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Ana de Oliveira Resende Pires Marques

Exploring the Secretome of Mesenchymal Like Stem Cells for Central Nervous System Regenerative Medicine: A Focus on Parkinson's disease

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Tese de Doutoramento em Ciências da Saúde

Trabalho realizado sob a orientação do
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e do
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Exploring the Secretome of Mesenchymal Like Stem Cells for Central Nervous System Regenerative Medicine: A Focus on Parkinson's Disease

ABSTRACT

In recent years, mesenchymal stem cells (MSCs) have emerged as strong therapeutic candidates for Central nervous system (CNS) regenerative medicine. Over the last decade, neuroregulatory molecules secreted by different tissue derived MSCs have shown to hold a tremendous therapeutic potential towards CNS protection and recovery in animal models of distinct CNS disorders. More recently, it has been discovered that MSCs also secrete microvesicles and exosomes which have been reported to act as reparative agents. Nevertheless, despite these progresses, is still not known if the MSCs secretome alone, without any further cell transplantation, induces similar therapeutic benefits. Moreover, it is still not known if the secretome of different tissue derived MSCs have similar or differential therapeutic impact on a neurodegenerative disease, such as Parkinson's disease (PD). Finally, an in-depth proteomic analysis to the secretome of MSCs is yet to be made. As a consequence of this, the scope of the present thesis was to explore the potential of the sole use of different tissue derived MSCs secretome, namely derived from bone marrow (BMSCs), adipose tissue (ASCs) and the Wharton jelly surrounding the vessels of the umbilical cord [(WJ-MSCs/human umbilical cord perivascular cells (HUCPVCs)] for CNS regenerative medicine, namely in PD. For this purpose, we first studied (Chapter 2) the effect of the BMSCs, ASCs and HUCPVCs secretome, in the form of conditioned media (CM) collected at different time points (24h,96h), on the survival and neuronal differentiation of a neuroblastoma cell line (SH-SY5Y cells). Results showed that the secretome of both BMSCs and HUCPVCs was capable of supporting SH-SY5Y cells survival, induce neurite outgrowth, as well as their differentiation into neuron-like cells. These experiences further indicated that the secretome of the two cell populations was inducing SH-SY5Y cells towards a different phenotype. In chapter 3 it was revealed that ASCs secretome induced a higher survival rate in ventral mesencephalic cells (VMCs). Moreover, when the secretome of the three MSC like cell populations was individually administrated into a 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat model of PD, it was observed that BMSCs secretome induced a higher functional recovery, as assessed by the stair case test, as well as an increase on the number of tyrosine hydroxylase (TH) positive cells in the substantia nigra. Finally, in chapter 4, an exhaustive proteomics approach based on liquid chromatography coupled with tandem mass spectrometry following information dependent and SWATH (sequential windowed

data independent acquisition of the total high-resolution mass spectra) acquisitions was performed in order to characterize the secretome of BMSCs, ASCs and HUCPVCs. Through this approach, we have been able to identify and quantify 121 proteins, from which 20 have been shown to be involved in events related with neural repair. The latter not only included neurotrophic, neurogenic, axon guidance, axon growth and neurodifferentiative proteins, but also proteins playing roles against distinct pathogenic processes, including oxidative stress, apoptosis, excitotoxicity, inflammation, glial scarring and toxic protein deposition, which have been shown to be involved in several CNS disorders/injuries. Importantly, the latter proteins were found to be differently expressed within the secretome of the MSCs populations in study. This result, not only demonstrates that effectively there are differences within the secretome of the MSCs populations in study, but also suggests that the secretome of different tissue derived MSCs may have a different impact in neuroprotection, neuroreparative and neurodifferentiation phenomena, which can explain the results obtained in the studies conducted throughout the present thesis.

In summary, the work developed in the present thesis adds new knowledge on the biological and molecular relevance of the secretome differences of different tissue derived MSCs in the context of CNS neuroprotection and neuron repair. Moreover, it also demonstrates that, although it is important to select the appropriate cell type for application, the sole use of MSCs secretome may be a promising cell free therapeutic tool for future application in CNS regenerative medicine.

O Papel do Secretoma das Células Estaminais Mesenquimais no campo da Medicina Regenerativa do Sistema Nervoso Central: Foco na doença de Parkinson

RESUMO

Nos últimos anos, as células estaminais mesenquimais (MSCs) surgiram como fortes candidatos terapêuticos para a medicina regenerativa do sistema nervoso central (SNC). Durante a última década, as moléculas com carácter neuroregulador secretadas pelas MSCs isoladas de diferentes tecidos têm demonstrado um imenso potencial terapêutico para a proteção e recuperação em modelos animais de diferentes doenças do SNC. Mais recentemente, descobriu-se que as MSCs também secretam microvesículas e exossomas, tendo estes sido descritos como agentes activos nas possíveis propriedades terapêuticas do secretoma. Contudo, apesar destes progressos, ainda não é conhecido se o secretoma das MSCs, por si só, é capaz de induzir benefícios terapêuticos. Para além disso, ainda não é conhecido se o secretoma de MSCs isoladas de diferentes tecidos têm impacto terapêutico semelhante ou diferencial na mesma doença neurodegenerativa. Finalmente, uma análise proteómica detalhada do secretoma das MSCs ainda se encontra por realizar. Como consequência, o objetivo da presente tese foi explorar o potencial do uso exclusivo do secretoma das MSCs, nomeadamente derivado da medula óssea (BMSCs), tecido adiposo (ASCs) e da geleia circundante dos vasos do cordão umbilical (HUCPVCs), para a medicina regenerativa do SNC, nomeadamente na doença de Parkinson (DP). Para este fim, foi estudado em primeiro lugar (Capítulo 2) o efeito do secretoma das BMSCs, ASCs e HUCPVCs, sob a forma de meio condicionado (MC) recolhido em diferentes alturas (24h, 96h), na sobrevivência e diferenciação neuronal de uma linha celular de células de neuroblastoma (células SH-SY5Y). Os resultados demonstraram que o secretoma das BMSCs e HUCPVCs foi capaz de suportar a sobrevivência das células SH-SY5Y, induzir o crescimento de neurites, bem como a sua diferenciação em células neuronais. Estas experiências indicaram ainda que o secretoma das duas populações celulares induziu a diferenciação das células SH-SY5Y em diferentes fenótipos. No capítulo 3, foi revelado que o secretoma das ASCs induziu um efeito superior na sobrevivência de células mesencefálicas ventrais. Para além disso, quando o secretoma das três populações de MSCs foi administrado individualmente num modelo de DP com lesão unilateral induzida com 6-hidroxidopamina (6-OHDA), observou-se que o secretoma das BMSCs induziu uma maior recuperação funcional, avaliada com recurso ao *staircase test*, assim como um aumento do numero de células positivas para tirosina hidroxilase (TH) na substancia

nigra. Finalmente no capítulo 4, uma análise proteómica exaustiva, baseada em cromatografia liquida acoplada à espectroscopia de massa em tandem seguindo aquisições de informação dependente e SWATH, foi realizada com o intuito de caracterizar o secretoma das BMSCs, ASCs e HUCPVCs. Através desta abordagem, foi possível identificar e quantificar 121 proteínas, das quais 20 demonstraram estar envolvidas em eventos relacionados com a reparação neuronal. Estas incluíram não só proteínas com carácter neurotrófico, neurogénico, de orientação de axónios e neuro-diferenciativo, como também proteínas que desempenham funções contra diferentes processos patogénicos, incluindo stress oxidativo, apoptose, excito-toxicidade, inflamação, cicatrização glial e deposição de proteínas tóxicas, que têm sido descritos como estando envolvidos em vários distúrbios/lesões do SNC. É ainda importante referir que as proteínas que foram encontradas no secretoma das populações de MSCs em estudo exibiram expressões diferentes. Este resultado, não só demonstra que efetivamente existem diferenças no secretoma das populações de MSCs em estudo, mas também sugere que o secretoma de MSCs derivado de diferentes tecidos pode ter um impacto diferente em fenómenos de neuroprotecção, neuro-reparação e neurodiferenciação, o que pode explicar os resultados obtidos nos estudos realizados ao longo da presente tese.

Em resumo, o trabalho desenvolvido na presente tese acrescenta novos conhecimentos acerca da relevância biológica e molecular do secretoma de MSCs derivado de diferentes tecidos no contexto de neuroprotecção e neuro-reparação do SNC. Adicionalmente, também demonstra que embora seja importante selecionar o tipo de células apropriado para aplicação, o uso exclusivo do secretoma das MSCs pode ser uma ferramenta terapêutica promissora, isenta de células, para aplicação futura na medicina regenerativa do SNC.

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LIST OF ABBREVIATIONS

#

°C: Degrees celsius

6-OHDA: 6-hydroxydopamine

A

AAAD: Aromatic L-aminoacid decarboxilase

AADC: Aromatic amino acid decarboxylase

AAV: Adeno-associated virus

AAV-AADC: Adeno-associated virus vector encoding for aromatic amino acid decarboxylase

AAV-GAD: Adeno-associated virus vector encoding for glutamic acid decarboxylase

AAV2-GAD: Adeno-associated virus serotype 2 vector encoding for glutamic acid decarboxylase

AAV2-hAADC: Adeno-associated virus serotype 2 vector encoding for human aromatic amino acid decarboxylase

AAV2-NTN: Adeno-associated virus serotype 2 vector encoding for human Neurturin

Aβ: Amyloid beta

ACN: Acetonitrile

AD: Alzheimer's disease

ADL: Activities of daily living

ADX88178: 5-Methyl-N-(4-methylpyrimidin-2-yl)-4-(1H-pyrazol-4-yl)thiazol-2amine

ANOVA: Analysis of variance

AP: Anterior-posterior

AS: Albumin serum

ASCs: Adipose stem/stromal cells

B

BASP-1: Brain acid soluble protein 1

BDNF: Brain-derived neurotrophic factor

bFGF: Basic fibroblast growth factor

BMSCs: Bone marrow mesenchymal stem/stromal cells

C

CADH2: Cadherin-2

CD11b: Cluster of differentiation 11b

CD14: Cluster of differentiation 14

CD19: Cluster of differentiation 19

CD34: Cluster of differentiation 34

CD45: Cluster of differentiation 45

CD73: Cluster of differentiation 73

CD79: Cluster of differentiation 79

CD90: Cluster of differentiation 90

CD105: Cluster of differentiation 105

cDNA: complementary deoxyribonucleic acid

C1-Inh: Plasma protease C1 inhibitor

CLUS: Clusterin

CM: Conditioned media

Cm^2: Square centimeters

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

CO: Cross-over

CO_2: Carbon dioxide

COMT: cathecol-O-methyltransferase

CT: computed tomography

CXCL-5: C-X-C motif chemokine ligand 5

CXCL-16: C-X-C motif chemokine ligand 16

CYPA: Cyclophilin A

CYPB: Cyclophilin B

CYSC: Cystatin C

D

DA: Dopamine

DAB: 3,3í-diaminobenzidinetetrahydrochloride

DAergic: Dopaminergic

DAPI: 4',6-diamidino-2-phenylindole, dihydrochoride

DAT: Dopamine transporter

DAT SPECT: Dopamine transporter imaging with single photo emission computed tomography

DB: Double-blind

DBS: Deep brain stimulation

DCN: Decorin

DMEM/F12: Dulbecco's modified eagles medium nutrient mixture F12

DNase: desoxirribonuclease

DRD2: Dopamine receptor D2

DRG: Dorsal root ganglia

DS: Delayed start

DV: Dorsal-ventral

E

EGF: Endothelium growth factor

ELISA: Enzyme-Linked Immunosorbent Assay

ER: Endoplasmatic reticulum

ES cells: Embryonic stem cells

eV: electron volt

F

FA: Formic acid

FBS: Fetal bovine serum

FCS: Fetal calf serum

FDA: Food and drug administration

FDR: False discovery rate

FGF 8: Fibroblast growth factor 8

FGF 20: Fibroblast growth factor 20

FVM: Fetal ventral mesencephalic

G

GABA: Gamma-aminobutyric acid

GAD: Glutamic acid decarboxylase

Gal-1: Galectin 1

GCH1: Guanosine 5´-triphosphate cyclohydrolase 1

G-CSF: Granulocyte colony-stimulating factor

GDN: Glia-derived nexin

GDNF: Glial cell-derived neurotrophic factor

GFs: Growth factors

GFP: Green fluorescent protein

GITR: Glucocorticoid-induced tumor necrosis factor receptor

GPe: External globus pallidus

GPi: Internal globus pallidus

GProX: Graphical Proteomix Data Explorer

H

h: Hour

hr: Human recombinant

hAADC: Human aromatic amino acid decarboxylase

hBMSCs: Human bone marrow mesenchymal stem/stromal cells

HGF: Hepatocyte growth factor

H_2O_2: Hydrogen peroxide

HSP27: Heat shock protein 27

HUCPVCs: Human umbilical cord perivascular cells

I

IDA: Information-dependent acquisition

IGF-1: Insulin-like growth factor 1

IGF 2: Insulin-like growth factor 2

IgG: Immunoglobulin G

IL-1β: Interleukin 1 beta

IL-2: Interleukin-2

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-10: Interleukin-12

iN cells: Induced neural cells

INF γ: Interferon gamma

iPS cells: Induced pluripotent stem cells

ISCT: International society for cellular therapy

K

Kg: Kilogram

kDa: Kilodaltons

L

LC: liquid chromatography

LC-MS/MS: Liquid chromatography coupled with tandem mass spectrometry

L-DOPA: Levodopa

L-DOPA-carbidopa: Levodopa-carbidopa

LSP1-2111:(2S)-2-amino-4-[hydroxy[hydroxy(4-hydroxy-3-methoxy-5-nitro-phenyl)methyl]phosphoryl]butanoic acid

M

M: Molar

MAO-B: Monoamine oxidase-B

MAP-2: Microtubule-associated protein 2

MC: Multicenter

MCP-1: Monocyte chemotactic protein 1

MD: Multi-dosage

MFB: Middle forebrain bundle

mGluRs: Metabotropic glutamate receptors

mGluR4: Metabotropic glutamate receptor 4

MHC: Major histocompatibility complex

Min: Minute(s)

miRNA: Micro-ribonucleic acid

mg: Milligram(s)

ML: Medial-lateral

ml: Milliliter(s)

mm: Millimeter(s)

mm^2: Square millimeters

MMTS: methanethiosulfanate

mRNA: Messenger ribonucleic acid

MS: Mass spectrometer

ms: milliseconds

MS/MS: Tandem mass spectrometry

MSCs: Mesenchymal stem cells

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI: Magnetic resonance imaging

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

m/z: Mass to charge

N

$NaHCO_3$: Sodium bicarbonate

NBM: Neurobasal media

NCT: National clinical trial

NAP-2: Neutrophil-activating protein-2

NAP-52: Neutrophil-activating protein-52

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartate

NMSS: Non-motor symptoms scale

Non-DAergic: Non-dopaminergic

NSCs: Neural stem cells

NT-3: Neurotrophin-3

NT-4: Neurotrophin-4

NTN: Neurturin

Nurr1: Nuclear receptor related-1

O

O.D.: Optical density

P

P6: Passage 6

PAI-1: Plasminogen activator inhibitor-1

PBS: Phosphate buffered saline

PC: Placebo controlled

PD: Parkinson's disease

PDGF-AA: Platelet-derived growth factor AA

PEDF: Pigment epithelium-derived factor

PET: Positron emission tomography

PG: Parallel group

PGF: placental growth factor

PHCCC: *N*-phenyl-7-(hydroxylimino)cyclopropa[*b*]chromen-1a-carboxamide

PRDX1: Peroxiredoxin-1

PPN: Pedunculo pontine nucleus

R

RA: Retinoic acid

RA-DCs: Retinoic acid differentiated cells

RA-differentiated cells: Retinoic acid differentiated cells

RD: Randomized

ROS: Reactive oxygen species

rpm: Rotations per minute

RT: Room temperature

RT-PCR: Real-time reverse-transcription polymerase chain reaction

RNase: Ribonuclease

S

s: Second

SAGE: Serial analysis of gene expression

SCF: Stem cell factor

SCI: Spinal cord injury

SD: Standard deviation

SDF-1: Stromal cell-derived factor 1

SDF-1α: Stromal cell-derived factor 1 alpha

SEM: standard error of the mean

SEM7A: Semaphorin 7A

SN: Substantia nigra

SNpc: Substantia nigra *pars compacta*

SPECT Single photo emission computed tomography

STN: Subthalamic nucleus

STN AAV-GAD: Adeno-associated virus vector encoding for glutamic acid decarboxylase in the Subthalamic nucleus

STN AAV2-GAD: Adeno-associated virus serotype 2 vector encoding for glutamic acid decarboxylase in the Subthalamic nucleus

SWATH: Sequential windowed data independent acquisition of the total high-resolution mass spectra

T

TCA: Trichloroacetic acid

TCEP: Tris(2-carboxymethyl)phosphine

TEAB: Triethylammonium bicarbonate buffer

TGF β1: Transforming growth factor beta 1

TH: Tyrosine hydroxylase

TNF α: Tumor necrosis factor alfa

TRX: Thioredoxin

U

UCHL1: Ubiquitin-carboxy-terminal hydrolase 1

µg: Microgram(s)

UKPDBB: United Kingdom parkinson's disease society brain bank

UKPDBBCDC: United Kingdom parkinson's disease society brain bank clinical diagnostic criteria

µm: Micrometer(s)

µM: Micromolar

µl: Microliter(s)

UPDRS: Unified Parkinson's disease rating scale

V

VEGF: Vascular endothelial growth factor

VEGF-R3: Vascular endothelial growth factor receptor 3

VU0155041: (+/-)-cis-2-(3,5-dichlorphenylcarbamoyl)cyclohexanecarboxylic acid

W

W: Week(s)

WJ: Wharton's jelly

WJ-MSCs: Wharton's jelly mesenchymal stem cells

X

XIC: Extracted ion chromatograms

Z

ZI: Zona incerta

Chapter 1

Introduction - Old and New Challenges in Parkinson's Disease Therapeutics (Manuscript status: to be submitted)

Old and New Challenges in Parkinson's Disease Therapeutics

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Abstract

Central nervous system (CNS) neurological disorders/injuries often pose a major challenge for treatment due to the limited capability of the CNS to self-renew and to regenerate. Among CNS disorders, Parkinson's disease (PD) is a debilitating disorder that affects millions worldwide. It is a neurodegenerative disease characterized by the loss of dopaminergic (DAergic) neurons in several dopaminergic networks, most intensively in the ventral tier of the substantia nigra *pars compacta* (SNpc) within the mesostriatal/nigrostriatal pathway. Current treatments of idhiopatic PD mainly rely on the use of pharmacologic agents to improve motor symptomatology of PD patients. Nevertheless PD remains a deleterious disease for which there is no cure. Thus, with the extension of life expectancy, global aging of populations and the increasing capacity of modern medicine to prolong human life, it is expected that the number of people affected by PD will double every 20 years. Therefore it is of the utmost importance to establish new therapeutic strategies for PD treatment. Over the last 20 years, several molecular, gene and cell/stem-cell therapeutic approaches have been developed to meet the clinical challenge of counteracting or retard PD progression. Among stem cell populations, the use of mesenchymal stromal/stem cells (MSCs) have been proposed as a possible therapeutic route to meet this challenge. Indeed, MSCs transplantation has already showed to promote neuroprotection and/or neurorecovery, and to improve motor deficits in PD animal models. Nowadays is commonly accepted that these effects are mostly mediated by MSCs secretome. Indeed, the latter has shown to mediate phenomena such as neural survival, neuroprotection and/or neurorecovery, as well as immune response modulation observed upon transplantation of these cells in rodent models of PD.

The scope of this review is to provide an overview of PD and the major breakthroughs achieved on PD field. For that, this review will start by focusing on PD characterization and current treatment options covering thereafter molecular, gene and cell/stem cell-based therapies that are currently being studied in animal models of PD or have recently been tested in clinical trials. Among stem cell-based therapies, those using MSCs as possible disease modifying agents for PD therapy and, specifically, the MSCs secretome contribution to meet the clinical challenge of counteracting or retard PD progression will be more deeply explored.

1. Introduction

Among CNS disorders, Parkinson's disease (PD) is the most common motor-related disorder in middle or late life affecting millions worldwide (Pereira and Aziz 2006). PD clinical syndrome was first described in 1817 by James Pakinson on his original "An essay on the shaking palsy" as "paralysis agitans" after observing signs of tremor, festinating gait and flexed posture in six patients (Parkinson 1817, Pereira and Aziz 2006). "Paralysis agitans" was later named as "maladie de Parkinson" or Parkinson's disease in 1888 by Charcot (Charcot 2002). The scope of this review is to provide an overview of PD and the major breakthroughs accomplished in PD field. For that, this review will first characterize PD and will focus thereafter on PD clinical features and diagnosis, as well as on current clinical approaches and emerging molecular and stem cell-based therapies, particularly those using mesenchymal stem cells (MSCs). An overview of new candidate drugs and current status of gene therapy for PD treatment will also be provided. All the therapeutic approaches outlined in this review aim at treating motor symptoms of PD, which still remain the main focus of therapy development, and are summarized in figure 1.

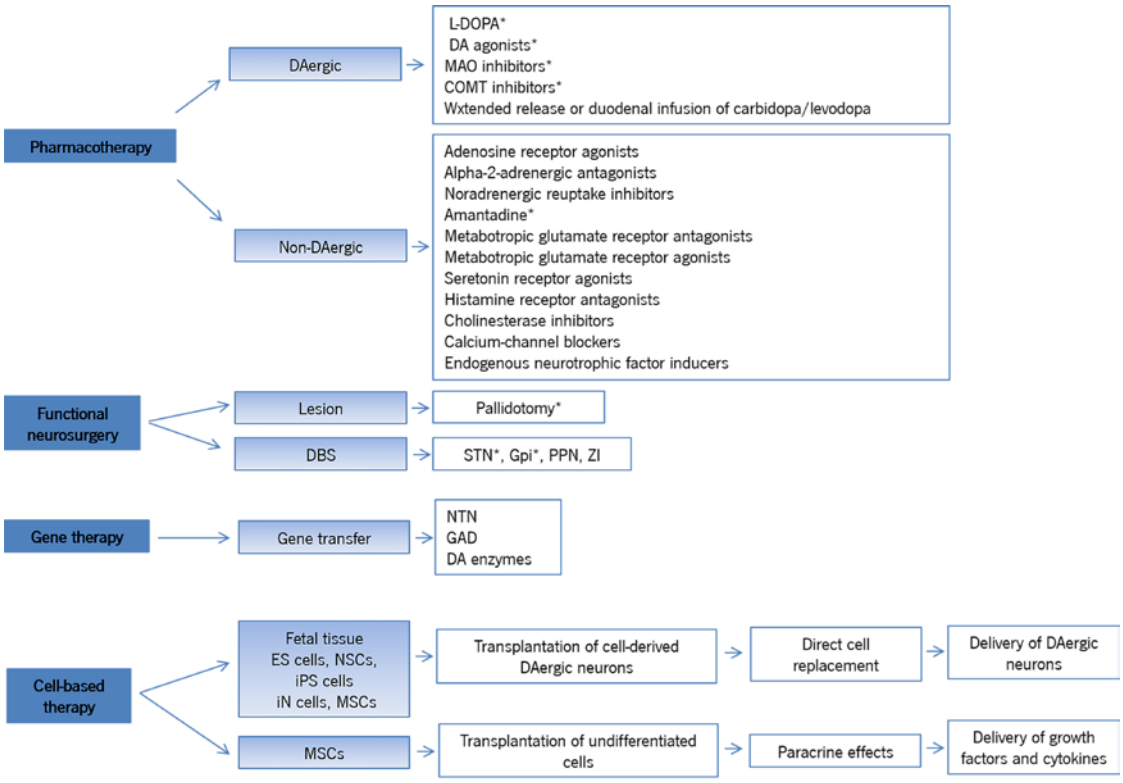

Figure 1: Summary of PD therapeutic approaches currently used in clinics or under research (* indicates current therapeutic approaches used in clinics).

2. Parkinson's disease

Parkinson's disease is a slowly progressive neurodegenerative disease that is primarily characterized by the progressive loss of dopaminergic (DAergic) neurons in several dopaminargic networks, most intensively in the ventral tier of the substantia nigra *pars compacta* (SNpc) within the mesostriatal/nigrostriatal pathway, and the presence of Lewy bodies (deposition of α-synuclein cytoplasmatic protein aggregates) in the remaining neurons (Koller 2003, Pereira and Aziz 2006, Cummins and Barker 2012, Teixeira et al. 2013). Loss of substantia nigra (SN) neurons leads to the loss of DAergic innervations and consequently to striatal dopamine (DA) deficiency, which is the responsible for the major sensory-motor symptoms of PD (Dauer and Przedborski 2003). By the time PD motor symptoms appear, 60% of DAergic neurons in SN and 80% of striatal DA terminals were lost (Bernheimer et al. 1973, McGeer et al. 1988, Hornykiewicz 1993, Akerud et al. 2001).

The etiology of SN degeneration is unknown. Approximately 5 to 10% of patients present the classical Mendelian inheritance form of PD ("familial PD"), with patients presenting mutations in the genes that have been related with neurodegeneration, such as genes encoding for α-synuclein, parkin, tau and ubiquitin c-terminal hydrolase (Kitada et al. 1998, Simon-Sanchez et al. 2009). However, the most common form of PD is sporadic and it is thought that the interaction of multiple genetic susceptibilities and environmental factors underlie the cause of idiophatic PD (Dawson and Dawson 2003, Di Monte 2003). In addition, biochemical abnormalities such as mitochondrial dysfunction, free radical mediated damage, excitotoxicity, inflammatory change and proteasomal dysfunction have also been reported to mediate PD pathogenesis (Dauer and Przedborski 2003, Schapira 2005).

2.1 Parkinson's disease clinical features

PD is the second most common neurodegenerative disorder and the most frequent movement disorder in middle or late life, affecting millions worldwide (Pereira and Aziz 2006, Sherer et al. 2012). PD clinical features include a variety of motor and non-motor features. From the motor point of view, PD is included in Parkinsonism clinical syndrome and, therefore, shares the same four motor cardinal features of Parkinsonism, which include: bradykinesia, resting tremor, rigidity, and postural instability (Koller 2003, Jankovic 2008, Massano and Bhatia 2012). In a typical

clinical picture of PD, at the onset of the disease, patients present asymmetrical-like tremor, more prominent in the upper extremity (Paulson and Stern 1997, Koller 2003). As the disease progresses, patients are affected bilaterally and bilateral bradykinesia becomes evident (Koller 2003). Later on, patients postural instability, gait dysfunctions (freezing and festination) and falls also become manifest (Koller 2003). Bradykinesia or slowness of movement is the most characteristic clinical feature of PD and is often easily recognized, even before any formal neurological examination (Jankovic 2008). Bradykinesia is manifested by difficulties in initiation, execution and arrest of a movement, or in any task requiring fine motor control (Koller 2003, Jankovic 2008). Tremor at rest, particularly in distal part of the extremities, is the typical parkinsonian tremor and is also one of the most recognizable symptoms of PD (Jankovic 2008). Yet, many patients also present postural tremor, which may be the initial manifestation of PD (Koller 2003, Jankovic 2008). Rigidity is characterized by increased resistance to muscle stretch and relaxation due to tightness and stiffness of muscles and may occur proximally (e.g., neck, shoulders, hips) and distally (e.g., wrists, ankles). Thus, rigidity is often associated with pain (e.g., painful shoulder) and, later in the disease, with postural deformities, such as flexed neck and trunk posture, as well as flexed elbows and knees (Koller 2003, Jankovic 2008). In later stages of PD, postural instability also becomes evident as a result of loss of postural reflexes. The latter is usually accompanied with freezing, a form of movement loss (akinesia) characterized by a sudden transient inability to move that contributes to loss of balance (postural instability) and subsequent falls (Giladi et al. 1997, Giladi et al. 2001, Koller 2003, Jankovic 2008). Therefore, although freezing does not occur universally is probably the most disabling of all parkinsonian symptoms in more advanced stages of PD. Finally, in certain circumstances, particularly in patients with postural instability and flexed trunkal posture, festination of gait (involuntary quickening of gait) may also occur (Koller 2003).

Although PD is generally considered as a motor control disorder and the cardinal signs of disease rely on motor defects, a variety of non-motor features also emerge in PD patients due to the degeneration of other neuronal pathways (Mayeux et al. 1992, Koller 2003). These non-motor features are commonly known as PD non-motor symptoms. Non-motor symptoms are very frequent in PD patients affecting 88% of PD patients after seven years of disease duration, which significantly contribute for the morbidity and impaired quality of life for those who suffer from this disease (Shulman et al. 2001, Hely et al. 2005, Schapira 2005). They are characterized by

neuropsychiatric, autonomic, sensory and sleep abnormalities (Koller 2003, Schapira 2005, Jankovic 2008). Most frequent neuropsychiatric comorbidities include apathy (anhedonia), dementia, anxiety disorders (e.g., panic attacks), depression, hallucinations, psychosis and impulse control disorders (e.g., obsessive-compulsive and impulse behaviors) (Koller 2003, Schapira 2005, Jankovic 2008). Autonomic/involuntary nervous system control functions affect about one third of PD patients, but in early disease are not severe (Koller 2003). Most common autonomic abnormalities are orthostatic hypotension (decrease in blood pressure), sweating dysfunction, bowel problems, constipation, dysphagia (swallowing difficulties), sialorrhoea (excessive production of saliva), sphincter and erectile dysfunction (Koller 2003, Schapira 2005, Jankovic 2008). Sensory disturbances also affect PD population, but often pass unrecognized as parkinsonian disturbances (Shulman et al. 2002, Koller 2003). Sensory disturbances include anosmia (lack of olfaction), akathisia (physical restlessness and subjective urge to move), paresthesias (abnormal sensation of the skin like burning, prickling and formication) and pain (Koller 2003, Jankovic 2008). Finally, sleep disturbances such as excessive sleepiness, sleep attacks, insomnia as well as rapid-eye movement sleep behavior are also common in PD patients and are considered to be a substantial risk factor for the development of PD (Gjerstad et al. 2006, Gjerstad et al. 2007, Jankovic 2008).

2.2 Diagnosis and assessment of Parkinson's disease impact in patients' motor impairments and disabilities

As there is no standard test for PD, the diagnosis of this disease is based on clinical criteria. In clinical practice, the clinicians typically evaluate patients' neurologic status, medical and familial history, along with the presence of PD cardinal clinical features, associated and exclusionary symptoms, and response to levodopa/L-DOPA (Jankovic 2008). The clinical United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria (UKPDBBCDC) is the most well accepted for PD diagnosis (NationalCollaboratingCentreforChronicConditions 2006) (Table 1). According with UKPDBBCDC, the first step (step 1) for PD diagnosis is presentation of bradykinesia and at least one more PD cardinal motor feature by the PD patients (Hughes et al. 1992, Koller 2003, Lees et al. 2009). However, although PD diagnosis may be very straightforward in those cases in which patients present the classical clinical picture, in PD mild-cases clinical diagnosis may be difficult. For this differential diagnosis, the United Kingdom Parkinson's Disease Society Brain Bank (UKPDBB) provides a list of exclusion criteria (step 2). Finally, for definite

diagnosis of PD (step 3), the UKPDBBCDC also provides supportive prospective positive criteria for Parkinson's disease, which focuses on asymmetry on onset, clinical progression, and L-DOPA response (Koller 2003).

Table 1: United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) clinical diagnostic criteria for idiopathic Parkinson's disease (from (Hughes et al. 1992)).

➢ **Step 1 Diagnosis of Parkinsonian syndrome**

- Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive actions)
- And at least one of the following:
- muscular rigidity
- 4-6 Hz rest tremor
- postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction.

➢ **Step 2 Exclusion criteria for Parkinson's disease**

- History of repeated strokes with stepwise progression of parkinsonian features
- History of repeated head injury
- History of definite encephalitis
- Oculogyric crises
- Neuroleptic treatment at onset of symptoms
- More than one affected relative
- Sustained remission
- Strictly unilateral features after 3 years
- Supranuclear gaze palsy
- Cerebellar signs
- Early severe autonomic involvement
- Early severe dementia with disturbances of memory, language, and praxis
- Babinski sign
- Presence of cerebral tumor or communicating hydrocephalus on CT scan
- Negative response to large doses of levodopa (if malabsorption excluded)
- MPTP exposure

➢ **Step 3 Supportive prospective positive criteria for Parkinson's disease**
(Three or more required for diagnosis of definite Parkinson's disease)

- Unilateral onset
- Rest tremor present
- Progressive disorder
- Persistent asymmetry affecting side of onset most
- Excellent response (70-100%) to levodopa
- Severe levodopa-induced chorea
- Levodopa response for 5 years or more
- Clinical course of 10 years or more

However, although UKPDBBCDC is a good tool for PD diagnosis, the maximum diagnostic accuracy of the diagnostic criteria developed by UKPDBB was estimated to be 91 to 92% (Hughes et al. 2001). Therefore, in addition to the clinical examination, neuroimaging techniques may also be

very helpful for PD differential diagnosis. For instance, dopamine transporter (DAT) imaging with single photo emission computed tomography (DAT SPECT) and positron emission tomography (PET) with fluorodopa may be used to verify if degenerative parkinsonism is the cause of symptoms, whereas computed tomography (CT) and magnetic resonance imaging (MRI) may be useful to reveal possible other diagnostic entities (Massano and Bhatia 2012). Following PD diagnosis, clinicians also use several rating scales to diagnose patients' motor impairment and the degree of disability caused by PD. The Unified Parkinson's disease rating scale (UPDRS) provided by the European's Parkinson's disease association is the most routinely used scale to evaluate mental cognition, behavior and mood disabilities (UPDRS I); quality of life/activities of daily life disabilities (UPDRS II); motor impairment (UPDRS III) and complications of therapy (UPDRS IV) (Ramaker et al. 2002, Goetz et al. 2007, Jankovic 2008). The UPDRS is usually accompanied by two other scales, Hoehn and Yahr, to grossly assess disease progression, and the Schwab and England activities of daily living (ADL) scale to evaluate quality of life. UPDRS is currently being updated to integrate new assessment of non-motor elements of PD (Goetz et al. 2007, Gallagher et al. 2012). Therefore, non-motor questionnaire developed and validated by the international Parkinson's disease non-motor group is currently being used in clinics to assess PD non-motor symptoms, such as cognitive status, mood and behavior PD manifestations (e.g., sleep disorders, depression, apathy, anxiety, fatigue, hallucinations, among others) (Chaudhuri et al. 2006, Chaudhuri and Quinn 2009). Thus, it is worth to mention that a novel non-motor symptoms scale (NMSS) has been proposed as a valid and precise tool for assessing the frequency and severity of non-motor symptoms in PD patients to be used as adjunctive with the recently validated non-motor questionnaire (Chaudhuri et al. 2007).

3. Clinical approaches on Parkinson's disease

3.1 PD pharmacotherapeutics

Pioneer works of Carlsson and colleagues (1957) on the discovery of DA as putative neurotransmitter (Björklund et al. 2010), together with the findings from Ehringer and Hornykiewicz (1960) which revealed that dopamine concentrations are markedly decreased in the striatum of PD patients (Ehringer and Hornykiewicz 1998), paved the way for the first use of dopamine precursor L-DOPA in 1961 (Pandey 2012, Smith et al. 2012). Indeed, L-DOPA dopaminergic drug truly revolutionized the treatment of cardinal motor symptoms of PD (rest tremor, rigidity,

bradykinesia and postural instability) following its approval in 1970, bringing about improved daily function, quality of life and survival to PD patients (Smith et al. 2012). L-DOPA has been largely used for the past fifty years, remaining today as the "gold standard" therapy for PD motor symptoms. However, chronic use of L-DOPA often leads to motor fluctuations and drug-induced dyskinesias (Schapira 2005, Factor and Weiner 2008, Smith et al. 2012). The mechanisms underlying these effects are not completely understood, but are most likely related with the pulsatile stimulation of DA receptors and the degree of striatal denervation (Obeso et al. 2000, Schapira 2005). In an attempt to solve these motor complications, dopamine receptor agonists started to be administrated either alone, in early course of the disease, or as combinatorial therapy with L-DOPA (Corrodi et al. 1973, Calne et al. 1974, Lang and Lees 2002, Schapira 2005, Smith et al. 2012). Nevertheless, the use of DA receptor agonists is not free of motor disturbances and often leads to major autonomic and psychiatric side effects that outweigh their beneficial effects in PD patients (Smith et al. 2012). Currently, two orally (pramipexole, ropinerole) and one injectable (apomorphine) DA receptors agonists are available for administration on PD patients (Smith et al. 2012). These DA receptors agonists act by stimulating specific postsynaptic DA receptors subtypes that remain in the striatum as well as other cortical and subcortical brain regions (Smith et al. 2012). They were developed to reduce prevalence of drug-induced dyskinesias in PD patients and their efficacy was later confirmed in large-scale randomized controlled trials (ParkinsonStudyGroup 2000, Rascol et al. 2000, Dewey et al. 2001). Indeed, besides having specific action in certain DA receptors subtypes, pramipexole and ropinerole have longer half-lives, which presumably avoid rapid fluctuations (pharmacokinetic curves) in DA receptors stimulation, thereby managing dyskinesias in PD patients (Schapira 2005, Smith et al. 2012). However, although pramipexole and ropinerole DA receptor agonists diminish the risk of dyskinesias in PD patients, they often generate major non-motor side effects like psychiatric symptoms (e.g., hypomania, euphoria, paranoia, confusion, delusions, hallucinations), psychiatric disorders (e.g., psychosis, depression, impulse control disorders), autonomic side effects (e.g., orthostatic hypotension) and sleep disorders, among others (Adler et al. 1997, Shannon et al. 1997, Factor 2008, Olanow et al. 2009b, Kalinderi et al. 2011, Smith et al. 2012). Thus, supplementation with L-DOPA will, sooner or later, be required along the course of the disease (Rascol et al. 2000, Schapira 2005). Therefore, pramipexole and ropinerole are currently administrated alone early in the course of PD or in combination with L-DOPA in more advanced stages of the disease. On other hand, apomorphine is one of the oldest non-ergot short-acting DA receptor agonists, that has receptor affinity (D1, D2,

D3) similar to DA (Factor 1999, Factor 2008, Smith et al. 2012). Apomorphine is also the only DA receptor agonist that has been shown to possess an antiparkinsonian efficacy similar to L-DOPA in double-blind clinical trials (Cotzias et al. 1970, Dewey et al. 2001, Koller 2003, Smith et al. 2012). This similarity with L-DOPA, together with the short-acting activity of apomorphine, may explain the appearance of autonomic side effects resembling those described for pramipexole and ropinerole, yet with less prevalence of psychiatric problems (Adler et al. 1997, Dewey et al. 2001, Pfeiffer et al. 2007, Smith et al. 2012). Apomorphine use for PD treatment has been avoided for several years due to its emetic (vomit-inducing) action (Dewey et al. 2001, Koller 2003). However, the development of the injectable form of apomorphine (apomorphine hydrochloride) and the following demonstration of its efficacy in clinical trials, led to the Food and Drug Aministration (FDA) approval for fluctuating PD symptoms, as adjunct to other antiparkinsonian medications (Dewey et al. 2001, Pfeiffer et al. 2007, Smith et al. 2012). In spite of apomorphine benefits, reported technical difficulties and cutaneous adverse effects after long-term subcutaneous infusions made of this drug an unattractive choice for PD treatment (Hughes et al. 1993, Smith et al. 2012). Therefore, injectable apomorphine is only used in unpredictable motor fluctuations (end-of-dose "wearing off") and "off" episodes in advanced PD patients (Koller 2003) (Figure 2).

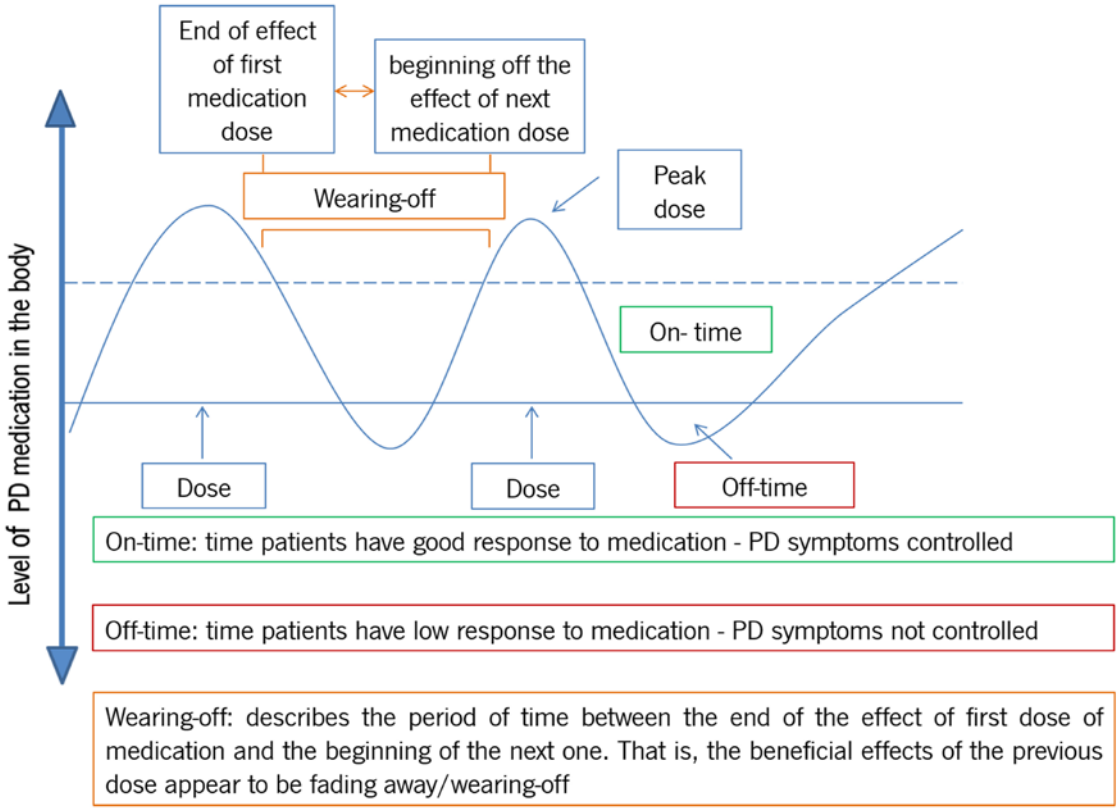

Figure 2: Wearing-off typical pattern during the day in PD patients.

Inhibitors of dopamine metabolizing enzymes, like monoamine oxidase-B (MAO-B), (peripheral) cathecol-O-methyltransferase (COMT) and aromatic L-aminoacid decarboxilase (AAAD) inhibitors, are other agents presently used in clinics for normalizing DAergic transmission. MAO inhibitors were found in 1962 to potentiate the antiparkinsonian effect of L-DOPA (Bernheimer et al. 1962, Koller 2003). The confirmation of the latter in subsequent clinical trials, together with the demonstration that selegiline and rasagiline were effective antiparkinsonian agents in early stages of PD, led to current adoption of these drugs as monotherapy in early stages of PD, or with L-DOPA supplementation to reduce motor fluctuations and "off" episodes in advanced PD patients (ParkinsonStudyGroup 1989, Shoulson 1998, ParkinsonStudyGroup 2002, ParkinsonStudyGroup 2005, Factor 2008, Olanow et al. 2008a, Olanow et al. 2009a, Olanow et al. 2009b, Kalinderi et al. 2011, Rascol et al. 2011). Thus, it is worth to mention that MAO-B inhibitors were also suggested to act as neuroprotective agents (Tatton and Greenwood 1991, Akao et al. 2002, Maruyama et al. 2002, Bar Am et al. 2004, Jenner 2004). However, their potential for displaying neuroprotective properties has been surrounded by controversy (Olanow et al. 2008a, Olanow et al. 2008b, Hart et al. 2009, Rascol 2009). On the other hand, COMT (e.g., tolcapone, entacapone), and AAAD inhibitors (e.g., carbidopa) have been developed to improve the L-DOPA therapeutic effect (Koller 2003). The demonstration of COMT inhibitors effectiveness in decreasing advanced PD patients motor fluctuations when combined with L-DOPA or with L-DOPA-carbidopa (Nutt et al. 1994, ParkinsonStudyGroup 1997, Rinne et al. 1998, Kieburtz and Hubble 2000, Heikkinen et al. 2002, Poewe et al. 2002, Larsen et al. 2003, Schapira 2005, Smith et al. 2012), led to the FDA approval and clinical use of entacapone or tolcapone, as adjunctive of L-DOPA and/or L-DOPA-carbidopa, respectively. Nevertheless, L-DOPA-carbidopa, which has been approved in 1998 by the FDA, is still used nowadays to prevent peripheral conversion of L-DOPA to DA and consequent nausea and vomiting (Koller 2003, Schapira 2005, Horstink et al. 2006).

Anticholinergics are among the oldest class of pharmaceuticals used as therapeutic agents for the management of PD (Koller 2003, Factor and Weiner 2008, Smith et al. 2012). They were developed to equilibrate striatal DA and acethylcoline activity (Kalinderi et al. 2011). However, in virtue of their modest clinical effects in comparison with L-DOPA, the elderly poor toleration and the development of severe side effects, they are currently clinically adopted mostly for the treatment of young patients with PD-associated tremor or dystonia (Koller 2003, Smith et al. 2012).

Amantadine is another FDA approved antiparkinsonian agent that acts by blocking hyperactivity of glutamatergic activity, which has been associated with PD pathology (Rascol et al. 2011, Smith et al. 2012). It is the only ionotropic glutamate receptor antagonist that has been shown to have good antiparkinsonian and anti-dyskinetic properties in the reduction of L-DOPA-induced dyskinesias (Schwab et al. 1969, Greenamyre and O'Brien 1991, Blanchet et al. 1998, Blandini and Greenamyre 1998, Factor and Molho 1999, Factor 2008, Olanow et al. 2009b, Kalinderi et al. 2011, Smith et al. 2012). Therefore is the only drug currently used capable of concomitantly reduce dyskinesia and improve PD symptoms (Ferreira and Rascol 2000, Koller 2003).

A summary of study design, number of patients as well as the primary outcome measure, main results and conclusions of trial studies above referenced is provided in table 2.

Table 2: Summary of key clinical trials conducted using current drug options for treatment of PD patients' motor symptomatology

References	Drug(s)	Study design	Patients	Nr of subjects	Primary outcome measure	Results and Conclusions
(ParkinsonStudyGroup 1989) DATATOP study	Deprenyl (Selegiline) Vs. Tocopherol	RD, DB, PC	Early untreated	800	Delay on the onset of a disability necessitating L-DOPA therapy	**Results:** Selegiline delayed the onset of L-DOPA therapy and slowed parkinsonism disability in comparison with placebo group; patients needed L-DOPA after 1 year treatment with selegiline **Conclusions:** Mild symptomatic effects of selegiline did not allowed to draw conclusions regarding any disease-modifying effects of selegiline
(Nutt et al. 1994)	Entacapone + L-DOPA	unblinded	Advanced fluctuating	15	Effect of entacapone in L-DOPA Pharmacokinetics and pharmacodynamics	**Results:** Entecapone reduced L-DOPA plasma elimination, increased "on" time, decreased daily L-DOPA during entacapone chronic treatment and increased L-DOPA single dose action duration **Conclusions:** Entacapone increases plasma half-life of L-DOPA and improves antiparkinsonian effects of both single and repeated doses of L-DOPA

Abbreviations: MC (multicenter), RA (randomized), DB (doube-blind), PC (placebo controlled), CO (cross-over), DS (delayed start), MD (multi-dosage), PG (Parallel group).

Table 2: Summary of key clinical trials conducted using current drug options for treatment of PD patients' motor symptomatology. (continued)

References	Drug(s)	Study design	Patients	Nr of subjects	Primary outcome measure	Results and Conclusions
(ParkinsonStudyGroup 1997) SEESAW study	Entacapone + L-DOPA	MC, DB, PC, PG , MD	Advanced fluctuating	205	Change in "on" time	**Results:** Entecapone increased "on" time, dyskinesia and nausea in patients treated with entacapone **Conclusions:** Entacapone is well tolerated, effective at increasing the response to L-DOPA and at relieving motor fluctuations in PD patients
(Rinne et al. 1998) Nordic NOMECOMT study	Entacapone + L-DOPA	RD, PC, DB, PG	Adnvanced fluctuating	171	Changes in "on" and "off" time	**Results:** Entacapone increased "on" time and decreased "off" time and L-DOPA daily dosage; increase in UPDRS scores; no major side effects, DAergic side effects decrease by diminishing L-DOPA dose **Conclusions:** Long-term treatment with entacapone prolonged beneficial response to L-DOPA in fluctuating ("wearing-off") PD patients and the improvement was L-DOPA dosage independent
(Rascol et al. 2000) part of 056 study	Ropinerole vs L-DOPA	RD,DB, PG	Early untreated	268	Occurence of dyskinesia	**Results:** Ropinerole prolonged the time for dyskinesia occurrence; the cumulative incidence in dyskinesias after 5 years follow-up was lower in ropinerole group; no differences in UPDRS II scores in both groups; adverse effects: higher incidence of hallucinations in ropinerole group and similar incidence of nausea among groups **Conclusions:** Ropinerole may be used as monotherapy for treatment of early PD patients for up to 5 years with a reduced risk of dyskinesias; supplementation with L-DOPA is advisable when necessary

Table 2: Summary of key clinical trials conducted using current drug options for treatment of PD patients' motor symptomatology. (continued)

References	Drug(s)	Study design	Patients	Nr of subjects	Primary outcome measure	Results and Conclusions
(ParkinsonStudyGroup 2000) CALM-PD study	Pramipexole vs L-DOPA	MC, RD, DB, PG	Early untreated	301	Time to occurrence of motor complications	**Results:** Pramipexole initially improved UPDRS scores, but patients follow-up revealed higher UPDRS scores for those treated with L-DOPA; adverse effects: somnolence, hallucinations, generalized and peripheral edema in patients treated with pramipexole **Conclusions:** Pramipexole delayed motor complications; L-DOPA provided higher efficacy on parkinsonian features
(Heikkinen et al. 2002) Orion pharma	Entacapone + L-DOPA/-Carbidopa	RD, DB, PC	Healthy males	1176	46	**Results:** Entacapone increased the area under the plasma concentration time-curve (AUC) of L-DOPA to a similar extent of L-DOPA/Carbidopa AUC; Entacapone increased half-life of L-DOPA plasma concentrations and did not haltered the pharmacokinetics of carbidopa. Similarly carbidopa did not affected the pharmacokinetics of entacapone **Conclusions:** Administration of entacapone significantly augments the AUC of L-DOPA by changing its metabolic balance
(ParkinsonStudyGroup 2002) TEMPO study	Rasagiline	MC, RA, DB, PC, PG, MD	Early untreated	404	Change in UPDRS (I,II,III,IV)	**Results:** Rasagiline induced an increase in UPDRS III independently of the used dose (1 or 2 mg) vs. placebo **Conclusions:** Rasagiline is efficient as monotherapy for early PD patients. Future studies are needed to evaluate Rasagiline long-term effect

Table 2: Summary of key clinical trials conducted using current drug options for treatment of PD patients' motor symptomatology. (continued)

References	Drug(s)	Study design	Patients	Nr of subjects	Primary outcome measure	Results and Conclusions
(Larsen et al. 2003) (extension of NOMECOMT study)	Entacapone + L-DOPA	RD, DB, PC, PG	Advanced fluctuating	152	Change in "off" time	**Results:** Entacapone increased the benefit of L-DOPA single dose and decreased "off" time; DAergic side effects were managed by L-DOPA dose control; adverse effects: diarrhea, insomnia, dizziness, nausea, aggravated parkinsonism and hallucinations **Conclusions:** Entacapone as adjunctive therapy of L-DOPA provides long-term safety and sustained efficacy in patients with motor fluctuations
(ParkinsonStudyGroup 2005) PRESTO study	Rasagiline	MC, RD, DB, PC, PG, MD	Advanced fluctuating	472	Change in hours "off"	**Results:** Rasageline decreased daily "off" time; improvement in UPDRS II, III and investigator-rated clinical global impression scores in patients treated with L-DOPA **Conclusions:** Rasagiline improves motor fluctuations and PD symptoms in L-DOPA treated PD patients
(Pfeiffer et al. 2007)	Apomorphine	RD, DB, PC	Advanced fluctuating	62	Change in UPDRS III	**Results:** Apomorphine increased UPDRS vs. placebo; no significant differences in adverse side effects; decrease in daily "off" times **Conclusions:** Support for apomorpphine as acute therapy for "off" episodes in advanced PD patients

Table 2: Summary of key clinical trials conducted using current drug options for treatment of PD patients' motor symptomatology. (continued)

References	Drug(s)	Study design	Patients	Nr of subjects	Primary outcome measure	Results and Conclusions
(Olanow et al. 2008a, Olanow et al. 2009a) ADAGIO study	Rasagiline	DB,DS, MD	Early untreated	1176	Change in UPDRS (I,II,III,IV)	**Reults:** Early treated PD patients with 1mg/day of rasagiline met all the three primary endpoints:1) increase in UPDRS scores between 12 and 36 weeks, 2) less worsening between baseline and 72 weeks and 3) non-inferiority between the early and delayed treated groups; patients administrated with 2mg/day did not met the three endpoints **Conclusions:** In consistency with a possible disease modifying effect, early treatment of PD patients with 1mg/day of rasagiline met all three primary endpoints. However, early treatment of rasagiline with 2mg/day did not. Therefore rasagiline disease modifying effects must be interpreted with caution

3.2 PD surgical interventions

Surgery approach is perhaps the oldest strategy used for movement disorders (e.g., extrapyramidal syndromes). First suggestion for surgery use to improve impaired nervous system came from James Parkinson on "An essay on the shaking palsy" (Parkinson 1817). However, first reports showing that surgery interventions within the basal ganglia could resolve tremor and rigidity came from the work reported by Russel Meyers in 1942 and 1951 (Meyers 1942, Meyers 1951). Following Russel Meyers work and the surgical precision advance (e.g., stereotaxic technique), several surgical groups conducted surgeries based in ablative techniques. At the time, surgical ablation was performed to lesion different basal ganglia locations, such as the anterodorsal and posteriomedial segments of the pallidum (pallidotomy), as well as the ventrolateral and the ventral intermediate segments of the thalamus (thalamotomy) (Svennilson et al. 1960, Kelly et al. 1987, Alexander et al. 1990, Jankovic et al. 1995, Koller 2003). Indeed, the surgical advance and the reported benefits of surgery in tremor and rigidity improvement gave rise to the use of ablative surgeries (pallidotomy, thalamotomy) as the standard procedure for the treatment of PD motor

symptoms between the 1950s and the 1960s, an epoch where no drugs were available for PD (Pandey 2012). In the 1960s, with the advent of L-DOPA as an effective drug for PD motor symptoms, surgery for PD gradually declined. Yet, several other events in the past twenty-five years contributed for the reemergence of neurosurgical interventions for PD management. For instance, reversible lessoning by deep brain stimulation (DBS), pioneered by Cooper in the early 1970s (Cooper 1973, Pereira and Aziz 2006), and the outcomes provided by non-human primate models of PD in understanding basal ganglia patophysiology (Burns et al. 1983, Bergman et al. 1990, Aziz et al. 1991, Rascol et al. 2011) were major contributors for the "renaissance" of neurosurgical therapies for PD. The latter findings, together with the onset of long-term motor complications related with chronic DAergic replacement treatment (e.g., L-DOPA) and the report of L-DOPA-induced dyskinesias relief in patients refractory to L-DOPA treatment following pallidotomy in the 1990s (Laitinen et al. 1992), led to the reemergence of neurosurgery for treatment of advanced stages of PD. In the last decade, DBS has overtaken pallidotomy in the developed countries due to the significant risks (e.g., hemorrhage, infarction, facial palsy, dysphagia, mortality) and adverse effects (e.g., affective disorders; visual fields, speech and cognitive deficits) of ablative surgeries (Pereira and Aziz 2006, Rascol et al. 2011). DBS of the subthalamic nucleus (STN) and the internal pallidal segment (internal globus pallidus/GPi) are currently the most commonly applied surgical treatment in patients with tremor, dyskinesias, rigidity and motor fluctuations refractory to the present available medication (Pereira and Aziz 2006, Benabid et al. 2009a, Benabid et al. 2009b, Rascol et al. 2011, Smith et al. 2012). DBS is an FDA approved (2002) procedure that consists in the implantation of internal and external electric stimulators with the aim of delivering continuous high frequency electric stimulation within the basal ganglia. While the internal implant is set into the STN or the GPi, the external device provides high-frequency electric stimulation of the internal electrodes (brain pacemaker) (Smith et al. 2012). Nevertheless, the ideal target for DBS is still a matter of debate in the research field, as some clinical trials have related STN DBS with significant cognitive and psychiatric side effects, such as depression, apathy, impulsivity, emotional instability and increased risk of suicide (Anderson et al. 2005, Schupbach et al. 2005, Soulas et al. 2008, Follett et al. 2010, Moro et al. 2010, Taba et al. 2010, Strutt et al. 2012, Okun 2013). The underlying cause of these events, which are commonly reported following DBS surgery either in STN or in the GPi has not yet been established. However, it is thought to involve stimulation of non-motor areas of these targets, along with the limbic structures and pre-surgical psychiatric conditions (Okun et al. 2009, Follett et al. 2010). A summary of the type of surgery, study design

and duration, number of patients as well as the primary outcome measure, main results and conclusions of trial studies above referenced is provided in table 3.

Table 3: Summary of key clinical trials conducted using functional neurosurgery for treatment of PD patients' motor symptomatology.

References	Study	Study design and duration	Number of subjects	Primary out come measure	Results
(Anderson et al. 2005)	Bilateral STN Vs. GPi DBS	Randomized, blinded, parallel group, 12 months	23	UPDRS III in off-medication	**Results:** Bradykinesia improved more in STN DBS than in GPi DBS groups; no improvement in "on" medication scores in either group; reduction in L-DOPA dose in STN group; dyskinesia decreased in both groups; cognitive behavioral complications were seen only in L-DOPA + STN DBS group **Conclusions:** STN and GPi DBS improve many PD features, further studies should be performed before excluding Gpi as an appropriate target for DBS in advanced PD patients
(Schupbach et al. 2005)	Bilateral STN DBS	Unblinded, 5 years	37	UPDRS III, UPDRS II (ADL)	**Results:** Improvement in UPDRS III and UPDRS II (ADL) scores **Conclusions:** The significant improvement of motor function was sustained 5 years after neurosurgery; moderate motor and cognitive decline are most likely related with disease progression
(Soulas et al. 2008)	Bilateral STN DBS	Unblinded, 6 months	200	Suicidal behavior	**Results:** Suicidal behavior was related with post-operative depression and/or altered impulse regulation **Conclusions:** Suicidal behavior is a potential hazard of STN DBS; careful pre-operative assessment and post-operative follow-up of psychiatric behavior should be provided

Table 3: Summary of key clinical trials conducted using functional neurosurgery for treatment of PD patients' motor symptomatology. (continued)

References	Study	Study design and duration	Number of subjects	Primary out come measure	Results
(Moro et al. 2010)	Bilateral STN Vs GPi DBS	Double-blinded, cross-over, 5 to 6 years	41	UPDRS III	**Results:** Post-operative improvement in off-medication UPDRS III motor scores in either group; significant improvement in dyskinesias in both groups; more frequent adverse effects in STN DBS group **Conclusions:** Both STN and GPi DBS provide long-term effects in advanced PD patients; motor signs tend to be better in STN DBS group; fewer adverse effects in GPi DBS group
(Follett et al. 2010)	Bilateral STN Vs. GPi DBS	Randomized, blinded, 24 months	299	UPDRS III	**Results:** No differences in UPDRS III for either STN or GPi DBS groups were observed; DAergic agents doses in STN DBS group decreased; increase in depression and decrease in speed processing in patients submitted to STN DBS; no differences in adverse side effects between STN and GPi DBS groups **Conclusions:** Similar improvement in motor function in both STN and GPi DBS targets; selection of DBS target should consider PD non-motor features
(Taba et al. 2010, Okun 2013)	Unilateral STN Vs. GPi DBS	Randomized, unblinded, 6 months	44	UPDRS III, UPDRS IV	**Results:** Bilateral DBS improved UPDRS III and/or ipsilateral scores at baseline and lower asymmetry index; bilateral implantation was more suitable for patients with higher scores of dyskinesia on/off motor fluctuations and gait problems; unilateral DBS was suitable for patients with asymmetric UPDRS III scores and moderate gait disturbances **Conclusions:** Unilateral DBS is effective in improving motor symptoms in many PD patients; bilateral DBS may be more appropriated in the future for patients with more symmetric PD, higher UPDRS III scores and severe gait dysfunction or bradykinesia

3.3 New drugs and surgical targets for PD

Motor complications of long-term L-DOPA treatment and the suggestion that some drugs (e.g., selegiline, rasageline) could have neuroprotective effect on PD, led to the development of new drugs for symptomatic or neuroprotective therapies. Symptomatic drug therapy for motor features of PD, that have already reached clinical trials, involve the use of DAergic drugs such as controlled-release formulation (IPX 066) and continuous duodenal infusions of carbidopa-levodopa (LCIG/Duodopa), sustained release formulation of L-DOPA (XP 21279), as well as drugs targeting non-DAergic neurotransmitter systems, such as those involving adenosine receptor antagonists (e.g., Preladenant; Tozadenant; caffeine); alpha-2-adrenergic antagonists (e.g., Fipamezole); noradrenergic reuptake inhibitors (e.g., Methylphenidate); ionotropic (e.g., Talampanel) or metabotropic glutamate receptor antagonists (e.g., mavoglurant, dipraglurant); serotonin receptor agonists (e.g., Sarizotan, Pardoprunox); nicotinic receptor agonists (e.g. nicotine) histamine receptor antagonists (e.g., Famotidine); cholinesterase inhibitors (e.g., Donepezil, Rivastigmine); calcium channel blockers (e.g., isradipine) and endogenous neurotrophic factor inducers (e.g., cogane) [for review see (Hauser 2011, Rodnitzky 2012, Kalia et al. 2013)]. Moreover, some of the above referred drugs (e.g., nicotine, preladenant) are also being investigated in clinical trials for the potential neuroprotective actions that they can hold towards PD (NCT01560754, NCT01155479) [for review see (Dunkel et al. 2012, Kalia et al. 2013)]. On other hand, new drugs targeting oxidative-stress (e.g., deferiprone), mitochondrial dysfunction and excitotxicity (e.g., creatine), which have been implicated in PD neuropathology (Schapira 2005, Rascol et al. 2011), are also currently being investigated in clinical trials (NCT01539837, NCT00449865) to determine their possible neuroprotective role in PD [for review see (Dunkel et al. 2012, Rodnitzky 2012, Kalia et al. 2013)].

In surgery research field, electrode implantation in patients has also provided insight into the pathoanatomy and pathophysiology of PD. These progresses led to the search of new targets for DBS such as pedunculo pontine nucleus (PPN) and caudal zona incerta (ZI) [for review see (Sackeim and George 2008)]. These targets generated interest for DBS in PD patients as some small clinical trials reported antiparkinsonian effects following the stimulation of either PPN (Plaha and Gill 2005, Ferraye et al. 2010) or ZI (Kitagawa et al. 2005, Plaha et al. 2006). Therefore, new

clinical trials are planned to study PPN (NCT01485276) and ZI (NCT01945567) as potential DBS targets for PD therapy.

4. Molecular therapies

To date, administration of dopamine precursor L-DOPA remains the gold-standard clinical therapy for PD treatment and management of motor symptoms. As discussed before, the long-term use of this drug has been associated with undesirable side effects such as motor fluctuations and dyskinesias along with non-total recovery of disease symptomatology (Schapira 2005, Smith et al. 2012, Teixeira et al. 2013). Thus, none of the current available therapies can retard or halt the disease progression. These limitations along with the significant advances made in the knowledge of the pathobiology and pathoanatomy of PD, led to the emergence of new pharmacologic agents and gene engineering approaches for the long-term outcome of PD patients. In this section, pharmacological agents targeting non-DAergic neurotransmission currently under research and the most advanced studies using gene engineering as promising tools for symptomatic and/or neuroprotective PD therapies will be explored.

4.1 Drug therapy

Presently, new candidate drugs under preclinical studies mainly focus on research of neuroprotective agents and on alternative non-DAergic therapies for PD treatment. Based on recent animal studies, among non-DAergic agents, particularly metabotropic glutamate receptor agonists, have raised special attention as potential anti-parkinsonian and neuroprotective targets in PD (Marino and Conn 2006). Among metabotropic glutamate receptors (mGluRs) agonists, subtype 4 of group III of mGluRs (mGluR4) are mainly localized in presynaptic terminals and mediate inhibitory effects on basal ganglia circuitry, namely on glutamatergic synapses in the striatum and the GABAergic synapses in the globus pallidus (Cartmell and Schoepp 2000, Nicoletti et al. 2011, Smith et al. 2012). DA depletion in PD has been associated with basal ganglia circuitry hyperactivation and electrophysiological studies have demonstrated that activation of mGluR4 significantly reduce excitatory synaptic transmission within the basal ganglia (Cartmell and Schoepp 2000, Bennouar et al. 2013). For these reasons interest has been raised in the use of mGluR4 agonists, and more recently in enhancers of the mGluR4 agonist effect (positive allosteric modulators), as potential anti-parkinsonian targets. Indeed, several studies have shown that drugs

which activate mGluR4, such as orthosteric agonists (e.g.,(2S)-2-amino-4-[hydroxy[hydroxy(4-hydroxy-3-methoxy-5-nitro-phenyl)methyl]phosphoryl]butanoic acid/**LSP1-2111**) and positive allosteric modulators [(e.g., *N*-phenyl-7-(hydroxylimino)cyclopropa[*b*]chromen-1a-carboxamide/**PHCCC**);(+/-)-cis-2-(3,5-dichlorphenylcarbamoyl)cyclohexanecarboxylic acid/ **VU0155041**, and 5-Methyl-N-(4-methylpyrimidin-2-yl)-4-(1H-pyrazol-4-yl)thiazol-2amine /**ADX88178**)], crossed brain-blood-barrier and alleviated PD motor symptoms such akinesya and drug-induced dyskinesias in animal models of PD (Marino et al. 2003, Niswender et al. 2008, Beurrier et al. 2009, Betts et al. 2012). Thus, allosteric modulators like PHCCC have showed to reduce loss of nigrostriatal dopamineric neurons in a mice model of PD following local injection of PHCCC in the external globus pallidus/GPe (Battaglia et al. 2006). Similar results regarding neuroprotection of DAergic neurons and motor benefits were reported by Betts *et al*. after injection of VU0155041 in the SNpc of a rat model of PD (Betts et al. 2012). These results suggest that mGluR4 allosteric modulators may play a dual role by relieving PD motor symptoms and providing neuroprotection of the nigrostriatal pathway. Thus, more recent developed positive allosteric modulators like ADX88178 showed to improve efficacy in a rodent PD model when combined with L-DOPA and adenosine receptor antagonists (Celanire and Campo 2012, Amalric et al. 2013). Taken together, current experimental data on mGluR4 agonists effects on both PD motor symptoms and on neuroprotection of the nigrostriatal pathway are promising. Importantly, non-motor side effects should also be assessed in future animal studies. In fact, the latter, which had not been previously assessed in animal studies, were reported in human studies following administration of several ionotropic glutamate receptor antagonists which intended to block increased glutamatergic transmission in the basal ganglia circuitry (Starr 1995, Blandini and Greenamyre 1998, Smith et al. 2012).

4.2 Gene therapy

Gene therapy in PD makes use of viral vectors to carry out gene transfer for targeted protein expression in specific brain regions within the brain nuclei from the basal ganglia. In the last decade, gene therapy has reached clinical trials to relieve PD motor symptoms essentially through three approaches: 1) induction of glutamic acid decarboxylase (GAD) enzyme in the STN; 2) delivery of synthetic enzymes to increase striatal DA levels, and 3) local infusion of neurotrophic factors to protect and restore nigral DAergic neurons (Cummins and Barker 2012, Rodnitzky 2012, Smith et

al. 2012). Loss of DA in the striatum induces the decrease of the inhibitory control driven by the GPe on the STN. These events affect the output of basal ganglia circuitry, thereby leading to the impairment of motor functions (Coune et al. 2012, Rodnitzky 2012).

Therefore, the rationale behind the first approach is to deliver the rate-limiting enzyme for GABA synthesis, GAD, into the glutamatergic neurons of the STN, using an adeno-associated virus (AAV) vector. By doing so, this gene therapy approach aims at modulating STN activity by modifying the phenotype of the STN neurons from predominantly excitatory (glutamatergic) to predominantly inhibitory (GABAergic), thereby restoring the normal function in striato-pallidal circuitry (Coune et al. 2012, Smith et al. 2012). Based on preclinical data provided in animal models of PD (During et al. 2001, Luo et al. 2002, Lee et al. 2005, Emborg et al. 2007) and the reported improvement of motor scores in a phase I open-label study involving 12 moderately advanced PD patients (Kaplitt et al. 2007), a phase II, double-blind, randomized, sham-controlled trial enrolling 45 advanced PD patients, was conducted (LeWitt et al. 2011). Results revealed a modest but significant improvement in UPDRS motor scores of patients bilaterally infused with STN AAV-GAD. Most common adverse effects reported were nausea, headache and depression. A five year follow up of adeno-associated virus serotype 2 (AAV2) vector encoding for glutamic acid decarboxylase in the subthalamic nucleus (STN AAV2-GAD) treated patients is currently on-going to evaluate long-term effects of AAV-GAD gene transfer and its long-term safety (NCT01301573).

The second gene therapy approach consists in the transfection of dopamine-synthesizing enzymes to induce DA synthesis in the striatum in order to alleviate PD motor symptoms. So far two different enzyme replacement therapies have been tested in clinical trials. In the first open label phase I/II trial conducted in 2007, a lentiviral viral vector containing the genes encoding for the enzymes required for DA biosynthesis (tyrosine hydroxylase/TH, guanosine 5´-triphosphate/GTP cyclohydrolase 1/GCH1 and aromatic amino acid decarboxylase/AADC), under the name of ProSavin, was injected into the striatum to evaluate the safety, efficacy and dosage of ProSavin in 15 mid-to-late stage PD patients for 6 months (NCT00627588). The Oxford biomedical company recently reported good tolerance of ProSavin and patients motor improvement at 6 months relative to baseline, as assessed by UPDRS motor scores (A). Currently the company is planning a multicenter, open label study for a ten year follow-up of patients who were treated with ProSavin to evaluate its long-term safety, tolerability and efficacy for PD treatment (NCT01856439). The

second enzyme replacement therapy tested in two phase I clinical trials consisted in bilateral intraputaminal or intrastriatal delivery of an AAV encoding human AADC (hAADC) gene to induce local conversion of peripheral administered L-DOPA into DA, in which the degree of DA production could be controlled through regulation of L-DOPA dosage (Christine et al. 2009). Although preclinical studies provided in a non-primate model of PD had previously showed that AAV-AADC could induce stable long-term expression of the vector, restore L-DOPA levels, improve motor deficits and reduce L-DOPA side effects in animals' (Bankiewicz et al. 2000, Bankiewicz et al. 2006), both phase I clinical trials reported only modest improvements in UPDRS scores of advanced PD patients, independently of the concentration of vector used. Moreover, in one of the clinical trials (Christine et al. 2009), some patients suffered from aggravated dyskinesias, most likely, due to non-regulated striatal neurons release of DA. In addition, in the latter clinical trial, a decrease of the effective L-DOPA dose was also observed during the course of the trial and several patients suffered hemorrhage during surgery (Christine et al. 2009). Nevertheless, a non-randomized open label trial to evaluate safety and efficacy of AAV2-hAADC injected through MRI-guided administration into the putamen of advanced fluctuating PD patients is currently being planned (NCT01973543). Yet, the future trials must be designed with caution to avoid excessive production of dopamine in the striatum, as GABAergic striatal neurons do not possess vesicular storage capability. This lack of vesicular structures can cause overproduction of extracellular or cytosolic DA which in turn may lead to oxidative-stress (Chen et al. 2008), dyskinesias (Bankiewicz et al. 2000, Bankiewicz et al. 2006) and even to degeneration of striatal neurons (Cyr et al. 2003, Chen et al. 2008).

The third gene therapy approach relies in the principle that neurotrophic factors, like glial cell-derived neurotrophic factor (GDNF), mediate growth, survival and protection of DAergic neurons within the midbrain (Sherer et al. 2006). Neurotrophic factor therapy for PD has gained interest for the past 20 years. Initially, effects of direct infusion of GDNF have been study in both preclinical and clinical studies due to GDNF trophism for the nigral DAergic neurons (Kearns and Gash 1995, Sauer et al. 1995, Grondin et al. 2002, Ai et al. 2003, Grondin et al. 2003). However, two double-blind trials reported no improvement of parkinson motor symptoms following infusion of GDNF, either in the lateral ventricles or in the putamen, with many patients suffering from diverse side effects (Nutt et al. 2003, Lang et al. 2006). Based on these results, the attention has shifted towards neurturin (NTN), a member of the GDNF ligands family that, similarly to GDNF, has shown

to promote survival and growth of midbrain DAergic neurons (Kotzbauer et al. 1996). Recently, the efficacy and safety of an adenovirus vector encoding for human NTN (AAV2-NTN), under the name CERE-120, has been evaluated in two clinical trials. Phase I clinical trial enrolling 12 PD patients demonstrated good toleration of patients to AAV2-NTN and an improvement in "off" medication symptoms (Marks et al. 2008), which led to the conduction of a phase II multicenter, randomized, double-blind, sham-controlled trial involving intraputaminal injection of AAV2-NTN in 58 patients with moderate to severe PD (Marks et al. 2010). However, conversely to phase I trial, results revealed no improvements in UPDRS motor "off" score in AAV2-NTN treated patients when compared with sham group. Thus, contrarily to the results observed in pre-clinical non-human primate studies (Kordower et al. 2006, Herzog et al. 2007, Herzog et al. 2008), post-mortem brain analysis of AAV2-NTN treated patients revealed the expression of NTN mainly in the striatum with minimal presence in the SNpc (Marks et al. 2010). This result has been associated with the lack of retrograde transportation of NTN to the SNpc and to the extensive loss of DAergic neurons in advanced PD patients (Bartus et al. 2011). Therefore, a new phase I/II trial in which AAV2-NTN was injected into the SN and the putamen is presently being conducted in earlier stage patients to evaluate safety and potential beneficial effects of AAV2-NTN over longer periods of time (NCT00985517). So far, phase I has been completed in 6 PD patients with no reported complications.

In spite of the encouraging preclinical data resulted from the different gene therapy approaches, clinical trials based on therapeutic trangenes delivery to basal ganglia neuronal populations failed to confirm the beneficial motor effects observed in animal studies (with the exception of the first approach). However, although genetic approaches remain to prove its long-term efficacy and long-term safety as alternative therapy for the existing symptomatic treatments, recent insights in understanding the genetic causes of PD pathology, together with the development of innovative gene delivery systems, have generated new hope for future gene therapy interventions aiming to slow or halt PD progression.

5. Cell-based therapies

The reports on PD patients increasing motor disabilities over time, related with the chronic use of L-DOPA (Schapira 2005, Smith et al. 2012, Teixeira et al. 2013), and the recognition that cell-

based therapies could be a good strategy to replace lost diseased DAergic neurons along the course of PD, led to the emergence of cell-based approaches to meet the clinical challenge of restoring the degenerated DAergic neural circuitries and provide long-lasting relief of patients symptoms. In this section, characterization, advantages and disadvantages of each of these cell-based therapies will be further explored. A summary of the advantages and disadvantages of different stem cell types for application in PD is provided in table 4.

Table 4: Advantages and disadvantages of different stem cell types for application in PD (bold sentences indicate advantages of MSCs over ES cells and NSCs).

Cell type	Advantages	Disadvantages	References
ES cells	High proliferative pluripotent cell source Retain pluripotency for long periods of *in vitro* expansion Can be differentiated into DAergic neurons ES cells-derived DAergic neurons were shown to: survive, integrate and reinnervate the striatum of the host, thus improving functional recovery of PD symptoms	Risk of tumor formation related with phenotypical instability of the grafts Ethical concerns No data in non-human primate models of PD	(Björklund et al. 2002, Kim et al. 2002, Brederlau et al. 2006, Roy et al. 2006, Salgado et al. 2006, Politis and Lindvall 2012)
Fetal NSCs	Expandable multipotent cell source Can be differentiated into DAergic neurons Midbrain NSCs-derived DAergic neurons were shown to survive, differentiate, migrate and induce funtional recovery in PD animals	Limited differentiation *in vivo* Technical problems related with acquisition of homogenous populations of DAergic neurons Safety issues related with the use of retroviral vectors for differentiating adult NSCs into DAergic neurons Ethical concerns	(Carvey et al. 2001, Sanchez-Pernaute et al. 2001, Sawamoto et al. 2001, Schwarz et al. 2006, Parish et al. 2008, Politis and Lindvall 2012)
iPS cells	Expandable cell source Can be re-programmed into pluripotent stem cells by retrovirus gene transfer Possibility of generating patient specific donor cells for autologous transplantation Less probability for immune rejection No ethical concerns iPS cells-derived DAergic neurons have shown to survive and induce functional benefits in PD animals	Differentiation pattern variability *in vivo* Risk for teratoma formation Autologous transplantation: risk of susceptibility to the original pathology related with possible genetic mutations present in patients fibroblasts No data in non-human primate models of PD	(Wernig et al. 2008, Hargus et al. 2010, Swistowski et al. 2010, Politis and Lindvall 2012)

Table 4: Advantages and disadvantages of different stem cell types for application in PD (bold sentences indicate advantages of MSCs over ES cells and NSCs). (continued)

Cell type	Advantages	Disadvantages	References
MSCs	Expandable multipotent cell source **Can be isolated from different tissue sources** **Obtained with minimal invasive procedures** Easy cultured and expanded in large numbers **Safe source for autologous transplantation** **Possess immunosuppressive function** **Secrete a vast panel of growth factors and cytokines** **Less prone for tumor formation** **Not hindered by ethical concerns** Virtual possibility for differentiation into DAergic neurons MSCs-derived DAergic neurons grafts have shown to survive for long periods of time, increase levels of DAergic markers and to improve animals' motor recovery **Undifferentiated MSCs have shown not only to survive, migrate toward the injured site and to promote both neuroregenerative and neurorestorative effects in PD animals', but also to attenuate animals motor deficits through the secretion a vast panel of growth factors and cyokines**	Full differentiation of MSCs-derived DAergic neurons remains to be proven No data of undifferentiated MSCs transplantation in non-human primate models of PD Transplantation of BMSCs provided only modest clinical improvement in humans	(Salgado et al. 2006, Weiss et al. 2006, Offen et al. 2007, Bouchez et al. 2008, Levy et al. 2008, McCoy et al. 2008, Kim et al. 2009, Sadan et al. 2009, Shetty et al. 2009, Blandini et al. 2010, Cova et al. 2010, Venkataramana et al. 2010, Wang et al. 2010, Kishk and Abokrysha 2011, Park et al. 2012, Hayashi et al. 2013, Teixeira et al. 2013, Wang et al. 2013, Zhou et al. 2013a)

5.1 Fetal ventral mesencephalic tissue

Fetal ventral mesencephalic (FVM) tissue is derived from the fetuses' midbrain (mesenchephalon), the region from which SN DAergic neurons develop (Shamekh et al. 2008). The rationale behind transplantation of fetal cells is that transplantation of healthy DAergic neurons could reinnervate the striatum, replace synapses and restore physiological DA transmission in the brain. In the late 1970s, several grafting studies were conducted using FVM tissue transplanted either in the lateral ventricle adjacent to the caudate (Perlow et al. 1979) or directly into the striatal parenchyma (Björklund et al. 1980b) of 6-hydroxydopamine (6-OHDA) models of PD. These studies showed that transplantation of DAergic tissue or cells induced the recovery of motor functions and that this recovery was associated with graft-derived reinnervation of most of the caudate-putamen. Subsequent studies consistently demonstrated that intrastriatal transplantation of rat fetal SN tissue could reinnervate rat striatum, secrete DA and induce substantial or even complete restoration of animals' motor deficits (Björklund and Stenevi 1979, Björklund et al. 1980a, Freed et al. 1980, Dunnett et al. 1981, Dunnett et al. 1983, Dunnett et al. 1988, Koller 2003, Björklund

and Kordower 2013). The extent of animals' motor recovery largely depended on the extent of nigrostriatal reinnervation and DA restoration following SN transplants. Thus, the observations that animal's behavioral recovery could only be achieved with striatal intraparenchyma grafting led to the large abandon of intraventricular transplantation (Björklund and Kordower 2013). In the late 1980s, the first human open trials took place and revealed quite promising results regarding the long-term survival of DAergic neurons, growth and secretion of DA following human FVM tissue transplantation (Lindvall et al. 1989, Lindvall et al. 1990, Lindvall et al. 1992, Widner et al. 1992, Lindvall et al. 1994, Kordower et al. 1996, Wenning et al. 1997, Hagell et al. 1999, Koller 2003). Motivated by these encouraging results, two National Institutes of Health funded double-blind placebo-controlled trials were conducted in advanced PD patients (Freed et al. 2001, Olanow et al. 2003). However, both studies failed to meet their primary outcome concerning long-term survival of DAergic neurons and some patients developed severe graft-induced dyskinetic side effects postoperatively. In addition, Lewy body degeneration has been observed in patients that have come to autopsy, ten to sixteen years after human FVM tissue transplantation (Kordower et al. 2008, Li et al. 2008, Rascol et al. 2011). Moreover, methodological and ethic related issues associated with human FVM tissue harvesting have also hindered the use of human FVM tissue allografts for PD treatment (Azari et al. 2010, Teixeira et al. 2013). Nevertheless, although different outcomes have been reported following human FVM tissue transplant trials, the reported improvement in striatal DAergic function and in functional outcome in some patients with PD resulted in a currently ongoing phase I European clinical trial (TRANSEURO-NCT01898390). For this trial, an optimized tissue preparation protocol to reduce the graft-induced dyskinesias has been provided. Nevertheless, due to ethical issues the future use of human FVM tissue is still a matter of intense debate.

5.2 Embryonic stem cells

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst and are considered pluripotent cells due to their capability to differentiate into the three germ layers (endoderm, mesoderm, ectoderm) of the embryo (Salgado et al. 2006). ES cells are high proliferative cells, able to maintain their pluripotency for long periods of *in vitro* expansion (Politis and Lindvall 2012). The possibility of obtaining large-scale production of ES cells and to differentiate them into DAergic neurons, led ES cells to be considered for PD treatment (Park et al. 2004, Perrier et al. 2004, Cho et al. 2008). Numerous animal experiments using either rodent or human ES cells-derived

dopaminergic neurons showed that ES cells could induce functional recovery in animal models of PD (Björklund et al. 2002, Kim et al. 2002, Ben-Hur et al. 2004, Brederlau et al. 2006, Yang et al. 2008b). Indeed, Björklund *et al.* and Kim *et al.*, based on histochemical and neuroimaging techniques, demonstrated that striatal grafted ES cells-derived DAergic neurons were able to survive, integrate and reinnervate the striatum, thus improving animals' behavior (Björklund et al. 2002, Kim et al. 2002). In addition, Björklund *et al.* observed that the degree of striatal reinnervation was correlated with animals' behavior improvement, an observation that has also been reported by Yang and colleagues (Yang et al. 2008b). Yet, despite the consistent description of animals' motor improvement following ES cells-derived DAergic neurons grafting, phenotypic instability of the grafts and consequent tumor formation in rats has also been reported (Brederlau et al. 2006, Roy et al. 2006). Therefore, issues related with ES cells inappropriate differentiation into midbrain neurons and safety have severely hampered clinical application of human ES cells. Nevertheless, Studer *et al.* has recently addressed these concerns, which might bring back ES cells for the clinical arena (Kriks et al. 2011). Still, human ES cells translation to clinics is surrounded by controversy related with possible immune rejection of the grafts as well as with safety and ethical issues.

5.3 Neural stem cells

Neural stem/precursor cells (NSCs) are multipotent cells that are capable of differentiating into the main phenotypes of the CNS, namely neurons, astrocytes and oligodendrocytes (Yi et al. 2013). NSCs can be isolated from the developing or adult CNS (Bennett et al. 2009, Meyer et al. 2010) and cultured *in vitro* as clusters of multicellular free-floating spheres (neurospheres) in the presence of endothelium growth factor (EGF) and/or basic fibroblast growth factor (bFGF) (Svendsen et al. 1996, Svendsen et al. 1997). These NSCs properties and the prospect of using NSCs for replacement of lost DAergic neurons and reconstitution of the DAergic transmission in the striatum raised great interest in NSCs as source for cell replacement therapy for PD.

The first studies using undifferentiated embryonic or fetal NSCs isolated from cortical and midbrain areas of rodent developing brain, not only reported poor survival and differentiation of striatal grafted cells into DAergic neurons in animal models of PD, but also only mild amelioration of lesion induction deficits (Svendsen et al. 1996, Svendsen et al. 1997). In addition, these and other studies have consistently reported that only NSCs isolated from the midbrain could differentiate into

DAergic neurons (Svendsen et al. 1996, Svendsen et al. 1997, Sanchez-Pernaute et al. 2001, Storch et al. 2001, Storch et al. 2004, Meyer et al. 2010). Since then, most transplantation studies use either rodent (Carvey et al. 2001, Sawamoto et al. 2001, Schwarz et al. 2006) or human fetal NSCs (Sanchez-Pernaute et al. 2001) isolated from the developing midbrain, differentiated into DAergic neurons *in vitro*, prior to *in vivo* transplantation. These studies showed that striatal transplantation of fetal NSCs-derived DAergic neurons resulted in histological, biochemical and functional recovery in animal models of PD. Moreover, one of these studies reported a more mild immune rejection and lower risk of tumor formation than ES cells (Schwarz et al. 2006). However, despite these encouraging results, a low percentage of the transplanted cells survived and/or adopted the DAergic phenotype *in vivo* after both short- and long-term *in vitro* expansion of NSCs (Carvey et al. 2001, Sawamoto et al. 2001, Schwarz et al. 2006). These results, together with the mild-benefits observed in functional recovery of PD animals' reported in earlier studies, where undifferentiated NSCs that had been used for transplantation (Svendsen et al. 1996, Svendsen et al. 1997), have been associated with NSCs dependence on the use of developmental signals (e.g., fibroblast growth factor 8/FGF8 and Sonic hedghoc) and transcription factors (e.g., nuclear receptor realated-1 protein/Nurr1) (Storch et al. 2004). The latter have been implicated in DAergic neurons development (Ye et al. 1998, Perrone-Capano and Di Porzio 2000, Storch et al. 2004). Moreover they have shown to improve the survival and modulate NSCs differentiation towards the DAergic phenotype, prior to *in vivo* transplantation (Kim et al. 2003a, Kim et al. 2003b, Meyer et al. 2010). More recently, Parish *et al*. took advantage of the lessons learned from these earlier studies and proposed an alternative method to address this issue through the culture of genetically engineered NSCs cells together with developmental signals necessary for inducing DAergic neurons differentiation *in vivo* (Parish et al. 2008). Using this alternative method, Parish and coworkers (Parish et al. 2008) reported the generation of higher yields of functional DAergic neurons *in vitro* along with an enhancement in TH-positive cells engraftment and in striatal reinnervation *in vivo*. In addition, these authors showed that these improvements were accompanied with complete behavior recovery of parkinsonian rodents without tumor formation (Parish et al. 2008). Nevertheless, long-term follow up of the animals' should be provided in order to ensure safety of these engineered cells. Thus, despite the progresses achieved with mesencephalic derived NSCs, the long-term survival and phenotype stability of grafted DAergic neurons in animal models of PD still remains to be demonstrated. Therefore, standardization of protocols ensuring the control of

NSCs differentiation into homogeneous populations of DAergic neurons should be provided and their transplantation effects in parkinsonian models further explored.

Taken together, these studies show that NSCs may provide functional benefits following transplantation in PD animals'. Nevertheless, it is unlikely that NSCs can reach clinical trials in a near future due to the lack of understanding of the signals needed to control NSCs conversion into DAergic neurons and the mechanisms underlying the observed *in vivo* functional benefits, in addition to the ethical concerns, particularly regarding the use of fetal NSCs.

5.4 Induced pluripotent stem cells

Induced pluripotent stem cells (iPS cells) recently emerged in the field of regenerative medicine following the pioneer studies provided by Takahashi and colleagues (Takahashi and Yamanaka 2006, Takahashi et al. 2007) in which the authors showed that fibroblasts derived from mice or humans could be re-programmed into pluripotent stem cells. These studies demonstrated that iPS cells presented morphological and phenotypical properties similar to the ES cells, such as pluripotency and generation of viable chimeras (Takahashi and Yamanaka 2006, Takahashi et al. 2007, Brundin et al. 2010). Subsequent studies showed that iPS cells not only exhibited genomic stability and transcription profiles similar to ES cells, but also that iPS cells are able to use the same transcriptional network and the same developmental patterning cues as ES cells to differentiate into DAergic neurons (Wernig et al. 2008, Brundin et al. 2010, Swistowski et al. 2010, Gibson et al. 2012). Thus, these cells can hold advantages over ES cells, such as the possibility of generating patient specific donor cells for autologous transplantation and, simultaneously, avoid both immune rejection and ethical concerns related with the use of ES cells (Wernig et al. 2008).

Studies on transplantation of iPS cells in the striatum of PD animals', either isolated from mice or PD patients somatic cells, provided proof-of-principle of the iPS cells ability to survive and induce functional benefits in PD animals' (Wernig et al. 2008, Hargus et al. 2010, Swistowski et al. 2010). However, these studies also reported variability on the differentiation pattern of iPS cells into DAergic neurons *in vivo*. Thus, it has been observed that tumor (teratoma) can occur if iPS cells grafts are not fully differentiated prior to transplantation (Wernig et al. 2008). Thus a problem that can emerge upon the use of iPS cells for autologous transplantation is patients' development of the original pathology due to its possible presence in patients' fibroblasts. Therefore, patients' susceptibility to develop the original disease along with risks of tumor formation either related with

incomplete and unsynchronized differentiation of iPS cells and/or with the possible degeneration of iPS cells derived from PD patients after grafting, are important complications that need to be circumvented before iPS cells can be safely applied in PD patients.

5.5 Induced neural cells

In an attempt to solve complications related with incomplete and unsynchronized differentiation of iPS cells observed *in vivo* (Wernig et al. 2008, Hargus et al. 2010) an interesting alternative approach, which consists in directly reprogram one somatic cell type to another, has been proposed five years ago by Melton and colleagues (Zhou et al. 2008). Recently, Vierbuchen *et al.* have reprogrammed fibroblasts into functional neurons *in vitro* using a similar combinatorial strategy of three neural-specific transcription factors (Brundin et al. 2010, Vierbuchen et al. 2010). The resulting cells were called induced neural cells (iN cells). These cells have shown the capability of generate DAergic neurons *in vitro*, while avoiding the reversion of cells to a pluripotent stage, therefore diminishing the risk for tumor formation. However the ability of these iN cells-derived DAergic neurons to induce benefits on PD animals' phenotype remains to be demonstrated (Caiazzo et al. 2011, Pfisterer et al. 2011, Gibson et al. 2012). Thus, a problem that would arise if iN cells could reach clinical trials would be the need of large numbers of patient reprogrammed neurons to obtain enough cells for transplantation. Moreover, similarly to iPS cells the use of iN cells for autologous transplantation could increase patients' susceptibility to the original pathology due to its possible presence in patients' somatic cells. Nevertheless, the use of iN cells is still an emerging field in regenerative medicine.

5.6 Mesenchymal stem cells

Mesenchymal stem/stromal cells (MSCs) research started in 1974 with the first report on the isolation of a population of cells derived from rodent bone marrow (BM) by Friedenstein and co-workers (Friedenstein et al. 1974b). Friedenstein *et al.* defined cells isolated from BM as plastic-adherent fibroblast colony-forming units with clonogenic capacity (Friedenstein et al. 1974a). These cells were named in 1991 as marrow "stromal cells" by Eaves *et al.*, on the basis on the possible use of these cells as a feeder layer for hematopoietic stem cells (Eaves et al. 1991, Glavaski-Joksimovic and Bohn 2013). In the same year, these cells became also known as mesenchymal stem cells on the basis of the report of their clonogenicity capacity and ability to undergo

multilineage differentiation published by Caplan and colleagues (Caplan 1991, Bluguermann et al. 2013). In fact, later on, MSC have been defined according with the International Society for Cellular Therapy (ISCT) criteria as multipotent cells, capable of self-renewal and to differentiate into adipocytes, osteoblasts and chondroblats. (Emsley et al. 2005). Additionally, MSCs have also been characterized by their ability to adhere to tissue culture flasks and to display the presence of cells surface markers (CD105, CD73, CD90), as well as the lack of hematopoietic cell surface markers (CD45, CD34, CD14 or CD11b, CD79a or CD19 and Human Leukocyte Antigen DR) (Dominici et al. 2006). So far, MSCs have been isolated from bone marrow (BMSCs), adipose tissue (ASCs), dental pulp, placenta, amniotic fluid, umbilical cord blood, umbilical cord Wharton´s jelly (WJ-MSCs), liver, lung and spleen (Teixeira et al. 2013). MSCs can be isolated with minimal invasive procedures; easily cultured and expanded *in vitro* for several passages; can be used for autologous transplantation in virtue of their hypoimmunogenicity (probably related with their surface expression of major histocompatibility complex antigens); have less probability of being tumorogenic and, as adult cells, are not hindered by ethical concerns (Salgado et al. 2006, Kishk and Abokrysha 2011, Seo and Cho 2012, Teixeira et al. 2013). These MSCs features have made them attractive tools for CNS neurodegenerative diseases. In the particular case of PD, these MSCs characteristics along with the focused loss of DAergic neurons and the recognition that MSCs could hold several advantages over embryonic or fetal neural stem cells (Table 4), made of them an appealing alternative for PD therapy. Indeed a considerable body of evidence has revealed the potential of MSCs to promote protection and/or recovery of DAergic neurons against neurotoxin-induced nigrostriatal degeneration following intrastriatal (Weiss et al. 2006, Bouchez et al. 2008, McCoy et al. 2008, Sadan et al. 2009, Blandini et al. 2010, Cova et al. 2010, Khoo et al. 2011) intranigral (Somoza et al. 2010, Mathieu et al. 2012), intratechal (Salama et al. 2012) intravenously (Chao et al. 2009, Wang et al. 2010) and intranasal (Danielyan et al. 2011) delivery of BMSCs, ASCs or WJ-MSCs in rodent or non-human primate models of PD. The mechanisms underlying *in vivo* functional recovery following MSCs transplantation are, however, a matter of an intense debate.

In line with the strategy followed for transplantation of FVM tissue and embryonic or neural stem cells, together with reports on the ability of MSCs to differentiate into neuronal lineages (Kopen et al. 1999, Brazelton et al. 2000, Jiang et al. 2002, Mitchell et al. 2003, Hermann et al. 2004, Munoz-Elias et al. 2004, Phinney and Prockop 2007, Baer and Geiger 2012), several groups continue to focus on the use of MSCs as replacers of injured DAergic neurons, using neuronally-

induced MSCs prior to transplantation in PD animal models. Indeed, several authors have reported that BMSCs-, ASCs- and WJ-MSCs-derived DAergic neurons transplantation into the striatum of both rodent and primate models of PD could survive for long periods, increase levels of DAergic markers (e.g., TH) and also improve animals' motor recovery (Offen et al. 2007, Levy et al. 2008, Shetty et al. 2009, Hayashi et al. 2013, Wang et al. 2013, Zhou et al. 2013a). In addition, two of these studies have also reported detectable levels of DA in culture medium following *in vitro* BMSCs differentiation into DAergic phenotype or DA release after depolarization by potassium stimulation (Shetty et al. 2009, Hayashi et al. 2013). In a recent study, similar outcomes regarding the increase TH expression levels and animals' motor recovery were also reported after intranigral transplantation of undifferentiated WJ-MSCs and WJ-derived DAergic neurons in a 6-OHDA rodent model of PD (Shetty et al. 2013). In this study, the authors further compared the effect of either naïve or differentiated BMSCs and WJ-MSCs in parkinsonian animals'. Results revealed that, although the differentiated WJ-MSCs further accelerated animals' motor improvement, both naïve and differentiated WJ-MSCs were able to significantly promote animals' motor recovery when compared with non-transplanted animals'. However, contrarily to the observed in the previous study, Bouchez *et al.*, reported similar beneficial effects on animals' behavioral recovery after intrastriatal transplantation of either BMSCs cultured in standard conditions or in neuronal differentiation medium in a 6-OHDA rat model of PD (Bouchez et al. 2008). McCoy *et al.* also showed that ASCs were able to protect DAergic neurons and ameliorate animals' functional deficits against neurotoxin-induced neurodegeneration without the need of DAergic differentiation (McCoy et al. 2008). Moreover, similarly to Bouchez *et al.* and McCoy *et al.*, other researchers have reported no (Chao et al. 2009, Khoo et al. 2011) *in vivo* differentiation of *in vitro* neural-induced MSCs after intrastriatal or intravenous transplantation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-OHDA rodent models of PD. Thus, functional data failed to provide robust evidence regarding MSCs differentiation into full functional neurons (Trzaska et al. 2007, Thomas et al. 2011, Liu et al. 2012). Therefore, it seems unlikely that MSCs differentiation into neuronal lineages may be the major contributor for MSCs-induced recovery in PD.

In recent years, MSCs-induced regenerative effects have been related with the MSCs secretome, that is, with the panel of bioactive soluble factors with neuroregulatory properties, released by these cells to the extracellular environment (Teixeira et al. 2013). Indeed, different tissue derived MSCs not only seem to sense the local environment, but have also shown to respond to signals that are

up-regulated under injury conditions by migrating to the damage site. Once there, they promote cell regeneration and limit the extent of tissue damage through the secretion of soluble growth factors (GFs), anti-inflammatory cytokines and microvesicles/exosomes (Li et al. 2002, Neuhuber et al. 2005, Caplan and Dennis 2006, Ding et al. 2007, Yang et al. 2008a, Meirelles Lda et al. 2009). The effect of such soluble factors can be generally classified into neuroprotective/anti-apoptotic, neurogenic, angiogenic, synaptogenic and scarring inhibitors. Such effects are commonly mediated by the secretion of the following factors by MSCs (Chen et al. 2000, Chen et al. 2001a, Chen et al. 2001b, Li et al. 2002, Neuhuber et al. 2005, Ding et al. 2007, Wright et al. 2007, Wei et al. 2009, Hu et al. 2010, Wakabayashi et al. 2010, Lin et al. 2011, Lopatina et al. 2011): brain-derived neurotrophic factor (BDNF), GDNF, nerve growth factor (NGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), VEGF-receptor 3 (VEGF-R3), angiopoietin 1, insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), EGF, bFGF, fibroblast growth factor 20 (FGF 20), granulocyte colony-stimulating factor (G-CSF), platelet-derived growth factor AA (PDGF-AA), chemokine ligand 16 (CXCL 16), neutrophil-activating-protein-2 (NAP 2) and neurotrophin-3 (NT-3) growth factors, as well as interleukin-6 (IL-6), interleukin-10 (IL-10), transforming growth factor beta 1 (TGF β1), stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1) and monocyte chemotactic protein 1 (MCP-1) cytokines. In the context of PD and regarding BMSCs, several studies have also demonstrated that BMSCs secretome protect and/or regenerate DAergic neurons in *in vitro* and *in vivo* models of PD, through the secretion of growth factors and cytokines (Weiss et al. 2006, Shintani et al. 2007, McCoy et al. 2008, Kim et al. 2009, Sadan et al. 2009, Blandini et al. 2010, Cova et al. 2010, Wang et al. 2010, Danielyan et al. 2011, Park et al. 2012). For instance, Shintani and coworkers demonstrated that BMSCs conditioned media (CM) was able to promote survival of TH-positive DAergic neurons in rat primary cultures of ventral mesencephalic cells (Shintani et al. 2007). Moreover, intrastriatal transplantation of fetal mesenchephalic cells treated with human BMSCs CM, during steps of donor preparation and implantation, induced survival of DAergic grafted cells and promoted functional recovery in a 6-OHDA rat model of PD (Shintani et al. 2007). The observed protection of DAergic neurons was attributed to BMSCs secretion of BDNF, GDNF and bFGF, all of which had previously shown to stimulate survival of DAergic neurons (Hyman et al. 1991, Lin et al. 1993, Mayer et al. 1993, Hyman et al. 1994, Björklund et al. 1997, Shintani et al. 2007). Similarly, Sadan *et al.* showed that human BMSCs (hBMSCs) cultured in the presence of growth factors, not only significantly increased the viability of the SH-SY5Y neuroblastoma cell line exposed to 6-OHDA, but also that

BMSCs transplanted into the striatum of a 6-OHDA rat model of PD, migrated to the lesion site, increased the numbers of TH-positive cells and DA levels (Sadan et al. 2009). These neuroprotective and neuro-regenerative effects were accompanied by an improvement in animals' behavioral impairments and were correlated with BMSCs secretion of significant amounts of BDNF and GDNF. Likewise, this expression pattern is in accordance with data published by Blandini and co-workers using the same animal model (Blandini et al. 2010). On other hand, Wang and colleagues associated rat-derived BMSCs expression of SDF-1α with the DAergic neurons protection against 6-OHDA neurotoxin, both *in vitro* and *in vivo*, through anti-apoptotic based mechanisms (Wang et al. 2010). Moreover, Cova *et al*. demonstrated that hBMSCs transplanted in the striatum of a 6-OHDA rodent model of PD were able to survive and interact with the lesion site surroundings, thus enhancing the survival of DAergic terminals and neurogenesis in the SVZ in a sustained manner (Cova et al. 2010). Importantly, BMSCs *in vitro* secretion of neurogenic (EGF, NT-3, BDNF), neurodevelopmental, neurorescuing and lesion home-mediating GFs (VEGF, HGF, bFGF), along with the active secretion of BDNF *in vivo*, were correlated with the activation of endogenous stem cells and striatal/nigral DAergic protection against neurodegeneration induced by 6-OHDA (Cova et al. 2010). Similarly, Park *et al*., using an MPTP mice model of PD, reported that BMSCs were able to modulate neurogenesis through the secretion of EGF (Park et al. 2012).

In addition to the capability of BMSCs to induce survival and restorative effects on the DAergic neurons, the BMSCs-induced neuroprotection effects on DAergic neurons has also been related with the BMSCs immunomodulatory and anti-inflammatory properties. In this context, Danielyan and coworkers recently showed that intranasally delivered rat BMSCs into 6-OHDA exposed rats, migrated toward the SN and the striatum, engrafted, survived for long periods of time, counteracted the loss of nigral DAergic neurons and striatal fibers, and prevented the decrease of DA in the lesioned brain areas (Danielyan et al. 2011). Moreover, a substantial improvement of animals' motor function was also observed. These neuroprotective effects and functional recovery of the DAergic system have been associated with the increase of BDNF levels in the lesioned hemisphere side as well as with the BMSCs capacity to modulate the host immune response and exert a strong anti-inflammatory activity. Indeed MSCs are known to modulate the response of inflammatory cells, decreasing the expression of pro-inflammatory cytokines, such as IL-1β (interleukin 1β), IL-2 (interleukin 2); IL-12 (interleukin 12); TNF-α (tumor necrosis factor alpha) and INF γ (interferon γ), in the lesioned brain halves (Meirelles Lda et al. 2009, Danielyan et al. 2011). Moreover, in yet

another study hBMSCs secretion of IL-6, IL-10 and TGF-β anti-inflammatory cytokines was related with protection of nigral DAergic neurons (Kim et al. 2009).

Although ASCs and WJ-MSCs application in PD still remain largely unexplored, some authors have already demonstrated that ASCs and WJ-MSCs secretome not only can induce DAergic neurons survival and protection in 6-OHDA models of PD, but also an improvement in animals' motor impairments (Weiss et al. 2006, McCoy et al. 2008). For instance, Weiss *et al.* showed that WJ-MSCs transplantation in a 6-OHDA model of PD could increase the numbers of TH-positive cells recovered in the midbrain and ameliorate PD animal behavior through the secretion of GDNF and FGF 20 (Weiss et al. 2006). Using the same animal model, McCoy *et al.* also demonstrated that intranigral transplantation of ASCs increased both the survival and protection of DAergic neurons in the lesioned area and ameliorated animals' motor deficits through the secretion of NGF, BDNF and GDNF (McCoy et al. 2008). Moreover, McCoy and colleagues also observed that ASCs attenuated microglial activation in the lesioned SNpc and suggested that this ASCs capacity to modulate microglial activity could be related with ASCs secretion of anti-inflamatory molecular mediators (McCoy et al. 2008).

From the above referred studies, it is clear that there is increasing evidence indicating that the neuroprotective and neuroregenerative effects of MSCs observed in PD are attributed to the secretion of soluble GFs and cytokines. The MSCs secretion of these factors not only protect DAergic neurons from further degeneration and enhance endogenous restorative processes (e.g., neurogenesis), but also act as inflammation and immune response modulators. Moreover, recent reports have shown that besides soluble GFs and cytokines, MSCs also secrete microvesicles and exosomes containing mRNA (messenger ribonucleic acid) or miRNA (microRNA), which are believed to mediate cell-to-cell communication and act as reparative agents (Baglio et al. 2012). Indeed, Xin *et al.* has already demonstrated *in vitro* that exosomes secreted by BMSCs not only mediate communication with neurons and astrocytes, but also that secreted exosomes may regulate neurite outgrowth by transfer of miRNA (miR-133b) to neural cells (Xin et al. 2012). Moreover, administration of either umbilical cord- or bone marrow-derived MSCs secreted vesicles have also showed to improve recovery from kidney or lung acute injuries (Zhou et al. 2013b, Zhu et al. 2013). But, can exosomes mediate the same regenerative effects in PD as the soluble fraction of MSCs secretome? The answer for this question remains to be unveiled. Therefore, more data

needs to be obtained on the MSCs therapeutic effects in non-human primate models and on the functional roles and specific effects of both the soluble and the vesicular fraction of MSCs in neuroprotection and neuroregeneration, so the true potential of MSCs and their secretome for novel PD therapeutic approaches can be unveiled.

6. Conclusions

PD is the second most prevalent neurodegenerative disorder, characterized by a variety of motor and non-motor features. From the motor point of view, PD cardinal features include bradykinesia, resting tremor, rigidity, and postural instability (Koller 2003, Jankovic 2008, Massano and Bhatia 2012). Once there are no definitive diagnostic tests for this disease and PD may often be confounded with other parkinsonian disorders (e.g., essential tremor, multiple system atrophy and progressive supranuclear palsy), clinicians require thorough knowledge of PD clinical manifestations to differentiate them from other conditions (Steele 1972, Stephen and Williamson 1984, Findley and Koller 1987, Golbe et al. 1988, Koller 2003, Tolosa et al. 2006, Jankovic 2008). In this sense, one of the future challenges to overcome in the future is the discovery of biomarkers that could not only allow clinicians to differentiate PD from other neurodegenerative conditions, but also to initiate neuroprotective therapy at an asymptomatic stage.

Current clinical therapeutic approaches focus on alleviating patients' motor symptoms with L-DOPA. With the significant advances made in the knowledge of the aetiology, pathobiology and pathoanatomy of PD, as well as the reports on PD patients manifestation of undesirable motor side effects along with non-total recovery of disease symptomatology upon chronic use of L-DOPA (Schapira 2005, Smith et al. 2012, Teixeira et al. 2013), new pharmacologic agents, gene engineering and cell replacement therapeutic approaches have arisen to meet the clinical challenge of treating or modify the course of PD. However, gene therapy approaches have shown to promote only mild beneficial effects in alleviating motor deficits in clinical trials (Christine et al. 2009, LeWitt et al. 2011). On other hand, transplantation of FVM has provided proof-of-evidence that cell-based transplantation techniques are promising candidates for PD treatment (Lindvall et al. 1989, Lindvall et al. 1990, Lindvall et al. 1992, Widner et al. 1992, Lindvall et al. 1994, Kordower et al. 1996, Wenning et al. 1997, Hagell et al. 1999). However, most of the cell-based therapies developed so far, namely stem-cell based therapies, aiming at replacing lost DAergic neurons by inducing differentiation of embryonic or fetal neural stem cells into the DAergic phenotype, are severely

hindered by limited or inappropriate differentiation into DAergic neurons *in vivo* as well as by safety and ethical concerns (Carvey et al. 2001, Sawamoto et al. 2001, Schwarz et al. 2006, Jensen et al. 2008). Thus, to date, none of the developed pharmacologic agents, gene engineering and cell replacement therapeutic approaches has been shown to slow or retard PD progression in clinical trials.

In recent years MSCs have been considered to overcome limitations related with ethical issues associated with ES cells and stem cell of fetal origin. In fact, they have already demonstrated to be a safe source for transplantation in PD patients (Venkataramana et al. 2010). Moreover, different tissue derived MSCs transplantation in rodent models of PD have revealed promising results in providing not only neuroprotection and/or neurorecovery, but also in promoting relief of PD motor symptomatology (Bouchez et al. 2008, McCoy et al. 2008, Mathieu et al. 2012, Salama et al. 2012). Indeed, MSCs have already been shown not only to mediate DAergic neurons survival/protection and induce endogenous restorative processes through the secretion of variety of neurotrophic factors, but also to modulate immune response through the secretion of anti-inflammatory cytokines (Weiss et al. 2006, Shintani et al. 2007, McCoy et al. 2008, Kim et al. 2009, Sadan et al. 2009, Blandini et al. 2010, Cova et al. 2010, Wang et al. 2010, Danielyan et al. 2011, Park et al. 2012). However, despite these encouraging results, there are still some challenges ahead that have to be addressed before MSCs can be considered for clinical trials. For instance, there is a clear need to fully characterize the secretome of different tissue derived MSCs in order to identify all the neuroregulatory molecules released by MSCs and to develop protocols to detect their presence *in vivo*. By doing so, one could clarify the specific effects of each of these molecules on different cell processes that can be responsible by the neural survival, neurorecovery or neuroprotection as well as immune response modulation observed phenomena upon transplantation of these cells in rodent models of PD. Similarly, in face of the recent demonstration that besides the soluble soluble GFs and cytokines, MSCs also secrete vesicles, which are believed to mediate cell-to-cell communication and act as reparative agents, the vesicular fraction of the secretome should also be characterized (Baglio et al. 2012, Xin et al. 2012, Zhou et al. 2013b, Zhu et al. 2013). Thus, the potential role of these vesicles to mediate the same cell processes that have been reported to be mediated by the soluble fraction of MSCs secretome should also be addressed in animal models of PD. On other hand, taking into account that MSCs isolated from different tissues have been suggested to exhibited different secretome compositions (Ribeiro et al. 2012), comparative studies on the secretome of different tissue derived MSCs (e.g., BMSCs, ASCs,

WJ-MSCs/HUCPVCs) should also be provided in both rodent and non-human primate PD models. These studies would elucidate about the potential differential therapeutic specificity of different tissue derived MSCs secretome towards PD. By meeting these challenges, it would be possible to clarify whether MSCs secretome can be considered an efficacious disease modifying tool for PD disease treatment and, if so, which of the current MSCs sources could have higher therapeutic specificity toward PD.

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Chapter 2

The Secretome of Bone Marrow and Wharton Jelly Derived Mesenchymal Stem Cells Induces Differentiation and Neurite Outgrowth in SH-SY5Y Cells (Stem Cells International, 2014:438352)

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Research Article

The Secretome of Bone Marrow and Wharton Jelly Derived Mesenchymal Stem Cells Induces Differentiation and Neurite Outgrowth in SH-SY5Y Cells

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The goal of this study was to determine and compare the effects of the secretome of mesenchymal stem cells (MSCs) isolated from human bone-marrow (BMSCs) and the Wharton jelly surrounding the vein and arteries of the umbilical cord (human umbilical cord perivascular cells (HUCPVCs)) on the survival and differentiation of a human neuroblastoma cell line (SH-SY5Y). For this purpose, SH-SY5Y cells were differentiated with conditioned media (CM) from the MSCs populations referred above. Retinoic acid cultured cells were used as control for neuronal differentiated SH-SY5Y cells. SH-SY5Y cells viability assessment revealed that the secretome of BMSCs and HUCPVCs, in the form of CM, was able to induce their survival. Moreover, immunocytochemical experiments showed that CM from both MSCs was capable of inducing neuronal differentiation of SH-SY5Y cells. Finally, neurite lengths assessment and quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis demonstrated that CM from BMSCs and HUCPVCs differently induced neurite outgrowth and mRNA levels of neuronal markers exhibited by SH-SY5Y cells. Overall, our results show that the secretome of both BMSCs and HUCPVCs was capable of supporting SH-SY5Y cells survival and promoting their differentiation towards a neuronal phenotype.

1. Introduction

Central nervous system (CNS) neurological disorders/injuries often pose a major challenge for treatment due to the limited capability of CNS to self-renew and to regenerate [1]. These CNS features have prompted the search for new therapies, such as those using mesenchymal stem cells (MSCs). MSCs have been defined as multipotent cells which are capable of self-renewal [2]. Additionally, they are known to adhere to tissue culture flasks and to display the presence of MSCs surface markers (CD105, CD73, and CD90), as well as the lack of hematopoietic MSCs cell surface markers (CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen DR) [2, 3]. Current sources of MSCs include bone marrow, adipose tissue, dental pulp, placenta, amniotic fluid, umbilical cord blood, umbilical cord Wharton's jelly, liver, lung, and spleen [3, 4].

MSCs isolated from different sources have been proposed for CNS related applications. Indeed, MSCs transplantation has shown to have a therapeutic effect in animal models of ischemia [5, 6], spinal cord injury (SCI) [7, 8], and Parkinson's disease (PD) [9, 10]. The underlying mechanisms by which the MSCs transplantation mediates the beneficial outcomes remain to be elucidated. Although the putative MSCs differentiation into neuronal lineages has been purposed as the major contributor for CNS regeneration in animal models of neurodegenerative diseases [11–15], MSCs differentiation into full functional neuronal lineages remains to be clarified [16–18]. In contrast, robust data indicates that CNS tissue restorative effects are mediated by MSCs secretome, that is, the panel of bioactive factors and vesicles, with neuroregulatory properties, released by these cells to the extracellular environment [10, 19–42].

For instance, we have demonstrated that human BMSCs secretome promotes cell survival and increases cell viability of rat postnatal hippocampal neurons and cortical glial cells [19]. Nakano et al. also showed that the secretome of BMSCs cultured in the supernatant of ischemic brain extracts was able to increase neuronal survival and neurite outgrowth of postnatal rat hippocampal neurons, through apoptosis suppression mechanisms [20]. These findings were correlated with the expression and secretion of IGF-1 (insulin-like growth factor 1), HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), and TGF β1 (transforming growth factor beta 1) by the BMSCs [20]. This was further confirmed by other studies in which, upon being cultured with extracts from ischemic and traumatic brain, BMSCs altered its gene expression profile when compared with uninjured control brain extracts [21, 22]. Moreover, significant improvements in functional recovery were also described in *in vivo* models of ischemia, upon intravenous injection of BMSCs [23–25]. In these studies, improvements in neurologic function were accompanied by a reduction of infarct size and/or with an increase in endogenous cell proliferation and a reduction of apoptosis. These neuroprotective and neurorecovery effects have thus been attributed to BMSCs secretion of interleukin-6 (IL-6) neurotrophic and anti-inflammatory cytokine as well as of growth factors (GFs) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), VEGF, TGF β1, IGF-1, insulin-like growth factor 2 (IGF 2), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF).

Similar findings were also reported in *in vitro* [26–29] and *in vivo* [30–32] models of spinal cord injury. For instance, Führman et al. [28] and Gu et al. [29] reported that coculture of BMSCs with dorsal root ganglia (DRG) explants and neurons significantly enhanced neuronal cell survival and neurite outgrowth, through the secretion of NGF, BDNF, bFGF, and CNTF (ciliary neurotrophic factor), HGF, SDF-1 (stromal cell-derived factor 1), VEGF, EGF, NT-3 (neurotrophin-3), and NT-4 (neurotrophin 4) GFs, as well as IL-1 (interleukin-1), IL-6, and IL-8 (interleukin-8) cytokines. This expression pattern is in accordance with data published by others upon BMSCs transplantation in animal models of SCI [30–32]. On the other hand, several authors have also reported that BMSCs expression of BDNF, GDNF, EGF, bFGF, VEGF, HGF, SDF-1, and NT-3 could be correlated with dopaminergic (DAergic) neurons protection against 6-hydroxydopamine (6-OHDA) neurotoxin both in *in vitro* and *in vivo* models of PD [33–35].

Similarly, the secretome of MSCs isolated from the Wharton's jelly of the umbilical cord (WJ-MSCs) also disclosed some interesting properties for CNS regenerative medicine. For instance, Ribeiro et al. [36] and Fraga et al. [37] revealed that the secretome of mesenchymal progenitors isolated from the Wharton's jelly of the umbilical cord increased neuronal cell viability and cell densities. These effects were attributed to the expression of NGF and the vesicular fraction of the secretome, respectively, which contained proteins typically involved in neuroprotection. Several studies also revealed that the expression of neuroprotective, neurogenic, and angiogenic GFs as well as of growth-associated cytokines, like BDNF, GDNF, bFGF, G-CSF (granulocyte colony-stimulating factor), SDF-1, PDGF-AA (platelet-derived growth factor AA), angiopoietin-2, VEGF receptor 3 (VEGF-R3), CXCL-16 (chemokine ligand 16), and NAP-2 (neutrophil-activating protein-2), could be correlated with WJ-MSCs beneficial outcomes towards ischemic stroke in rats [38–40]. On the other hand, Yang et al. [41] and Hu et al. [42] linked the improvement of locomotor function, the neuroprotection, and the axon regeneration in a rat SCI model with the WJ-MSCs secretion of NT-3, GDNF, bFGF, VEGF-R3, NAP-52 (neutrophil-activating protein-52), and GITR (glucocorticoid-induced tumor necrosis factor receptor). Finally, Weiss et al. [10] showed that WJ-MSCs transplantation could also ameliorate the condition of a hemi-Parkinsonian rat model through the secretion of GDNF and FGF 20 (fibroblast growth factor 20) DAergic trophic factors.

Despite all these studies, there are few reports where the effects of the secretome of MSCs, isolated from different sources, on neuronal cell populations are directly compared. Therefore, in the present study we aimed to determine to which extent the secretome of MSCs isolated from the bone marrow and the connective tissue surrounding umbilical cord vessels affected the survival and differentiation of a human neuroblastoma cell line. Our results show that the secretome of BMSCs and HUCPVCs, in the form of CM, is able *per se* to induce SH-SY5Y cells survival, differentiation into neuron-like cells, and neurite outgrowth. Moreover, the secretome of BMSCs and HUCPVCs, collected at different time points, was capable of increasing SH-SY5Y neuronal differentiation at the same extent as the retinoic acid (RA), which is commonly used to differentiate SH-SY5Y cells [43]. Finally, CM from BMSCs and HUCPVCs displayed different temporal profiles regarding stimulation of neurite outgrowth and the gene expression of neuronal markers exhibited by SH-SY5Y cells.

2. Materials and Methods

2.1. Cell Culture

2.1.1. Bone Marrow Tissue Derived Stem Cells. BMSCs were acquired from PROMOCELL (Heidelberg, Germany). Cells were thawed and expanded according to the protocol previously described by Silva et al. [44]. Briefly, BMSCs were cultured in α-MEM (GIBCO, Grand Island, NY, USA) supplemented with sodium bicarbonate ($NaHCO_3$, MERCK, USA), 10% of fetal bovine serum (FBS, BIOCHROM AG, UK), and 1% of penicillin-streptomycin antibiotic (GIBCO). Confluent cells were trypsinised, plated in new T75 tissue culture flasks (NUNC, Denmark), at a density of 4.000 cells/cm^2, and incubated at 37°C in a 5% humidified CO_2 atmosphere. The culture medium was changed every two to three days. BMSCs were used for experiments during passage 6 (P6).

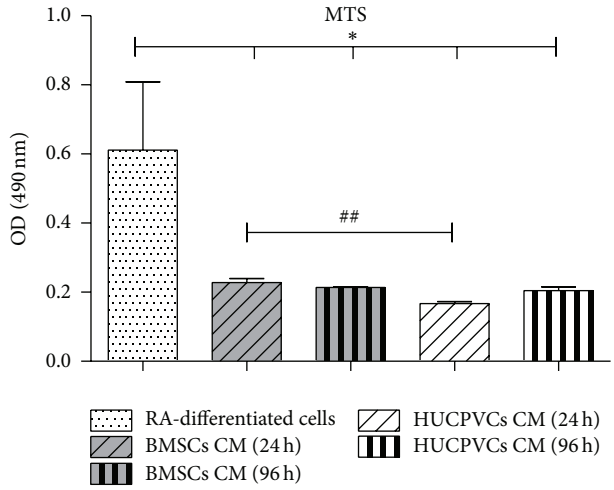

Figure 1: Metabolic viability (MTS test) of SH-SY5Y cells seven days after incubation with BMSCs and HUCPVCs CM. Results revealed that, for all CM tested conditions, the secretome of both MSCs populations is able to support neuronal-like cell viability without the use of any other exogenous growth factors. The differences observed towards RA-differentiated cells ($P < 0.05$) are considered to be natural as these cultures were supplemented with 1% of FBS. Concerning statistical differences among time points, BMSCs CM 24 h promoted a significant increase in SH-SY5Y cells viability when compared with the HUCPVCs CM 24 h group ($P < 0.01$) (values are shown as mean ± SEM, $n = 3$). Symbols correspondence to statistical signification: (1) $*$ refers to comparisons between RA-differentiated cells and MSCs CM; (2) # regards the correlation between MSCs CM from the same time point ($^{\#\#}P < 0.01$, $^{*}P < 0.05$).

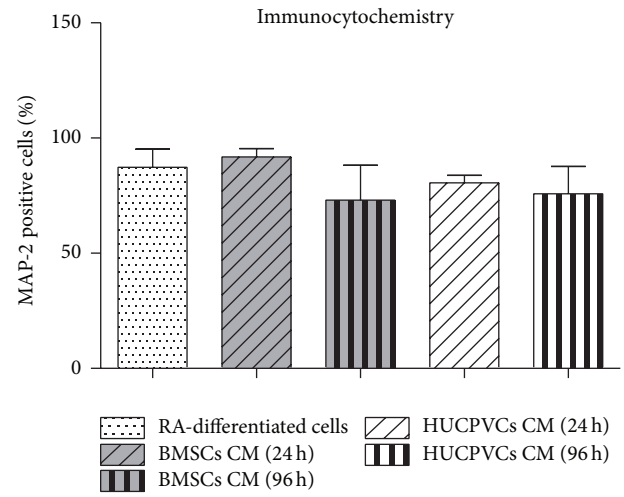

Figure 2: Cell densities for MAP-2 positive cells presenting neurites seven days after incubation with BMSCs and HUCPVCs CM. Immunocytochemistry assessment (using Cell-P software and 20x magnification micrographs) revealed that all CM tested conditions presented similar percentages of MAP-2 positive cells when compared to RA-differentiated cells, the positive control for SH-SY5Y differentiation ($P > 0.05$). The later effect was more noticeable for the BMSCs CM 24 h. Therefore, these results show that the secretome of both BMSCs and HUCPVCs is capable of inducing SH-SY5Y cells differentiation into neuronal-like cells (values are shown as mean ± SEM, $n = 5$, and statistical significance was defined as $P < 0.05$).

2.1.2. Human Umbilical Cord Perivascular Cells. HUCPVCs were kindly provided by Professor J. E. Davies (University of Toronto, Canada). Cells isolation from umbilical cord was performed according to the procedures described by Sarugaser and coworkers [45]. Expansion of cells was performed according to the protocol described above for BMSCs. HUCPVCs were used for experiments during P6.

2.1.3. Human Neuroblastoma Cell Line. SH-SY5Y cells were cultured following the methods previously published by Lopes et al. [46]. Briefly, cells were thawed and grown in T75 flasks (NUNC) containing Dulbecco's modified Eagles medium nutrient mixture F12 (DMEM/F-12, PAA, LABCLINICS, M, Spain), to which were added 1% of glutamax (GIBCO), 10% of FBS (BIOCHROM AG), and 1% of kanamycin sulfate (GIBCO). Confluent cells were trypsinized and plated at a density of 42.105 cells/cm^2 in 13 mm glass coverslips, double precoated with both poly-D-lysine (SIGMA-ALDRICH, St. Louis, MO, USA) and pig skin gelatin (SIGMA-ALDRICH), inserted in 24-well plates (NUNC) for cell metabolic viability, immunocytochemical, and neurite outgrowth assays. For analysis of SH-SY5Y cells gene expression regarding several neuronal markers, cells were plated in 6-well plates (NUNC) at a density of 42.105 cells/cm^2. Afterwards, cells were incubated with the same medium described above in a 5% humidified CO_2 atmosphere at 37°C for 24 h, after which media were changed and the experiments were performed as described below. SH-SY5Y cells were used for experiments between passages 11 and 15.

2.1.4. Conditioned Media Collection and Experiments. CM was collected from P6 BMSCs and HUCPVCs as previously reported by Fraga et al. [37]. Shortly, cells were plated at a density of 4.000 cells/cm^2 and allowed to grow for 3 days in a 5% humidified CO_2 atmosphere at 37°C. Culture medium was then renewed and collected 24 h and 96 h thereafter (cell culture was not renewed or added during this time period). Collected CM were frozen and thawed only in the day of experiments. For CM collection, DMEM/F-12 media supplemented with 1% of glutamax and 1% of kanamycin sulfate were used.

For differentiation assays, SH-SY5Y cells were incubated with BMSCs or HUCPVCs CM and respective positive control for neuronal differentiation (SH-SY5Y cells were cultured with DMEM/F-12 (PAA, LABCLINICS) supplemented with 1% of glutamax (GIBCO), 1% of FBS (BIOCHROM AG), 1% of kanamycin sulfate (GIBCO), and 10 μM of RA (SIGMA-ALDRICH)). An additional group, SH-SY5Y proliferative/undifferentiated cells, was also carried out (see Supplementary Data in Supplementary Material available online at http://dx.doi.org/10.1155/2014/438352). The culture medium was changed every day for 7 days, in the end of which cell metabolic viability, differentiation, and neurite outgrowth were assessed in parallel with all other experimental conditions.

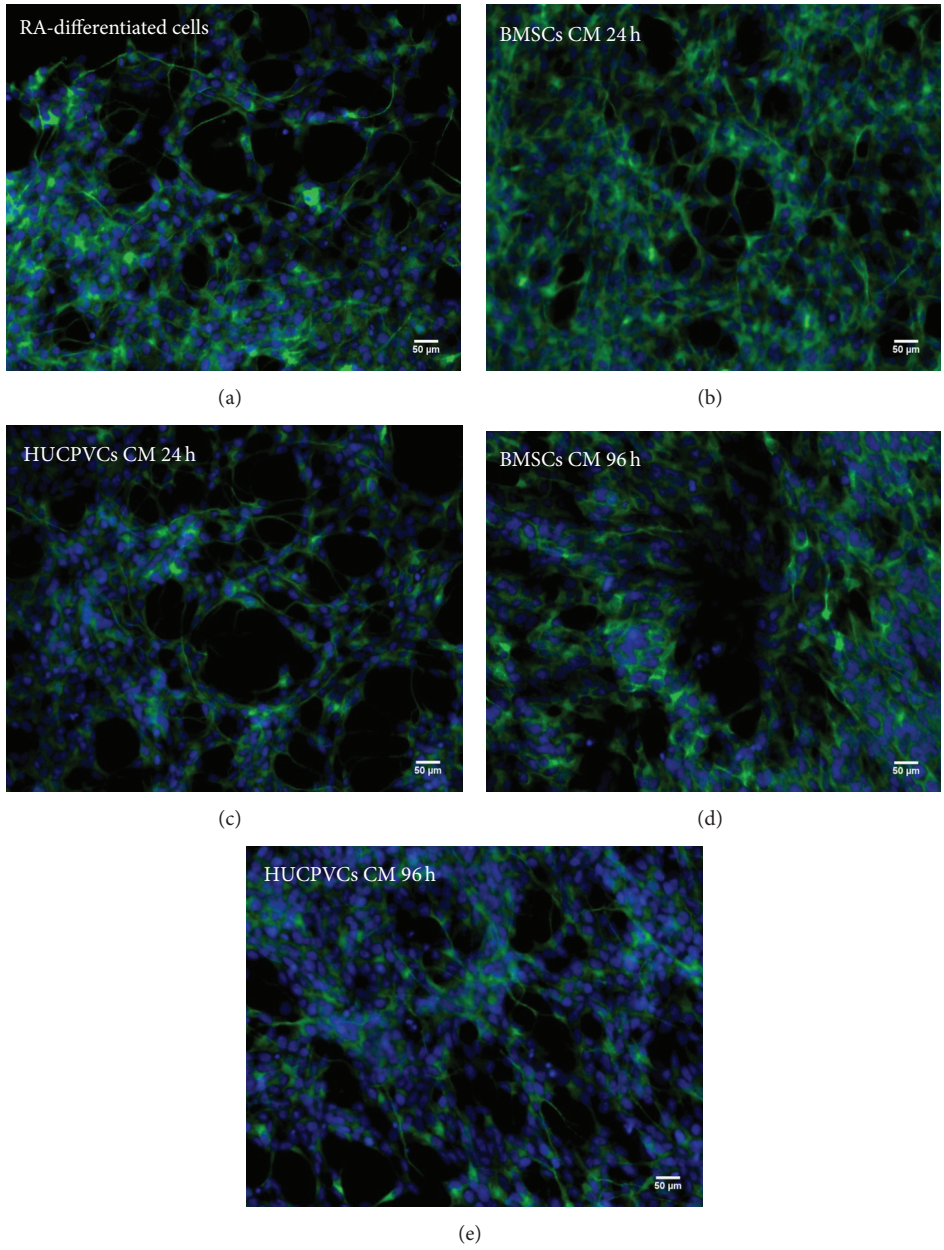

Figure 3: Fluorescence microscopy micrographs of SH-SY5Y cells immunostained with MAP-2 seven days after incubation with RA (a), BMSCs CM (24 h, 96 h: (b), (d)), and HUCPVCs CM (24 h, 96 h: (c), (e)). As it can be observed, the secretome of both BMSCs and HUCPVCs was able to induce SH-SY5Y cells differentiation into neuronal-like cells.

2.2. Cell Viability Assessment. Cell metabolic viability was assessed by the MTS test. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] test (PROMEGA, Madison, WI, USA) is a cell viability assay based on the bioreduction of the substrate (MTS) to a brown formazan product. Cell culture coverslips ($n = 3$ replicates) were set in culture medium containing MTS in a 5 : 1 ratio and incubated at 37°C with 5% humidified CO_2 atmosphere. Three hours after incubation, 100 μL of each sample was transferred to 96-well plates ($n = 3$ replicates) and optical density (OD) was measured at a 490 nm.

2.3. Immunocytochemistry. *Cells were cultured in double pre-coated coverslips ($n = 5$)*, fixed with 4% paraformaldehyde (MERCK, USA), and incubated for 30 min at room temperature (RT). After incubation, cells were permeabilised by incubation with 0.3% triton X-100 (MERCK)/PBS 1x (GIBCO). Membrane receptors were then blocked for 60 min (RT) with 10% FBS (BIOCHROM AG)/PBS. Afterwards, cells were incubated (60 min) with mouse anti-rat microtubule-associated protein 2 (MAP-2) antibody (SIGMA-ALDRICH) to detect mature SH-SY5Y neurons. Cells were washed thereafter with 0.5% FBS/PBS solution and incubated for 60 min

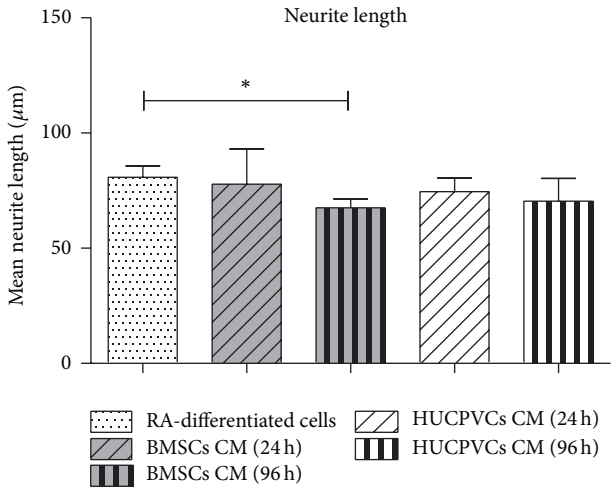

Figure 4: Quantitative analysis of SH-SY5Y neurite outgrowth seven days after incubation with BMSCs and HUCPVCs CM. Quantification of neurite lengths showed that for all CM conditions, with exception of SH-SY5Y cells cultured with BMSCs CM 96 h, the CM of BMSCs and HUCPVCs induced a neurite outgrowth and length very similar to the RA-differentiated cells group ($P > 0.05$). The decrease in the mean neurite length observed in the BMSCs CM 96 h group ($P < 0.05$) suggests that the neuronal differentiation inducement of SH-SY5Y cells may be associated with the temporal profile of MSCs CM collection (values are shown as mean ± SEM, $n = 5$, $^{*}P < 0.05$).

(RT) with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Finally, samples were incubated for 5 min with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), to stain cells nuclei (THERMO SCIENTIFIC, Rockford, USA), and observed under an OLYMPUS IX-81 fluorescence microscope (OLYMPUS, Germany).

2.4. Cell Counts. For cell counts, five representative fields of each coverslip condition ($n = 5$ replicates) were selected with 20x magnification and analyzed using Cell-P software (OLYMPUS, Germany). In addition, according to the literature, MAP-2 positive cells with one or more neurites were counted as differentiated cells [47].

2.5. Neurite Lengths Assessment. For neurite lengths assessment in SH-SY5Y neuron-like cells, multiple representative fields of cells morphology stained with MAP-2 labeling were photographed with an IX-81 OLYMPUS fluorescence microscope (OLYMPUS, Germany) fitted to a DP-711 digital camera (OLYMPUS, Germany). Captured images were labeled with a scale according to the correspondent microscope magnification (40x). The images scale was used to convert pixels units into micrometers (μm), using for this purpose the NIH Image J (Rasband WS, Image J, NIH), version 1.41. In addition, the channels were extracted to grey scale and the length of 5 to 10 neurites per field was traced and measured, thereafter, from the distal end of neuron growth-cone, using the neurite tracer plugin of NIH Image [48, 49].

2.6. Quantitative Real-Time RT-PCR. Total cellular ribonucleic acid (RNA) was extracted from SH-SY5Y differentiated cells with RA or MSCs CM ($n = 3$ replicates), using Trizol reagent (APPLIED BIOSYSTEMS, Life Technologies, CA, USA) for cell lysis and chloroform (MERCK)/isopropanol (THERMO SCIENTIFIC) for RNA isolation. The amount of RNA extracted and its purity were determined by measuring OD at 260 nm and 280 nm in ND-1000 spectrophotometer (ALFAGENE, PT). RNA was then treated with ribonuclease (RNAse) free desoxirribonuclease (DNAse, THERMO SCIENTIFIC) and 1 μg of total RNA was reverse-transcribed using Superscript kit (BIO-RAD, CA, USA) to obtain complementary deoxyribonucleic acid (cDNA). After obtaining cDNA, 1 μg of cDNA per reaction was amplified by quantitative real-time PCR in a CFX96 detection system (BIO-RAD) by means of SSOfast Evagreen supermix (BIO-RAD) and the primers sequences (concentration of 1 μM) previously described, using an annealing temperature of 60°C [50]. Each aliquot of cDNA was subjected to 40 PCR amplification cycles (94°C for 20 s, primer annealing at 60°C for 30 s, extension at 72°C for 40 s). Primers sequences used corresponded to several genes, namely, synaptophysin, βIII tubulin, MAP-2, DRD2 (dopamine receptor D2), and DAT (dopamine transporter). The expression levels of neuronal markers were determined as previously reported [51].

2.7. Statistical Analysis. Statistical evaluation was performed using one-way ANOVA followed by *Bonferroni's* post hoc test to assess statistical correlation between retinoic acid-differentiated cells (RA-differentiated cells) and conditioned media groups (for statistical evaluation, 3 replicates of each sample were used to perform the MTS test and RT-PCR, whereas five replicates were used to assess immunocytochemical and neurite outgrowth data ($n = 3/n = 5$; RA-differentiated cells/CM time point ± SEM)). These statistical tests were complemented with student's t-test to determine statistical correlation between RA and conditioned media groups ($n = 3/n = 5$; RA-differentiated cells/CM time point ± SEM) or between conditioned media groups corresponding to the same time point ($n = 3/n = 5$; CM time point ± SEM). Statistical significance was defined as $P < 0.05$ for a 95% confidence interval.

3. Results and Discussion

In the present study we aimed to determine and compare how the secretome of two MSCs populations, derived from either the bone marrow or the Wharton Jelly surrounding umbilical cord vessels, could impact the viability and neuronal differentiation of a human neuroblastoma cell line. For this purpose, SH-SY5Y cells incubated with a combination of low percentage of FBS and RA treatment were used as positive control of SH-SY5Y cells differentiation (RA-differentiated cells). Results revealed that cells incubated with both BMSCs and HUCPVCs CM had similar levels of metabolic viability after 7 days of culture (Figure 1). However, the values were significantly lower ($P < 0.05$) than those obtained for control samples. These differences were within the expected

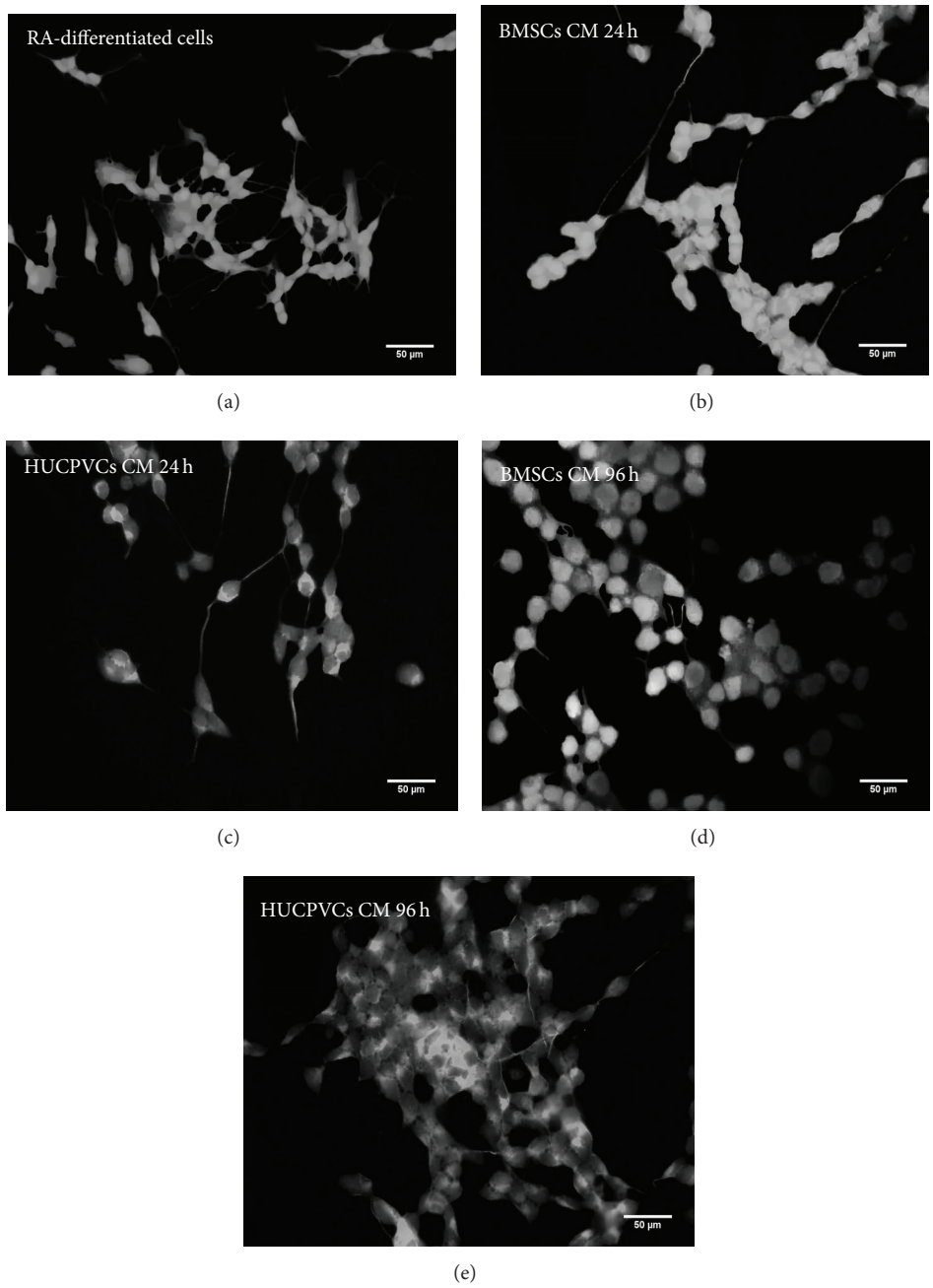

Figure 5: Representative micrographs (magnification: 40x) used to quantify neurite outgrowth in SH-SY5Y cells seven days after incubation with RA (a), BMSCs CM (24 h, 96 h: (b), (d)), and HUCPVCs CM (24 h, 96 h: (c), (e)) through the use of neurite tracer plugin from Image J.

as control cultures were incubated with 1% of FBS, which can increase their metabolic viability. Of note is the fact that SH-SY5Y cells were not able to survive for more than 5 days of *in vitro* culture when incubated in plain neurobasal media, without the addition of any other supplements (CM control; data not shown). This fact is a strong indicator that, *per se*, the secretome of both MSCs populations is able to support neuronal-like cell viability, without the use of any other exogenous growth factors.

Following this initial cell viability assay, an analysis on the differentiation of SH-SY5Y cells incubated with BMSCs/HUCPVCs CM or RA was performed by determining the percentage of cells positive for the neuronal marker MAP-2 that displayed one or more neurites (Figure 2). This criterion was established according to what was previously described by Encinas et al. [47]. Results revealed that all CM incubated groups had similar percentages of differentiated SH-SY5Y cells when compared to the positive control for cell

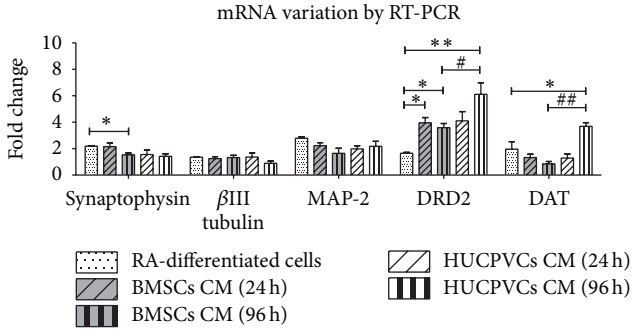

Figure 6: Variation of neuronal markers seven days after incubation with BMSCs and HUCPVCs CM. Levels of mRNA for different neuronal markers were quantified by quantitative real-time RT-PCR and normalized to both undifferentiated/proliferative cells (reference level: 1) and HBMS housekeeping gene. Quantification of neuronal markers expression revealed that BMSCs CM 24 h and 96 h displayed a significant increase in the SH-SY5Y cells expression of DRD2 gene when compared to RA-differentiated cells ($P < 0.05$). However, BMSCs CM 96 h simultaneously induced a decrease in synaptophysin in comparison with RA-differentiated cells ($P < 0.05$). On the other hand, for all the neuronal markers studied, no statistically significant differences were found between HUCPVCs CM 24 h and RA groups ($P > 0.05$). Yet, HUCPVCs CM 96 h significantly elevated mRNA levels of DRD2 and DAT genes when compared with both BMSCs 96 h ($P < 0.05$; $P < 0.01$) and RA-differentiated cells ($P < 0.01$; $P < 0.05$). The later results suggest that HUCPVCs CM 96 h is inducing SH-SY5Y cells towards the DAergic phenotype. In addition, differences between BMSCs and HUCPVCs from the same time point indicate that different tissue derived MSCs secretome have distinct effects in SH-SY5Y cells differentiation with respect to neuronal phenotype. For all the other neuronal markers studied, no significant statistical differences were observed between all CM tested conditions and RA-differentiated cells ($P > 0.05$), which strongly indicates that the CM from BMSCs and HUCPVCs are capable of inducing SH-SY5Y cells neuronal differentiation. Moreover, the different expression pattern of neuronal markers exhibited by SH-SY5Y cells among CM time points of collection indicates that the effects mediated by MSCs secretome in SH-SY5Y cells differentiation is related with the temporal profile of CM collection (values are shown as mean ± SEM, $n = 3$). Symbols correspondence to statistical signification: (1) $*$ refers to comparisons between RA-differentiated cells and MSCs CM and (2) # regards the correlation between MSCs CM from the same time point ($^{**/\#\#}P < 0.01$, $^{*/\#}P < 0.05$).

differentiation (RA-differentiated cells, $P > 0.05$) (Figure 2 and Figures 3(a) to 3(e)). This effect was more noticeable for the BMSCs CM 24 h (Figure 2 and Figure 3(b)). Thus, from the data obtained, it is possible to state that the secretome of both BMSCs and HUCPVCs is capable of inducing SH-SY5Y cells neuronal differentiation.

In order to further understand the role of the CM of HUCPVCs and BMSCs on SH-SY5Y neuronal differentiation, a quantitative analysis of neurite lengths was carried out (Figure 4). As it can be observed, BMSCs CM 24 h as well as HUCPVCs CM 24 h and 96 h had very similar results to those of the RA-differentiated cells group ($P > 0.05$) (Figure 4 and Figures 5(a) to 5(c) and 5(e)), which is a strong indicator of the differentiation effects of the secretome of both BMSCs and HUCPVCs. Finally, the decrease in the mean neurite length observed in the BMSCs CM 96 h group ($P < 0.05$) (Figure 4 and Figures 5(a) and 5(d)) may be related with the half-life of neurotrophic factors present in the CM at the point of their collection [19, 36, 37].

To confirm SH-SY5Y cells differentiation towards neuronal phenotype, seven days after incubation with CM from BMSCs and HUCVCs, mRNA expression of several neuronal specific markers was assessed by quantitative RT-PCR. According to the literature, mRNA levels of dopamine transporter and receptor D2 as well as levels of vesicle proteins (e.g., synaptophysin), neuronal specific cytoskeletal proteins (e.g., MAP-2), and globular proteins (e.g., βIII tubulin) were found increased in SH-SY5Y cells upon differentiation with RA [50, 52]. As it can be observed in Figure 6, the mRNA level of DRD2 was significantly increased in SH-SY5Y cells differentiated with BMSCs CM 24 h when compared to RA-differentiated cells ($P < 0.05$). Similarly, DRD2 gene expression was significantly elevated in SH-SY5Y cells differentiated with BMSCs CM 96 h when compared to RA-differentiated cells ($P < 0.05$). On the other hand, for all the neuronal markers studied, no statistically significant differences for SH-SY5Y cells gene expression were found between HUCPVCs CM 24 h and RA groups ($P > 0.05$). Interestingly, SH-SY5Y cells differentiation with HUCPVCs CM 96 h resulted in a significant increase in DRD2 and DAT genes expression in comparison with RA-differentiated cells ($P < 0.01$, $P < 0.05$). As DAT is a gene expressed only by DAergic neurons [53], this result suggests that GFs present in HUCPVCs CM 96 h may be inducing SH-SY5Y cells towards the DAergic phenotype. Indeed, it has been reported that SH-SY5Y cells differentiate into the cholinergic, adrenergic, or DAergic phenotype depending on media conditions [52]. In addition, the different SH-SY5Y cells expression pattern observed between HUCPVCs CM 24 h and HUCPVCs 96 h further reinforces the hypothesis that different temporal profiles of CM collection have distinct effects on SH-SY5Y cells differentiation. For all the other neuronal markers studied, no significant differences were found among the different tested CM conditions and RA groups ($P > 0.05$). These results further reinforce that the secretome of both BMSCs and HUCPVCs induces neuronal differentiation of SH-SY5Y cells. RT-PCR results also revealed differences regarding mRNA levels of DRD2 and DAT genes between BMSCs and HUCPVCs, collected at the same time point (96 h). Indeed, SH-SY5Y cells differentiated with HUCPVCs CM 96 h exhibited significant greater expression of both DRD2 and DAT genes than cells differentiated with BMSCs CM 96 h ($P < 0.05, P < 0.01$). The later result suggests not only that the different secretome composition of different tissue derived MSCs induces SH-SY5Y cells differentiation into different neuronal phenotypes but also that the effects mediated by the secretome of MSCs in neuronal differentiation are associated with the temporal profile of CM collection [19, 36, 37].

4. Conclusions

The present study has shown that the secretome of both BMSCs and HUCPVCs was capable of supporting SH-SY5Y

cell survival, while promoting their differentiation towards a neuronal phenotype. Furthermore, it was also observed that the secretome collected from both MSCs populations may induce SH-SY5Y cells differentiation into different neuronal phenotypes, which is an indicator of possible differences within the secretome of the two cell populations. Therefore, future studies should not only provide full characterization of factors secreted by MSCs derived from different microenvironments/sources but also assess the impact that different temporal profile of secretome collection can hold towards different CNS pathologies/injuries. Additionally the effects of cell passaging on MSCs secretome should also be assessed.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Supplementary data

Supplementary methods

Proliferative cells culture and experiments

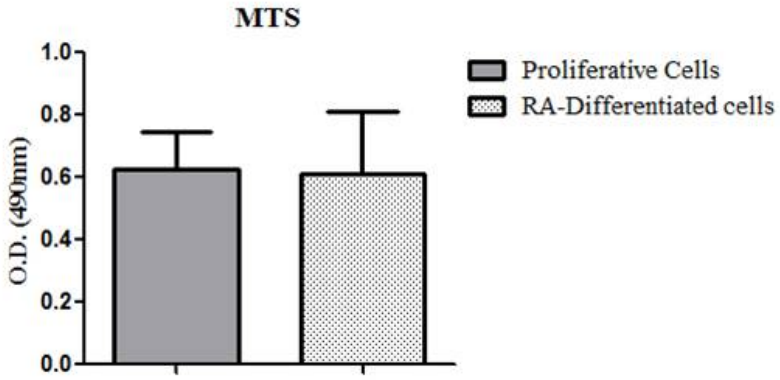
A control of SH-SY5Y proliferative/undifferentiated cells was also performed during the seven days differentiation of SH-SY5Y cells with MSCs CM. For this purpose, SH-SY5Y cells were cultured in DMEM/F-12 (PAA, LABCLINICS®, M, Spain) supplemented with 10 % of FBS (BIOCHROM AG®), 1% of glutamax (GIBCO®, Grand Island, NY, USA) and 1% of kanamycin sulfate (GIBCO®) for seven days. The culture medium was changed every day during this period of time. In addition, seven days later, cell metabolic viability (MTS test), immunocytochemical, RT-PCR and neurite outgrowth assays were performed at the same time as all the other tested conditions, following the same procedures described in the section of Materials and Methods. In addition, SH-SY5Y proliferative/undifferentiated cells were compared with RA-differentiated cells regarding cells metabolic viability, differentiation and neurite outgrowth, in order to demonstrate that RA-differentiated cells provide a good positive control for the objectives of this work.

Supplementary notes

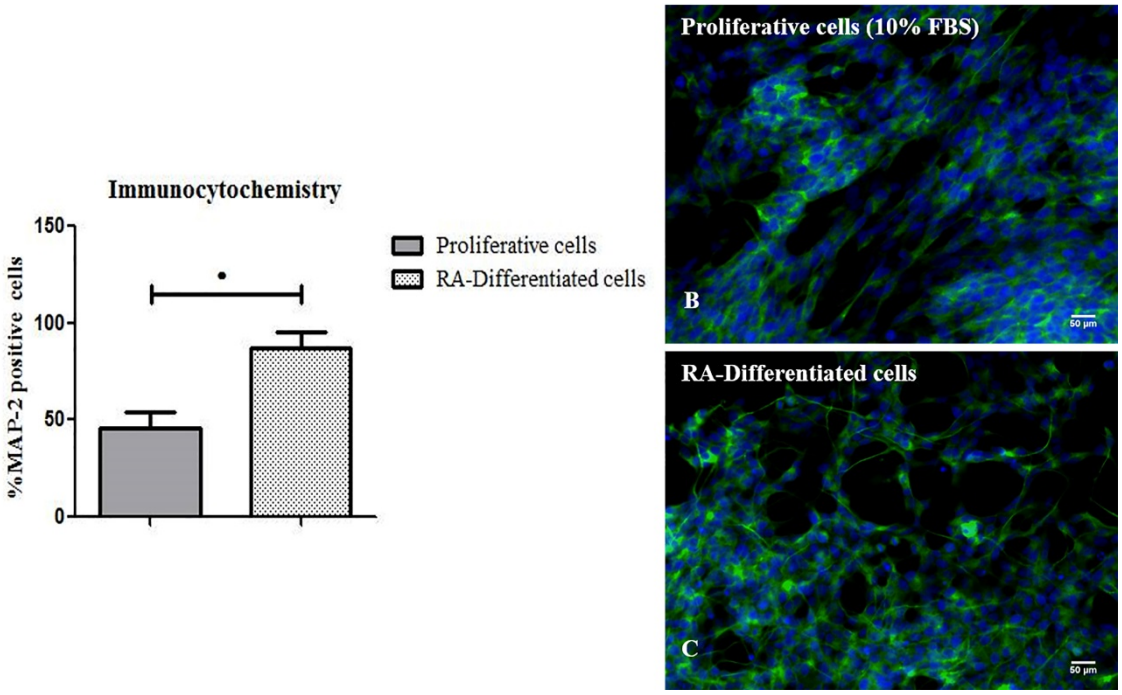
Statistics

Statistical evaluation was performed using *t-test* to assess statistical differences between proliferative cells and RA-differentiated cells (for statistical evaluation 3 replicates of each sample were used to perform the MTS test, whereas five replicates were used to assess immunocytochemical and neurite lengths data; n = 3 / n = 5; proliferative cells /RA-differentiated cells ±SD).

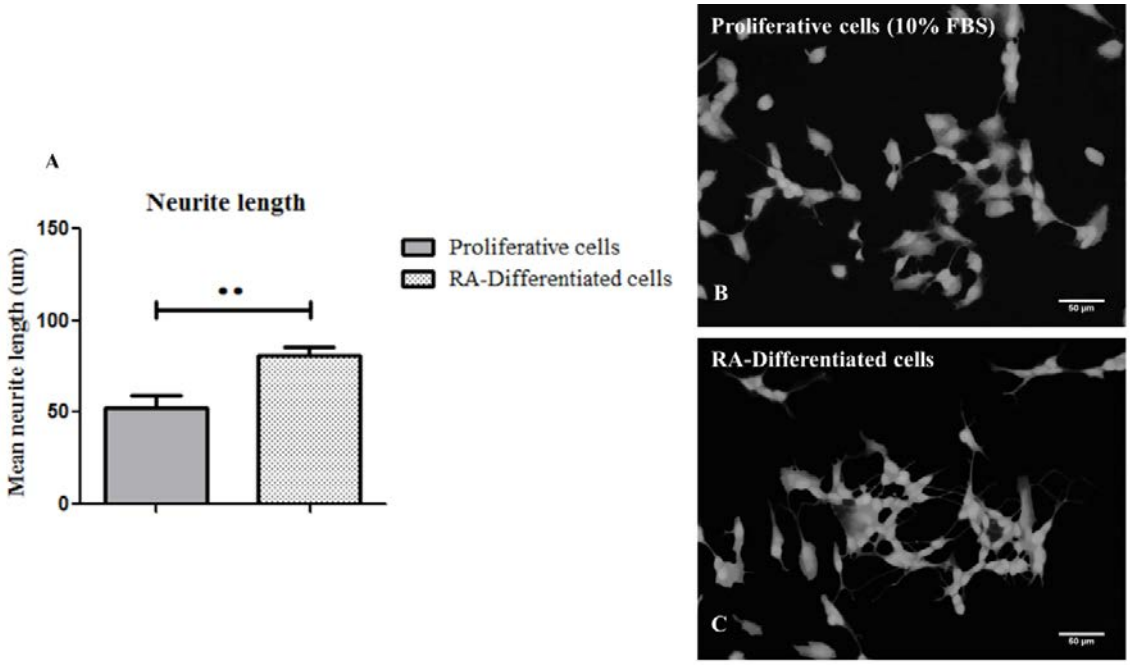
Supplementary figures



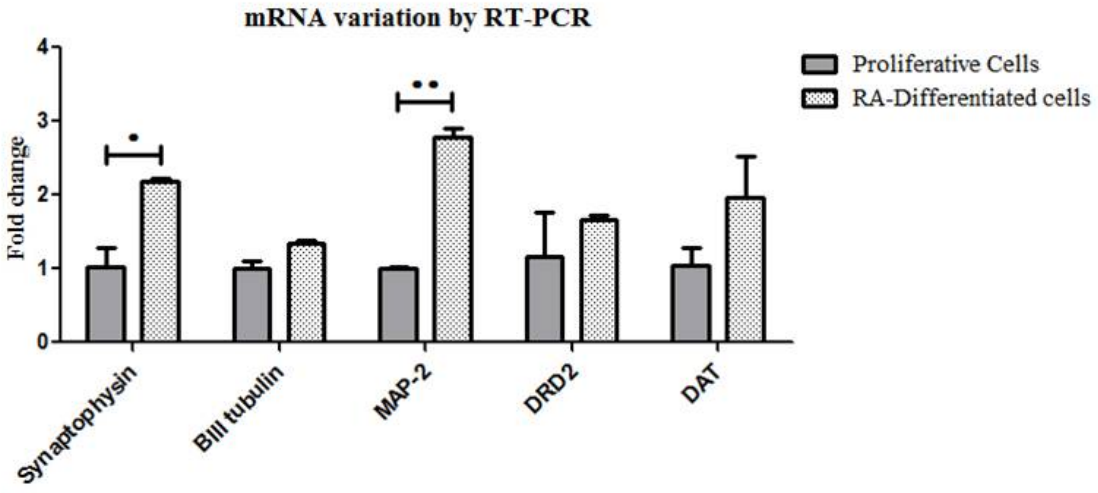
Suppl. Fig. 1: Metabolic Viability of SH-SY5Y cells seven days post-incubation with FBS and RA. Results revealed that RA-differentiated cells were able to support SH-SY5Y cells viability at the same extension as SH-SY5Y proliferative cells (Values are shown as mean ± SD, n = 3, statistical significance was defined as $p<0.05$).



Suppl. Fig. 2: Cell densities for MAP-2 positive cells presenting neurites seven days post-incubation with FBS and RA. Cell densities assessment revealed that the densities of MAP-2 positive cells after culture with RA (A, C) where significantly higher than the ones presented by SH-SY5Y proliferative cells (A, B) (Values are shown as mean ± SD, n = 5, • $p<0.05$).



Suppl. Fig. 3: Quantitative analysis of SH-SY5Y neurite outgrowth seven days post-incubation with FBS and RA. Neurite lengths assessment revealed that RA-differentiated cells (A, C) stimulated neurite outgrowth at a greater extent than SH-SY5Y proliferative cells (A, B) (Values are shown as mean ± SD, n = 5, • • $p<0.01$).



Suppl. Fig. 4: Variation of neuronal markers seven days post-incubation with FBS and RA. Levels of mRNA for different neuronal markers was quantified by quantitative real-time RT-PCR, normalized to undifferentiated/proliferative cells (reference level: 1) and HBMS housekeeping gene. Quantification of neuronal markers expression revealed that the mRNA levels of Synaptophysin and MAP-2 were significantly increased in RA-differentiated cells when compared to the expression levels of SH-SY5Y proliferative cells ($p<0.05$, $p<0.01$). For all the other neuronal markers, an increase in expression was also noticed, although not at statistically significant level (Values are shown as mean ± SD, n = 3, • $p<0.05$, • • $p<0.01$).

Supplementary discussion

It has been largely documented that a combination of low percentage of FBS and RA induces SH-SY5Y cells differentiation into neuron-like cells [29-31, 33]. Therefore, in the present study, a control of SH-SY5Y proliferative/undifferentiated cells was provided to confirm that SH-SY5Y cells were differentiated seven days post-incubation with 1% FBS and RA. Results revealed that RA-differentiated cells presented similar cell viability as SH-SY5Y proliferative cells (Figure S 1; $p>0.05$). Also, immunocytochemical and neurite lengths data revealed that SH-SY5Y cells differentiated with RA exhibited significant higher densities of MAP-2 positive cells (Figure S 2; $p<0.05$,) and mean neurite lengths (Figure S 3; $p<0.01$). Moreover, Synaptophysin and MAP-2 gene expression levels were significantly increased in RA-differentiated cells in comparison with SH-SY5Y proliferative cells (Figure S 4; $p<0.05$, $p<0.01$). For all the other neuronal markers, an increase SH-SY5Y cells genes expression was also noticed although not at a statistically significant level.

Taken together, results show that the differentiation protocol, using low percentage of FBS and RA, effectively induced SH-SY5Y proliferative cells differentiation into neuron-like cells. Therefore RA-differentiated cells provide a good positive control for the objectives of this work.

Chapter 3

Effects of Mesenchymal Stem Cells Secretome on Dopaminergic Neuronal Populations: *In vitro* and *In vivo* Assays (Manuscript status: to be submitted)

Effects of Mesenchymal Stem Cells Secretome on Dopaminergic Neuronal Populations: *In vitro* and *In vivo* Assays

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Abstract

Over the last decade, mesenchymal stem cells (MSCs) have been suggested as potential candidates for Parkinson's disease (PD) treatment in virtue of their availability in multiple adult tissues, their immunomodulatory profile, their immune-privileged character and the absence of ethical concerns when compared with fetal or embryonic stem cell sources. Several studies have already demonstrated that transplantation of different tissue derived MSCs promote histological and motor recovery in animal models of PD. An increasing body of literature attributes these MSCs-induced functional improvements to the MSCs secretome. Therefore, we aimed to determine whether: 1) the MSCs secretome could be a potential tool for PD therapy, without the need for cells to be transplanted and, 2) the secretome of MSCs isolated from different sources could display different therapeutic specificity towards PD. For this purpose, ventral mesencephalic cell (VMC) cultures were incubated with the secretome (in the form of conditioned media/CM) of MSCs derived from human bone marrow (BMSCs), adipose tissue (ASCs) and the Wharton Jelly of the perivascular zone of the umbilical cord (HUCPVCs). Subsequently, it's possible *in vivo* therapeutic potential was assessed by a single intranigral injection and multiple intrastriatal injections in a hemiparkinsonian model. Immmunocytochemical results revealed that ASCs CM was able to significantly increase the survival of mature ventral midbrain dopaminergic (DAergic) neurons when compared to both, the control of the experiment and VMCs incubated with HUCPVCs CM. On the other hand, immunohistochemical and behavioral data revealed that only BMSCs CM treatment induced the survival of nigral DAergic neurons and animals' motor recovery. Overall, the *in vitro* data showed that the secretome of different tissue derived MSCs displayed distinct effects on the survival of VMC cultures, which is indicative of the existence of different secretome profiles among MSCs populations. Moreover, *in vivo* results showed that only the BMSCs secretome displayed therapeutic specificity towards PD, which once again indicates that different tissue derived MSCs synthesize and release distinct biomolecules that contribute to the observed differential therapeutic potential of BMSCs, ASCs and HUCPVCs secretome towards PD.

1. Introduction

Parkinson's disease is a slowly progressive neurodegenerative disorder, primarily characterized by the progressive loss of dopaminergic (DAergic) neurons in the substantia nigra *pars compacta* (SNpc) within the nigrostriatal pathway, that leads to the loss of DAergic innervations and consequently to striatal dopamine (DA) deficiency (McGeer et al. 1988, Jin et al. 2008). The marked loss of SNpc DAergic neurons (50% to 60%) (Bernheimer et al. 1973) and striatal DA levels (80%) (Hornykiewicz 1993) leads to the appearance of motor impairments, such as bradykinesia, resting tremor, rigidity, and postural instability, which are the main cardinal motor symptoms of PD (Koller 2003, Jankovic 2008). Levodopa (L-DOPA) and DA agonists have been largely used for treatment of PD (Smith et al. 2012, Teixeira et al. 2013). Indeed, these DA normalizing agents have shown to enhance motor symptoms, thus improving the quality of life and survival of PD patients. However, their application has been associated with severe side effects and none of these treatments has shown to provide total recovery of PD symptomatology, or to induce either recovery of lost DAergic neurons or their protection from further degeneration (Smith et al. 2012, Teixeira et al. 2013).

In order to overcome these limitations, cell-based therapies have arisen to meet the clinical challenge of restoring physiological DA transmission and provide long-term relief of PD patients' symptomatology (Koller 2003, Teixeira et al. 2013). Indeed, open trials conducted in the late 1980s provided proof-of-principle that cell-based transplantation techniques are promising candidates for PD, following the demonstration that ventral mesencephalic tissue transplantation could improve striatal DAergic function and functional outcome of patients (Lindvall et al. 1989, Lindvall et al. 1990, Lindvall et al. 1992, Widner et al. 1992, Kordower et al. 1996). Yet, issues related with donor availability, tissue manipulation and ethical concerns (Vidaltamayo et al. 2010, Teixeira et al. 2013) led to the search of alternative cell sources such as mesenchymal stem cells (MSCs). MSCs are plastic adherent multipotent cells with fibroblast-like morphology, capable to differentiate into mature mesenchymal cells (osteoblasts, adipocytes and chondroblasts). They are further characterized by the expression of cells surface markers (CD105, CD73 and CD90), as well as by the absence of hematopoietic cell surface markers (CD45, CD34, CD14 or CD11b, CD79a or CD19) and Human Leukocyte Antigen DR (Dominici et al. 2006). MSCs are particularly interesting candidates for PD therapy in virtue of their availability in multiple adult tissues, immunomodulatory profile and the absence of ethical constraints when compared with fetal or embryonic stem cell

sources (Salgado et al. 2006, McCoy et al. 2008, Troyer and Weiss 2008, Kishk and Abokrysha 2011). In fact, MSCs have been shown to exhibit relevant properties for PD therapeutics. For instance, an *in vitro* study conducted by Jin and colleagues demonstrated that, upon co-culture, MSCS derived from bone marrow (BMSCs) were able to increase both the expression levels of tyrosine hydroxylase (TH) and the DA content in embryonic ventral mesencephalic cells/VMCs (Jin et al. 2008). Moreover, several *in vivo* studies have already reported that besides BMSCs, transplantation of MSCs derived from adipose tissue (ASCs) and the Wharton jelly (WJ-MSCs) were able to increase TH levels and induce functional improvements in both 6-OHDA (6-hydroxydopamine) and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) animal models of PD (Li et al. 2001, Bouchez et al. 2008, McCoy et al. 2008, Chao et al. 2009, Hayashi et al. 2013, Shetty et al. 2013, Wang et al. 2013). Furthermore, two of these studies have reported an increase of DA levels post-transplantation (Bouchez et al. 2008, Chao et al. 2009). The mechanisms underlying these MSCs-induced neuroprotective and/or neuroregenerative effects along with motor recovery phenomena observed in animal models of PD are not completely understood. Some of the above referred authors suggest that MSCs-induced beneficial outcomes are related with MSCs differentiation into DAergic neurons (Hayashi et al. 2013, Wang et al. 2013), but undisputable evidence of MSCs differentiation into the neuronal phenotype remains to be demonstrated (Thomas et al. 2011, Liu et al. 2012). On the other hand, an increasing body of literature indicates that the observed MSCs-induced functional benefits seen in animal models of PD are attributed to MSCs secretome, that is, to the MSCs secretion of bioactive molecules and vesicles with neuroregulatory properties to the extracellular environment (Weiss et al. 2006, Shintani et al. 2007, McCoy et al. 2008, Sadan et al. 2009a, Sadan et al. 2009b, Cova et al. 2010, Wang et al. 2010). In fact, several studies have already documented that MSCs secretome was able to protect and/or regenerate DAergic neurons both in *in vitro* (Shintani et al. 2007, Sadan et al. 2009a, Sadan et al. 2009b) and in *in vivo* (Weiss et al. 2006, Shintani et al. 2007, McCoy et al. 2008, Sadan et al. 2009a, Sadan et al. 2009b, Cova et al. 2010, Wang et al. 2010) models of PD. For instance, Shintani *et al.* showed that human BMSCs CM improved the survival of DAergic neurons in 6-hydroxydopamine (6-OHDA) injured rat primary cultures of VMCs (Shintani et al. 2007). In addition, the authors showed that transplantation of VMCs pre-treated with BMSCs CM into the striatum of a 6-OHDA model of PD promoted both the survival of VMCs and animals' functional recovery (Shintani et al. 2007). These *in vitro* and *in vivo* results were related with BMSCs expression of factors responsible for the survival and protection of DAergic neurons, like brain-derived

neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) (Shintani et al. 2007). Similar results were also reported by others using *in vitro* and *in vivo* 6-OHDA rat models of PD (Sadan et al. 2009a, Sadan et al. 2009b). Furthermore, it has also been shown that BMSCs are able to protect or rescue DAergic neurons from 6-OHDA neurotoxin-induced nigrostriatal degeneration through MSCs secretome mediated anti-apoptotic (Wang et al. 2010) and neurogenic (Cova et al. 2010) mechanisms. For instance, Wang *et al.* (Wang et al. 2010) has associated BMSCs-induced neuroprotective effects against 6-OHDA-induced nigrostriatal degeneration to the BMSCs expression of the anti-apoptotic stromal cell-derived factor 1 alpha (SDF-1α). On other hand, Cova *et al.* (Cova et al. 2010) related the counteraction of the nigrostriatal degeneration to the BMSCs activation of neurogenesis and rescue of DAergic neurons through MSCs secretion of neurogenic and neurotrophic factors like BDNF, endothelial growth factor (EGF), neurotrophin-3 (NT-3), bFGF, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).

ASCs and WJ-MSCs have also been shown to disclose important effects in 6-OHDA models of PD. For instance, McCoy *et al.* (McCoy et al. 2008) demonstrated that intranigral transplantation of ASCs attenuated the loss DAergic neurons through ASCs synthesis of nerve growth factor (NGF), BDNF and GDNF, whereas Weiss *et al.* (Weiss et al. 2006) reported the rescue of dying nigrostriatal neurons and animals' behavior recovery, upon intrastriatal transplantation of WJ-MSCs, and related the observed effects with WJ-MSCs release of GDNF and fibroblast growth factor 20 (FGF 20).

The above referred studies highlight the contribution of MSCs secretome for DAergic neuronal survival, neuroprotection and/or neurorecovery observed phenomena, as well as to the functional improvement of animals' motor deficits, in *in vitro* and *in vivo* models of PD. However, to our knowledge, a comparative study on the effects that different tissue derived MSCs secretome can have on the survival of midbrain DAergic neurons in uninjured/intact cultures remains to be provided. Moreover, the potential differential therapeutic effect that different tissue derived MSCs secretome, *per se*, can hold towards PD remains to be unveiled. Finally, it is also important to assess if the secretome alone could represent a viable therapeutic route to follow in *in vivo* models of PD. Herein, in the present study we intended to evaluate the impact of BMSCs, ASCs and HUCPVCs secretome, in the form of CM, in the survival of primary cultures of VMCs. Moreover, we also aimed to assess the possible therapeutic efficacy of the secretome of these cell populations in a rat model of PD.

2. Materials and Methods

2.1 Mesenchymal stem cells culture

2.1.1 Bone marrow tissue derived stem cells

BMSCs were acquired from PROMOCEL (Heidelberg, Germany). Cells were thawed and expanded according to the protocol previously described by Silva *et al.* (Silva et al. 2013). Briefly, BMSCs were cultured in α-MEM (Gibco, Grand Island, NY, USA) supplemented with Sodium bicarbonate ($NaHCO_3$, Merck, USA), 10% of fetal bovine serum (FBS, Biochrom AG, UK) and 1% of penicillin-streptomycin antibiotic (Gibco). Once confluent, cells were trypsinised, plated at a density of 4.000 cells/cm^2 in new tissue culture flasks (Nunc, Denmark), and incubated at 37°C in a 5% humidified CO_2 atmosphere. The culture medium was changed every two to three days. BMSCs were used for experiments during passage 6 (P6).

2.1.2 Human umbilical cord perivascular cells

HUCPVCs were kindly provided by Professor J. E. Davies (University of Toronto, Canada). Cells isolation from the umbilical cord was performed according to the procedures described by Sarugaser and co-workers (Sarugaser et al. 2005). Expansion of cells was performed according with the protocol described above for BMSCs. HUCPVCs were used for experiments during P6.

2.1.3 Adipose tissue derived stem cells

ASCs were kindly provided by Professor J. M. Gimble (University of Tulane, USA). Cells isolation from adipose tissue was performed according to the previously described procedures reported by Dubois and colleagues (Dubois et al. 2008). Expansion of cells was performed according with the protocol described above for BMSCs. ASCs were used for experiments during P6.

2.1.4 Ventral mesencephalic neuronal cultures

Ventral mesencephalic cells were cultured following similar methods to the ones previously published by Campos *et al.* (Campos et al. 2012). Briefly, embryonic neurons were obtained from

the ventral mesencephalon tissue of time pregnant Wistar-Han rats at 15 to 16 days of gestation. Female pregnant rats were anesthetized under ketamine (87.5 mg/Kg; Merial, Chile) and xylazine (12.5 mg/Kg; Calier, Portugal). The ventral mesencephalon of rat embryos was dissected thereafter according with the procedures described by Dunnett and Björklund (Dunnett and Björklund 1992). After the latter procedure, the dissected tissue was digested with trypsin (2.5mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and DNase (2.5mg/ml; Sigma-Aldrich) in PBS (0.1M), and incubated in a 37°C water-bath for 15min. Tissue digestion was then stopped by adding 10% FCS/PBS (Biochrom AG). After centrifugation (3K18C Bioblock Scientific, Sigma-Aldrich) and supernatant removal, tissue was washed, centrifuged and mechanically dissociated with a micropipette (Gilson, Middleton, WI, USA). Obtained cell suspensions were resuspended in neurobasal media/NBM (Gibco) supplemented with 2% of B27 (Gibco), 25µM of glutamate (Sigma-Aldrich), 0.05mg/ml of glutamine (Sigma-Aldrich) and 50µg/ml of gentamicine (Sigma-Aldrich). Following this procedure, cells were counted, plated at a density of 210.526 cells/cm^2 in 24 multi-well plates (Nunc), containing poly-D-lysine (Sigma-Aldrich) pre-coated coverslips, and incubated at 37°C in a 5% humidified CO_2 atmosphere. Neuronal cultures were used in the sixth day of culture.

2.2 Conditioned media collection and concentration

CM was collected from P6 BMSCs, ASCs and HUCPVCs as previously reported by Fraga *et al.* (Fraga et al. 2013). Shortly, cells were plated at a density of 4.000 cells/cm^2 and allowed to grow for 3 days at 37°C in a 5% humidified CO_2 incubator. Upon PBS (PBS without ca^{2+} Mg^{2+}, Gibco) washes, culture medium was renewed and collected 24h thereafter (cell culture was not renewed or added during this time period). For CM collection, NBM supplemented with 1% of Kanamycin sulfate (Gibco) was used. For *in vivo* experiments, MSCs CM 24h were further processed: collected CM were 100 times concentrated by centrifugation through the use of ultrafiltration spin columns (Vivaspin) with 5 kDa cut-off polyethersulfone membranes, according with the manufacturer's guidelines (Sartorius Stedim Biotech, Goettingen, Germany). Collected CM were frozen and thawed only in the day of experiments.

2.3 *In vitro* experiments

To determine the effect of MSCs CM in the survival of ventral mesencephalic cell cultures, after 6 days of *in vitro* culture, cells were washed to remove supplements added for VMCs culture and were thereafter incubated with BMSCs, ASCs or HUCPVCs CM 24h (n = 3) for 24h. VMCs incubated with NBM containing 1% of Kanamycin sulfate (n = 3) were used as control of the experiment. After VMCs incubation with MSCs CM 24h or with NBM, DAergic cell densities were determined by microtubule-associated protein 2 (MAP-2)/TH double immunostaining.

2.3.1 Immunocytochemistry

Cells cultured in pre-coated coverslips (n = 3) were fixed with 4% paraformaldehyde (Merck) and incubated for 10 min at room temperature (RT). After incubation, cells were permeabilised with 0.1% Triton X-100 (Merck)/PBS (0.1M). Non-specific binding to membrane receptors was then blocked for 60 min (RT) with 20% of FBS (Biochrom AG) and 0.1% of tween 20 (Fisher Bioreagents, Portugal) in PBS (0.1M). Cells were incubated (60min) thereafter with mouse anti-rat MAP-2 (Sigma-Aldrich) and rabbit anti-TH (Millipore, USA) antibodies diluted in 1% FCS/PBS-Tween 20 (0.1%) to detect mature dopaminergic neurons in VMC cultures (in some coverslips, primary antibodies were omitted to assess specificity of MAP-2 and TH immunostaining). Cells were then washed with 0.1% tween 20/PBS (0.1M) solution and incubated for 60 min (RT) with both Alexa Fluor 568 goat anti-mouse immunoglobulin G (IgG) (Sigma-Aldrich) and Alexa Fluor 488 goat anti-rabitt IgG (Sigma-Aldrich) antibodies. Finally, after washing cells, samples were incubated for 10 min with Hoescht (Sigma-Aldrich), to stain cells nuclei, and observed under an inverted Zeiss fluorescence microscope (Axiobserver Z1, Zeiss, Portugal).

2.4 Cell counts

For cell counts, twenty representative fields of each coverslip condition (n = 3) were selected with a magnification of 60× and both MAP-2 single-stained and MAP-2/TH double-immunostained cells were counted using the image J program (Rasband WS, Image J, NIH), version 1.41. Results are presented as the percentage of MAP-2/TH positive cells in proportion to the control (% MAP-2/TH positive cells ±SD).

2.5 *In vivo* experiments

2.5.1 6-OHDA hemiparkinsonian rat model

Eight week old Wistar-Han male rats (Charles River, Barcelona) were housed according with the standard laboratory controlled conditions previously reported by Carvalho *et al*. (Carvalho et al. 2013): animals' were housed in pairs, with 12h light-dark cycle, 22°C RT, 55% relative humidity, food and water available *ad libitum*. All animal manipulations and procedures were performed in accordance with the European Union regulations (directive: 2010/63/EU) and NIH guidelines for animal care and experimentation.

Animals' (n = 28) were anesthetized with an intraperitoneal injection of ketamine (75 mg/Kg) and medetomidine (0.5mg/kg). Anesthetized animals' were placed on a stereotaxic frame (Stoelting, USA) and were unilaterally injected in the right hemisphere middle forebrain bundle (MFB) [coordinates related to Bregma: AP = -4.4 mm; ML = -1.0mm; DV = -7.8mm; according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 1998)] with either vehicle (Sham group, n = 8) or 6-OHDA hydrochloride (6-OHDA group, n = 20). Animals' from the saline group were injected with 2µl of 0.2mg/ml of ascorbic acid (Merck) in 0.9% sodium chloride (Merck), whereas 6-OHDA animals' were injected with 2µl of 6-OHDA hydrochloride (4µg/µl) (Sigma-Aldrich) diluted in 0.2mg/ml of ascorbic acid and in 0.9% sodium chloride, by means of a 30-gauge needle Hamilton syringe (Hamilton, Switzerland), at a rate of 1µl/min. After 6-OHDA injection, the syringe was left in the injection site for four minutes to allow 6-OHDA or saline diffusion. After surgery, animals' were allowed to recover for three weeks. At the fourth week post-surgery, animals' forelimb skills, were assessed through the staircase test (see below) under bilateral and unilateral (forced choice) conditions. Following behavioral assessment, animals' were allocated to four groups [BMSCs CM 24h (n = 5), ASCs CM 24h (n = 5), HUCPVCs CM 24h (n = 5) or NBM (control group, n = 5)] for stereotaxic injection of CM 24h or NBM.

2.5.2 Stereotaxic injection of MSCs CM

Six weeks after unilateral injection of 6-OHDA in the MFB of the animals', animals' were unilaterally injected into the striatum and the SNpc of the right hemisphere (ipsilateral side of the 6-OHDA lesion) with 100× concentrated BMSCs CM 24h (n = 5), ASCs CM 24h (n = 5), HUCPVCs CM 24h

(n = 5) or NBM (control group, n = 5). For MSCs CM and NBM delivery into the striatum, 8 µl of BMSCs CM 24H, ASCs CM 24H, HUCPVCs CM 24h or NBM were individually injected and distributed at a rate of 1µl/min, according with the stereotaxic coordinates, related to Bregma, described by E.M. Torres *et al.* (Torres et al. 2008): AP = +1.3mm, ML = -2.7mm, V= -4.0mm; AP = +1.3mm, ML = -2.7mm, V= - 4.5mm; AP = +0.4mm, ML = -3.1mm, V= -4.0mm; AP = +0.4mm, ML = -3.1mm, V= -4.5mm; ; AP = -0.4mm, ML = -4.3mm, V= -4.0mm; AP = -0.4mm, ML = -4.3mm, V= -4.5mm; AP = -1.3mm, ML = -4.7mm, V= -4.0mm and AP = -1.3mm, ML = -4.7mm, V= -4.5mm. In addition to the intrastriatal delivery of MSCs CM or NBM, animals' were also grafted unilaterally with 4µl of either BMSCs CM 24H, ASCs CM 24H, HUCPVCs CM 24h or NBM into the SNpc at the following coordinates related to Bregma: AP = -5.3mm; ML= -1.8mm; V= -7.4mm (Paxinos and Watson 1998). One week after MSCs CM 24h or NBM injections and every two weeks thereafter (for a total period of 7 weeks), motor recovery of the animals' was assessed by the staircase test, both under bilateral and unilateral conditions (see below).

2.5.3 Behavioral assessment

2.5.3.1 Skilled paw reaching test

Two staircase boxes were used to assess animals' lateralized deficits in skilled paw reaching. The staircase boxes used in this study were similar to the ones produced by Montoya (Montoya et al. 1991). The staircase apparatus consists of a chamber, with a hinged lid, connected with a narrower compartment with a central raised platform to which a removable holed double staircase with seven steps on each side can be attached. Sugar pellets can be placed in holed stairs between the platform and the chamber walls (Abrous and Dunnett 1994). A pre-training of the test was performed in the first two days at the fourth week post-6-OHDA lesion according with the procedures reported by Campos *et al.* (Campos et al. 2013). Shortly, for pre-training of the test, three pellets were placed in each step hole of the double staircase and animals' were allowed to reach, retrieve and eat sugar pellets for 5 min in the first day and 10 min in the second day of the training sessions. First testing session took place at the fourth week post-6-OHDA lesion (before MSCs CM 24h injection). Subsequent sessions started 1 week post-delivery of MSCs CM 24h or NBM and were performed every 2 weeks for a total period of 7 weeks. The testing sessions were carried out every day for 4 days, with the pellets being available for 15min in each step of the

double staircase. In addition to the 4 days of testing, animals' were tested for 2 additional days under forced choice conditions, i.e., pellets were placed unilaterally in either the left or right side of the double staircase under the same pellet configuration. All the testing sessions were performed at the same time of the day, with 23h food restricted animals' and following similar procedures to the ones previously reported by Cordeiro *et al*. (Cordeiro et al. 2010) and Campos *et al*. (Campos et al. 2013). At the end of each session, the number of pellets remaining in the holes or retrieved but dropped by each animal was counted separately to calculate the number of pellets eaten by each animal. Data are expressed as the average number of pellets eaten by each group of animals', either with the contralateral paw (contralateral side of the lesion) or the ipsilateral paw (ipsilateral side of the lesion), under bilateral or forced choice conditions (average number of pellets ±SEM).

2.6 Histology

2.6.1 Immunohistochemistry

At the end of behavioral assessment, animals' were sacrificed with sodium pentobarbital (Ceva-Saúde animal, Portugal) and transcardially perfused with 4% paraformaldehyde (Merck) diluted in 0.1M of PBS (Gibco). After brains removal, coronal slices (40µm) of brains imbedded in 3% agarose (GeneOn, Ludwigshafen am Rhein, Germany)/PBS containing the mesencephalon and prosencephalon were obtained with a vibrotome (VT1000S, Leica, Germany). Four series and twelve series of the mesencephalon and prosencephalon, respectively, were collected and one series was further processed for free-floating TH immunohistochemistry to detect DAergic neurons and fibers present in the SNpc and the striatum, respectively. TH immunostaining was performed according to the protocol previously described by Carvalho *et al*. (Carvalho et al. 2013). Shortly, slices were immersed in 3% hydrogen peroxide (H_2O_2, Panreac, Barcelona, Spain)/PBS (0.1M) for 20min to inhibit endogenous peroxidase. After washing with tween 20 (Fisher Bioreagents)/PBS (0.1M), slices were incubated with 5% fetal calf serum (FCS, Biochrom AG)/PBS (1M) at RT for 120min to block antibody non-specific binding. Slices were then incubated at 4°C overnight with rabbit anti-mouse TH primary antibody diluted in 2% FCS/PBS (1M). Following this procedure, slices were incubated (RT) with biotinylated secondary anti-rabbit antibody (Thermo Scientific, Rockford, USA) for 30 min. Slices were then incubated with avidin-biotin complex (Thermo Scientific) at RT for 30min and the reaction product was visualized thereafter using 3,3i-

diaminobenzidinetetrahydrochloride (DAB, Sigma-Aldrich) (25mg DAB in 50ml Tris-HCl 0.05M, pH 7.6 with 12.5µl H_2O_2). Finally, slices were mounted on superfrost slides (Thermo Scientific), dehydrated and coversliped (Menzel-Glaser, Braunschweig, Germany). In the case of mesencephalon slices, they were also subjected to thionine counter-coloration before being coversliped.

2.6.2 Determination of TH positive cells and fibers

The sections processed for immunohistochemistry were used to determine the total number of TH immunoreactive cell bodies in the SNpc and fiber densities in the striatum.

Determination of the total number of SNpc TH positive neurons was performed as previously described by Carvalho *et al.* (Carvalho et al. 2013). Shortly, six TH-stained slices containing the entire mesencephalon, including the SNpc region were selected under a bright-field microscope (BX51, Olympus, USA), fitted to a digital camera (PixeLINK PL-A622, Canimpex Enterprises Ldt, Canada), and the boundaries of SNpc were outlined (4× objective) after anatomic identification of the SNpc region with the help of the Visiomorph software (V2.12.3.0, Visiopharm, Denmark) and the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998). The total number of TH positive cell bodies present in the full extent of the SNpc of both hemispheres was thereafter counted (40× objective). Data is presented as % of the remaining neurons in the MSCs CM or NBM injected side over the control side (% TH positive neurons ± SEM).

For estimation of TH immunoreactive striatal fibers, the total immunoreactivity of TH fibers was measured by densitometry as described by Febbraro *et al.* (Febbraro et al. 2013). For this purpose, four TH-immunostained prosencephalon sections representing the coordinates of injection sites within the striatum were selected and photographed (1× objective) with an SZX 16 Microscope (Olympus, Germany) fitted to a DP-72 digital camera (Olympus, Germany). The photos were thereafter converted to gray scale using the Image J program (1.42 version) and analyzed for gray intensity after calibrating the image J program, through the use of the "optical density step tablet", to determine the optical density (O.D.) of the selected sections, according with the program instructions. The striatum O.D. values were thereafter determined in both hemispheres using a 1.1 mm^2 rectangular grid, encompassing the injection sites (Coordinates related to Bregma: AP = +1.3mm, ML = -2.7mm; AP = +0.4mm, ML = -3.1mm; AP = -0.4mm, ML = -4.3mm; AP = -1.3mm,

ML = -4.7mm at two different depths, V= -4.0mm and V= -4.5mm), as determined by anatomical reference and the rat brain atlas (Paxinos and Watson 1998). The corpus callosum (internal control) O.D. was also measured in both hemisphere sides, to avoid nonspecific background, and the TH striatal fiber densities were determined thereafter by calculating the O.D. difference between the striatum injected with CM or NBM and the corpus callosum, as well as, between the intact striatum and the corpus callosum. Data is presented as % of the remaining TH fibers in the injected side over the control side (% TH positive fibers ± SEM).

2.7 Statistical Analysis

Statistical evaluation of experiments involving only one variable was performed using one-way ANOVA followed by Bonferroni *post-hoc* test, to assess statistical differences between CM and NBM groups or among CM groups. *t-student* test was used to evaluate the magnitude of change in animals' behavior in unilateral staircase test between the 6-OHDA and sham groups. To determine the effect of 6-OHDA lesion and MSCs CM treatments on unilateral staircase test, statistical analysis was performed using two-way repeated measures analysis of variance followed by Bonferroni *post-hoc* test. For statistical analysis, three replicates of each sample from three experiments were used for cell culture experiments, whereas at least 3 animals' were used to assess data from *in vivo* experiments. Data are presented as the mean ±SEM or the mean ±SD, and statistical significance was defined as $P < 0.05$, for a 95% confidence interval.

3. Results and discussion

In the present study, we aimed to understand whether the sole use of MSCs secretome could be used as a tool for PD therapy. To accomplish this aim, embryonic ventral mesencephalic cells, which are the precursors of midbrain DAergic neurons (Jin et al. 2008), were incubated with CM from BMSCs, ASCs or HUCPVCs. As it can be observed in figure 1, analysis on midbrain differentiated DAergic neurons, through MAP-2/TH double immunostaining, revealed that the ASCs CM 24h significantly increased the percentage of MAP-2/TH cell densities when compared with both the control of the experiment (VMCs incubated with NBM) (Figure 1 A, B, D, $p < 0.05$) and those incubated with HUCPVCs CM 24h (Figure 1 A, D, E, $p < 0.05$). No significant differences

were observed between VMCs incubated with BMSCs CM 24h or HUCPVCs CM 24h and the control (Figure 1 A, B, C, E, $p > 0.05$). These results indicate that the secretome of BMSCs, ASCs and HUCPVCs most likely contain different biomolecular profiles that are mediating the observed differential effects of MSCs secretome on the survival of VMC cultures.

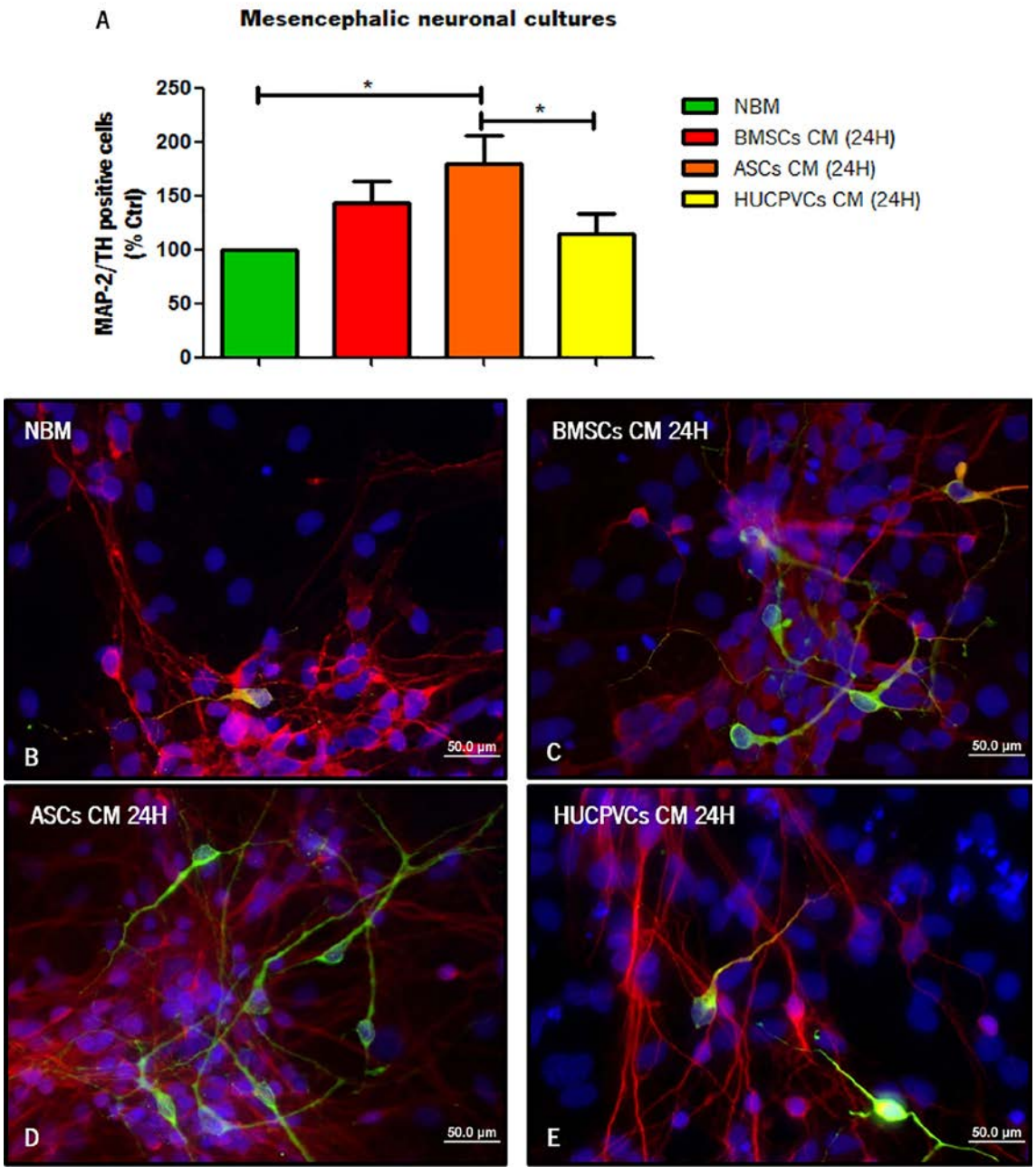

Figure 1: Ventral mesencephalic cell densities for MAP-2/TH positive cells, at the sixth day of culture, 24h post-incubation with BMSCs, ASCs or HUCPVCs CM 24h. Immunocytochemical data revealed that ASCs CM 24h was able to increase the percentage of MAP-2/TH positive cells when compared with both the control/NBM (A, B, D, $p < 0.05$), and VMCs incubated with HUCPVCs CM 24h (A, B, D, E, $p < 0.05$). For all the other CM tested conditions no significant statistical differences were found when compared to the control (A, B, C, E, $p > 0.05$). Therefore, these results show

that the secretome of ASCs 24h induced a greater survival of mature ventral midbrain DAergic cells when compared to the control, and further indicate that the secretome of BMSCs, ASCs and HUCPVCs probably has distinct compositions that may be the responsible for the differential effects observed in VMCs survival (Values are shown as mean ± SD, n = 3, statistical significance was defined as $p < 0.05$).

To further understand their potential therapeutic role in PD, we investigated the effects of BMSCs, ASCs and HUCPVCs secretome in an *in vivo* neurotoxin-induced model of dopaminergic neurodegeneration. For this purpose, we have unilaterally injected 6-OHDA, in the middle forebrain bundle of Wistar-Han rats to induce the neurodegeneration of the nigrostriatal pathway. The latter is a well-established protocol that is considered to better mimic the extent of neurodegeneration observed in humans with later stage PD, that corresponds to the time that PD motor symptoms appear, and is known to cause several lateralized motor impairments in rats skilled limb performance in motor tasks (Cenci et al. 2002, Deumens et al. 2002). To determine whether the unilaterally 6-OHDA injected animals' exhibited lateralized deficits, animals' were tested for skilled forelimb motor impairments for a period of four days through the staircase test. This test is useful to assess the lateralized deficits induced by the unilateral 6-OHDA lesion in the MFB, once forelimb skills of the animals' is dependent on a balance in dopamine transmission (Cordeiro et al. 2010).

Results regarding the forelimb motor skills of animals' under bilateral conditions [Figure 2 and Table 1 (annexes section)] revealed that animals' treated with 6-OHDA exhibited a significant decrease in the ability to use the contralateral paw to retrieve and eat the sugar pellets, at the fourth day of testing, when compared with animals' injected with saline (Figure 2 A: $F_{1.78} = 5.669$, $p= 0.0249$; *post-hoc*: $p < 0.01$). This result was indicative of animals' acquisition of skilled limb motor impairments post-6-OHDA lesion. To better determine whether animals' injected with 6-OHDA exhibited forelimb motor deficits in the contralateral paw, animals' forelimb motor performance was tested under forced choice conditions using the same pellet configuration. Results confirmed that injured animals' displayed a significant impairment of forelimb motor skills when compared to sham animals' (Figure 2 C, $p < 0.05$). This result is in accordance with the data published by Cordeiro and colleagues (Cordeiro et al. 2010). In contrast with these results, analysis of animals' ipsilateral forelimb motor skills on the staircase test under both bilateral and unilateral conditions (Figure 2 and table 1), showed that the lesion did not affect the forelimb motor performance of the animals' (Figure 2 B and table 1: $F_{1.78} = 0.0641$, $p = 0.8020$; *post-hoc*: $p >$

0.05; Figure 2 D: $p > 0.05$). Indeed, as it can be observed in figure 2), no statistically significant differences between the 6-OHDA and sham groups (B, D, $p > 0.05$) were found in animals' ipsilateral paw performance, either in bilateral or unilateral staircase test.

The obtained behavioral results were within the expected, as rats injected with 6-OHDA unilaterally into the MFB typically exhibit skilled forelimb reaching performance impairment in the contralateral paw (Jeyasingham et al. 2001, Cordeiro et al. 2010).

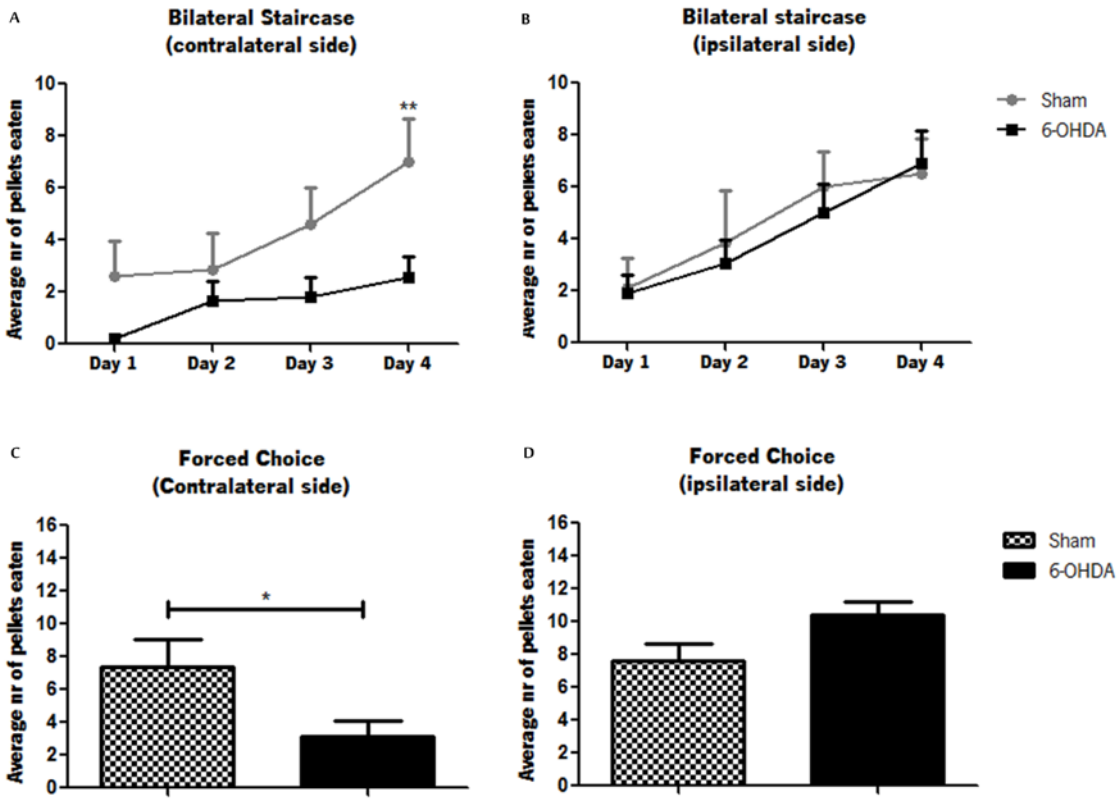

Figure 2: Effects of 6-OHDA injection in bilateral and unilateral staircase test (A to D). Evaluation of the forelimb skills of animals' under bilateral conditions showed that at the fourth day of the staircase test, animals' injured with 6-OHDA (n = 20) presented a significant decrease in the ability of using the contralateral paw to retrieve and eat sugar pellets when compared to the control animals' (Sham group, n = 8) (Figure 2 A, $p < 0.01$). Similarly, unilateral (forced choice) staircase test (Figure 2 C, D) also revealed that injured animals' displayed a significant impairment of the contralateral forelimb skills when compared to sham animals' (C, $p < 0.05$), as assessed by the number of pellets eaten by each group of animals'. In contrast, no statistically significant differences were found in animals' ipsilateral paw performance, either in bilateral or unilateral staircase test, between the 6-OHDA and sham groups (B, D, $p > 0.05$). Herein, results showed that, as expected, unilaterally 6-OHDA injured animals' presented the typical skilled forelimb reaching performance impairment in the contralateral paw (Values are shown as mean ± SEM, statistical significance was defined as $*p < 0.05$, $**p < 0.01$).

Upon confirmation of the injury, the secretome of BMSCs, ASCs and HUCPVCs were independently injected in both the SNpc and the striatum of animals' right hemisphere. To assess animals' motor behavior post-operatively, animals' were tested one week post-MSCs CM delivery and every two weeks thereafter (for a total period of 7 weeks) using the staircase test, both under bilateral and forced choice conditions. Although the bilateral staircase test provides separate measures of the animals' forelimb skills, it does not avoid animals' lesion-induced forelimb preference to use the paw ipsilateral to the lesion to reach, retrieve and eat the sugar pellets. Therefore, results are expressed as the average number of pellets eaten by each group of animals' under forced choice conditions, which allows a more sensitive measure of unilateral injured animals' motor skills.

Results revealed that the secretome of MSCs isolated from different sources induced different effect on animals' motor behavior (Figure 3 and Table 1). Results showed that only the group of animals' treated with BMSCs CM 24h presented a significant increase in motor performance 7weeks post-delivery of BMSCs secretome when compared with the control group [Table 1 and Figure 3A: $F_{1,32} = 7.473$, $p = 0.0257$; *post-hoc* (7^{th} week): $p < 0.05$]. Additionally they have also presented a significant increase on the number of pellets eaten on weeks 1, 3 and 7, ($p < 0.05$) respectively, when compared to the values obtained before the injection of the secretome. In contrast, and as expected, results regarding comparisons between the different MSCs CM 24h and control groups showed that the better ipsilateral motor performance exhibited by the animals' was not affected, in any case, by the MSCs (Table 1 and Figure 3, B: $F_{1,32} = 3.158$, $p = 0.1135$; D: $F_{1.28} = 3.841$, $p = 0.0908$; F: $F_{1,24} = 1.546$, $p = 0.2601$; *post-hoc* for all comparisons: $p > 0.05$).

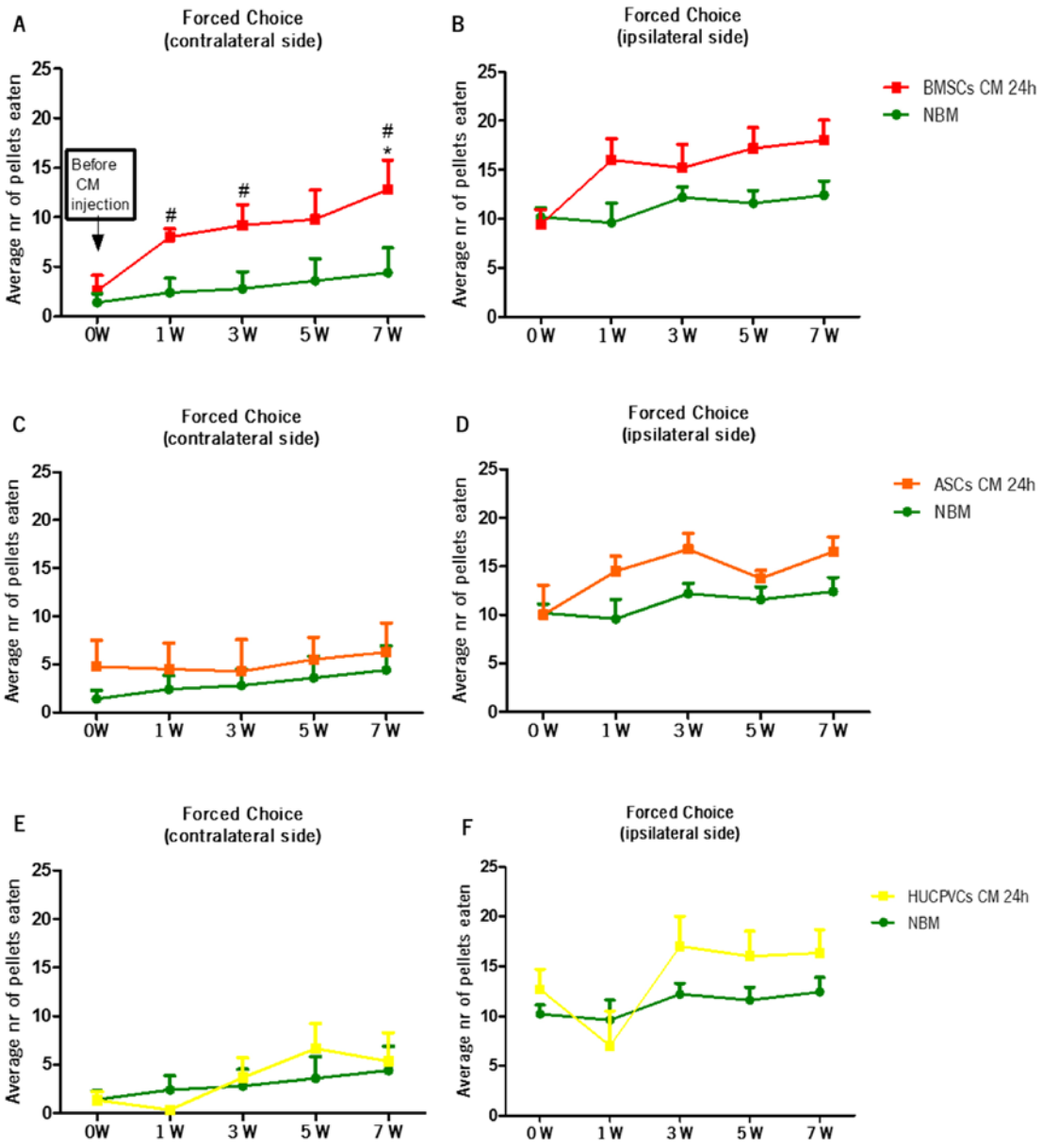

Figure 3: Effects of MSCs CM injection in the unilateral staircase test (A to F). Analysis of the forelimb skills of animals' under unilateral (forced choice) conditions showed that seven weeks post-MSCs CM delivery, animals' treated with BMSCs CM 24h (A, n = 5) exhibited a statistically significant effect on the improvement of the motor skills of 6-OHDA injected animals' when compared with the control (n = 5, $p < 0.05$). Additionally it was also observed that after 1, 3 and 7 weeks there was a statistically significative increase on the number of pellets eaten, when compared to the start of the experimental protocol. As expected, BMSCs CM 24h (B, n = 5), ASCs CM 24h (D, n = 4) or HUCPVCs CM 24h (F, n = 3) treatment did not affect the motor performance of animals' regarding the ipsilateral paw skills to retrieve and eat sugar pellets (Values are shown as mean ± SEM). Symbols correspondence to statistical signification: (1) ∗ refers to comparisons on the number of pellets eaten between BMSCs CM 24 and NBM; (2) # regards the correlation between the number of pellets eaten between 1,3 and 7 weeks and the start of the experimental protocol (* $p < 0.05$, # $p < 0.05$).

In order to determine if the observed animals' forelimb motor behavior post-MSCs CM injection could be associated with histological data, a quantitative analysis of the TH immunoreactivity in the SNpc and in the striatum of animals' brains was carried out seven weeks post-MSCs secretome delivery. Quantification of TH positive cell densities (Figure 4) demonstrated that animals' treated with BMSCs CM 24h presented a significant higher survival of TH positive cell bodies when compared with control animals' (Figure 4 A, B,C, $p < 0.05$).

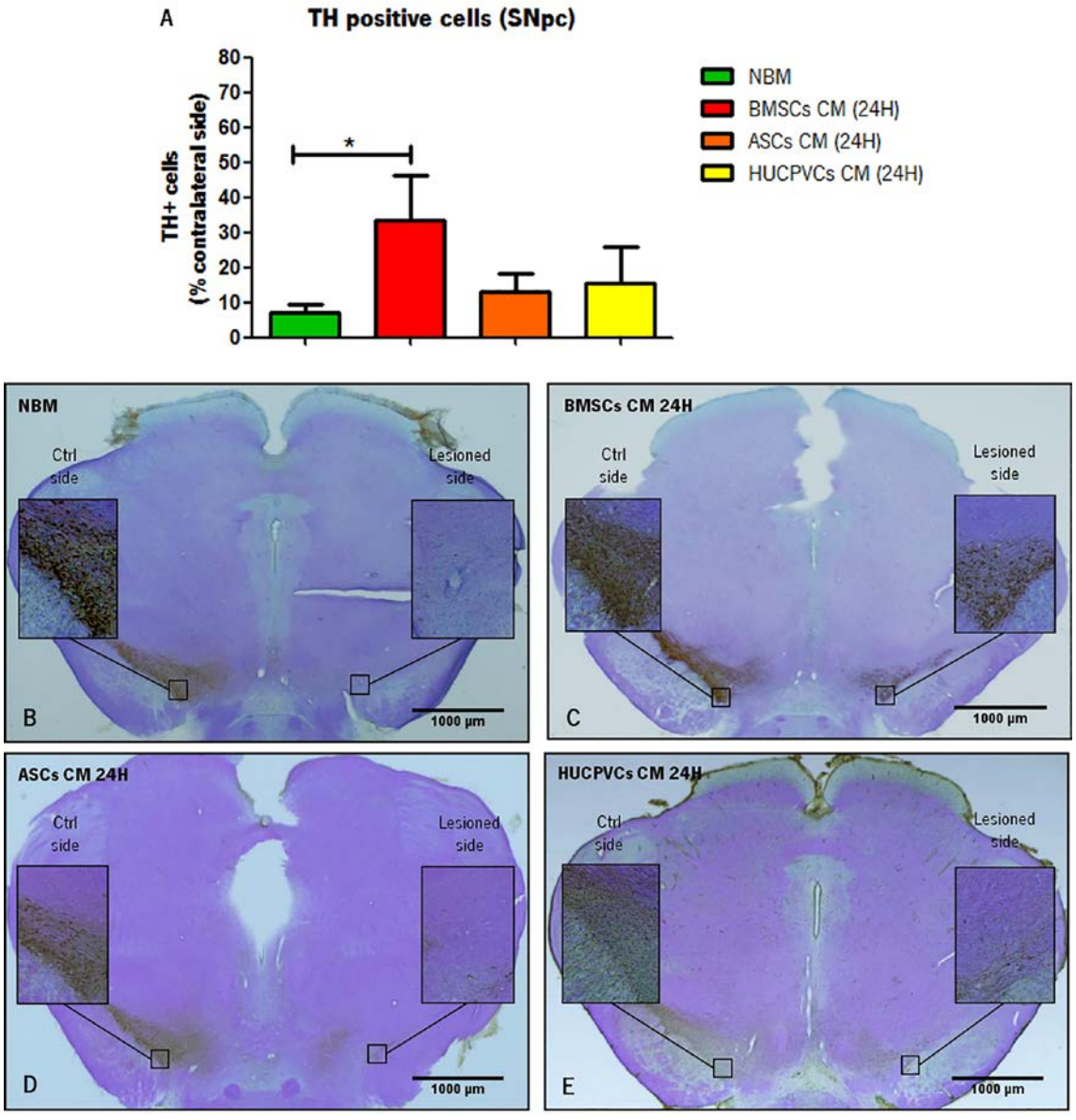

Figure 4: SNpc cells densities for TH positive cells seven weeks post-injection of BMSCs, ASCs and HUCPVCs CM (A to E). Immunohistochemistry assessment revealed that BMSCs CM 24h injection in hemiparkinsonian animals' significantly increased DAergic nigral cell densities when compared with the control animals' (A, B, C, $n = 4$, $p < 0.05$). For ASCs ($n = 4$) and HUCPVCs ($n = 3$) CM treatment conditions, no significant statistical differences were found (A, B, D, E, $p > 0.05$). Therefore, these results show that BMSCs CM 24h was the only treatment condition that

was able to significantly induce nigral DAergic neurons survival. (Values are shown as mean ± SEM, statistical significance was defined as * $p < 0.05$).

On the other hand, assessment of TH fiber densities in the striatum (Figure 5) showed that although animals' treated with BMSCs CM 24h presented a more pronounced density of TH positive fibers in the striatum, no statistically significant differences were found (Figure 5 A, B, C, $p > 0.05$).

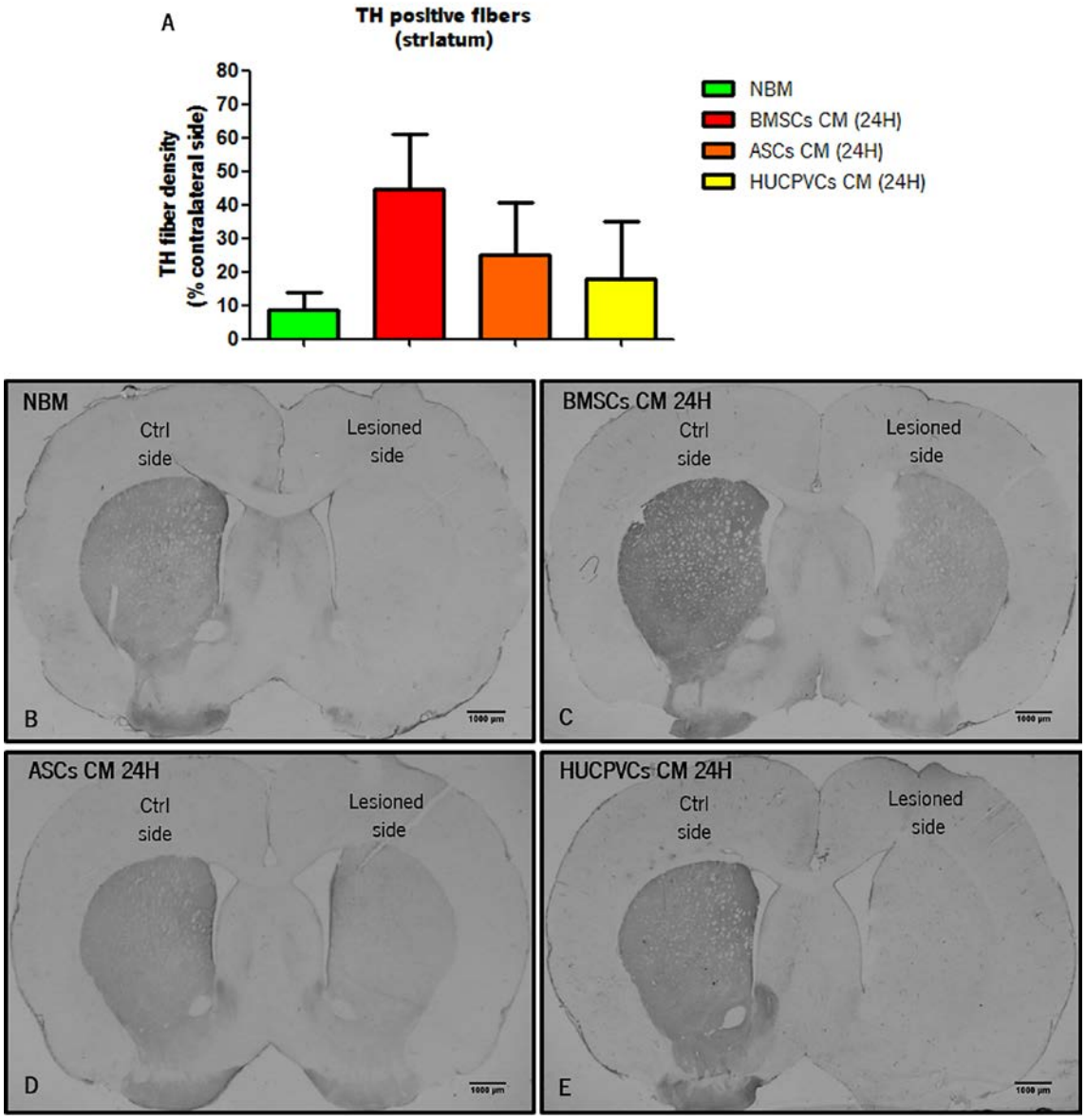

Figure 5: TH immunoreactivity of striatum fibers seven weeks post-injection of BMSCs, ASCs and HUCPVCs CM (A to E). Results of immunohistochemical analysis showed no statistically significant differences in striatal TH positive densities between BMSCs CM 24h (n = 4), ASCs CM 24h (n = 4) or HUCPVCs (n = 3) CM 24h and the NMB control (n = 4) group (A to E) (Values are shown as mean ± SEM, statistical significance was defined as $p < 0.05$).

Taken together, histological data revealed that the secretome of BMSCs, but not the one of ASCs or HUCPVCs, was able to significantly induce the survival of SNpc DAergic neurons when compared to the NBM control group. Moreover, results showed that the higher spare of TH positive nigral cell bodies seven weeks after animals' treatment with BMSCs CM 24h (Figure 4 A, B, C) was accompanied by a significant recovery of animals' contralateral forelimb deficits (Figure 3 A), when compared with control animals'. This recovery could be correlated with BMSCs CM-induced neuroprotection and/or neurorecovery of nigral DAergic neurons against 6-OHDA-induced neurodegeneration. Indeed this is a topic that should be further addressed in the future.

4. Conclusions

In present study we have performed for the first time a comparative study on how the secretome of different tissue derived MSCs, namely BMSCs, ASCs and HUCPVCs, could independently impact in the survival of intact cultures of mature ventral midbrain DAergic neurons and in a 6-OHDA unilateral animal model of PD. Results showed that the different tissue derived MSCs had a different impact on the survival of ventral midbrain DAergic neuronal populations *in vitro* and *in vivo*, which suggests that the secretome of different tissue derived MSCs exhibit distinct biomolecular cues. ASCs secretome had a greater impact in VMCs survival, while the secretome of BMSCs was the one which has induced a significant increase in SNpc DAergic neurons survival *in vivo.* Importantly, the observed increase in the nigral TH positive cell densities was accompanied by a significant improvement of animals' forelimb motor deficits. In summary, the behavioral and histological data obtained in this work showed that the BMSCs secretome alone was able to promote both the survival of nigral DAergic neurons and the functional recovery of animals' forelimb motor impairments. As so, BMSCs secretome may be considered as a promising cell-free therapeutic candidate for future treatment of PD.

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Chapter 4

A comparative Proteomics Analysis on the Secretome of Bone Marrow, Adipose Tissue, and Wharton's Jelly derived Mesenchymal Stem Cells (Manuscript status: to be submitted)

A comparative Proteomics Analysis on the Secretome of Bone Marrow, Adipose Tissue, and Wharton's Jelly derived Mesenchymal Stem Cells

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Abstract

Recent studies have revealed that although the secretome of different tissue derived MSCs populations, namely derived from bone marrow (BMSCs), adipose tissue (ASCs) and the Wharton's jelly of the umbilical cord (WJ-MSCs/HUCPVCs), exhibit phenotypical similarities and pro-regenerative potential, there are also important differences within its composition. So far, most of these studies make use of targeted proteomics approaches, such as Enzyme-Linked Immunosorbent Assay (ELISA) and multiplex immunological techniques to characterize the proteins present in the CM. Considering the vast functional therapeutic capabilities attributed to MSCs it has become clear that, besides the classical growth factors and cytokines released by MSCs, there are other proteins that might be related with neuroprotective/neuro-reparative profile of MSCs secretome. In the present work, an exhaustive proteomics analysis based on liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) (LC-MS/MS) following information dependent and SWATH (sequential windowed data independent acquisition of the total high-resolution mass spectra) acquisitions was carried out. The latter analysis was conducted to identify and quantify the expression of all the proteins present in MSCs CM derived from BMSCs, ASCs and HUCPVCs that could be involved in neuroprotection, neuro-reparative and neurodifferentiation phenomena. For this purpose, the CM from 3 different donors of ASCs and HUCPVCs, as well as, from 2 donors of BMSCs in passage six, were collected in serum-free media following strict and equal controlled culture conditions. Following this, the MSCs CM was concentrated, and the protein samples were submitted to precipitation and proteolysis before the LC-MS/MS was carried out. Our results revealed that BMSCs, ASCs and HUCPVCs secrete neurotrophic, neurogenic, axon guidance, axon growth and neurodifferentiative proteins, as well as, proteins with neuroprotective character against oxidative stress, apoptosis, excitotoxicity, inflammation, glial scarring and toxic protein deposition, which have been shown to be involved in several CNS disorders/injuries. Moreover, our results suggest that the secretome of different tissue derived MSCs may have a different impact in protecting against the above mentioned pathogenic processes and also in inducing neurite outgrowth and neuron differentiation as mediators of neuron repair.

1. Introduction

Mesenchymal stem cells (MSCs) are considered potential candidates for future applications in central nervous system (CNS) regenerative medicine. Nowadays, it is commonly accepted that the secretome of MSCs plays a crucial role in mediating several cell processes that contribute for CNS protection and/or regeneration in different CNS pathological conditions. Indeed, preclinical *in vitro* (Mackay et al. 2003, Kamei et al. 2007, Shintani et al. 2007, Wright et al. 2007, Sadan et al. 2009a, Sadan et al. 2009b, Fuhrmann et al. 2010, Gu et al. 2010, Nakano et al. 2010, Wang et al. 2010, Egashira et al. 2012) and *in vivo* (Li et al. 2002, Mackay et al. 2003, Lu et al. 2005, Neuhuber et al. 2005, Himes et al. 2006, Weiss et al. 2006, Ding et al. 2007, Kang et al. 2007, Shintani et al. 2007, Koh et al. 2008, McCoy et al. 2008, Yang et al. 2008a, Sadan et al. 2009a, Sadan et al. 2009b, Cova et al. 2010, Hu et al. 2010, Lee et al. 2010a, Wakabayashi et al. 2010, Wang et al. 2010, Arboleda et al. 2011, Lin et al. 2011, Zilka et al. 2011, Egashira et al. 2012, Kim et al. 2012b, Bobkova et al. 2013) studies conducted so far showed that the growth factors and cytokines secreted by different MSCs populations, namely those isolated from bone marrow (BMSCs), adipose tissue (ASCs) and the Wharton jelly surrounding the vessels of the umbilical cord [(WJ-MSCs/human umbilical cord perivascular cells (HUCPVCs)], are able to: 1) promote neuronal survival and neurite outgrowth, 2) increase levels of neurogenesis and angiogenesis; 3) inhibit apoptosis and scarring, 4) modulate immune response, and 5) improve functional outcomes in different models of CNS injury and disease, such as brain ischemia, spinal cord injury (SCI), Parkinson's disease (PD) and Alzheimer's disease (AD). In addition, these studies also revealed that these MSCs secretome-mediated cell processes contribute for the improvement of animals' functional recovery upon MSCs transplantation.

Nevertheless, although these different MSCs populations share similar phenotypical characteristics (Chamberlain et al. 2007, Shetty et al. 2010, Nakanishi et al. 2011, Ong and Sugii 2013) and exhibit pro-regenerative potential, they reside in different anatomic parts of the body and, therefore, it is most likely that they present differences within their secretome. In fact, Ribeiro and colleagues (Ribeiro et al. 2012) conducted a screening on the presence of some neuronal survival and differentiation growth factors in ASCs and HUCPVCs secretome, through an antibody-based multianalyte Bio-Plex platform analysis, and revealed important differences in the secretome (in the form of conditioned media/CM) composition between these two populations. Indeed, While ASCs CM was positive for the presence of hepatocyte growth factor (HGF), vascular endothelial

growth factor (VEGF), stem cell factor (SCF) and nerve growth factor (NGF), only NGF and VEGF were detected in HUCPVCs CM. On the other hand, Nakanishi *et al.* (Nakanishi et al. 2011) making use of Enzyme-linked immunosorbent assay (ELISA) to investigate differences between the secretome of rat derived ASCs and BMSCs, demonstrated that there were significant differences in the growth factors and cytokines secreted between these two MSCs populations. In this work, Nakanishi and co-workers showed that while ASCs secreted higher amounts of angiogenenic and anti-apoptotic growth factors, like HGF and VEGF, as well as, interleukin-6 (IL-6), BMSCs secreted larger amounts of the cell migration-related chemokine stromal cell-derived factor 1 alpha (SDF-1α). Hsieh *et al.* (Hsieh et al. 2013), using the same approach as Nakanishi and co-workers (Nakanishi et al. 2011), also revealed that although both supernatants collected from WJ-MSCs and BMSCs contained angiogenesis-related factors, the secreted factors were distinct. In fact, Hsieh and co-workers showed that while the CM from WJ-MSCs contained significantly higher amounts of angiogenesis promoter C-X-C motif chemokine ligand 5 (CXCL5), BMSCs CM contained higher amounts of placental growth factor (PGF), a pivotal molecule in mediating angiogenesis and vasculogenesis (Hsieh et al. 2013).

Nevertheless, although all of the above referred studies were extremely important to demonstrate that there are important differences among the secretome of different MSCs populations, the fact is that so far, only targeted proteomic approaches have been used for this purpose. As so, these studies provide only a short overview of the soluble factors secreted by MSCs. Given the vast panel of functional roles attributed to MSCs in mediating paracrine actions through the release of soluble factors and vesicles that ultimately contribute for CNS repair, a more detailed based proteomics approach would better clarify the potential complexity of MSCs secretome. In this sense, a shotgun/discovery proteomics-based approach in which LC- is used to identify and quantify proteins present in MSCs secretome, offers a more broad knowledge of the MSCs secreted proteins. In fact, Fraga and colleagues (Fraga et al. 2013), making use of LC-MS/MS, were able to detect, identify and quantify the expression of several proteins within the HUCPVCs secretome (in the form of CM) that, up to date, were not known to be secreted by these cells. Importantly, in this study, the authors identified proteins, such as 14-3-3, ubiquitin-carboxy-terminal hydrolase 1 (UCHL1), heat shock protein 70 and peroxiredoxin-6, that had been previously shown to mediate neuronal cell survival/protection, proliferation and differentiation phenomena (Fraga et al. 2013).

Having this in mind, the objectives of the present work were: to: 1) identify and quantify the expression of proteins with neuroregulatory character, in addition to the reported classical growth factors and cytokines released by different tissue derived MSCs, that might be related with the MSCs secretome-mediated processes that contribute for neuroprotection, neuron repair and neurodifferentiation, and 2) evaluate at what extent the secretome of different MSCs populations can diverge in neuroprotection, neuron repair and neurodifferentiation phenomena. For this purpose, the CM from 3 different donors of ASCs and HUCPVCs, as well as, from 2 donors of BMSCs was collected, after MSCs culture in strict controlled conditions, and subjected to an exhaustive shotgun proteomic analysis based on LC-MS/MS to identify the maximal number of proteins present in the MSCs CM. This analysis was further complemented with SWATH-MS to accurately quantify both the proteins present at higher and lower abundance in the MSCs CM.

2. Materials and Methods

2.1 Mesenchymal stem cells culture

2.1.1 Bone marrow tissue derived stem cells

BMSCs were acquired from Stem cell Technologies (Grenoble, France). Cells were thawed and expanded according to the protocol previously described by Silva *et al.* (Silva et al. 2013). Briefly, BMSCs were cultured in α-MEM (Gibco, USA) supplemented with Sodium bicarbonate ($NaHCO_3$, Merck, USA), 10% of fetal bovine serum (FBS, Biochrom AG, UK) and 1% Penicillin-Streptomycin antibiotic (Gibco). After reaching confluence, cells were trypsinised, plated at a density of 4.000 cells/cm^2 in new tissue culture flasks (Nunc, Denmark) and incubated at 37°C in a 5% humidified CO_2. The culture medium was changed every two to three days. BMSCs were used for experiments during passage 6 (P6).

2.1.2 Human umbilical cord perivascular cells

HUCPVCs were kindly provided by Professor J. E. Davies (University of Toronto, Canada). Cells isolation from umbilical cord was performed according to the procedures described by Sarugaser

and co-workers (Sarugaser et al. 2005). Expansion of cells was performed according with the protocol described above for BMSCs. HUCPVCs were used for experiments during P6.

2.1.3 Adipose tissue derived stem cells

ASCs were kindly provided by Professor J. M. Gimble (University of Tulane, USA). Cells isolation from adipose tissue was performed according to the previously described procedures reported by Dubois and colleagues (Dubois et al. 2008). Expansion of cells was performed according with the protocol described above for BMSCs. ASCs were used for experiments during P6.

2.2 Conditioned media collection and concentration

CM from 3 different donors of ASCs and HUCPVCs, as well as, from 2 donors of BMSCs was collected from P6 MSCs as follows: cells were plated at a density of 12.000 cells/cm^2 in T175 tissue culture flasks (Nunc) and allowed to grow for 3 days in 5% humidified CO_2 incubator, at 37°C. Cells were then washed five times with neurobasal serum-free media (Gibco), to remove the FBS used for MSCs culture, and were thereafter incubated with neurobasal media supplemented with 1% of Kanamycin sulfate (Gibco). After 24h, MSCs CM was collected. BMSCs 24h, ASCs 24h and HUCPVCs CM 24h were then 100× concentrated by centrifugation through the use of ultrafiltration spin columns (Vivaspin) with 5 kDa cut-off polyethersulfone membranes by centrifugation, according to manufacturer's guidelines (Sartorius Stedim Biotech, Goettingen, Germany). All Collected CM were frozen and thawed only in the day of experiments.

2.3 LC-MS/MS

2.3.1 Sample preparation for LC-MS/MS

2.3.1.1 MSCs CM protein precipitation

Previously concentrated MSCs CM 24h were precipitated with trichloroacetic acid (TCA, Sigma, USA)-Acetone (Sigma) (Manadas et al. 2006). Briefly, samples were incubated at -80°C with TCA [final concentration of 20% (v/v)] for 30min and centrifuged (20,000g) for 20 min at 4°C. Protein

pellets were then solubilized in ice-cold (-20°C) acetone (Sigma), aided by ultrasonication (VC750, Vibracell-Sonics&Materials, USA), and centrifuged (20,000g) for 20min. The obtained pellets were thereafter ressuspended in triethylammonium bicarbonate buffer (TEAB, 1M, Sigma) aided by ultrasonication and centrifuged (20,000g) for 5min to remove the insoluble material.

2.3.1.2 Protein digestion and sample cleanup

For liquid digestion, samples were reduced by the addition of 4 µl of tris(2-carboxymethyl)phosphine (TCEP, 50mM, Sigma] to 45 µl of each sample followed by a ultrasonication step for 2 min. Then, 2 µl of the cysteine blocking agent methanethiosulfanate (MMTS, 600mM, Sigma) were added and samples were allowed to react for 10min at room-temperature. Following adjustment of samples volume with TEAB (final volume of 100 µl), samples were digested into peptides with trypsin (2 µg/sample) in a thermomixer (Eppendorf AG, Germany) at 560rpm, 37 °C and over-night. Formic acid (FA, 2 µl, Amresco, USA) was added to stop protein digestion and the resulting peptides were dried (rotatory evaporation) under vacuum. Samples were spiked with green fluorescent protein (GFP, 2 µg) prior to protein digestion in order to evaluate samples loss during sample processing. After proteins digestion, samples were cleaned/desalted through the use of bond elut OMIX pipette tips containing C18 stationary phase (Argilent technologies, USA), according with the manufacturer's instructions. Eluted peptides spiked with iRT peptides (Biognosys AG, Switzerland) were dried (rotator evaporation) and ressuspended in a mobile phase containing 0.1% FA and 2% of acetonitrile (ACN) aided by ultrasonication (20% intensity). To remove insoluble material, samples were centrifuged (14,000g; 5min) prior to the LC-MS/MS analysis.

2.3.2 Protein identification and quantification by LC-MS/MS

For LC-MS/MS, peptide samples were first separated by liquid chromatography, on the basis of peptides hydrophobicity, using for this purpose a C18 AR reverse phase column (ChromXP, 300 µm inner diameter with 15 cm length, 3 µm particle size, 120 Å pore size; Eksigent, USA). Samples were resolved at 5µl/min and eluted from the column, using a 25min ACN linear gradient (from 2 to 35%) in 0.1% FA, into the mass spectrometer (Triple TOF 5600 system, AB SCIEX, USA) through

and electrospray ionization source (DuoSpary, AB SCIEX). For tandem mass spectrometry (MS/MS) analysis, samples were analyzed in two phases. First, each sample was analyzed with the mass spectrometer operating in information-dependent acquisition (IDA) to detect and identify the maximal number of proteins within sample mixtures. Then, samples were analyzed using the SWATH acquisition method, which allows the detection and an accurate quantification of the identified proteins. These two methods differ in the mass spectrometric operating mode. Specifically, when the MS instrument operates in IDA mode, all the ions are first detected in a survey and then these precursor ions are selected/isolated based on their intensities for fragmentation. SWATH-MS is a data independent acquisition method that does not require the selection of precursor ions based on their intensity for acquisition of peptides fragmentation spectra. In this method, the MS operates by consecutively selecting peptides across several user-defined precursor ion isolation windows, after which all the ions within each window are fragmented, thereby allowing the acquisition of the fragment ion spectra of all precursor ions within each defined window (Gillet et al. 2012, Collins et al. 2013, Lambert et al. 2013).

For IDA, the mass spectrometer was set to scan full spectra of ions in the 350-1250 m/z range, during 250ms, followed by 20 ions fragmentation spectra (MS/MS) scans (100-1500 m/z range), with 1 MS/MS being acquired for 100ms before adding those ions to the exclusion list for 20 s). The selection/isolation criteria for ions fragmentation comprised intensity, where ions had to meet a minimum threshold of 70 counts/s with a charge state between +2 and +5. Ions were fragmented in the collision cell (rolling collision) using a collision energy spread of 5 eV.

Peptide identification was performed with Protein Pilot software (version 4.5, ABSciex®). Mass spectra were queried against a database composed by *human and bovine* species from SwissProt database (released in February 2014), GFP and iRT peptides sequences, considering the following criteria: trypsin and MMTS alkylated cysteines. To monitor the quality of identifications, an independent false discovery rate (FDR) analysis based on the target-decoy approach was performed using the Protein Pilot software and positive identifications were considered when the identified proteins and peptides achieved a 5% local FDR (Tang et al. 2008, Sennels et al. 2009).

For SWATH acquisition, the same chromatographic conditions as the ones chosen for IDA experiments were followed and the SWATH setup was set as reported by Gillett *et al.* (Gillet et al. 2012). Briefly, for SWATH-MS based experiments, the mass spectrometer was adjusted to specifically permit a quadrupole resolution of 25 m/z selection. For this purpose, the instrument

was set to isolate ions with a 26 m/z width, in a loop mode, over the precursor mass range of 350-1100 m/z and 30 overlapping windows were constructed. For instrument calibration, the survey scan was set to scan full spectra of peptide ions in the 350-1250 m/z range (50ms), at the beginning of each cycle. SWATH fragmentation spectra was collected from 100-1500 m/z using an accumulation time of 100ms for all fragment-ion scans, which resulted in a cycle time of 3.25s. For optimal fragmentation of precursors within the isolation windows, a 15 eV spread of collision was applied.

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 (version 4.5, ABSciex®) with the same parameters as described above. The generated library was used for SWATH data processing, using for this purpose the SWATH processing plug-in for PeakView (2.0.01 version, AB SCIEX). Briefly, peptides (up to 15 peptides were selected per protein) and target fragment ions (up to 5 target fragment ions), were automatically selected from the library, as previously described (Lambert et al. 2013). Peak groups from selected fragment ions belonging to each of the selected peptides were scored as described by Lambert *et al*. (Lambert et al. 2013). The peak group score of each peptide was obtained by combining all scores from fragment ions. The confidence of peak group was determined through the target-decoy approach and the presence of peptides was considered when peptides scores met a 1% FDR threshold. Finally, for quantification of protein levels, the peak areas of target fragment ions from the peptides, that were considered to be present, were extracted across SWATH-MS runs through the use of a 1.5min extracted ion chromatogram (XIC) window, the fragments from all the peptides belonging to a determined protein were summed (an adaptation of (Collins et al. 2013)) and normalized to the more stable internal standard.

Quantification results are expressed as the average protein intensity that corresponds to the relative protein intensity in proportion to the internal control (GFP). These are represented in the form of heatmap (Figure 1) generated through the use of the Graphical Proteomix Data Explorer (GProX) program (http://gprox.sourceforge.net/) (Rigbolt et al. 2011) and in the numerical form (Figures 2 to 6).

2.4. Statistical Analysis

Statistical Analysis was performed using one-way ANOVA followed by Bonferroni *post-hoc* test to assess statistical correlation among and between CM groups. This statistical analysis was further complemented with student's *t*-test to determine statistical correlation between CM groups (for statistical evaluation, 3 donors were used to quantify proteins present in HUCPVCs and ASCs CM, whereas 2 donors of BMSCs CM were used (n = 3/n = 2; CM time point ±SD). Statistical significance was defined as $P < 0.05$ for a 95% confidence interval.

3. Results and discussion

Upon LC-MS/MS and SWATH acquisition analysis, a heat-map (figure 1), representing the ratios of proteins present in MSCs CM was obtained. From this analysis, it was possible to identify and quantify 121 proteins present in the CM of the three MSCs populations in study. Among these 121 proteins, 20 proteins were found of particular interest in virtue of their roles in the recovery of CNS models (*in vitro* and *in* vivo) of injury and disease, and other processes such as neurite growth and/or neuronal survival and differentiation.

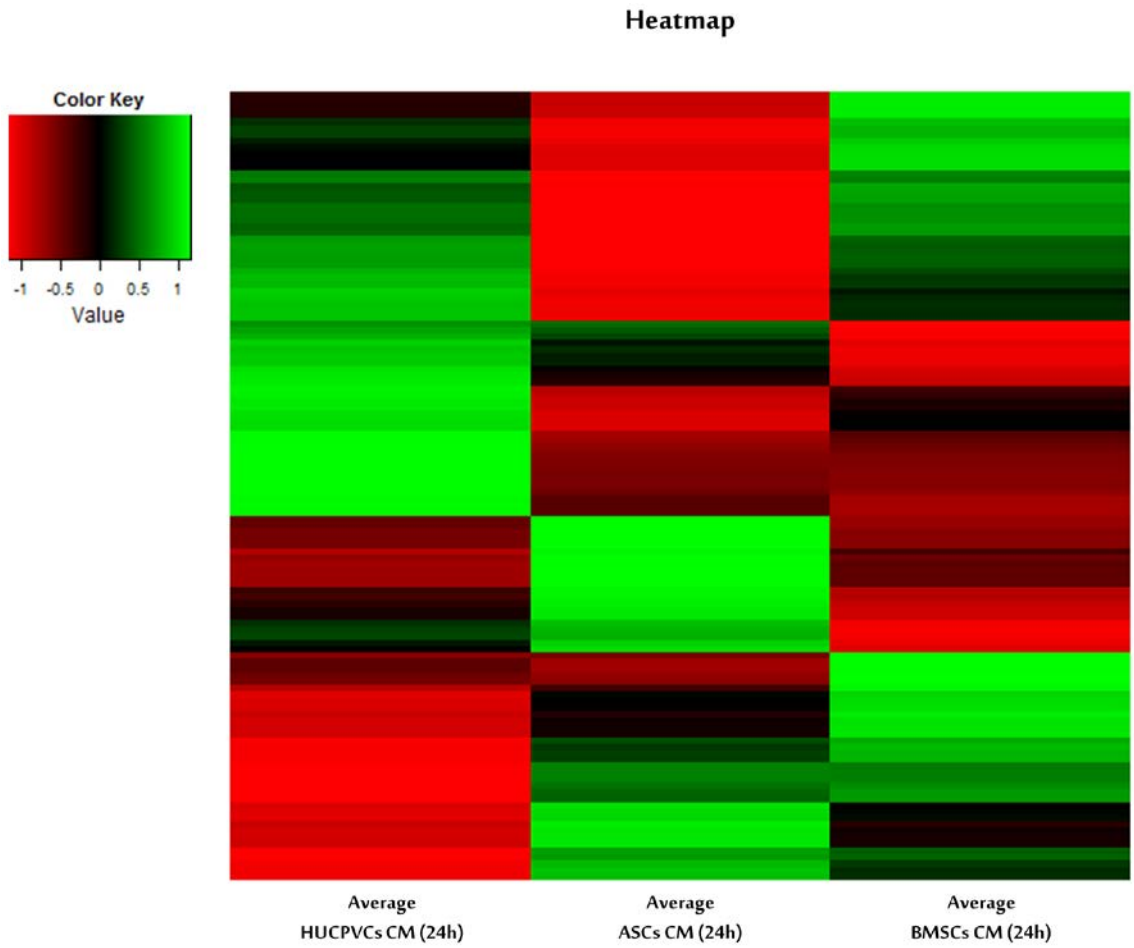

Figure 1: Heatmap representing the complete set of HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) expression of secreted proteins generated through the use of GProx. The protein intensity was quantified through LC-MS/MS and normalized to the internal control (GFP). Protein intensity representing the average protein intensity of each protein, of a total of 121 proteins secreted by HUCPVCs, ASCs and BMSCs is represented in colors (Color key: green: high intensity; black: middle intensity; red: low intensity). As it can be observed, the heatmap indicates that there are differences within the CM groups regarding protein expression between the secretome of MSCs derived from different sources.

From the 20 proteins considered of interest for this study, 11 (Cystatin C/CYSC, albumin serum/AS, Interleukin-6/IL-6, Pigment epithelium-derived factor/PEDF, Plasminogen activator inhibitor-1/PAI-1, Plasma protease C1 inhibitor/C1-Inh, Decorin /DCN, Clusterin/CLUS, Cadherin-2/CADH2, Semaphorin 7A/SEM7A and Glia-derived nexin/GDN) are typically known by their extracellular role (see cell component in table 1). All of them are characterized for the presence of an N-terminal signalling peptide, which is essential for proteins to be secreted through the endoplasmatic reticulum (ER)-golgi classical pathway (table 1). However, there are also reports that indicate that all of them can be exocitized through exosomes (Gonzales et al. 2009, Gonzalez-

Begne et al. 2009, Buschow et al. 2010, Welton et al. 2010, Atay et al. 2011, Inder et al. 2012, Tauro et al. 2012) or microvesicles (Skog et al. 2008, Hong et al. 2009) by different cell types. The other 8 proteins, known by their intracellular role (CyclophilinA/CYPA, CyclophilinB/CYPB; DJ-1, Thioredoxin/TRX, Peroxiredoxin-1/PRDX1, Heat shock protein 27/HSP27, UCHL1, and Brain acid soluble protein 1/BASP-1) have been reported to be secreted through exosomes (Gonzales et al. 2009, Gonzalez-Begne et al. 2009, Kesimer et al. 2009, Buschow et al. 2010) or microvesicles (Skog et al. 2008, Hong et al. 2009) by different cell types or tissues, including by human BMSCs in the form of microvesicles (Kim et al. 2012a) (table 1). Finally, Galectin 1 (Gal-1) is known to have both extracellular and intracellular roles (Camby et al. 2006) and has shown to be secreted through the same pathway as the latter 8 proteins (Gonzales et al. 2009, Gonzalez-Begne et al. 2009), including by human BMSCs (Kim et al. 2012a).

Table 1: Summary of proteins main cell component(s), secretory pathways by different cells or tissues and proteins already detected in MSCs secretome through LC-MS/MS analysis or immunological assays.

Protein	Main cell component	ER signal	Exossome/micro-vesicles	secretion cells/tissues	Refs	MSCs CM presence	Refs
Gal-1	Cytoplasm, EXT	NO	YES	T cells CCCs (m.v.); BCCs (ex)	(Yang et al. 2008b, Hong et al. 2009, Welton et al. 2010)	hBMSCs (m.v.)	(Kim et al. 2012a)
CLUS	EXT	YES	YES	RPE; CE	(Dota et al. 1999, An et al. 2006)	mBMSCs	(Li et al. 2010)
CADH2	membrane	YES	YES	GBCs (m.v.)	(Skog et al. 2008)	hBMSCs	(Choi et al. 2010)
SEM7A	EXT, membrane	YES	YES	U87 cells; PCCs (ex)	(Formolo et al. 2011, Inder et al. 2012)	hBMSCs	(Choi et al. 2010)
GDN	EXT, membrane	YES	YES	astrocytes; neurons; fibroblasts; CCCs (ex)	(Reinhard et al. 1994, Tauro et al. 2012)	hBMSCs, hASCs	(Choi et al. 2010, Lee et al. 2010b, Kim et al. 2012a)
BASP-1	membrane	NO	YES	TCBCs (ex)	(Kesimer et al. 2009)	hBMSCs (m.v.)	(Kim et al. 2012a)

Abbreviations: BCCs: Bladder cancer cells; CCCs: Colorectal cancer cells; CE: Corneal epithelium; ex: Exosomes; ER: Endoplasmatic reticulum EXT: Extracellular-meaning extracellular space or extracellular matrix; GBCs: Glioblastoma cells; hASCs: human ASCs; hBMSCs: human BMSCs; hWJ-MSCs: human WJ-MSCs; mBMSCs murine; m.v.: Microvesicles; NBCs: Neuroblastoma cells; RECs: Renal epithelial cells; RPE: Retinal pigment epithelium; RPECs: Retinal pigment epithelial cells; PCCs: Prostate cancer cells; PGCs: Parotid gland cells; TPBCs: Trophoblast cells; TCBCs: Tracheobronchial cells; VSCs: Vascular smooth cells.

Table 1: Summary of proteins main cell component(s), secretory pathways by different cells or tissues and proteins already detected in MSCs secretome through LC-MS/MS analysis or imunological assays. (continued)

Protein	Main cell component	ER signal	Exossome/micro-vesicles	secretion cells/tissues	Refs	MSCs CM presence	Refs
DJ-1	cytoplasm	NO	YES	RECs (ex); PGCs(ex)	(Gonzales et al. 2009, Gonzalez-Begne et al. 2009)	hBMSCs (m.v.)	(Kim et al. 2012a)
TRX	Cytoplasm	NO	YES	B cells (ex); RECs(ex)	(Buschow et al. 2010)	hBMSCs (m.v.)	(Kim et al. 2012a)
CYPA	Cytoplasm	NO	YES	VSCs; RECs (ex); PGCs (ex); B cells (ex)	(Gonzales et al. 2009, Gonzalez-Begne et al. 2009, Buschow et al. 2010)	hBMSCs (m.v.)	(Kim et al. 2012a)
CYPB	ER	YES	YES	Keratinocytes RECs (ex); PGCs (ex); B cells (ex)	(Gonzales et al. 2009, Gonzalez-Begne et al. 2009, Buschow et al. 2010, Fearon et al. 2011)	hBMSCs (m.v.)	(Kim et al. 2012a)
CYSC	Ext	YES	YES	RPECs; OGCs (ex), GBCs (ex)	(Gauthier et al. 2011)	hBMSCs	(Choi et al. 2010, Kim et al. 2013)
PRDX1	Cytoplasm	NO	YES	B cells (ex); PGCs (ex)	(Gonzalez-Begne et al. 2009, Buschow et al. 2010)	hBMSCs (m.v.)	(Kim et al. 2012a)
SA	Ext	YES	YES	Liver; PGCs (ex); B cells (ex)	(Gonzalez-Begne et al. 2009, Buschow et al. 2010, Prajapati et al. 2011)	hBMSCs (m.v.)	(Kim et al. 2012a)
HSP27	Cytoplasm; Nucleus	NO	YES	BCCs (ex); CCCs (m.v.)	(Hong et al. 2009, Welton et al. 2010)	hBMSCs (m.v.)	(Kim et al. 2012a)
IL-6	Ext	YES	YES	Astrocytes; PCCs (ex); GBCs (m.v.)	(Maeda et al. 1994, Skog et al. 2008, Inder et al. 2012)	hBMSCs, hASCs; hWJ-MSCs	(Nakanishi et al. 2011, Fong et al. 2012)
PEDF	Ext	YES	YES	CCCs (m.v.); BCCs (ex); NBCs (m.v.)	(Skog et al. 2008, Gonzales et al. 2009, Hong et al. 2009)	hBMSCs, hASCs	(Chiellini et al. 2008, Choi et al. 2010)
PAI-1	Ext	YES	YES	adipose tissue; astrocytes; TPBCS (ex)	(Morange et al. 1999, Docagne et al. 2002, Atay et al. 2011)	hBMSCs, hASCs	(Chiellini et al. 2008, Choi et al. 2010, Nakanishi et al. 2011)
UCHL1	Cytoplasm, ER	NO	YES	NBCs (m.v.)	(Skog et al. 2008)	hBMSCs (m.v.), hWJ-MSCs	(Kim et al. 2012a)
C1 Inh	EXT	YES	YES	Astrocytes; BCCs (ex);	(Veerhuis et al. 1998, Welton et al. 2010)	hASCs	(Zvonic et al. 2007)
DCN	EXT	YES	YES	Myofibroblasts; GBCs (m.v.)	(Honda and Munakata 2004, Skog et al. 2008)	hBMSCs, hASCs; hWJ-MSCs	(Chiellini et al. 2008, Choi et al. 2010, Arufe et al. 2011)

For discussion purposes, it should be highlighted that some of these 20 MSCs secreted proteins have been shown to play roles in more than one biological process and some of them appear represented more than one time in the results presented in graphs in the following subsections.

3.1 Proteins involved in CNS protection and/or regeneration

The results from LC-MS/MS analysis revealed the presence of 16 proteins within the MSCs CM, which have been shown to play protective roles either against oxidative stress (Bai et al. 2002, Gum et al. 2004, Hattori et al. 2004, Inden et al. 2006, Boulos et al. 2007, Lee et al. 2008, Yanagisawa et al. 2008, Ge et al. 2009, Oh et al. 2011, Ma et al. 2012, Teramoto et al. 2013, Shimada et al. 2014), apoptosis (Matsuda et al. 1996, Loddick et al. 1998, Xu et al. 2005, Yamashita et al. 2005, Ge et al. 2009, Qu et al. 2010, Tizon et al. 2010a, Tizon et al. 2010b, Oh et al. 2011, Teramoto et al. 2013, Shimada et al. 2014), excitotoxicity (Toulmond et al. 1992, Yamada and Hatanaka 1994, Taniwaki et al. 1997, DeCoster et al. 1999, Docagne et al. 2002), abnormal proteasomal degradation (Gong et al. 2006, Setsuie and Wada 2007), inflammation (De Simoni et al. 2003, Heydenreich et al. 2012), glial scarring (Davies et al. 2004, Davies et al. 2006, Minor et al. 2008, Ahmed et al. 2014) and toxic protein deposition (Boggs et al. 1996, Yerbury and Wilson 2010) pathogenic processes, or to induce neurogenesis (Ishibashi et al. 2007, Tian et al. 2014) in CNS disorders/injuries.

Regarding **oxidative stress**, studies indicate that 8 of the found proteins, namely DJ-1, TRX, CYPA, CYPB, CYSC, PRDX1, AS and HSP27 work as anti-oxidative factors (Bai et al. 2002, Gum et al. 2004, Hattori et al. 2004, Inden et al. 2006, Boulos et al. 2007, Lee et al. 2008, Yanagisawa et al. 2008, Ge et al. 2009, Oh et al. 2011, Ma et al. 2012, Teramoto et al. 2013, Shimada et al. 2014). In fact, **DJ-1**, which is a multifunctional protein from the peptidase C56 family of proteins, has been demonstrated to play a protective role against oxidative stress-induced cell death *in vitro* upon exogenous addition to culture media. Similar evidences were found in *in vivo* models of PD and focal cerebral ischemia after intranigral or intrastriatal injection, respectively (Inden et al. 2006, Yanagisawa et al. 2008). Regarding **TRX**, which is a cytoplasmatic redox-active protein, at least three studies indicate that it may act as neuroprotective both in an *in vitro* model of PD (Bai et al. 2002) and in *in vivo* rodent models of transient focal ischemia (Hattori et al. 2004, Ma et al. 2012) through its antioxidant function upon overexpression and/or administration (exogenous or

intravenous/i.v.) of human recombinant (hr) TRX. **CYPA and CYPB** are proteins from the immunophilin family of peptidyl-prolyl cis-trans isomerases (Galat 1993) whose role in CNS remains largely unknown. Nevertheless, they have already shown to act as neuroprotectants against amyloid beta (Aβ)-induced neurotoxicity upon overexpression and/or administration in pheochromocytoma PC12 cells (a cell line susceptible to Aβ) or cortical neurons through suppression of reactive oxygen species (ROS) formation (Boulos et al. 2007, Ge et al. 2009, Oh et al. 2011). **CYSC,** a cysteine protease inhibitor, has also been demonstrated to protect cortical neurons against hydrogen peroxide (H_2O_2)-induced oxidative stress upon exogenous administration of rh-CYSC (Tizon et al. 2010b). In the case of **PRDX1**, as a cytoplasmatic thioredoxine-dependent peroxidase reductase, its overexpression in a dopaminergic (DAergic) neuronal cell line has shown to counteract 6-OHDA-induced DAergic cell death by acting as ROS (superoxide anion and H_2O_2) scavenger (Lee et al. 2008). Human **SA**, a multifunctional protein and the most abundant protein in plasma, as antioxidant, has been found to reduce the death of cortical neurons induced by H_2O_2 and copper/ascorbic acid oxidants after administration in culture medium (Gum et al. 2004). Finally, **HSP27** from the subfamily of small HSP, which is mostly known by its role in providing thermotolerance to cells (Landry et al. 1989) and chaperone activity in proteins (Ellis 1990), has demonstrated to attenuate ischemic brain damage in an *in vivo* mouse model of focal cerebral ischemia after i.v. administration of human-derived physiological HSP27 by inhibiting oxidative stress (Teramoto et al. 2013, Shimada et al. 2014).

From these 8 above mentioned proteins, expression quantification results (Figure 2) revealed that 5 proteins, namely DJ-1, TRX, CYPA, CYPB and CYSC revealed statistically significant differences between the CM of MSCs populations, while no statistically significant differences were found among MSCs CM tested conditions concerning PRDX1, AS and HSP27. From these proteins, CYPB was the only protein found to be upregulated in BMSCs CM over HUCPVCs ($p < 0.05$) and ASCs CM ($p < 0.01$). Regarding comparisons between MSCs CM groups, results revealed that in addition to CYPB, CYPA was also significantly more expressed in BMSCs CM when compared ASCs CM ($p < 0.05$). Additionally, it was also found that DJ-1 was upregulated in BMSCs CM when compared with HUCPVCs CM ($p < 0.05$). Therefore, these results indicate that BMSCs secretome might have a better anti-oxidative profile than ASCs or HUCPVCs. On the other hand, upon comparison of proteic profile between HUCPVCs and ASCs CM, results showed that TRX and CYPA expressions were significantly elevated for the first cell population, whereas the opposite was observed regarding CYSC expression ($p < 0.05$). Nevertheless, as different protein intensities were found

between the tested groups, the functional outcomes of these analysis must be tested in future both *in vitro* and *in vivo*, in order to identify the possible roles on the neuro-protective character of these molecules in oxidative stress.

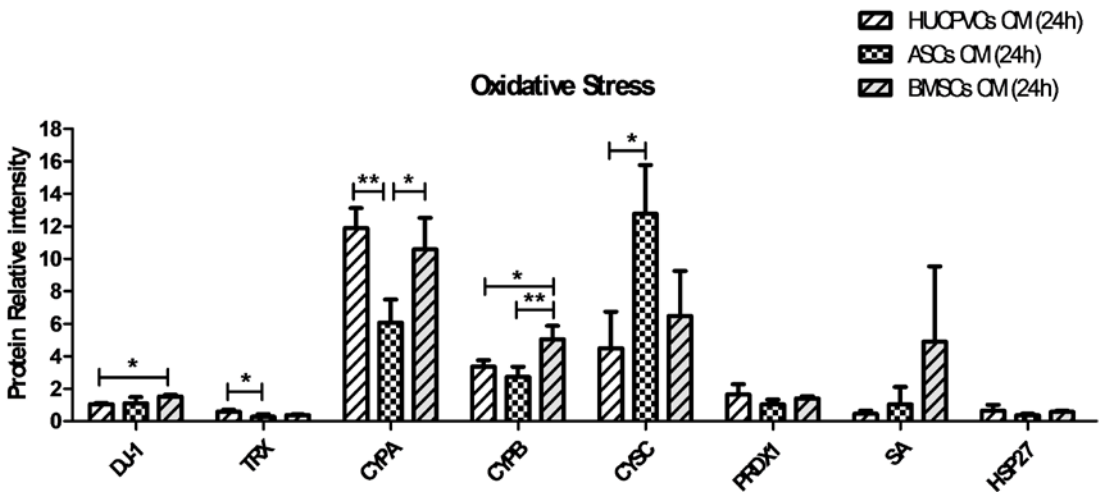

Figure 2: Quantitative expression of proteins present in HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) with antioxidative function. LC-MS/MS analysis revealed that CYPB was significantly more expressed in BMSCs CM when compared with HUCPVCs CM ($p < 0.05$) and ASCs CM ($p < 0.01$). On the other hand, no statistically significant differences were found among or between CM tested conditions regarding the expressions of PRDX1, AS and HSP27 ($p > 0.05$). Concerning statistical differences between CM groups, CYPA and CYPB expressions were significantly elevated in BMSCs CM over ASCs CM ($p < 0.05$, $p < 0.01$, respectively). Similarly, comparisons between BMSCs CM and HUCPVCs CM, showed that CYPB and DJ-1 were significantly more expressed in BMSCs CM ($p < 0.05$). On the other hand, statistical differences between HUCPVCs CM and ASCs CM revealed that expression of TRX and CYPA were significantly increased in HUCPVCs CM ($p < 0.05$, $p < 0.01$, respectively), whereas CYSC was significantly upregulated in ASCs CM ($p < 0.05$). Taken together, results indicate that BMSCs secretome might have a more prominent role in providing neuroprotective activity related with oxidative stress.

In what concerns to **apoptosis**, 6 of the found proteins, namely CYPA, CYPB, CYSC, IL-6, Gal-1 and HSP27, have been described as having anti-apoptotic roles (Matsuda et al. 1996, Loddick et al. 1998, Xu et al. 2005, Yamashita et al. 2005, Ge et al. 2009, Qu et al. 2010, Tizon et al. 2010a, Tizon et al. 2010b, Oh et al. 2011, Teramoto et al. 2013, Shimada et al. 2014). CYPA, CYPB and CYSC have shown to have a dual role in CNS, that is, to play anti-oxidative and anti-apoptotic functions in *in vitro* models of AD and/or PD. For instance, Ge *et al.* (Ge et al. 2009) and Oh *et al.* (Oh et al. 2011) reported that administration of hr**CYPA** or **CYPB** overexpression in PC12 cells exposed to Aβ peptides protected PC12 cells against Aβ-mediated apoptotic death. On the other

hand, **CYSC** has demonstrated to play a neuroprotective role against Aβ- and 6-OHDA-induced apoptotic neuron death by different pathways, upon exogenous administration in hippocampal and mesencephalic neurons, respectively (Xu et al. 2005, Tizon et al. 2010a, Tizon et al. 2010b). **IL-6** has also shown to have a protective role within the CNS. For instance, intracerebral administration of rhIL-6 has been reported to promote a marked delay/reduction of neuronal death in transient and permanent rodent models of cerebral ischemia (Matsuda et al. 1996, Loddick et al. 1998). These results were later attributed to its anti-apoptotic function (Yamashita et al. 2005). **Gal-1**, is an endogenous soluble mammalian lectin that belongs to the galectin family of carbohydrate-binding proteins and is expressed in reactive astrocytes following CNS injury, (Qu et al. 2010). As anti-apoptotic, rGal-1 infusion into the cerebello-medullar cistern has shown to reduce neuronal apoptosis in a rat model of focal cerebral ischemia by inducing the expression of brain-derived neurotrophic factor (BDNF) (Camby et al. 2006, Qu et al. 2010), also known for its anti-apoptotic nature (Schabitz et al. 2000). Finally, **HSP27** has also shown similar properties to the herein discussed factors (Teramoto et al. 2013, Shimada et al. 2014).

The quantification of the relative expression of these 6 proteins (Figure 3) revealed that CYPA, CYPB, CYSC and IL-6 were differently expressed between the CM tested conditions, whereas Gal-1 and HSP27 were not ($p > 0.05$). CypB was found to be significantly upregulated in BMSCs CM ($p < 0.05$; $p < 0.01$), whereas IL-6 was found to be highly expressed in HUCPVCs CM ($p < 0.05$). These results indicate that both BMSCs and HUCPVCs secretome exhibit similar anti-apoptotic profile. Concerning differences between HUCPVCS and ASCs CM, IL-6 and CYPA expressions were significantly elevated in HUCPVCs CM when compared with ASCs CM ($p < 0.05$; $p < 0.01$, respectively), whereas the opposite was observed regarding CYSC expression ($p < 0.05$). Taken together, these results indicate that both BMSCs and HUCPVCs secretome may exhibit a similar, and increased when compared to ASCs, anti-apoptotic profile.

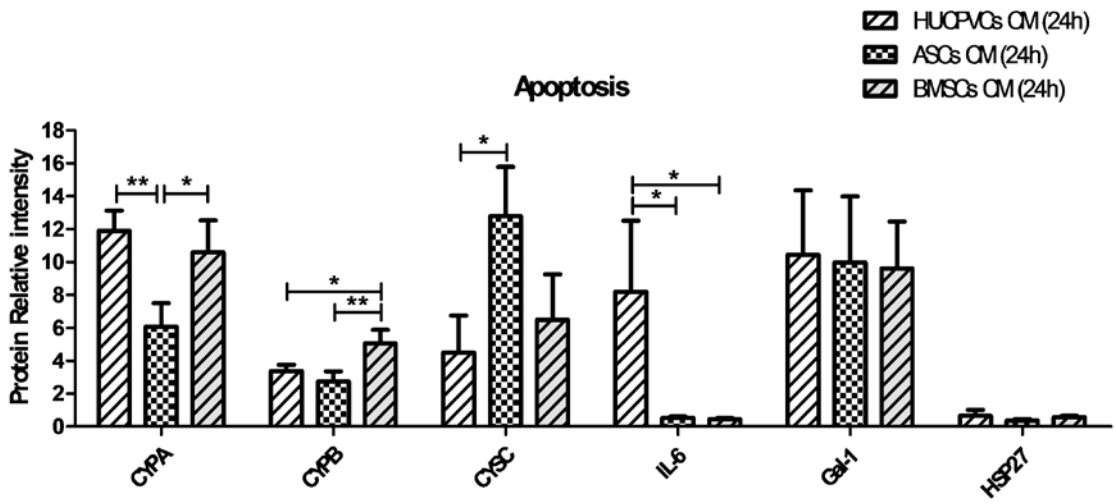

Figure 3: Quantitative expression of proteins present in HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) with anti-apoptotic function. LC-MS/MS analysis revealed that CypB was significantly upregulated in BMSCs CM ($p < 0.05$) when compared to HUCPVCs CM and ASCs CM ($p < 0.05$, $p < 0.01$, respectively). On the other hand, IL-6 was found to be highly expressed when compared with HUCPVCs and ASCs CM ($p < 0.05$). CYSC was found upregulated in ASCs CM when compared to HUCPVCS CM ($p < 0.05$). In opposition, ASCs CM was found to be downregulated concerning expression of CYPA when compared with both HUCPVCs CM and BMSCs CM ($p < 0.01$, $p < 0.05$, respectively). Collectively, results indicate that BMSCs and HUCPVCs secretome might display similar profiles in mediating neuroprotective functions associated with apoptotic cell death.

Concerning **excitotoxicity**, literature shows that 3 proteins, including IL-6, PEDF and PAI-1, display neuroprotection against glutamate induced-excitotoxicity (Toulmond et al. 1992, Yamada and Hatanaka 1994, Taniwaki et al. 1997, DeCoster et al. 1999, Docagne et al. 2002). Actually, **IL-6** in addition to its anti-apoptotic action has also been reported to reduce neuronal damage induced by glutamate in hippocampal neurons *in vitro* (Yamada and Hatanaka 1994) and striatal colhinergic neurons in *vivo* (Toulmond et al. 1992). Similarly, **PEDF**, which is a multifunctional protein and a non-inhibitory member of the serine protease inhibitor (SERPIN) gene family (Yaba et al.,2010), has also been demonstrated to protect cerebellar granule cells and hippocampal neurons against glutamate cytotoxicity *in vitro* (Taniwaki et al. 1997, DeCoster et al. 1999). Finally, **PAI-1**, also a member of the serine protease inhibitor (Serpin E1) superfamily (Dupont et al. 2009), in the recombinant form, was able to exert a protective role against N-methyl-D-aspartate (NMDA) excitotoxicity in cortical neurons through modulation of NMDA-Ca^{2+} influx upon exogenous addition (Docagne et al. 2002).

Regarding these 3 proteins, quantitative results of their expression (Figure 4) revealed that IL-6 and PEDF were differently expressed in HUCPVCs (upregulated, $p < 0.05$) and ASCs (upregulated, $p < 0.01$), which indicates that their secretome can play a role in mediating neuroprotection induced by excitotoxicity phenomena.

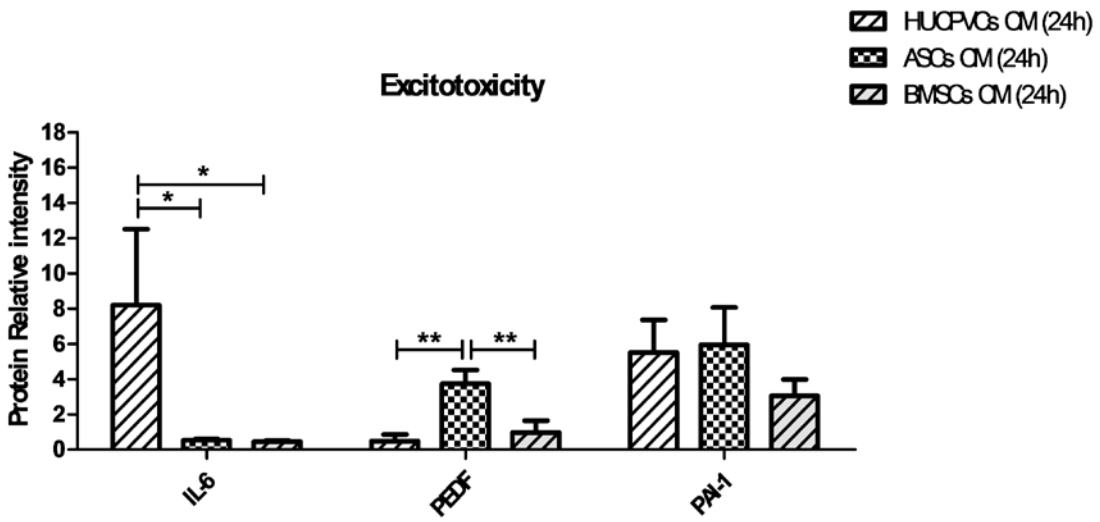

Figure 4: Quantitative expression of proteins present in HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) with anti-excitotoxicity function. LC-MS/MS analysis revealed that IL-6 was found to be significantly more expressed in HUCPVCs CM when compared to both ASCs CM ($p < 0.05$) and BMSCs CM ($p < 0.05$). On the other hand, results showed that PEDF expression was significantly elevated when compared with HUCPVCs CM ($p < 0.01$) and BMSCs CM ($p < 0.01$). Conjointly, the data obtained indicates that HUCPVCs and ASCs secretome may have a similar role in neuroprotective functions related with excitotoxicity cell death caused by increased concentration of neurotransmitters (e.g., glutamate).

As mentioned above, in addition to the ability of proteins within MSCs secretome to regulate processes such as oxidative stress, apoptosis and glutamate-mediated excitotoxicity, LC-MS/MS analysis also revealed the presence of proteins within the MSCs CM involved in the regulation of proteasomal degradation (Gong et al. 2006, Setsuie and Wada 2007), neurogenesis (Ishibashi et al. 2007, Tian et al. 2014), inflammation (De Simoni et al. 2003, Heydenreich et al. 2012), glial scarring (Davies et al. 2004, Davies et al. 2006, Minor et al. 2008, Ahmed et al. 2014), and toxic protein deposition (Boggs et al. 1996, Yerbury and Wilson 2010) (Figure 5). One of these proteins was UCHL1, which is both a ubiquitin (UB) hydrolase (Wilkinson et al. 1989) and UB-ligase enzyme (Liu et al. 2002), mostly localized in neurons (Setsuie and Wada 2007). **UCHL1** has been shown to be involved in regulation of proteasomal degradation (Tai and Schuman 2008), which has been linked with neurodegenerative diseases like AD and PD (Layfield et al. 2003). For instance,

administration of UCHL1 protein fused with a cell penetrating peptide was shown to restore Aβ-amyloid-induced synaptic dysfunction in both *in vitro* and *in vivo* mice models of AD, and to alleviate memory loss *in vivo* (Gong et al. 2006). As it can be observed in figure 5, results show that UCHL1 is significantly upregulated in HUCPVCs CM ($p < 0.05$). For **TRX**, beyond its already described antioxidant activity, it has been recently reported its possible role in promoting neurogenesis in the hippocampus of hrTRX-treated adult mice, as well as in facilitating cognitive recovery in a cerebral ischemia model (Tian et al. 2014). Its expression was found to be significantly increased in HUCPVCs CM ($p < 0.05$).

Another protein that was found in MSCs secretome was plasma protease **C1-Inh**, a glycoprotein that belongs to the superfamily of serine protease inhibitors (Serpin G1) (van Gent et al. 2003). It is an endogenous inhibitor of the complement classical pathway and the contact-kinin systems (Wagenaar-Bos and Hack 2006, Heydenreich et al. 2012). The former system is involved in a variety of immune inflammatory responses, whereas contact-kinin system is involved not only in inflammation, but also in coagulation and blood pressure control, both of which have been shown to play crucial roles in the pathophysiology of ischemic stroke (De Simoni et al. 2003, Kleinschnitz et al. 2007). As it can be observed in figure 5, C1-Inh was found to be upregulated in BMSCs CM ($p < 0.05$). The role of this protein in the CNS is still largely unknown but it would be interesting to evaluate its effects on the modulation of the activity of microglial cells.

LC-MS/MS analysis also revealed the presence of a small leucine proteoglycan protein named **DCN** (Minor et al. 2008), which was the only anti-scarring quantifiable molecule found in the analysis of MSCs CM. Indeed, hr-DCN administration has been reported to promote axon regeneration, even across the lesions by acting as an anti-scarring agent both in *in vitro* and in *in vivo* models of SCI (Davies et al. 2004, Minor et al. 2008). In these studies, DCN anti-scarring effect was attributed to the reduction of inflammatory fibrosis, astrogliosis and levels of several scar-related elements (e.g. Chondroitin sulfate proteoglycans). The expression of the latter (figure 5) was found to be increased in ASCs secretome ($p < 0.001$, $p < 0.01$) and also in BMSCs in comparison with HUCPVCs ($p < 0.01$).

Finally, regarding proteins involved in neuroprotection phenomena, within the MSCs secretome we have also found **CLUS**, which is an extracellular chaperone found in all human fluids (Nilselid et al. 2006). It has previously shown to have a protective function against Aβ-induced neurotoxicity *in vitro* (Boggs et al. 1996, Yerbury and Wilson 2010). Although the exact mechanism by which

secreted CLUS protects neuronal cells from Aβ neurotoxicity is unclear, it is thought to be related with CLUS-Aβ complex formation, attenuation of Aβ aggregation, Aβ removal via receptor-mediated endocytosis and subsequent degradation by lysosomes (Hammad et al. 1997, Pucci et al. 2008, Dabbs et al. 2013). Results showed that although a much more noticeable expression of CLUS was found in ASCs CM, CLUS was similarly expressed by the three CM tested groups. This result makes difficult to conclude which of the MSCs secretome sources would be advantageous for a treatment aiming to prevent and clear excessive levels of toxic deposits without conducting *in vitro* experiments for individual MSCs CM test in *in vitro* models of toxic protein deposits (e.g., Aβ, α-synuclein).

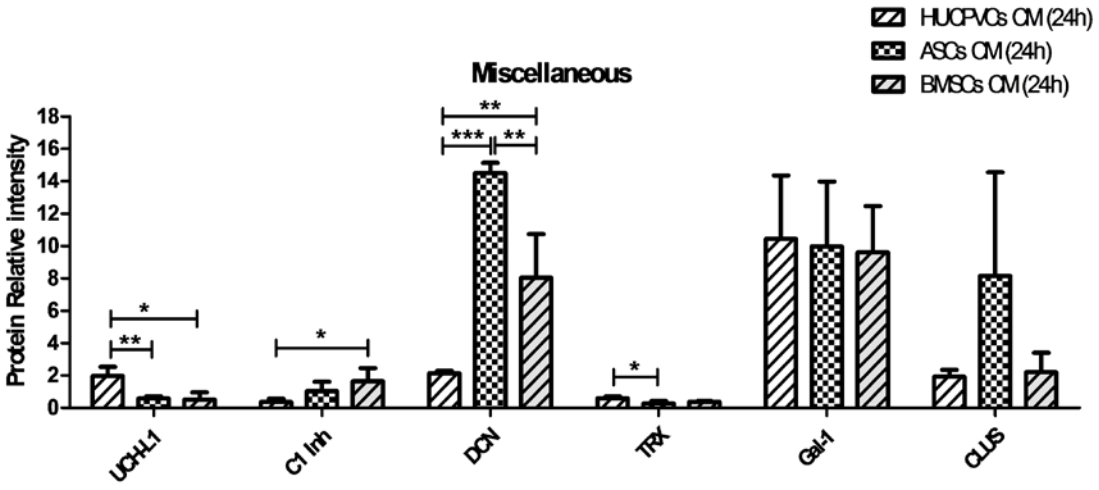

Figure 5: Quantitative expression of proteins present in HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) involved in the regulation of proteasome degradation (UCHL1), neurogenesis (TRX, Gal-1), inflammation (c1-Inh), glial scarring (DCN) or toxic protein deposition (CLUS). LC-MS/MS analysis revealed that UCHL1, expression was significantly elevated in HUCPVCs CM when compared to both ASCs CM ($p < 0.01$) and BMSCs CM ($p < 0.05$). TRX expression was also significantly elevated in HUCPVCs CM when compared with ASCs CM ($p < 0.05$), whereas no significant differences were found regarding Gal-1expression ($p > 0.05$). C1-inh was significantly increased in BMSCs CM when compared with HUCPVCs CM ($p < 0.05$). On the other hand, results showed that DCN expression was significantly upregulated when compared to both HUCPVCs CM ($p < 0.001$) and BMSCs CM ($p < 0.01$) and also in BMSCs CM when compared to ASCs CM ($p < 0.01$). Finally, Regarding CLUS expression, no statistical significant differences were found ($p > 0.05$). Collectively, results indicate that HUCPVCs CM seem to have a more pronounced role in mediating neuroprotective activity associated with abnormal proteasomal degradation, and also in promoting neurogenesis when compared with ASCs CM. In contrast, the major strengths of HUCPVCs CM as neuroprotective agent do not seem to be related with prevention of scar formation and inflammation.

3.2 Proteins related with neurite outgrowth and neurodifferentiation

LC-MS/MS results also revealed the presence of 6 proteins (Figure 6) which have been related to processes such as neurite outgrowth and neuron differentiation (Studzinski 2001, Hollenbeck and Bamburg 2003). From these 6 proteins, PEDF, CADH2, IL-6 have been reported to promote neuron differentiation (Tombran-Tink et al. 1991, Chader and Schwartz 1995, Houenou et al. 1999, Gao et al. 2001). Indeed, besides PEDF role as neuroprotective factor it has been shown to be a neurotrophic factor with both neuron survival and neurodifferentiation activity (Tombran-Tink et al. 1991, Chader and Schwartz 1995, Houenou et al. 1999). In fact, exogenous addition of PEDF to human Y-79 retinoblastoma cells and embryonic chick spinal cord motor neurons has been described not only to promote survival but also the establishment of a dense meshwork of neurites (Tombran-Tink et al. 1991, Chader and Schwartz 1995, Houenou et al. 1999). Similarly, IL-6 has also demonstrated to induce neuronal differentiation in PC12 cells (Satoh et al. 1988). On the other hand, CADH2, a neuronal cell adhesion glycoprotein, has been found to induce both morphological and biochemical features of differentiated neurons in embryonic carcinoma P19 cells (Gao et al. 2001). Regarding quantification results (Figure 6), IL-6 and CADH2 were found significantly elevated in HUCPVCs CM when compared to ASCs ($p < 0.05$), whereas the contrary was observed concerning PEDF ($p < 0.01$). Nevertheless, conjointly LC-MS/MS analysis and current available experimental data indicate that all the three MSCs populations present in its secretome composition proteins which promote neurodifferentiation. These data further reinforce the neurodifferentiation properties of the secretome of these cells.

With regard to proteins identified and quantified through LC-MS/MS analysis that have been documented to play roles in axon guidance (Pasterkamp et al. 2003) and/or neurite outgrowth (Zurn et al. 1988, Farmer et al. 1990, Gurwitz and Cunningham 1990, Pasterkamp et al. 2003, Korshunova et al. 2008), these included SEM7A, GDN and BASP-1 (Figure 6). In fact SEM7A which belongs to the semaphorin family of axon guidance proteins has been described to enhance axon growth from olfactory bulb explants in both its soluble and membrane bound forms (Pasterkamp et al. 2003). GDN, a serine protease inhibitor from the serpin family (Serpin F2) (Reinhard et al. 1994) has also been shown to promote neurite outgrowth in neuroblastoma (NB2a) cells, chick sympathetic neurons and rat hippocampal cells upon exogenous addition (Zurn et al. 1988, Farmer et al. 1990, Gurwitz and Cunningham 1990). Finally, BASP-1, a major protein of neuronal lipid rafts, when overexpressed in PC12E2 cells (a subclone of PC12 cells) and rat primary hippocampal

neurons, was found to strongly stimulate neurite outgrowth in both cell types (Korshunova et al. 2008). As it can be observed in figure 6, HUCPVCS CM expression of GDN and SEM7A, was found significantly downregulated when compared to both BMSCs CM ($p < 0.01$) and ASCs CM ($p < 0.05$). No statistically relevant expression differences or tendencies between BMSCs and ASCs CM were observed. Thus, from the obtained data, BMSC and ASCs CM might exhibit similar profile in promoting neurite outgrowth when compared to HUCPVCs.

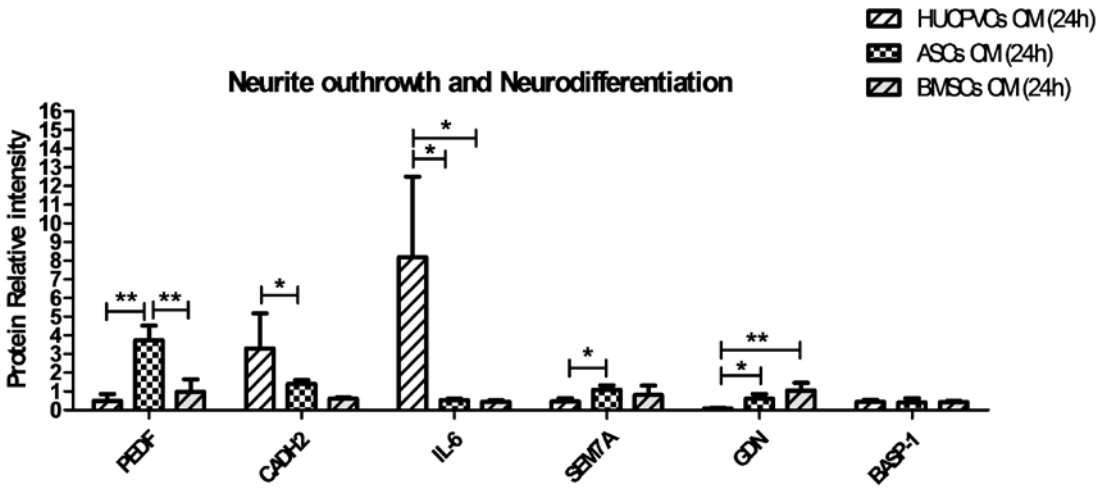

Figure 6: Quantitative expression of proteins present in HUCPVCs CM (24h), ASCS CM (24h) and BMSCs CM (24h) with neurite outgrowth and/or neuron differentiation functions. LC-MS/MS analysis revealed that for proteins involved in neurodifferentiation (PEDF, CADH2 and IL-6), IL-6 and PEDF were significantly upregulated in HUCPVCs CM and ASCs CM, respectively, when compared with the other CM groups ($p < 0.05$, $p < 0.01$, respectively). Concerning statistical differences between CM groups, IL-6 and CADH2 were significantly increased in HUCPVCs CM when compared with ASCs CM ($p < 0.05$). In contrast, PEDF was found significantly downregulated in HUCPVCs CM and BMSCs CM when compared with ASCs CM ($p < 0.01$). On the other hand, LC-MS/MS analysis revealed that for proteins involved in neurite outgrowth (SEM7A, GDN and BASP-1), GDN expression was significantly elevated in BMSCs CM when compared to both HUCPVCs CM ($p < 0.01$) and ASCs CM ($p < 0.05$). Concerning statistical differences between CM groups, HUCPVCs CM exhibited significantly lower expression of SEM7A and GDN when compared with both ASCs CM ($p < 0.05$) and BMSCs CM ($p < 0.01$), whereas no significant differences were found between ASCs CM and BMSCs CM ($p > 0.05$). Taken together, the data obtained regarding neurite outgrowth and neurodifferentiation as mediators of neuron repair indicates that BMSCs CM might have a least distinguished role in inducing neurodifferentiation, whereas BMSCS CM and ASCs CM seem to play similar roles in inducing neurite outgrowth.

In summary, quantification of proteins expression involved in neurite outgrowth or neuron differentiation indicate that HUCPVCs CM and BMSCs CM exhibit the least indicated profile for neurite outgrowth or neuron differentiation purposes, respectively. Nevertheless, although it is

important to select the appropriate cell type for application, collectively, the obtained results and current available experimental *in vitro* approaches making use of cell cultures show that MSCs secrete a subset of neuroregulatory molecules that positively affect neuronal repair and could contribute for the reconstruction of the CNS neuronal circuitry.

4. Conclusions

In the present work we have: 1) characterized the secretome of BMSCs, ASCs and HUCPVCs based on an exhaustive LC-MS/MS proteomics analysis, and 2) quantified the protein expression differences among MSCs populations concerning molecules previously described as having a role in neuroprotection and/or neurite outgrowth and neuronal differentiation. Through this analysis we have been able to identify 121 proteins within MSCs secretome, 20 of which had previously been reported to induce neuroprotection, axon growth and/or neurodifferentiation. Based on the differential expression of proteins within MSCs CM, our results show that the different tissue derived MSCs may have a different impact in protecting against distinct pathogenic processes involved in various CNS disorders/injuries. Similar observations were found for neurite outgrowth and neuron differentiation. Indeed, evaluation of MSCs CM secretion profile, based on the differential expression of proteins with neuroprotection character, indicated that BMSCs CM might be the most advantageous choice for a therapy aiming to reduce oxidative stress, while HUCPVCs and ASCs could have a more leading role in the protection against excitotoxic phenomena. On the other hand, results suggest that as anti-scarring HUCPVCs CM can be the least indicated, whereas it might be the most indicated to a therapy aiming to target abnormal proteasomal degradation. Regarding apoptosis, both BMSCs and HUCPVCs CM might be advantageous as anti-apoptotic agents, as opposed to ASCs CM. Our results suggest that the secretome of different tissue derived MSCs might contribute differently for protecting against several pathogenic processes involved in various CNS disorders/injuries, as well as in neuron repair. Yet, it is important to highlight that the obtained results in this study are just indicative on the possible advantage or disadvantage to use the secretome of one or two MSCs sources in detriment to another for future therapeutic application. Therefore, future studies should focus on validating the efficacy of the CM of each individual MSCs source in promoting neurogenesis, neuroprotection, and neurite/axonal growth in *in vitro* and *in vivo* models of injury and disease. This validation would provide preliminary evidence of the

importance to choose the secretome from the appropriate MSCs source for future administration as a cell free therapy according to the therapeutic application.

5. Acknowledgments

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Chapter 5

General Discussion and Future Perspectives

General Discussion and Future Perspectives

Collectively, the low efficacy of the current treatments associated with the limited capability of the CNS to self-renew and to regenerate functional neurons, the global aging and increasing human life expectancy impose an urgent need for alternative strategies aiming to treat CNS neurological disorders or injuries. In recent years, MSCs have called the attention of the scientific community as potential candidates in the field of CNS regenerative medicine due to their unique characteristics. As mentioned in chapters 1 and 3 of this thesis, MSCs have been seen as interesting candidates for CNS therapy in virtue of their availability in multiple human adult tissues, relative simple isolation with minimal invasive procedures, easy culture and expansion *in vitro* for several passages, low immunogenicity and the absence of ethical constraints when compared with other stem cell sources. The mechanisms underlying MSCs-mediated therapeutic benefits observed upon transplantation in animal models of CNS damage are not completely understood and are still a matter of debate. Some scientists suggest MSCs differentiation into neuronal lineages as the principal effector of CNS regeneration, but robust and solid evidence of MSCs differentiation into fully differentiated and functional neurons *in vivo* remains to be demonstrated (Phinney and Prockop 2007, Maltman et al. 2011, Thomas et al. 2011, Liu et al. 2012). On the other hand, in recent years, MSCs-induced regenerative effects have been attributed to their paracrine activity, that is, to the MSCs ability to secrete bioactive soluble factors and microvesicles/exosomes that assist cell regeneration and repair of damaged tissue (Li et al. 2002, Neuhuber et al. 2005, Caplan and Dennis 2006, Ding et al. 2007, Yang et al. 2008, Meirelles Lda et al. 2009). Taken together, the current body of data indicates that the MSCs secretome underlies the restorative effects observed in distinct CNS disorders, such as neurodegenerative diseases. Yet, despite these progresses, the MSCs secretome, as well as its potential, per se, to induce the reported therapeutic benefits remains largely unexplored. For instance, there is paucity of studies where the effects of the secretome of MSCs isolated from distinct microenvironments/tissue sources in promoting neuronal survival, neurite outgrowth, influence neuron differentiation, and to induce neuroprotection and/or neurorecovery have been directly confronted. Moreover, to date it was not verified whether the secretome alone derived from distinct MSCs populations can hold therapeutic specificity towards a CNS pathology, such as Parkinson's disease. Finally, although classical growth factors and cytokines released by different tissue derived MSCs have been ascribed to play a role in the phenomena reported so far, there are probably other secreted bioactive molecules and vesicles with neuroregulatory character that can better explain their role in the above mentioned

phenomena. Hence the scope of the present thesis was to explore the potential of the sole use of different tissue derived MSCs secretome, namely BMSCs, ASCs and WJ-MSCs/HUCPVCs for CNS regenerative medicine. As referred in chapters 2, 3 and 4, the studies conducted throughout this thesis aimed at exploring the potential of different tissue derived MSCs secretome for CNS regeneration, namely BMSCs, ASCs and WJ-MSCs/HUCPVCs. The choice of these specific sources was based on the global consideration of bone marrow as the gold standard source of MSCs for regenerative medicine and the subsequent demonstration that ASCs and WJ-MSCs/HUCPVCs besides sharing many phenotypical similarities with BMSCs could hold attracctive characteristics for clinical purposes, such as the possibility of being harvested with less invasive procedures and to provide higher yields of MSCs when compared with BMSCs (De Ugarte et al. 2003, Sarugaser et al. 2005, Fraser et al. 2006, Weiss et al. 2006, Baksh et al. 2007, Fraser et al. 2008).

5.1 Impact of MSCs secretome from different MSCs populations in neurite outgrowth and/or neuronal differentiation

Previous work of our laboratory demonstrated that HUCPVCs and BMSCs secretome, in the form of CM, were able to increase metabolic viability and differentiation in primary cultures of postnatal hippocampal neurons (Salgado et al. 2010, Ribeiro et al. 2011). More recently Wright *et al.* (Wright et al. 2010) also demonstrated that BMSCs CM was able to induce neurite outgrowth in a human neuroblastoma cell line and in explants of chick dorsal root ganglia as contributor for neuronal repair. Even more recently, a comparative study on the secretome of HUCPVCs and ASCs revealed that these MSCs populations acted differently in increasing metabolic viability and differentiation in hippocampal neuronal cultures. These differences were related with the presence of distinct growth factors within their secretome (Ribeiro et al. 2012). At the time the authors hypothesized that these differences could be related to the different microenvironments from where they were isolated. In this sense, the primary aim of this work (Chapter 2) was to use an experimental approach that allowed to directly compare the impact of BMSCs, ASCs and HUCPVCs secretome on a neuron-like cell population and determine the ability of the secretome of these MSCs populations to promote survival and neuronal differentiation. In order to accomplish these aims, we have used a neuroblatoma cell line (SH-SY5Y cells). The latter is a widely used model of neuron differentiation due to its potential to: 1) differentiate in functionally mature neuron-like cells in response to a variety of biologic stimulus, 2) exhibit biochemical, morphological and electrophysiological similarity

to neurons and, 3) its interesting particularity to differentiate towards different phenotypes according to culture conditions (Xie et al. 2010). CM collected from distinct time points, 24h and 96h, were used, as previous findings have revealed that cell viability, proliferation and cell densities of neuronal cell cultures could be modulated by them (Ribeiro et al. 2012, Fraga et al. 2013). Based on cell metabolic viability and immunocytochemical assays (chapter 2), our results showed that the secretome of both BMSCs and HUCPVCs CM (24h, 96h) was able to support SH-SY5Y cells survival and differentiation into neuron-like cells without the addition of any other exogenous supplements (a condition where these cells were not able to survive). On the other hand, cells incubated with ASCs CM (24h, 96h) were not able to survive. Assessment of neurite outgrowth and mRNA expression of specific markers of SH-SY5Y cells neuronal differentiation revealed interesting results. While our findings regarding mRNA expression of specific markers of SH-SY5Y cells differentiation upon incubation with BMSCs CM (24h, 96h) and HUCPVCs CM 24h further supported the capability of the CM from these populations to induce SH-SY5Y cells differentiation into neurons, the results regarding HUCPVCs 96h were surprising. The transcriptomics analysis of specific markers of SH-SY5Y cells differentiation suggested that the HUCPVCs CM 96h was inducing a similar SH-SY5Y cells differentiation towards the DAergic phenotype to that obtained for the positive control of the experiment and BMSCs CM group from the same time point. The differences in the expression of a gene (DAT), which is present only in DAergic neurons (Constantinescu et al. 2007), was concomitant with the decrease in neurite length displayed by SH-SY5Y cells after incubation with BMSCs CM 96h. Together, differences in neurite lengths and mRNA levels of neuronal markers were not only indicative of the possibility that BMSCs CM 96h and HUCPVCs CM 96h were inducing SH-SY5Y cells towards different neuronal phenotypes, but also that there were differences in the secretome composition between the two MSCs populations. Additionally, the temporal profile of MSCs CM collection was apparently influencing SH-SY5Y cells differentiation. Thus, the data obtained in this work further reinforced our beliefs and called for the attention that there are differences within the secretome of different tissue derived MSCs that need to be addressed. As our results also suggested the possibility that BMSCs CM 96h and HUCPVCs CM 96h were inducing SH-SY5Y cells towards different neuronal phenotypes, it would be interesting in the future to: 1) conduct immunocytochemical experiments using DAergic, cholinergic and adrenergic antibodies to further confirm that HUCPVCs CM 96h could effectively induce differentiation of SH-SY5Y cells towards the DAergic phenotype and determine in which phenotypes BMSCs collected from distinct time points (24h, 96h) and HUCPVCs CM 24h would differentiate

SH-SY5Y cells and, 2) confirm the functionality of the differentiated neurons. Moreover, it would also be interesting to reproduce this study (including ASCs secretome) and further complement it with the above suggested approaches on primary cultures of neural stem cells derived from neurogenic niches (e.g., SVZ and hippocampus). By doing so, we would confirm if the results and suggestions of this work would be verified in a cell population that is found within the CNS and, this way, ensure that MSCs secretome could contribute for neuronal cell differentiation by its paracrine activity. Finally, as we also observed that different temporal profile of CM collection might have distinct effects in SH-SY5Y cells differentiation, it would be interesting to study in the future the impact that the temporal profile of CM collection can hold towards distinct CNS injuries/disorders.

5.2 Therapeutic effect of the secretome derived from different MSCs populations towards a CNS pathology: *in vitro* and *in vivo* assays

Pre-clinical studies on the transplantation of MSCs in models of CNS disorders/injuries, such as cerebral ischemia, Parkinson´s disease, Alzheimer´s disease, amyotrophic lateral sclerosis traumatic brain injury, spinal cord injury, among others, have shown their possible use in CNS regenerative medicine (Seo and Cho 2012, Glavaski-Joksimovic and Bohn 2013, Paul and Anisimov 2013, Teixeira et al. 2013). The main objective of the work described in chapter 3 was to determine whether the MSCs secretome alone could be a promising tool for future treatment of CNS pathologies, namely Parkinson's disease. Initially the effect of the individual CM 24h of BMSCs, ASC and HUCPVCs was studied in embryonic ventral mesencephalic cells, which are the precursors of midbrain DAergic neurons (Jin et al. 2008). ASCs CM 24h had the most positive effect on the survival of mature ventral midbrain DAergic neurons when compared with both the control of the experiment (plain neurobasal media) and VMCs incubated with HUCPVCs CM 24h. For *in vivo* assays, the 6-OHDA unilateral rat model was used. Immunohistochemical and behavioral data (forced choice test) showed that BMSCs secretome was the one which had a more robust effect in promoting the survival of DAergic neurons and the functional recovery of hemiparkinsionian animals. Based on this data it would be interesting to further complement these studies in the future by: 1) using a higher number of animals, 2) increasing the amount of intrastriatal delivery of MSCs CM in order to establish an optimal dosage for secretome administration, 3) further complement the staircase test with other behavioral motor tests, such as

rotarod, open field, fixed ratio bar-pressing task, and forelimb use asymmetry test, and 4) quantify the levels of dopamine (e.g. by high-performance liquid chromatography). By doing so, it would be possible not only to clarify any remaining questions, but also grant further robustness to the hypothesis of using MSCs secretome as a therapeutic tool in PD regenerative medicine. Nevertheless, with this work (chapter 3), it was possible to show that cell-free therapies based on the use of the MSCs secretome may be considered for the future treatment of PD. Similarly, the secretome of ASCs and HUCPVCs might be promising candidates for other CNS neurological disorders, in fact this is a topic that should be addressed in the future. Of note is also the fact, that the disparity of results obtained *in vitro* and *in vivo* regarding the most positive effect of ASCs CM 24h in inducing the survival of midbrain/nigral DAergic neurons in intact ventral mesencephalic cultures and BMSCs CM 24h higher effect in promoting the survival of DAergic nigral neurons was not surprising. These discrepancies may be explained based on the fact that our *in vitro* approach was based in uninjured conditions, whereas the *in vivo* approach was based in lesion conditions. In other words, the CM of one MSCs population may be more apt for neuroprotection in virtue of its ability to respond to all the phenomena that occur in a situation of injury, whereas in the absence of lesion the CM of another MSCs population might be better to promote cell survival. Finally, the different impact of different tissue derived MSCs secretome on the survival of mature nigral neurons observed in uninjured VMCs *in vitro* and the different therapeutic effects towards PD seen *in vivo* further reinforce the hypothesis of having selective therapies according to the CM that is being used.

5.3 Proteomics characterization of the MSCs secretome derived from different tissue sources

In the last decade researchers have focused on the characterization of MSCs secretome derived from different sources to unveil the potential relevance of MSCs secretome for future application in a clinical setting. There is a vast array of proteomic techniques to unveil MSCs secretome content. To our knowledge, studies on the MSCs secretome based on proteomics techniques conducted so far included antibody-based techniques (e.g., ELISA, multiplex) gene expression-based techniques, such as serial analysis of gene expression (SAGE) and DNA microarrays, and LC-MS/MS (Kupcova Skalnikova 2013, Mukherjee and Mani 2013). Antibody-based techniques provides a characterization of MSCs secretome, in the form of CM, mainly based in targeted proteomics, that is, on the detection of growth factors and cytokines with known roles in the

biological processes being studied by employing ELISA and multiplex immunological techniques (Crigler et al. 2006, Nakanishi et al. 2011, Kupcova Skalnikova 2013, Lavoie and Rosu-Myles 2013). On the other hand, SAGE and DNA microarrays are based in gene expression (Mukherjee and Mani 2013) using MSCs extracts as starting material instead of MSCs CM. Therefore, it provides a prediction on which factors will MSCs secrete. In this sense, a proteomic characterization of MSCs secretome through a shotgun/discovery-based approach in which LC-MS/MS is employed instead of proteomics strategies based on gene expression or target/candidate-based approach allows a more detailed knowledge on the MSCs released factors. This technique allows the identification and quantification of a vast number of soluble proteins or factors released in extracellular vesicles (Kupcova Skalnikova 2013, Lavoie and Rosu-Myles 2013). Studies conducted so far making use of LC-MS/MS have identified numerous proteins within different tissue derived MSCs secretome (Zvonic et al. 2007, Chiellini et al. 2008, Choi et al. 2010, Lee et al. 2010, Arufe et al. 2011, Kim et al. 2012, Kim et al. 2013). However, they were only focused on the characterization of MSCs secretome from one single population. In fact those that have compared distinct MSCs populations were based on gene expression approaches or based in cell extracts instead of the secretome itself (Roche et al. 2009, Nakanishi et al. 2011, Hsieh et al. 2013, Roche et al. 2013). Herein, in the study provided in chapter 4 of the present thesis we intended to identify and quantify the expression of all the proteins present in MSCs CM derived from BMSCs, ASCs and HUCPVCs that could be involved in neuroprotection, neuroreparative and neurodifferentiation phenomena, and evaluate at which extent the secretome of these different MSCs population could diverge. In order to pursue these aims, we have performed an exhaustive proteomics analysis based on LC-MS/MS. For the latter analysis, we have collected CM from the 24h time point, since results from our lab based on immunocytochemical assays and LC-MS/MS secretome analysis using CM collected from 24h and 96h time points showed that the first had an increased effect in cell densities of mature cortical neurons, as well as a higher content of proteins (e.g., 14-3-3, heat shock protein 70) involved in neuron survival and neuroprotection phenomena (Fraga et al. 2013). The LC-MS/MS analysis was performed following the traditional IDA method, which allows to detect and identify the maximal number of proteins within sample mixtures. Importantly, this analysis was further complemented with the recently introduced SWATH technique to overcome the more limited capability of the classical LC-MS/MS based on IDA acquisition to quantify expression of proteins present in lower abundance in complex sample mixtures (Gillet et al. 2012, Lambert et al. 2013). From the 121 identified and quantified in the

CM 24h of BMSCs, ASCs and HUCPVCs, we have found 20 proteins of interest, which have been reported to be involved in neuroprotection, neuroreparative and neurodifferentiation phenomena. These included neurotrophic, neurogenic, axon guidance, neurite growth and neurodifferentiative proteins, as well as, proteins with neuroprotective character against oxidative stress, apoptosis, excitotoxicity, inflammation, glial scarring and toxic protein deposition, which have been shown to be involved in several CNS disorders/injuries, such as cerebral ischemia, AD, PD and SCI (Yankner 1996, Dauer and Przedborski 2003, Schapira 2005, Doyle et al. 2008, Ahmed et al. 2014). Moreover these proteins were differentially expressed in the secretome of the distinct MSC populations under analysis, which can explain the results obtained in chapters 2 and 3. Regarding the study conducted in chapter 2, it was observed through quantification of neurite lengths, MAP-2 immunostaining and quantification of mRNA levels of SH-SY5Y cells neuron differentiation markers that BMSCs CM 24h and HUCPVC CM 24h exhibited similar profiles in inducing neurite outgrowth and neurodifferentiation. Secretome analysis for these cells revealed the presence of SEM7A, BASP-1 and GDN, which are known to be involved in these processes. From these, the first two were similarly expressed in both CM, whereas only the GDN was found upregulated in BMSCs CM 24h ($p < 0.01$), which may indicate that SEM7A and BASP-1 have a more prominent role in the observed phenomena. In order to validate this possibility, a possible experimental approach to conduct would be to exogenously add the latter proteins to neuroblatoma cells serum-free media and in parallel conduct the same experimental approach with additionally added GDN. The same analysis also provided some information on possible proteins present in the secretome of BMSCs and HUCPVCs that could modulate neuronal cell differentiation, namely PEDF, CADH2 and IL-6. Again, from these three, two, PEDF and CADH2, were found to have similar expression levels in BMSCs CM 24h and HUCPVCs CM 24h. In contrast, IL-6 was found significantly upregulated in HUCPVCs CM 24h ($p < 0.05$). However, the latter is mostly known for its role in regulation of the immune system response (Ulich et al. 1991, Xing et al. 1998, Nishimoto and Kishimoto 2006) and although it has already been shown to act as anti-apoptotic and anti-excitotoxicity upon brain insult/injury (Toulmond et al. 1992, Yamada and Hatanaka 1994, Yamashita et al. 2005), IL-6 is not particularly abundant within the CNS, neither its roles within the CNS are well established (Landreth 2006). Hence, promoting neurodifferentiation apparently is not IL-6 main function and, therefore, is most likely that IL-6 is not conferring an additive effect in neuroblastoma cells differentiation. To validate this hypothesis a possible approach to conduct, would be a similar one to the above mentioned for proteins with neurite outgrowth properties.

Regarding the study conducted in chapter 3, TH immunostaining revealed that ASCs CM 24h had a higher effect on the survival of mature DAergic neurons when compared to HUCPVCs CM 24h. Thus, BMSCs CM 24h and ASCs CM 24h displayed similar profiles in inducing survival of nigral neurons. LC-MS/MS analysis revealed that CYSC, which has been reported to induce survival of DAergic neurons in VMCs (Xu et al. 2005) was found upregulated in ASCs CM 24h when compared to HUCPVCs CM 24h ($p < 0.05$), whereas no significant differences were found when compared with BMSCs CM 24h, which is in accordance with our results. On the other hand, our *in vivo* data showed that BMSCs secretome was the only capable to promote the survival of DAergic neurons and the functional recovery of a unilateral model of dopaminergic depletion. LC-MS/MS analysis revealed the presence of proteins which are typically involved in neuroprotection phenomena in such models, namely DJ-1, CYSC, PEDF and PRDX1 (Xu et al. 2005, Inden et al. 2006, Lee et al. 2008, Falk et al. 2009). From these proteins PEDF and PRDX1 have been shown to be neuroprotective in *in vitro* 6-OHDA models of PD (Lee et al. 2008, Falk et al. 2009), but so far there are no insights on their *in vivo* function. Therefore, from the four mentioned proteins, only DJ-1 and CYSC may account for the obtained results once they were the only studied in *in vivo* 6-OHDA hemiparkinsonian rat models of PD (Xu et al. 2005, Inden et al. 2006). Studies using these proteins reported distinct results. Xu and colleagues (Xu et al. 2005) using a preventive approach, by injecting CYSC prior to 6-OHDA, reported that CYSC partially rescued DAergic neurons from apoptosis. On the other hand, Inden and co-workers (Inden et al. 2006) reported that intranigral co-injection of the antioxidant DJ-1 with 6-OHDA dramatically increased nigral DAergic neurons survival, as well as DA and DAT levels in the striatum. Upon intranigral post-injection of DJ-1, a similar protection of SNpc neurons to the one reported by Xu and colleagues was revealed. These studies provide evidence on the higher efficacy of DJ-1 as neuroprotective agent for PD. Therefore, although the expression levels of CYSC were found significantly elevated in ASCs CM 24h, DJ-1 expression, which was found significantly upregulated in BMSCs CM 24h when compared with HUCPVCs CM 24h ($p < 0.05$), may better explain our results obtained *in vivo*. Yet, no expression differences were found between BMSCS CM 24h and ASCs CM 24h, which indicates that there are most likely other proteins within BMSCs CM 24h that may have contributed for the observed higher therapeutic specificity of BMSCs CM 24h towards PD. In fact, within the BMSCs secretome, there are proteins with both anti-apoptotic and anti-oxidative roles (CYPA, CYP B) which are upregulated when compared to the other two cells lineages. Therefore it would be interesting to study the individual therapeutic effect of CYPA and CYPB, to combine CYPA with CYPB, as well as

each one and both with DJ-1 in an *in vitro* 6-OHDA model of PD. Subsequently the best combination with DJ-1 could be injected in the same sites and using the same *in vivo* 6-OHDA model of PD used in our *in vivo* study in order to verify if the presence of these proteins within the BMSCs secretome can add therapeutic effect and hence validate our results.

5.4 Relevance of the novel findings and concluding remarks

With the work developed in the present thesis, we added new knowledge on the biological relevance of the secretome differences of different tissue derived MSCs, by demonstrating that MSCs secretome derived from different microenvironment/sources differently: 1) induced neurite outgrowth and neurodifferentiation of a neuroblastoma cell line (chapter 2), as well as 2) neuron survival in a neuronal cell line and in distinct CNS populations (chapter 2 and chapter 3). Moreover, the former demonstration further reinforce the neurite elongation and neurodifferentiation properties of the MSCs secretome, which are known to mediate neuronal repair and contribute for the reconstruction of the CNS neuronal circuitry (Crigler et al. 2006, Hardy et al. 2008). Furthermore, we demonstrated for the first time, using an *in vivo* approach, that the MSCs secretome alone, namely BMSCs secretome, was capable of promoting the survival of nigral DAergic neurons and the functional recovery of animals in an animal model of PD (chapter 3). Moreover, it was also possible to show that the secretome of MSCs from different sources have distinct therapeutic specificity towards a CNS pathology, which pinpoint for the importance of choosing the appropriate source of MSCs secretome for future clinical application in distinct CNS pathologies.

At the molecular level, the characterization of BMSCs, ASCs and HUCPVCs secretome, through an exhaustive proteomics analysis, demonstrated that in the context of neuroprotection and neuron repair these MSCs populations secrete a vast array of proteins with potential for CNS therapeutic applications. The identification of proteins residing within the MSCs secretome revealed a multitude of therapeutic mechanisms offered by the MSCs secretome ranging from anti-oxidative, anti-apoptotic, anti-excitotoxicity, anti-inflammatory, anti-scarring and anti-toxic protein deposition to neurotrophic, neurogenic, axon guidance, axon/neurite outgrowth and neurodifferentiation factors that contribute to endogenous neuronal repair. Furthermore, the quantification of MSCs secreted bioactive molecules with neuroregulatory character demonstrated differences within the expression

of BMSCs, ASCs, and HUCPVCs, suggesting that the secretome of different MSCs populations may have a different impact in protecting against injury and disease in the CNS. Yet, based on literature documentation, the role of a wide range of proteins identified in MSCs secretome within the CNS remains largely unknown or poorly explored in the CNS context, whereas others remain to be applied in distinct CNS disorders, such as PD. For these reasons, the cross-over of proteomics obtained data (chapter 4) with the work developed in chapters 2 and 3, based on proteins expression levels within BMSCs, ASCs, and HUCPVCs secretome and on current available literature, did not allowed to fully validate our results. Therefore, addressing the functional roles and specific effects of MSCs secreted factors under physiological and pathological conditions is crucial for future clinical application of MSCs secretome alone for CNS regenerative medicine.

Overall, the work developed in the present thesis adds important knowledge on the biological and molecular relevance of the MSCs secretome derived from different tissues. Moreover, the work herein presented further demonstrates that BMSCs secretome alone is capable of inducing survival of DAergic neurons within the SNpc and functional recovery *in vivo*, thereby supporting the MSCs secretome as the main mechanism underlying therapeutic benefits reported in pre-clinical *in vivo* studies of neurological disorders/injuries. The latter finding paves the way for the opportunity to exploit the therapeutic potential of different tissue derived MSCs secretome towards distinct CNS pathological conditions and open new avenues on the possible future application of MSCs secretome as a cell free therapy for CNS regenerative medicine. Finally, the perception that the role of a wide range of proteins within the CNS that have been identified in MSCs secretome remains largely unknown, elicits new and substantial challenges so that in the future the secretome of the appropriated cell source can be selected and tailored for a specific neurological disorder.

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Annexes

Annexes

Table 1: Results from two-way repeated measures ANOVA regarding animals' forelimb motor performance in the bilateral and unilateral staircase test.

Figure	staircase	Paw side	Source of variation	$F_{(Df,r)}$ = F value	P value	*Post-hoc* analysis
2A	Bilateral	C.S.	Time:	$F_{3,78} = 4.428$	0.0058	Saline vs. 6-OHDA (4th day): Saline > 6-OHDA , $p < 0.01$
			Treatment:	$F_{1,78} = 5.669$	0.0249	
			Interaction	$F_{3,78} = 2.195$	0.0953	
2B	Bilateral	I.S.	Time:	$F_{3,78} = 15.12$	< 0.0001	Saline vs. 6-OHDA (for all time points): $p > 0.05$
			Treatment:	$F_{1,78} = 0.0641$	0.8020	
			Interaction	$F_{3,78} = 0.3498$	0.7894	
3A	Unilateral	C.S.	Time:	$F_{4,32} = 4.428$	0.0058	BMSCs CM 24H vs. NBM: BMSCs CM 24H (7th week) > NBM, $p < 0.05$
			Treatment:	$F_{1,32} = 7.473$	0.0257	
			Interaction	$F_{4,32} = 1.331$	0.2796	
3B	Unilateral	I.S.	Time:	$F_{4,32} = 10.76$	< 0.0001	BMSCs CM 24H vs. NBM (for all time points): $p > 0.05$
			Treatment:	$F_{1,32} = 3.158$	0.1135	
			Interaction	$F_{4,32} = 5.394$	0.0020	
3C	Unilateral	C.S.	Time:	$F_{4,28} = 0.9992$	0.4245	ASCs CM 24H vs. NBM (for all time points): $p > 0.05$
			Treatment:	$F_{1,28} = 0.5732$	0.4737	
			Interaction	$F_{4,28} = 0.1512$	0.9609	
3D	Unilateral	I.S.	Time:	$F_{4,28} = 3.890$	0.0123	ASCs CM 24H vs. NBM (for all time points): $p > 0.05$
			Treatment:	$F_{1,28} = 3.841$	0.0908	
			Interaction	$F_{4,28} = 1.318$	0.2877	
3E	Unilateral	C.S.	Time:	$F_{4,24} = 2.506$	0.0689	HUCPVCs CM 24H vs. NBM (for all time points): $p > 0.05$
			Treatment:	$F_{1,24} = 0.07465$	0.7938	
			Interaction	$F_{4,24} = 0.6533$	0.6302	
3F	Unilateral	I.S.	Time:	$F_{4,24} = 7.397$	0.0005	HUCPVCs CM 24H vs. NBM (for all time points): $p > 0.05$
			Treatment:	$F_{1,24} = 1.546$	0.2601	
			Interaction	$F_{4,24} = 2.407$	0.0775	

Abbreviations: C.S.: contralateral side; I.S.: ipsilateral side; DF: degree of freedom; r: residual.