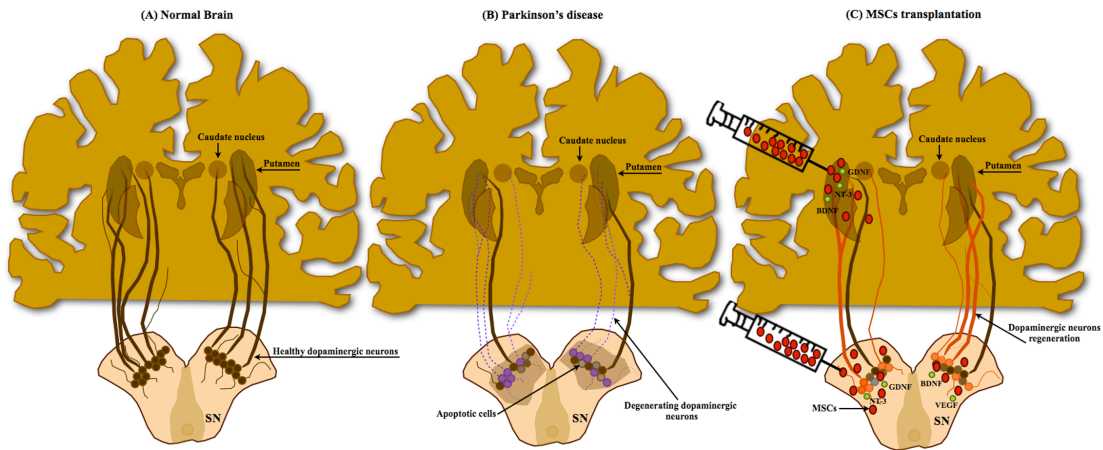
Figure 3. Mesenchymal stem cell-based therapy for PD. PD is characterized by a progressive neuronal death of dopaminergic neurons in multiple dopaminergic networks, most intensively in the nigrostriatal pathway leading to motor complications (B). The transplantation of MSCs has emerged as possible therapeutic tool due to their proliferation and differentiation capacity (C). The ability to release growth and trophic factors seems to be one of the reasons for their contribution to the protection/survival of the preexisting dopaminergic neurons in lesioned areas, leading to functional amelioration and improvement of motor function.

Due to these limitations, and based on the rationale that cell transplantation approaches could be beneficial in restoring degenerated DA pathways and ameliorating behavioral outcome, some clinical trials were conducted in the 90's [136-139]. These were based on the transplantation of human fetal mesencephalic tissue and the results were quite promising, with patients displaying, increases in DA synthesis, improved motor function and reduction of L-dopa doses required [132]. These studies confirmed the relevance and feasibility of cell-based transplantation techniques to treat PD, but because of methodological and ethical related with manipulation of human fetal tissue other cell sources needed to be found [140].

MSCs cell-based applications have thus emerged as possible potential therapy for PD [141-143] (Figure 3C). Although the literature continues to look carefully on its application as a tool for the

treatment of PD in humans, several studies in PD animals' models have shown that transplantation of BM-MSCs, ASCs or WJ-MSCs, seems to contribute to neuroprotection and/or neural recovery [144-146]. Indeed, it was already demonstrated that after transplantation, these cells were able to increase the levels of *tyrosine hydroxylase* (TH) and dopamine levels when compared with untransplanted animals [147, 148]. For instance, with ASCs, McCoy and colleagues [145] demonstrated that, after autologous transplantation these cells were able to attenuate 6-OHDA-induced nigrostriatal pathway degeneration and behavioral deficits even without dopaminergic differentiation. Recently, Thomas and colleagues [149] reported that ideally, MSCs could only be considered an alternative and a credible source of DA neuronal replacement cells, when their ability to transdifferentiate into neuronal lineages is clarified not only in terms of morphology but also functionally. Thus, while some studies propose the differentiation capacity of MSCs into DA neurons or neural lineages as principal outcome for PD recovery, nowadays, in addition to this idea, it has also been suggested that this functional recovery promoted by MSCs in PD can also be caused by the release of trophic factors *in vivo* [33, 47]. For instance, Cova and colleagues [47] using a 6-OHDA model demonstrated that BMSCs have the capacity to interact with the surrounding of the lesion site, which indicates their ability to maintain their phenotype even under non-physiological conditions. In addition to this finding, these authors also observed an active secretion of trophic factors like EGF, VEGF, NT3, FGF-2, HGF, and BDNF for a long period of time *in vivo*, demonstrating that BM-MSCs did not require the acquisition of neuronal phenotype to contribute to the maintenance of dopaminergic phenotype [47]. Wang *et al.* [150] demonstrated that BM-MSCs could exert neuroprotection against 6-OHDA-exposed dopaminergic neurons both *in vitro* and *in vivo* through anti-apoptotic mechanisms promoted by the expression of SDF-1. Likewise, using the same model, Weiss and colleagues demonstrated that WJ-MSCs are also able to secrete trophic factors *in vivo* [151]. Contrary to the observed in the previous study, these authors associate the recover of TH-stained cells and behavioral recovery to the significant secretion of GDNF and FGF-20 [151]. In line with this, the protection and survival of dopaminergic neurons through the secretion of GDNF, BDNF and NGF was also achieved with ASCs [145]. Indeed, studies show that intrastriatal transplantation of hMSCs as a good method for the restoration of the function of nigrostriatal dopaminergic networks, which associated to their secretion capacity *in vivo* seems to be one of the main causes to the improvement of behavioral impairments in PD models [152, 153]. For that reason, it is strongly suggested that hMSCs may really represent a valid tool in the neuroprotection and survival of the dopaminergic neurons through the release of a multiple panel of trophic factors

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[154]. Nowadays, studies have suggested the genetic modification of hMSCs as a new strategy to secrete specific trophic factors such as GDNF into the striatum and SNc, having in view the long-term restoration of PD [155, 156].

1.3. Bioreactors: an insight in the MSCs cell culture, growth, metabolism, secretome and clinical applications

Stem cell plasticity it is one of the most important characteristics that makes MSCs a therapeutic potential tool for CNS applications [27, 157-160]. Indeed, the capacity to adapt and grow into different culture conditions (i.e. different oxygen concentrations; matrix of adherence, etc.) indicates that MSCs could be able to change/modulate their own secretome according with the conditions in where they could be implemented [161-163].

One of the big limitations of the clinical therapeutic use of MSCs is the requirement of large number of cells [163]. Although different percentages of cells could be isolated from the different sources of the body (bone marrow, adipose tissue or umbilical cord), a robust and good manufacturing practice (GMP) associated to a favorable scalable MSC expansion process should be used for the development of an off-shelf therapeutic product [164, 165]. The use of static culture flasks is still used as the *gold standard* methodology in the expansion of MSCs. However the use of this method of expansion presents several limitations such as the requirement of an extensive manual handling, limited available surface area, gas diffusion, scale-up, automation, process control and reproducibility [164]. In order to overcome these limitations imposed by the static culture, the use of bioreactors has been introduced as a technology able to improve the quality of expansion of MSCs as scalable, cost-efficient and regulated process [163, 166-168]. Bioreactor has been defined as any device able to provide the physiological requirements of the cell (e.g. nutrients, growth factors and mechanical environment) that allows studying cellular functions, scaling production of cells as well as their products [166]. Different devices have been used in the expansion and study of MSCs namely, stirred bioreactors, rotating bioreactors, wave bioreactors as well as perfusion bioreactors (e.g. column, parallel plate, hollow fibre and micro-fluidic), being their use dependent of the application requirements [168, 169]. However, the use of the stirred spinner devices is the most common for scalable expansion of MSCs [163, 170]. Indeed, this kind of vessels offers attractive advantages over other culture devices, allowing a ready scalability and higher homogeneity, minimizing concentration gradients (e.g. pH, gas diffusion and metabolites) [163, 171]. In addition to this, these stirred bioreactors can be easily operated and scaled-up and thus the generation of a

desired number of cells can readily achieved [163]. This kind of strategy avoids vessel-to-vessel variability, saving time and labor [163, 172]. Associated to this, this equipment's' can be readily linked to a computer-controlled online monitoring system, enabling a tight control of the majority variables present in culture such as pH, temperature, dissolved oxygen and carbon dioxide concentrations [163, 165, 172, 173]. According with Jung and colleagues [163] this kind of process could be operated in multiple modes (batch, fed-batch or perfusion), allowing the implementation of a well-mixed environment that can ensure an appropriate supply to cells, reducing the levels of waste metabolic products such as lactate and ammonium, which ate normally associated with undesired effects on cell proliferation and differentiation. This idea was also defended by Eibes and colleagues [165], who indicated that the MSCs energy metabolism under dynamic culture was significantly more efficient than the conventional culture flasks. Nevertheless, on the basis of these advantages promoted by the use of stirred bioreactors in the expansion and scale-up of MSCs is the use of microcarriers (MC) [165, 170, 173]. At the same time, the use of scaffolds as a matrix of adherence to MSCs has been also described [166, 174-177]. However, studies have shown that the use of MC is more common in the study of MSCs properties [165, 173]. Indeed, these small beads (100-400 μm of diameter) can be placed in the cell culture medium, providing an available surface where cells could attach and subsequently grow as a pseudosuspension culture condition [163]. In addition to this, it was also defended that the use of these beads could offer a superior means of producing large quantities of adherent cells. Moreover, as defended by Schop and colleagues [178], the use of this beads are less likely to foul filtration devices used to separate cells from the media and secreted products [178, 179].

Regarding the effects of using bioreactors in the MSCs expansion, studies have demonstrated that these devices are able to modulate their behavior when compared to the static culture conditions. Indeed, as demonstrated by Hupfeld and colleagues [164], the MSCs expansion process in the bioreactors significantly influenced the characteristics of MSCs when compared to the static culture flasks. On the basis of this modulation, is the dynamic environment that is created through mechanical stimulation, shear stress, flow and mass transport [168]. Together, all these conditions are important to ensure that cell metabolism is kept within the physiological range by provision of metabolic substrates and removing the toxic degradation products [172, 178]. Meanwhile, in the static culture systems these conditions are limited [164, 171].

It was recently demonstrated that MC expansion processes into bioreactors significantly influenced the characteristics of MSCs when compared to the flask-expanded cells [164]. Indeed, it was

showed that after expansion in the bioreactors MSCs were able to grow as well as to differentiate in the three main characteristic lineages (osteogenic, adipogenic and chondrogenic) [164, 180-183]. In addition to this, it was also demonstrated that the use of dynamic culture condition through MC expansion does not affect the expression of MSCs surface characteristic markers [164, 184]. The use of bioreactors have also demonstrate to modulate the secretion profile of MSCs, when compared to static culture conditions [164]. Indeed, in the only study published so far it was demonstrated that the use of a dynamic culture condition increased significantly the angiogenic capacity of UC and amniotic membrane (AM) MSCs once. In fact higher concentration of VEGF, FGF-2, MCP-1 and SDF-1 were observed when compared to the static culture condition [164]. Thus, it is logical to hypothesized that bioreactors may act as modulators of MSCs secretome [164]. Therefore, to consider the use of bioreactors as a potential tool for the development of new MSCs-based therapies (including their secretome as well), optimal designs must be performed in order to understand the effects of these dynamic culture conditions in the modulation of MSCs properties such as growth requirements, metabolism, differentiation potential and secretome. Once adequately addressed, these findings will be useful for the generation of MSCs products with tailored properties for future clinical applications.

1.4.   Conclusions and perspectives

Neurodegenerative diseases are indeed chronic and acute insults against the homeostasis of the CNS, capable of promoting a large amount of cell death in neural populations in the brain and spinal cord. Thus, as a result of the limited capacity of self-repair, the design of new therapeutical strategies represents a major challenge for CNS regenerative approaches. Due to their capacity of self-renew and multilineage differentiation potential, MSCs have been suggested as possible therapeutic tools for regenerative medicine, representing a promising cell source for the creation of new cell-based therapies [7, 140, 159, 187]. When compared to other sources they do not imply the ethical and moral questions raised by embryonic stem cells (ESCs) or the technical issues regarding the isolation and further *in vitro* expansion of neural stem cells (NSCs). Throughout the years it has become evident that MSCs might have a role in future stem cell based therapeutic strategies for CNS regeneration [188]. Initially these effects were attributed to a possible neural differentiation of MSC-like cells (Figure 4) [189]. Nevertheless, these apparently neuronal differentiation capacities both *in vitro* and *in vivo* conditions still remain under discussion [190]. In

addition to this assumption, authors also suggest that cell fusion could also be another phenomenon that could lead to a false immunopositive characterization of MSCs as neural cells [32]. Nowadays, there is ample evidence that most of the effects promoted by MSCs may reside in their secretome (Figure 4) [46, 53, 59, 191].
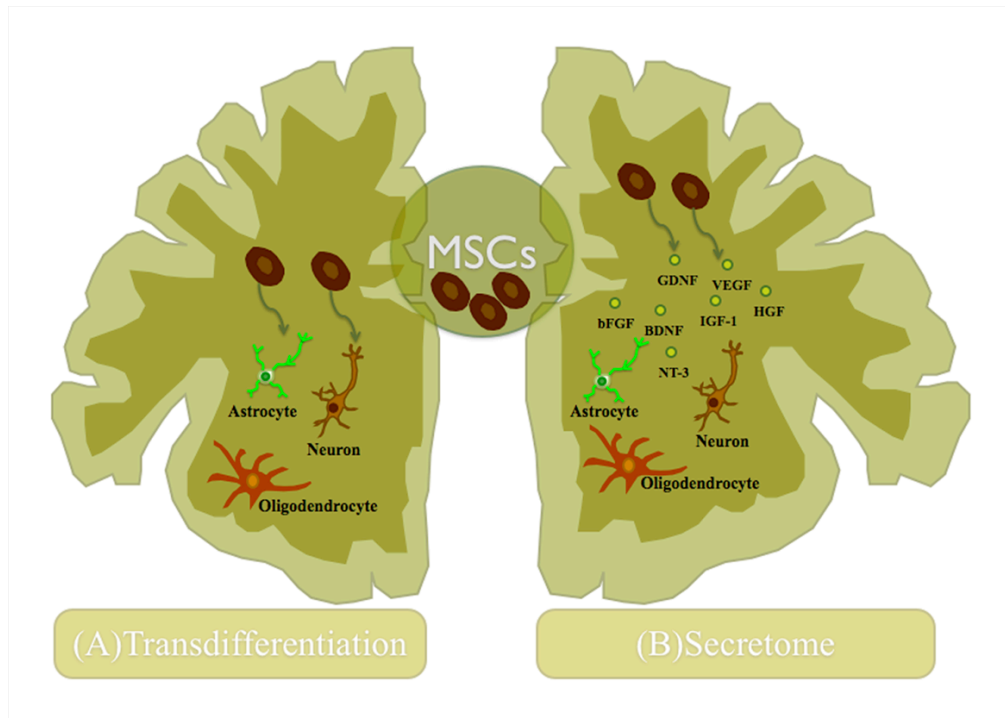
Figure 4. Mechanisms of action of MSCs in the CNS. (A) The transdifferentiation capacity of MSCs into neuronal and glial lineages both *in vitro* and *in vivo* was described over the years as the probable explanation by their beneficial outcomes after transplantation in the CNS, although this concept remains still unclear. (B) The trophic action of MSCs has been increasingly accepted nowadays as a new concept to the regeneration of the CNS. The secretion of growth and neurotrophic factors by these cells has been described as an assistant in the nervous tissue regeneration through the activation/modulation of some endogenous processes like the promotion of neurogenesis, angiogenesis and immunomodulation, contributing in this way to the neuroprotection and regeneration of the CNS.

Indeed it was already shown both *in vitro* and *in vivo* that MSCs secrete a variety of neurotrophic factors such as IGF-1, BDNF, VEGF, GM-CSF, FGF2 and TGF-B, having a prominent role in the inhibition of scarring, apoptosis, immune response modulation, neurogenesis and angiogenesis [9, 42, 140, 187]. Concerning the clinical application of MSCs, few studies were done so far and only in stroke and SCI (Table 1). However, there are still many variables regarding its application as a new therapy for neurological disorders that need to be addressed. Despite the promising results already

described, the type of MSCs, culture conditions, transplantation parameters (e.g. cell numbers and site), timing of treatment as well as the route of delivery represent some of the issues that need to be clarified in order to create a safe therapy [192]. Although the neural differentiation of MSCs is still considered a possible explanation to some authors, their secretome seems to be nowadays the main reason of their therapeutic effect after transplantation [32, 47, 104, 126]. Studies have shown that the molecules secreted by MSCs seem to assist the nervous tissue regeneration through the activation/modulation of endogenous neurorestorative processes [126, 193-195].

Table 1. Examples of clinical approaches using mesenchymal stem cells for stroke and SCI repair and regeneration.

Kind of injury	Outcomes	Reference
Stroke	No adverse cell response; reduction of infarct size; neurological function improvement	[103]
	Safe application of MSCs after long period; no zoonoses after treatment; increase of functionality and survival; clinical improvement correlated with the increase of SDF-1α plasma levels	[104]
Spinal cord injury	No adverse reaction to the transplantation in the CSF; the release of some trophic factors was associated with neuronal/glial neuroprotection	[124]
	Patients followed up for 1–4 years did not present any kind of adverse response; BM–MSCs were highly effective, promoting a remarkable recovery in the patients; intrathecal administration of MSCs is a safe method	[125]
	No adverse reaction to the transplantation such as fever or headache; most of the patients showed amelioration in their neurological function after transplantation	[123]

In this sense, a thorough characterization of these MSCs' secretome becomes necessary not only to identify the full scope of factors released but also to clarify if in fact the molecules released are able to modulate not only the immune response but also, different cell processes such as cell proliferation, differentiation and survival into different physiological conditions [153, 196, 197]. At the same time, new protocols must be developed in order to examine the MSCs secretome *in vivo*, as well as strategies to modulate it [191]. By doing this, it will be possible to understand if in fact the secretome of these cells may be used as a new therapeutic strategy in CNS regenerative medicine.

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SECTION II

Chapter 2

Teixeira FG., Carvalho MM., Neves-Carvalho A., Panchalingam KM., Behie LA.,

Pinto L., Sousa N., Salgado AJ. (2014)

Secretome of Mesenchymal Progenitors from the Umbilical Cord Acts as Modulator of Neural/Glial Proliferation and Differentiation

Secretome of Mesenchymal Progenitors from the Umbilical Cord Acts as Modulator of Neural/Glial Proliferation and Differentiation

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Abstract

It was recently shown that the conditioned media (CM) of Human Umbilical Cord Perivascular Cells (HUCPVCs), a mesenchymal progenitor population residing within the Wharton Jelly of the umbilical cord, was able to modulate *in vitro* the survival and viability of different neuronal and glial cells populations. In the present work, we aimed to assess if the secretome of HUCPVCs is able to 1) induce the differentiation of human telencephalon neural precursor cells (hNPCs) *in vitro*, and 2) modulate neural/glial proliferation, differentiation and survival in the dentate gyrus (DG) of adult rat hippocampus. For this purpose, two separate experimental setups were performed: 1) hNPCs were incubated with HUCPVCs-CM for 5 days after which neuronal differentiation was assessed and, 2) HUCPVCs, or their respective CM, were injected into the DG of young adult rats and their effects assessed 7 days later. Results revealed that the secretome of HUCPVCs was able to increase neuronal cell differentiation *in vitro*; indeed, higher densities of immature neurons (DCX$^+$ cells) and mature neurons (MAP-2$^+$ cells) were observed when hNPCs were incubated with the HUCPVCs-CM. Additionally, when HUCPVCs and their CM were injected in the DG, results revealed that both cells or CM were able to increase the endogenous proliferation (BrdU$^+$ cells) 7 days after injection. In the group injected with HUCPVCs-CM it was possible to observe an increased number of newborn neurons (DCX$^+$ cells) and astrocytes (GFAP$^+$ cells) when compared to sham group. Despite proteomic/secretome studies performed on HUCPVCs are still scarce, through Western blot, we demonstrated that after CM or HUCPVCs transplantation, there was an increase of fibroblast growth factor-2 (FGF-2) and, to a lesser extent, of nerve growth factor (NGF) in the DG tissue. Concluding, our results have shown that HUCPVCs and/or their secretome were able to potentiate neuronal survival and differentiation *in vitro*, as well as *in vivo* adult-hippocampal neurogenesis.

1. Introduction

It is well established that the mammalian adult brain has the ability to generate new neurons [1]. In adult mammals, the active neurogenic process occurs mainly in the subependymal zone (SEZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [2, 3]. Nevertheless, the fact that adult neurogenesis is restricted to specific brain regions implies a low regeneration capacity of the central nervous system (CNS) [4]. Therefore, there has been an increasing interest on the development of therapies to promote CNS regeneration. Several studies have proposed the use of adult stem cells as a possible tool for CNS regeneration, and within it, mesenchymal stem cells (MSCs) [4-6] have emerged as a promising therapeutic option [7-9]. In fact, over the last decade, there has been a substantial effort to assess the impact of MSCs for CNS repair [10-12]. Different studies have demonstrated that bone-marrow (BM-MSCs) and adipose stem cells (ASCs) produce a significant recovery of neurological impairments in animal models of stroke [13, 14], demyelination [15, 16], Parkinson's disease (PD) [17, 18] and spinal cord injury (SCI) [19, 20].

The stem/progenitors cells present in the Wharton Jelly of the umbilical cord, known as Wharton Jelly Stem Cells (WJ-MSCs) and Human Umbilical Cord Perivascular Cells (HUCPVCs), have been suggested as possible populations of interest for CNS applications [21-23]. Like BM-MSCs and ASCs, these populations are also defined as MSCs [24-26]. They are plastic adherent, positive for mesenchymal stem cell markers (CD73, CD90 and CD105), negative for hematopoietic markers (CD45, CD34, CD14) and they also have the capacity to differentiate towards osteogenic, chondrogenic, and adipogenic lineages [25-27]. Concerning its application into the CNS, Weiss and coworkers [28] revealed that MSCs isolated from the bulk of the porcine WJ matrix, were able to survive in the brains of rats after transplantation without concomitant immunosuppressive therapy. In addition, it was also shown that these stem cells were able to improve the condition of hemiparkinsonian rats [28], to modulate the inflammatory brain reaction after global ischemia [29] as well as, to potentiate axonal regeneration on spinal cord injury (SCI) [30, 31]. However, little is known regarding the real mechanisms underlying the interactions between umbilical cord stem cell populations and the residing neuronal and glial cells. Recently, it has been suggested that most of the effects that may be promoted by these cells could be mediated by their secretome [23, 32-34]. For instance, in a stroke model, Koh *et al.* [35] and Ding *et al.* [36] showed that after transplantation

MSCs were able to express granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), glial-derived neurotrophic factor (GDNF), stem cell-derived factor 1 (SDF-1) and brain-derived neurotrophic factor (BNDF). Moreover, the authors considered that these growth factors were the main responsible for the neuroprotection and reduction of the lesion site as well as, for the improvement of animal behavior and neuroplasticity [35]. Similar findings were also reported into animal models of PD, demonstrating that after cell transplantation there was an increase on the local tissue expression of GDNF and fibroblast growth factor 20 (FGF-20), leading to the protection of tyrosine hydroxylase (TH) cells and to behavioral amelioration [28]. Adding to this, we have recently shown recently that the secretome of these cells was able to increase, *in vitro*, the survival of neuronal and glial populations through the secretion of different trophic factors and, possibly, extracellular vesicles [33, 37]. Thus, based on these results, we wanted to further explore if the secretome of mesenchymal progenitors, isolated from the Wharton Jelly found within the perivascular regions of the umbilical cord (HUCPVCs), would be able to: 1) induce neuronal differentiation of human telencephalon neural progenitors (hNPCs) and 2) modulate phenomena such as neuronal/glial survival and differentiation in the hippocampal neurogenic niche (DG) upon an injection in the form of conditioned media (CM) or after HUCPVCs transplantation.

Results revealed that the secretome of HUCPVCs was able to induce neuronal differentiation of hNPCs *in vitro*. Moreover, animals that were injected with the secretome of the cells under study into the hippocampal DG, displayed similar levels of proliferation, neuronal/glial survival and differentiation comparing to those transplanted with MSCs. Finally the injection of the secretome also lead to an enhanced expression of FGF-2 in the resident DG.

2. Materials and Methods

2.1. Isolation and primary culture of human umbilical cord perivascular stem cells (HUCPVCs)

HUCPVCs were obtained from Prof. John E. Davies (University of Toronto, Toronto Canada). Cells were isolated from umbilical cords collected after c-section, with the consent of parenting entities. The isolation of HUCPVCs was performed based on the protocol established by Sarugaser *et al.* [27]. Briefly, the umbilical cord was cut into small segments (4-5 cm) to expose both arteries and

vein, rejecting the epithelium. Once isolated the vessels, with a sterilized suture, the ends of the vessels were fixed creating a short loop in order to reduce the contamination by erythrocytes. Vessels were then placed in a collagenase solution (0.5 mg/ml for arteries and 0.75 mg/ml for veins; Sigma, USA) and incubated at 37 ºC for a period of 16-18 hours. Completed this period, the vessels were removed from the suspension and diluted in PBS without Mg^{2+}/Ca^{2+} (Invitrogen, USA). Finally, HUCPVCs were ressuspended in Alpha-Mem medium (Invitrogen, USA) supplemented with 1% of antibiotic/antimycotic (Invitrogen, USA) and 10% of fetal bovine serum (FBS) (Invitrogen, USA), and plated on culture at a density of 4.0×10^3 cells/cm². Subsequently, the culture medium was renewed every three days and the culture maintained at 37 ºC, 5% CO_2, 95% air and 90% relative humidity until confluence.

Mesenchymal phenotype of HUCPVCs was screened by differentiating them towards osteogenic and adipogenic lineages. These cells were incubated with Mesencult® MSC Basal medium with Mesencult® adipogenic stimulatory supplements and Mesencult® osteogenic kit (Stem Cell Technologies, Canada) during 2 weeks. At the end of this period, osteogenic differentiation was assessed through Alizarin Red staining (Sigma, USA), while adipogenic differentiation was assessed through Oil Red O staining (Sigma, USA).

2.2. HUCPVCs Conditioned Media (HUCPVCs-CM) collection

The conditioned media (CM) used was collected from HUCPVCs cultures in passage five (P5) previously plated and kept at a density of 4.0×10^3 cells/cm² for three days in Alpha-Mem medium (Invitrogen, USA) supplemented with 1% of antibiotic/antimycotic (Invitrogen, USA) and 10% FBS (Invitrogen, USA). After this, the flasks were washed three times in Neurobasal A medium (Invitrogen, USA) for five minutes and then, washed five times in PBS without Mg^{2+}/Ca^{2+} (Invitrogen, USA). Neurobasal A medium supplemented with kanamycin (1%) (Invitrogen, USA) was added and 24 hours after, the medium was collected and frozen at -80 ºC until use.

2.3. Primary culture of Human Telencephalon Neural Progenitors (hNPCs) and incubation with HUCPVCs-CM

hNPCs were obtained from Prof. Leo A. Behie (University of Calgary, Calgary, Canada). Cells were isolated from the telencephalon region according with the protocols and strict ethical guidelines previously established [38-40]. hNPCs were thawed at 37ºC and the content placed in a T-25 flask

(Nalge Nunc, Rochester, NY) containing 5 mL of a serum-free medium PPRF-h2 [38]. After two days, the cells were harvested and mechanically dissociated into a single cell suspension, and subcultured into fresh cell growth medium (PPRF-h2). Every 4 days, the T-flaks were fed by replacing 40% of the spent medium with fresh growth medium. After 14-20 days of growth in the culture flasks, hNPCs were passaged and plated on a pre-coated (poly-D-lysine hydrobromide (100 μg/mL) and laminin (10 μg/mL) - Sigma, USA) 24-well plate at a density of 1.0×10^5 per well during 5 days with the HUCPVCs-CM at 37 ºC, 5% CO_2, 95% air and 90% relative humidity. Neurobasal A medium with 1% of kanamycin was used as control group.

2.4. Stereotaxic surgeries

All experiments were conducted using 8 weeks old male Wistar rats (Charles River, Spain), and obtained previous consent from the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council. The animals were housed and maintained in a controlled environment at 22-24°C and 55% humidity, on 12h light/dark cycles and fed with regular rodent's chow and tap water ad libitum. Animals were handled for 1 week prior to the beginning of the injections, in order to reduce the stress induced by the surgical procedures. For the cerebral injections three experimental groups were used (n=5/group): 1) Sham, 2) HUCPVCs and 3) HUCPVCs-CM. Adult rats were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg). Using a stereotaxic system (Stoelting, USA) and a Hamilton syringe (0.5μl Hamilton, Switzerland) all injections made in these three groups were bilateral according to previously determined coordinates (Anterior/Posterior (AP)= 3.5mm; Dorsal/Ventral (DV)= 3.5/3.1mm; Lateral (L)= 2.0mm) [41]. Coordinates had been previously confirmed by a preliminary surgery with methylene blue injected into the desired area (data not shown). The volume injected per DG was 0.5 μl with a rate of injection of 0.25 μl per minute. Two minutes were allowed after each injection in order to avoid any backflow up the needle tract. Sham group was only injected with 0.5μl of Neurobasal A medium; HUCPVCs group with 5.0×10^4 cells (in 0.5μl of Neurobasal A medium) and finally, the CM group were injected with 0.5μl of HUCPVCs-CM. At the

end, the animals were sutured and then injected with 100µl of anti-sedating (Orion Pharma, Finland) in order to recover from surgical procedure.

2.5. BrdU administration and histological procedures

Adult rats received intraperitoneally (i.p.) 100mg/kg of BrdU (Sigma, USA) 24h before the sacrifice. 7 days after of the stereotaxic injection animals were anaesthetized with sodium pentobarbital (Eutasil, 60 mg/kg i.p.; Ceva Saúde Animal, Portugal) and perfused with paraformaldehyde (4%) (Merck, Lisbon, Portugal) diluted in PBS (0,1x) in order to fix the tissues. Subsequently, the brains were stored in sucrose solution (30%) before heading to histological processing.

2.6. Immunostaining

hNPCs were fixed in 4% paraformaldehyde for 15 min, and then permeabilized by incubation with 0.1% Triton X-100 in PBS for 5 min at room temperature, and washed three times in PBS. hNPCs were then blocked with 10 % of fetal calf serum (FCS, Invitrogen, USA) in PBS, being followed by a 1h incubation (at room temperature) with the primary antibodies: rabbit anti-doublecortin (DCX; 1:500, Abcam, USA) to detect immature neurons and mouse anti-rat microtubule associated protein-2 (MAP-2; 1:500, Sigma, USA) to detect mature neurons. hNPCs were then washed in PBS three times and incubated with the secondary antibodies namely, Alexa Fluor 488 *goat anti-rabbit* (IgG, Invitrogen, USA) and Alexa Fluor 594 *goat anti-mouse immunoglobulin* G (IgG; Invitrogen) during 1h at room temperature and then 10 minutes in *4-6-diamidino-2-phenylindole-dihydrochloride* (DAPI; Invitrogen, USA). Samples were then observed under an Olympus BX-61 Fluorescence Microscope (Olympus, Germany). For this purpose, three coverslips and ten representative fields per condition were chosen and analyzed. Results are shown as percentage of DCX or MAP-2 positive cells over the total number of cells per field of observation (n=3).

Regarding the *in vivo* experiment, coronal sections were obtained by vibrotome (VT1000S, Leica, Germany) with a thickness of 40µm and processed as free-floating sections. As first approach, sections were permeabilized in PBS-T (0.2%) (3 times for 10 minutes), and were subsequently

incubated with 10% FCS/PBS during two hours for endogenous blocking. After that, sections were incubated overnight at 4°C with primary antibodies namely, DCX (1:300) for immature neurons, *rabbit anti-rat glial fibrillary acid protein* (GFAP; 1:200 Millipore, USA) for astrocytes detection; *Human nuclear antigen* (HNA; 1:100, Millipore, USA) for HUCPVCs detection; and *5bromo-2-deoxyuridine* (BrdU; 1:100, Abcam, USA) for proliferation. Sections were then incubated with secondary antibodies: Alexa Fluor 568 *goat anti-mouse immunoglobulin* G (IgG; Invitrogen, USA), Alexa Fluor 488 *goat anti-rabbit* (IgG, Invitrogen, USA) during 2h at room temperature and then 10 minutes in *4-6-diamidino-2-phenylindole-dihydrochloride* (DAPI; Invitrogen, USA). Images were obtained with a confocal microscope (Olympus FV1000, Germany) using the software FV10-ASW 2.0c (Olympus, Germany), presenting the hippocampal DG (five sections per animal were analyzed for a n=5/group). Then, SGZ/GCL area was defined and the cell counts were made in these areas.

2.7. Western Blot

Rat hippocampi (n=4/group) were homogenized in cold RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% NP-40, PMSF and a protease inhibitors cocktail (Complete; Roche). Protein concentration was determined using the Bradford assay (BioRad). Samples were sonicated for 5 min, boiled for 5 min at 100°C, and centrifuged for 10 sec before loading. Fifty micrograms of total protein were loaded into 15% SDS-Page gels and then transferred to nitrocellulose membranes. After overnight incubation at 4°C with the primary antibodies: rabbit anti-FGF2 (1:100, Abcam), rabbit anti-NGF (1:100, Abcam), the secondary antibodies were incubated 1h at room temperature at the following dilutions: anti-rabbit (1:10.000, BioRad) and anti-mouse (1:15.000, BioRad). Antibody affinity was detected by chemiluminescence (ECL kit, BioRad). Band quantification was performed using ImageJ software according to the manufacturer's instructions using alpha-tubulin as the loading control.

2.8. Statistical analysis

Statistical evaluation was performed using One-way ANOVA and Student's t-test through the program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Data is presented as mean ± S.E.M. Significance value was set at $p < 0.05$.

3. Results

3.1. Isolation and characterization of HUCPVCs

The isolation of HUCPVCs from the perivascular layer of the human umbilical cord has previously been reported [27]. In the present study, it is possible to observe HUCPVCs in culture, presenting the morphological characteristics of MSCs (Figure 1A, fibroblast predominantly long and spindle shaped). The differentiation into different mesodermal lineages is considered another basic characteristic of MSCs [42, 43]. We were also able to observe the multipotent character of HUCPVCs, obtained through the differentiation towards the osteogenic (Figure 1B, formation of bone nodules) and adipogenic (Figure 1C, formation of fat droplets) lineages. These results confirm that HUCPVCs are capable to differentiate into different lineages similarly to other MSC populations [26, 44, 45].
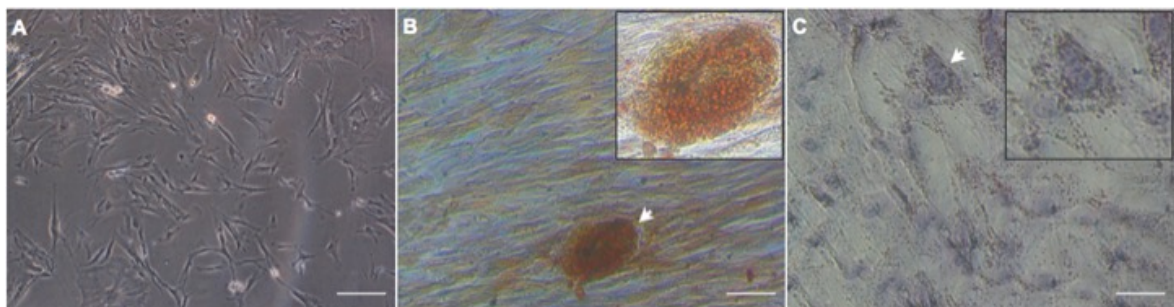
Figure 1. Multipotent character of HUCPVCs *in vitro.* (A) HUCPVCs under standard culturing conditions, and differentiated into (B) osteogenic and (C) adipogenic lineages. Osteogenic differentiation was assessed using Alizarin Red staining, while adipogenic phenotype was assessed through the use of Oil Red O staining (Scale bar: (A) 100 μm, (B, C) 50 μm).

3.2. HUCPVCs-CM induced neuronal differentiation of hNPCs in vitro

hNPCs grow as neurospheres in a serum-free medium, PPRF-h2 (Figure 2A). However, when the growth medium is removed and replaced by the HUCPVCs-CM or Neurobasal A medium, hNPCs lose the neurosphere state and start to differentiate (Figure 2B). Immunocytochemistry analysis revealed that when hNPCs were incubated for 5 days with the HUCPVCs-CM there was a clear increase (p<0.01) of DCX positive (immature neurons; Figure 3B/C) and MAP-2 positive cells (mature neurons; Figure 3E/F) densities when compared to control group (incubation with

Neurobasal A medium), as the latter had almost undetected numbers of DCX and MAP-2 positive cells (Figure 3A/D).
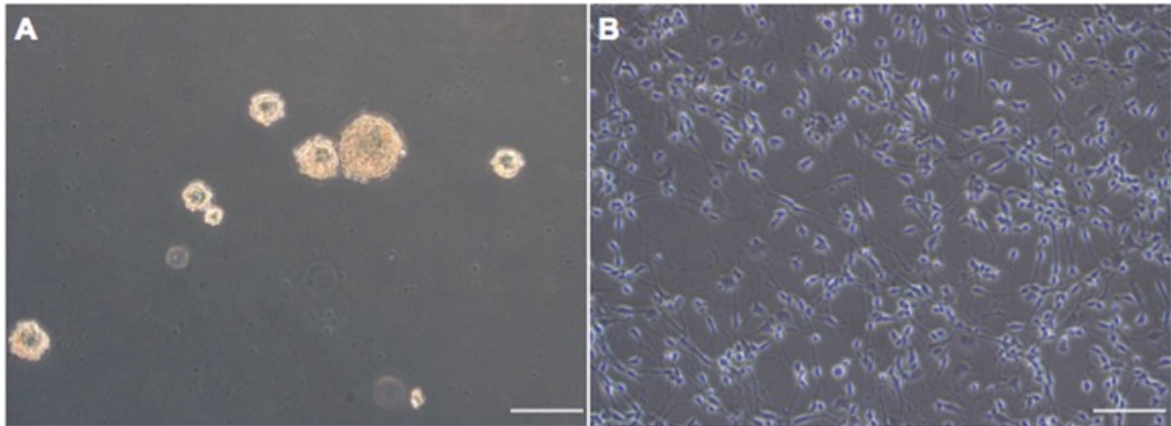
Figure 2. Expansion of Human Telencephalon Neural Precursor Cells (hNPCs) *in vitro.* (A) hNPCs standard expansion as neurospheres in the presence of their growth medium PPRF-h2 and B) spontaneous differentiation into neural phenotypes upon PPRF-h2 removal. (Scale bar: 50 μm).
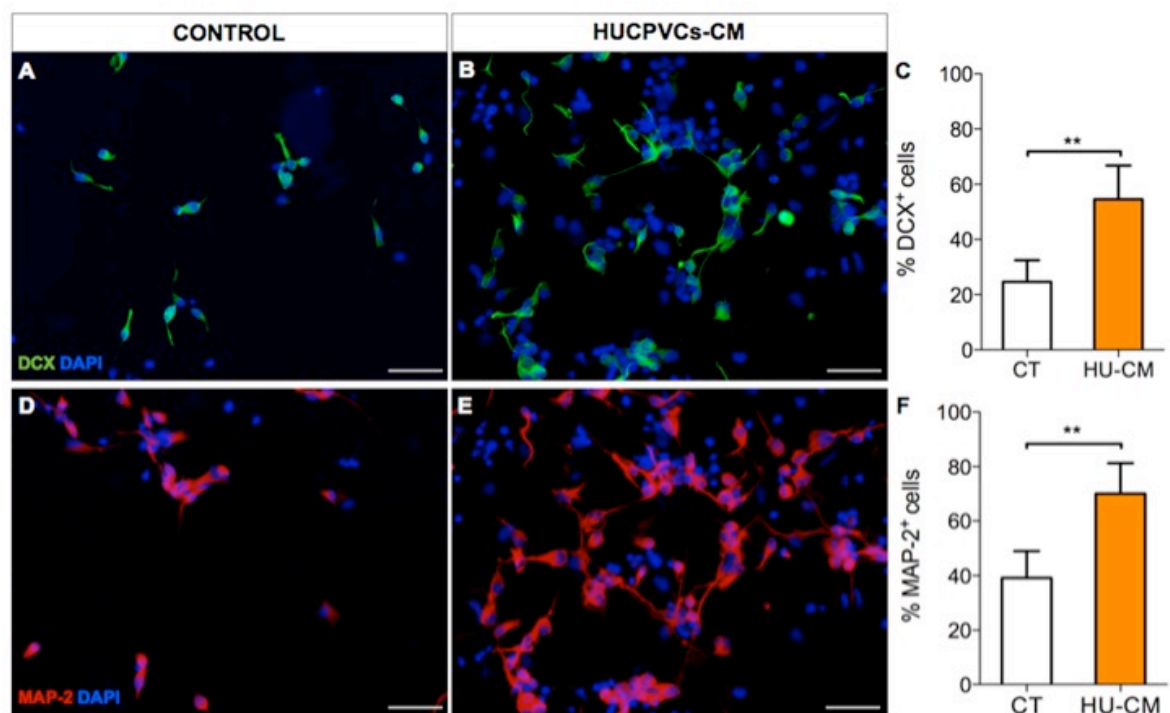
Figure 3. *In vitro* neuronal differentiation of hNPCs. HUCPVCs-CM was able to significantly increase the survival and differentiation of hNPCs into neuronal phenotypes such as (B) immature (DCX$^+$cells) and (E) mature (MAP-2$^+$ cells) neurons when compared to control conditions (A/D) (C/F, mean ± SEM, n=3, p < 0.05). CT: Control (Neurobasal A media), HU-CM: HUCPVCs conditioned media. (Scale bar: 50 μm).

3.3. HUCPVCs and their CM promoted proliferation and differentiation of endogenous progenitor cells

To assess their role *in vivo* HUCPVCs (50.000 cells/DG) and HUCPVCs-CM (0.5 µL/DG) were bilaterally implanted into the hippocampal DG of adult rats without any concomitant immunosuppression therapy. As a first approach, we determined if HUCPVCs were able to survive into the DG 7 days after the transplantation. As it can be observed in Figure 4A, the transplanted cells detected by immunostaining for *human nuclear antigen* (HNA), were able to survive in the DG 7 days after the transplantation.

In order to confirm proliferation and the cell type of the newly-born cells in the hippocampal DG, sections were immunostained with different markers. BrdU-positive cells were found mainly in the SGZ of the hippocampus. We first verified if the proliferating cells could be derived from HUCPVCs, but no positive co-labeling between BrdU and HNA was observed (Figure 4B). Nevertheless, the analysis of the cell proliferation in the DG, revealed a significant increase in the number of BrdU$^+$ cells within the HUCPVCs-injected DG ($p<0.05$; Figure 4C) when compared to the control group (Sham, Figure 4C). Similar findings were observed in the group injected with HUCPVCs-CM, ($p<0.05$, Figure 4C).
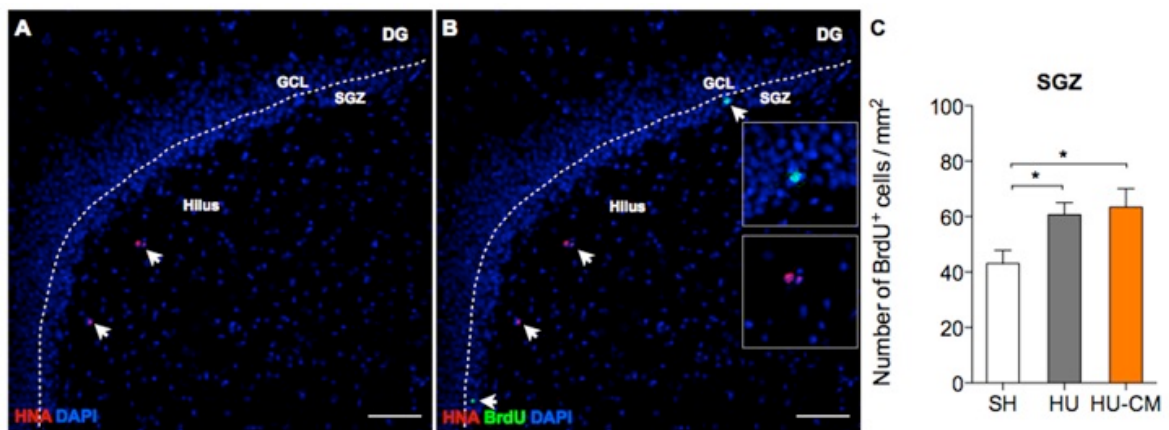
Figure 4. Transplantation of HUCPVCs and their CM into the hippocampal DG. HUCPVCs were able to survive *in vivo* after 7 days of transplantation (HNA$^+$ cells, red) (A). However, they were not proliferating, as there was no co-localization between HNA$^+$ and BrdU$^+$ cells. Cell counts revealed that 7 days post-injection both (C) HUCPVCs and CM transplanted groups were able to increase the number of BrdU-labeled cells in the DG, when compared to control (Sham group; mean ± SEM, n=5, $p < 0.05$). SH: Sham, HU: HUCPVCs, HU-CM: HUCPVCs conditioned media. (Scale bar: 100 µm).

After observing that HUCPVCs and their respective CM were able to stimulate the proliferation in the DG, we next aimed to determine their effects on the differentiation of DG resident cells. Newborn neurons expressed markers such as DCX, and 7 days post-injection we observed that both HUCPVCs (Figure 5B) and their CM (Figure 5C) were able to increase ($p<0.05$) the number of DCX-expressing cells in the SGZ (Figure 5D) when compared to the Sham group (Figures 5A). For the GCL, only differences were observed for the HUCPVCs-CM face to the control group (Sham, Figure 5E). Regarding the effects on the astrocytic cell densities (Figure 6A-C), we just observed differences ($p<0.05$) for the HUCPVCs-CM in the SGZ when compared to the control group (Figure 6D). In the GCL, no differences were observed in the tested groups.
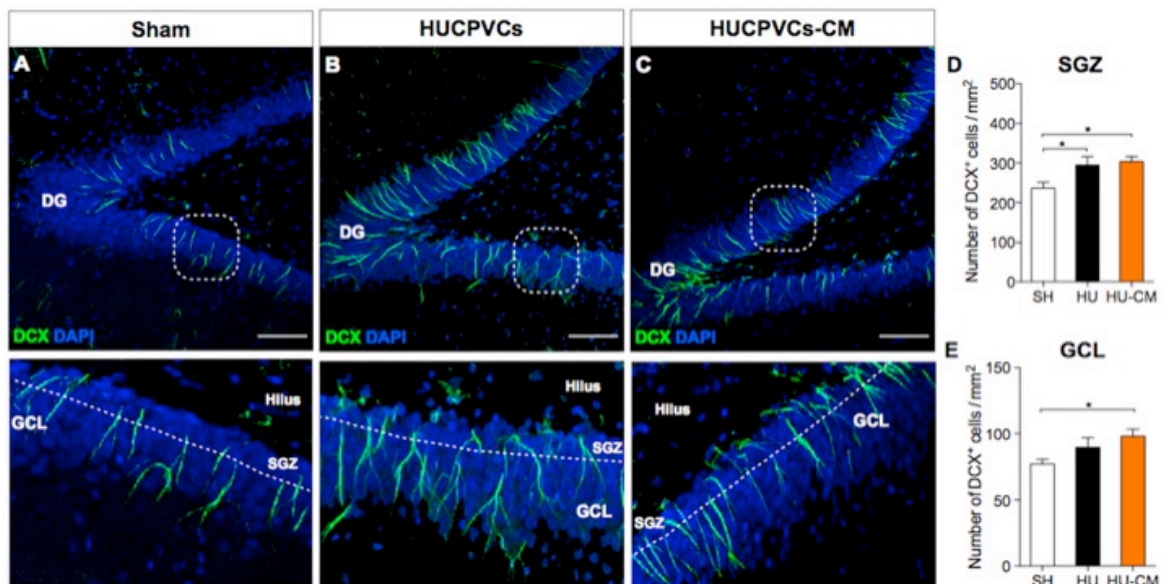
Figure 5. HUCPVCs-CM enhances neuronal densities in the DG of hippocampus. Immunohistochemistry for DCX (newborn neurons: A/B/C) revealed that the CM of HUCPVCs was able to increase the number of newborn neurons (C, statistically significant to the Sham group; mean ± SEM, n=5, $p < 0.05$) both in the (D) SGZ and (E) GCL. HUCPVCs were also able to increase the number of immature neurons (B, statistically significant to the Sham group; mean ± SEM, n=5, $p < 0.05$) into the (D) SGZ. SH: Sham, HU: HUCPVCs, HU-CM: HUCPVCs conditioned media. (Scale bar: 100 μm).
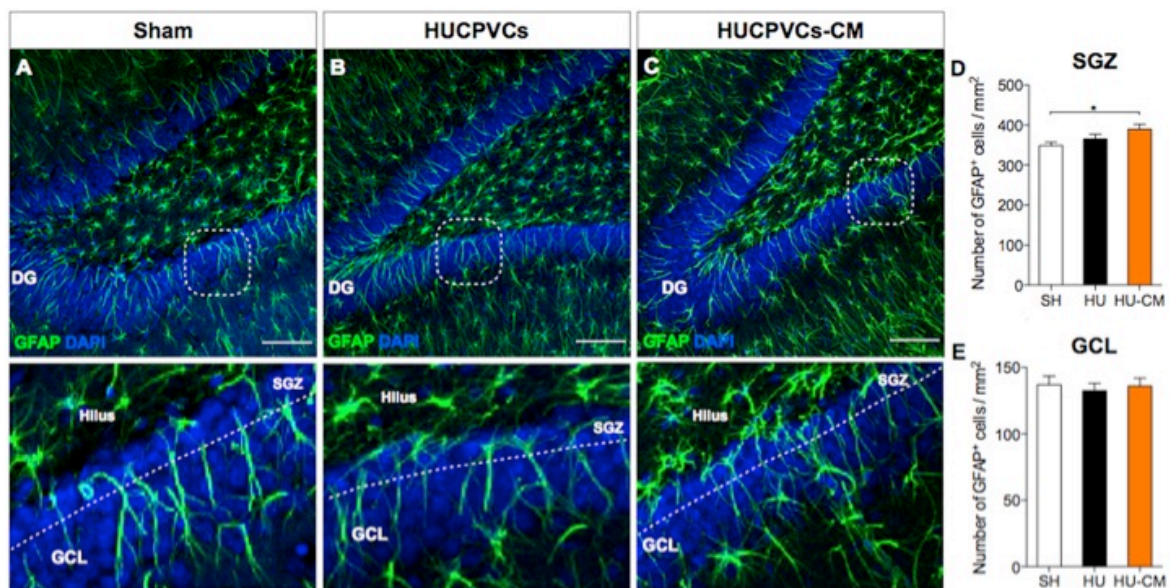
Figure 6. HUCPVCs-CM enhances astrocytic densities in the DG of hippocampus. Immunohistochemistry to GFAP (Astrocytes: A/B/C) revealed increased numbers promoted by the injection of HUCPVCs-CM (C, statistically significant to the Sham group; mean ± SEM, n=5, p < 0.05) in the (D) SGZ. In the (E) GCL no differences were observed 7 days post-injection. SH: Sham, HU: HUCPVCs, HU-CM: HUCPVCs conditioned media (Scale bar: 100 µm).

3.4. Expression of trophic factors after HUPCVCs/HUCPVCs-CM Implantation

In order to stimulate the endogenous proliferation as well as to increase the number of neuronal/glial cell populations, HUCPVCs or their CM must be able to modulate the surrounding environment through direct interaction or paracrine action [45]. To understand the underlying mechanisms through which HUPCVCs/HUCPVCs-CM implantation stimulates adult hippocampal neurogenesis we measured the levels of growth factors such as FGF-2 and NGF in the DG. Western blot analysis (Figure 7) revealed that levels of FGF-2 were significantly increased when compared to those obtained by the Sham group (p<0.05; Figures 7A/B); however, despite the absence of significant effects, the expression of NGF was also increased (Figures 7A/C).
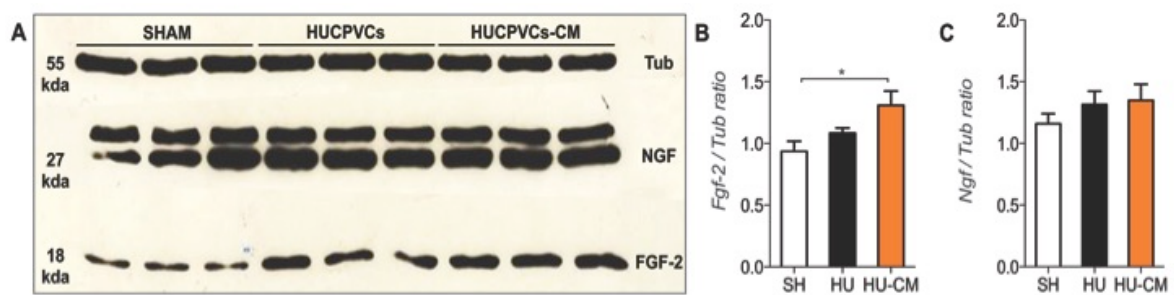
Figure 7. Injection of HUCPVCs-CM increased the expression of neurotrophic factors *in vivo*. By Western blot assay it was possible to observe that HUCPVCs-CM was able to increase the secretion of (A) FGF-2 in the DG tissue (B, statistically significant to the Sham group; mean ± SEM, n=4, $p < 0.05$). It was also observed a slightly increase in the levels of (C) NGF although, no statistically significant differences were observed when compared to the Sham group. SH: Sham, HU: HUCPVCs, HU-CM: HUCPVCs conditioned media.

4. Discussion

The use of MSCs-based therapies as a new strategy for CNS regeneration constitutes one of the biggest challenges in the regenerative medicine field. Different reports have shown that the release of paracrine factors and extracellular vesicles is the main route by which MSCs can mediate improvements in the CNS [34]. Within the different MSCs based populations, HUCPVCs have been described as an attractive and readily available alternative source of MSCs for cell therapy [27]. Indeed, studies have shown that HUCPVCs have a higher clonogenic frequency, containing a good self-renewing capacity. Additionally, they have also shown to possess multilineage differentiation [46, 47], as confirmed in the present report (Figure 1). Moreover, previous results from our laboratory had revealed that the secretome of these cells was able to induce and increase the cell survival and densities of different (hippocampal, cortical and cerebellar) neuronal and glial cells [23, 33, 37, 48]. The initial *in vitro* experiments performed in the present report revealed that the secretome of HUCPVCs could also modulate survival and differentiation of CNS derived cells. Indeed, as shown in Figure 3, when hNPCs were incubated with HUCPVCs-CM an increased differentiation of hNPCs into neuronal lineages (immature (DCX[+]) and mature (MAP-2^{+}) neurons) was observed. Previous work from Ribeiro *et al.* [33] had already shown that these cells have a strong expression of NGF, which is most likely playing a strong role on the observed effects.

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More relevant were the *in vivo* experiments, which revealed that HUCPVCs were able to boost cell proliferation within the DG 7 days after injection, as assessed by the number of BrdU+ cells (Figure 4C). Similar outcomes were reported by Munoz and colleagues [49] after BM-MSCs transplantation in the DG, being this finding attributed to the secretion of growth factors and chemokines; this hypothesis was, in fact, confirmed in the present study. Indeed, as shown in Figure 4C, the application of CM discloses similar levels of cell proliferation to those obtained by the cell transplanted group, which is a strong indicator that the secretome, *per se*, regulates such phenomena and by the demonstration of the increased expression of FGF2 and NGF in the transplanted DG.

Similar effects were also observed regarding neuronal differentiation. Indeed, 7 days after injection of HUCPVCs, or their respective CM, it was possible to observe a significantly increase the number of immature neurons (DCX-expressing cells) in the SGZ (Figure 5D). This is in line with the findings of Tfilin and colleagues [50], who transplanted BM-MSCs into the hippocampal DG, and observed that after the transplantation there were increasing numbers of DCX-expressing cells, indicating the differentiation of newly formed neurons. Curiously, in the present study it was observed, that the CM, and not the cells, was also able to increase significantly the number of DCX-expressing cells into the GCL (Figure 5E), suggesting that the CM also stimulates the migration of these newly-born neurons within the DG. We hypothesized that these effects are related with the increased expression of FGF-2 in the CM injected group (Figure 7A/B). Indeed, the latter is considered to be a potent modulator of the early phases of neurogenesis, being able to support not only the proliferation as well as, to enhance the survival and neuronal/glial maturation [45, 50]. The small increase of NGF in the DG tissue (Figure 7A/C) could also impact on these phenomena [49, 51-53]. Nevertheless, although proteomic studies are still scarce to explain the functional effects of MSCs secretome (including HUCPVCs as well), previous studies from our group have revealed through proteomic-based techniques that HUCPVCs are really capable to secrete important molecules with actions in the CNS physiology [33, 37]. For instance, Ribeiro and colleagues [33] demonstrated that HUCPVCs were able to secrete large amounts of NGF, an important neurotrophin in the support of neuronal/glial cell survival [51, 52]. In addition to this, Fraga and colleagues [37] through mass spectrometry observed that HUCPVCs are also able to secrete important intercellular proteins such as 14-3-3 proteins, Hsp70 and UCHL1, which are well known by their important roles in the induction of cell proliferation, survival and differentiation, including in cells derived from the CNS

[54-56]. However, future studies should be promoted in order to address the temporal effects of the HUCPVCs secreted factors, as well as their interactions or inhibitory actions in order to understand if the above-described molecules could be related with the results herein reported.

5. Conclusions

In the present work it was demonstrated that the secretome of HUCPVCs, a MSC based population in the Wharton Jelly of the umbilical cord, was able to induce neuronal differentiation of human neural progenitors into neurons in different stages of maturation. Additionally, *in vivo* injections into the hippocampal DG, a known neurogenic niche, revealed that the secretome of HUCPVCs, in the form of conditioned media, induced increased levels of BrdU$^+$ cells (proliferation), newly differentiated neurons (DCX-expressing cells) and GFAP$^+$ cells, when compared to HUCPVCs transplanted groups. These outcomes were associated with higher expression of FGF-2 in the group injected with HUCPVCs-CM. These results suggest that the secretome (alone) of an MSC-like population is able to modulate neural progenitor proliferation and differentiation, which may open new therapeutic opportunities in the future.

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Chapter 3

Teixeira FG., Panchalingam KM., Jung S., Serra S., Anjo S., Manadas B., Sousa N., Behie LA., Salgado AJ. (2014)

Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors Increases Neuronal Cell Proliferation, Survival and Differentiation

Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors Increases Neuronal Cell Proliferation, Survival and Differentiation

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Abstract

The administration of human mesenchymal stem cells (hMSCs) into different animal models of disease, including those of the central nervous system (CNS), has shown important therapeutic benefits, which have been mainly attributed to their secretome. Current hMSC production bioprocesses use conventional static culture flasks for their expansion. However, the latter suffer from variable culture conditions (i.e. heterogeneous culture environment, limited growth surface area per culture, among others), and thus are not acceptable procedures to meet the expected future demand of quality-assured hMSCs for human therapeutic use. The use of dynamic culturing environments such as those provided by computer-controlled suspension bioreactors can overcome these limitations. Additionally, as hMSCs actively respond to dynamic environments, there is the hypothesis that one can modulate its secretome through the use of such systems. Herein, we present data indicating that the use of a dynamic condition was able to lead to a rapid and efficient expansion of hMSCs, enhancing their neurotrophic secretory profile (e.g. conditioned medium - CM), which can support the survival and differentiation of human neural progenitor cells into neurons *in vitro*. In addition, *in vivo* injections of the dynamic cultured CM into the hippocampal dentate gyrus (DG) enhanced neuronal and astrocytic survival and differentiation. Proteomic analysis also revealed that the dynamic culturing of hMSCs increased the secretion of important neuroregulatory molecules, thus modulating its secretome. Our conclusion is that the production of an enhanced hMSC secretome in a serum-free bioreactor culture condition, may present a better avenue for the clinical application of hMSCs in regenerative medicine.

1. Introduction

Human mesenchymal stem cells (hMSCs) are of great interest in the field of regenerative medicine. Their therapeutic properties are mediated by their secretome, which has shown to modulate several processes *in vitro* and *in vivo,* such as cell proliferation, survival, differentiation, immunomodulation, anti-apoptosis, angiogenesis and stimulation of tissue adjacent cells [1-3]. For instance, it has already been suggested that their secretome may open future therapeutic options for cell-free based therapies [4-6]. Indeed, it was already demonstrated that hMSCs are able to secrete important trophic molecules such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), glial-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF-2), stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF) and stromal cell-derived factor (SDF-1) both *in vitro* and *in vivo* [1, 7-12]. In addition to these paracrine soluble factors, recent findings on hMSCs secretome research also suggested that its vesicular fraction plays an important role in mediating the processes referred above [13, 14]. Indeed, it has been shown that hMSCs are able to secrete large amounts of vesicles (microvesicles, exosomes), either constitutively or after activation of signals [13]. Although proteomic studies continue still be scarse on this field, studies have already demonstrated promising results of the hMSCs-derived exosomes application in a wide panel of diseases [15-22]. Therefore, despite some research gaps regarding the exosomal/vesicular fraction of the hMSCs secretome (i.e. precise definition and mechanism of interaction in the tissues), it has been suggested that MSCs-derived exosomes, when compared to cells transplant, are more stable, presenting a lower risk of aneuploidy and immune rejection after *in vivo* allogenic administration, representing in this way a potential therapeutic tool for the treatment of different diseases [23].

Typically the most utilized source of hMSCs is the bone marrow, which is a safe and readily available source of cells. However, due to the low frequency of primary bone marrow-derived hMSCs, it is critical to expand *in vitro,* a clinically relevant number of cells for clinical trials [24]. Currently, hMSCs are expanded using conventional static culture flasks in the presence of fetal bovine serum (FBS) or human-sourced supplements. However, these expansion platforms lead to variable culture conditions (i.e. ill-defined medium components, heterogeneous culture environment and limited growth surface area per volume) and thus are unsafe and not ideal to meet the expected future demand of quality-assured therapeutic cells for wide implementation of hMSC-related therapies. Previous studies from our group revealed that the use of a serum-free medium condition (e.g. PPRF-

msc6) was able to support rapid and efficient isolation and expansion of hMSCs from different sources [25-27]. In addition to developing a well-defined medium we have also developed a scalable, computer-controlled stirred suspension bioreactor-based microcarrier-mediated bioprocess that can be translated to operate in a closed system [27]. Using stirred suspension bioreactors, a number of advantages can be achieved including: (1) a large number of cells can be expanded in one vessel (minimizing vessel-to-vessel variability and minimizing cost related to labor and consumables), (2) the bioreactors can be operated in a fed-batch or perfusion mode of operation and (3) the bioreactors can be set up with computer-controlled online monitoring instruments to ensure tight control of process variables such as pH, temperature and dissolved oxygen concentration. Additionally, it has been shown that hMSCs respond to changes in their physiological environment [28], namely by using dynamic culturing environments, such as those provided by bioreactors [28-30]. Therefore it is possible to hypothesize that the modulation, and further enrichment with growth factors/vesicles, of their secretome could be achieved by using these dynamic culturing systems. Having this in mind in the present work we aimed to characterize and analyze the effects of the hMSCs secretome collected from dynamic culture conditions (suspension bioreactors) to that obtained from standard culturing conditions. Results revealed that the dynamic obtained secretome was able to induce higher levels of neuronal differentiation *in vitro* when compared to static condition. A similar trend was observed *in vivo* (DG of the hippocampus) in which the dynamic hMSCs secretome revealed a more robust increase of the proliferation levels and neuronal densities in the DG, when compared to static conditions. The proteomic analysis revealed that the use of a dynamic condition is able to modulate the secretome profile of hMSCs, enhancing the secretion of neuroregulatory molecules, such as Cystatin-C, Glia-derived nexin, Galectin-1 and Pigment epithelium-derived factor when compared to static secretome, which could open in this way future perspectives of application to central nervous system diseases.

2. Methods

2.1. Media and cell culture

The serum-free medium used to isolate and expand the hMSCs (derived from bone marrow) was developed at the Pharmaceutical Production Research Facility (PPRF, University of Calgary, Alberta, Canada). The preparation of PPRF-msc6 has previously been described in detail [26]. Briefly, a 1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Life Technologies, Grand Island, NY) was supplemented with L-glutamine (final concentration 4.0 mM) and 0.1% v/v

lipid concentrate (Life Technologies), 20.5 mM sodium bicarbonate, 4.9 mM HEPES, 4.01 uM insulin, 0.318 uM apo-transferrin, 55.9 uM putrescine, 17.8 nM progesterone, 1.0 mg/mL fetuin, 100 nM hydrocortisone and 197.6 uM L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO), 4.0 mg/mL human serum albumin (HSA, InVitroCare, Fredrick, MD), 2.0 ng/mL basic fibroblast growth factor (bFGF) and 1.0 ng/mL transforming growth factor-b1 (both from R&D Systems, Minneapolis, MN). No antibiotics were used for the culture of our hMSCs. Thus, for all the experiments, hMSCs (derived from three donors) were expanded until passage five (P5) into our PPRF-msc6 serum-free medium.

2.2. Expansion of hMSCs in static culture and collection of static conditioned medium

Passage 2 (P2) hMSCs were thawed and inoculated into gelatin-coated tissue culture flasks (Nunc, Roskilde, DO) at 5,000 cells/cm^2 in growth medium (at a volume/area ratio of 0.32 mL/cm^2). The cell cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2. After 3 days, 50% of the medium was replaced with fresh growth medium. When the cells reached 80-90% confluence, the cells were harvested by incubation with 0.05% trypsin-EDTA (Life Technologies) at 37°C for 3-5 min, and then FBS-DMEM was added to neutralize the reaction. The harvested cells were then centrifuged at 300 g for 10 min and re-suspended in fresh growth medium and re-plated into new, gelatin-coated tissue culture flasks at 5000 cells/cm^2. At P5, after 72 hours of growth, the cell culture growth medium was removed and the cells were washed twice with Neurobasal®-A medium (Life Technologies), and Neurobasal®-A medium was added to the tissue culture flasks at the same ratio was the growth medium (i.e. 0.32 mL/cm^2). The cells were then placed in a humidified incubator, operating at 37°C and 5% CO_2, for 24 h. After 24 h, the medium was removed, centrifuged at 300 g for 10 min to remove any cell debris, and then stored at -80°C until it was required for further experiments. This medium is referred to in the paper as conditioned medium (CM) as it contains the varying factors secreted by the hMSCs during the 24 h of incubation.

2.3. Expansion of hMSCs in dynamic (bioreactor) conditions and collection of conditioned medium.

2.3.1. Preparation of 500 mL Suspension Bioreactors

The DASGIP Parallel Bioreactor system (DASGIP, Julich, DK, http://www.dasgip.com) was used for the expansion of hMSCs in dynamic conditions. Prior to inoculating hMSCs in the DASGIP

bioreactors, the 500 mL suspension bioreactors and modified Teflon 4-paddle impellers (designed and manufactured at PPRF) were siliconized using Sigmacote (Sigma) to minimize cell attachment to the sides the bioreactor vessel and the impeller. After siliconization and autoclaving of the vessels, the DASGIP system was calibrated according to procedures provided by the manufacturer. The bioreactors were maintained at (1) 37°C using a heating jacket, (2) 100% dissolved oxygen (corresponding to oxygen saturation of the medium at 37°C exposed to 21% O_2 in the headspace), (3) a pH of 7.4, controlled by a gas mixture hooked up to oxygen, nitrogen, carbon dioxide and air tanks that was introduced into the headspace, and (4) agitated at 52 rpm using a magnetic stir plate under the bioreactors.

2.3.2. Preparation of Microcarriers and Inoculation of hMSCs

Cytodex 3 microcarriers (GE Healthcare) were used for this study and were prepared as follows. 1.0 g of microcarriers were weighed out and hydrated in 50 mL of 1X PBS (Life Technologies) in a 125 mL, pre-siliconized, Erlenmeyer flask, at room temperature overnight. To this flask 2-3 drops of Tween 80 (United States Biochemical Corporation, Cleveland, OH) was added to break the surface tension and ensure proper wetting and sedimentation of the microcarriers. The microcarriers were then washed 3X with 1X PBS, and autoclaved. Following autoclaving the microcarriers were incubated with fetal bovine serum (FBS, Life Technologies) for 6 h at 37°C to coat the microcarriers with serum-attachment factors, and agitated every 30 min. After 6 h, the FBS was removed, the microcarriers were washed twice with our serum-free medium, and then inoculated into our 500 mL DASGIP bioreactors in 275 mL of medium for 4h at the controlled culture conditions. hMSCs were expanded in static culture for two passages before inoculation into the DASGIP bioreactors. The cells were harvested using trypsin-EDTA, and then inoculated into the bioreactors at a density of 24,000 cells/mL (based on the final volume of 500 mL) and the volume of the bioreactors was maintained at 325 mL for the first 24 h to increase cell attachment. After 24 h the culture volume was increased to 500 mL to bring the final microcarrier density to 2.0 g/L. The cells were cultured on the microcarriers for 72 h, after which time the bioreactors were removed from the DASGIP system, and placed in a biosafety cabinet for 10 min to allow the microcarriers to settle. The supernatant was removed from the bioreactors, and the microcarriers were washed once with 100 mL of Neurobasal®-A medium. Following this, 500 mL of Neurobasal®-A medium was added to the bioreactors and the bioreactors were placed back into the DASGIP control system for 24 h. After 24 h the bioreactors were again removed from the system, placed in a biosafety cabinet for 10 min to

allow the microcarriers to settle, the supernatant was harvested and centrifuged at 300 g for 10 min to remove any cell debris. This supernatant, called the dynamic CM, was then placed at -80ºC until it was required.

2.4. Growth of hNPCs and incubation with hMSCs CM

Human telencephalon-derived neural precursor cells (hNPCs) were isolated from the telencephalon region of a 10-week post-conception fetus according with the protocols and strict ethical guidelines previously established [31-33]. Pre-isolated and cryopreserved hNPCs were thawed at 37ºC and the contents placed into a T-25 flask (Nunc) containing 5 mL of a serum-free medium PPRF-h2 [31]. After two days, the cells were harvested and mechanically dissociated into a single cell suspension, and subcultured into fresh cell growth medium (PPRF-h2). Every 4 days, the T-flasks were fed by replacing 40% of the spent medium with fresh growth medium. After 14-20 days of growth in the culture flasks, hNPCs were passaged and plated onto pre-coated [(poly-D-lysine hydrobromide (100 μg/mL) and laminin (10 μg/mL) - Sigma)] 24-well plates at a density of 4.0×10^4 cells per well for 5 days with the hMSCs CM obtained from either the static or dynamic condition and placed in an incubator operating at 37 ºC, 5% CO_2, 95% air and 90% relative humidity. Neurobasal®-A medium supplemented with 1% kanamycin (Life Technologies) was used as control group.

2.5. Transplantation of hMSCs CM: Stereotaxic surgery

Consent was obtained from the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457). All stereotaxic surgeries were conducted using 8 weeks old male *Wistar* rats (Charles River, Barcelona, Spain). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council. Animals were housed and maintained in a controlled environment at 22-24°C with 55% humidity, on a 12 h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Animals were handled for 1 week prior to the beginning of the injections, in order to reduce the stress induced by the surgical process. Three experimental conditions (n=5/condition) were evaluated with cerebral injection of either: 1) Neurobasal®-A medium without MSC factors (Sham), 2) Static-conditioned media (CMs) or 3) Dynamic-conditioned media (CMd). Adult rats were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.30 mg/kg). Using a stereotaxic system (Stoelting, Wood Dale, IL) and a Hamilton

syringe (0.5 µl Hamilton Bonaduz AG, Bondauz, CH) all injections made in these three groups were bilateral according to previously determined coordinates (Anterior/Posterior (AP) = 3.5 mm; Dorsal/Ventral (DV)= 3.5/3.1 mm; Lateral (L)= 2.0 mm) [34]. The volume injected per DG was 0.5 µl with a rate of injection of 0.25 µl/min. Two minutes were allowed after each injection in order to avoid any backflow up the needle tract. Sham group was only injected with 0.5 µl of Neurobasal®-A medium; CMs and CMd groups were injected with 0.5 µl of hMSC CM from the respective growth conditions. At the end, the animals were sutured and then injected with 100 µl of anti-sedating (Orion Pharma, Espoo, FIN) in order to recover from surgical procedure.

2.6. Immunostaining

2.6.1. *In Vitro* Immunostaining of hNPCs

hNPCs were fixed in 4% paraformaldehyde (Mallinckrodt, Paris, KY) for 15 min, and then permeabilized by incubation with 0.1% Triton X-100 (Sigma) in 1X PBS for 5 min at room temperature, and washed three times in 1X PBS. hNPCs were then blocked with 10% of fetal calf serum (FCS, Life Technologies) in 1X PBS, followed by a 1 h incubation (at 37℃) with the primary antibodies: rabbit anti-doublecortin (DCX; 1:500, Abcam, Cambridge, MA, http://www.abcam.com) to detect immature neurons and mouse anti-rat microtubule associated protein-2 (MAP-2; 1:500, Sigma) to detect mature neurons. hNPCs were then washed with 1X PBS three times and incubated with the secondary antibodies namely, Alexa Fluor 488 *goat anti-rabbit* (IgG, Life Technologies) and Alexa Fluor 594 *goat anti-mouse immunoglobulin* G (IgG, Life Technologies) for 1 h at 37℃ and then 10 min with *4-6-diamidino-2-phenylindole-dihydrochloride* (DAPI; Life Technologies). After staining samples were observed under an Olympus BX-61 Fluorescence Microscope (Olympus, Hamburg, DK). For this purpose, three coverslips and ten representative fields per condition were chosen and analyzed. Results are shown as percentage of DCX or MAP-2 positive cells over the total number of cells per field of observation (n=3).

2.6.2. *In Vivo* Immunostaining

Regarding the *in vivo* experiment, frozen coronal sections were obtained by cryostat with a thickness of 20 µm. Sections were then permeabilized by incubation with 0.2% Triton X-100 in 1X (PBS-T, 10 minutes at 37℃), and were subsequently washed with 1X PBS (3 times with 5 min wait time). For Ki-67 staining the permeabilized sections were incubated with citrate buffer treatment (15-20 min) and placed in the microwave for antigen retrieval. After that, sections were washed with 1X PBS (3

times with 5 min wait time), and then incubated with 10% FCS/PBS-T for two hours for endogenous blocking. After that, sections were incubated overnight at 4ºC with primary antibodies namely, Ki-67 (1:100, Millipore, Billerica, MA) for proliferation, DCX (1:300, Abcam) for immature neurons, and rabbit anti-rat glial fibrillary acid protein (GFAP; 1:200, Dako) for astrocytes detection. Sections were then incubated with secondary antibodies: Alexa Fluor 568 goat anti-mouse immunoglobulin G (IgG, Life Technologies), Alexa Fluor 488 goat anti-rabbit (IgG, Life Technologies) for 2 h at room temperature and then 10 min with 4-6-diamidino-2-phenylindole-dihydrochloride (DAPI; Life Technologies). Images were obtained with a confocal microscope (Olympus FV1000) using the software FV10-ASW 2.0c (Olympus), presenting the hippocampal DG (five sections per animal were analyzed for n=5/group). Then, all granular cell layer (GCL) area was defined and the cell counts were made in these area.

2.7. Proteomics – Mass Spectrometry and SWATH Acquisition
2.7.1. Liquid Digestion/Sample Preparation

hMSCs CM was firstly concentrated using a Vivaspin 20 sample concentrator (GE Healthcare) by ultracentrifugation at 3000 g during 45 minutes. Then, the CM was precipitated with Trichloroacetic acid (TCA) – Acetone. TCA was added to each sample at a final concentration of 20% (v/v), and incubated for 30 min at -80°C and subsequently centrifuged at 20 000 g for 20 min. Protein pellets were then solubilized with ice-cold (-20°C) acetone, aided by ultra-sonication, followed by a centrifugation at 20 000 g for 20 min. The washed pellets were then ressuspended in 1.0 M Triethylammonium bicarbonate buffer (TEAB, Sigma), aided by ultra-sonication, followed by a centrifugation at 20 000 g for 5 min to remove insoluble material [35].

Samples were quantified using the 2D-Quant Kit (GE Healthcare) and 100 μg of each sample were subjected to liquid digestion. Briefly, 4 μL of 50 mM TCEP was added to 45 μL of sample, followed by ultra-sonication for 2 min. Next, 2 μL of 600 mM MMTS was added and samples were left to react for 10 min, at room temperature. TEAB was then added to bring the final volume of each sample to 100 μL, and the samples were digested with trypsin overnight (2 μg trypsin/sample), at 37 °C, with swirling at 650 rpm. Reactions were stopped by the addition of 2 μL of formic acid (FA, Sigma) and the peptides were dried by rotary evaporation under vacuum. Before performing the MS/MS analysis the peptide mixtures were cleaned/desalted-using OMIX tips with C18 stationary phase (Agilent Technologies, Santa Clara, CA, http://www.agilent.com) as recommended by the

manufacturer. Eluates, spiked with iRT peptides (Biognosys, Schlieren/Zurich, CH, http://www.biognosys.ch), were dried by rotator evaporation,. The samples were ressuspended to 23 µL in a solution of 2% ACN and 0.1% FA followed by vortex, spin and sonication in water bath [2 min with pulses of 1 sec – 1 sec sonication followed by 1 sec break pulse – at 20% intensity, in a sonicator Vibra-Cell™ 750 watts, (Sonics & Materials, Newtown, CT, http://www.sonicsandmaterials.com)]. In order to remove insoluble material the peptide mixture were then centrifuged for 5 min at 14 000 g and collected into the proper vial for LC-MS injection.

2.7.2. SWATH acquisition

For the SWATH acquisition, samples were analyzed on a Triple TOF™ 5600 System (AB SCIEX, Framingham, MA, http://www.absciex.com) in two phases: information-dependent acquisition (IDA) was followed by SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition on the same sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent, Redwood City, CA, http://www.eksigent.com) on a Halo Fused-Core™ C18 reverse phase column (300 µm ID × 15 cm length, 2.7 µm particles, 90 Å pore size, Eksigent) at 5 µL/min. Peptides were eluted into the mass spectrometer with an acetonitrile gradient in 0.1% FA (2% to 35% ACN, in a linear gradient for 25 min), using an electrospray ionization source (DuoSpray™ Source, AB SCIEX).

For information dependent acquisition (IDA), the mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 30 MS/MS scans (100–1500 m/z for 75 ms each). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 70 counts per second were isolated for fragmentation and one MS/MS spectra was collected for 75 ms before adding those ions to the exclusion list for 15 sec (mass spectrometer operated by Analyst® TF 1.6, AB SCIEX). Rolling collision was used with a collision energy spread of 5. Peptide identification was performed with Protein Pilot software (v4.5, AB SCIEX). Search parameters used were the following: SwissProt database, against a database composed of *human and bovine* species from SwissProt database (released in February 2014), GFP and iRT peptides, and using MMTS alkylated cysteines as fixed modification. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR [36, 37]. The SWATH setup was described in Gillet *et al.* [38], with the

same chromatographic conditions used as in the IDA run described above. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode. The instrument was specifically tuned to allow a quadruple resolution of 25 m/z mass selections. Using an isolation width of 26 m/z (containing 1 m/z for the window overlap), a set of 30 overlapping windows was constructed covering the precursor mass range of 350–1100 m/z. A 250 ms survey scan (350-1500 m/z) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from 100–1500 m/z for 90 ms resulting in a cycle time of 3 sec from the precursors ranging from 350 to 1100 m/z. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with a collision energy spread of 15. A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments, and used for subsequent SWATH processing. Libraries were obtained using Protein Pilot™ software (v4.5, AB SCIEX) with the same parameters as described above. Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, AB SCIEX) briefly peptides were selected automatically from the library using the following criteria: (i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and (ii) peptides that contained biological modifications and/or were shared between different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH™ quantitation was attempted for all proteins in library file that were identified below 5% local FDR from ProteinPilot™ searches. In SWATH™ Acquisition data, peptides were confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide.

Target fragment ions, up to 5, were automatically selected and peak groups were scored following the criteria described in Lambert *et al.* [39]. Peak group confidence threshold was determined based on a FDR analysis using the target-decoy approach and 1% extraction FDR threshold was used for all the analyses. Finally, peptides that met the 1% FDR threshold in one of the samples were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 4 min. The levels of the human proteins were estimated by summing all the transitions from all the peptides for a given protein (an adaptation of [40]) and normalized to the total response for the given sample.

2.8. Statistical Analysis

Statistical evaluation was performed using One-way ANOVA and Student's t-test through the program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Data is presented as Mean ± S.E.M. Significance value was set at $p < 0.05$.

3. Results

3.1. Expansion of hMSCs in Static and Bioreactor Conditions

We have shown previously that by utilizing a serum-free medium, PPRF-msc6, we can rapidly and efficiently isolate and expand hMSCs, compared to using conventional growth medium (i.e. 10% FBS-DMEM) [4,5]. We report herein that using PPRF-msc6 we were able to rapidly expand cells in both static cultures as well in our 500 mL suspension bioreactors (dynamic). Over 4 days in culture, hMSCs in both the static and dynamic culture approached confluence. Moreover, it was also observed that, in the dynamic condition, cells were present on the majority of the microcarriers (Figure 1A). The doubling time (i.e. during the exponential growth phase) of the hMSCs in static culture was 37.8±6.0 h, which was similar to the doubling time of hMSCs in dynamic culture (36.4±4.9 h). Additionally, flow cytometry analysis of static and dynamic culture expanded hMSCs revealed that both types of cells expressed the standard hMSCs markers CD13, CD73, CD90 and CD105 at >99.9% and was negative (<2.0%) for CD34, CD45 and HLA-DR (Figure 1B). In the dynamic bioreactor environment, the dissolved oxygen, pH and temperature were well controlled within the preset set points during the expansion phase and the CM collection phase for all three hMSC donors in Figure 1C.

3.2. The secretome of hMSCs induced neuronal differentiation of hNPCs *in vitro*

As was previously shown, hNPCs grow as neurospheres in a serum-free medium PPRF-h2 as was previously shown [31, 41]. Upon removal of the growth medium, and plating the cells in adherent plates in hMSC CM, the cells adhere and start to differentiate. Immunocytochemistry analysis revealed that when hNPCs were incubated for 5 days with the hMSC CMs there was a significant increase in the cell population expressing markers of DCX (immature neurons; p<0.001; Figure 2B-D) and MAP-2 (mature neurons; (p<0.01); Figure 2F-H) when compared to control group (incubation with Neurobasal A medium (Figure 2A/E). Additionally it was possible to observe the secretome

collected under dynamic culture condition induced an increased differentiation of hNPCs ($p<0.05$), as assessed by DCX and MAP-2 immunostaining, when compared to the group incubated with the secretome collected under static conditions (Figure 2B-H).
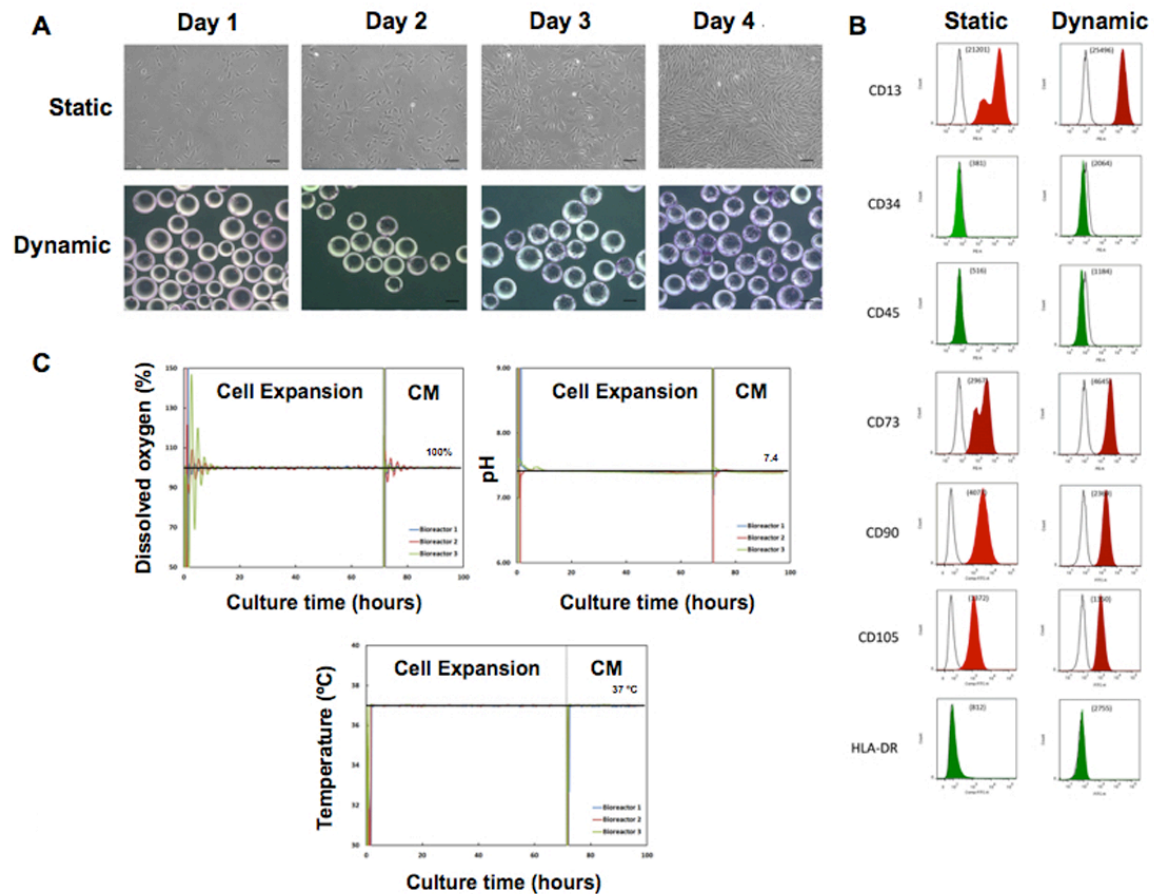
Figure 1. Expansion and characterization of hMSCs in static culture and 500 mL computer-controlled bioreactors. hMSCs adhered to both the tissue culture flasks and the microcarriers in the suspension bioreactors on day 1, and proliferated up to 90% confluence by day 4 (A). FACS analysis for hMSC markers, CD13, CD73, CD90, CD105 were >99.9% while non-hMSC markers were expressed <2.0% (B). Additionally, key process parameters (i.e. dissolved oxygen, pH and temperature) in our computer-controlled bioreactor system were well maintained at pre-determined setpoints for the culture period (C). Scale bars represent 100 μm.

3.3. The secretome of hMSCs increase the levels of proliferation and induced neuronal differentiation *in vivo*

To assess the role hMSCs CM from static and dynamic conditions *in vivo*, animals were bilaterally injected (0.5 μL of CM) into the hippocampal DG without any concomitant immunosuppression therapy. To access proliferation, Ki-67 marker was used. Most of the proliferating cells (Ki-67$^+$) were found mainly in the SGZ of the hippocampus (Figure 3B-C). Moreover, the analysis of the cell proliferation in the DG, revealed a significant increase in the number of KI-67$^+$ cells within the CM-injected DG ($p<0.05$; Figure 3A-C, J) when compared to the control group (Sham).

After observing that both CM (from the static and dynamic conditions) were able to stimulate cell proliferation in the DG, we next aimed to determine their effects on the differentiation of DG resident cells. 7 days post-injection of both CM (Figure 3E-F), we observed an increase in the number of DCX-expressing cells (newborn neurons) in all DG granular layers ($p<0.05$), namely in the SGZ (Figure 3D-F, K) when compared to the Sham group (Figures 3D). However, injection of the dynamic CM resulted in a significantly higher number of DCX-expressing cells ($p<0.05$) when compared to the static-CM group (Figure 3E-F, K). After analyzing the co-expression of Ki-67 and DCX, an indication of neuronal differentiation, it was observed that both CM were able to significantly increase the number of differentiating neurons when compared to the Sham group (Figure 3G-I, L). At the same time, although without statistical differences, the dynamic CM presented a positive trend when compared to the static CM (Figure 3L). Similar effects were also obtained for astrocytic cells (Figures 4A-C), in which, both CM were able to significantly increase GFAP-positive cell densities ($P<0.05$, Figure 4D) when compared to the Sham group (Figure 4A).
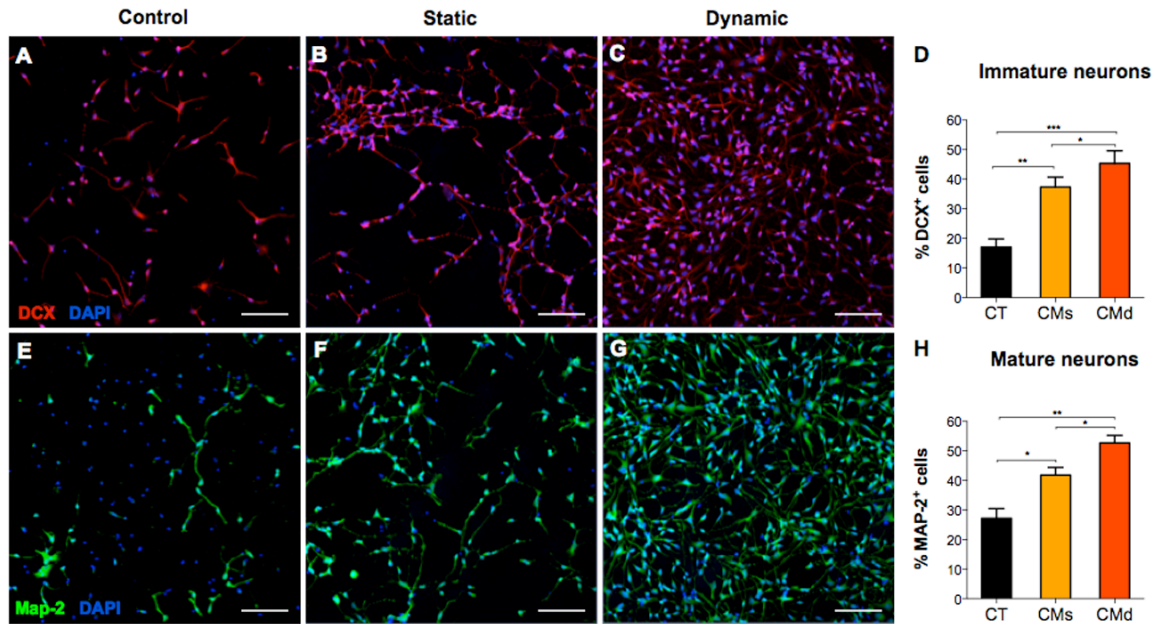
Figure 2. *In vitro* differentiation of hNPCs. hMSCs CM collected from static and dynamic culture conditions was able to significantly increase the survival and differentiation of hNPCs into (B, C) immature (DCX$^+$ cells) and (F, G) mature (Map-2$^+$ cells) neurons when compared to the (A, E) control group (D, H; mean ± S.E.M., n=3, p < 0.001). At the same time, the (C, G) hMSCs CM collected from the dynamic culture conditions also showed significantly higher levels of immature and mature neurons compared to the (B, F) static hMSCs CM in the induction hNPC differentiation (D, H; mean ± S.E.M., n=3, p < 0.05). CT: control group (Neurobasal®-A media); CMs: hMSCs Static conditioned media; CMd: hMSCs Dynamic bioreactor conditioned media (Scale bar: 50 µm).
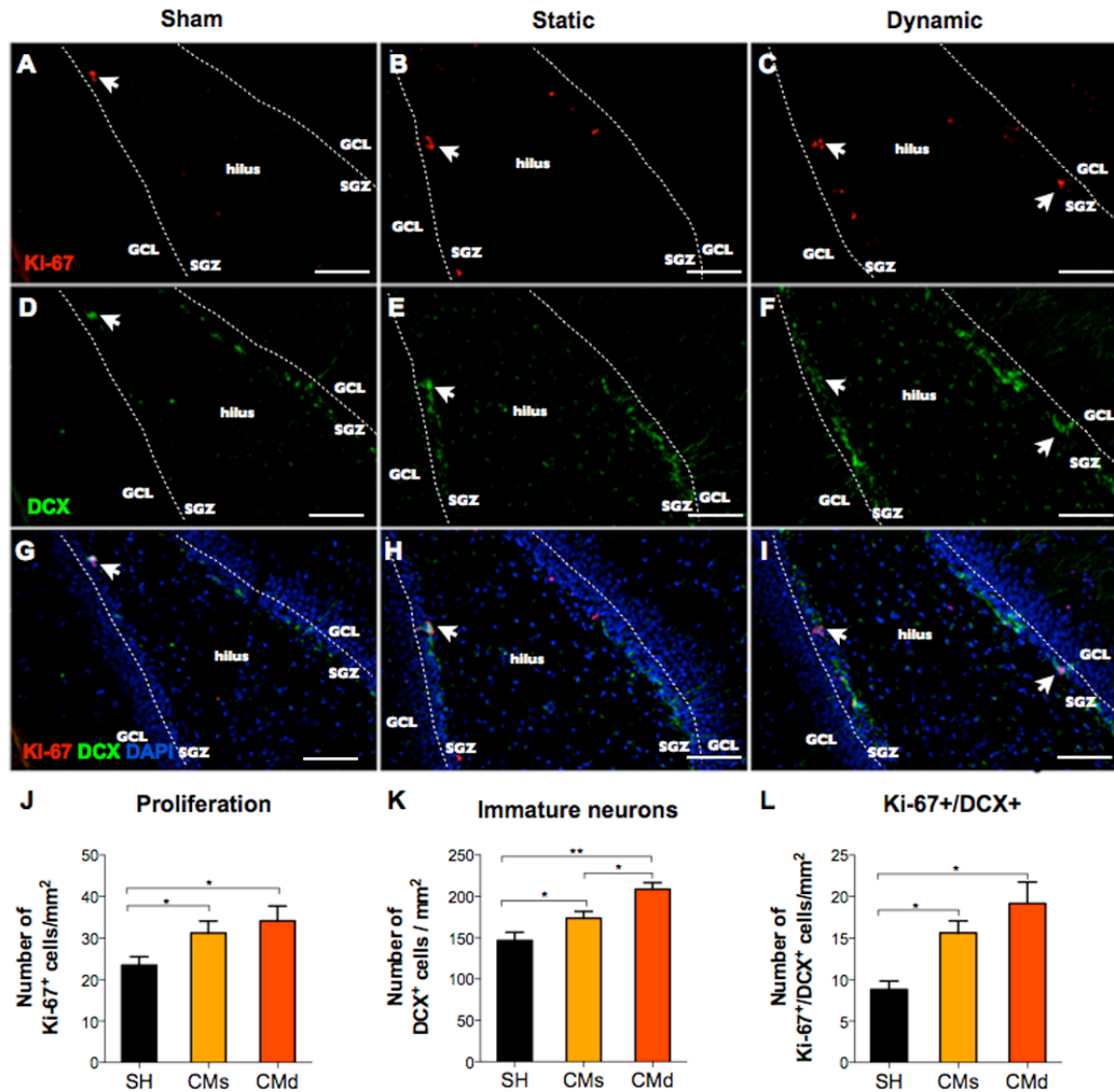
Figure 3. *In vivo* injection of CM increases proliferation and neuronal differentiation. Injection of the hMSC CM from static and dynamic conditions into the DG of adult rat hippocampus. After 7 days post-injection, both CM (static and dynamic) were able to increase the (B, C) levels of endogenous proliferating cells (Ki-67$^+$ cells) in the DG when compared to the (A) sham group (J, mean ± S.E.M., n=5, $p < 0.05$). Moreover, although both media were able to increase the number of newborn neurons (DCX$^+$ cells) compared to the (D) sham group, the dynamic CM showed higher numbers of the newborn (neurons) cell densities (K, mean ± S.E.M., n=5, $p < 0.05$) compared to the static CM. For the induction of neuronal differentiation, both CM (from the static and dynamic conditions) were able to increase significantly the number of Ki-67$^+$/ DCX$^+$ cells when compared to the control group (L, mean ± S.E.M., n=5, $p < 0.05$), SH: Sham (injected with Neurobasal®-A media), CMs: hMSCs Static conditioned media group. CMd: hMSCs Dynamic bioreactor conditioned media group (Scale bar: 100 μm).
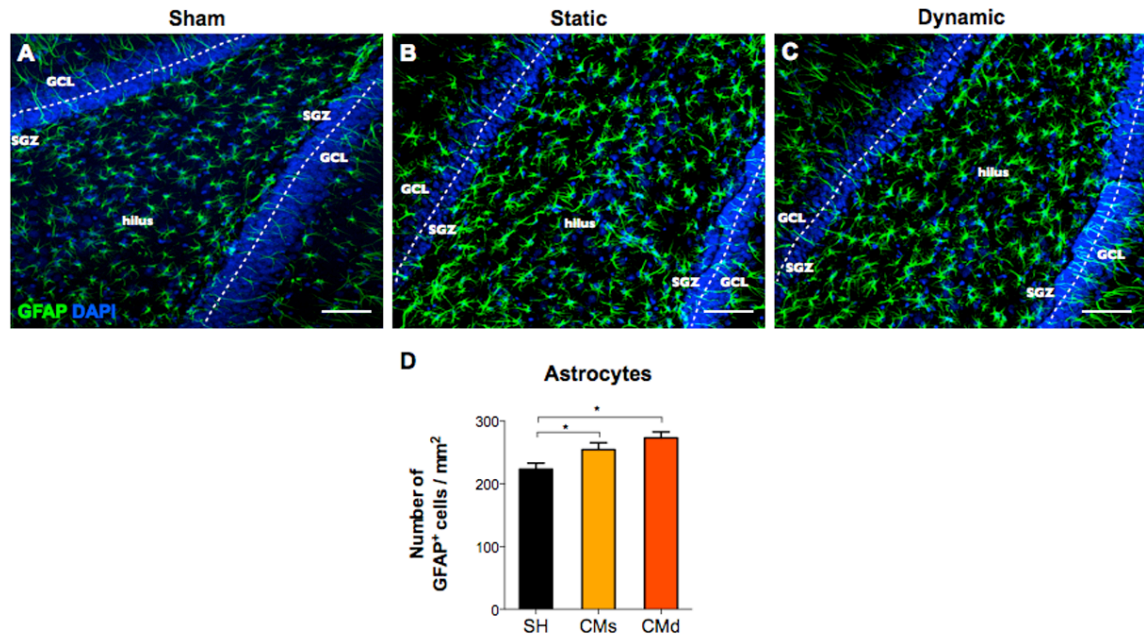
Figure 4. *In vivo* injection of CM increases astrocytic cell densities. hMSC CM from static and dynamic conditions increased astrocytic densities in the DG of hippocampus. Immunohistochemistry to GFAP (Astrocytes: A/B/C) revealed increased numbers promoted by the injection of hMSCs CM (D, statistically significant to the Sham group. (D, mean ± S.E.M., n=5, $p < 0.05$) 7 days post-injection. SH: Sham group (injected with Neurobasal®-A media), CMs: hMSCs Static conditioned media group. CMd: hMSCs Dynamic bioreactor conditioned media group (Scale bar: 100 μm).

3.4. hMSCs secretome proteomic analysis

In order to further understand the differences that were evidenced in the *in vitro* and *in vivo* studies, hMSCs secretome (from both the static and dynamic conditions) was characterized through a proteomic approach based analysis. From these results, we observed that the bioreactor-based bioprocess (dynamic condition) modulated the hMSCs secretome to produce a different pattern of protein expression when compared to the CM collected from the static condition (Figure 5A-B). In line with this, through the use of the Venn diagram software (http://bioinformatics.psb.ugent.be/webtools/Venn/) we were able to identify 120 proteins in the static and 130 proteins in the dynamic condition (Figure 5C, D), in which 102 proteins were common to the two conditions (static and dynamic; Figure 5E). From these, when we analyzed the expression levels of the two hMSC secretomes for specific proteins with actions in CNS physiology, we were able to find that molecules such as Cystatin-C (Cys C), Glia-derived nexin (GDN), Galectin-1, and Pigment epithelium-derived factor (PEDF), were upregulated in the dynamic conditions (Figure 6). Moreover it was also found that some other proteins with important roles in CNS regulations

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were only found in the CM from MSCs cultured in the bioreactor, namely Ezrin, Radixin, Thymosyn-β proteins, Beta-1,4-galactosyltransferase and connective tissue growth factor (CTGF).
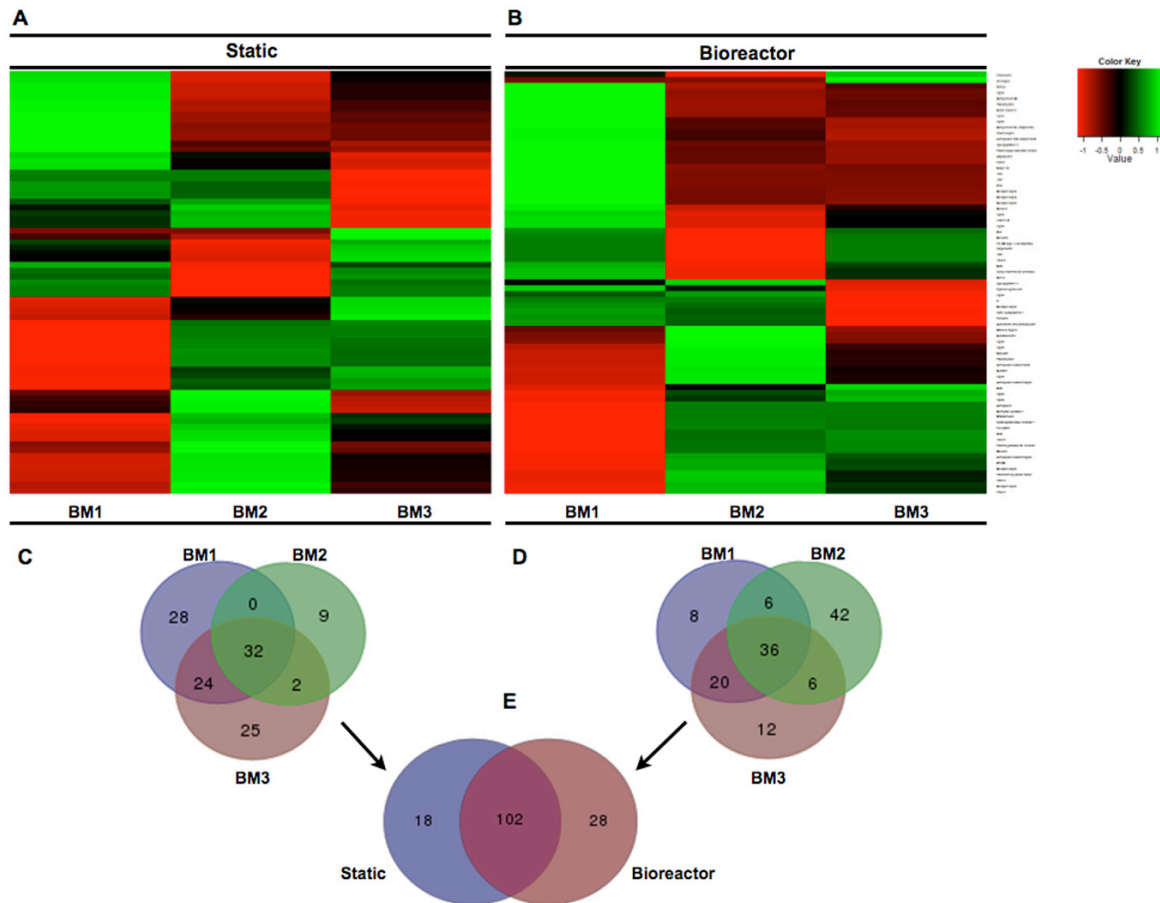
Figure 5. Proteomics - Heatmap and Venn diagram. Graphical representation of hMSC CM proteomic analysis by mass spectrometry. Peaks detected after CM analysis show that the patterns of protein expression is modulated when we change from (A) static to (B) dynamic culture condition. Indeed, the (D) Venn diagram indicates more proteins were identified in dynamic CM (130 proteins) when compared do the (C) static CM (120 proteins), in which (E) 102 proteins were common to the two conditions.
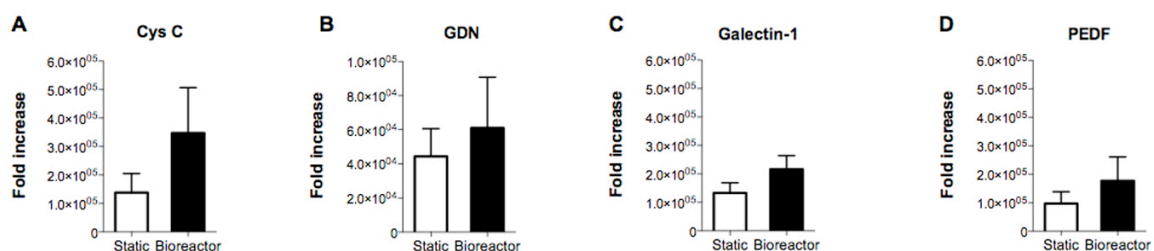
Figure 6. Graphs of Specific hMSC CM Proteins. Comparative analysis of the secreted paracrine factors with known neuregulatory actions in the CM collected from static and dynamic conditions.

4. Discussion

The use of bioreactors has been suggested as a promising alternative to conventional culture flasks for hMSC expansion [27, 30, 42]. So far, different bioreactors have been used for hMSC expansion, including fixed bed bioreactors, perfusion bioreactors, and the cultivation of hMSCs as aggregates or through the use of microcarriers (MCs) in stirred suspension bioreactor systems [43-47]. In the present work, we have expanded hMSCs on MCs in computer-controlled stirred suspension bioreactor. This technology allows us to: (1) expand a large number of cells in one vessel, (2) monitor and ensure tight control of process variables such as pH, temperature and dissolved oxygen concentration, and (3) develop a process which allows for scale-up to larger bioreactor systems. Numerous studies have evaluated the use of microcarriers and suspension bioreactors for the expansion of hMSCs [29, 30, 48-50]. Using our serum-free medium (PPRF-h2), we have shown that we can minimize this lag phase while achieving an 18-fold cell expansion in our microcarrier-mediated suspension bioreactors [27]. Herein, we have shown that by expanding the hMSCs in a computer-controlled suspension bioreactor system, we can achieve similar cell doubling times and expression of hMSC surface antigens. This is the first work that we know of, that has shown that hMSCs can be isolated and expanded in a serum-free medium in computer-controlled stirred-suspension bioreactors.

The *in vitro* application of the hMSC secretomes obtained from static and dynamic conditions revealed that both were able to induce the survival and differentiation of human CNS-derived cells. Indeed, as shown in Figure 2, when hNPCs were incubated with the two CM an increased differentiation of hNPCs to the neuronal lineages – both immature (DCX-positive cells) and mature (MAP-2 positive cells) neurons - was observed compared to the control group. This is in line to what Sart and colleagues [51] had already reported regarding the effects hMSC secretome, and the bioactive molecules within it, on NPCs differentiation and maturation. Moreover, they observed that the hMSCs secretome was able to enhance the proliferation, migration and neurite extension of NPCs [51]. Moreover, we also observed that the dynamic hMSCs secretome was significantly better in promoting neural differentiation/survival when compared to the static secretome (Figures 2B-D and 2F-H). It has been shown that the secretion of neurotrophic factors like FGF-2, TGF-β1, NGF and BDNF by hMSCs plays a role in promoting growth, as well as glial and neuronal differentiation of NPCs [12, 52-55]. Moreover, previous results from our laboratory have revealed through proteomic-based techniques that in addition to the classical growth factors, hMSCs are also capable to secrete

proteins such as 14-3-3 and hsp70, in which the significant increase of neuronal densities was correlated with their presence in the CM of human umbilical cord perivascular cells (HUCPVCs) [56]. The proteomic analysis performed in the present work further revealed that hMSCs produce other kind of molecules, than those already reported for this type of studies, with a neuroregulatory potential. From these Cys C, GDN, Galectin-1 and PEDF were found to be upregulated in the dynamic conditions (Figure 6). They have been reported to have important roles in the migration, differentiation and neuroprotection of neural progenitors and neural cells both *in vitro* and *in vivo* [57-62]. For instance, Cys C and GDN (upregulated in the dynamic CM, Figure 6A, B) are known to play crucial roles in the enhancement on neurite outgrowth and neuroprotection through the prevention of oxidative stress [62-67]. On the other hand, Galectin-1 and PEDF (upregulated in the dynamic CM, Figure 6 C, D) have been described as important regulators involved in neurogenesis, playing a role on neural stem cells self-renewal and differentiation as well as into neuroprotection and functional recovery after the occurrence of CNS disorders [60, 68-73]. Interestingly, in addition to this, we also identify the presence of 28 specific molecules only in the dynamic CM (Figure 5E). From these, besides muscular and skeletal proteins, we were able to identify more neuregulatory potential molecules such as Ezrin, Radixin, β-Thymosin proteins, β-1, 4-galatosyltransferase and Connective tissue growth factor, which have been described as important regulators of neurite outgrowth, neuroregeneration and angiogenesis [74-81].

The *in vivo* experiments revealed that the application of both hMSC CM (dynamic and static) were able to boost cell proliferation within the DG 7 days after their injection, as assessed by the number of Ki-67 positive cells (Figure 3A-C, J). Indeed, this was also noted (with BrdU positive cells) in one of our previous works where we compared injection of MSCs isolated from the umbilical cord tissue or their secretome (CM) into the DG [41]. Similar effects were also observed regarding neuronal differentiation. 7 days after the injection of the both secretomes (static and dynamic), it was possible to observe that both of them were able to increase significantly the number of immature neurons (DCX-positive cells) in all the granular layer (particularly in the SGZ) compared to the Sham group (Figure 3D-F, K). Moreover, we also observed that the dynamic hMSC secretome injected group displayed a significantly increase in neuronal cell densities (DCX$^+$ cells) in the DG when compared to the static group (Figure 3K). However, when we compared the level of neuronal differentiation, by double staining for Ki-67 and DCX, we observed that both secretomes (static and dynamic) were able to increase similar numbers of newly formed neurons (Figure 3G-I, L). Thus, these results indicate that *in vivo*, the secretome of dynamically cultured hMSCs induces a high survival rate of

neuronal cells. From the proteomic data (Figure 6) we hypothesize that this could be related with the increased expression of PEDF and Galectin-1 in the secretome of these cells.

5. Conclusions

In the present work, we have demonstrated that the use of computer-controlled stirred suspension bioreactors improves the therapeutic potential of the hMSC secretome. In fact, we have been able to induce a significantly higher number of human neural progenitors to differentiate into neurons at different stages of maturation when compared to the hMSC secretome collected under static conditions. Additionally, *in vivo* injections into the hippocampal DG revealed that hMSC dynamic secretomes (i.e. conditioned media) induced increased levels of neuronal cells densities (DCX$^+$ cells) when compared to both static and sham group. These outcomes were associated with the presence of neuroregulatory molecules within the dynamic secretome constitution such as PEDF and Galectin-1, considered to be important regulators/modulators of the neurogenic and neural differentiation processes. Thus, our results suggest that the use of a dynamic culture condition (i.e. computer-controlled stirred suspension bioreactors) improves the action of the hMSC secretome to modulate neural progenitor proliferation and differentiation, which may open novel therapeutic opportunities in the future.

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Chapter 4

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Behie LA. (2014)

Does Hypoxia/Normoxia Culturing Conditions Change the
Neuroregulatory Profile of Wharton Jelly Mesenchymal Stem Cells
Secretome?

Does Hypoxia/Normoxia Culturing Conditions Change the Neuroregulatory Profile of Wharton Jelly Mesenchymal Stem Cells Secretome?

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Abstract

The use of human umbilical cord Wharton Jelly-derived mesenchymal stem cells (hWJ-MSCs) has been considered a new potential source for future safe applications in regenerative medicine. Indeed, the application of hWJ-MSCs into different animal models of disease, including those from the central nervous system (CNS), has shown remarkable therapeutic benefits mostly associated with their secretome. Conventionally, hWJ-MSCs are cultured and characterized under normoxic conditions (21% oxygen tension), although the oxygen levels within tissues are typically much lower (hypoxic) than these standard culture conditions. Therefore, oxygen tension represents an important environmental factor that may affect the performance of MSCs *in vivo*. However, the impact of hypoxic conditions on distinct MSCs characteristics, such as the secretome, remains still unclear. Consequently, in the present study we examined the effects of normoxic (21% O_2) and hypoxic (5% O_2) conditions on the hWJ-MSCs secretome. We present data indicating that the hWJ-MSCs secretome collected from normoxic and hypoxic conditions displayed similar effects in supporting neuronal differentiation of human neural progenitors *in vitro*. However, proteomic analysis revealed that the use of hypoxic preconditioning led to the upregulation of several proteins within the hWJ-MSC secretome. The optimization of parameters, such as hypoxia, may lead to the development of strategies that enhance the therapeutic effects of the secretome for future regenerative medicine studies and applications.

1. Introdution

The use of adult stem cells as a possible therapeutic tool in the regenerative medicine field has been widely assessed, that includes human mesenchymal stem cells (hMSCs) [1-3], which have emerged as a promising therapeutic cell type [4-6]. The stem/progenitors cells present in the human Wharton Jelly (WJ) of the umbilical cord, known as human Wharton Jelly Mesenchymal Stem Cells (hWJ-MSCs) have been suggested as a possible population of interest for future clinical applications [7-9]. Like bone marrow MSCs (BM-MSCs) and adipose stem cells (ASCs), these populations are also defined as MSCs [10-12]. The secretion of trophic bioactive molecules (i.e. MSCs secretome) has now been considered as the most probable route for their therapeutic efficacy when applied both *in vitro* and *in vivo* [13-16]. Indeed, over the last decade, there has been a substantial effort to assess the impact of MSCs (including hWJ-MSCs) and its secretome into different disorders, such as those affecting the central nervous system (CNS) [14]. However, despite promising results of hMSCs and their paracrine activity, the low number of cells that normally are obtained after isolation still continues to be one of the big limitations of their application into the clinic [17]. Moreover, studies have shown that it is crucial to create new strategies that allow for the generation *in vitro* of a clinically-relevant number of cells in the clinic [18, 19]. One strategy that looks very promising is to focus on the modulation of culture conditions in which the dissolved oxygen concentration may play an important role in the behavior of MSCs [17].

Normally, hMSCs are cultured *in vitro* in static culture and in a 21% of oxygen tension environment. However, studies have demonstrated that the physiological niches from where hMSCs are isolated in the human body are at much lower oxygen tensions than 21% [20-22]. Indeed, it has been hypothesized that a hypoxic state could maintain the stemness of hMSCs, [20]. Other studies have also shown that hypoxic culture conditions affect the therapeutic properties of hMSCs [23, 24]. For instance, Rhijn and colleagues [17] demonstrated that hypoxic pre-conditioning enhances the regenerative potential of MSCs, maintaining their immunosuppressive capacities under these conditions. In addition, Tsai and colleagues [23] demonstrated that the use of 1% oxygen reduces hMSCs senescence while it increases their proliferation levels and maintains their differentiation properties. Similar outcomes were also described for hMSCs obtained from adipose tissue and Wharton Jelly [20, 25, 26].

Also in the secretome, the oxygen tension seems to play an important role [27, 28]. Previous studies have shown that by changing the oxygen concentration it was possible to modulate the angiogenic potential of MSCs namely through the increase of the secretion of vascular endothelial growth factor

(VEGF), b-fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) [27-29]. However, there are still reports examining carefully applications of different oxygen tensions on the performance of hMSCs [20, 30, 31]. Regarding hypoxic, Volkmer and colleagues [32] observed that prolonged exposure to hypoxia leads to cell death. On the other hand, under normoxic conditions studies have seen that higher levels of oxygen could be toxic, causing oxidative stress due to the generation of reactive oxygen species (ROS) that could alter the metabolic efficiency of the cells [21, 33]. Nevertheless, the real impact of oxygen on key hMSCs characteristics is still unclear.

Thus, based on these observations, in the present work we aimed to characterize and analyze the effects of the hWJ-MSCs secretome collected from hypoxic culture conditions to that obtained from normoxic culturing conditions. Results revealed that the use of different oxygen conditions (i.e. hypoxic and normoxic) led to a different secretome profile for hWJ-MSCs. In line with this, we further observed that hWJ-MSCs were able to secrete important neuroregulatory molecules such as Glia-derived nexin (GDN) and Cystatin C (Cys C), which were upregulated in the normoxic condition. In the hypoxic condition, the proteins Ubiquitin carboxy-terminal hydrolase L1 (UCHL1), Clusterin, Peroxiredoxin-1 (Prx1), 14-3-3 proteins, Thymosin-beta proteins and elongation factor 2 (EF-2), were found to be upregulated in the hWJ-MSC secretome. Additionally, we have also found Vitronectin, Cadherin-2 and Multidrug resistance-associated protein 1 (MRP1) expressed only in the normoxic conditions, while Pigment epithelium-derived factor (PEDF), Insulin growth factor 2 (IGF-2), Semaphorin-7A, Macrophage migration inhibitory factor (MIF), Hsp70 and Moesin were just found to be present in the hypoxic conditions. Finally, we also observed that the obtained secretomes were able to induce and support neuronal differentiation of hNPCs *in vitro,* in which the previously identified proteins in the hWJ-MSCs CM may explain our results.

2. Methods

2.1. Expansion of hWJ-MSCs under hypoxic and normoxic conditions (in dynamic bioreactors) and collection of conditioned medium

2.1.1. Preparation of 500 mL Suspension Bioreactors

A DASGIP Parallel Bioreactor system (DASGIP, Julich, DK) was used for the expansion of hWJ-MSCs in dynamic conditions. Prior to inoculating hWJ-MSCs in the DASGIP bioreactors, the 500 mL suspension bioreactors (containing modified Teflon 4-paddle impellers) were siliconized using Sigmacote (Sigma) to minimize cell attachment to the sides of the bioreactor vessel and the impeller.

After siliconization and autoclaving of the vessels, the DASGIP system was calibrated according to procedures provided by the manufacturer. The bioreactors were maintained at - (1) 37°C using a heating jacket, (2) at 100% dissolved oxygen (corresponding to oxygen saturation of the medium at 37°C exposed to 21% O_2 in the headspace) for normoxic conditions, (3) at 21% dissolved oxygen (corresponding to oxygen saturation of the medium at 37°C exposed to 5% O_2 in the headspace) for hypoxic conditions, (4) a pH of 7.4, controlled by a gas mixture connected to oxygen, nitrogen, carbon dioxide and air tanks that was introduced into the headspace, and (5) an agitation of 52 rpm using a magnetic stir plate under the bioreactors.

2.1.2. Preparation of Microcarriers and Inoculation of hWJ-MSCs

Cytodex 3 microcarriers (GE Healthcare) were used for this study and were prepared as follows: 1.0 g (per condition) of microcarriers were weighed out and hydrated in 50 mL of 1X PBS (Life Technologies) in two 125 mL, pre-siliconized, Erlenmeyer flasks, at room temperature overnight. To this flask 2-3 drops of Tween 80 (United States Biochemical Corporation, Cleveland, OH) was added to break the surface tension and ensure proper wetting and sedimentation of the microcarriers. The microcarriers were then washed 3X with 1X PBS, and autoclaved. Following autoclaving the microcarriers were incubated with fetal bovine serum (FBS, Life Technologies) for 6 h at 37°C to coat the microcarriers with serum-attachment factors, and agitated every 30 min. After 6 h, the FBS was removed, the microcarriers were washed twice with our serum-free medium (PPRF-msc6), and then inoculated into our 500 mL DASGIP bioreactors in 275 mL of serum-free medium for 4 h at the controlled culture conditions. Cryopreserved human Wharton Jelly MSCs (hWJ-MSCs) at passage 2 (P2), and derived from three donors (WJ1, WJ2, and WJ3) were expanded in serum-free medium, PPRF-msc6 [34] in static culture for two passages before inoculation into the DASGIP bioreactors. The cells were harvested using trypsin-EDTA, and then inoculated into the bioreactors at a density of 24,000 cells/mL (based on the final volume of 500 mL) and the volume of the bioreactors was maintained at 325 mL for the first 24 h to increase cell attachment. After 24 h the culture volume was increased to 500 mL to bring the final microcarrier density to 2.0 g/L. The cells were cultured on the microcarriers for 72 h, after which time the bioreactors were removed from the DASGIP system, and placed in a biosafety cabinet for 10 min to allow the microcarriers to settle. The supernatant was removed from the bioreactors, and the microcarriers were washed once with 100 mL of Neurobasal®-A medium. Following this, 500 mL of Neurobasal®-A medium was added to the bioreactors and the bioreactors were placed back into the DASGIP control system for 24 h. After 24h,

the bioreactors were again removed from the system, placed in a biosafety cabinet for 10 min to allow the microcarriers to settle, the supernatant was harvested and centrifuged at 300 g for 10 min to remove any cell debris. This supernatant [called dynamic conditioned medium (CM)] collected from normoxic and hypoxic conditions, was then stored at -80°C until it was required for further experiments.

2.2. Growth of hNPCs and incubation with hWJ-MSCs CM collected from normoxic and hypoxic conditions

Human telencephalon-derived neural precursor cells (hNPCs) were isolated from the telencephalon region of a 10-week post-conception fetus according with the protocols and strict ethical guidelines previously established [35-37]. Pre-isolated and cryopreserved hNPCs were thawed at 37°C and the contents placed into a T-25 flask (Nunc) containing 5 mL of a serum-free medium PPRF-h2 [35]. After two days, the cells were harvested and mechanically dissociated into a single cell suspension, and subcultured into fresh cell growth medium (PPRF-h2). Every 4 days, the T-flasks were fed by replacing 40% of the spent medium with fresh growth medium. After 14-20 days of growth in the culture flasks, hNPCs were passaged and plated onto pre-coated [(poly-D-lysine hydrobromide (100 μg/mL) and laminin (10 μg/mL) - Sigma)] 24-well plates at a density of 4.0×10^4 cells per well in the presence of the hWJ-MSC CM obtained from either hypoxic or normoxic conditions. The plates were placed in an incubator operating at 37 °C, 5% CO_2, 95% air and 90% relative humidity for 5 days.

2.3. Immunostaining

hNPCs were fixed in 4% paraformaldehyde (Mallinckrodt, Paris, KY) for 15 min, and then permeabilized by incubation with 0.1% Triton X-100 (Sigma) in 1X PBS for 5 min at room temperature, and washed three times in 1X PBS. hNPCs were then blocked with 10% of fetal calf serum (FCS, Life Technologies) in 1X PBS, followed by a 1 h incubation (at 37°C) with the primary antibodies: rabbit anti-doublecortin (DCX; 1:500, Abcam, Cambridge, MA) to detect immature neurons and mouse anti-rat microtubule associated protein-2 (MAP-2; 1:500, Sigma) to detect mature neurons. hNPCs were then washed with 1X PBS three times and incubated with the secondary antibodies namely, Alexa Fluor 488 goat anti-rabbit (IgG, Life Technologies) and Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG, Life Technologies) for 1.0 h at 37°C and then 10 min with 4-6-diamidino-2-phenylindole-dihydrochloride (DAPI; Life Technologies). After staining,

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samples were observed under an Olympus BX-61 Fluorescence Microscope (Olympus, Hamburg, DK). For quantification purposes, three coverslips and ten representative fields per condition were chosen and analyzed. Results are shown as percentage of DCX or MAP-2 positive cells over the total number of cells per field of observation (n=3).

2.4. Proteomics – Mass Spectrometry and SWATH Acquisition
2.4.1. Liquid Digestion/Sample Preparation

hWJ-MSCs CM was firstly concentrated using a Vivaspin 20 sample concentrator (GE Healthcare) by ultracentrifugation at 3000 g for 45 min. Then, the CM was precipitated with Trichloroacetic acid (TCA) – Acetone. TCA was added to each sample to a final concentration of 20% (v/v), followed by 30 min incubation at -80^0C and centrifugation at 20 000 g for 20 min. Protein pellets were washed with ice-cold ($-20°$C) acetone, briefly the pellets were solubilised in acetone, aided by ultrasonication, followed by a centrifugation at 20 000 g for 20 min. The washed pellets were ressuspended in 1.0 M Triethylammonium bicarbonate buffer (TEAB, Sigma), aided by ultrasonication, followed by a centrifugation at 20 000 g for 5.0 min to remove insoluble material [38].

Samples were quantified using the 2D-Quant Kit (GE Healthcare) and 100 µg of each sample were subjected to liquid digestion. Briefly, 4 µL of 50 mM TCEP was added to 45 µL of sample, followed by a ultrasonication step for 2 min. Next, 2 µL of 600 mM MMTS was added and samples were left to react for 10 min, at room temperature. TEAB was then added to adjust the volume of sample to 100 µL, and the samples were digested with trypsin overnight (2 µg trypsin/sample), at 37 °C, with swirling at 650 rpm. Reactions were stopped by the addition of 2 µL of formic acid (FA) and the peptides were dried by rotary evaporation under vacuum. Before digestion, the samples were spiked with 2 µg of green fluorescent protein (GFP) to monitor samples loss during the procedure.

Before performing the MS/MS analysis the peptide mixtures were cleaned/desalted-using OMIX tips with C18 stationary phase (Agilent Technologies) as recommended by the manufacturer. Eluates, spiked with iRT peptides (Biognosys), were dried by rotator evaporation, avoiding to totally evaporating the samples. The samples were ressuspended to 23 µL in a solution of 2% ACN and 0.1% FA followed by vortex, spin and sonication in water bath (2 min with pulses of 1.0 s – 1.0 s sonication followed by 1.0 s break pulse –, at 20% intensity, in a sonicator VibraCell 750 W,

Sonics® (Sonics&Materials)]. In order to remove insoluble material the peptide mixture were then centrifuged for 5 min at 14 000g and collected into the proper vial for LC-MS injection.

2.4.2. SWATH acquisition

Samples were analyzed on a Triple TOF™ 5600 System (ABSciex®) in two phases: information-dependent acquisition (IDA) was followed by SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition on the same sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a ChromXP™ C18AR reverse phase column (300 μm ID × 15cm length, 3 μm particles, 120 Å pore size, Eksigent®) at 5μL/min. Peptides were eluted into the mass spectrometer with an acetonitrile gradient in 0.1% FA (2% to 35% ACN, in a linear gradient for 25 min), using an electrospray ionization source (DuoSpray™ Source, ABSciex®).

For information dependent acquisition (IDA), the mass spectrometer was set to scanning full spectra (350-1250m/z) for 250 ms, followed by up to 30 MS/MS scans (100–1500 m/z for 75 ms each). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 70 counts per second were isolated for fragmentation and one MS/MS spectra was collected for 75 ms before adding those ions to the exclusion list for 15 s (mass spectrometer operated by Analyst® TF 1.6, ABSciex®). Rolling collision was used with a collision energy spread of 5. Peptide identification was performed with Protein Pilot software (v4.5, ABSciex®). Search parameters used were the following: SwissProt database, against a database composed by *human and bovine* species from SwissProt database (release at February 2014), GFP and iRT peptides, and using MMTS alkylated cysteines as fixed modification. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR [39, 40].

The SWATH setup was essentially that used by Gillet et al [41], with the same chromatographic conditions used as in the IDA run described above. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode. The instrument was specifically tuned to allow a quadruple resolution of 25 m/z mass selection. Using an isolation width of 26 m/z (containing 1 m/z for the window overlap), a set of 30 overlapping windows was constructed covering the precursor mass range of 350–1100 m/z. A 250 ms survey scan (350-1500 m/z) was

acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from 100–1500 m/z for 90 ms resulting in a cycle time of 3 s from the precursors ranging from 350 to 1100 m/z. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with a collision energy spread of 15.

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments, and used for subsequent SWATH processing. Libraries were obtained using Protein Pilot™ software (v4.5, ABSciex®) with the same parameters as described above. Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®), briefly peptides were selected automatically from the library using the following criteria: (i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and (ii) peptides that contained biological modifications and/or were shared between different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH™ quantitation was attempted for all proteins in library file that were identified below 5% local FDR from ProteinPilot™ searches. In SWATH™ Acquisition data, peptides are confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide. Target fragment ions, up to 5, were automatically selected and peak groups were scored following the criteria described in Lambert et al [42]. Peak group confidence threshold was determined based on a FDR analysis using the target-decoy approach and 1% extraction FDR threshold was used for all the analyses. Peptide that met the 1% FDR threshold in one of the samples were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 3.0 min. The levels of the human proteins were estimated by summing all the transitions from all the peptides for a given protein (an adaptation of [43]) and normalized to the more stable internal standard.

2.5. Statistical Analysis

Statistical evaluation was performed using One-way ANOVA and Student's t-test through the program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Data is presented as mean ± S.E.M. Significance value was set at $p < 0.05$.

3. Results

3.1. Expansion of hWJ-MSCs under normoxic and hypoxic conditions on Cytodex 3 microcarriers in dynamic conditions

PPRF-msc6, has been shown to support the rapid and efficient isolation and expansion of hBM-MSCs from bone marrow mononuclear cells. Additionally, higher cell-yields were obtained in comparison to hBM-MSCs that were isolated and expanded in classical serum-based medium over the same culture period [19, 34]. Moreover, this serum-free medium was shown to support the isolation and expansion of hMSCs derived from adipose, pancreatic and umbilical cord in conventional static culture [44]. Herein, we report that hWJ-MSCs were able to attach to and grow on Cytodex 3 microcarriers in computer-controlled stirred suspension bioreactors in normoxic and hypoxic conditions (Figure 1A, B We also set and controlled key parameters in this dynamic environment and observed that dissolved oxygen [21% (normoxic) and 5% (hypoxic)], pH and temperature were well controlled within the bioreactor at the set points during the expansion and conditioning phase. The collection hWJ-MSCs CM was performed at the end of the conditioning phase illustrated in Figure 1 C, D.

3.2. Hypoxic and Normoxic hWJ-MSCs secretomes induced neuronal differentiation of hNPCs *in vitro*

Human telencephalon-derived neural precursor cells (hNPCs) were grown as neurospheres in a serum-free medium PPRF-h2 [35]. The impact of hWJ-MSCs secretomes (from hypoxic and normoxic conditions) on the differentiation of hNPCs was evaluated. Upon the removal of PPRF-h2, and plating in adherent dishes with hWJ-MSCs normoxic or hypoxic secretomes, hNPCs adhered and started to differentiate. Immunocytochemistry analysis revealed that when htNPCs were incubated for 5 days with the hWJ-MSCs CM of both conditions there was a similar rate of neuronal differentiation into different stages of maturation namely immature neurons (DCX$^+$ cells) and mature neurons (MAP-2$^+$ cells; Figure 2), suggesting that both secretomes (from normoxic and hypoxic conditions) favoured hNPCs differentiation.
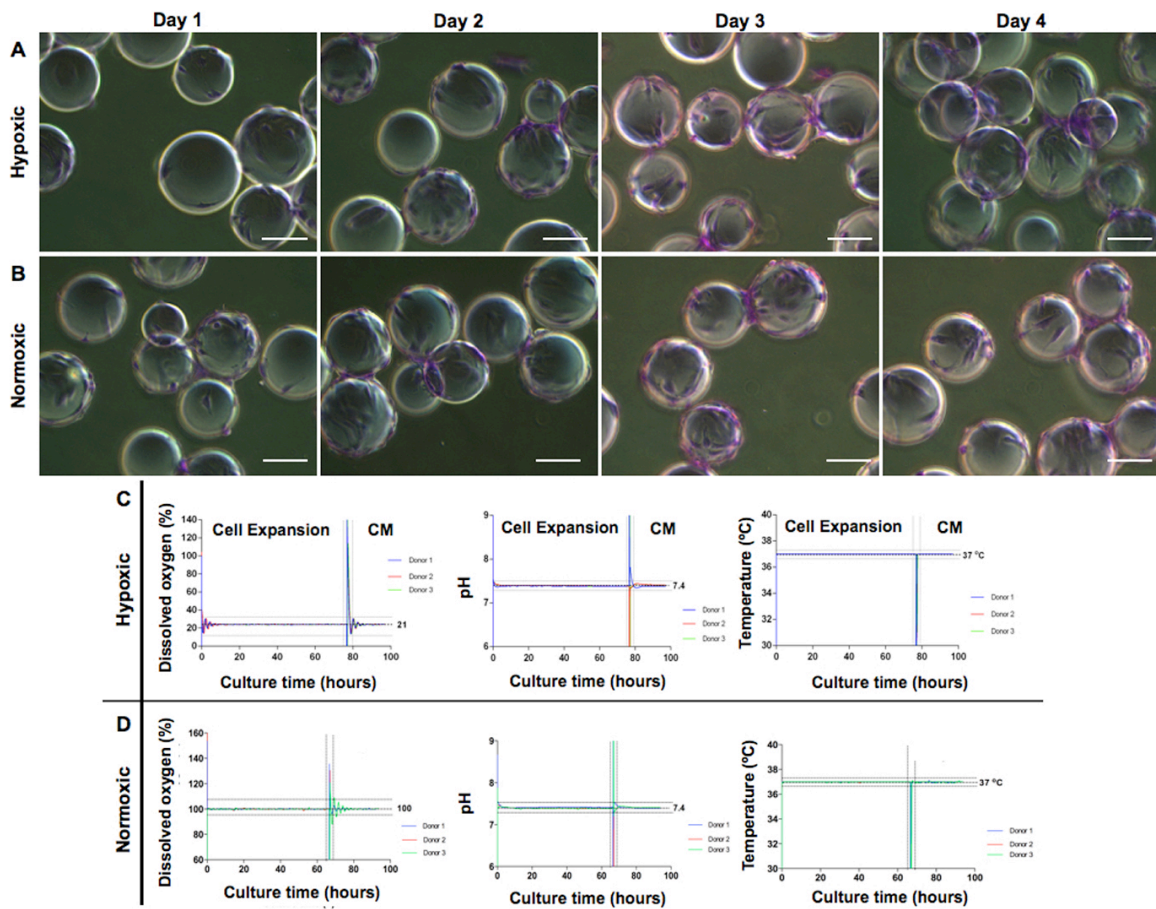
Figure 1. Expansion and adherence of hWJ-MSCs on Cytodex-3 microcarriers in computer-controlled bioreactors. hWJ-MSCs were able to adhere to microcarriers under - (A) hypoxic, and (B) normoxic conditions in the suspension bioreactors, and were well maintained at pre-determined setpoints for the culture period (C). Scale bars represent 200 μm.
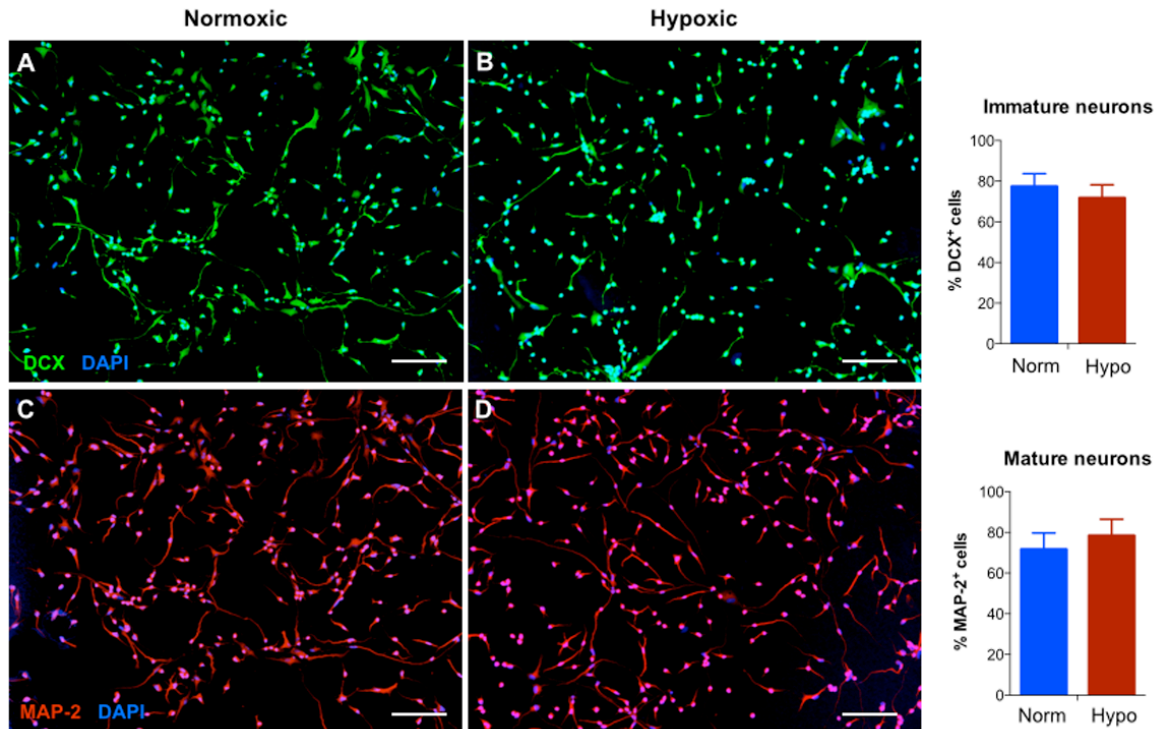
Figure 2. *In vitro* differentiation of hNPCs into immature and mature neurons. hWJ-MSCs Conditoned Medium (CM) collected from hypoxic and normoxic conditions was able to support and induce neuronal differentiation of hNPCs into (A, B) immature (DCX^+ cells) and (C, D) mature (Map-2^+ cells) neurons. Norm: Normoxic condition; Hypo: hypoxic condition. Scale bar: 100 μm.

3.3. Hypoxic and Normoxic conditions affect the profile of hWJ-MSCs secretome

In order to further understand the effects of using different percentages of oxygen (i.e. normoxic and hypoxic conditions) on the secretome of hWJ-MSCs, a proteomic-based analysis was performed. From the results, we observed that the use of different oxygen percentages modulated the hWJ-MSCs secretome to produce a different pattern of expression (Figures 3A, B), in which the hypoxic preconditioning led to an increased secretion profile of hWJ-MSCs when compared to the normoxic preconditioning. Indeed, we were able to identify under normoxic conditions 104 proteins (Figure 3C) whereas under hypoxic conditions we have identified 166 proteins (Figure 3D). After this proteomic analysis identification, when we analyzed both hWJ-MSCs secretomes for specific proteins with possible neuroregulatory actions on the CNS physiology (including on their derived cells), we were able to observe that hWJ-MSCs secreted important molecules such as Glia-derived nexin (GDN) and Cystatin C (Cys C), which were upregulated in the normoxic conditions (Figure 4A, B). As well, Ubiquitin carboxy-terminal hydrolase L1 (UCHL1), Clusterin, Peroxiredoxin-1 (Prx1), 14-3-3 proteins, Thymosin-beta proteins (significantly expressed, p<0.01), and elongation factor 2 (EF-2; significantly

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expressed, $p<0.05$), were found to be upregulated in the hypoxic conditions (Figure 4C-I). In addition to this, we discovered specific proteins that were restricted to each condition, presenting important roles in CNS regulation. Under normoxic conditions we were able to identify the presence of Vitronectin, Cadherin-2 and Multidrug resistance-associated protein 1 (MRP1) whereas, on the hypoxic conditions we were able to identify Pigment epithelium-derived factor (PEDF), Insulin growth factor 2 (IGF-2), Semaphorin-7A, Macrophage migration inhibitory factor (MIF), Hsp70 and Moesin.

Figure 3. hWJ-MSCs CM proteomic analysis. Graphical representation of WJ-MSC CM proteomic analysis by mass spectrometry. Peaks detected after CM analysis show that the patterns of protein expression is modulated when we change from - (A) normoxic to (B) hypoxic culture condition. Indeed, the Venn diagrams indicated that more proteins were identified in the hWJ-MSCs CM collected from (D) hypoxic conditions (166 proteins) when compared to the (C) normoxic hWJ-MSCs CM (104 proteins dynamic).
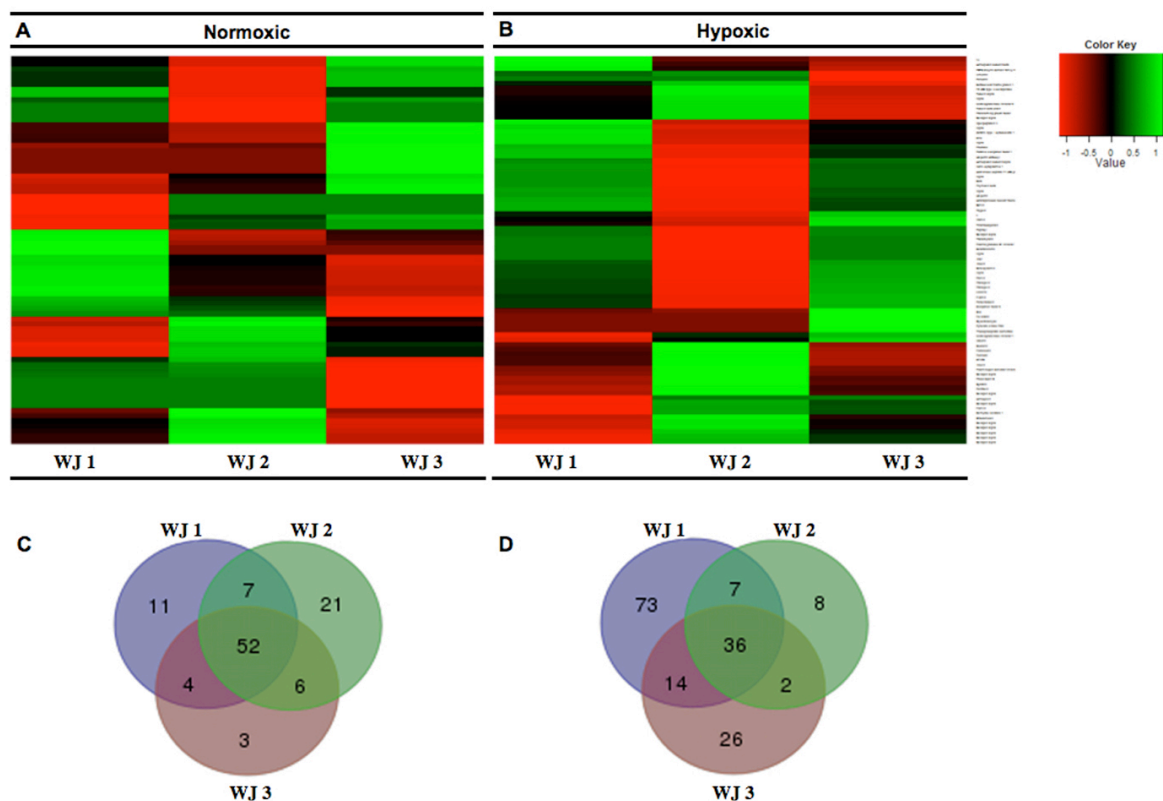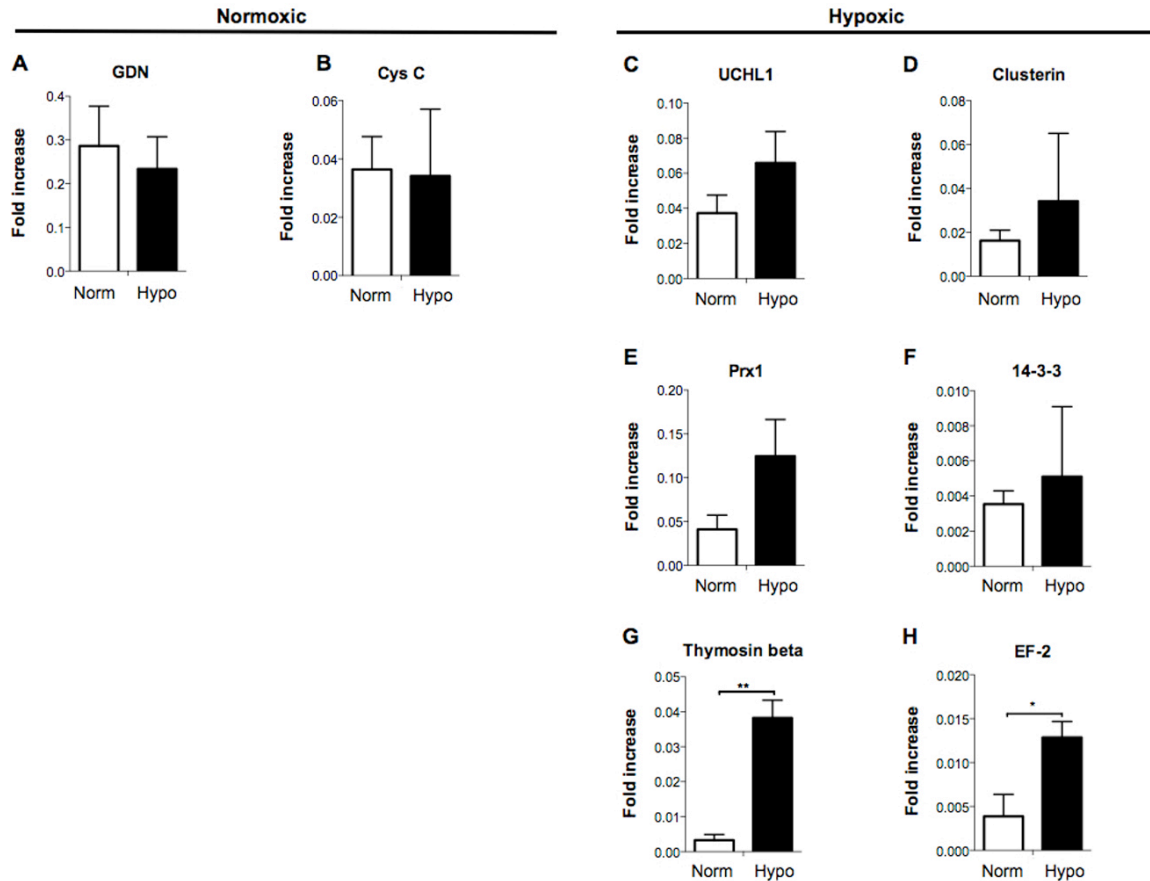
Figure 4. Specific WJ-MSCs CM proteins with neuroregulatory potential on CNS physiology. Comparative analysis of the secreted paracrine factors in the hWJ-MSC CM collected from normoxic (proteins: GDN, Cystatin C) and hypoxic conditions (proteins: UCHL1, Clusterin, Prx1, 14-3-3, Thymosin Beta, EF-2) with known neuregulatory actions (mean ± S.E.M., n=3, *p < 0.05; **p < 0.01). Norm: Normoxic condition; Hypo: Hypoxic condition.

4. Discussion

Reports have defended the proposition that the stem cell niche represents and plays an important role in stem cell biology and fate, in which oxygen concentration is an important component and regulator [20, 45]. Indeed, it has been suggested that low levels of oxygen (i.e. hypoxia) play an important role in the maintanance of the plasticity and proliferation of stem cells [22]. According to Cicione *et al.* [21] and Grant *et al.* [46] the conventional *in vitro* cultures are often carried out under ambient oxygen tension corresponding to 21%, called normoxic condition. However, the *in vivo* physiologic oxygen concentration is lower than this, varying from tissue to tissue and ranging from 1-13% [46]. In this way, the use of 21% oxygen tension exceeds the normal pressure that exists in

most of the mammalian tissues, which indicates that the oxygen concentration used during standard *in vitro* cultures of primary human cells, like MSCs, does not mimic the *in vivo* environment [21, 47]. Additionally, it has been hypothesized that culturing hMSCs under normoxic conditions could lead to a reduction in their therapeutical potential [48]. Therefore, in the present work, we have expanded hWJ-MSCs under different environmental oxygen concentrations (i.e. normoxic-21% and hypoxic-5%) and evaluated their impact on the secretome profile of hWJ-MSCs.

The *in vitro* application of the hWJ-MSCs secretome obtained from normoxic and hypoxic conditions revealed that both were able to induce the survival and differentiation of human CNS-derived cells. As seen in Figure 2, when hNPCs were incubated with normoxic and hypoxic secretomes similar levels of neuronal differentiation [namely, immature (DCX$^+$ cells) and mature (Map-2$^+$ cells) neurons], were observed. This is in line with what Sart and colleagues [49] had already reported regarding the effects of hypoxic and normoxic hMSCs CM on the NPCs survival, differentiation and maturation. In addition, they also observe that both CM (from normoxic and hypoxic conditions) were also able to enhance the proliferation, migration and neurite outgrowth of NPCs [49]. In fact, studies have shown that the secretion of BDNF, NGF, FGF-2, SDF-1α and TGF-β1 by MSCs plays a role in promoting growth and neural differentiation of hNPCs [13, 50-52]. In addition to this, it has been also hypothesized that hMSCs secretome could be a promoter of endogenous extracellular matrix (ECM) proteins secretion by NPCs [49]. Moreover, it has been suggested that the hMSCs secretome (either from normoxic or hypoxic conditions) acts as a modulator of NPCs behavior, influencing the ratio of adherence and differentiation [49]. This clearly suggests that the hMSCs secretome may be able to enhance the neural survival and differentiation of NPCs due to the regulation of the molecular milieu, including ECM proteins and growth factors [49, 53, 54].

The proteomic analysis performed in the present work further revealed that hWJ-MSCs produce other kind of different molecules than those already reported in the literature to this cells, with neuroregulatory potential. Of these, GDN and Cys C were found to be upregulated on in normoxic conditions (Figures 4A, B) whereas, UCHL1, Clusterin, Prx1, 14-3-3 proteins, Thymosin-β proteins and EF-2 were upregulated under hypoxic conditions (Figure 4C-H). Together, all these proteins have been reported to have important roles in neurite outgrowth, inhibition of apoptosis, neuroprotection, antioxidant activities and angiogenic effects both *in vitro* and *in vivo* [55-62]. For instance, GDN and Cys C (upregulated in normoxic condtions, Figure 4A, B) are known to play crucial roles in the enhancement on neurite outgrowth and neuroprotection through the prevention of oxidative stress [55, 63-66]. On the other hand, UCHL1 and Clusterin (Figures 4C, D) have been described as

important enhancers of neuroprotection, neurogenesis (e.g. neuronal process formation, elongation, and plasticity) as well as a potential target into some neurodegenerative disorders such as Alzheimer's disease (AD) [57, 67-70]. Prx1 and 14-3-3 proteins (Figures 4E, F) are known by their important roles on regeneration, cell migration, axonal growth as well as into neurite outgrowth and neuroprotection [58, 59, 71-73]. Finally, Thymosin-β proteins (significantly expressed, p <0.01; Figure 4G) and EF-2 (significantly expressed; p<0.05; Figure 4H) has been associated to important roles on the regulation of neurite outgrowth as well as neuroprotective actions into AD [61, 62, 74, 75]. Interestingly, in addition to this, we have also discovered specific proteins that were strictly expressed in each condition. From these, Vitronectin, Cadherin 2 and MRP1 (just present in the normoxic hWJ-MSCs CM) have been described as important modulators of neuronal differentiation, axonal growth and neuroprotection [76-81]. On the other hand, PEDF, IGF-2, Semaphorin-7A, MIF, Hsp70 and Moesin (only present in the hypoxic hWJ-MSCs CM) are known by their roles in the enhancement of neuroprotection, axonal growth, neurite outgrowth and neuronal cell survival and differentiation [81-90]. Moreover, previous results from our group have also identified the presence of some of these above-referred proteins, such as UCHL1, 14-3-3 proteins and Hsp70. Their presence was correlated with an increase, in cortical and cerebellar primary cultures, of neuronal cell densities (*in vitro*) [73].

Like Sart and colleagues [49], we have also observed that the hypoxic CM displayed similar cellular responses on hNPCs differentiation when compared to normoxic conditions. This was surprising as the proteomic analysis revealed that there was a robust increase on most of the proteins referred above for the secretome collected under hypoxic conditions. We believe that this could indicate that the differences within concentrations of the proteins that were detected may not be sufficient to induce significant variation on the functional differentiation of hNPCs. Nevertheless it was evident that in a dynamic culturing environment, such as that here in studies, the use of hypoxic conditions leads to significant changes in the composition of WJ-MSCs secretome. Taking all this into consideration, future standardized experiments should be developed in order to analyze the use of different hypoxic oxygen concentrations (for instance, below than 5%) and compared to the normoxic state - in order to get an insight of the real action of low levels of oxygen on the hMSCs secretome physiology.

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5. Conclusions

In the present work, we have demonstrated that the secretomes of hWJ-MSCs collected from normoxic and hypoxic conditions were able to induce neuronal differentiation of hNPCs into neurons in different stages of maturation. These outcomes were associated with the presence of important neuroregulatory molecules (differently expressed) within the secretomes constitution such as GDN, Cys C, UCHL1, Clusterin, Prx1, 14-3-3 proteins, Thymosin-β proteins and EF-2. These are important molecules involved in the promotion of neuroprotection, inhibition of the apoptosis, angiogenesis and neuronal cell survival and differentiation. Thus, our results suggest that the use of hypoxic preconditioning enhances the hWJ-MSCs secretome when compared to the normoxic precondition, indicating that hWJ-MSCs differ in their sensitivity when exposed to different oxygen concentrations, which may open the development of new therapeutic strategies in the future. However with conditions tested in the present study there were no changes on the neuroregulatory (neural differentiation) profile of their secretome. In the future different hypoxia oxygen concentrations should be tested in order to identify the optimal parameters for enriching WJ-MSCs secretome under a dynamic and hypoxic environment.

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Chapter 5

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(2014)

Secretome of Human Mesenchymal Stem Cells Improves the
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Parkinson's Disease

Manuscript to be submitted

Secretome of Human Mesenchymal Stem Cells Improves the Behavioral Outcomes and Neuronal Cells Densities in a Rat Model of Parkinson's Disease

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Abstract

The use of human Mesenchymal Stem Cells (hMSCs) has been proposed as a possible therapeutic tool for the treatment of CNS neurodegenerative disorders such as Parkinson's Disease (PD). Research in the last decade strongly suggests that MSC-mediated therapeutic benefits are primarily due to their secretome. Indeed, previous reports from our group have shown that the secretome of hMSCs from different sources increased neurogenesis and cell survival, inhibited apoptosis and had numerous neuroprotective actions in different conditions. Recently, we have found that the use of dynamic culturing conditions, using computer-controlled suspension bioreactors, can further modulate the hMSC secretome thereby generating a more potent neurotrophic factor cocktail. As a consequence, in the present work we investigated the possible therapeutic effects that the hMSCs secretome, collected from dynamic and static culture conditions, has on the physiological recovery of a PD rat model (6-OHDA). For this purpose we injected the hMSC secretome into the substantia nigra (SNc), and then characterized the behavioural performance of the injected animals. Results revealed that the injection of hMSC secretome (from either static or dynamic conditions) was able to improve the motor coordination (rotarod) and the paw reaching motor function of the animals (staircase behavioural tests) when compared to the 6-OHDA-control group. Additionally, we also observed that the injection of the hMSC secretome potentiated the recovery of dopaminergic neurons and supported the motor recovery in the PD rats. Overall, we concluded that the use of the hMSC secretome alone (i.e. no cells) may be a novel therapeutic in PD regenerative medicine.

1. Introduction

Parkinson's Disease (PD) represents the second most prevalent neurodegenerative disorder, which is clinically characterized by a progressive degeneration of dopaminergic neurons (DAergic) in several dopaminergic networks. This process is most intensively observed in the mesostriatal pathway at the level of the substantia nigra pars compacta (SNc) [1-5]. As a result of this neuronal loss, patients develop several motor complications including rigidity, bradykinesia, and postural instability [6]. In terms of treatment, the use of levodopa (L-DOPA) has been considered over the years the standard treatment for PD as well as for the reduction of its major symptoms [7, 8]. At the same time, together with L-DOPA treatment, the use of dopamine reuptake inhibitors, DA agonists and muscarinic antagonists also have positive clinical effects [7, 9]. However, despite these pharmacological advances in PD related therapies, most of these treatments have been shown to be insufficient, presenting some undesirable side effects, long-term inefficiency, as well as the inability to recover lost DAergic neurons or to protect the those remaining ones [10-14]. Surgical treatments, such as deep-brain stimulation (DBS), have been as alternative in patients where the pharmacological treatment is no longer effective [12, 15, 16]. However, as with drug treatments, the apparent clinical recovery after surgery does not last, and the progression of the degenerative process is not avoided [17, 18].

To overcome this, the use of a human Mesenchymal Stem Cells (hMSCs) cell-based strategy has emerged as possible potential therapy for PD [19-21]. In fact, several studies in PD animal models have already shown that the transplantation of hMSCs acts as a promoter of neuroprotection and/or neural function [22-24]. For instance, Venkataramana and colleagues [25] showed that hMSCs that survived transplantation integrated into the brain parenchyma and migrate towards the ipsilateral nigra. However, the specific mechanism by which hMSCs are able to improve the motor performance either in animal models or patients is still not known [25]. While some reports propose that the differentiation of hMSCs into DAergic neurons or neural lineages is the principal outcome for PD recovery, others indicate that this functional recovery is promoted by the hMSC secretome [26, 27]. Indeed, the secretome hypothesis has inspired an alternative outlook on the use of hMSCs as potential therapeutic tool for CNS regeneration [26, 28]. The role that the hMSC secretome plays *in vivo* has been investigated in other studies that have shown that upon transplantation, hMSCs (from different tissue sources) were able to secrete important trophic factors such as epidermal growth factor (EGF), vascular endothelial-growth factor (VEGF), neurotrophin-3 (NT3), fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), stromal

derived factor 1 (SDF-1), glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 20 (FGF-20), which exert neuroprotection against 6-OHDA-exposed DAergic neurons both *in vitro* and *in vivo*, leading to substantial improvements in the behavioral performance of PD models [23, 29-34]. For this reason, it is strongly suggested that hMSCs may represent a valid tool for the neuroprotection and survival of DAergic neurons through their secretome [35-38]. However, the direct transplantation of MSCs into the CNS still presents some drawbacks, namely: 1) low survival rate when transplanted into the brain, 2) a very high number of cells is needed for transplantation, which will delay their application into the patient, 3) long periods of expansion of hMSCs *in vitro* prior to transplantation, that can lead to phenotypic alterations, affecting the potency of the secretome.

The use of the secretome alone (i.e. instead of transplanting hMSC cells) as a therapeutic tool could obviate some of the problems mentioned above. In fact, we have previously shown that the administration of a single secretome injection into the rat hippocampus produced a similar effect when compared to animals that were transplanted with hMSCs into the same regions [39]. Moreover, we have also observed that the dynamic culture of hMSCs in computer-controlled suspension bioreactors further enriched the neuroregulatory properties of their secretome. Therefore, in the present work, we aimed to analyze the effects of the hMSCs secretome collected from dynamic culture conditions (i.e. computer-controlled suspension bioreactors) and standard (i.e. static T-flasks) culturing conditions on the DAergic neuronal cell survival and in the motor behavior of an animal model of PD. Results revealed that both hMSCs secretomes were able to improve the animals' motor behavior when compared to the 6-ODHA-control group. Additionally, we also observed that both hMSCs secretomes were able to increase the survival of TH-positive cells, which could open new approaches for the application of the hMSC secretome in the treatment of PD.

2. Methods

2.1. Expansion of hMSCs in static culture and collection of static conditioned medium

Passage 2 (P2) human bone marrow MSCs (hMSCs, Lonza) were thawed and inoculated into gelatin-coated tissue culture flasks (Nunc, Roskilde, DO) at 5,000 cells/cm^2 in serum-free hMSCs expansion medium – PPRF-msc6 (at a volume/area ratio of 0.32 mL/cm^2). The formulation and preparation of PPRF-msc6 has been disclosed elsewhere [40]. The cell cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2. After 3 days, 50% of the medium was replaced with

fresh growth medium. When the cells reached 80-90% confluence, the cells were harvested by incubation with 0.05% trypsin-EDTA (Life Technologies) at 37℃ for 3-5 min, and then FBS-DMEM was added to neutralize the reaction. The harvested cells were then centrifuged at 300 g for 10 min. After centrifugation, the supernatant was removed, and the cells were re-suspended in fresh growth medium and centrifuged again at 300 g for 10 min (one wash). After centrifugation, the supernatant was removed and the cells re-suspended in fresh growth medium and re-plated into new, gelatin-coated tissue culture flasks at 5000 cells/cm^2. At P5, after 72 hours of growth, the cell culture growth medium was removed and the cells were washed twice with Neurobasal®-A medium (Life Technologies), and Neurobasal®-A medium was added to the tissue culture flasks at the same ratio was the growth medium (i.e. 0.32 mL/cm^2). The cells were then placed in a humidified incubator, operating at 37℃ and 5% CO_2, for 24 h. After 24 h, the medium was removed, centrifuged at 300 g for 10 min to remove any cell debris, and then stored at -80℃ until it was required for further experiments. This medium is referred to as conditioned medium (CM) as it contains the varying factors secreted by the hMSCs during the 24 h of incubation, as we prev[s]ously described [41].

2.2. Expansion of hMSCs in dynamic (bioreactor) conditions and collection of conditioned medium (CM)

2.2.1. Preparation of 500 mL Suspension Bioreactors

The DASGIP Parallel Bioreactor system (DASGIP, Julich, DK, http://www.dasgip.com) was used for the expansion of hMSCs in dynamic conditions. Prior to inoculating hMSCs into the DASGIP bioreactors, the 500 mL suspension bioreactors and modified Teflon 4-paddle impellers (designed and manufactured at PPRF) were siliconized using Sigmacote (Sigma) to minimize cell attachment to the sides of the bioreactor vessel and the impeller. After siliconization and autoclaving of the vessels, the DASGIP system was set up and calibrated according to procedures provided by the manufacturer. The bioreactors were maintained at - (1) 37℃ using a heating jacket, (2) 100% dissolved oxygen (corresponding to oxygen saturation of the medium at 37℃ exposed to 21% O_2 in the headspace), (3) a pH of 7.4, controlled by a gas mixture connected to oxygen, nitrogen, carbon dioxide and air tanks that was introduced into the headspace, and (4) agitated at 52 rpm using a magnetic stir plate under the bioreactors [41].

2.2.2. Preparation of Microcarriers and Inoculation of hMSCs

Cytodex 3 microcarriers (GE Healthcare) were used for this study and were prepared as follows: 1.0 g of microcarriers were weighed out and hydrated in 50 mL of 1X PBS (Life Technologies) in a 125 mL, pre-siliconized, Erlenmeyer flask, at room temperature overnight. To this flask 2-3 drops of Tween 80 (United States Biochemical Corporation, Cleveland, OH) was added to break the surface tension and ensure proper wetting and sedimentation of the microcarriers. The microcarriers were then washed 3X with 1X PBS, and autoclaved. Following autoclaving the microcarriers were incubated with fetal bovine serum (FBS, Life Technologies) for 6 h at 37°C to coat the microcarriers with serum-attachment factors, and agitated every 30 min. After 6 h, the FBS was removed, and the microcarriers were washed twice with our serum-free medium, and then inoculated into our 500 mL DASGIP bioreactors in 275 mL of medium for 4 h at the controlled culture conditions. hMSCs were expanded in static culture (as described in the previous section) for two passages before inoculation into the DASGIP bioreactors. The cells were harvested using trypsin-EDTA, and then inoculated into the bioreactors at a density of 24,000 cells/mL (based on the final volume of 500 mL) and the volume of the medium in the bioreactors was maintained at 325 mL for the first 24 h to increase cell attachment. After 24 h, the culture volume was increased to 500 mL to bring the final microcarrier density to 2.0 g/L. The cells were cultured on the microcarriers for 72 h, after which time the bioreactors were removed from the DASGIP system, and placed in a biosafety cabinet for 10 min to allow the microcarriers to settle. The supernatant was removed from the bioreactors, and the microcarriers were washed once with 100 mL of Neurobasal®-A medium. Following this, 500 mL of Neurobasal®-A medium was added to the bioreactors and the bioreactors were placed back into the DASGIP control system for 24 h. After 24 h the bioreactors were again removed from the system, placed in a biosafety cabinet for 10 min to allow the microcarriers to settle, the supernatant was harvested and centrifuged at 300 *g* for 10 min to remove any cell debris. This supernatant, called the "dynamic CM", was then placed at -80°C until it was required.

2.3. Stereotaxic surgeries

2.3.1. 6-OHDA lesions

Ten week old *Wistar-Han* male rats (Charles River, Barcelona) were housed (two per cage) and maintained in a controlled environment at 22-24°C and 55% humidity, on 12 h light/dark cycles and fed with regular rodent's chow and tap water ad libitum. Animals were handled for 1 week prior to the beginning of the injections, in order to reduce the stress induced by the surgical procedures.

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All the manipulations were done after the consent from the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457) and in accordance with the local regulations on animal care and experimentation (European Union Directive 2010/63/EU). Thus, for the surgical procedures: under ketamine-medetomidine [75 mg/kg; 0,5 mg/kg, intraperitoneally (i.p.)] anesthesia the animals were placed on a stereotaxic frame (Stoelting, USA), and unilaterally injected, using of an 30-gauge needle Hamilton syringe (Hamilton, Switzerland), with either vehicle (Sham group, n=11) or 6-OHDA hydrochloride (Sigma, USA) (6-OHDA group, n= 37) directly into the medial forebrain bundle (MFB) (coordinates related to Bregma: AP= -4.4 mm; ML= -1.0 mm; DV= -7.8 mm [42]; according to Paxinos and Watson [43]). Sham animals received 2 μl of 0.2 mg/ml of ascorbic acid in 0.9% of NaCl and, the 6-OHDA animals were injected with 2 μl of 6-OHDA hydrochloride (4 μg/μl) with 0.2 mg/ml of ascorbic acid in 0.9% of NaCl at a rate of 1.0 μL/min. After each injection, the needle was left in place for 4 min in order to avoid any backflow up the needle tract. Three weeks after the surgery, behavioral assessment was performed.

2.3.2. Surgical treatment – injection of hMSC CM

Five weeks after the injection with 6-OHDA, we proceeded inject the hMSCs secretome (from static and dynamic conditions). As previously described, for the surgical procedures: under ketamine-medetomidine [75 mg/kg; 0,5 mg/kg, intraperitoneally (i.p.)] anesthesia the animals were placed on a stereotaxic frame (Stoelting, USA), and unilaterally injected, using of an 30-gauge needle Hamilton syringe (Hamilton, Switzerland), with either vehicle (Neurobasal®-A medium; 6-OHDA-control group; n=13), static CM (6-OHDA_CMs; n=12) or dynamic CM (6-OHDA_CMd; n=12) directly in the SNc (coordinates related to Bregma: AP= - 5.3 mm; ML= -1.8 mm; DV= -7.4 mm; according to Paxinos and Watson [43]). 6-OHDA-control group received 4.0 μL of Neurobasal®-A medium and the CM-injected animals received 4.0 μL each (from static and dynamic conditions) at a rate of 1.0 μL/min. After each injection, the needle was left in place for 8 min in order to avoid any backflow up the needle tract. At 1 week, 4 weeks and 7 weeks following surgery, behavioral assessment was performed.

2.4. Behavioral assessment
2.4.1. Rotarod

The motor coordination and balance of the animals was evaluated through the use of the Rotarod apparatus (3376-4R; TSE Systems, USA), under an accelerating protocol previously described

(Monville C., 2006). The first 3 days of testing served as training. Then, the animals underwent a four trial test under an accelerating protocol starting at 4.0 rpm and reaching 40 rpm in 5 min. The animals were allowed to rest for at least 20 min between each trial. On day 4, using the same protocol, the animal latency to fall was recorded.

2.4.2. Skilled paw reaching test (staircase test)

The skilled paw reaching (also named staircase test) was assessed trough the use of double staircase boxes (80300, Campden Instruments Ltd.) as previously described [5]. The shape and dimensions of the boxes were similar to the ones described by [44]. This type of apparatus consists of a clear chamber with a hinged lid that was developed to assess the independent forelimb use in skilled reaching and grasping task. A narrow compartment, with a central platform running along its length is connected to this chamber. The removable double staircase with 7 steps on each side can be inserted in the space between the platform and the box walls. Five pellets were placed into each well of the double staircase apparatus. On the first two days, the rats were familiarized with the test, with the pellets being available for 5 and 10 min, respectively. During the test session, animals were kept inside the box, having 15 min to reach, retrieve, and eat the food pellets present on the steps. All sessions were performed at the same time of the day and with food-restricted animals. After each test interval, animals were removed from the staircase boxes and the remaining (left over) pellets were counted.

2.4.3. Apomorphine turning behavior

In order to test the apomorphine-induced turning behavior, animals were injected in the neck with a subcutaneous solution of 0.05 mg/kg apomorphine hydrochloride (Sigma, USA) dissolved in 1% of ascorbic acid 0.9% NaCl, and then placed on metal testing bowls (MED-RSS, Med Associates) for 45 min. After this, the number of contralateral rotations was digitally recorded, which allowed us to assess the effects of the injection vehicle (0.2 mg/mL of ascorbic acid in 0.9% of NaCl) and 6-OHDA-hydrochloride (with 0.2 mg/mL of ascorbic acid in 0.9% of NaCl).

2.5. TH immunohistochemistry

After 13 weeks (including the development of the lesion and consequent treatment) animals were sacrificed with sodium pentobarbital (Eutasil, 60 mg/kg i.p., Ceva Saúde Animal, Portugal), and transcardially perfused with 4% paraformaldehyde (Merck, Lisbon, Portugal) in 0.1 M of PBS. Striatal

and mesencephalon coronal sections, 30 μm thick were obtained with a vibratome (VT1000S, Leica, Germany). Four series of consecutive slices were obtained and one was processed as free-floating TH-immunohistochemistry. The slices were immersed for 20 min into 1M PBS with 3% of H_2O_2, being followed by blocking [2 h with 5% fetal calf serum (FCS, Life Technologies) in 1M PBS]. After this, slices were incubated overnight (at 4 ºC) with the rabbit anti-mouse TH primary antibody [1:2000 (Millipore, USA), in 2% of FCS in 1M PBS], followed by incubation for 30 min with a biotinylated secondary anti-rabbit antibody (LabVision, USA), and another 30 min incubation with an Avidine/Biotine complex (LabVision). The antigen visualization was performed using 3,3í-diaminobenzidine tetrahydrocloride (DAB, Sigma, USA) (25 mg of DAB in 50 mL of Tris-HCL 0.05 M, pH 7.6 with 12.5 μL of H_2O_2) and stopped at the desired time. Then, slices were mounted on superfrost slices and thionin counter-coloration was performed.

In order to assure a representative sampling between all the animals, six identical TH-labeled slices spanning the entire mesenphalon were chosen, including all the portions of the SNc. Using a bright-field microscope (BX51, Olympus, USA) equipped with a digital camera (PixeLINK PL-A622, CANIMPEX Enterprises Ltd., Canada), and with the help of Visiomorph**TM** software (V2.12.3.0, Visiopharm, Denmark), the boundaries of SNc area was drawn. The delineation of this region was performed through identification of anatomic standard reference points and with the help of the rat brain atlas [43]. Counting of total TH+ cells in the SNc area was performed on both hemispheres and the data presented as the percentage (%) of remaining TH+ cells in the injected side, compared to the control side.

2.6. Statistical analysis

All data was analyzed using the program Graphpad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using Student t-test, Mann-Whitney nonparametric test and two-way (repeated measures) analysis of variance as appropriate. Differences between groups were further analyzed by Bonferroni's post-test. All data shown has mean ± S.E.M. Significance value set at $p < 0.05$.

3. Results

3.1. Phenotypic characterization of 6-OHDA lesions

To further evaluate the functional integrity of the dopaminergic system after the injection of 6-OHDA, the apomorphine-induced turning test was performed at the end of the behavioral assessment. 3

weeks after the 6-OHDA injections, statistical analysis revealed differences in the apomorphine-induced turning behavior (t = 6.431; p < 0.0001), resulting from a significantly higher number of rotations in the 6-OHDA injected animals when compared to the Sham group (Figure 1A). Indeed, the assessment of motor performance also revealed deficits after the 6-OHDA injections. Motor coordination, assessed by the rotarod test, was observed to be impaired in the animals injected with 6-OHDA ($F_{(1,163)}$ = 24.34; p<0.01; Figure 1B). In the staircase test (used to assess the forelimb use and skilled motor function) we also observed that the 6-OHDA-injected animals were clearly affected when compared to Sham animals ($F_{(1,206)}$ = 159.3; p<0.0001; Figure 1C). Moreover, in the forced-choice task (in which animals are forced to choose one of the steps-side), the 6-OHDA-injected animals were observed to be significantly impaired compared to the Sham animals (p<0.0001, Figure 1D).
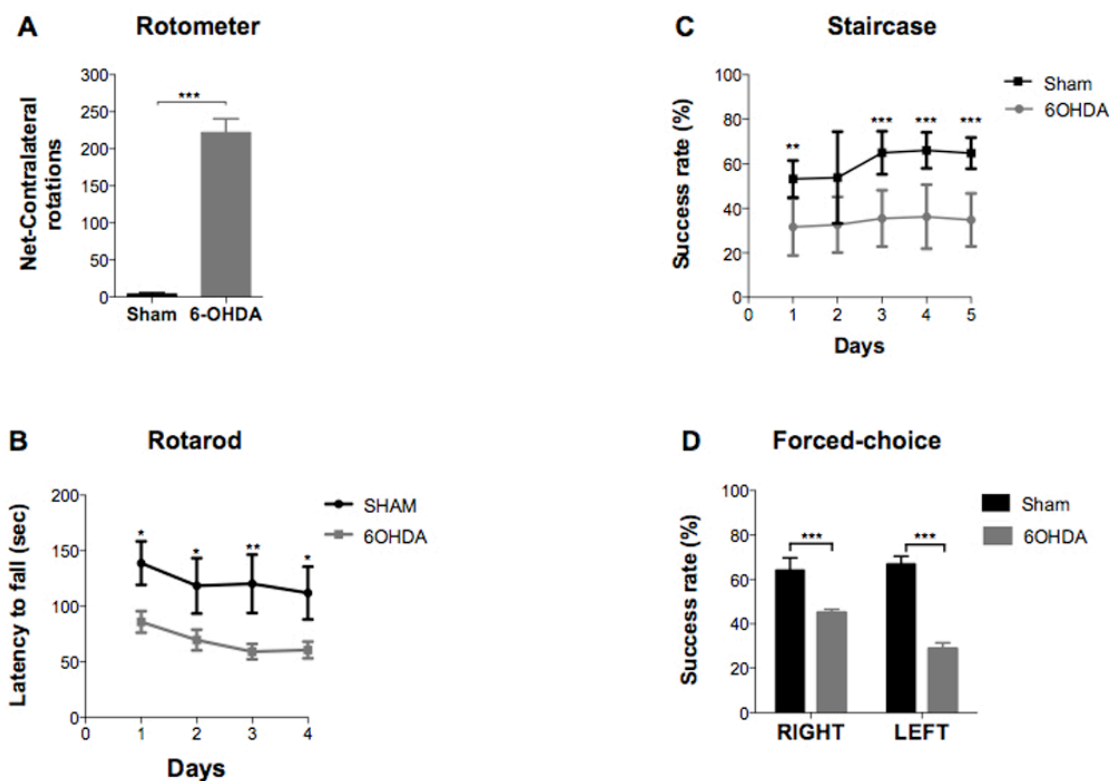
Figure 1. Behavioral characterization of 6-OHDA induced lesions. (A) Apomorphine-induced turning behavior revealed that 6-OHDA-injected animals displayed intense turning behavior when compared to Sham group. 6-OHDA-injected animals also presented significant impairment in motor coordination on the (B) rotarod test and in (C, D) the paw-reaching test performance. Sham n= 9, 6-OHDA n= 25. Data presented as mean ± S.E.M. * p < 0.05, ** p < 0.01, *** p < 0.001.

3.2. Transplantation of hMSCs CM from both static and dynamic conditions attenuate motor deficits of 6-OHDA-injected animals

In order to evaluate the effects of the hBM-MSC CM (from static and dynamic conditions) in 6-OHDA-injected animals, the animals motor performance was assessed at 1, 4 and 7 weeks after CM injection through the rotarod and staircase tests as previously described.

3.2.1. Rotarod

One week after CM injection, statistical analysis showed an effect for the factor treatment ($F_{(3,108)}$ = 10.51; p<0.001), but no effect for the factor time ($F_{(3,108)}$ = 0.5452; p = 0.6526) as well as no interaction between these factors ($F_{(9,108)}$ = 0.3551; p<0.9532). Comparing the CM-injected groups with the 6-OHDA-control group, *post hoc* testing revealed that both CM (from static and dynamic conditions) were able to significantly improve the motor performance of the CM-injected animals (p < 0.05; Figure 2A). The same tendency was also observed after 4 weeks after CM injection. Statistical analysis revealed a significant effect of the factor treatment ($F_{(3,108)}$ = 5.213; p < 0.001), but no effect for the factor time ($F_{(3,108)}$ = 0.4848; p = 0.6935) as well as no interaction between these two factors ($F_{(9,108)}$ = 0.8295; p = 0.5904). Comparing the injection of the hMSCs CM with the 6-OHDA-control group, statistical analysis revealed that both hMSCs CM continued to significantly improve the motor performance of the injected animals (p < 0.05; Figure 2B). Finally, after 7 weeks, we were able to observe that the factor treatment continued to be significant ($F_{(3,108)}$ = 21.32; p < 0.0001), but no effect was observed on the factor time ($F_{(3,108)}$ = 1.138; p = 0.3370) as well as no interaction between the factors ($F_{(9,108)}$ = 0.8192; p = 0.5997). Besides the significant performance of the sham group (i.e. for the injection of the CM), both CM-injected groups displayed a positive trend on motor coordination when compared to 6-OHDA-control group, although no statistical differences were observed (Figure 2C).
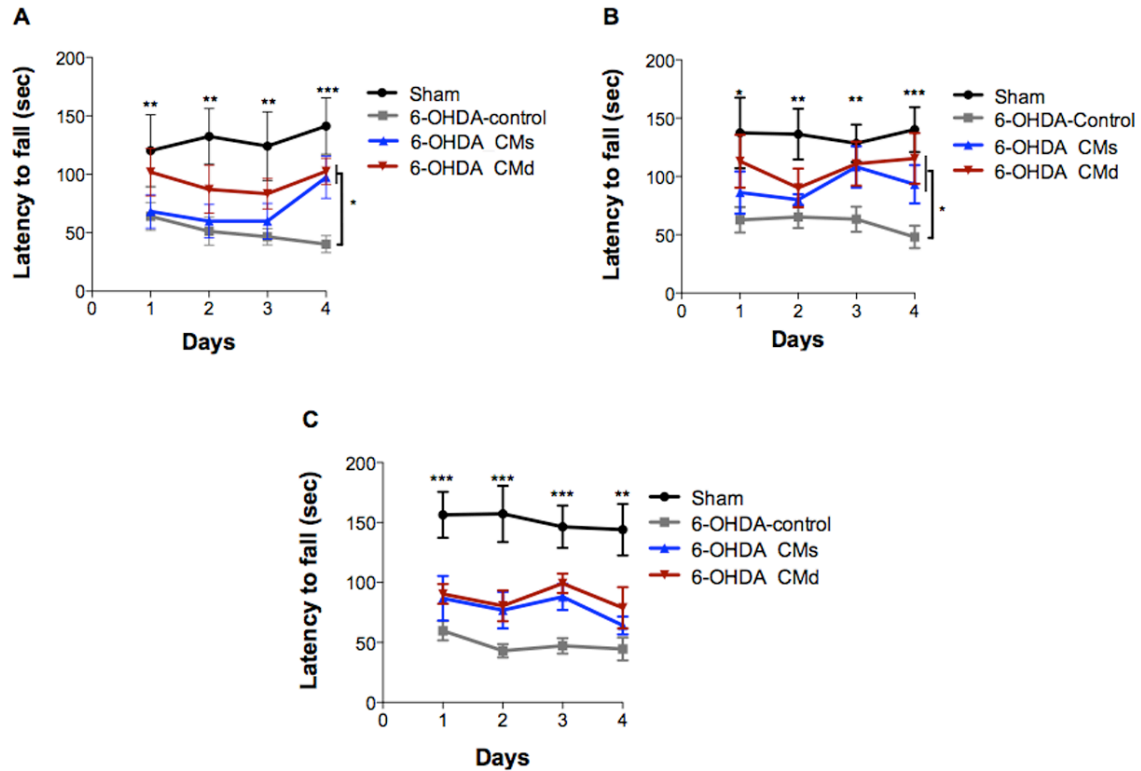
Figure 2. Motor coordination performance after (A) 1 week, (B) 4 weeks and (C) 7 weeks of hMSC CM injection in the SNc. Latency to fall was measured in the accelerating rotarod test. Sham n= 9, 6-OHDA-control n= 9, CMs n= 8, CMd n= 8. Data presented as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sham: Control group; 6-OHDA-control: Animals injected with Neurobasal A; CMs: Animals injected with static CM; CMd: animals injected with dynamic CM.

3.2.2. Staircase test

The staircase test, as previously described [5], used to address the forelimb use, in which the success rate of eaten pellets was evaluated. One week after hMSCs CM injection, statistical analysis revealed a significant effect for the treatment ($F_{(3,144)}$ = 9.948; $p < 0.0001$), but no effect for the factor time ($F_{(4,144)}$ = 0.8153; $p = 0.5174$) as well as no interaction between these factors ($F_{(12,144)}$ = 0.7014; $p = 0.7482$). For the CM-injected groups, *post-hoc* analysis revealed that both CM conditions appeared to ameliorate the performance in the injected animals when compared to the 6-OHDA-control group (Figure 3A). After 4 weeks, the factor treatment continued to be significant ($F_{(3,144)}$ = 11.22; $p < 0.0001$), but no effect was observed for the time ($F_{(4,144)}$ = 0.8643; $p = 0.4871$), and no interaction between these two factors was observed as well ($F_{(12,144)}$ = 0.8186; $p = 0.6311$). Comparing the effects of the CM-injected animals to the 6-OHDA-control group, statistical analysis revealed that 4 weeks after the injection of both CM, there was a significant improvement on the

success rate of eaten pellets ($p < 0.05$; Figure 3B). Similar observations were also found at 7 weeks after the injections. The factor treatment continued to display a significant effect ($F_{(3,144)} = 8.588$; $p = 0.0002$), but no effects on the factor time ($F_{(4,144)} = 0.2405$; $p = 0.9150$) as well as no interaction between these factors was observed ($F_{(12,144)} = 0.4147$; $p = 0.09568$). Concerning the effects of the hMSCs CM injection, we were able to observe that there was a significant improvement in success rate of eaten pellets of the CM-injected animals when compared to 6-OHDA-control group ($p < 0.05$; Figure 3C). In addition, in the forced-choice task, the hMSC CM-injected animals had an improved performance. Statistical analysis revealed an effect for the factor treatment ($F_{(3,108)} = 15.17$; $p < 0.0001$), but no effect for the factor time ($F_{(3,108)} = 1.724$; $p = 0.1641$) as well as no interaction between these factors ($F_{(9,108)} = 0.3254$; $p = 0.9657$) (Figure 4B). Concerning the effects of the hMSCs CM, *post-hoc* testing revealed an increase success rate of eaten pellets for the animals treated with the dynamic CM at 4 weeks ($p < 0.05$) and a positive trend after 7 weeks ($p = 0.0674$) of injection.
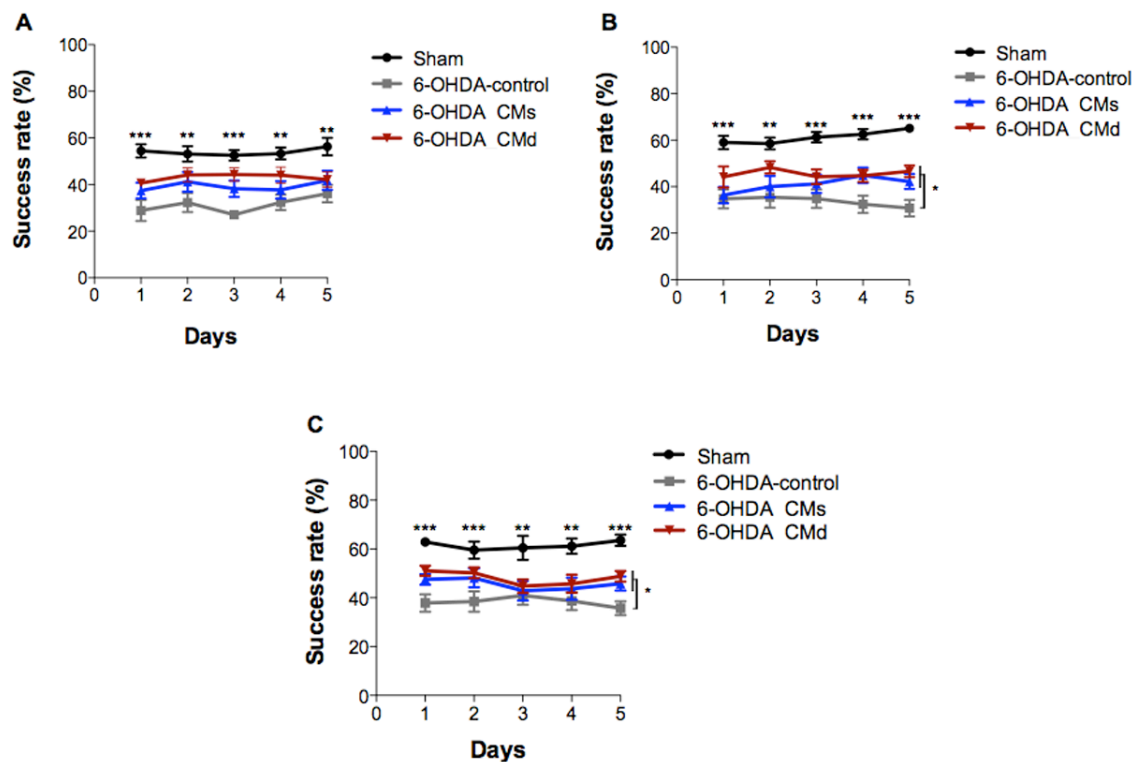
Figure 3. Paw reaching performance after (A) 1 week, (B) 4 weeks and (C) 7 weeks of hMSC CM injection in the SNc. Performance of rats in the paw reaching test is expressed as mean ± S.E.M. success rate of eaten pellets. Sham n= 9, 6-OHDA-control n= 9, CMs n= 8, CMd n= 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sham: Control group; 6-OHDA-control: Animals injected with Neurobasal A; CMs: Animals injected with static CM; CMd: animals injected with dynamic CM.

Figure 4. Paw reaching forced performance task after hMSC CM injection in the SNc. Performance of rats in the paw reaching test at (A) right and (B) left side is expressed as mean ± S.E.M. success rate of eaten pellets. Sham n= 9, 6-OHDA-control n= 9, CMs n= 8, CMd n= 8; * p < 0.05, ** p < 0.01, *** p < 0.001. Sham: Control group; 6-OHDA-control: Animals injected with Neurobasal A; CMs: Animals injected with static CM; CMd: animals injected with dynamic CM.
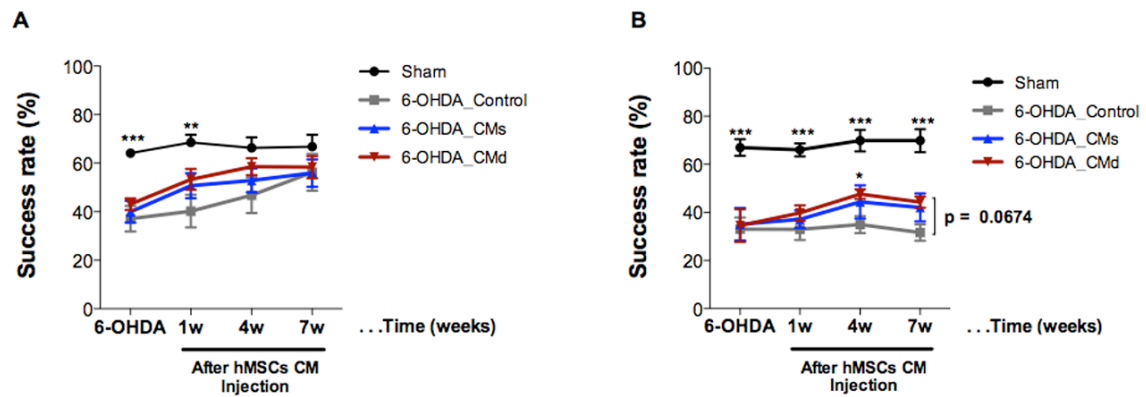
3.3. Assessment of the extension of the lesion

In order to further analyze the effects of the 6-OHDA injections as well as the resulting treatment with hMSCs CM, histological analysis for tyrosine hydroxylase (TH) was performed. From the results we were able to observe that a significant loss of DAergic neurons was obtained after 6-OHDA injections into the SNc. On the other hand, the injection of both hMSCs CM (from static and dynamic conditions) appeared to play a role on the survival of DAergic neurons since a significant number of TH-positive cells was observed after the injection of both CM [CMs: 20.09% ± 4.70%; CMd: 25.36% ± 5.45%] (p < 0.01) when compared to the 6-OHDA-control group [5.08% ± 1.60%] (Figure 5).
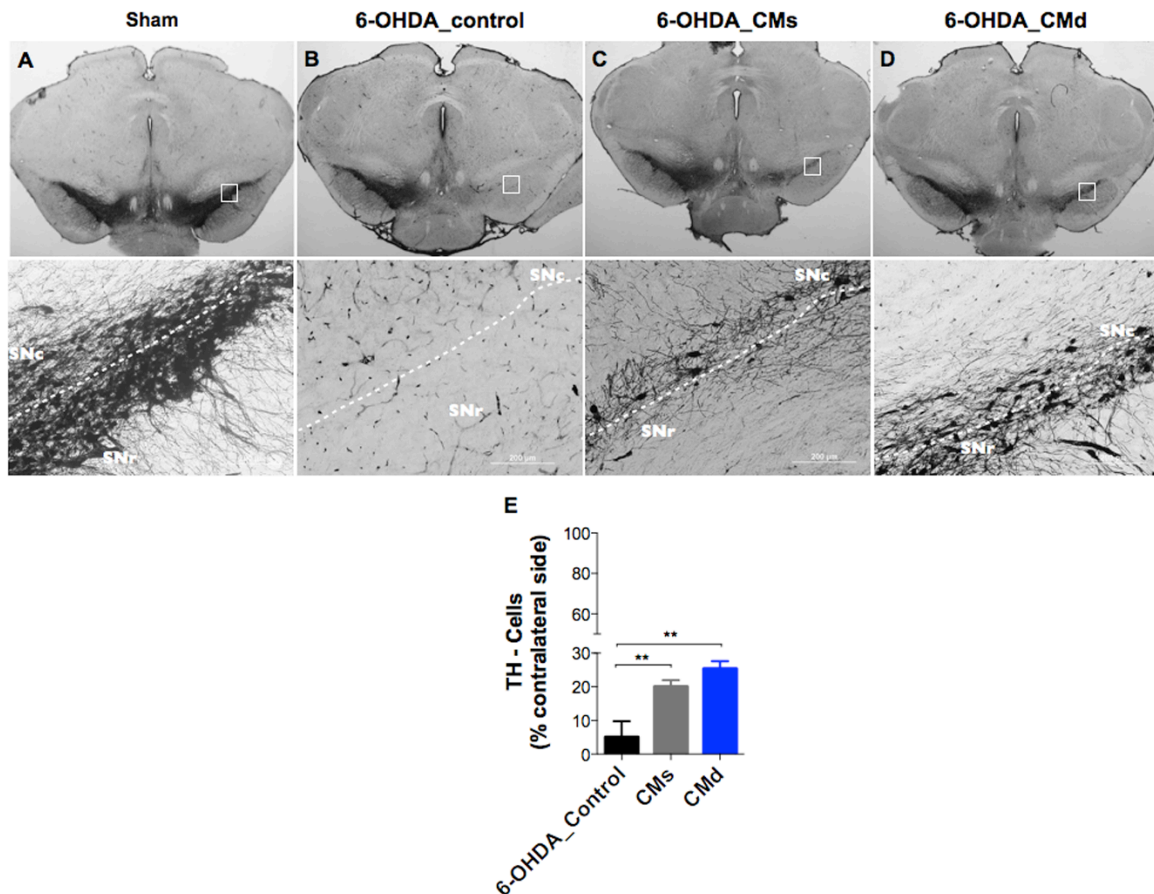
Figure 5. Representative photomicrographs of brain slices stained for TH.
Compared to the **(A)** Sham group, all animals that were submitted to 6-OHDA injection presented a reduction on the TH-staining. However, animals injected with **(C)** hMSC static CM and **(D)** hMSC dynamic CM presented a significant TH-positive staining when compared to **(B)** 6-OHDA-control group. **(E)** Quantification of TH-positive cells revealed ≈95% of degeneration into the 6-OHDA-control group and ≈80% and ≈75% in the CM-injected animals. Data presented as mean ± S.E.M. Sham n= 9, 6-OHDA-control n= 9, CMs n= 8, CMd n= 8; ** $p < 0.01$. Sham: Control group; 6-OHDA-control: Animals injected with Neurobasal A; CMs: Animals injected with static CM; CMd: animals injected with dynamic CM. Scale-bar: 200 μm.

4. Discussion

Parkinson's disease (PD) represents one of the most common CNS neurodegenerative disorders, and is characterized by the progressive cell death of the midbrain SNc dopaminergic neurons (DAergic) [45]. In the present study, we have used a rat model of PD, based on unilateral injections of 6-OHDA into the medial forebrain bundle (MFB) [1, 46]. The main characteristic of this model is that it resembles the progressive nature of the degeneration process in human PD, through the

progressive degeneration of DAergic neurons, leading to the appearance of the main motor symptoms of the disease [1, 47]. Indeed, as shown in the apomorphine-turning behavior (Figure 1A), 6-OHDA-injected animals displayed an intense turning behavior (p < 0.0001) when compared to the Sham group, indicating a decline on the functional integrity of the DAergic system. In addition to this, we also verified that the animal motor coordination was also affected (Figure 1B-D) which correlates nicely with previous reports showing impaired coordination and skilled motor function in animals with DAergic lesion [46, 48]. In the present work, we aimed to analyze the effects of hMSC secretome (collected from static and dynamic conditions) on the animal motor coordination and on the DAergic neuronal cell survival after 6-OHDA injections. Regarding the effects on motor coordination (rotarod test), we were able to observe that the injection of the hMSC CM (either from static and dynamic conditions) was able to improve the motor performance of the CM-injected animals when compared to the 6-OHDA-control group (Figure 2). Similar outcomes were also observed into the staircase test (assessing the paw reaching motor function), in which we observed that the injection of the hMSCs CM was also able to improve the success rate of eaten pellets in the CM-injected animals when compared to the 6-OHDA-control group (Figure 3). Moreover, in the forced-choice task, the injection of hMSC CM was observed to be an enhancer (in the affected side) of the paw reaching motor performance when compared to the 6-OHDA-control group (Figure 4B). Based on these observations, we can conclude that this may be due to the significant number of TH-positive cells that were observed after the CM injection when compared to the 6-OHDA-control group (Figure 5). This is in line with what has been described in the literature regarding the trophic action of hMSCs on PD [26, 28, 49]. For instance, Cova and colleagues [29] showed that human bone marrow-derived MSCs transplanted into a 6-OHDA animal model were able to survive and interact with the are surrounding the lesion site, and were able to secrete large amounts of trophic factors such as EGF, VEGF, NT3, FGF-2, HGF and BDNF for a long period of time *in vivo*. On the other hand, Weiss and colleagues [31] associated the recovery of TH-stained cells as well as animal behavior improvements to the significant secretion of GDNF and FGF-20. In addition to these findings, it was also shown that in fact the bioactive molecules secreted by hMSCs, such as SDF-1α, are able to display anti-apoptotic effects in 6-OHDA *in vitro* models (PC12 cells), leading increased dopamine release in the cultured cells [26, 30]. *In vivo,* the expression of SDF-1α by hMSCs was also shown to be effective as an anti-apoptotic agent on host DAergic neurons, and thereby improving the animals behavioral recovery when compared to the control lesioned groups [30].

In addition to these results, we have shown that the hMSC secretome (from our static and dynamic conditions) contains other neuroregulatory molecules in which the pigment epithelium-derived factor (PEDF) was found to be an important neurotrophic and neuroprotective molecule on PD [41, 50, 51]. This has been sustained by Falk and colleagues, [51] who when comparing other factors (e.g. GDNF family) used in the treatment of PD, stated that PEDF has advantages in the ease of delivery and functional outcomes. Indeed, recent reports have suggested that up-regulation of PEDF occurs in response to acute insults in the dopaminergic system, suggesting that PEDF may act as an endogenous neuroprotective molecule, in which an increase in its concentration may lead to and higher amount of neuronal survival and behavioral improvements in animal models of PD [51, 52]. Moreover previous studies from our group has demonstrated that the hMSC CM (collected from static and dynamic conditions) were able to induce higher rates of neuronal differentiation *in vitro* as well as to enhance the proliferation levels and neuronal differentiation *in vivo*. We have hypothesized that this increase could be correlated with the increased expression of PEDF in the secretome of these cells [41].

The present results indicate that the hMSC secretome is able to induce the survival of TH-positive cells in the SNc, a fact that could explain the improvements on the animal motor coordination. Indeed, as stated by Nikkhah and colleagues [53], although the reinnervation in the striatum appears to be important for the restoration of rotational symmetry, the reinnervation of the SNc may be necessary for more complex sensorimotor tasks. Clearly future studies need to be carried out in order to better understand the temporal effects of the hMSCs secreted factors. In addition, we would like to know whether or not the presence of PEDF (in the secretome of these cells) can support the results of this report.

5. Conclusions

Following the injection of the hMSC secretome (i.e. CM), we observed a substantial improvement in the animal behavior when compared to the 6-OHDA-control group. In addition to this, we also found that the injection of the hMSCs secretome (from either from the static or dynamic conditions) was also able to increase the survival levels of DAergic neurons - that could explain the improved behavioral performance of the CM injected animals. In addition to the many important neuroregulatory molecules present in the hMSC CM, PEDF might be partly for the outcomes since it

has been described as an important neurotrophic and neuroprotective molecule on PD. Overall, our results suggest strongly that the use of the hMSCs secretome may be a new tool for the treatment of PD since the secretome is able to modulate the DAergic neuronal survival and animal behavior. This could open novel therapeutic approaches for the treatment of PD.

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SECTION III

Chapter 6

General Discussion and Perspectives

General Discussion and Perspectives

The repair of CNS lesions is probably one of the biggest challenges of the regenerative medicine field [1]. Indeed, efforts have been done in order to develop strategies that allow the protection and repair of different CNS injuries [1]. Due to the type and nature of the injury, it has been suggested that the success of a functional recovery cannot be only associated to a single therapeutic approach but most likely to the development and combination of multiple strategies [1-5]. Thus, the work developed on the scope of the present thesis was centered on establishing a route to restore, maintain or improve the CNS tissue function and repair, trough a cell-free based therapy. Based on this idea, the objective was to generate a novel potential therapeutic tool through the use of hMSCs secretome for PD therapeutics.

Firstly, we have started by comparing the effects of the hMSCs cell transplantation with the injection of its secretome, in order to address if in fact the secretome of hMSCs *per se* could mediate similar actions to those originated by cell transplants. Furthermore this study could also address the real mechanism underlying the therapeutic benefits of hMSCs in the CNS [6-10]. Studies have proposed the multilineage differentiation capacity of hMSCs as the most probable reason of their therapeutic effects after transplantation [8]. Indeed, several studies have demonstrated that hMSCs are capable to differentiate into osteoblasts, chondrocytes, adipocytes and myoblasts [11, 12]. In addition to this, it was also showed that hMSCs could also differentiate into neuronal and epithelial populations [11, 13-16]. Nonetheless, while the epithelial differentiation is accepted, the neuronal differentiation of MSCs remains still under intense discussion [11, 17]. In that way, the secretion of bioactive molecules (trophic factors, growth factors, cytokines and vesicles) has been proposed as the most probable modulator of a wide panel of physiological processes such as immune/inflammatory responses, protection/regeneration systems, apoptosis, angiogenesis, neurogenesis and cell-signaling [6, 18-20]. From our experiments (on Chapter 2), we were able to observe that the use of hMSCs secretome *per se* was able to display similar levels of endogenous cell proliferation and neuronal/glial cell densities when compared to hMSCs transplants. This is an indicator that the secretome, by itself, might be used as a therapeutic agent which could obviate some of the disadvantages of cell transplantation, namely, their low survival rates when transplanted into the brain and the high numbers of cells needed for these procedures, which delays the application on the patient [7].

In order to further potentiate the use of the secretome as a therapeutic agent, and reduce the time for MSCs culture to collect it we have proposed the use of dynamic culture conditions through the use of bioreactors. Indeed, crucial advantages can be retrieved using these systems such as: a large number of cells can be expanded in one vessel (minimizing vessel-to-vessel variability and minimizing cost related to labor and consumables); the bioreactors can be operated into different modes of operation, as well as they can be set up with computer-controlled online monitoring instruments to ensure tight control of process variables such as pH, temperature and dissolved oxygen concentration [21-24]. Moreover, as hMSCs are highly sensitive to dynamic environments, such as those provided by bioreactors [25-27], it could be possible to modulate and improve the hMSCs secretome. Data from proteomic-based analysis revealed that the use of dynamic culturing conditions to modulate the secretome profile of hMSCs, enhancing the secretion of important neuroregulatory molecules such as Cys C, GDN, Galectin-1 and PEDF. Moreover it was also possible to identify the presence of specific neuroregulatory molecules that were only in the dynamic CM such as Ezrin, Radixin, β-Thymosin proteins, β-1, 4-galatosyltransferase[28] and Connective tissue growth factor, which have been described as important regulators of neurite outgrowth, neuroregeneration and angiogenesis [28-32]. These findings were then further correlated with the higher rates of neuronal differentiation of hNPCs (*in vitro*) and rat resident cells of the DG *(in vivo)*. With it was possible to show that, in fact, the use of bioreactors modulates hMSC activity, which then translates into a more enriched secretome in neuroregulatory molecules.

Another explored for the modulation of hMSCs secretome was the variation of oxygen concentration. From the literature several studies have demonstrated that oxygen concentration is an important component in the regulation and maintanance of hMSCs plasticity [33-35]. However, the conventional *in vitro* cultures are often carried out under standard ambient oxygen tension, corresponding to 21%, commonly denominated as normoxic condition. However *in vivo,* the oxygen concentration is lower than the previous value, varying from tissue to tissue among 1-13%. Thus *in vivo* cells are usually under hypoxic condition [36]. Thus the use of a 21% tension of oxygen exceeds the normal pressure existent in most of the mammalian tissues, which indicates that the oxygen concentration used during the standard *in vitro* cultures of primary human cells, including MSCs, is often not adapted to the *in vivo* situation [37, 38]. For that, we have exposed hMSCs to different oxygen concentrations (e.g. 21% O_2 – normoxic; 5% O_2 – hypoxic), addressing the impact of this

environmental change in their paracrine activity. Through proteomic-based analysis, we were able to observe that hMSCs were able to respond differently to different oxygen tensions, in which the hypoxic preconditioning led to an upregulation of several neuroregulatory proteins in their secretome. This is in line to what had already been described in other tissues/cells. For instance, Ohnishi and colleagues [39] described that many genes were upregulated when MSCs were cultured for 24h in 1% of oxygen when compared to normoxic. Focused on the encoding secretory proteins, most of the molecules upregulated were involved into cell proliferation and survival [39]. Into another study, Hung and colleagues [42] were able to found increased amounts of IL-6, MCP1 and VEGF in the CM after two days under hypoxic conditions [42]. Similar results were also reported by Chang *et al.* [40] and Hsiao *et al.* [43], which demonstrated that the hypoxic preconditioning of MSCs enhances the therapeutic potential of the secretome, playing important roles in the recovery of traumatic brain injury (TBI). However, when the hypoxic/normoxic secretome was delivered to hNPCs similar levels of neuronal differentiation was obtained for both conditions. This was surprising as the proteomic analysis revealed that there was a robust increase on most of the proteins identified for the secretome collected under hypoxic conditions. Although no conclusive data was obtained, we believe that this could indicate that the differences within concentrations of the proteins that were detected may not be sufficient to induce significant variation on the functional differentiation of hNPCs. Nevertheless this hypothesis should be further explored in future works

In the last work developed on the scope of this thesis, we have studied the possible therapeutic effect of hMSCs secretome, collected from dynamic and static culture conditions, on the recovery of a rat model (6-OHDA) of Parkinson's disease (PD). For this purpose we have injected the secretome of hMSCs (either from static and dynamic conditions) in the substantia nigra (SNc) of PD animals. From the results, we were able observe that injection of both CM was able to improve the motor performance of the animals (through the rotarod and staircase test analysis) when compared to 6-OHDA-control animals (Chapter 5). The significant increase on the survival of TH-positive in the hMSCs injected groups, it is most likely related with that. This is in line to what has been described in the literature, in which the action of the hMSCs secreted factors has been correlated with beneficial actions on PD [55, 56, 58-60]. However in the present thesis the same effects were originally reported for secretome and not transplanted, injected animals. One of the possible molecules related with this found through the described proteomic analysis is PEDF. Indeed, it has been proposed that this molecule acts as an endogenous neuroprotective molecule in response to

insults on the DAergic system, in which the enhancement of its levels leads was associated with the increase of TH-positive cells survival and behavioral improvements on PD [62, 63]. Interestingly, it was curious not observe statistical differences between the static and dynamic CM, as it was observed in the chapter 3, even though most of the proteins found in the proteomic analysis were correlated with neurogenic actions. We could hypothesize that in this 6-OHDA PD model, in which neurodegeneration is caused by oxidative stress, the recovery of TH-positive cells was not dependent on neurogenic-related proteins/actions, but rather of neuroprotective or antioxidant-related molecules. PEDF seems to be involved in these actions. However, future studies should be promoted in order to address the real effects of PEDF on PD, as well as to analyze its levels in the SNc resident tissue before and after disease, as well as after CM treatment. By doing so it would be possible to address if the PD improvements could be correlated with the presence of this factor in the hMSC secretome constitution. In addition to this future studies should also be promoted in order to compare the effects of hMSCs cell transplantation and its secretome. Nevertheless, despite these important considerations, our present results suggest that the use of hMSCs secretome could be a new promising therapeutic tool for the treatment of PD, able to modulate the DAergic neuronal survival and animal behavior, in which the PEDF might be at least partly involved on these outcomes, which could open novel therapeutic opportunities for PD.

As final remarks it can be stated that the work performed and included in this thesis provided interesting approaches regarding the potential use of hMSCs secretome as a future therapeutic tool for the CNS regenerative medicine field. Future studies should be performed in order to improve and validate this data. Importantly, the knowledge about the use of dynamic culture conditions (through bioreactors) associated to proteomic-based analysis was originally demonstrated in this thesis and may have a stronger impact in the field. Moreover, the association of dynamic culturing conditions with different percentages of oxygen revealed to be another way to modulate and improve the hMSCs secretome constitution. Finally, in a context of disease it was important to observe that the transplantation of hMSCs secretome (with no cell transplantation) was able to ameliorate the motor deficits of an animal model of PD, opening in these way future therapeutic opportunities for CNS regenerative medicine approaches.

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