Catarina Filipa da Cruz Barbosa

Assessing the Impact of induced mesenchymal stem cells secretome in a 3D in vitro model of Parkinson’s disease

Faculty of Medicine and Biomedical Sciences

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Master's degree in Biomedical Sciences – Disease mechanisms

This work was done under the supervision of:

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Faculty of Medicine and Biomedical Sciences

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Authorship statement:

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

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(Catarina Filipa da Cruz Barbosa)

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ABSTRACT:

Parkinson's disease (PD) is a complex disease characterized by a loss of dopaminergic (DA) neurons that leads to lifelong motor and non-motor impairments, still with no efficient treatment that can halt or revert its progression. Mesenchymal stem cells (MSCs) have been proposed as a promising therapeutic strategy to prevent dopaminergic neurons degeneration, particularly due to their paracrine action. Induced MSCs (iMSCs), obtained from induced pluripotent stem cells (iPSCs) differentiation, present an advantageous cell source for obtaining large amounts of secretome due to their superior proliferative capacity. In this project, we aimed to (1) develop a 3D *in vitro* model for the study of PD, (2) compare the long-term culture of iMSCs in either a commercial serum-free (SF) medium or a human platelet lysate (hPL) supplemented medium, (3) compare the effects of secretome from iMSCs expanded in different media and from early and late passages on dopaminergic differentiation, and (4) compare the effects of these secretomes on neuroprotection of a 3D *in vitro* model of PD. Results showed that the developed 3D collagen model demonstrated a robust network of neurons, that, when challenged with a toxin, resulted in a suitable PD model. hPL supplementation induces a higher proliferative capacity and lower levels of replicative senescence of iMSCs. The proteomic analysis reveals a secretory profile similar between iMSCs cultured in these two media, with a smaller portion of proteins that could be relevant for neuroregenerative processes being differentially expressed between them. Secretome from early passage iMSCs expanded in hPL supplemented medium had a positive impact on dopaminergic differentiation that other secretomes. The impact of these secretomes on the developed 3D model of PD did not show differences. Results show that the modulation of iMSCs secretome through expansion in different media and collection at different cell passages can influence therapeutic potential for PD.

Keywords: induced mesenchymal stem cells; Cell culture media; Cell passage; Parkinson's Disease; Secretome; Dopaminergic differentiation

RESUMO:

A Doença de Parkinson (PD) é uma doença neurodegenerativa complexa que está associada à perda de neurónios dopaminérgicos, levando a uma progressiva perda da capacidade motora. O tratamento para esta doença é maioritariamente sintomático, pelo que é necessário encontrar uma estratégia terapêutica eficaz que permita parar ou até reverter a progressão desta doença. Diversos estudos experimentais com base no uso de secretoma de células estaminais mesenquimatosas (MSCs) têm vindo a demonstrar diversos benefícios da sua utilização, tais como na neurogénese, na imunomodulação, na neuroprotecção e até na redução de agregados de α-sinucleína, uma das marcas características de PD. Estes resultados suportam o potencial do secretoma de MSCs como uma possível estratégia terapêutica para PD, no entanto, as MSCs retiradas de tecido adulto raramente são suficientes para a posterior utilização em ensaios clínicos e pré-clínicos, dada a sua capacidade proliferativa limitada. As MSC derivadas a partir de células estaminais pluripotentes induzidas (iMSCs) representam uma forma não exaustiva de obter elevados números de MSCs, removendo adicionalmente a necessidade de efetuar procedimentos invasivos para a sua colheita. Estudos prévios demonstraram que a expansão de MSCs em diferentes meios pode resultar na alteração de propriedades destas células, tais como a sua morfologia, capacidade proliferativa e o processo de senescência. Estas alterações podem ter implicações nas aplicações terapêuticas do secretoma coletado de MSCs expandidas em diferentes meios, pelo que se torna também necessário avaliar estas condicionantes relativamente às iMSCs. Desta forma, neste trabalho pretendeu-se: em primeiro lugar, desenvolver um modelo *in vitro* 3D e proceder à sua caracterização com vista ao seu emprego no estudo de PD; de seguida comparar a cultura a longo prazo de iMSCs em diferentes meios, correspondendo um a meio comercial sem soro e outro a meio suplementado com lisado de plaquetas humanas (hPL); posteriormente comparar os efeitos dos secretomas de iMSCs em diferentes passagens celulares e expandidas em diferentes meios na diferenciação dopaminérgica; e por fim comparar os efeitos destes secretomas na neuroprotecção de um modelo *in vitro* 3D onde foi induzida a degeneração dopaminérgica característica de PD.

Um modelo *in vitro* 3D foi desenvolvido através da cultura de corpos embrioides orientada para a diferenciação dopaminérgica em hidrogéis de colagénio e posteriormente caracterizado qualitativamente e quantitativamente. Esta caracterização resultou na revelação de um modelo com a presença de uma rede neuronal robusta e com uma

organização espacial 3D que mais se assemelha ao cérebro do que a organização refletida pela cultura celular 2D realizada. Uma porção das células obtidas foi identificada como neurónios dopaminérgicos (DA) maduros, o que revelou uma diferenciação bem-sucedida de células estaminais embrionárias de murganho (mESCs) em neurónios DA. Para além do uso de hidrogéis de colagénio, um *scaffolds* de origem natural, para o desenvolvimento deste modelo 3D, um outro *scaffold* alternativo, de origem sintética (baseado em Ormocomp), foi testado também. Os respetivos resultados sugeriram que ambos são capazes de suportar uma cultura celular 3D direcionada para a diferenciação dopaminérgica, apesar de o modelo 3D em colagénio ter tido uma capacidade superior para tal. A otimização da cultura 3D nestes dois *scaffolds* é, no entanto, necessária para futuros trabalhos experimentais, principalmente na cultura na estrutura baseada em Ormocomp. Devido à maior capacidade do modelo 3D em colagénio para originar neurónios dopaminérgicos, esse foi o modelo utilizado para posteriormente explorar os efeitos do secretoma coletado de iMSCs expandidas em diferentes meios e de diferentes passagens celulares.

Os resultados obtidos acerca da cultura a longo prazo de iMSCs revelaram que as propriedades destas células podem ser moduladas através do uso de diferentes meios, tendo sido utilizados neste caso um meio comercial quimicamente definido e livre de componentes animais e um meio suplementado por hPL. A expansão de iMSCs em meio suplementado com hPL demonstrou uma maior capacidade proliferativa destas células e menores níveis de senescência na fase inicial da cultura assim como na fase final. A avaliação do perfil secretório das iMSCs expandidas nestes dois meios demonstrou a presença de proteínas com relevância terapêutica, incluindo, por exemplo, proteínas com atividade catalítica ou antioxidante, e proteínas relacionadas com a matriz extracelular (ECM) e interação célula-a-célula que podem ter funções na regeneração de tecidos e neuroprotecção. O perfil secretório analisado demonstrou-se, na maior parte, semelhante para as duas condições, sendo que apenas uma pequena parte das proteínas se encontrava significativamente diferencialmente expressa entre as duas condições. Algumas das proteínas detetadas como diferencialmente expressas estavam associadas à matriz extracelular com funções relacionadas com adesão celular e outras com a linhagem neuronal e neurogénese.

A avaliação dos efeitos do secretoma de iMSCs expandidas em diferentes meios e de diferentes passagens celulares foi realizada recorrendo ao modelo 3D em colagénio. Esta

revelou que secretoma de iMSCs de passagem inicial expandidas em meio suplementado com hPL é capaz de promover crescimento axonal e diferenciação dopaminérgica com sucesso, tendo os seus efeitos sido em parte similares aos promovidos pelo meio positivo de diferenciação dopaminérgica. Secretoma de iMSCs de passagem tardia expandidas em meio suplementado com hPL teve efeitos menos positivos nestes mesmos aspetos. Secretoma de iMSCs de passagem inicial expandidas em meio comercial de composição definida sem componentes animais também teve uma menor capacidade relativamente à promoção de diferenciação dopaminérgica. Estes resultados confirmam que não só o meio em que as iMSCs são expandidas, como também a passagem celular em que o secretoma é coletado, podem modular as propriedades terapêuticas destas células.

A exposição do modelo 3D em colagénio a uma toxina, 6-OHDA, levou ao desenvolvimento de um modelo *in vitro* capaz de refletir a degeneração dopaminérgica presente em PD. Os efeitos do secretoma de iMSCs na neuroprotecção foram testados neste modelo de PD. Os resultados não demonstraram diferenças visíveis ou estatisticamente significativas na viabilidade da cultura entre o grupo veículo e o grupo sujeito à toxina.

Em suma, estes resultados demonstram que as iMSCs podem ser moduladas por forma não só a aumentar o número de células passíveis de serem obtidas como também de forma a potenciar algumas das suas aplicações terapêuticas, como a sua capacidade de diferenciação dopaminérgica, relevando o seu potencial terapêutico em doenças como PD.

Palavras-chave: Células estaminais mesenquimais induzidas; Meio de cultura celular; Passagem celular; Doença de Parkinson; Secretoma; Diferenciação dopaminérgica

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Abbreviations list

#

2D – two-dimensional

3D – tridimensional

6-OHDA – 6-hydroxydopamine

A

AA - Ascorbic acid

AAV - Adeno-associated virus

ANOVA - Analysis of variance

ASCs - Adipose-derived MSCs

B

BDNF - Brain-derived neurotrophic factor

BM-MSCs - Bone marrow-derived MSCs

C

CD - Cluster of differentiation

CDK – Cyclin-dependent kinase

CNS – Central nervous system

CO_2 – Carbon dioxide

COL6 – Collagen VI

COMT - Catechol-O-methyltransferase

CS – Calf serum

D

DA – Dopaminergic

DAPI - 4-6-diamidino-2-phenylindole-dhydrochloride

DAT – Dopamine transporter

DBH - Dopamine beta-hydroxylase

DBS – Deep brain stimulation

DCN – Decorin

DCX - Doublecortin

DMEM - Dulbecco's Modified Eagle Medium

E

EBs – Embryoid bodies

ECM – Extracellular matrix

ECM1 - Extracellular matrix protein 1

F

FBS – Fetal bovine serum

FGF2 - Fibroblast growth factor 2

FGF8 – Fibroblast growth factor 8

FSTL1 - Follistatin-like 1

G

GAP43 - Growth Associated Protein 43

GDNF - Glial cell line-derived neurotrophic factor

GFAP - Glial fibrillary acidic protein

GFP – Green fluorescent protein

GMP - Good Manufacturing Practice

GWAS - Genome-wide association studies

H

HGF – Hepatocyte growth factor

HLA – Human leukocyte antigen

hPL – human Platelet lysate

I

IGF1 – Insulin-like growth factor-1

IGFBP4 - Insulin growth factor binding protein-4

IGFBP6 - Insulin growth factor binding protein-6

IL-6 – Interleukin-6

IL-8 – Interleukin-8

iMSCs - MSCs derived from induced pluripotent stem cells

iPSCs - induced pluripotent stem cells

L

LAS - Lysosomal autophagy system

LTBP2 - Latent-transforming growth factor β-binding protein 2

M

MAP2 - Microtubule-associated protein 2

MCP-1 - Monocyte chemoattractant protein-1

MDS - Movement Disorder Society

mEF – mouse embryonic fibroblasts

mESC – mouse embryonic stem cells

MMPs - Matrix metalloproteinases

MPP^+ - Pro-parkinsonian molecule

MSCs – Mesenchymal stem cells

MTPT - 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTS – [(5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt)]

N

NO – Nitric oxide

NRTN – Neurturin

NSCs - Neural stem cells

NPCs - Neural progenitor cells

NT-3 – Neurotrophin-3

P

PBMCs - Peripheral blood mononuclear cells

PBS – Phosphate buffered saline

PD – Parkinson’s disease

PDL – Poly-D-lysine

PFA – Paraformaldehyde

PSD95 - Postsynaptic density protein 95

Q

qRT-PCR - quantitative real-time polymerase chain reaction

R

RBD - Rapid eye movement sleep behaviour disorder

RNA – Ribonucleic acid

ROI – Region of interest

ROS - Reactive oxygen species

RT – Room temperature

S

SA-β-Gal - Senescence-associated β-galactosidase

SAG – Smoothened agonist

SASP - Senescence-associated secretory phenotype

SEM - Standard error of the mean

SF – Serum-free

SHH – Sonic hedgehog

SN – Substantia nigra

SNc - Substantia nigra pars compacta

SPARC - Secreted protein acidic and rich in cysteine

T

TH – Tyrosine hydroxylase

U

UC-MSCs - Umbilical cord mesenchymal stem cells

UPS - Ubiquitin–proteasome system

UV – Ultraviolet

V

VEGF - Vascular endothelial growth factor

X

X-Gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1 – INTRODUCTION

1. Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease that was first referred to as a *shaking palsy* by James Parkinson, in 1817, when he described a motor disturbance pattern of involuntary tremor, bended posture and festinating gait in his patients (reviewed in Parkinson, 2002). Since then, it has been discovered that, although PD is commonly referred to as a movement disorder, there are a variety of non-motor aspects that are also part of the disease. The effort put into PD research resulted in a much wider understanding of its pathophysiology, progression, symptoms and has enabled the search for new therapies that aim to decrease symptoms and delay disease progression. However, PD has revealed itself to have more complexity than what was thought in the first place, not only by being a multifactorial disease but also because its progression correlates with an increase in the severity of treatment-resistant motor and non-motor symptoms (Rukavina et al., 2021). This makes finding disease-modifying therapies that delay further disability an important need.

1.1. Epidemiological and demographic characteristics

The number of people affected with PD globally has been rapidly increasing through the years, having gone from 2.5 million individuals affected, in 1990, to 6.1 million, in 2016 (Ray Dorsey et al., 2018). This increase can be due to: (1) having a worldwide aged population, although from 1990 to 2016 there has also been a 22% increase on age-adjusted prevalence; (2) an improvement in the diagnostic methods; (3) a longer disease duration that comes with higher life expectancy; and (4) environmental factors (Ray Dorsey et al., 2018).

The incidence of PD varies according to different reports, with annual values ranging from 5 to >35 cases per 100 000 individuals (Twelves et al., 2003). Even though PD is unusual before the age of 50, typically representing monogenic forms of PD, there is a dramatic increase in the incidence rate of PD after the age of 65, to 160 per 100 000 (Ascherio & Schwarzschild, 2016; Tysnes & Storstein, 2017).

Even though in the first decade after PD onset patients' mortality remains similar to general population, after that, it increases substantially, being implied that the longer the disease duration, the higher the mortality (Pinter et al., 2015).

Based on a study made in Portugal, PD has been estimated to affect around 180 per 100 000 Portuguese inhabitants (Ferreira et al., 2017). Within western Europe and from 1990 to 2016,

Portugal holds one of the highest increases in terms of age-standardized prevalence (31.9%) and death counts (34.3%) (Ray Dorsey et al., 2018).

The different ethnicities, genotypes and environments also influence PD numbers. Studies on different ethnic populations within the same country show that incidence rates of PD are higher on Hispanic and Caucasians than in Asians and Africans, possibly reflecting a genetic susceptibility (Abbas et al., 2018). On the other side, African American and Japanese American populations have higher occurrence of PD than in similar populations in their native countries, supporting a role of environmental factors (Abbas et al., 2018).

1.2. Risk factors

PD is considered a multifactorial disease, being caused not only by genetic factors but also by environmental factors, which makes PD a very complex disorder. In that regard, identifying the diverse risk factors and understanding how they interplay is of great importance. Although age is the major risk factor for PD, other non-modifiable factors like genetics and gender also contribute for developing PD.

The genetic component of this disease started being uncovered through the study of cases of familial PD, unveiling rare variants with high penetrance that caused monogenic PD (Table 1.1) (Day & Mullin, 2021). These forms of monogenic PD are associated to autosomal dominant (e.g.: SNCA, LRRK2 and VPS35), as well as autosomal recessive inheritance (e.g. PRKN, PARK7 and PINK1) (Blauwendraat et al., 2020; Day & Mullin, 2021).

Recently, genome-wide association studies (GWAS) have allowed the detection of common genetic variants with low penetrance that can have a combined effect and contribute to the risk of developing sporadic PD (Blauwendraat et al., 2020; Day & Mullin, 2021; Maraganore et al., 2005).

Table 1.1 – List of genes with high penetrance variants causing Parkinson's Disease.

Gene	Locus symbol	Protein	Function(s)	Inheritance	PD Phenotype
SNCA	PARK1	α-synuclein	Synaptic vesicle Dynamics Intracellular trafficking Mitochondrial function	Autosomal dominant	Early-onset
VPS35	PARK17	Vacuolar protein sorting 35	Retromer and endosomal trafficking	Autosomal dominant	Classic
PRKN	PARK2	Parkin	E3 ubiquitin ligase	Autosomal recessive	Early-onset
DJ-1	PARK7	DJ-1	Putative antioxidant	Autosomal recessive	Early-onset
PINK1	PARK6	PTEN-induced putative kinase 1	Mitochondrially targeted serine-threonine kinase	Autosomal recessive	Early-onset

Besides non-modifiable risk factors, important modifiable risk factors have also been identified, such as the regular exposure to pesticides and solvents or the non-use of caffeine and nicotine (Hernán et al., 2002; Pezzoli & Cereda, 2013).

1.3. Clinical features

PD is also heterogenous and multifaceted at the clinical level, comprising not only classical motor clinical features, but also nonmotor manifestations (Obeso et al., 2017). This disease shows progressive cell death, only becoming clinically evident when there's a depletion of approximately 70–80% of striatal dopamine and the loss of 30–50% of the dopaminergic neurons (H. C. Cheng et al., 2010). PD progression has been divided in three stages, the Movement Disorder Society (MDS): preclinical, prodromal and clinical, as seen in Figure 1.1 (Berg et al., 2015).

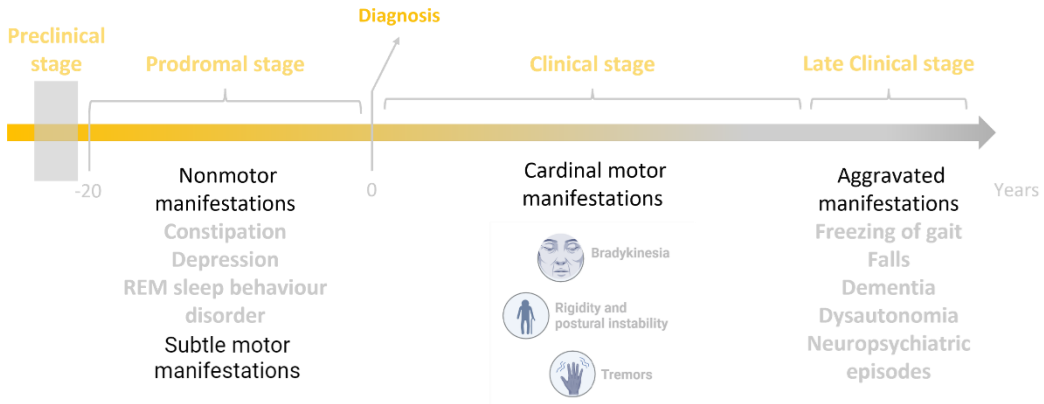

Figure 1.1 – Representation of the different stages of Parkinson's Disease and related clinical manifestations. *Created with BioRender.com*

Preclinical

The preclinical stage corresponds to the phase where neurodegeneration starts progressively occurring, but no symptoms are manifested (Berg et al., 2015), being only potentially evaluated using biomarkers (Meles et al., 2021).

Prodromal

In the prodromal stage, a set of nonmotor and motor manifestations can be used to assess the probability of the disease being present (Berg et al., 2015). Although the order in which motor and nonmotor manifestations develop varies among patients, frequently certain nonmotor manifestations appear first (Berg et al., 2015). Examples of early nonmotor signs include constipation, depression, rapid eye movement sleep behaviour disorder (RBD) and olfactory dysfunction. Even though the prodromal phase has a predominance of nonmotor manifestations, database studies have shown that motor alterations can also occur before PD diagnosis, such as voice and face akinesia, rigidity, gait abnormalities, limb bradykinesia and tremor (Postuma et al., 2012; Schrag et al., 2015).

Clinical

PD clinical phase starts with a diagnosis of the disease, which is characterized by four cardinal motor manifestations - tremor at rest, rigidity, bradykinesia and postural instability (Poewe et al., 2017). Even though clinical features are well described, PD diagnosis remains a challenge due to parkinsonism being a set of manifestations common with other neurodegenerative conditions (Rajput & Rajput, 2014).The challenge persists even after diagnosis, given that this disease is highly heterogeneous in terms of underlying mechanisms, clinical presentation, rate of progression, prognosis and treatment response (Tolosa et al., 2021). This highlights the need

to subtype PD according to these characteristics, in order to develop personalised treatment approaches (Tolosa et al., 2021).

Disease progression over the years culminates with late-stage PD, marked as a period that goes from an unstable advanced phase to a palliative phase (Rukavina et al., 2021). This progression is characterized by an aggravation of both treatment-resistant motor symptoms, such as freezing of gait, postural instability, falls and dysphagia, and nonmotor symptoms, observing cognitive decline that develops into overt dementia, neuropsychiatric symptoms, sleep disturbances, and dysautonomia aspects, for instance (Rukavina et al., 2021). This last phase of disease progression occurs in an exponential manner, culminating with death within approximately 5 years (Coelho & Ferreira, 2012).

1.4. Pathophysiology and neuropathology

PD is commonly referred to as a movement disorder, although it is already known that nonmotor alterations also occur in this disease. Even so, the main focus of studies has been to identify the neural network involved in the motor pathophysiology, leaving the nonmotor network poorly understood.

The neuropathological features of PD consist in moderate to severe neuronal loss in the Substantia nigra pars compacta (SNc) together with abnormal deposition of intracellular α-synuclein, resulting in the formation of Lewy bodies (Gelb et al., 1999). Although these aspects can be present in other pathologies, the identification of both these neuropathologies is necessary for definitive diagnosis of idiopathic PD (Dickson et al., 2009). Additionally, further neuronal assessment can show fibrillary astrocytosis and extraneuronal neuromelanin (Dickson et al., 2009).

Nigrostriatal dopaminergic neurons degeneration

In early stage of the disease the loss of dopaminergic neurons happens mainly in the lateral ventral tier of the SNc, whereas in a more advanced stage this neurodegeneration becomes widespread (Poewe et al., 2017). In early-stage disease, striatal dopaminergic loss exceeds susbtantia nigra (SN) neuronal death, which suggests that the degeneration of the nigrostriatal pathway is retrograde (Hernandez et al., 2019).

Several reasons have been put forward for SNc dopaminergic neurons vulnerability to cell damage, including: (1) a high requirement of energy supply due to having long, unmyelinated

axons with large number of synapses, (2) the production of highly reactive species by the dopaminergic metabolism, which results in oxidative stress and mitochondrial dysfunction, and (3) autonomous pacemaking activity, leading to calcium-mediated toxicity (Bolam & Pissadaki, 2012; Mosharov et al., 2009; Obeso et al., 2010).

Striatum and SNc are part of basal ganglia nuclei, which are involved in thalamo–cortico–basal ganglia circuits responsible for the control of actions, motor learning, executive functions, goal-directed behaviour, and emotions (Alexander et al., 1986, 1991). Therefore, SNc cell loss decreases dopaminergic transmission in the striatum, disrupting the balance between neuronal pathways controlling movement (Neumann et al., 2018).

α-synuclein abnormal accumulation

Another neuropathology characteristic of PD is the abnormal deposition of α-synuclein, in the form of Lewy bodies when present in somatodendritic compartments of certain neurons and, in the form of Lewy neurites, when observed in the neurites (Volpicelli-Daley et al., 2014). Lewy bodies, mostly made of aggregated α-synuclein, were the first form of aggregates described, being observed in several different brain regions (GIBB & POEWE, 1986; Poewe et al., 2017). Other forms of α-synuclein aggregates have been described, such as diffuse and granular deposits of α-synuclein, extracellular dot-like α-synuclein structures and α-synuclein spheroids in axons (Poewe et al., 2017).

It is suggested that Lewy pathology has a progressive spreading during the development of PD, where it first appears in the olfactory bulb causing olfactory dysfunction, then it progresses to SNc where it results in the onset of motor symptoms and, lastly, it accumulates in neocortical regions, causing cognitive decline and dementia (Braak et al., 2003; Kon et al., 2020).

1.5. Cellular and molecular pathways affected in PD

The knowledge on PD-associated gene mutations provided clues on the cellular and molecular mechanisms underlying PD neuropathology. These and other proteins have been linked to pathways related to α-synuclein proteostasis and degradation, mitochondrial dysfunction, oxidative stress, impaired calcium homeostasis, and neuroinflammation (Figure 1.2) (Poewe et al., 2017).

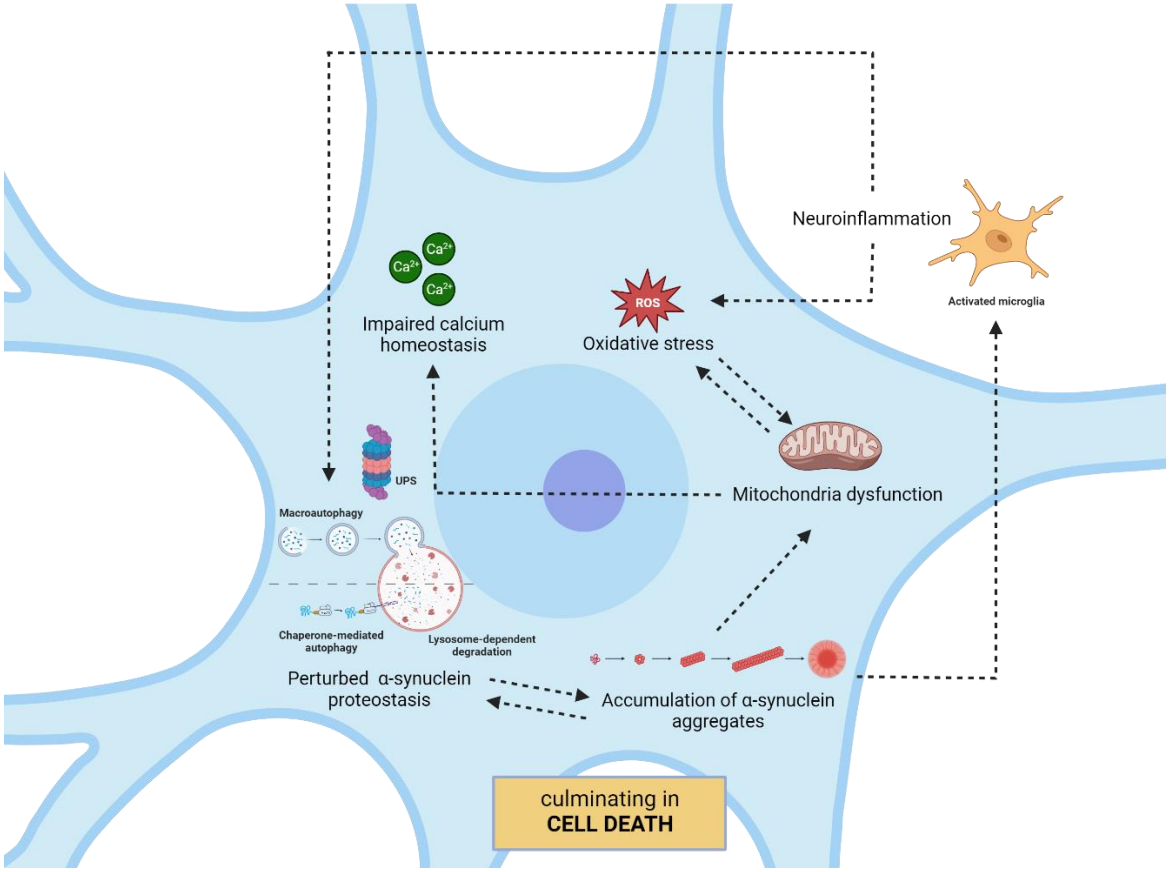

Figure 1.2 – Different cellular pathways associated with Parkinson's Disease neurodegeneration. *Created with BioRender.com*

α-synuclein proteostasis and degradation

α-synuclein is a small protein composed of 140 amino acids that can be either in a soluble form or in a membrane-bound state, being predominantly expressed in the brain (Burré et al., 2018). Even though α-synuclein is commonly associated to being localized bound to synaptic vesicles in presynaptic terminals, studies have reported that it can also be found in other subcellular localizations such as the mitochondria or the nucleus (Burré et al., 2010; Li et al., 2007; Yu et al., 2007).

α-synuclein aggregation is thought to occur in a progressive manner, where misfolded monomers are firstly combined into dimers, which then stabilize into small oligomeric species. Eventually, these oligomers cluster and form insoluble protofibrils, which in turn form amyloid-like fibrils that give rise to Lewy bodies (Winner et al., 2011). The aggregation of α-synuclein can have many causes, such as aging or genetic mutations that either lead to the overproduction of this protein or its misfolding and/or to impairments in molecular pathways that culminate in the imbalance between its production and degradation (Poewe et al., 2017). Once α-synuclein

is accumulated, a pathological vicious cycle is developed, in which the protein aggregates induce further impairment of the pathways involved in its degradation (Lindersson et al., 2004).

The major intracellular proteolytic systems responsible for α-synuclein degradation are the ubiquitin–proteasome system (UPS) and the lysosomal autophagy system (LAS) (Poewe et al., 2017). Parkin and UCH-L1, two PD-linked genes, were found to encode proteins from the UPS, suggesting that this degradation system is involved in the pathogenesis of PD (Kitada et al., 1998; Maraganore et al., 2004). Nevertheless, the UPS seems to only degrade specific species of α-synuclein, such as small, soluble oligomers, while LAS, on the other side, acts on pathological conditions (Xilouri et al., 2013).

From LAS, two pathways contribute to the degradation of α-synuclein: (1) chaperone-mediated autophagy, where specific chaperones target certain proteins to lysosomes and (2) macroautophagy, which involves the formation of autophagosomes that engulf intracellular constituents and later fuse with perinuclear lysosomes, forming the autophagolysosome (Xilouri et al., 2013). The study of experimental PD models and the analysis of postmortem PD patients' brains revealed reduced levels of lysosomal enzyme, fewer markers of chaperone-mediated autophagy and accumulated autophagosomes (Alvarez-Erviti et al., 2010; Anglade, 1997; Chu et al., 2009).

Many mutations that eventually lead to perturbations in the LAS were identified in familial and sporadic cases of PD. The G2019S mutation in LRRK2 gene, for example, increases the susceptibility of α-synuclein to form inclusions and causes impairment of LAS, by increasing the number of autophagosomes and decreasing the number of acidic lysosomes (Gómez-Suaga et al., 2012; Volpicelli-Daley et al., 2016).

Mitochondrial dysfunction

The involvement of mitochondrial dysfunction in the pathogenesis of PD has been suspected ever since MTPT consumption induced severe parkinsonism on drug addicts (William Langston et al., 1983). When MPP^+, an inhibitor of part of the electron transport chain (complex I), was administered to animal models, a selective death of DA neurons occurred, recreating the context of PD (Dauer & Przedborski, 2003; William Langston et al., 1983). Moreover, the analysis of PD patients' brains revealed a deficit in mitochondrial complex I activity, which supports the involvement of mitochondria in PD (Pollard et al., 2016). It is suggested that α-synuclein interacts with the mitochondrial complex I in two ways: through direct association with the complex, inhibiting it; or indirectly, by interacting with cardiolipin, a protein necessary for

complex I function (Devi et al., 2008; Zhang et al., 2002). This leads to the idea that α-synuclein accumulation and mitochondrial dysfunction exacerbate each other, creating a cycle relevant to PD pathogenesis (Poewe et al., 2017).

Additionally, the involvement of mitochondria in PD pathogenesis was also supported by the discovery of recessive genes associated to familial PD, PINK1 and PARK2, that code for proteins responsible for the mitochondrial network maintenance (Narendra et al., 2012). Mutations regarding these genes result in the accumulation of non-functional mitochondria, presenting cellular toxicity (Pickrell & Youle, 2015).

Oxidative stress

More than just a secondary response to the progressive neurodegeneration, oxidative stress has been proposed to also be a causative factor for DA neuron death, although this remains unclear (Trist et al., 2019). An imbalance between the production of reactive oxygen species (ROS) and their clearance by antioxidant enzymes culminates in ROS accumulation (Schieber & Chandel, 2014). This imbalance can be caused by an overproduction of ROS, due to increased levels of cytosolic dopamine, for example, and/or because the activity of antioxidant enzymes is compromised, when, for example, there are mutations in the PARK7 gene (Guzman et al., 2010; Obeso et al., 2010). The analysis of brain tissue from PD patients reveals oxidative damage of lipids, proteins, and nucleic acids, with increased oxidative stress (Bosco et al., 2006; Nakabeppu et al., 2007; Schieber & Chandel, 2014).

It has been shown that impairment of mitochondria and increased oxidative stress can result in the disruption of lysosomal structural integrity, leading to a defective clearance system (Dehay et al., 2010). This depletion of lysosomes ends up causing an accumulation of autophagosomes and of altered proteins and organelles, which can cause cell dysfunction and death (Dehay et al., 2010). On the other hand, it is hypothesized that, when oxidative stress is combined with intracellular iron overload, two events commonly present in PD, there can be the production of a severely toxic hydroxyl radical that induces cellular damage, lipid peroxidation and can trigger apoptosis (H. Jiang et al., 2017; Sian-Hülsmann et al., 2011).

Impaired calcium homeostasis

Calcium homeostasis is particularly important in DA neurons, since it is crucial for their pacemaking activity and because these cells have a low calcium buffering capacity (Michel et al., 2016). Phenomena related to PD such as α-synuclein aggregation, mitochondrial and

endoplasmic reticulum dysfunction can indirectly cause a dysregulation of calcium homeostasis, particularly affecting this type of neurons (Michel et al., 2016).

Studies have shown that an overload of Ca^{2+} can lead to the increase of DA synthesis, post-translational modifications of α-synuclein and activation of calpains, posing harmful effects for DA neurons and contributing to PD progression (Michel et al., 2016). On the other side, levels of Ca^{2+} under the threshold can also compromise the survival of DA neurons, possibly because these neurons highly rely on proteins regulated by calcium (Michel et al., 2016).

Neuroinflammation

Postmortem, genetic and imaging studies have revealed that, even though chronic neuroinflammation is most likely not the initial cause for PD, it probably contributes for its pathogenesis (Moehle & West, 2015; Poewe et al., 2017).

The study of evidence from both experimental models and patients places the hypothesis that α-synuclein aggregation in PD induces an innate and adaptive immune response in the body (Poewe et al., 2017). For instance, microglia activation plays an important role in the homeostasis of the brain, since it is responsible for the phagocytosis and degradation of extracellular α-synuclein aggregates during PD (Rocha et al., 2018). However, this microglia activation involves proinflammatory alterations that eventually lead to oxidative stress, such as the production of nitric oxide (NO) through the activation of astrocytes (Hewett et al., 1993). Even though this nitrosative stress is indirectly a result of the inflammatory environment established by the α-synuclein, NO has been identified to cause an impairment of the UPS system, contributing for α-synuclein accumulation (Gu et al., 2005). This causes a cycle of protein accumulation, microglia activation and neuronal damage that ends up playing a part in PD progression (Rocha et al., 2018).

To conclude, through the years the know-how on different cellular and molecular mechanisms involved in PD pathogenesis has abruptly increased, showing the complexity of this disease. Nonetheless, most of the studies focus on mechanisms underlying alterations in dopaminergic neurons, while the mechanisms related to other cells are also damaged in PD, such as non-dopaminergic neurons and glial cells, remain unknown (Rocha et al., 2018).

2. Experimental models of Parkinson's Disease

To have a wider knowledge on PD pathology, molecular pathogenesis, discover possible diagnostic tools and potential biomarkers, several studies are directed to the development of reliable *in vitro* and *in vivo* experimental models, conceived to mimic specific features of PD. Although many aspects interfere with the translation of the model characteristics into the human being itself, the achievement of representative models for this disease can open doors for the discovery of efficient therapies.

Resorting to animal models allows a more global view of the pathological and behavioural aspects of the disease, since it captures the full complexity of an organism, including the interactions between different functional systems and the phenotypical consequences of intrinsic alterations. To then evaluate and validate the phenotype of PD, behavioural tests are performed, being mostly directed to the motor aspect of the disease, since motor manifestations are considered the cardinal signals of PD and are easier to observe and measure (Brooks & Dunnett, 2009; Olsson et al., 1995; Ungerstedt & Arbuthnott, 1970). Although animal models provide important knowledge to better understand the disease and are necessary to test and validate *in vitro* studies in a whole organism, there are other aspects that cannot be covered only by animal models, as for example performing large-scale pre-clinical studies.

The use of *in vitro* models to study PD brings numerous advantages (Figure 1.3). Cell culture allows an easier manipulation of both the cells and their microenvironment, and a quicker and more scalable way to experimentally study diverse conditions (Chia et al., 2020). Additionally, a more detailed cellular and molecular analysis can be performed in *in vitro* models, yielding a deeper understanding of PD pathogenesis and even to the effects of candidate therapies on the disease-specific cells (Chia et al., 2020).

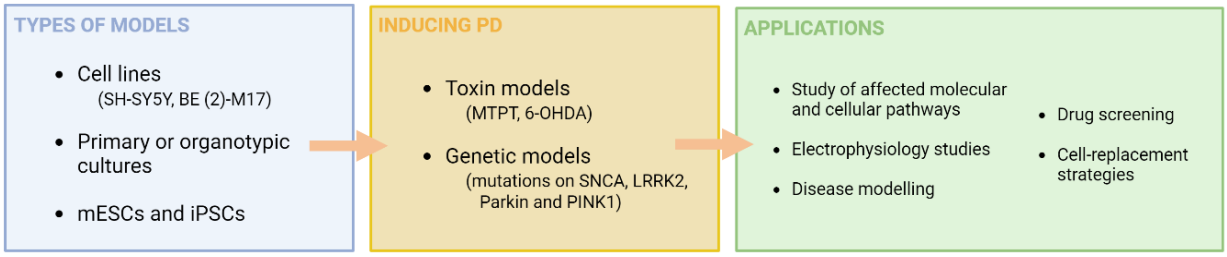

Figure 1.3 – Schematic organization of *in vitro* experimental models of Parkinson's disease and their applications. PD-like phenotypes can be induced in i*n vitro* models with the use of toxins or genetic manipulation. Cellular models involve cells lines, primary or organotypic cultures, and disease-specific cell types differentiated from mouse embryonic stem cells (mESCs) or from induced pluripotent stem cells (iPSCs). The developed models can then be used with different purposes as to deepen knowledge regarding specific molecular pathways affected in PD or to find a potential drug candidate

that halts or decreases the progression of this disease (Finkbeiner & Skibinski, 2011; Y. Wang et al., 2021). *Created with BioRender.com*

2.1. Inducing Parkinson's Disease

2.1.1. Toxin models

Toxin models can be created through the administration of classical neurotoxins, as 6-hydroxydopamine (6-OHDA) and MPTP, or agrochemicals, as the herbicide paraquat and the pesticide rotenone (Raza et al., 2019).

6-OHDA

6-OHDA is a neurotoxin, structurally analogue of dopamine, that leads to the selective loss of DA neurons. Due to its similar chemical structure to dopamine, it can be transported into dopaminergic neurons, where it gets rapidly oxidized and leads to the production of free radicals and hydrogen peroxide (Fulceri et al., 2006). This results in oxidative stress which then initiates cellular stress, culminating with dopaminergic cell death (Blum et al., 2001). Besides, 6-OHDA has been associated with having a role in initiating caspase-dependent apoptosis (Y. M. Ding et al., 2004)

This toxin has been highly reported as capable of inducing a PD phenotype on not only *in vivo* but also *in vitro* models (Y. M. Ding et al., 2004; Lopes et al., 2010; Sadan et al., 2009). The development of *in vitro* models based on the use of 6-OHDA constitutes a valuable tool for the study of specific components of the pathogenesis of PD and to evaluate the effects of experimental therapies on the restoration of the DA system (Simola et al., 2007).

MTPT

This classical neurotoxin can efficiently and selectively cause DA degeneration in the nigrostriatal tract, with known mechanisms underlying PD pathogenesis being evident, such as oxidative stress and mitochondrial dysfunction (P. Jiang & Dickson, 2018). However, there is a failure to produce a slow, progressive neurodegeneration and to showcase the typical intraneuronal inclusions, as seen in PD (Duty & Jenner, 2011).

Paraquat and rotenone

These agrochemicals have proven to be able to induce Lewy pathology on DA neurons, one of PD's hallmarks (Blesa & Przedborski, 2014). In terms of DA degeneration, the results regarding both agrochemicals show variable loss in striatal DA content (Blandini & Armentero, 2012).

2.1.2. Genetic models

Toxin-based models show a difficulty to reproduce the α-synuclein pathology observed in PD patients, requiring the use of other models to complement the research (Raza et al., 2019). Genetic models for PD can be obtained either through overexpression strategies for autosomal dominant genes such as SNCA and LRRK2, or through knockdown/knockout strategies for autosomal recessive genes, as parkin, PINK1 and DJ-1. These models emerge as opportunities for the study of mechanisms underlying the pathogenesis of genetic forms of PD (Raza et al., 2019). To obtain these cell specific mutations *in vitro*, different procedures can be applied, such as collecting fibroblasts from patients with monogenic forms of PD and then reprogramming them into the desired cells for their study.

Genetic models based on α-synuclein

The accumulation of α-synuclein fibrils can lead to neuronal loss and recreate characteristics of PD pathology (Luk et al., 2012; Wu et al., 2019). In this regard, to develop an *in vitro* model of PD, a research group prepared pre-formed fibrils of human α-synuclein and then added them to differentiated SH-SY5Y cells, obtaining significant pathological α-synuclein accumulation in these human neural cells (Ross et al., 2020).

Another research group was able to produce multiple induced pluripotent stem cell lines derived from a patient with an aggressive form of PD, consisting of a triplication of the SNCA gene (Devine et al., 2011). After differentiation into midbrain DA neurons, these cell lines reflected the overexpression of α-synuclein, recapitulating an important hallmark of PD in the patients.

These studies uncovered new experimental *in vitro* models for the study of neurodegenerative mechanisms underlying α-synuclein accumulation and of potential compounds that alleviate the α-synuclein burden.

Genetic models based on LRRK2

In order to further study the role of LRRK2 in PD pathogenesis, a research group proposed the transient overexpression of LRRK2 G2019S in HEK293 cells that had been previously transfected with α-synuclein (Hu et al., 2018). This study revealed that the G2019S mutation of LRRK2 disrupts the autophagic-lysosomal pathway and culminates in the accumulation of α-synuclein, providing a good PD model for this genetic form.

Another possibility for generating an *in vitro* genetic model based on LRRK2 consists of harvesting fibroblasts from PD patients harbouring the LRRK2 G2019S mutation (Thomas et

al., 2020). This model allowed deeper knowledge on the role of this mutation in mitochondrial and lysosomal dysfunction, uncovering new mechanisms underlying PD.

Genetic models based on Parkin and PINK1

To further understand the mechanisms through which mutations in the Parkin and PINK1 genes can lead to PD pathogenesis, an *in vitro* study performed DA differentiation of iPSCs derived from cells belonging to PD patients with either recessively inherited Parkin or PINK1 mutations (Chung et al., 2016). The results suggested that these mutations lead to a pathological loop consisting of mitochondrial defects, α-synuclein accumulation, synaptic dysfunction, increased oxidative stress and ROS, reflecting PD phenotypes in this model.

Taking into consideration the current models for PD, it seems necessary to combine toxin and genetic models, in order to achieve a more complete characterization of this disease, from its aetiology to its pathophysiology. At the same time, refining the existent models for PD would allow their usage for disease-modifying therapies with higher confidence on the translation from the model to the human being.

2.2. Types of models for in vitro studies

2.2.1. Cell lines

Although the neuronal phenotype does not include a high proliferation capacity, the cell lines used to study PD are either naturally or artificially immortalized, offering reproducible testing and the rapid screening of drug candidates (Lopes et al., 2017). The SH-SY5Y human neuroblastoma cell line (ATCC CRL-2266) is widely used in PD research, due to its useful capacity to differentiate into neuron-like cells (Xicoy et al., 2017). Nonetheless, a recently published article has demonstrated that SH-SY5Y cells do not successfully differentiate into functional DA neurons and, consequently, are not suitable for the study of the DA component of PD (Carvajal-Oliveros et al., 2022). Alternatively, it suggests that BE (2)-M17 (ATCC CRL-2267) cells are more adequate for this research, reliably differentiating into DA neurons with the use of a simple protocol (Carvajal-Oliveros et al., 2022).

2.2.2. Primary or organotypic cultures

Primary cultures of neurons, usually obtained from embryonic rodent brain tissue, represent valuable models for mimicking the DA neuron phenotype, closely resembling their morphology and physiology (Gaven et al., 2014). These models allow the induction of PD pathology and,

later, the investigation of DA cell survival, neurite retraction and cellular regeneration (Schlachetzki et al., 2013). Organotypic cultures, on the other hand, can maintain certain *in vivo-like* scenarios, such as the nigrostriatal circuitry and the brain region cytoarchitecture (Daviaud et al., 2013). This model is advantageous for experiments that require long term survival as, for example, chronic drug administrations or electrophysiology studies (Daviaud et al., 2014). Notwithstanding, both models present a low reproducibility of experiments, taking to consideration that the involved technical procedures are demanding and there is considerable cellular heterogeneity (Lopes et al., 2017).

2.2.3. Pluripotent stem cell-derived dopaminergic neurons

Pluripotent stem cells are a valuable group of cells with a self-renewal capacity that can give rise to cells from the three primary germ cell layers of the early embryo (Romito & Cobellis, 2016). For this reason, across the last decades, these cells have been explored in multiple fields of research, as for instance, on the development of *in vitro* models for diseases or for regenerative medicine.

Human embryonic stem cells (hESCs) are a source of pluripotent cells that can be obtained from blastocyst-stage embryos. Although these source of cells as revealed itself to be promising for not only the *in vitro* study of degenerative diseases but also for the replacement of damaged tissue from patients affected with these diseases, there are major ethical issues concerning their use, since the harvesting of these cells leads to the destruction of the blastocyst (Lo & Parham, 2009). mESCs can be an alternative to human embryonic stem cells since have a non-human origin, eliminating the ethical burden concerning the utilization of hESCs (King & Perrin, 2014). Besides, they still allow a robust study of the disease, including the mechanisms underlying it, and enable the testing of potential therapies (Yamashita et al., 2006).

In 2007, a breakthrough in stem cells was made, with the discovery of the possibility to originate pluripotent, embryonic-like, stem cells by accessible reprogramming somatic cells (Takahashi et al., 2007). It was shown that this reprogramming was achievable by introducing four transcription factors in these cells: Oct4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). The resulting iPSCs reveal similar morphological and phenotypical properties to human embryonic stem cells (hESCs), with the capacity to self-renewal and the potential to generate cells from the three germ layers (Shi et al., 2017; Takahashi et al., 2007). They are a valuable alternative to the use of hESCs, which brings ethical concerns and likewise, can be differentiated into basically any cell type, when given a specific combination of factors (Mertens et al., 2016).

The differentiation of pluripotent stem cells, independently of their origin, into dopaminergic neurons is achieved through the addition of soluble factors in specific timepoints to recapitulate what is known about their embryonic development (Mammadov et al., 2012). Still, the choice of these factors, as well as their concentration and timing of addition, needs to be optimized for each species and cell line. The general concept consists of firstly directing the pluripotent stem cells into a neuronal lineage. Afterwards, to mimic *in vivo* neural floor-plate patterning, the sonic hedgehog (SHH) pathway should be activated and fibroblast growth factor 8 (FGF8) should be added to the neural stem cells (Ye et al., 1998). Finally, cells can be directed into terminal differentiation either through the withdrawal of mitogens and/or by adding certain growth factors and molecules as, for example, ascorbic acid (AA), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Kriks et al., 2012; Playne & Connor, 2017).

The development of protocols for 2D *in vitro* cultures that give rise to DA neurons has already been achieved, for example, through the differentiation of iPSCs into midbrain DA neurons (B. Song et al., 2020; Xi et al., 2012). This differentiation allows the development of *in vitro* models for PD, where the disease mechanisms can be more closely studied, and provides a more scalable process to perform drug tests, increasing chances to find an efficient therapy for this disease. Notwithstanding, the use of iPSCs to obtain DA neurons needs to be further optimized to efficiently achieve an adequate number of DA neurons on the cultures of cells in a reproducible manner (Gilmozzi et al., 2021).

2.3. Substrates and scaffolds

Although these cell culture systems have already provided some knowledge on molecular pathways that lead to DA loss, thereby contributing to the better understanding of PD, they still present certain limitations that are a setback on further advancement. For instance, formed 2D cultures are composed of a monolayer of cells, where interactions cell-cell and cell-matrix are not well revised, and maintained on rigid surfaces, which do not recapitulate the *in vivo* neuronal environment (Edmondson et al., 2014). Therefore, cells grown in 2D culture systems will not only exhibit non-physiological responses that affect cell survival, migration and differentiation, but will also demonstrate variable levels of neuron maturation and different functionality from the one observed in *in vivo* DA neurons (Edmondson et al., 2014; Geckil et al., 2010; Knight & Przyborski, 2015). It is then of high importance to develop alternative culture systems that more closely resemble the human brain. To achieve this, culture systems

should have the presence of a supporting scaffold, capable of mimicking the native neural extracellular matrix (ECM). This would potentially promote further neuronal maturation, with higher levels of functionality, developing models that recapitulate the cellular and structural organization of midbrain tissue, thereby allowing a better platform to study the disease and to test potential therapies.

Different types of scaffolds have been tested on their capacity to support 3D culture systems. These scaffolds can have a natural origin, such as collagen, cellulose nanofibrils and fibrinogen, or a synthetic origin, based on polyethylene glycol or nanostructured materials, for instance (Cai et al., 2014; Estrada et al., 2014; McManus et al., 2007; Shan et al., 2014). Additionally, natural-synthetic hybrids, metals, ceramics and glass-based or carbon-based nanotubes can also be potential scaffolds for 3D culture (Murphy et al., 2017). These materials have different properties, such as the degree of porosity and their stiffness, hence there is a necessity to choose the appropriate scaffold type that better suits the desired application.

For instance, natural hydrogel scaffolds are polymers that (1) present a highly porous nature, which facilitates the transport of oxygen, nutrients, cell waste and soluble factors, (2) are composed of proteins that naturally occur in the ECM, providing structure and support, and (3) have the capacity to be modified to better support a 3D cell culture (Y. H. Kim et al., 2015; Ma et al., 2004, 2005; Murphy et al., 2017). These characteristics make natural hydrogel scaffolds a suitable platform for neural cell culture applications, while mimicking the natural ECM. An experimental study, performed in 2015, revealed that a 3D culture based on collagen hydrogels can be an inexpensive method to have 3D models that exhibit realistic cellular responses (Leung et al., 2015). This can be achieved because these gels enable cells to generate tension within the matrix, replicating the typical tension cells are under within the body. These results indicated that a collagen 3D culture system can be a necessary improvement to the conventional 2D cultures regarding the testing of drug candidates on physiologically accurate models (Leung et al., 2015). However, collagen hydrogels are limited by batch-to-batch variability, stability and long-term storage issues (Murphy et al., 2017).

On the other hand, synthetic solid porous scaffolds, can be manufactured in a reproducible fashion while closely controlling and tailoring their porous structure, thereby eliminating variability, and providing an optimal support to study differentiation of neural tissues *in vitro* (Hayman et al., 2005). Besides, the mechanical stability provided by this type of scaffolds allows for an easier handling (Chwalek et al., 2015). A recent article described the generation of functional 3D neuronal models using peptide scaffolds (Abdelrahman et al., 2022).

Compared to poly-D-lysine(PDL)-coated plates, a 2D culture system, the fabricated scaffolds allowed the formation of a higher number of neurite branches both in DA and non-dopaminergic neurons. Additionally, the 3D generated neurons not only exhibited more stimulated activity overall than the 2D counterpart, but also demonstrated spontaneous activity for a longer period, surpassing four weeks (Abdelrahman et al., 2022). Although the manufacturing of these scaffolds is scalable and leads to long-term stable structures, solid porous scaffolds may not reflect the mechanobiological aspects of the central nervous system, thereby questioning its reliability to provide realistic tissue and disease models (Murphy et al., 2017; Tyler, 2012).

The use of scaffolds has been demonstrating the capacity to yield cultures that closer resemble the *in vivo* development and maturation of neurons, allowing the development of more robust 3D disease cellular models, including for the study of PD (Estrada et al., 2014). For instance, a 3D cell culture based on the use of carbon fibers as a new type of scaffold for midbrain organoid culture allows an increase of the survival of these cultures, enabling, for example, the screening of drugs for longer periods of time (Tejchman et al., 2020).

3. Potential application of MSCs secretome therapy in PD

The complexity and heterogeneity of PD slows down the process of achieving models that represent the whole picture of this neurodegenerative disease. In turn, the treatments developed on the existent models for PD cannot be translated into clinically proven disease-modifying therapies, delaying advancements on how to slow, halt or reverse PD progression (Vijiaratnam et al., 2021). Despite the existence of symptomatic treatment for PD patients, as the disease progresses the symptoms start being more serious and to accumulate, leading to a loss of independence from the patient. Thereby, several therapeutic approaches are being explored in preclinical and clinical studies, aiming to either repair the brain in a neurochemical or structural manner, or modify the disease by targeting mechanisms underlying PD pathogenesis or even its genetic aetiology (Dawson & Dawson, 2019; Poewe et al., 2017; Vijiaratnam et al., 2021).

3.1. Symptomatic treatments

One of the major PD hallmarks is the loss of DA neurons in SNc, resulting in striatal dopamine depletion (Gelb et al., 1999). This explains why most symptomatic treatments for PD revolve around DA pharmacological targets.

Since the beneficial effects of the dopamine precursor amino acid L-DOPA on PD were discovered in 1967, L-DOPA has remained the most effective treatment for PD-related motor

symptoms, being considered the gold standard (Cotzias et al., 1967; Gray et al., 2014). Other DA targets are used together with L-DOPA to enhance its bioavailability and half-life, such as catechol-O-methyltransferase (COMT) inhibitors, monoamine oxidase type B (MAOB) inhibitors and dopamine agonists (Poewe et al., 2017). While being the gold standard for PD symptomatic treatment in the moment, L-DOPA therapy brings complications, including motor fluctuations and dyskinesia consequent from chronic therapy, the persistence of L-DOPA-resistant motor features, as well as non-motor symptoms that don't respond to L-DOPA therapy or are even aggravated by it (Aquino & Fox, 2015). Some of these troublesome non-motor symptoms include cognitive dysfunction, depression and autonomic impairment, which can be treated parallelly through therapies that target non-dopaminergic pharmacological systems, such as the cholinergic, serotonergic or noradrenergic systems (Poewe et al., 2017). When patients have a great response to L-DOPA treatment but exhibit subsequent motor fluctuations and dyskinesia, a surgical approach like DBS, consisting of deep brain stimulation, can present positive effects and improve quality of life (Bronstein et al., 2011).

3.2. Experimental molecular strategies

Some of the current therapies under development for PD are based on gene delivery through either non-disease modifying strategies, like dopamine-augmentation, or disease modifying strategies, like the enhancement of nigral neurotrophic support and the correction of disease-specific genetic mutations (Merola et al., 2020). For example, the delivery of the correct version of the gene GBA to PD carriers of GBA mutation is now being tested in a clinical trial, in the hopes to reduce deposition of α-synuclein and to rescue dopamine neuron loss, as observed in promising preclinical studies in transgenic mouse models (Morabito et al., 2017; Rocha et al., 2015).

Along the course of PD, DA neurons are lost, disrupting the neural circuitries. A strategy that could revert this situation is the replacement of DA neurons in the striatum, restoring the normal physiological network of dopamine neurotransmission. For this reason, some experimental therapies pursue a cell-based strategy for the treatment of PD. Pioneer clinical studies that explored this strategy in the 1990s, revealed that the transplantation of fetal immature DA neurons into the striata of PD patients had promising results on the long-term survival of DA neurons and on dopamine synthesis (Lindvall et al., 1989, 1990). Even so, the use of fetal ventral mesencephalon cells in PD therapeutics has been hindered, due to various reasons, such as not achieving long term survival of DA neurons in later clinical trials, where individuals

even developed adverse effects, and the ethical problems regarding the collection of human fetal tissue (Freed et al., 2001; Olanow et al., 2003). The discovery of iPSCs allowed the continuation of experiments based on cell-replacement strategies, replacing the use of human fetal cells. Nevertheless, current therapies based on the use of iPSCs for DA neurons restoration cannot cover the whole spectrum of degeneration, commonly leaving extranigral degeneration aside, even though it also contributes for motor and nonmotor symptoms. This issue could possibly be avoided if the use of iPSCs was conjugated with other therapies.

Through the last years, mesenchymal stem cells (MSCs) have shown to be promising cell sources with applications in regenerative medicine. MSCs were initially designated as clonogenic fibroblast precursor cells, having been found in mouse bone marrow in 1976 (Friedenstein et al., 1976). Only later, when these cells were found to have a linkage to the development of various mesenchymal tissues, were they named *mesenchymal stem cells* (Caplan, 1991). With the deepening of knowledge on the characteristics of these cells, it was discovered, in recent years, that they have a beneficial role in the treatment of PD, being considered an alternative to other cell-based therapies (Figure 1.4). (Barker et al., 2015; Pires et al., 2017).

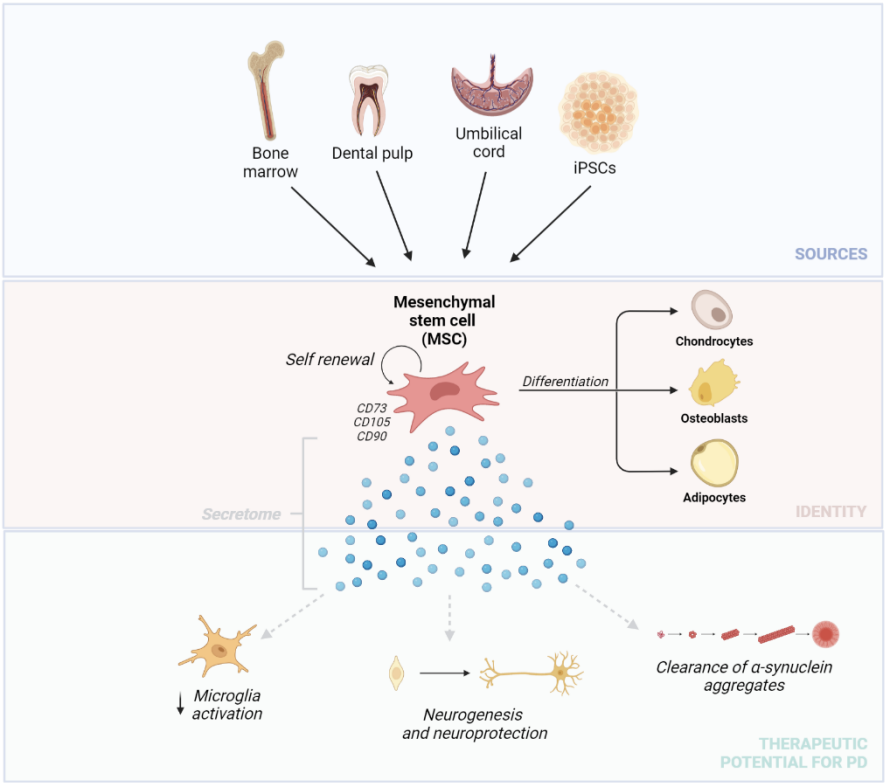

Figure 1.4 - Schematical representation of MSCs sources, their identity and potential applications for Parkinson's Disease. *Created with BioRender.com*

MSCs characterization

In a general perspective, MSCs are known to have a spindle-shaped morphology as well as a high proliferative potential (Uccelli et al., 2011). Nonetheless, to be identified and characterized as MSCs, these isolated cells need to fulfil minimal criteria according to the International Society for Cellular Therapy (Dominici et al., 2006). These criteria comprises the need for MSCs to be adherent to plastic in standard culture conditions, to have a specific pattern of cluster of differentiation (CD) cell surface markers that involves the presence of CD73, CD90 and CD105 and the lack of CD14, CD19, CD34, CD45 and Human leukocyte antigens (HLA)-DR, along with the ability to differentiate *in vitro* into adipocytes, chondroblasts and osteoblasts (Dominici et al., 2006).

iMSCs as a source

MSCs can be isolated from fetal, neonatal and adult tissues, including sources like bone marrow, adipose tissue, dental pulp, placenta, amniotic fluid, umbilical cord blood, liver and lung (Pires et al., 2017; Teixeira et al., 2013).

In the last years, there has been an increased interest concerning the use of MSCs to prevent or treat diverse medical conditions (Wei et al., 2013). In order to achieve therapeutic efficacy, large numbers of cells need to be collected for administration. However, MSCs from human adult tissue sources present major limitations that make this number difficult to achieve (Bonab et al., 2006; Chambers et al., 2007; Zhou et al., 2008). For instance, adult MSCs exhibit limited proliferation potential and lose their therapeutical potential when in long term *in vitro* culture (Eto et al., 2018). Besides, MSCs therapeutical potential is donor and age dependent, which, together with the fact that harvesting MSCs from bone marrow or adipose tissues implies invasive procedures, makes these cell sources impractical (Kretlow et al., 2008; Neuhuber et al., 2005).

These limitations can be overcome by using MSCs derived from induced pluripotent stem cells (iMSCs). These cells fulfil the minimal criteria defining MSCs, demonstrating adherence to plastic, spindle-shaped morphology, expression of specific cell surface markers and lineage commitment (Lian et al., 2010; Marote et al., 2023; Villa-Diaz et al., 2012).

An experimental study previously performed in our lab group comparing iMSCs to the gold-standard bone marrow derived MSCs demonstrated that iMSCs present a more rejuvenated phenotype with higher proliferation rates (Marote et al., 2023). This supports the idea that this emerging MSCs source can provide a larger number of cells before attaining senescence, as

desired for therapeutical purposes. Moreover, the analysis of secretome collected from both iMSCs and BM-MSCs revealed a similar secretory profile, both in pre-senescent and senescent states, meaning that they possibly carry similar therapeutical effects in regenerative applications (Marote et al., 2023). This alternative source of MSCs also avoids the age dependent therapeutical potential limitation observed with adult MSCs, since the reprogramming of somatic cells into iPSCs implies their rejuvenation (C. Zhao & Ikeya, 2018).

MSCs' replicative senescence

Another factor that becomes relevant when characterizing MSCs is their replicative senescence, associated with a finite *in vitro* lifespan, the "Hayflick limit" (Hayflick, 1965). The importance of taking this factor into consideration relies on the fact that, depending on which phase of senescence the cells are encountered in, they will have different biological and functional properties, which can interfere with their therapeutical purpose (Schmeer et al., 2019). Besides affecting the morphological and functional properties of MSCs, cell proliferation arrest is also associated with the acquisition of senescence-associated secretory phenotype (SASP) (Kumari & Jat, 2021). The main SASP components include soluble signaling factors, such as pro-inflammatory cytokines, chemokines, growth modulators, angiogenic factors, extracellular matrix components and matrix metalloproteinases (MMPs) (Lopes-Paciencia et al., 2019). Although many studies have already identified SASP components in different cell types, its composition varies substantially according to factors such as environment, cell type and duration of senescence (Maciel-Barón et al., 2016; Özcan et al., 2016).

There can also be an interplay between the chosen cell culture medium and the replicative senescence of MSCs. For instance, culturing human UC-MSCs and BM-MSCs in two media only differing on the chosen serum, fetal bovine serum (FBS) or calf serum (CS), is enough to result in cell cultures with different timepoints of senescence induction (Y. Liu et al., 2015). Additionally, previous studies have revealed that replacing the use of FBS with options without animal-derived components (xeno-free) or with the use of media compatible with Good Manufacturing Practice (GMP) guidelines has consequences in MSCs properties, including their replicative senescence (Azouna et al., 2012; Oikonomopoulos et al., 2015).

Therefore, given the increased use of MSCs for therapeutical applications, it is of great importance to monitor the state of senescence of each given cell culture, and to take into consideration that, if a cell-free approach with the use of iMSCs secretome is chosen, the cell passage in which this secretome is collected might influence the secretome composition and,

therefore, the same experimental study might have different outputs depending on this factor (Turinetto et al., 2016).

The assessment of the state of senescence of a cell culture can be done through the use of different tools, such as analysis of the p53/$p21^{CIP1}$ pathway and the $p16^{INK4A}$/pRB pathway, that have been associated with having a central role in regulating senescence, complemented with the senescence associated β-galactosidase activity assay (Kumari & Jat, 2021; Turinetto et al., 2016).

The influence of cell culture media

There has been a lot of effort to achieve a uniform characterization of MSCs, however there are many factors that interfere with this goal, as, for example, the MSCs origin properties and the culture conditions to which they are exposed, which may differ (Ankrum et al., 2014; Fan et al., 2020). In this regard, experimental studies have started to aim at deeply understanding the effects of the use of different cell culture conditions on MSCs properties.

The use of two different media, both supplemented with serum, on the culture of bone marrow-derived MSCs (BM-MSCs) resulted in a large number of genes being significantly differentially regulated and in different proteomic profiles between the two conditions, supporting the idea that MSCs properties depend on the conditions they are submitted to (Wagner et al., 2006). Additionally, an experimental study comparing the properties of MSC-derived exosomes depending on cell culture media revealed that exosomes derived from human umbilical cord mesenchymal stem cells (UC-MSCs) cultured on a serum-free (SF) chemically defined media (CellCor™ CD MSC) provided better wound healing and angiogenic effects than when cultured on serum containing media (J. Y. Kim et al., 2021). When cultured in serum-free media, MSC-derived exosomes showcased increased levels of regeneration-related cytokines and decreased levels of pro-inflammatory cytokines (J. Y. Kim et al., 2021).

In order to use iMSCs for future clinical applications it is essential that differentiation from iPSCs into iMSCs is pursued in xeno-free conditions and in accordance with GMP guidelines (McGrath et al., 2019). This requires the optimization of differentiation procedures and of xeno-free culturing systems, while still allowing good quality iMSCs to be obtained. Human Platelet lysate (hPL) is a substitute supplement for FBS that allows *ex vivo* expansion of cells. It is obtained from platelet lysis, which can be induced through repeated freeze and thaw cycles, sonication or by solvent/detergent treatment, releasing a cocktail of growth factors (Oeller et al., 2021). This supplement has shown to be capable of inducing higher rates of proliferation of

MSCs when compared to FBS, while maintaining the identity of these cells (Astori et al., 2016; Doucet et al., 2005).

Further experimental studies are also necessary in order to evaluate the effect of different culture media on the performance of iMSCs in regenerative applications, since media has already been proven to influence the properties of other sources of MSCs, such as BM-MSCs and UC-MSCs (J. Y. Kim et al., 2021; Wagner et al., 2006).

MSCs secretome

Although there is still a need to clarify the best procedures concerning the handling of MSCs in order to obtain a uniform population, research on MSCs properties and functions has allowed the beginning of the study of their therapeutic applications on various diseases.

Underlying MSCs therapeutic potential is, on one side, the ability of these cells to regulate their immunomodulatory functions according to the inflammatory conditions of their micro-environment, which makes MSCs a flexible therapeutic strategy. On another side, MSCs differentiation potential has also been explored on tissue regeneration strategies by using these cells in, for example, synthetic scaffolds (Bagher et al., 2016).

Even so, over the years, it has been progressively accepted that the positive consequences of MSCs on the proposed therapies is due to their paracrine activity (Caplan & Dennis, 2006; Teixeira et al., 2013). Such is thought because MSCs release soluble factors and vesicles, referred to as *secretome*, that have immunomodulatory, angiogenic, trophic, antiapoptotic and antioxidant effects on their surroundings (Pinho et al., 2020). It has been identified that MSCs secretome has a soluble fraction, that includes growth factors and cytokines, and a vesicular fraction, composed of exosomes and microvesicles loaded with specific combinations of coding and non-coding ribonucleic acid (RNA), proteins and lipids (Marote et al., 2016).

The composition of micro and nano-vesicles secreted by MSCs varies according to intrinsic features, such as its tissue source, and extrinsic aspects, like the surrounding microenvironment (Hass et al., 2011; Levy et al., 2020). This knowledge led to the emergence of novel therapeutic approaches either testing the differentially sourced MSCs effects on various diseases or aiming to modulate the release of paracrine factors by pre-conditioning or genetically manipulating MSCs (Bagno et al., 2018). Other examples of therapeutic applications being explored involve the combination of MSCs with other cell types or culturing MSCs in substrates made of biomaterials (Bagno et al., 2018).

MSCs therapeutic effects in PD

One of the diseases in which the application of MSCs has reverted positive effects is PD (J. Wang et al., 2020). Although the mechanisms behind these positive effects are not yet well-elucidated, there is currently a considerable number of findings regarding the use of MSCs in PD animal models that provide knowledge on the different aspects in which MSCs may act. The main mechanism of action is proposed to occur through the secretory factors released by MSCs, with effects on neurogenesis, neuroprotection, cell survival, immunomodulation and clearance of α-synuclein aggregates (Danielyan et al., 2011; Oh et al., 2017; Sadan et al., 2009; Venkatesh & Sen, 2016).

Neurogenesis

Neurogenesis is a process that consists of the formation of new neuronal cells, involving a multitude of steps from the cell division until the integration of the new cell into the neuronal network, with adequate synaptic connections (Salgado et al., 2015). Although in most brain regions neurogenesis is restricted to a short developmental period, being called the prenatal neurogenesis, there are two brain regions, described in the mammalian brain, that retain the ability to generate neurons after this period and even during the adulthood: the lateral subventricular zone and the subgranular zone of the hippocampal dentate gyrus (Eriksson et al., 1998; Kumar et al., 2019).

MSCs and, more concretely, the factors present in its secretome have the capacity to modulate these neurogenic niches, by increasing the survival, proliferation and differentiation of neural stem cells (NSCs) (Munoz et al., 2005). The factors thought to have a role on neurogenesis include the BDNF, the vascular endothelial growth factor (VEGF), GDNF, fibroblast growth factor 2 (FGF2) and neurotrophin-3 (NT-3) (Kot et al., 2022; Volkman & Offen, 2017).

Neuroprotection and cell survival

Aside from the role of MSCs secretome on neurogenesis in PD, its secretory factors also exert function on neuronal survival and protection. Various *in vitro* studies have shown that neurotrophic factors, present on the secretome, can protect against 6-OHDA toxicity (Cova et al., 2012).

Along the years, a part of the factors present in MSCs secretome and potentially involved on neuroprotection and cell survival were identified, such as SDF-1α, prostaglandin E2 receptors, VEGF, BDNF, interleukin-6 (IL-6), GDNF, DJ-1, cystatin C, glial-derived nexin, galectin-1

and pigment epithelium-derived factor (Mendes-Pinheiro et al., 2019; Parga et al., 2018; F. Wang et al., 2010).

Besides the use of MSCs secretome to obtain positive effects regarding neuroprotection and recovery of PD models, strategies exploring the modulation of the secretion profile of MSCs, or the overexpression of particular factors, are also being employed in the research of therapies for PD (Sadan et al., 2009). For instance, in a 6-OHDA rat model of PD, the transplantation of genetically modified MSCs, that overexpress GDNF, promotes the reduction of amphetamine-induced rotations and the rejuvenation of dopamine fibers, allowing recovery (Glavaski-Joksimovic et al., 2010).

The positive results from studies involving MSCs secretome and its neuroprotective potential propelled its conjoint use with cell-based therapies, with the goal to provide trophic support to the transplanted cells. When transplanting NSCs treated with secretome into rat models of PD, a higher activity in cell survival and migration is identifiable, as well as a behavioural improvement (Yao et al., 2016).

Immunomodulation and inflammation

MSCs act not only on neurogenesis and neuroprotection, but also on immunomodulation and anti-inflammation, suggesting the potentially beneficial use of these properties on therapies (Ankrum et al., 2014). Although MSCs secrete both pro and anti-inflammatory cytokines, the positive effects of secretome on PD animal models are related to its anti-inflammatory properties: (1) capacity to decrease the expression of pro-inflammatory cytokines by inflammatory cells, such as interleukin-1β, interleukin-2, interleukin-12, tumor necrosis factor α and interferon γ, and (2) to secrete anti-inflammatory cytokines such as interleukin-6, interleukin-10 and transforming growth factor β (Danielyan et al., 2011; Y. J. Kim et al., 2009; Pires et al., 2017). MSCs secretome also leads to microglial suppression in PD animal models, even though the mechanism underlying this action is not yet understood (Y. J. Kim et al., 2009).

Clearance of α-synuclein aggregates

The study of the effects of MSCs in *in vitro* and *in vivo* models of α-synuclein aggregation showed that these cells have the capacity to contradict the destabilization of microtubules and axonal deficits induced by α-synuclein and that would lead to further α-synuclein accumulation (Oh et al., 2017). The presence of eukaryotic elongation factor 1A-2 on MSCs secretome stabilizes microtubules by reducing tau phosphorylation and stimulates autophagolysosome

fusion, suggesting a protective role against α-synuclein aggregation that could be further explored in therapeutic applications for PD (Oh et al., 2017).

4. Conclusions

PD is a highly complex disease that affects millions of people worldwide but, unfortunately, there is still no efficient treatment capable of delaying or halting its progression (Rukavina et al., 2021; Vijiaratnam et al., 2021). Since a considerable number of dopaminergic neurons is already lost at the time of onset of this disease, it becomes relevant to find a therapy that promotes regeneration and provides neuroprotection to the cells that remain viable (Marques et al., 2018a). During recent years, the potential use of MSCs as a therapeutical tool for PD has been explored. Previous reports have shown that, most positive effects exerted by MSCs, associated with the rescue of degenerating DA neurons and promotion of neurogenesis, are linked to their paracrine action, the secretome (Cova et al., 2010; Marques et al., 2018a; Teixeira et al., 2015).

Even so, MSC secretome composition and therapeutical potential highly depends on the source of these cells and on their *in vitro* manipulation, since these factors impact MSCs properties (Assunção-Silva et al., 2018; J. Y. Kim et al., 2021). Additionally, although human MSCs can be harvested from different tissues, the need to obtain a large number of these cells for therapeutical applications raises an obstacle to their use (Eto et al., 2018). This obstacle can be surpassed through the use of MSCs derived from iPSCs, a source which avoids the invasive procedures related to the harvesting of MSCs from tissues and provides a large number of these cells (Marote et al., 2023). However, this source of cells might also be subject to influences of the different conditions they are expanded in, such as cell culture media and serum supplementation, as well as the cell passage in which their secretome is collected might affect the therapeutical outcome of their secretome. All these aspects should be explored before proceeding to clinical applications of iMSCs secretome.

CHAPTER 2 – RESEARCH OBJECTIVES

In this project, we aimed to develop and characterize a 3D *in vitro* model of DA neurons that could address the PD phenotype, using collagen hydrogel droplets as scaffold. A recent and innovative synthetic scaffold, fabricated from Ormocomp, was also tested for its capacity to provide support and promote DA differentiation of the cellular model. Additionally, we aimed at studying the long-term culture of iMSCs in either a commercial serum-free medium or a hPL supplemented medium, in order to assess the effects of different media and senescence on iMSCs' properties and, furthermore, on their secretome applications when applied to the 3D *in vitro* model of DA neurons. For that purpose, two main objectives were defined:

1) Develop and characterize a 3D model of dopaminergic neurons and how it can be used to address PD phenotype.
2) Long-term culture of iMSCs on two different media and explore the secretome effects on neuronal differentiation and neuroprotection.

CHAPTER 3 – MATERIALS AND METHODS

1. iMSCs culture

Human iMSCs from one donor were thawed at passage (P) 7 (P7) and expanded in two different mediums for comparison purposes: (1) αMEM-5%hPL medium: minimum essential medium α (αMEM, Invitrogen, USA) supplemented with 1% Penicillin-Streptomycin (Pen-Strep, Invitrogen, USA), 5% human platelet lysate (hPL; Stemmatters, Portugal) and 1.8 mM heparin (B. Braun, Germany); (2) complete MesenCult™-Animal Component-Free(ACF) Plus Medium (STEMCELL Technologies, Canada): MesenCult™-ACF Plus Medium supplemented with MesenCult™-ACF Plus 500X Supplement and 2 mM L-Glutamine. Medium was renewed every 3 days and, once a confluency of 80-90% was obtained, cells were dissociated. This passage was performed by enzymatic dissociation with 0.05% trypsin-EDTA (Invitrogen, USA) for iMSCs cultured in αMEM-5%hPL medium and with ACF Cell Dissociation Kit (STEMCELL Technologies) for iMSCs cultured in complete MesenCult™-ACF Plus Medium. Cell cultures were maintained at 37ºC in a humidified atmosphere with 5% carbon dioxide (CO_2).

2. Cumulative population doublings in long-term iMSCs culture

From P7 onwards, iMSCs were seeded in αMEM-5%hPL medium and in complete MesenCult™-ACF Plus Medium at 2,000 cells/cm^2. Cell passaging was continuously performed and the number of cells counted at the end of each passage was registered, until proliferation ceased.

For each condition from P7 onwards, cumulative population doublings (cPD) were calculated with the following formula:

$cPD = cPD(0) + 3.322 * (\log(Nf) - \log(Ni))$, where cPD(0) represents the cPD from the previous passage, Nf the number of cells counted at the end of each passage and Ni the number of seeded cells.

3. Senescence-associated β-galactosidase assay

To evaluate senescence, cells at P8 and at a later passage (P14 for iMSCs cultured in hPL supplemented medium and P12 for iMSCs cultured in MesenCult medium) were plated in 12-well plate wells, following the culturing conditions described in “iMSCs culture” section, until

a confluency of 80-90% was reached. To perform the senescence-associated β-galactosidase (SA-β-Gal) assay, a SA-β-Gal activity assay kit (Abcam) was used, in consonance with manufacturer's instructions. Cells were first washed with PBS and fixed with the kit's fixative solution for 10 min. After completion of this period, cells were washed with PBS and 500 µL of a staining solution, prepared following manufacturer's instructions and containing 20 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), were added to each well. After sealing the plate with parafilm, cells were incubated at 37 ºC. In the following day, cells were washed with PBS and nuclei counterstaining with 4-6-diamidino-2-phenylindole-dhydrochloride (DAPI; 1 µg/mL, Invitrogen, USA) for 5 min at room temperature (RT) was performed. Cells were washed again and conserved with PBS at 4 ºC until fluorescence images were acquired. Ten brightfields images and the corresponding fluorescence images were obtained with a widefield inverted microscope (IX53, Olympus). In order to obtain the percentage of SA-β-Gal positive cells the number of nuclei and the number of SA-β-Gal positive cells was manually counted in each image.

4. Analysis of cell area

The previously prepared cells for the SA-β-Gal activity assay were permeabilized with PBS 0.1 % Triton X-100 (Sigma) for 5 min. After washing with PBS, cells were stained for 45 min with phalloidin (0.1 µg/mL, Sigma, USA). Finally, cells were washed and stored at 4ºC. For fluorescence photo acquisition, ten random fields were captured using a widefield inverted microscope (IX81, Olympus). Average cell area was calculated by dividing the positive area for phalloidin by the number of nuclei in each image.

5. Quantitative RT-PCR

To evaluate the expression of target genes, cells at P8 and at a later passage (P14 for iMSCs cultured in hPL supplemented medium and P12 for iMSCs cultured in MesenCult medium) were plated in 6-well plate wells following the culturing conditions previously described. Once a confluency of 80-90% was reached, total RNA was extracted using TripleXtractor (Grisp, Portugal), according to manufacturer's instructions. RNA quantification was performed with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA). Afterwards, 500 ng of each sample was converted into cDNA through the Xpert cDNA Synthesis Supermix (Grisp, Portugal), according to manufacturer's instructions. Primers had previously been designed by

lab group members using Primer-BLAST (NCBI, United States) taking into consideration the respective GenBank sequence. Using the XPert fast SYBR mastermix (Grisp, Portugal) and 125 ng of cDNA per condition the quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 7500 Fast Real-Time PCR System (ThermoFisher Scientific, USA). The resulting melting curves exhibited a single sharp peak at the expected temperature. Through the method $2^{-\Delta Ct}$ expression of target genes ($p16^{INK4}$, $p21^{CIP1}$ and p53) was normalized to the housekeeping gene GAPDH.

6. Proteomic Bioinformatics

Previously in our laboratory iMSCs originated from 3 donors had been cultured either in hPL supplemented medium or MesenCult medium. At P9, secretome was collected from these cells and a proteomic analysis was performed. Data regarding the results of this proteomic analysis was then used for the present project.

A protein-protein interaction network analysis was done with the online STRING database version 12.0, comprising physical and functional associations between proteins with a medium confidence level (0.4). These proteins were organized in five clusters, through k means clustering method. This procedure was applied first to all proteins identified in both secretome from iMSCs expanded in hPL supplemented medium and secretome from iMSCs expanded in MesenCult™ medium, and, afterwards, only to proteins statistically differentially expressed in these two conditions.

7. Mouse embryonic fibroblasts culture

Irradiated mouse embryonic fibroblasts (mEF) (CF-1 MEF IRR, Gibco, USA) were seeded in a T25 flask coated with gelatin (0.1% gelatin; Milipore, USA) to form a feeder layer. The medium utilized was Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco, USA) supplemented with 10% FBS (Biochrom, Germany) and 1% Penicillin-Streptomycin (Gibco, USA). Once mEF culture appeared well developed and formed a consistent support network it was time for mESC culture.

8. Mouse embryonic stem cells culture

To prepare a mESC culture a reporter line of mESC was used. This line was obtained from heterozygous transgenic mice with expression of green fluorescent protein (GFP) under the transcriptional control of the rat tyrosine hydroxylase (TH) promotor (Chumarina et al., 2017a). mESC were expanded on a mEF-feeder layer and the utilized medium was DMEM GlutaMAX, 10% FBS, 1x Nucleosides (Millipore, USA), 2 mM L-glutamine (Life Technologies, USA), 1% Penicillin-Streptomycin and 0.1 mM β-mercaptoethanol (Sigma, USA), supplemented with leukaemia inhibitory factor (LIF; 1:1 000; Millipore, USA).

9. Mouse embryonic stem cells culture differentiation towards dopaminergic fate

9.1. EBs generation

To promote the formation of embryoid bodies (EBs) followed by differentiation in DA neurons, the steps described below were performed.

Once mESC cultures exhibited an adequate size and morphology, enzymatic dissociation with 0.05% trypsin solution (Life Technologies, USA) was effectuated, followed by centrifugation at 300 x g for 5 min. Around 14,000 cells/cm^2 were then seeded in low attachment culture surfaces. At this point the added medium was ADNFK, consisting of Advanced DMEM-F12 medium (Gibco) and Neurobasal medium (Gibco) in a proportion of 1:1 ratio, 10% Knockout Serum Replacement (Life Technologies), 1% GlutaMAX^{TM} Supplement (Gibco), 1% Penicillin-Streptomycin and 0.1 mM β-mercaptoethanol. On the next day, medium was replaced by resorting to centrifugation at 300 x g for 5 min, obtaining a pellet of small aggregates. At day 2 of differentiation and every other day, ADNFK medium was replaced and supplemented with Smoothened agonist (SAG; 0.5 µM; Tocris Bioscience, UK). From day 8 of differentiation and every other day until day 14, instead of replacing and supplementing the medium with a single factor, a combination of different factors to induce neuronal differentiation was added to the ADNFK medium: SAG 0.5 µM, BDNF (10 ng/mL; Peprotech, UK), GDNF (10 ng/mL; Peprotech) and AA (200 µM; Sigma).

9.2. 2D model

To have a 2D model, round glass coverslips were added to wells from a 24-well plate and then coated with PDL (100 µg/mL; Sigma) and left at RT for 2 h, after which 3 washes were performed. A laminin coating (10 µg/mL; Sigma) was applied once wells were dry, and then the plate was kept for 2 h at 37º C. Excess laminin was removed and wells were ready to receive

cells. During the 2 h period of laminin coating, EB dissociation was performed. Firstly, all content from the low attachment surface containing the EBs was transferred to a 15 mL falcon and then the medium was removed. EBs were carefully washed twice with Phosphate Buffered Saline (PBS) 0,4 % glucose, to prevent adhesion in between them. 0.05% trypsin-EDTA was added to the EBs and left for 3-5 min at 37º C, while mixing the contents every 1 min. Mechanical dissociation was then performed with the help of P200 pipette. Trypsin was neutralized with FBS and a centrifugation at 300 x g per 5 min was done. Supernatant was removed and ADNFK medium added to then proceed to cell count. 80 000 cells were then plated per each well of the 24-well plate and kept in ADFNK medium supplemented with the four factors described above (SAG, BDNF, GDNF and AA) for 5 additional days.

9.3. 3D model – collagen hydrogel and Ormocomp structure

To develop a 3D cellular model, after obtaining DA differentiated EBs, they were placed in collagen hydrogel droplets, that function as a matrix to facilitate EB adhesion and neurite outgrowth. The preparation of this matrix consisted of the mixture of rat tail collagen type I (3.61 mg/mL; BD Biosciences, USA) with 10x concentrated DMEM medium (Gibco, USA) and a 7.5(m/v) % solution of $NaHCO_3$. It is important to refer that the preparation was performed in ice to avoid collagen polymerization. Collagen droplets of 30 µL each were then placed in the center of each well of a 48-well tissue culture plate and, to induce polymerization, they were then incubated for 1 hour at 37°C, 5% CO_2. When the incubation period was completed similar sized EBs were selected and transferred onto the top of the polymerized collagen droplets. Every other day, medium changes with ADNFK supplemented with the differentiation factors (0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF, 200 µM AA) were performed, until day 6 of culture within this 3D system.

Besides being placed in collagen hydrogel droplets, DA differentiated EBs were also placed in Ormocomp structures, kindly provided by the International Iberian Nanotechnology Laboratory (INL), located in Braga. To accomplish that, structures were initially sterilized by passing the slides containing the structures through 70 % alcohol, followed by 3 washes with sterile water. When the structures were dry, 2 well silicone inserts were placed in order to keep the structure in the center of the well. Structures were then subjected to a minimum of 30 min of ultraviolet (UV) sterilization.

Once sterilization was concluded, structures were coated with PDL (100 µg/mL) and laminin (10 µg/mL). After covering the structures with PDL and leaving them at RT for 2 h, 3 washes were performed. The laminin coating was applied only once structures had dried, and then left

for 2 h at 37º C. In the end of this period, excess laminin was removed and structures were ready for EB placement. To accomplish this, ADNFK medium supplemented with differentiation factors (0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF, 200 µM AA) was added to each well and then a DA differentiated EB was placed on top of each structure. Just as EBs seeded on collagen hydrogels, every other day, medium changes with ADNFK supplemented with differentiation factors (0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF, 200 µM AA) were performed to EBs seeded on Ormocomp structures, until day 6 of culture within this 3D system.

10. Exposure of 3D collagen model to 6-OHDA

At the end of 6 days of culture on collagen hydrogel, EBs already presented well-developed neurites. Dopaminergic cell death was then induced through the use of 6-OHDA hydrochloride (Sigma, USA). For that, a stock solution with 100 mM of 6-OHDA was prepared in 0.2 % ascorbic acid solution and kept in dark to avoid oxidation. Using this stock solution, Neurobasal A medium (Gibco, USA) containing 500 µM of 6-OHDA was prepared. To have a vehicle group, Neurobasal A medium containing 0.2 % ascorbic acid, was prepared. Differentiation medium was removed from the wells and cells were washed with Neurobasal A medium. The 6-OHDA solution was added to a subset of wells while the vehicle solution was added to the other subset of wells. After a 4 h incubation at 37 ºC, cells were washed with Neurobasal A medium. On the 48-multiwells plate destined to perform the [(5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt)] (MTS) assay, two conditions were maintained on both the wells exposed to 6-OHDA and wells kept in vehicle: (1) incubation in Neurobasal A medium and (2) incubation in supplemented ADFNK. On the 48-multiwells plate destined to perform an immunocytochemistry, wells where the vehicle solution had been added were maintained in supplemented ADFNK, whilst wells where 6-OHDA solution had been added were maintained in Neurobasal A medium.

Cells were incubated in the respective media for 48 h until further analysis.

11. Incubation of 3D model with iMSCs secretome

Neuronal differentiation assay

By the end of the 14 days of differentiation as embryoid bodies, the differentiated EBs were placed on top of collagen hydrogel droplets, in a 48-well tissue culture plate. At this point EBs

were incubated with 1) basal medium, consisting of neurobasal medium without further supplementation; 2) basal medium supplemented with 0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF, 200 µM AA; 3) differentiation medium, consisting of ADNFK medium supplemented with 0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF, 200 µM AA; 4) conditioned medium from early passage iMSCs cultured in hPL supplemented medium; 5) conditioned medium from late passage iMSCs cultured in hPL supplemented medium and 6) conditioned medium from early passage iMSCs cultured in MesenCult^{TM} medium. EBs were maintained in these conditions for 6 additional days, with a medium change halfway though.

Neuroprotection assay

At the end of the 6 days of EB culture on the collagen hydrogels, Neurobasal medium 0.2 % ascorbic acid containing 500 µM of 6-OHDA was added to a subset of wells. Meanwhile, to the other subset of wells Neurobasal medium 0.2 % ascorbic acid was added, representing the vehicle. Cells were exposed to this toxin for 4 h, after which the medium was removed from both subsets of wells and cells were incubated with 1) basal medium, consisting of neurobasal medium without further supplementation; 2) differentiation medium, consisting of ADNFK medium supplemented with 0.5 µM SAG, 10 ng/mL BDNF, 10 ng/mL GDNF and 200 µM AA; 3) conditioned medium from early passage iMSCs cultured in MesenCult^{TM} medium; 4) conditioned medium from early passage iMSCs cultured in hPL supplemented medium; and 5) conditioned medium from late passage iMSCs cultured in hPL supplemented medium. This incubation had the duration of 48 h, after which further analysis on cell viability was performed.

12. Immunocytochemistry to characterize 2D model

An immunocytochemistry was performed to identify the types of cells present in the 2D model and their degree of maturity.

Medium was removed from all wells and cells were fixed in 4% paraformaldehyde (PFA; Sigma) for 15 min at RT. After washing, cells were permeabilized with PBS 0.3 % Triton X-100 (Sigma) for 5 min. After washing, a blocking solution composed of PBS 10 % FBS was added to block non-specific binding and left incubating for an hour at RT. Cells were incubated for 1 hour at RT with different primary antibodies (Table 2.1) diluted in blocking buffer. After washing the cells, secondary antibodies antibodies (Table 2.1) diluted in PBS 10 % FBS were added for 1 h at RT in the dark, followed by wash and incubation with DAPI for 5 min at RT.

After washing the cells, slides were mounted. Images were acquired with confocal microscopy (LSM 780, ZEISS, Germany) using the software Zen black (ZEISS).

Table 3.1 - List of primary and secondary antibodies used for the 2D model characterization

	Antibody	Host	Reference	Dilution
Primary antibodies	anti-MAP2	mouse	Sigma, Cat#M4403	1:500
	anti-DCX	rabbit	Abcam, Cat#ab18723	1:300
	anti-Nestin	mouse	Sigma, Cat#MAB5326	1:200
	anti-GFAP	rabbit	DAKO, Cat#z0334	1:200
	anti-β-III tubulin	mouse	Promega, Cat#g712a	1:1000
	anti-TH	rabbit	Abcam, Cat#ab152	1:500
Secondary antibodies	anti-mouse alexa 488	goat	Invitrogen, Cat# A32723	1:1000
	anti-rabbit alexa 594	goat	Invitrogen, Cat# A32740	1:1000

13. Immunocytochemistry to characterize 3D model

The different immunohistochemistry assays performed to evaluate the 3D model followed the same general protocol, differing only in the primary and secondary antibodies used.

Medium was removed from all wells and EBs were fixed in 4% PFA for 45 min at RT. After washing, EBs were permeabilized with PBS 0.3 % Triton X-100 for 10 min. After washing, a blocking solution composed of PBS 10 % FBS was added and left incubating for 1 hour at RT. EBs were incubated overnight at 4 ºC with different combinations of primary antibodies diluted in blocking buffer. After washing the cells, secondary antibodies diluted in PBS 10 % FBS were added for 2 h at RT in the dark, followed by wash and incubation with DAPI for 15 min at RT. Images were acquired with confocal microscopy (FV3000, Olympus, Japan) using the software FV10-ASW 2.0c (Olympus). Since the EBs develop large areas of neurite growth, images were taken with the mosaic function, forming a large image of the specimen composed of single images.

To make a phenotypic characterization of the 3D model developed on the collagen hydrogel regarding to aspects like what types of cells are present and their degree of maturity, an immunocytochemistry using the primary and secondary antibodies present in Table 2.2 was performed.

Table 3.2 - List of primary and secondary antibodies used for the 3D model phenotypic characterization

	Antibody	Host	Reference	Dilution
Primary antibodies	anti-MAP2	mouse	Sigma, Cat#M4403	1:500
	anti-DCX	rabbit	Abcam, Cat#ab18723	1:300
	anti-β-III tubulin	mouse	Promega, Cat#g712a	1:1000
	anti-DBH	rabbit	Invitrogen, Cat#PA5-34664	1:200
	anti-synaptophysin	rabbit	Abcam, Cat#ab32127	1:400
	anti-PSD95 [6g6-1c9]	mouse	Abcam, Cat#ab2723	1:1000
	anti-MAP2	mouse	Sigma, Cat#M4403	1:500
	anti-GFAP	rabbit	DAKO, Cat#z0334	1:200
	anti-NeuN	rabbit	Cell Signal, Cat#D4G40	1:200
	anti-GAP43	mouse	Abcam, Cat#ab129990	1:300
	anti-TH	rabbit	Abcam, Cat#ab152	1:500
	anti-DAT	rat	Santa Cruz, Cat#sc32259	1:50
Secondary antibodies	anti-mouse alexa 488	goat	Invitrogen, Cat#A32723	1:1000
	anti-rabbit alexa 594	goat	Invitrogen, Cat#A32740	1:1000
	anti-rat alexa 647	goat	Invitrogen, Cat#A21247	1:1000

In order to measure the neuronal differentiation and, more precisely, the DA differentiation present in the 3D collagen model and in the 3D structure model, an immunocytochemistry using a single pair of primary antibodies, anti-β-III tubulin and anti-TH, identified on Table 2.2, was performed. Secondary antibodies are also identified on Table 2.2: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit.

To quantify the presence of DA neurons on the 3D collagen model after 6-OHDA exposure, an immunocytochemistry using the primary antibodies mouse polyclonal β-III tubulin antibody (1: 1000; Promega), rabbit polyclonal TH antibody (1:500; Sigma) and rat monoclonal dopamine transporter (DAT) antibody (1:50; Santa Cruz) were used. The secondary antibodies were Alexa Fluor 488 goat anti-mouse (1:1000; Invitrogen), Alexa Fluor 594 goat anti-rabbit (1:1000; Invitrogen) and Alexa Fluor 647 goat anti-rat (1:1000; Invitrogen).

To evaluate the secretome effects on DA differentiation of the 3D collagen model, an immunocytochemistry using a single pair of primary antibodies, anti-β-III tubulin and anti-TH, identified on Table 2.2, was performed. Secondary antibodies are also identified on Table 2.2: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit.

14. Image analysis

14.1. Expression of β-III tubulin and TH

The positive area for β-III tubulin, TH and DAPI staining on the images acquired from the 3D model on collagen and on the structure was analysed using Fiji, an open-source platform for image processing, through the function «Analyse Particles» (Schindelin et al., 2012). Data obtained was used to calculate the ratios of β-III tubulin/DAPI, TH/DAPI and TH/β-III tubulin.

14.2. Neurite outgrowth

Images acquired from the neuronal differentiation assay were used to evaluate DA neurite outgrowth on the different conditions. This evaluation was performed resorting to Fiji, by delimiting, for each individual EB, four consecutive rings with a thickness of 300 µm starting around the nucleus of the EB (0-300 µm; 300-600 µm; 600-900 µm; 900-1200 µm). This way, the positive area for TH fluorescence could be measured within each interval of length and compared within conditions. To do neurite segmentation, firstly all fluorescence signal present outside of the examined ring was discarded. After this step, the image was binarized via one of three thresholding algorithms to get an appropriate segmentation of neurites: 1) the Li algorithm, when high density of neurites was present, 2) the Triangle algorithm, when medium neurite density was present, and 3) the Moments algorithm, when low density of neurites was present. At this point, the positive area for TH could be measured, using the *Measure* function, and normalized to the area of the region of interest (ROI), consisting of the delimited ring.

14.3. Neurite fragmentation

Images acquired from a vehicle and a 6-OHDA subset of EBs were analysed with the goal to evaluate fragmentation of TH^+ neurites and the DAT^+ area within TH^+ area, with the help of Fiji.

To analyse fragmentation of DA neurites both in the vehicle and in the toxin group, a freehand selection resembling a partial ring-like shape with 250 µm thickness was drew. This shape was then carefully positioned in order to frame a region with less neurite density, to facilitate analysis. To measure the average size of particles within each delimited area, a sequence of steps was taken: 1) the fluorescence signal present outside of the drawn area was discarded, 2) the image was binarized via Triangle thresholding to get an appropriate segmentation of neurites, 3) the function *Close-* was applied to the binary image obtained, 4) the function

Analyse Particles… was used, measuring particles with a size range of 0.50-Infinity, to eliminate noise, and pixel circularity of 0.00-1.00.

It is important to denote that three technical replicates were analysed per individual EB and that the drawn shape was the same for every analysis, avoiding variations in the delimited area.

14.4. DAT quantification

Images acquired from a vehicle and a 6-OHDA subset of EBs were analysed with the goal to evaluate DAT expression within TH^+ neurites. This analysis was performed resorting to Fiji.

The fluorescence signal present in the main body structure of the EB was discarded since in this region there is a majority of neuronal cellular bodies which are not the target of this evaluation. Afterwards, TH expression was binarized via Li thresholding to get a mask and TH^+ area was measured. Using the *Paste Control* function in the Transfer mode *Transparent-white* this mask was applied to the DAT channel, making it possible to measure the positive area of DAT within the TH mask.

15. MTS assay

In order to test the impact of the 6-OHDA exposure on the EBs viability and, later on, to test the effect of conditioned medium, from iMSCs cultured in hPL supplemented medium or MesenCult^{TM} medium, on neuroprotection of DA neurons exposed to 6-OHDA, an MTS assay was used. This assay relies on the reduction of a tetrazolium salt by mitochondrial activity of living cells, generating a coloured formazan dye that is soluble in cell culture media.

After the end of the 48 h of incubation post 6-OHDA exposure, medium was replaced with Neurobasal A medium containing MTS in a 5:1 ratio and cells were incubated for 2 h at 37 ºC and 5 % CO_2. At the end of the 2 h incubation, two technical replicates of each sample were disposed in wells of a 96-well plate and samples were taken to a microplate reader (Tecan, Switzerland) to measure optical density through spectrometry.

16. Statistical analysis

Statistical analysis was performed using the GraphPad Prism v 8.0.1 software (GraphPad Software, USA). To perform comparisons between two groups unpaired Student's t-test was used. One-way analysis of variance (ANOVA) was used to perform multiple comparisons

between groups. All quantitative data were expressed as mean ± standard error of the mean (SEM) and significance was set at *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

1. Development and characterization of *in vitro* neuronal models and their potential application as PD models

The deepening of the understanding on how to direct differentiation from pluripotent stem cells into specific cell types as allowed the development of new *in vitro* disease models. The majority of the *in vitro* neurodegenerative disease modelling studies so far rely on conventional 2D cell culture systems, although 3D cell culture models are also starting to be developed for the study of these diseases (Geckil et al., 2010). In order to have a culture system that more closely resembles the *in vivo* cellular organization and physiology, culture systems can have the presence of a supporting scaffold, that mimics the native ECM. In this project, we aimed at developing and characterizing cellular models that could be used, in a quick and cheap manner, as platforms for initial drug screenings for PD and, further on, to perform studies that deeply evaluate the molecular mechanisms of PD.

Our laboratory group has previously optimized a protocol, that was in part based on the protocol described by Lee et al., 2000, to direct the differentiation of mESCs to a DA fate, through the formation of embryoid bodies. The first step performed based on this protocol consisted of culturing a reporter line of mouse embryonic stem cells that expresses GFP under the promoter of TH (mESCs TH::GFP) on a mEF-feeder layer (Chumarina et al., 2017b). At the end of the following 14 days of DA differentiation, EBs presented a characteristic morphology with around 250 µm of diameter.

2D developed model does not sustain the growth of a robust dopaminergic network

At the end of the 14 days of suspension culture, the EBs were dissociated and cultured in PDL and laminin coated wells (Figure 4.1A). This initial 2D cell culture served as a mean to visually compare the influence of the substrates on the development of mESC-derived DA neurons.

At the end of the 6 days of culture post EB dissociation, an immunocytochemistry was performed to test different aspects: (1) the presence of neural progenitor cells in the culture, (2) the capacity of the performed protocol to originate neurons and promote their migration and (3) the presence of DA neurons.

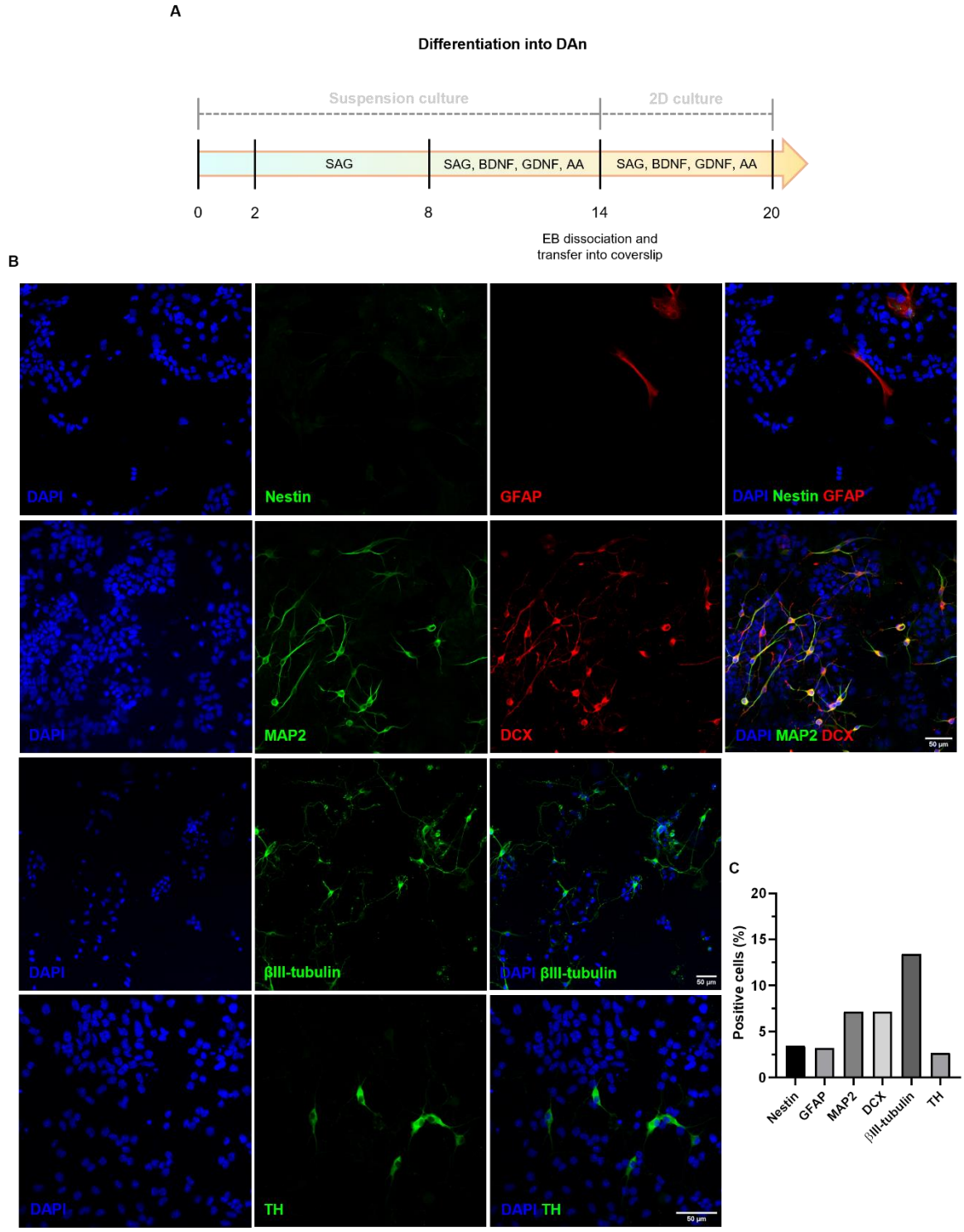

Figure 4.1 – Assessment of 2D culture resulting from dissociation of mESC-derived embryoid bodies. (A) Timeline of the differentiation protocol carried out in a suspension culture for the first 14 days and in a 2D monolayer in the last 6 days. **(B)** Confocal images of the expression of different neural-associated markers. Cells were immunostained with antibodies against Nestin and GFAP, MAP2 and DCX, β-III tubulin, TH. Nuclei were counterstained with DAPI (blue). **(C)** Percentage of positive cells for the aforementioned markers. *n* = 1-2; scale bar = 50 µm

To evaluate the presence of neural progenitor cells on the originated 2D cell culture, a co-labeling with a Nestin antibody and a Glial fibrillary acidic protein (GFAP) antibody was performed. Nestin is a class IV intermediate filament expressed in the early central nervous system (CNS), which presence can be firstly identified in the neural tube and somites. For this reason, it is considered a valuable marker to detect neural progenitor cells (Lendahl et al., 1990). GFAP on the other hand is a class III intermediate filament specific to not only mature astrocytes but also neural stem cells (Mamber et al., 2012; Yang & Wang, 2015). The images acquired (Figure 4.1B) reveal that GFAP immunoreactivity exhibits a fibrillar pattern, associated with the presence of intermediate filaments. Nestin expression seems to have a more diffuse and somewhat punctiform pattern. It is visible that there is expression of both of these markers in the cell culture, but only a very small portion of the cells is positive to GFAP (3.19 ± 1.78 %), or Nestin, (3.46 ± 2,74 %) (Figure 4.1 B and C). Some cells are positive for both GFAP and Nestin, which can denote the presence of neural stem cells, while other cells are only positive to GFAP, which potentially means that these consist of astrocytes.

To evaluate neuron formation and migration, a co-labeling with an antibody marking microtubule-associated protein 2 (MAP2) and an antibody marking doublecortin (DCX) was used. While MAP2 is a cytoskeletal protein that is specific to neurons, allowing the detection of neuronal populations in the culture, DCX is a marker of cells committed to the neuronal lineage, both in developing and adult brains, and has been associated to having a crucial role in neuronal migration (Walker et al., 2007). As observed in Figure 4.1B, MAP2 and DCX exhibit a closely overlapping distribution, mainly surrounding the nucleus and extending into proximal neurites, a result that is consistent with other reports on the literature (Gleeson et al., 1999). Even so, in neurite regions more distal from the soma there seems to be only presence of DCX and not MAP2, which can be related to DCX having a role in axonal growth, promoting neuronal migration (Gleeson et al., 1999). These markers exhibit a very similar % of positive cells within all cells, with the value for MAP2 being of 7.16 ± 0.38 and for DCX 7.18 ± 0.38 (Figure 4.1C).

Finally, to assess the presence of differentiated neurons and, more specifically, DA neurons, to thereby verify if this protocol can in fact give rise to this type of neurons, a labelling with β-III tubulin antibody and a labelling with TH was performed. β-III tubulin is a commonly used marker specific for neuronal cells and has been associated with playing an important role in neuronal differentiation, hence the importance to evaluate its expression on this cell culture (Mundhara & Panda, 2022). On the other hand, TH is an enzyme involved in the synthesis of

catecholamines that is regularly used as a marker to identify DA neurons. The results of the immunocytochemistry, evidenced in Figure 4.1 B and C, showcase the presence of β-III tubulin positive cells (13.42 ± 6.35 %), where the neuronal cell body and neurites can easily be identified. Dopaminergic neurons can also be identified in the cell culture, although the quantitative analysis reveals a low percentage of TH positive cells within all cells present in the culture, reaching only 2,7 % (Figure 4.1C).

Overall, the 2D cell culture, resulting from the DA differentiation protocol applied, revealed the presence of cells positive to different markers associated with the neural lineage (Nestin, GFAP, MAP2, DCX and β-III tubulin). These markers reveal the presence of neural stem cells, astrocytes and neurons in the obtained monolayer culture. Even so, the percentage of TH^+ cells in the 2D cell culture is rather low and does not allow the formation of a robust neuronal network, which is not the ideal, given that the employed protocol was optimized to promote a DA fate. The reasoning behind this low rate of DA neurons could be related to (1) only one technical replicate having been used for this evaluation, (2) the EB dissociation process performed before the plating might have been too aggressive for the cells, or (3) the monolayer culture system is not a propitious environment for the growth of DA neurons.

Since the behaviour of cells is impacted by the mechanical properties of the surrounding microenvironment and it has already been shown that soft substrates enhance neurogenic differentiation, it can be deduced that the stiffness of the surface provided for this 2D culture might have a negative effect on the desired neuronal and DA differentiation (Keung et al., 2012; Saha et al., 2008). Moreover, cells grown on 2D surfaces do not fully maintain cell-to-cell and cell-to-matrix interactions, which can lead to non-physiological responses regarding cell growth, migration, differentiation and survival (Edmondson et al., 2014; Geckil et al., 2010; Knight & Przyborski, 2015).

Culture of embryoid bodies in collagen hydrogel to develop 3D model allows a robust network of neurons to be formed

Since the developed 2D cell culture did not result in the formation of either a culture with an adequate number of TH nor a robust neuronal network, we decided to move onto 3D models.

The native ECM plays an important role on tissue organization and on the regulation of cellular function, being an essential part of the extracellular environment (Nicolas et al., 2020). In order

to obtain cell culture systems that take this into account, the brain ECM can be partially modelled *in vitro* by the use of natural, synthetic or even hybrid scaffolds (Murphy et al., 2017).

Since collagen is a natural protein abundantly present in the ECM, a collagen-based hydrogel was chosen for the development of this 3D model. Thus, at the end of the 14 days of mESCs differentiation into DA neurons, the resulting EBs were placed on collagen hydrogel droplets and differentiation was modulated, taking advantage of the presence of a more favourable extracellular environment for neuronal differentiation (Figure 4.2A). Along the six days of EB culture on collagen, the extension of neurites from all around the body structure spreading into the surrounding collagen was visible, forming a robust neuronal network (Figure 4.2B).

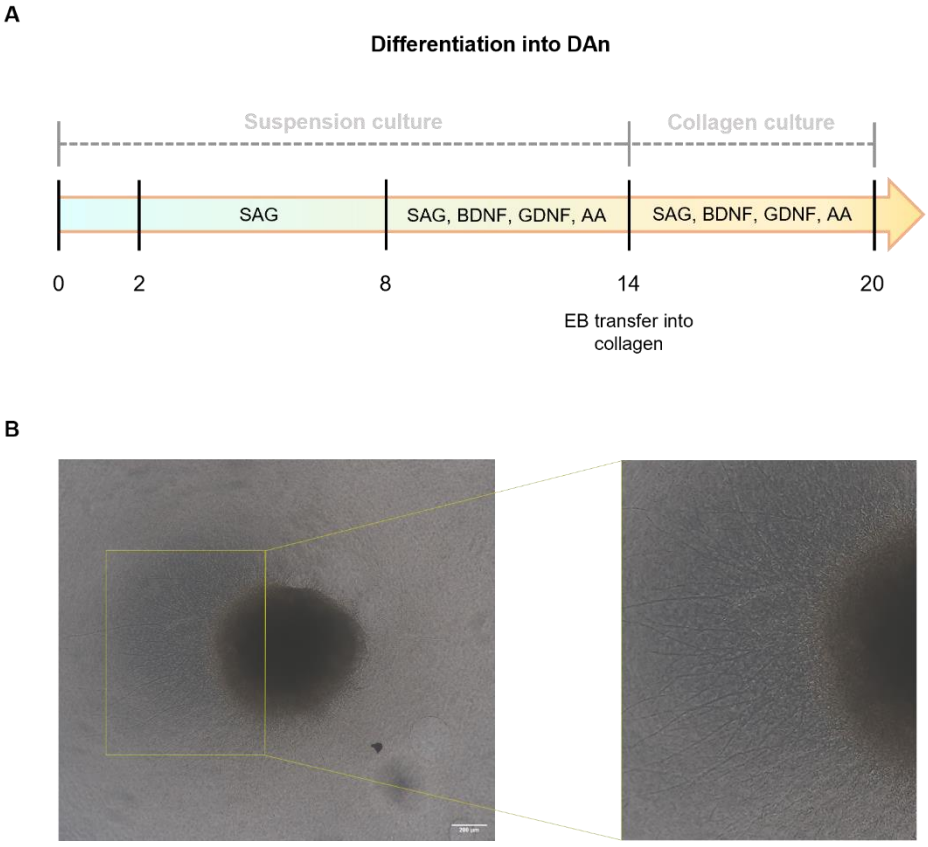

Figure 4.2 – Seeding of mESC-derived embryoid bodies on collagen hydrogel. (A) Timeline of the differentiation protocol carried out in suspension culture for the first 14 days and on collagen hydrogel in the last 6 days. **(B)** Bright-field image of an EB extending its neurites on top of the collagen hydrogel. Scale bar = 200µm

To further evaluate this neuronal differentiation and thereby characterize the generated EBs, an immunocytochemistry analysis was performed. Different sets of markers were used, including structural markers (MAP2, β-III tubulin, GAP43, PSD95 and synaptophysin), markers for

neuronal development and maturation (GFAP, DCX and NeuN), and monoamine markers (TH, DBH and DAT) (Figure 4.3 and 4.4).

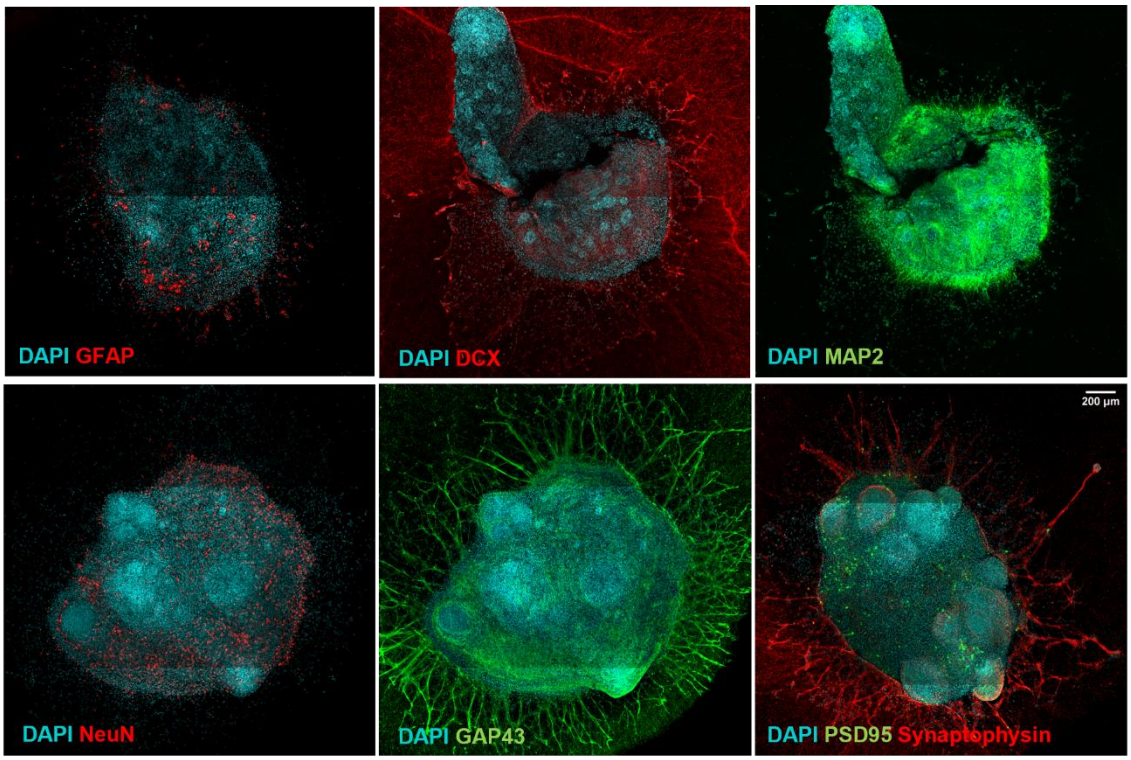

Figure 4.3 – Qualitative assessment of expression of different neural-associated markers in a 3D model based on the seeding of EBs on collagen hydrogel. Confocal images showing cells immunostained with antibodies against GFAP, DCX, MAP2, NeuN, GAP43, PSD95 and Synaptophysin. Nuclei were counterstained with DAPI (cyan). Scale bar = 200µm

The expression of the GFAP and DCX markers allows the identification of neuronal cells with a lower degree of maturation (Figure 4.3). The results concerning GFAP reveal the presence of GFAP positive cells in the 3D model. While a portion of these GFAP positive cells enhances a morphology similar to that of neural progenitor cells, the other portion presents a characteristic astrocyte morphology. This occurs because GFAP marks both neural progenitor cells and astrocytes. This result indicates the possibility that the performed differentiation protocol leads to the development not only of neural progenitor cells, but also of other types of cells, such as astrocytes. However, to confirm the presence of astrocytes, the evaluation of GFAP by itself is not sufficient, since previous reports have shown that this marker leaves a big portion of astrocytes undetected (Bushong et al., 2002; Walz & Lang, 1998). DCX expression is present in the axonal shaft and growth cone of young neurons (Gleeson et al., 1999). In concordance to this, the expression of DCX in the developed model reveals the axons of immature neurons. Its expression is noticeable all around the EB body structure.

The expression of the MAP2, NeuN, Growth Associated Protein 43 (GAP43), Postsynaptic density protein 95 (PSD95) and synaptophysin markers allows the identification of neuronal cells with a higher degree of maturation (Figure 4.3). The analysis of the structural marker MAP2 evidences a strong presence of MAP2 expression across the EB main structure. Since MAP2 is expressed in the cell bodies and dendrites of mature neurons, this could indicate that neuron cell bodies are mostly present on the EB center. The expression of NeuN also supports this idea, since this marker of mature neurons has a nuclear expression and, when analysing the figure, its expression can be encountered within the main structure of the EB. On the other hand, the marker GAP43, with known expression in axon outgrowth, is a protein associated with regulation of presynaptic vesicular function, axonal growth, and plasticity (Holahan, 2017). Thereby, during neuronal development and maturation, since axonal structural plasticity is required, the expression of this protein can increase (Holahan, 2017). Its expression is observed in great quantity in the images acquired irradiating from the EB surface to its periphery, revealing the presence of a 3D model with noticeable axon growth and plasticity, promoting the generation of mature and functional neurons.

Lastly, the expression of synaptophysin, a presynaptic protein, and PSD95, a postsynaptic protein, allows to evaluate the synaptic density of the developed model. The respective images acquired (Figure 4.3) reveal that there is presence of both markers which can suggest that the differentiated EBs are able to give rise to neurons with synaptic connections to each other.

Two additional co-labeling evaluations were performed, with the goal to identify adrenergic, noradrenergic and DA neurons in the model. Since β-III tubulin marks the axons of all types of neuronal cells it was used either in combination with Dopamine beta-hydroxylase (DBH), a marker of adrenergic and noradrenergic neurons, or with TH, a marker of DA neurons.

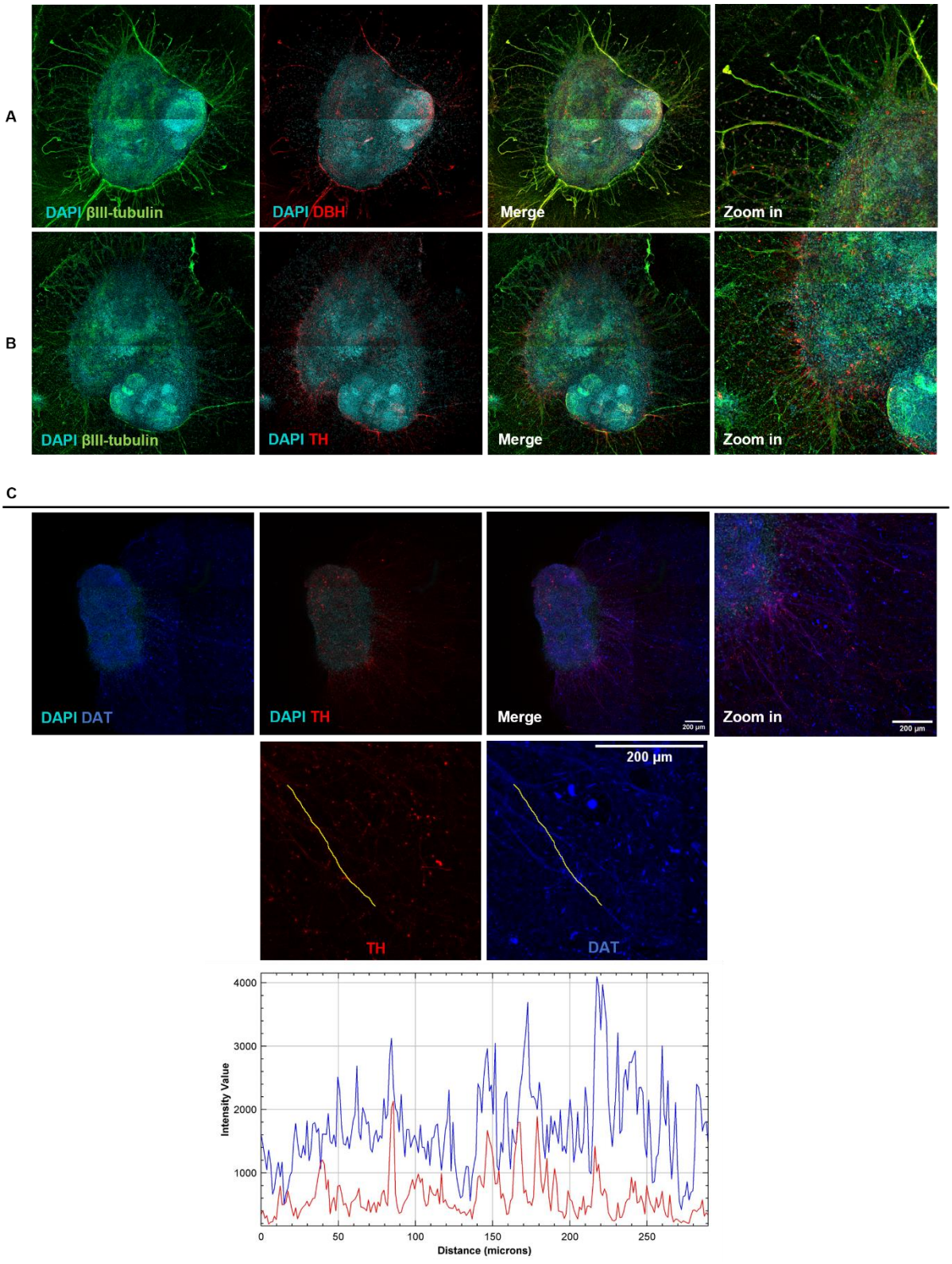

Figure 4.4 – Qualitative assessment of the presence of mature neurons in a 3D model based on the seeding of EBs on collagen hydrogel. (A) Confocal images of an β-III tubulin$^+$ and DBH$^+$ embryoid body. Nuclei were counterstained with DAPI (cyan). **(B)** Confocal images of an β-III tubulin$^+$ and TH$^+$ embryoid body. Nuclei were counterstained with DAPI. **(C)** Confocal images of an DAT$^+$ and TH$^+$ embryoid body (above). Zoom in enhancing a neurite (indicated with a line) and respective intensity values of TH and DAT immunostaining across the enhanced neurite (below). Scale bar = 200µm

The expression of DBH, which can be found in punctiform shape (Figure 4.4A), marks synaptic vesicles where the conversion of dopamine into norepinephrine occurs. Since β-III tubulin identifies all types of neurons, an overlap between β-III tubulin and DBH can be seen, revealing the presence of adrenergic and noradrenergic neurons in this 3D culture. Additionally, the co-labeling of β-III tubulin and TH (Figure 4.4B) reveals that a portion of the cells positive to β-III tubulin are also TH positive, thus evidencing a successful differentiation of mESCs into DA neurons.

A final qualitative characterization of this collagen model was performed, by assessing the expression of TH and DAT. This final co-labeling had the main goal of making sure that the obtained DA neurons had the expression of DAT. This information would become relevant further on this project, given that to induce the DA degeneration characteristic of PD 6-OHDA, a DA neurons' selective neurotoxin, would be used (Simola et al., 2007). For this reason, the expression of DAT in DA neurons is required, since the selective effects of 6-OHDA in DA neurons results from an intracellular action after its uptake is done through DAT (Y. M. Ding et al., 2004). As shown in Figure 4.4C, the presence of DAT in the develop 3D model can be confirmed. Furthermore, the expression of DAT seems to be overlapping, in its majority, with the expression of TH. Nonetheless, to further analyse if there is in fact co-localization of both markers, a zoom in was performed on the acquired images, to individualize a singular TH positive neurite and afterwards plot its profile, with the help of fiji (Figure 4.4C) (Schindelin et al., 2012). This procedure allows the measurement of the intensity values of the TH and DAT in the underlined neurite. The results obtained showcase the presence of both TH and DAT on the neurite, suggesting that DAT can be found in the DA neurons present in the developed model.

Altogether, although this first analysis of the developed 3D model was merely qualitative, it provided relevant aspects regarding its characterization. For instance, it gave clues about the spatial organization of neurons on this 3D model, since dendrites, identified by MAP2 expression, could be found within the EB body structure while the axons, identified by β-III tubulin and GAP43, were visible extending from the EB surface onto its periphery. Besides, other markers, such as DCX, NeuN, GFAP and DBH, revealed the presence of diverse types of cells, including neural stem cells and astrocytes, the presence of both young neurons and mature neurons, and even the presence of different types of neurons, including noradrenergic and adrenergic neurons.

This qualitative overview also demonstrated, through the expression of TH and DAT, the capacity of this differentiation protocol to give rise to mature DA neurons in a 3D scenario, with a higher degree of complexity than what seen in the 2D cell culture. Furthermore, the presence of DAT with some degree of overlapping distribution with TH in the developed 3D model was validated, demonstrating that the model can be used later on for 6-OHDA exposure. Nonetheless, an additional assessment to this 3D model should be performed in the future, consisting of performing its electrophysiological characterization, to functionally assess the DA neuronal maturation. This electrophysiological study could be performed through whole-cell patch-clamp recording, as this technique has already shown to be capable of successfully measuring neuronal activity in a 3D culture of DA neurons. Its use provided clues that a 3D culture system might be more capable of supporting the generation of mature functional mDA neurons than a 2D culture (Gilmozzi et al., 2021).

It is important to refer that this analysis reveals that the specificity of the used DA differentiation protocol is not very high and, although it successfully gives rise to mature DA neurons, it also gives rise to other types of cells. This possibly happens because, although EBs are exposed to a set of soluble factors that promote their DA differentiation (SAG, BDNF, GDNF and AA), only the cells that are on the most peripheral region get a good access to them, making it difficult for cells within the EB interior to be influenced by these factors. Even so, this characteristic could be interesting for a 3D model of PD, since the presence of different types of cells would allow the evaluation of the impact of DA cell death on a wider panorama, exploring not only how this affects the remaining viable DA neurons but also how this damage affects other neural cells, mimicking what is seen in the brain. Moreover, the presence of astrocytes in this 3D model could be advantageous, since glial support has revealed to be beneficial to the generation of functional DA neurons (Roy et al., 2006; H. Song et al., 2002).

Regarding the use of collagen hydrogel as a scaffold for this 3D model, the qualitative characterization provided clues that the nature and the reduced stiffness of this material have positive impacts on neurite outgrowth and DA differentiation, in concordance with previous reports on the use of this material (Leung et al., 2015; Ma et al., 2005). Besides this, the collagen hydrogel droplets were able to maintain the integrity of the cultured EBs, thus increasing viability of generated neurons.

Use of an alternative scaffold to develop 3D model also allows a robust network of neurons to be formed

Besides the development of a 3D model on collagen hydrogel, this project also explored the use of an innovative Ormocomp structure as an alternative synthetic scaffold. Ormocomp consists of a hybrid organic-inorganic polymer, which can be used to produce scaffolds through non-linear direct laser. Experimental studies regarding the use of this material have shown that it has the capacity to promote cell adherence and maturation (Käpylä et al., 2014; Koufaki et al., 2011). Additionally, it can be modified in order to satisfy certain characteristics, such as allowing neuronal cell adhesion and growth by modifying the Ormocomp surface (Kidwell et al., 2019). In this regard, we tested the use of Ormocomp-based structures (Figure 4.5A) as synthetic 3D scaffolds to support EB culture and promote DA differentiation.

Since neurons tend to not adhere well to untreated materials it was important to prepare the scaffold with PDL, to confer positive charge and promote initial cell adhesion, and with laminin, to enhance cell adhesion and to influence cell differentiation and migration (Kidwell et al., 2019). Thereby, structures were previously sterilized and coated with PDL [100 µg/mL] and laminin [10 µg/mL]. At the end of the 14 days of DA differentiation, performed accordingly to the described protocol (Figure 4.5B), a single EB was placed on the center of each structure, and differentiation was modulated for 6 additional days.

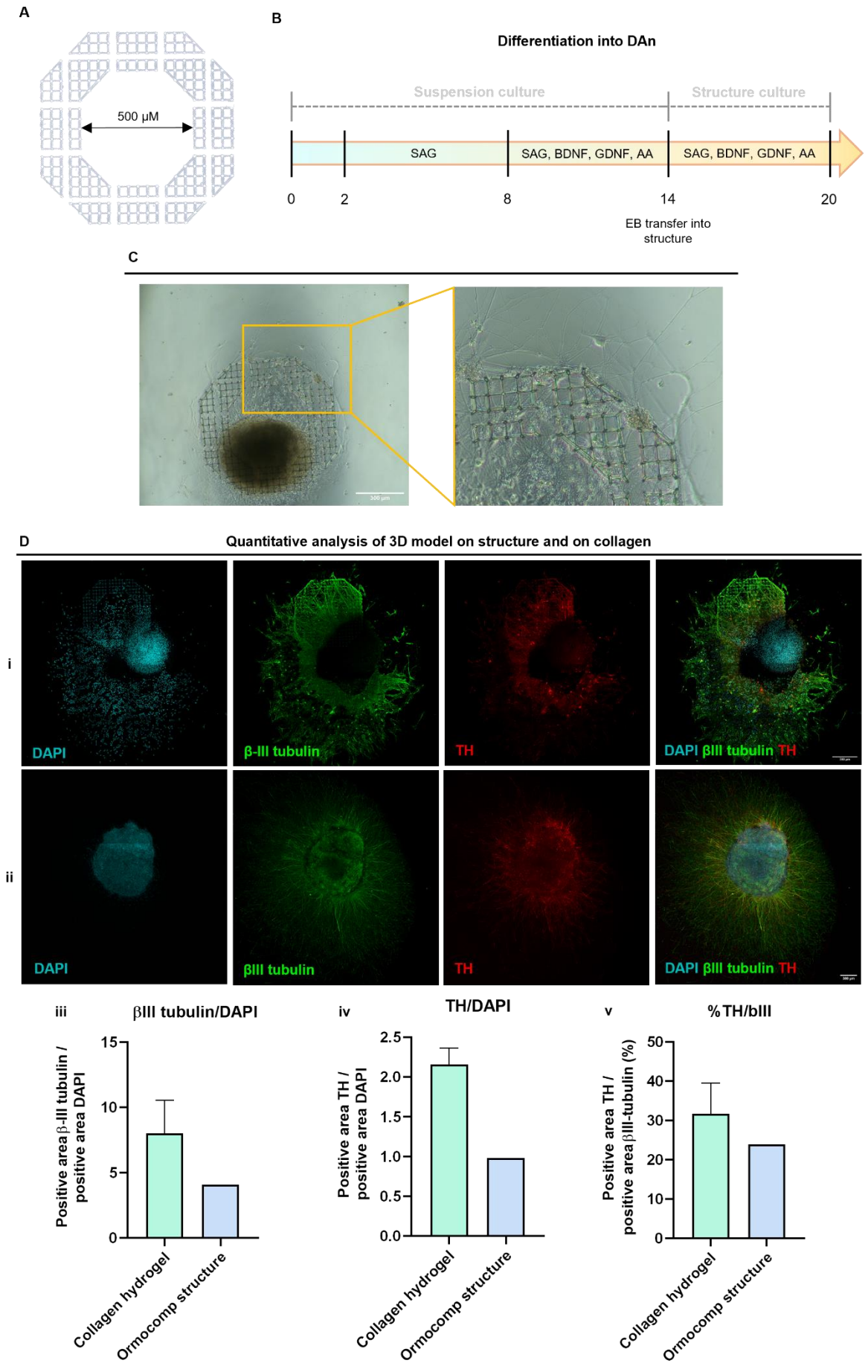
A
500 µM
B
Differentiation into DAn
Suspension culture
Structure culture
SAG
SAG, BDNF, GDNF, AA
SAG, BDNF, GDNF, AA
0
2
8
14
20
EB transfer into structure
C
D
Quantitative analysis of 3D model on structure and on collagen
i
ii
DAPI
β-III tubulin
TH
DAPI βIII tubulin TH
βIII tubulin
iii
βIII tubulin/DAPI
Positive area β-III tubulin / positive area DAPI
Collagen hydrogel
Ormocomp structure
iv
TH/DAPI
Positive area TH / positive area DAPI
v
%TH/bIII
Positive area TH / positive area βIII-tubulin (%)

Figure 4.5 – Seeding of mESC-derived embryoid bodies on Ormocomp structures and comparison of β-III tubulin and TH expression with collagen model. (A) Ormocomp structure for EB seeding. **(B)** Timeline of the differentiation protocol carried out in a suspension culture for the first 14 days and on an Ormocomp structure in the last 6 days. **(C)** Bright-field image of an EB extending its neurites on top of the Ormocomp structure. **(D)** Quantitative analysis of 3D model on structure and on collagen. (i) Confocal images of an β-III tubulin$^+$ and TH$^+$ embryoid body on top of an Ormocomp structure. (ii) Representative images of an β-III tubulin$^+$ and TH$^+$ embryoid body on collagen hydrogel. Nuclei were counterstained with DAPI (cyan). (iii-v) indicate ratios of β-III tubulin/DAPI, TH/DAPI and TH/β-III tubulin measured for both 3D models. n = 1 for Ormocomp structure model and n = 6 for collagen model. Scale bar = 300μm.

Even though the handling of the EBs on top of the structures was very demanding and medium changes frequently resulted in the displacement of the EB body, preliminary results on this model were positive. The growth of neurites extending from the EB body and spreading along the structure was visible (Figure 4.5C), suggesting that this 3D polymeric scaffold can facilitate higher-order cellular organization and is able to direct the neurites to desired locations.

Quantitative characterization of 3D collagen model and 3D Ormocomp model reveals successful dopaminergic differentiation

To have an idea of the rates of neuronal and DA differentiation present in each one of the developed 3D models and perform an initial comparison, the expression of the neuronal marker β-III tubulin and the DA marker TH was evaluated and then quantified (Figure 4.5D).

The results relative to the use of the Ormocomp structure reflect a high level of adherence and neuronal differentiation, demarked by the presence of a robust network of β-III tubulin positive neurites that spreads from the EB structure, including in the direction of the structure, involving it (Figure 4.5D i). When focusing on the expression of TH, the presence of cells TH positive is visible on the whole periphery of the EB but mainly surrounding the structure.

The 3D model developed in collagen, subjected to the same protocol conditions as the structure model, demonstrated a tendency to increased values in the calculated ratios (β-III tubulin/DAPI, TH/DAPI and TH/β-III tubulin) (Figure 4.5D iii-v). The analysis of the percentage of positive TH area within the positive area of β-III tubulin reveals a value of 31.78 ± 10.85 for the 3D collagen model and a value of 23.95 % for the 3D structure model (Figure 4.5D v). The observed differences between scaffolds cannot be considered significant due to only one sample having been evaluated for the structure group. Therefore, this experiment should be repeated in the future with a higher number of samples per group to get more trustworthy results and

confirm if, in fact, there are differences between the use of these two scaffolds and if they are significant.

Even so, the calculated ratios suggest that the 3D collagen model would be a better option when the goal is DA differentiation, for example, for the *in vitro* study of PD. Nevertheless, it is important to enhance the fact that the 3D structure model is still at a very initial stage of development, meaning that the future optimization of the structure model could lead to more satisfactory results.

Exposure of the 3D collagen model to 6-OHDA leads to dopaminergic degeneration

Since the 3D collagen model was the developed model with the best performance in terms of dopaminergic differentiation, we decided to proceed with its use for the induction of PD phenotype. To induce DA degeneration in the model we performed an incubation with 6-OHDA, a toxin that leads to the production of free radicals and hydrogen peroxide, causing selective DA cell death (Fulceri et al., 2006).

Exposure to 6-OHDA was done incubating the 3D collagen model previously obtained with a dosage of [500 µM] for a 4 h period. At the end of this period, the subset of EBs exposed to 6-OHDA was incubated in Neurobasal medium and the control subset of EBs was incubated in supplemented ADFNK medium, for 48 h additional hours.

To analyse the morphology of TH neurites after 6-OHDA exposure, an immunocytochemistry was performed.

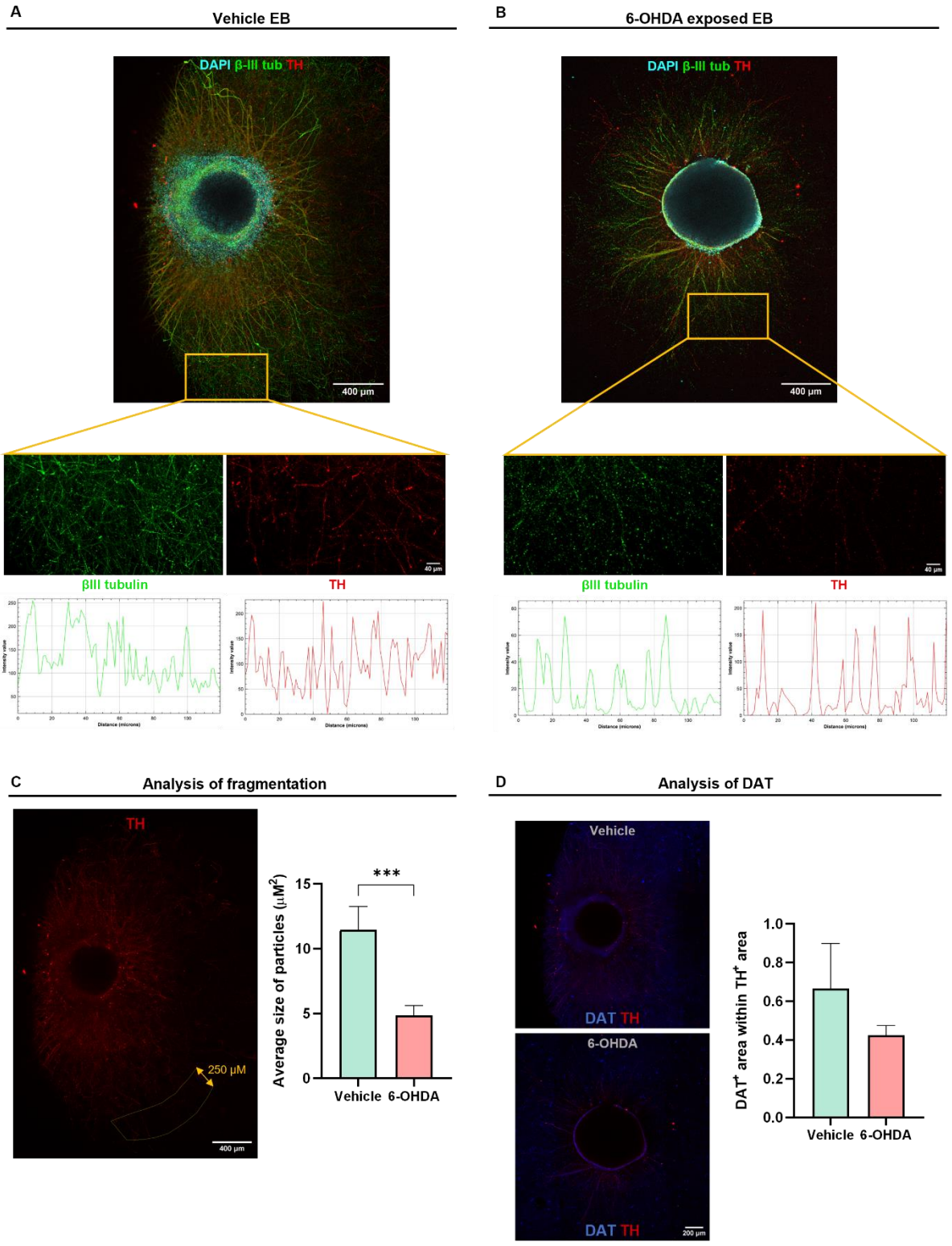

Figure 4.6 – Assessment of 6-OHDA-induced dopaminergic degeneration on the developed 3D model on collagen hydrogel. (A) Representative confocal image of 3D model vehicle group (above). Cells were immunostained with antibodies against β-III tubulin and TH. Nuclei were counterstained with DAPI (cyan). Scale bar = 400µm. Below can be seen a zoom in enhancing neurites immunostained with antibodies against β-III tubulin and TH and intensity values for these markers measured across a single neurite. Scale bar = 40µm. **(B)** Representative confocal image of 3D model exposed to 6-OHDA (above). Cells were immunostained with antibodies against β-III tubulin and TH. Nuclei were

counterstained with DAPI. Scale bar = 400µm. Below can be seen a zoom in enhancing neurites immunostained with antibodies against β-III tubulin and TH and intensity values for these markers measured across a single neurite. Scale bar = 40µm. **(C)** Confocal image with representation of the drawn area with 250µM thickness to measure average size of neurite portions (left) and quantification of these results for both the vehicle group and the 6-OHDA group (right). Scale bar = 400µm. **(D)** Representative confocal images of vehicle group and 6-OHDA group immunostained with antibodies against DAT and TH (left), and respective quantification of expression of these markers (right). Scale bar = 200 µm. Data is presented as mean ± SEM. $n = 4$. ***$p<0.001$

A general analysis over the images acquired (Figure 4.6 A and B) reveals that EBs exposed to 6-OHDA present an atrophied appearance, where the neuronal network is not as easily identifiable as the one present in the vehicle group. To further explore these differences, images were zoomed in on a neurite area, revealing that, while the vehicle group presents well defined neurites, proven by a more stable expression of TH along the neurites analysed, the 6-OHDA group does not showcase well defined neurites, revealing an irregular expression of TH along the neurites, composed of high and low extremes of intensity (Figure 4.6 A and B – bottom). To complement this analysis, fragmentation and levels of DAT were measured for the vehicle and the 6-OHDA group (Figure 4.6 C and D). For fragmentation analysis, the average size of TH^+ particles was measured within a defined polygon area with 250 µM thickness, revealing a statistically significatively decreased size of the TH^+ particles present in the 6-OHDA group, when compared to the vehicle group (Figure 4.6C). To analyse DAT levels of expression, the DAT^+ area within TH^+ area was quantified for the vehicle and the 6-OHDA group (Figure 4.6D). The results, exposed in the graph, reveal a correlation between the exposure to 6-OHDA and a decrease in DAT^+ area. Altogether, the evidenced results demonstrate the presence of DA degeneration when the cellular model is exposed to 6-OHDA, evidenced by an increase of dopaminergic neurite fragmentation.

It is important to mention that this experiment had the limitation of the 6-OHDA group having been kept in Neurobasal medium on the 48 h post exposure while the vehicle group was kept in supplemented ADFNK medium. This could mean that some of the DA degeneration observed in the toxin group could be due to the medium they were kept in not being supportive, thereby the importance to repeat this experiment in future studies using the same medium for both conditions.

Overall, the results regarding the development and characterization of a model of dopaminergic neurons for the study of PD demonstrated that (1) the 2D cell culture developed did not meet our goals, not leading to the formation of a neuronal network and revealing a low number of

dopaminergic neurons, (2) by providing a scaffold for the cell culture to grow in we were able to obtain a 3D model with a robust neuronal network and successful dopaminergic differentiation, being suitable for the study of PD, (3) the use of an Ormocomp structure as an alternative scaffold also revealed the capacity to develop a 3D model with dopaminergic differentiation, although not as evident as in the 3D collagen model, and (4) the exposure of the developed 3D collagen model to 6-OHDA leads to the formation of a culture model suitable for the study of PD.

2. iMSCs and the influence of cell expansion media and passage number on secretome therapeutical potential

Previous reports have related positive effects of MSCs secretome on promoting neurogenesis and neuroprotection, revealing its potential as a therapeutical strategy for PD (Marote et al., 2016; Munoz et al., 2005; Pires et al., 2017; Vilaça-Faria et al., 2021). With the emergence of iMSCs, it is now possible to obtain a larger number of cells for therapeutical applications. However, before proceeding to clinical applications with iMSCs secretome, certain aspects should be addressed, concerning how the modulation of the cell culture expansion conditions of these cells influences their properties. This evaluation is relevant, since, as shown for other MSCs sources, iMSCs might also be subject to influences of different conditions, such as cell culture media and serum supplementation, as well as the cell passage in which their secretome is collected might affect the therapeutical outcome of their secretome.

Medium in which iMSCs are expanded and cell passage have effects in cell growth and senescence

Although a previous report from our group has already revealed that the expansion of iMSCs in hPL supplemented medium is capable of maintaining their identity and leads to a high proliferative capacity of these cells (Marote et al., 2023), it is important to directly compare the expansion of iMSCs in these two different media, MesenCult^{TM} and hPL supplemented medium. This allows to better understand the influence of different cell culturing media on iMSCs properties and if the presence of a supplement from human origin can result in an equal to higher proliferation of these cells.

MesenCult^{TM}-ACF Plus Medium is a commercially available ACF medium for the expansion of human MSCs. Although its chemical composition is known, which is a positive point in

regards for a translation into preclinical and clinical sets, this medium is expensive, which makes its use in large-scale expansion of MSCs highly costly.

hPL provides an animal component-free supplement for the culture of MSCs that can also be produced under GMP conditions, providing a potentially more cost-effective approach than MesenCult™ (Becherucci et al., 2018; Oeller et al., 2021).

It is important to also denote that the iMSCs used for this purpose were previously generated in our laboratory in a commercially defined medium without animal components (Marote et al., 2023). It would be important to explore in the future if there are other cheaper GMP-compatible approaches that are appropriate for generating iMSCs.

To evaluate the growth of iMSCs in either hPL supplemented medium or MesenCult™ medium, iMSCs obtained from a single donor were expanded in these two media from P7 until proliferation arrest. To facilitate reading, iMSCs expanded in hPL supplemented medium will be referred to as iMSCs-hPL and iMSCs expanded in MesenCult™ medium as iMSCs-MesenCult™.

Various aspects, such as morphology and replicative senescence were evaluated in both conditions and in different time points, one corresponding to an earlier stage (P8) and one at a later stage (P14 for iMSCs-hPL and P12 for iMSCs-MesenCult™).

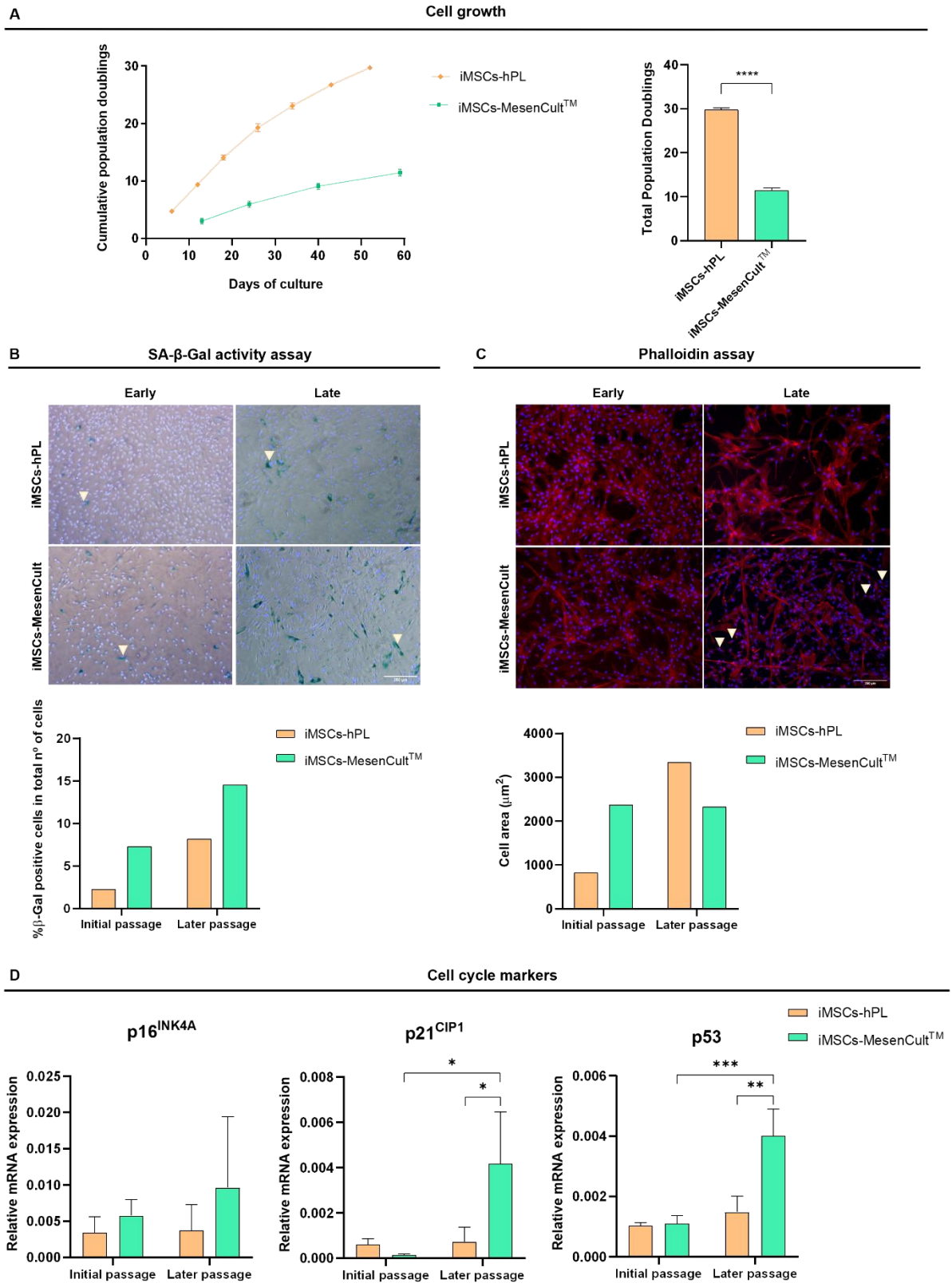

Figure 4.7 – Proliferation and replicative senescence assessment of iMSCs expanded under hPL supplementation or in MesenCult™ medium. (A) Cumulative population doublings over long-term culture expansion of iMSCs expanded in the two media (left) and respective total population doublings (right). **(B)** Phase contrast images of the cells acquired after the SA-β-Gal assay (above) which leads to the formation of a blue precipitate that indicates senescent cells (arrow heads). Ratio of SA-β-Gal

positive cells, both in early and later cell passage (bellow). **(C)** Representative fluorescence images of phalloidin and DAPI staining, both in early and later cell passage (above). The arrow heads highlight the presence of very small sized cells in late passage iMSCs-MesenCult^{TM}. Cell are measurements for iMSCs both in early and later cell passage (bellow). **(D)** Relative expression of p16^{INK4A}, p21^{CIP1} and p53 genes, normalized to GAPDH. Data is presented as mean ± SEM, n = 2-3; scale bar = 250µm; *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Cell growth was expressed as cPD and was measured from the initial plating of iMSCs (P8) until cell growth arrest (P15 for iMSCs-hPL and P12 for iMSCs-MesenCult^{TM}). Figure 4.7A (left) showcases the cell growth curve obtained at the end of the long-term experiment. iMSCs-hPL exhibit a higher growth rate in comparison to iMSCs-MesenCult^{TM}. iMSCs-hPL exhibit a significantly higher number (29,72 ± 0,31) of total PD than when cultured in MesenCult medium (11.44 ± 0,63), derived from the same donor (Figure 4.7A right). These results indicate a higher proliferative capacity of iMSCs-hPL in comparison to iMSCs-MesenCult^{TM}. As referred above, since iMSCs used for this experiment were obtained from a single donor, the possibility that the observed differences in proliferative rates between the two conditions are caused by donor variability is eliminated. Therefore, taking into consideration that the only parameter varying between these two conditions is the cell culture media in which iMSCs are cultured in, it can be concluded that the media influences cell growth and, in this case, supplementation with hPL benefits proliferation of iMSCs.

MSCs, just like most somatic cells, have a finite lifespan *in vitro* (Bonab et al., 2006). This phase is associated with a decrease in proliferation that culminates in stable cell-cycle arrest, also known as replicative senescence. Although multiple pathways and predisposing factors can lead to senescence making it a complex process, there are common markers of MSC senescence that allow the monitoring of replicative senescence in cell cultures (Al-Azab et al., 2022). Some of these are the enlargement of the cells, an upregulation of the p53 and the p16^{Ink4a} pathway, and the presence of SASP (Al-Azab et al., 2022). The process of senescence is part of the normal physiology of human cells, however, an accumulation of senescent cells can contribute, for instance, to chronic inflammation and age-related diseases (Di Micco et al., 2021). Moreover, properties attributed to MSCs secretome can be altered negatively by senescence, resulting in a decreased immunosuppressive ability related to an enhanced profile in pro-inflammatory cytokines and a decreased angiogenic potential with downregulation of factors such as insulin-like growth factor-1 (IGF1), VEGF and Hepatocyte growth factor (HGF) (Gnani et al., 2019; Khan et al., 2011).

Therefore, to evaluate replicative senescence of iMSCs at the beginning and at the end of the long-term culture in each medium, a SA-β-Gal assay was employed. This assay measures β-galactosidase activity in senescent cells, by quantifying the number of cells in which its substrate, X-Gal, is converted to a blue-dyed precipitate (Dimri et al., 1995)(Figure 4.7B). The results of this assay, for iMSCs at early stage revealed a percentage of 2.23 ± 0.42 β-gal positive cells out of the total number of cells cultured in medium supplemented with hPL and a percentage of 7.29 ± 1.69 β-gal positive cells out of the total number of cells cultured in MesenCult medium (Figure 4.7B). Surprisingly, at this point, a difference in the percentage of β-gal positive cells between the two conditions could already be seen. Taking into consideration that only the medium in which iMSCs were expanded varied, a possible explanation for this difference at such an early stage of the evaluation could rely on the fact that, since iMSCs-hPL proliferate at higher rates, the number of non-senescent cells on this condition increases quickly, decreasing thereby the percentage of β-gal positive cells within the total number of cells in the culture.

The assay was then repeated on later passages: P14 for iMSCs-hPL and P12 for iMSCs-MesenCult^{TM}. At this point, while iMSCs-hPL exhibited a percentage of 8.20 ± 2.41 β-gal positive cells out of the total number of cells, iMSCs in SF medium showed a percentage of 14.56 ± 2.41 β-gal positive cells out of the total number of cells. As expected, alongside with the increase of cell passage, the percentage of β-gal positive cells also increased, in both conditions. However, even though iMSCs-hPL underwent a higher number of cell passages than iMSCs-MesenCult^{TM}, they still exhibited a lower ratio of β-gal positive cells at the final passage, suggesting that, when cultured in hPL supplemented medium, iMSCs may present a more rejuvenated profile.

Although the SA-β-Gal assay can provide some clues on the senescence state of a cell culture, its use alone is not reliable to detect cellular senescence specifically and efficiently, due to β-gal activity also being increased in consequence of other internal and external parameters, such as serum starvation (Knabe et al., 2021).

An increment in cell area is another senescence-associated phenotype (Al-Azab et al., 2022), which can be evaluated using phalloidin-mediated actin filaments staining. The calculation of the average cell area for iMSCs-hPL from initial passage resulted in a value of 827.89 ± 1.68 μm^2, while the same method applied for iMSCs-MesenCult^{TM} from initial passage resulted in a value of 2368.90 ± 98.02 μm^2 (Figure 4.7C). Similarly to what happened in the cell growth analysis, at this point, a difference between the two conditions could also already be seen, with

iMSCs-hPL showing a cell area almost 3 times smaller than iMSCs-MesenCult™. Since small cell size is correlated with higher proliferative capacities (Lian et al., 2010), these results support the idea of iMSCs cultured in hPL supplemented medium having a higher proliferation rate.

Conversely, the calculation of the average cell area for iMSCs-hPL from late passage resulted in a value of 3338,94 ± 223,23 μm^2, while iMSCs-MesenCult™ revealed an average cell area value of 2327,64 ± 562,73 μm^2. These results suggest that, for iMSCs-MesenCult™, average cell area was similar between early and late passage, whereas iMSCs cultured in hPL supplemented medium displayed an abrupt increase of cell area in late passage. The results regarding iMSCs-hPL suggest a certain degree of senescence at a later passage, since there is an enlargement of cell size. On the other hand, at first sight, results relative to iMSCs-MesenCult™ would suggest that senescence did not increase from early to later passage, coming in contradiction with the results from the SA-β-Gal assay, where an increase in β-gal positive cells was denoted. However, there are two possible reasons that could explain the non-increase of cell area value for iMSCs-MesenCult™ within cell passages. The first one relies on the fact that, at an early passage, the value of iMSCs-MesenCult™ cell area was much higher than for iMSCs-hPL, alongside the percentage of β-gal positive cells, which could indicate that, at P8, some degree of senescence could already be present. The second one is related to the morphology of iMSCs-MesenCult™ being very heterogenous at P12: while a larger portion of the cells exhibit an enlarged flattened shape, the other portion reveals a smaller and more round shape (indicated by arrow heads in Figure 4.7C). This could be an indicative of asymmetric cell division, occurring the formation of a self-renewing cell and of a non-dividing cell that eventually becomes senescent in culture (Colter et al., 2001; J. Liu et al., 2020; Mets & Verdonk, 1981). This cell culture exhibits thereby the presence of different subpopulations at this stage, where the small sized subpopulation might be masking the "real" cell area value of the senescent subpopulation. Moreover, it would be interesting for future studies to fractionate these subpopulations to understand if they give raise to different secretory profiles, since this heterogeneity could be a limitation for the collection of secretome from these cells.

To further complement the evaluation of the replicative senescence, the analysis of the expression of specific molecular markers, $p16^{INK4}$, $p21^{CIP1}$ and p53, was also performed by qRT-PCR. The pathways involved with senescence, particularly in human cells, comprehend the upregulation of $p21^{CIP1}$, a cyclin-dependent kinase (CDK) inhibitor, induced mainly by p53, and the upregulation of another CDK inhibitor, $p16^{INK4}$, both resulting in cell cycle arrest (Hall

et al., 2017; Rufini et al., 2013). The results (Figure 4.7D) demonstrate that the levels of the cell cycle markers from iMSCs-hPL do not change significantly from initial passage to later passage. In contrast, iMSCs-MesenCult^{TM} show a statistically significant increase of expression of both p21^{CIP1} and p53 from initial passage to later passage, but not of p16^{INK4}. Although the results above concerning β-galactosidase activity and cell area reveal that there is presence of some degree of senescence at P14 for iMSCs-hPL, expression of p16^{INK4} and p21^{CIP1} is not increased at P14, being only denoted a slight increase in p53 expression. This could mean that at P14, iMSCs-hPL are still at an initial stage of senescence, an idea supported by the fact that the cPD graphic, exhibited in Figure 4.7A, reveals an exponential cell growth curve that does not reach the plateau, a phase in cell growth more associated with senescence (H. Zhao & Darzynkiewicz, 2013).

Altogether, these results point out a higher rejuvenated profile of iMSCs cultured in hPL supplemented medium. Therefore, it might be more advantageous to expand iMSCs in hPL supplemented medium than in MesenCult medium when the goal is to obtain a higher number of iMSCs along time. When resorting to the literature, it is noticeable that some studies report that supplementation with hPL has an increased impact on senescence of MSCs (Becherucci et al., 2020; Schallmoser et al., 2010), while others reveal the opposite (Griffiths et al., 2013). These contradictory results indicate that there is a necessity to evaluate senescence features in each MSC population and, given the complexity of senescence itself, this assessment should analyse multiple markers, and not only one (Young et al., 2013).

Medium in which iMSCs are expanded has effects on their secretory profile

The therapeutical potential of iMSCs, as well as for MSCs from different sources, has been mainly related to their paracrine activity (Cova et al., 2010; Marques et al., 2018a; Teixeira et al., 2015). As already described before, in studies conducted in BM-MSCs and UC-MSCs, media can influence the secretory profile of MSCs and, therefore, secretome collected from MSCs cultured in different media can have different therapeutical outcomes (J. Y. Kim et al., 2021; Wagner et al., 2006). Since there were evident differences in iMSCs-hPL versus iMSCs-MesenCult^{TM}, it was important to also evaluate secretome derived from both conditions to analyze if there were differences in its profile and, therefore, in its therapeutical applications.

Previously to this project, in our laboratory, iMSCs originated from 3 donors were cultured either in hPL supplemented medium or MesenCult medium, and secretome was collected at P9

for proteomic analysis. To further dissect the influence of cell culture media on iMSCs secretory profile, we used the dataset generated from this previous analysis to construct a protein-protein interaction network analysis with the online STRING database.

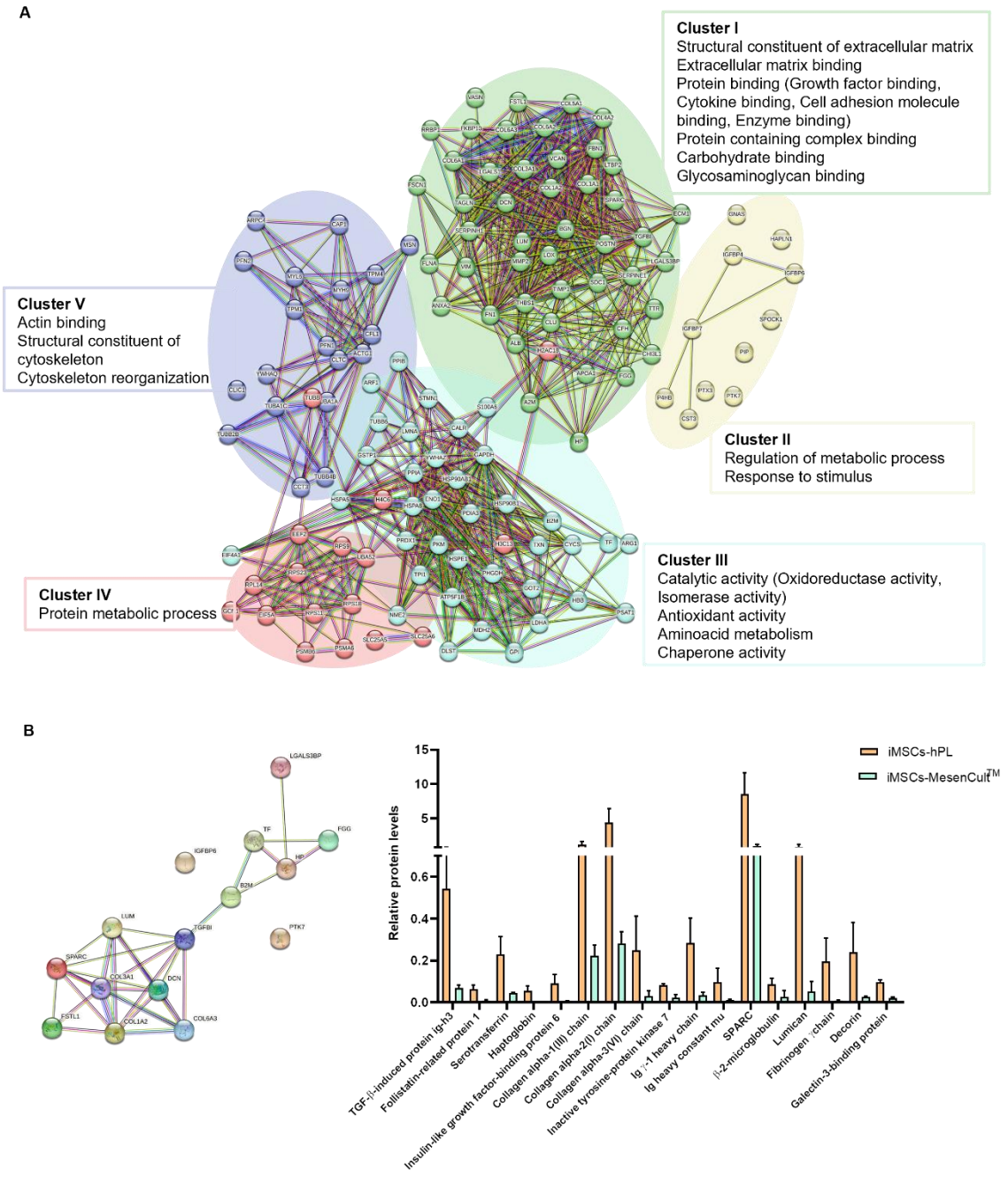

Figure 4.8 – Proteomic analysis of secretome from iMSCs expanded under hPL supplementation or in MesenCult™ medium. (A) Protein-protein interaction network analysis of all 128 identified proteins in secretome from both conditions, using the STRING online software with a medium level confidence of 0.4. Proteins organized in 5 clusters accordingly to their distinct molecular functions. **(B)** Protein-protein interaction network analysis, using STRING online, of 17 differently concentrated proteins in secretome from both conditions (left), and relative protein levels of the differentially concentrated proteins in secretome from both conditions (right). n = 3. For all significant differences $p \leq 0.05$.

A total of 128 proteins were identified in both conditions, which were then grouped into five clusters, according to their main molecular functions or biological processes (Figure 4.8A). This proteomic analysis revealed the presence of groups of proteins with potential therapeutical applications for PD. To start, cluster I contains 45 items mainly associated with constituents of the ECM, such as different collagen subunits, extracellular matrix protein 1 (ECM1), and proteins related to cell-to-cell or cell-to-matrix interactions, indicating a possible positive outcome regarding tissue regeneration and wound healing (Ahangar et al., 2020; Nazarie et al., 2021). Other than these, some proteins from cluster I and cluster II were identified as having binding functions to growth factors, such as Insulin growth factor binding protein-4 (IGFBP4), Follistatin-like 1 (FSTL1) and Latent-transforming growth factor β-binding protein 2 (LTBP2), enhancing the secretome capacity to regulate the bioavailability of trophic molecules (Caplan & Dennis, 2006; H. Ding & Wu, 2018).

Another cluster highly enriched in proteins found in the secretome from both conditions is cluster III, containing proteins with catalytic and antioxidant activity, proteins involved in folding and proteins with chaperone activity. In terms of therapeutical potential for PD, proteins involved in folding and with chaperone activity (e.g.: Protein disulfide-isomerase A3 and Heat shock cognate 71 kDa protein) could be relevant to prevent α-synuclein misfolding, oligomerization and aggregation, which is one of the hallmarks of PD (Chaari et al., 2013). Moreover, proteins with catalytic and antioxidant activity (e.g.: Glutathione S-transferase P and Thioredoxin) could suggest a potential capacity to protect DA neurons from oxidative stress-induced cell death in PD (Sun et al., 2017).

Even though a multitude of proteins with potential beneficial effects was found in the secretome from iMSCs-hPL and iMSCs-MesenCult^{TM}, some relatively important proteins that are usually reported in MSC secretome were not identified. Examples of these are the growth factors BDNF and GDNF, that have been identified as inducers of neuronal differentiation and could be potentially positive for a PD directed therapeutical application (Allen et al., 2013). Further experimental studies resorting to other protein detection techniques should be performed in order to evaluate if, in fact, these proteins are not present in secretome from iMSCs-hPL and iMSCs-MesenCult^{TM}, given that BDNF and GDNF are normally found in low concentrations and, therefore, the mass spectrometry performed to gather these data could have hindered their detection.

While a big proportion of the 128 identified proteins reveals similar levels of expression between both conditions, 17 proteins were found to be significatively differentially expressed

between iMSCs-hPL and iMSCs-MesenCult™ secretome (Figure 4.8B). Interestingly, all identified proteins were present in higher levels in secretome from iMSCs-hPL (Figure 4.8B right). Levels of ECM related proteins, such as lumican, Secreted protein acidic and rich in cysteine (SPARC), collagen subunits (COL1A2, COL3A1 and COL6A3) and Decorin (DCN), were significantly increased in secretome from iMSCs-hPL. The presence of higher levels of expression of ECM-related proteins could indicate a better capacity for tissue regeneration, angiogenesis and even axonal growth (N. C. Cheng et al., 2020; Oliveira et al., 2017). Interestingly, collagen VI (COL6) has been recently described as having a role in the proper maintenance of dopamine circuitry function, thereby its increased expression in secretome from iMSCs-hPL could potentially have a beneficial effect for PD (Gregorio et al., 2022). Additionally, DCN, an extracellular matrix glycoprotein, has been previously found to promote neurite outgrowth of dorsal root ganglion neurons (Minor et al., 2008). Besides proteins related to the ECM, other interesting proteins were also identified has having higher levels of expression in iMSCs-hPL secretome, such as insulin growth factor binding protein-6 (IGFBP6), that has been associated with roles on neuroprotection, neurogenesis, synaptogenesis and neuron maturation, and FSTL1, which was capable of decreasing neuronal death on a rat model of brain ischemic injury (Chen et al., 2020; Liang et al., 2014).

Altogether, even though significant differences were observed in proliferation and senescence of iMSCs-hPL and iMSCs-MesenCult™, a great degree of similarity regarding the secretory profile of iMSCs-hPL and iMSCs-MesenCult™ can be seen, with only 13% of the proteins being secreted in a different level. However, this portion of proteins significatively differentially expressed could have a functional impact on the secretome, which could predict a different therapeutic capacity in the context of PD (Abdelrazik et al., 2011).

Secretome from iMSCs-hPL and secretome from iMSCs-MesenCult™ have different impact on neurite outgrowth and dopaminergic differentiation

Secretome obtained from MSCs has revealed to have a multitude of applications within the biomedical field (Bagno et al., 2018; Pinho et al., 2020). As it has been already suggested in literature, factors present in the secretome have been found to have the capacity to modulate neurogenic niches, by increasing the survival, proliferation and differentiation of neural stem cells (Munoz et al., 2005).

Since PD leads to degeneration of DA neurons and, eventually, to their death, therapies for PD need to take DA differentiation into consideration to promote regenerative effects on the DA system (Marques et al., 2018b; Poewe et al., 2017).

The capacity of MSCs secretome to promote differentiation and maturation of neural progenitor cells (NPCs) could be an indicative of the potential beneficial effects that it could have as a therapeutic tool for PD (Pires et al., 2017; Teixeira et al., 2016; Vilaça-Faria et al., 2021). However, many aspects remain to be addressed, such as the effects of secretome obtained from MSCs obtained from iPSCs when cultured in different media and collected in different cell passages. Thereby, it became of our interest to test the effects of secretome collected in different cell passages from iMSCs cultured in hPL supplemented medium or MesenCult medium on neurite outgrowth and DA differentiation of a 3D cellular model.

For that, at day 14 of DA differentiation, EBs were placed in collagen hydrogel droplets and each subset of EBs was maintained for 6 additional days in different media: (1) Neurobasal medium (NB), (2) Neurobasal medium supplemented with SAG, BDNF, GDNF and AA in the same concentrations as described for the 3D collagen model (NB+factors), (3) supplemented ADFNK medium (ADFNK+factors), (4) conditioned medium from low passage iMSCs-MesenCult^{TM} (MesenCult), (5) conditioned medium from low passage iMSCs-hPL (hPL P6) and (6) conditioned medium from late passage iMSCs-hPL (hPL P15). At the end of this period, an immunocytochemistry was performed to evaluate the expression of TH and β-III tubulin in the different conditions.

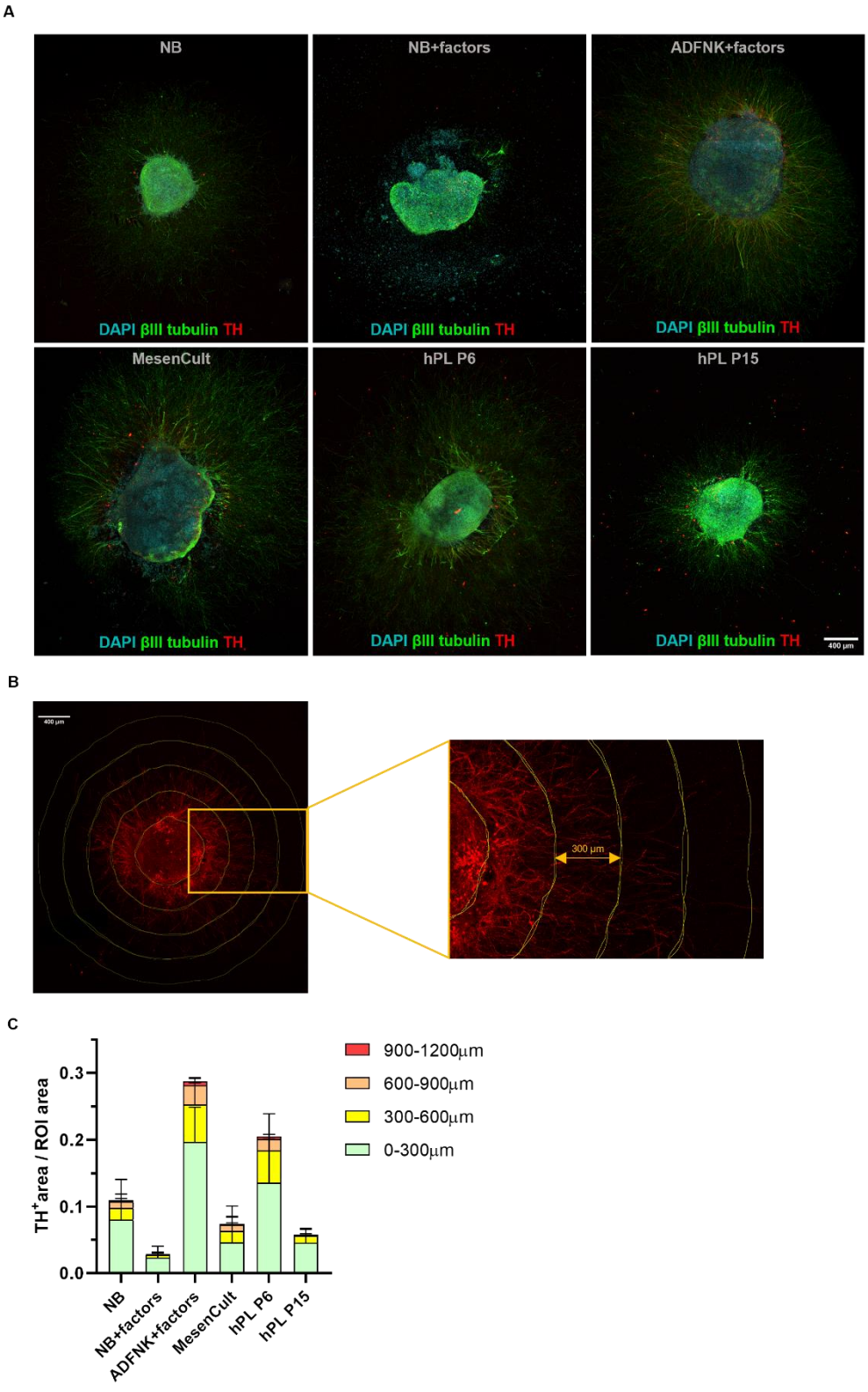

Figure 4.9 – Assessment of the effects of secretome from iMSCs expanded in different media and collected at different cell passages on dopaminergic differentiation of a 3D model on collagen. (A) Representative confocal images of the different conditions. Cells were immunostained with antibodies against β-III tubulin and TH. Nuclei were counterstained with DAPI (cyan). **(B)** Representation of the analysis performed to evaluate TH expression on the different conditions. Four rings with 300µm

thickness each were drawn in a consecutive manner starting adjacent to the EB main body structure and TH expression was evaluated within each length. **(C)** Quantification of TH expression on the different conditions and on the different lengths. Data is presented as mean ± SEM, *n* = 4-7; scale bar = 400µm

In Figure 4.9 A, a representative image for each condition is presented. It reveals that the conditions where there seems to be a higher neurite outgrowth, similar to the positive condition (ADFNK+factors), correspond to EBs cultured in conditioned medium from low passage iMSCs-MesenCult^{TM} and EBs cultured in conditioned medium from low passage iMSCs-hPL. The remaining conditions show a lower capacity to sustain the formation of neurites, showcasing either shorter length neurites (NB and hPL P15 condition) or non-visible neurites (NB+factors).

To take more precise conclusions regarding DA differentiation, the area of TH positive neurites present within different distances from the EB center in each condition was measured, with the help of Fiji (Schindelin et al., 2012). Four rings with 300 µm thickness each were drawn, starting from the area closer to the EB center and progressing to its periphery (Figure 4.9B). Afterwards, TH positive area was measured for each ring and normalized to the area of the drawn ring. The results, presented in Figure 4.9C, allow a comparison between the different conditions and within the different lengths evaluated. It is visible that, although at distance 0-300 µm from the EB center all conditions present TH positive area, at the furthest distance (900-1200 µm) the EBs cultured in either supplemented Neurobasal medium or in conditioned medium from late passage iMSCs-hPL do not show presence of TH positive area. Interestingly, EBs cultured in secretome from early passage iMSCs-hPL showcase a similar TH positive area to the one presented by EBs cultured in supplemented ADFNK medium, the positive medium of DA differentiation, in all the distances analysed.

Moreover, EBs cultured in conditioned medium from iMSCs-hPL early passage have a significatively increase in the positive area for TH when compared to EBs cultured in conditioned medium from iMSCs-MesenCult^{TM}, at the length of 0-300 µm.

Additionally, it is important to refer that secretome collected from iMSCs-hPL at early passage results in a significatively increased TH positive area when compared to secretome collected from iMSCs-hPL at late passage, for the lengths of 0-300 µm, 600-900 µm and 900-1200 µm (Figure 4.9C).

Secretome from iMSCs has been already demonstrated to have a positive effect on axonal growth (Alawdi et al., 2017). However, as it was shown with these results, the media in which

the iMSCs are expanded before harvesting the secretome has an impact on its capacity to promote axonal growth.

Our results from iMSCs expanded in different media revealed differences related to iMSCs properties. These reports also showed that in function of MSCs properties changing, their therapeutical applications can also change. Our evaluation of secretome from iMSCs cultured in different media resulted in 13 % of proteins being differentially expressed and, although initially these differences seemed minor it is now visible that, in fact, these differences lead to different outcomes in the capacity to promote axonal growth and DA differentiation. An increased expression of certain proteins detected in the proteomic analysis of secretome of iMSCs-hPL could be behind these different outcomes. For instance, an interplay between proteins that lead to neurogenesis, such as IGFBP6 and DCN, and proteins that are more connected to the DA lineage, such as COL6A3, could be creating a permissive environment for the differentiation into DA neurons to occur (Chen et al., 2020; Gregorio et al., 2022; Minor et al., 2008). Moreover, DCN has also been previously associated with a role in promoting axon growth across spinal cord injuries, which could imply that, in this experiment, its increased expression in iMSCs-hPL secretome could also be leading to axonal outgrowth (Minor et al., 2008).

Even the secretome collected from iMSCs expanded in hPL supplemented media, collected at different cell passages (early and late passage) resulted in visible differences not only in terms of neurite outgrowth (Figure 4.9A), but also in different capacities of inducing DA differentiation (Figure 4.9C). A previous report from our laboratory group, aiming at understanding the replicative senescence-associated alterations on BM-MSCs and iMSCs secretory profile, revealed that, in both cell sources, there is an increase in the secretion of characteristic SASP markers, such as IL-6, IL-8 and MCP-1, when MSCs are senescent, coupled to a reduced immunomodulatory capacity (Marote et al., 2023). Furthermore, another experimental study reported that, after long-term cultivation of adipose-derived MSCs (ASCs), SASP modifications lead to an attenuation of the angiogenic potential of their conditioned medium (Ratushnyy et al., 2020). Even though these studies pinpoint that SASP is associated with a negative effect on the therapeutical potential of secretome, there are also indications that SASP can have a beneficial effect in tissue regeneration (Demaria et al., 2014; Ritschka et al., 2017). In this project, results indicated a negative effect of replicative senescence-associated alterations in the application of iMSCs-hPL secretome for neurite outgrowth and DA differentiation. Taking into consideration that the referred above report from our group analysed

the secretory profile of senescent iMSCs expanded in hPL and that iMSCs expansion was performed in a similar manner in this project, it is possible that the SASP markers IL-6, IL-8 are also elevated in the secretome of senescent iMSCs used for this experiment. However, an elevated expression of IL-6 has been associated with positive roles in the recovery of DA neurons from toxin-induced neurodegeneration and from lesion (Hakkoum et al., 2007; Kummer et al., 2021; Spittau et al., 2012). Additionally, monocyte chemoattractant protein-1 (MCP-1) is a cytokine whose expression has already been associated with the neurodegenerative process present in various central nervous system diseases, including Alzheimer's disease, meaning that the decrease of expression of this protein should be beneficial for neuron growth (Fenoglio et al., 2004; Pae, 2014; Semple et al., 2010). Although the individual analysis of the function of each differentially expressed protein present in senescent iMSCs secretome does not explain why on this project its effects were detrimental to neurite outgrowth and DA differentiation, there could be an interplay of the different proteins that are altered that could be negatively affecting these processes. To confirm these hypothesis, further experiments would be necessary in order to address the secretory profile of senescent iMSCs, including when expanded in different media. Unfortunately, the evaluation of the impact of secretome from iMSCs-MesenCult^{TM} from late cell passage on neurite outgrowth and DA differentiation was not possible to perform and, thereby, it would be interesting to repeat this experiment in the future including that condition, to assess if the cell passage also influences the performance of secretome from iMSCs-MesenCult^{TM}.

Additionally, the further modulation of the conditions to which iMSCs are expanded in should be explored, since it could help targeting their secretory profile into the desired therapeutical applications.

Secretome from iMSCs-hPL and secretome from iMSCs-MesenCult^{TM} do not seem to have an impact on neuroprotection

The results from our proteomic analysis gave us some clues on the positive effects that secretome from iMSCs could have on the neuroprotection of a 3D model of PD. For instance, in both secretome from iMSCs-hPL and secretome from iMSCs-MesenCult^{TM} the expression of proteins with catalytic and antioxidant activity, such as Glutathione S-transferase P and Thioredoxin, was detected, which could indicate a potential capacity to provide neuroprotection to DA neurons after exposure to 6-OHDA (Sun et al., 2017). Multiple reports have already

described a neuroprotective role of Glutathione S-transferase against the oxidative stress seen in PD (Chauhan et al., 2019; Harish et al., 2010; Smeyne & Smeynen, 2013).

For this reason, we decided to test the effects of iMSCs' secretome on neuroprotection, using the 3D model of PD previously obtained. For this, at the end of the incubation with the toxin, cells were incubated with either: 1) Neurobasal medium; 2) supplemented ADNFK medium; 3) conditioned medium from early passage iMSCs-hPL; 4) conditioned medium from late passage iMSCs-hPL; and 5) conditioned medium from early passage iMSCs-MesenCult^{TM}. This incubation had the duration of 48 h. The results of an MTS assay, performed to evaluate cell viability in the different conditions, are exhibited in Figure 4.10.

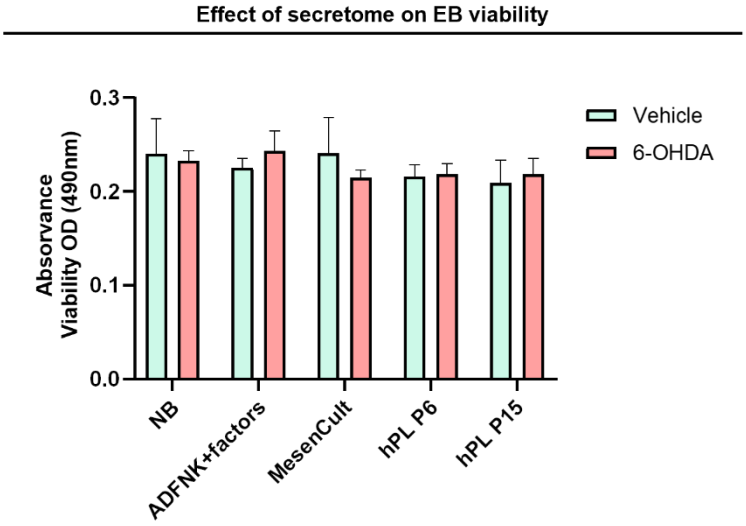

Figure 4.10 - Assessment of the effects of secretome from iMSCs expanded in different media and collected at different cell passages on neuroprotection of a 3D model on collagen. Effects of 6-OHDA exposure on cell viability of the different conditions, measured by the MTS assay. Data is presented as mean ± SEM. *n* = 6-8

Surprisingly, the results from this assay revealed no statistical differences between the different conditions tested, including when comparing the vehicle group kept in supplemented ADFNK medium with the 6-OHDA group kept in Neurobasal medium, which revealed visible and significant differences in the previous experiment regarding the development of the PD model. This inconsistency could be caused by the fact that the MTS assay is not a specific assay to measure the viability of DA neurons, instead measuring the global viability of the culture. Considering that 6-OHDA affects specifically DA neurons and not other cells, this assay is

possibly not the best choice for this purpose, revealing the necessity to find a more adequate assay to test if iMSCs secretome can rescue this degeneration in future studies.

It is important to note that a different impact on neuroprotection could be expected from the use of secretome from iMSCs-hPL and secretome from iMSCs-MesenCult^{TM}, since some of the proteins that are significatively increased in secretome from iMSCs-hPL consist of proteins that have been associated to a role in neuroprotection, as for example IGFBP6 and FSTL1 (Chen et al., 2020; Liang et al., 2014).

CHAPTER 5 – CONCLUSIONS AND FUTURE PERSPECTIVES

This project had the final objective of exploring the therapeutical potential of iMSCs secretome in the context of PD, by firstly developing an *in vitro* cellular model that could mimic this disease.

Results regarding the development and characterization of a model of dopaminergic neurons for the study of PD revealed that the 2D cell culture obtained did not provide a good platform for this goal, resulting in a low number of dopaminergic neurons that did not form a neuronal network. The use of collagen hydrogel as a scaffold enabled to get a 3D model with a robust neuronal network and successful dopaminergic differentiation, that, when challenged with 6-OHDA gave rise to a suitable model for the study of PD. The use of an alternative scaffold, the Ormocomp structure, also demonstrated the capacity develop a 3D model with dopaminergic differentiation, although less evident than in the 3D collagen model.

The long-term culture of iMSCs revealed that these cells' characteristics can be modulated through the use of different media, since their expansion in hPL supplemented medium resulted in a higher proliferative capacity and lower levels of replicative senescence, comparing to iMSCs expanded in MesenCult medium. These results suggest that the culture of iMSCs in hPL supplemented medium might be more advantageous when the goal is to obtain a considerate amount of secretome for preclinical and clinical applications, since this results in a higher amount of iMSCs obtained. Nonetheless, the secretory profile of iMSCs expanded in hPL supplemented medium was, in most part, similar to the one from iMSCs expanded in MesenCult medium, although a smaller portion of the proteins were differentially expressed.

The testing of secretome collected from iMSCs expanded in different conditions revealed that secretome from iMSCs in early cell passage expanded in hPL supplemented medium is capable of successfully promoting DA differentiation, with effects similar to the ones induced by the positive medium for DA differentiation. Thereby, once again, these results confirm that iMSCs can be modulated by the media they are expanded in, and, furthermore, by the cell passage from which secretome is collected, with the possibility to alter their therapeutical applications.

The neuroprotective effects of iMSCs secretome were also tested, however, results were not the expected, since the MTS results showed no statistical differences in the viability of the culture.

In order to have a more robust study surrounding the effects of iMSCs secretome on a 3D model of PD, in the closest future we should try to address some matters. Regarding the 3D model, a functional characterization of the 3D collagen model should be performed to assure the

presence of functional mature DA neurons, which could be done through electrophysiology studies, such as the whole-cell patch-clamp recording. Regarding the neuroprotection effects of iMSCs secretome, which remain to be explored, we should not only repeat the MTS assay performed, to make sure that there were not technical issues involved, but we should also perform other types of analysis, such as evaluating the production of ROS, which could provide a better insight on the neuroprotective effects of iMSCs secretome. After having a better understanding of the general effects of iMSCs secretome on dopaminergic neuroprotection, we could try to comprehend the molecular mechanisms of the effects of iMSCs secretome on dopaminergic differentiation and neuroprotection by, for example, blocking the receptors of certain factors present in the secretome, which would allow to understand if these factors are involved in the observed effects.

Overall, although further studies and repetition of some of the experiments performed in this project are needed, this work showed that iMSCs can be modulated in order to increase the number of cells obtained and even to potentiate some of their therapeutical applications such as their DA differentiation capacity. Thus, it is suggested that the use of iMSCs secretome could be in the future a promising therapeutical strategy in the context of PD.

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