Utjecaj uvjeta uzgoja i pojačane ekspresije odabranih gena na diferencijaciju ljudskih dopaminergičnih neurona

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Petra Terešak

Influence of cell culture conditions and enhanced expression of genes of interest in optimization of dopaminergic neuron generation

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Ovaj rad izrađen je na institutu Centro de Biología Molecular Severo Ochoa (CBMSO) u Madridu u Laboratoriju za molekularnu neuropatologiju pod vodstvom Dr. sc. Alberta Martinez- Serrana, prof. i Dr. sc. Marta Pereira. Rad je predan na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja magistre molekularne biologije.

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Utjecaj uvjeta uzgoja i pojačane ekspresije odabranih gena na diferencijaciju ljudskih dopaminergičnih neurona

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Parkinsonova bolest (PB) je neurološki poremećaj koji je karakteriziran gubitkom mezencefaličkih dopaminergičkih (DA) neurona. Proizvodnja ovog tipa stanica omogućit će novi terapijski pristup koji trenutno nedostaje. Naime, trenutni farmakološki i kirurški tretmani ublažavaju simptome Parkinsonove bolesti, ali niti jedna terapija ne može spriječiti ili zaustaviti progresivni patološki učinak. Živčane matične stanice izolirane iz ventralnog mezencefaličkog ljudskog tkiva predstavljaju snažan istraživački alat i kandidati su za terapiju transplantacije stanica u PD. Istraživačka grupa dr. Martinez-Serrana razvila je besmrtnu staničnu liniju hNSCs uvođenjem gena *v-myc* ptičjeg retrovirusa u stanice. Ove stanične linije sadrže sva izvorna svojstva neuralnih matičnih stanica koje su u mogućnosti proliferirati u DAn. Ranije je pokazano da se prisiljenom ekspresijom gena Bcl-XL povećava razina dopaminergičnih neurona, no anti-apoptotička priroda Bcl-XL čini ga protoonkogenom pa se ne može koristiti u kliničke svrhe. Stoga je potrebno pronaći sigurnije gene sličnog učinka. Testirani geni od interesa su CDKN1C, GADD45G, GFRA1, INSM1 i NHLH1. Imunocitokemijska analiza pokazala je da geni GADD45G, GFRA1, INSM1 induciraju ekspresiju DA biljega, tirozin hidroksilaze i predstavljaju obećavajuću zamjenu za Bcl-xL. Dodatci za medije, B-27, N-2 i SCM005, testirani su kako bi se optimiziralo kultiviranje hNSC-a. Nije bilo značajnih razlika u učinkovitosti navedenih medijskih dodataka.

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Influence of cell culture conditions and enhanced expression of genes of interest in optimization of dopaminergic neuron generation

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Parkinson's disease (PD) is a neurological disorder characterized by the loss of midbrain dopaminergic (DA) neurons. The generation of this cell type will fulfill a currently unmet therapeutic need. Current pharmacological and surgical treatments alleviate parkinsonian symptoms, but none can prevent or stop the progressive pathology effect. Human neural stem cells derived from the ventral mesencephalon are powerful research tools and candidates for cell therapies in PD. The Dr. Martinez-Serrano research group were able to develop an immortalized cell line of hNSCs by introducing the avian retroviral v-myc gene into the cells. The cell lines provide all the genuine properties of NSC that act as precursors of human DAn. Previous studies proved that a forced expression of Bcl-xL enhance the generation of dopaminergic neurons. The anti-apoptotic nature of Bcl-xL makes it a protooncogene that cannot be used for clinical applications. Therefore it is necessary to find an alternative and safer genes which can work in a similar way. The selected genes of interest that were tested were CDKN1C, GADD45G, GFRA1, INSM1 and NHLH1. Immunocytochemical analysis demonstrated that GADD45G, GFRA1, INSM1 genes induced the expression of the DA marker, tyrosine hydroxylase, and could be good substitutes of Bcl-xL. Media supplements, B-27, N-2 and SCM005, were tested to optimize hNSC culturing. No substantial difference was recorded between media supplements.

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Abbreviations

PD, Parkinson disease; 6-OH-DA, 6-hydroxydopamine; DA, dopamine; DAn, dopaminergic neuron(s); GOI, Gene of interest; CRT, Cell Replacement Therapy; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; MAO, Monoamine oxidase; COMT, Catechol-O methyltransferase; ESC, Embryonic Stem Cells; iPSC, induced Pluripotent Stem Cells; iN, induced Neuronal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hNSC, human neural stem cell; NSC, neural stem cell; DAT, dopamine transporter; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VM, ventral mesencephalon; PBS, phosphate-buffered saline; GABA, γ -aminobutyric acid; MSNs, medium spiny projection neurons ; GFAP, glial fibrillary acidic protein; GADD45G, growth arrest and DNA-damage-inducible 45 gamma; ST-18, Suppression of tumorigenicity 18; NHLH1, Nescient helix loop helix; ELAVL3, ELAV like neuron-specific RNA binding protein 3; INSM1, insulinoma-associated 1; GFRA1, GDNF family receptor alpha 1; CDKN1C, cyclin-dependent kinase inhibitor 1C; ICC, immunocytochemistry; GFP, Green Fluorescent Protein

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1. Introduction

1.1. Parkinson's disease and current therapies

Parkinson's disease is a chronic neurodegenerative age-associated disorder (de Rijk et al., 2000). It was first medically described as a neurological syndrome in 1817 in a detailed medical essay entitled "An Essay on the Shaking Palsy" published by London doctor James Parkinson to whom it owes its name (Goetz, 2011). There are six cardinal symptoms of Parkinson's disease, all related to motor behavior: tremor at rest, rigidity (increased resistance to passive movement of a patient's limbs), akinesia (absence of normal unconscious movements), bradykinesia (slowness of movement), hypokinesia (reduction in movement amplitude), postural instability. Commonly cognitive symptoms are also present, e.g. dementia, depression (Jankovic, 2008). The lifetime risk of developing Parkinson's disease is relatively high at 1.5%, however, though life-expectancy is reduced compared to healthy subjects, it's not considered a fatal disease (Ishihara et al., 2007). As is commonly known, the main neuropathological features of Parkinson's disease are twofold: motor-symptoms arise from the progressive loss of dopaminergic neurons of the ventral midbrain structure substantia nigra pars compacta (SNpc) which may correlate with the increased susceptibility to oxidative stress, and evolving processes of development of Lewy bodies and neurites, corresponding with the protein misfolding, polymerization, and abnormal proteostasis at the cellular level (Jankovic 2008).

Although more than a century passed before the central pathological feature of Parkinson's disease (PD) was found to be the loss of neurons in the substantia nigra pars compacta (SNpc), the pace of discovery accelerated following Arvid Carlsson's 1958 discovery of dopamine (DA) in the mammalian brain. SNpc neurons were then found to form the nigrostriatal dopaminergic pathway, and this line of research culminated with key discoveries. One of them being that the loss of SNpc neurons leads to striatal DA deficiency, which is responsible for the major symptoms of PD (Dauer and Przedborski, 2003; Kandel et al., 2003; Smith et al. 2004). The dopaminergic neurons in the midbrain are located in three cell groups: nucleus A8 cells in the retrorubral field,

nucleus A9 cells in the substantia nigra, and nucleus A10 cells in the ventral tegmental area and related nuclei (Berger et al, 1991; Porrino and Goldman-Rakic 1982; Williams and Goldman-Rakic, 1998). Parkinson's disease involves the chronic and selective depletion of mesencephalic dopaminergic neurons (DAns) of the A9 subgroup localized in the SNpc, projecting to the striatum (Mendez et al, 2005; Martinez-Serrano et al., 2011; Thompson et al., 2005).

Under normal conditions, dopaminergic neurons of the SNpc project to the striatum, where terminally released dopamine (DA) activates excitatory D_1 as well as inhibitory D_2 metabotropic dopamine receptors on subsets of striatal γ-Amino Butyric Acid (GABA) signaling medium spiny projection neurons (MSNs) (Onn et al., 1994.). As the MSNs expressing D_1 and D_2 receptors project to different primary targets within the basal ganglia, the DA depletion causes hyper activity in the D_2-regulated pathway through lack of inhibition, and hypo activity in the D_1-regulated pathway through lack of activation (Bolam et al., 2000). The consequential signaling imbalance is manifested throughout the basal ganglia and results in a reduced overall input to the motor cortex, causing the before mentioned motor symptoms. Environmental factors are considered to increase the risk of developing the pathogenesis of PD. They range from behavioral features, lifestyle (coffee and cigarette consumption), chronic exposure to pesticides and metals, to the hormonal status, ethnicity, gender and age (Litvan et al., 2007). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and its hydroxylated derivatives, a particular nigrostriatal neurotoxin, has provided powerful evidence to support the theory of environmental risks, which develops a relatively selective destruction of DAn in the substantia nigra and causes similar symptoms with PD in both animals and humans. In the last decades, new discoveries in the neurogenetics of PD have suggested that genetic factors may play equally important roles in the ethiology of PD. Several studies have demonstrated that major familial PD results from mutations in certain single genes and monogenic forms, including autosomal dominant and recessive disorders, which may account for around 5–10% of all PD cases (the rest being of unknown origin, the so called idiopathic PD). The G2019s mutation in the Leucine-Rich Repeat Kinase (LRRK2/PARK8 locus) and mutations in the alpha-synuclein (SNCA/PARK1 locus) gene, which generate intraneuronal inclusions containing α-synuclein protein known as Lewy bodies and carry progressive degeneration of the neuron in the substantia nigra and then trigger clinical

motor symptom, are two of the most common autosomal dominant missense genes. Lewy bodies are abnormal aggregates of protein that develop inside nerve cells. They are identified under the microscope when histology is performed on the brain. They appear as spherical masses that displace other cell components. There are two morphological types: classical (brain stem) Lewy bodies and cortical Lewy bodies. A classical Lewy body is an eosinophilic cytoplasmic inclusion that consists of a dense core surrounded by a halo of 10-nm wide radiating fibrils, the primary structural component of which is alpha-synuclein. In contrast, a cortical Lewy body is less well-defined and lacks the halo. Nonetheless, it is still made up of alpha-synuclein fibrils. A Lewy body is composed of the protein alpha-synuclein associated with other proteins such as ubiquitin, neurofilament protein, and alpha B crystallin. It is believed that Lewy bodies represent an aggresomal response in the cell (Popescu, et al., 2004; Ishizawa et al., 2003).Other autosomal dominant mutations, including PAPK3, PAPK13, UCH-L1, among others, are relatively rare. Autosomal recessive mutations in genes PTEN-induced putative kinase 1 (PINK1), Parkin, DJ-1 (Daisuke-Junko-1), ATP13A2, PLA2G6, FBXO7, DNAJC6, and SYNJ1 usually cause early onset and lesser clinical manifestation. For example, PD patients with a Parkin/PAPK2 mutation generally present with early-onset Parkinsonism, slow disease progression, and a better response to L-DOPA. In addition, the majority of PD cases are sporadic (over 90%), known as idiopathic PD, possibly caused by a complex interaction among genetically susceptibility variants and environmental factors, which some geneticists and epidemiologists have explored as the G × E model in the pathogenesis of sporadic PD. A large proportion of these sporadic forms have been identified in the monogenic form linked to familial PD. In other words, those genes responsible for monogenic aberrations are also susceptibility factors, such as polymorphism variants in Mendelian genes, such as α-synuclein (SNCA), LRRK2, Parkin, PINK1, and DJ-1, and heterozygous mutations in susceptibility genes, such as microtubule-associated protein tau and glucocerebrosidase beta acid (GBA), identified by genome-wide association studies (GWAS) (Klein et al., 2006).

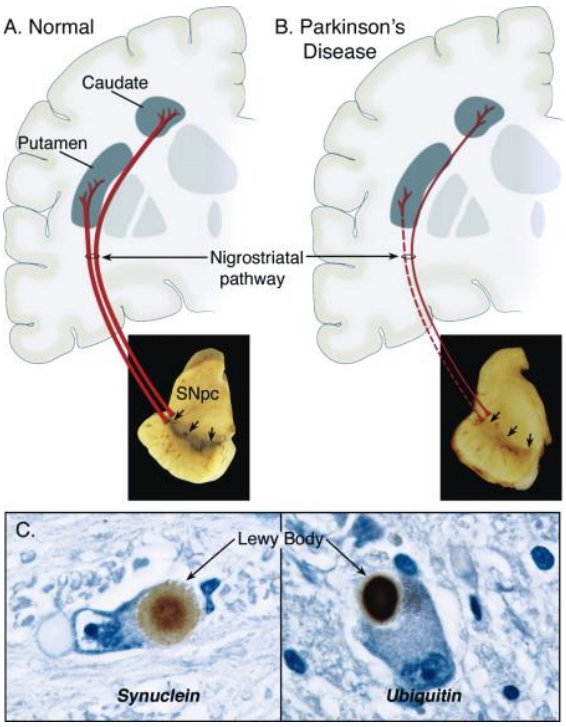


Figure 1. Neuropathology of Parkinson's Disease

(A)The schematic shows a normal nigrostriatal pathway (in red), while the photograph demonstrates the normal pigmentation of the substantia nigra pars compacta (SNpc; see arrows), produced by neuromelanin within the dopaminergic neurons. The nigrostriatal pathway consists of dopaminergic neurons whose cell bodies are located in the SNpc. These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum. (B) The illustration shows the diseased nigrostriatal pathway (in red), and the photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; arrows) of the SNpc due to the marked loss of dopaminergic neurons. In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). (C) Immunohistochemical images of Lewy bodies, in a SNpc dopaminergic neuron. Immunostaining with an antibody against α-synuclein reveals a Lewy body with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (left photograph). Conversely, immunostaining with an antibody against ubiquitin yeilds more diffuse immunoreactivity within the Lewy body (right photograph). (Dauer and Przedborski, 2003)

In PD, the progressive depletion of dopaminergic neurons results in deficient levels of dopamine, an endogenous neurotransmitter belonging to the catecholamine family. Its synthesis starts from L-Phenylalanine an amino acid which is converted into L-tyrosine by the enzyme phenylalanine hydroxylase, with molecular oxygen (O2) and tetrahydrobiopterin as cofactors. L-Tyrosine is converted into L-DOPA by the enzyme tyrosine hydroxylase (TH), with tetrahydrobiopterin, O2, and iron (Fe2+) as cofactors. This step is considered to be the limiting one and thus, TH is used as a marker for dopaminergic neurons (DAns). The L-DOPA is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase (also known as DOPA decarboxylase), with pyridoxal phosphate as the cofactor (Musacchio, 2013). After synthesis, dopamine is transported from the cytosol into synaptic vesicles by a solute carrier—a vesicular monoamine transporter, VMAT2 (Eiden et al., 2004).Dopamine is stored in these vesicles until it is released into the synaptic cleft. In most cases, the release of dopamine occurs through a process called exocytosis which is caused by action potentials, but it can also be caused by the activity of an intracellular trace amine-associated receptor, TAAR1 (Grandy et al., 2016). This mechanism is necessary in order to prevent dopamine from being metabolized by Monoamine oxidase (MAO) or by Catechol-O-methyltransferase (COMT, before having fulfilled its task as a neurotransmitter (Daubner et al., 2011; Meiser et al., 2013).

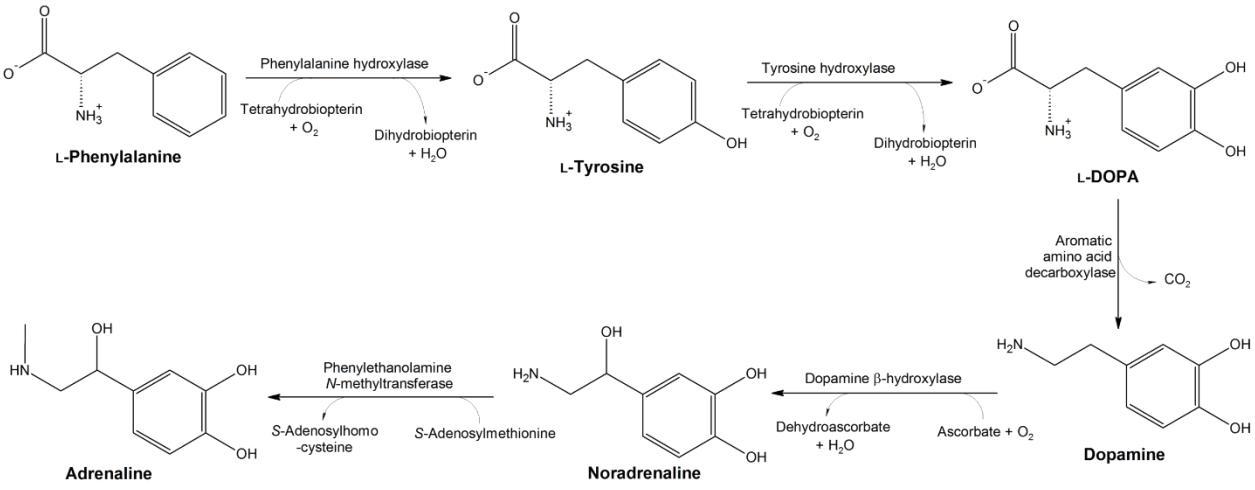


Figure 2. Conversion of phenylalanine and tyrosine to its biologically important derivatives. (https://en.wikipedia.org/wiki/Tyrosine)

Current clinical therapies for Parkinson's disease offer strictly symptomatic relief, aiming at maintaining motor functionality of patients as dopaminergic neurodegeneration progresses. From the late 1960's up until today the dopamine precursor L-3,4-dihydroxy-phenyl-alanine, levodopa, remains the most potent drug for controlling PD symptoms. Levodopa is metabolized and released by surviving dopaminergic neurons, thereby increasing the quantitative release per cell, compensating for the degenerated dopaminergic neurons (Melamed et al., 1982.) The addition of carbidopa, a peripheral dopa decarboxylase inhibitor, enhances the therapeutic benefits of levodopa. In patients who are particularly sensitive to peripheral side effects such as nausea and vomiting, additional carbidopa (Lodosyn®) may be added to the conventional carbidopa/levodopa preparation. A majority of patients treated with levodopa experience motor fluctuations, dyskinesias or other complications after 5 years of treatment (Jankovic, 2005). Since motor fluctuations and dyskinesias are primarily related to the dose and duration of levodopa treatment (Schrag and Quinn, 2000), most parkinsologists advocate therapeutic strategies designed to delay the onset of levodopa therapy in order to delay the onset of levodopa-related motor complications (Jankovic 2002). There are three strategies designed to improve levodopa-induced dyskinesias: 1) reduce the dosage of levodopa, 2) use drugs known to ameliorate dyskinesias, and 3) surgery. Several drugs, including amantadine, have been reported to improve levodopa-induced dyskinesias without necessitating the reduction in levodopa dosage (Verhagen Metman et al., 1999). The addition of a COMT inhibitor, MAO-I inhibitor or a dopamine agonist inhibitor may be used in the management of levodopa-induced motor complications (Jankovic et al., 2007). Other drugs with antidyskinetic effect include clozapine, fluoxetine, propranolol, the cannabinoid receptor agonist nabilone, and fipamezole. Some of the new antiepileptic drugs are being investigated as potential therapies for levodopa-induced dyskinesias. For example, levetiracetam (Keppra®) was found to significantly reduce levodopa-induced dyskinesias in MPTP-lesioned marmosets (Hill et al., 2003). In patients with severe motor fluctuations, subcutaneous apomorphine, a dopamine agonist, may be used as rescue therapy (Pietz et al., 1998).

In the adult brain, two areas are recognized to harbor populations of dividing neural stem cells, giving rise to new neurons throughout life. One area is the sub-granular zone of the dentate gyrus of the hippocampus, where newly formed neurons migrate only shortly and integrate in the immediately overlaying granule cell layer (Alvarez-Buylla and Lim, 2004). The other region is the sub-ventricular zone of the striatum, where newly formed neurons migrate to integrate in the olfactory bulb (Merkle et al., 2004). Despite this ability of the adult brain to produce new neurons throughout life, the potential of the brain to repair itself is highly limited (Thored et al., 2006). This means that once neurons are lost in other regions than the olfactory bulb or the granule cell layer, they're generally not replaced. It is the aim of the neural stem cell field to design and produce stem cells in the laboratory, which can be transplanted into the brain to replace or support compromised neurons in disease states (Lindvall et al., 2004). By introducing genes of interest in stem cells prior to transplantation, the therapeutic potential of the cells can be increased, either by enhancing existing properties of the cells, or by introducing new ones.

1.2. PD animal models

To date, the animal model of PD has progressively matured, and includes the 6-hydroxydopamine (6-OHDA) stereotaxic injection model and the 1-methyl-4-phenyl-1,2,5,6- tetrahydropyridine (MPTP) intraperitoneal injection model, providing gross specimen models to mimic DAn loss in SNpc and striatal dopamine depletion. However, these gross experimental PD models cannot recapitulate the intrinsic neuropathological features of PD, since they do not replicate the special vulnerability to neurodegeneration of DAn and the natural process of Lewy bodies and Lewy neurites (Dauer and Przedborski , 2003).

Currently, the most common model of PD replicated in the animal adult brain involves the intraperitoneal injection of MPTP and the stereotactic injection of 6-OHDA. The administration of these neurotoxins could induce specific damage of DAn to study pathophysiology, clinical features, and pathogenesis of PD (Langston et al., 1983; Rodriguez et al., 2001). For MPTP, its metabolic product 1-methyl-4-phenylpyridinium ion (MPP+) in vivo, which can enter DAn through the dopamine transporter (DAT) and then block mitochondrial complex I activity, deplete

intracellular ATP, and enhance oxidative stress, induces the specific neurotoxicity of DAn in SNpc (Nicklas et al., 1985). For 6-OHDA, which can contribute to the accumulation of ROS in the mitochondria and the inhibition of the mitochondrial respiratory enzymes, this leads to gradual neuron death and selective degeneration of DAn.

1.3. Neural stem cells

A stem cell is a non-specialized cell with the capacity to give rise to more stem cells, self-renewal, for an extensive period of time and produce progeny that in the end will terminally differentiate into major cell types of tissue of origin, a property known as multipotency (Seaberg and van der Kooy, 2003). Stem cells can be arranged in a hierarchy depending on their self-renewal and differentiation capacity. Totipotent stem cells can give rise to all cells that will make up an embryo, for example the fertilized egg. Pluripotent stem cells can be found in the inner cell mass of the blastocyst and can generate all cells of the three germ cell layers which will give rise to all tissues in the body. Stem cells obtained from this source are called embryonic stem (ES) cells. Multipotent stem cells can give rise to all mayor mature cell types in the tissue from which they were originally obtained (Gage, 2000). Stem cells divide, symmetrically, producing daughter cells that either are two identical stem cells or two progenitor cells, or asymmetrically, producing one stem cell and one progenitor cell. The progenitor cell is frequently defined to be more lineage restricted, and can be bipotent or even unipotent. It has less self-renewal capacity than the stem cell. The progenitor cells will eventually generate more specialized cells that are committed towards a particular lineage, and, in the case for neural progenitor cells, eventually give rise to neurons and glia. Precursor cells is a term collectively used for both stem and progenitor cells and defines unspecifically a cell earlier in development than the progeny it gives rise to (McKay, 1997).

Neural stem cells (NCSs) have attracted major research and public interest during recent years. One explanation is the potential use of NSCs in treating or reducing the impairment for patients suffering from various neurodegenerative diseases such as Parkinson's disease, Huntington's

disease, Alzheimer's disease and stroke (Lindvall and Kokaia, 2006). NCSs generally refer to stem cells derived from the central nervous system (CNS) or from the inner cell mass of the blastocyst, which maintain the capacity for self-renewal, and can generate neurons, astrocytes and oligodendrocytes (Temple, 2001). There are three main sources of human NSCs for in vitro culture expansion. Pluripotent ES cells derived from the inner cell mass of the blastocyst and multipotent somatic stem cells that can be generated from either the developing fetal or mature adult CNS.

Cellular plasticity is a major focus of investigation in developmental biology. The major breakthrough came in 2006 when Takahashi and Yamanaka introduced the concept of induced pluripotent stem cells (iPSCs) by generating stem cells with qualities remarkably similar to embryonic stem cells. iPSCs were generated by using a combination of 4 reprogramming factors, including Oct4 (Octamer binding transcription factor-4), Sox2 (Sex determining region Y)-box 2, Klf4 (Kruppel Like Factor-4), and c-Myc and were demonstrated both self-renewing and differentiating like ESCs, and thus, could be used as an alternative for hESCs in various clinics/research (Singh et al., 2015).

The recent discovery that induced neuronal (iN) cells can be generated from mouse and human fibroblasts by expression of defined transcription factors suggested that cell fate plasticity is much wider than previously anticipated, also indicating that direct lineage conversions are possible between very distantly related cell types (Vierbuchen and Wernig, 2011). Importantly, iN cells can also be derived from defined endodermal cells. The reprogramming process both induces neuronal properties and extinguishes prior donor cell identity, and therefore represents a complete and functional lineage switch as opposed to generation of a chimeric phenotype. The examples of direct reprogramming may very well become important tools for both basic biology and regenerative medicine (Gaspard et al., 2009). It remains to be seen whether in direct somatic lineage conversions the reprogramming is truly complete, or whether any epigenetic memory of the previous cell fate remains, as has been seen with nuclear transfer (Ng and Gurdon, 2005).and induced pluripotent stem cells (Kim et al., 2010) .

1.4. Cell replacement therapy

Experimental therapies, based on the cell replacement of the loss of substantia nigra pars compacta (SNpc) DAn using neural stem cells (NSCs) from fetal brain tissues, human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs) and directly induced dopamine neurons (iDA neurons) have been touted as the future of regenerative medicine, with a heavy burden of promise and expectation placed upon them to deliver an unprecedented number of cell-based therapies (Han et al., 2015). According to the studies carried out, each potential source of dopaminergic neurons has to comply with some essential characteristics for a correct cell replacement therapy (Martinez- Serrano et al., 2011). A number of crucial issues that need to be addressed in preclinical studies before these cells can be considered for clinical use are; firstly, it is important to verify that their functional efficacy is robust, reproducible, and stable over significant time periods; secondly, that the transplanted cells have the capacity to grow axons and reinnervate the DA-denervated host striatum over distances that are relevant for the size of the human brain; and finally, that they function with equal potency to human fetal VM DA neurons that have previously been used in clinical trials (Barker, 2014).

There are two limitations of cell transplantation therapy. The first is that traditional reprogrammed dopamine neurons (i.e. from iPSCs, or as iNs) are usually achieved by transfecting the transcription factors, which result in transgene integration, spontaneous insertional mutagenesis, (Datta et al., 1995) and resultant tumorigenesis (Hu K., 2014). The second is that the transplanted patient-derived dopamine neurons may carry transcriptomic or epigenetic parental disease-causing memory, and the graft cells will show this tendency to the original disease phenotypes. New advances in stem-cell technology herald the dawn of solutions for these problems.

Initially, the use of human fetal ventral mesencephalic (VM) tissue provided proof of principle of a therapeutic effect of the transplants on a long term basis (Mendez et al., 2008). However, in addition to ethical problems related to fetal tissue procurement, practical limitations were found, like the need for large amounts of VM tissue, including immunological rejection, limited cell sources, verified complications, and the elevated cell death rate of the transplanted cells. There

are therefore many reasons to explore better cell sources (Martinez-Morales and Liste, 2012). Another stem cell source, the human embryonic stem cells (hESCs), required long and difficult differentiation protocols as well as neuronal and DAn progenitor selection to obtain high amounts of DAn, also the problems were: the phenotypic stability of hESC-derived dopamine neurons after transplantation, and the worry about residual undifferentiated hESCs within the large numbers of cells that need to be injected for human therapy (Lindvall et al., 2009). The residual undifferentiated hESCs might lead to tumor formation even if this is not observed anymore in rodent experiments. In addition, some ethical concerns and problems of immune rejection also limited the clinical applications of hESCs. As an alternative, induced pluripotent stem cells (iPSCs), derived in the Yamanaka lab using the human orthologs of four transcription factors (OCT4, SOX2, c-MYC, KLF4), were tested. Several aspects of iPSCs need to be resolved before they go to clinical use. These include low yields of DA neurons, genetic and epigenetic abnormalities, and the safety of iPSC-derived cells (Kim K et al. 2010; Lister R et al. 2011; Han et al., 2015). Also, human neural stem cells (hNSCs) derived from the developing and adult central nervous system were used, but they were inefficient for DAn generation, since the cultures proliferated poorly and rapidly entered senescence producing some DAn at early passages but mostly glia at later ones (Christophersen et al., 2006; Martinez- Serrano et al., 2011). Previous transplantation studies established that the generation of functional SNpc DAn *in vivo* was highly dependent on the regional tissue origin, the VM being the optimal region (Kim et al., 2007), and that only DAn with SNpc properties (meaning adequate patterning, transcription factor, and differentiated protein profile) were able to reinervate the striatum and induce a therapeutic effect (Thompson et al., 2005).

Much effort has been put into identifying human and mouse precursors of VM DAn and into understanding the genetic cues and cascades controlling their development. These understandings improved the capacity to generate and engineer correctly differentiated human A9-Dan in vitro from their stem cells (Ungerstedt et al., 1974). The key discovery that nigral dopaminergic neurons were in fact uniquely of floor plate origin, allowed for effective differentiation protocols to be developed, heralding the generation of genuine A9 dopaminergic nigral neurons that expressed floor plate markers. This recognition of the floor plate origin of

mesencephalic dopaminergic neurons was a crucial step toward the development of dopamine-producing neurons that could potentially be used in clinical trials (Lindvall et al., 2009). With this new insight, the differentiation process was refined, and dopamine-producing neurons could now be generated from both human ES cells and iPSCs with high efficiency, and increased graft survival and functionality compared with the neuroepithelial-patterned cells that had originally been described. The derived dopaminergic neurons conveyed comparable function to fetal dopaminergic neurons, and the potential for tumorigenesis also seemed to have been circumvented, meaning stem cell-derived neurons had become the leading candidates for future clinical trials (Barker, 2014).

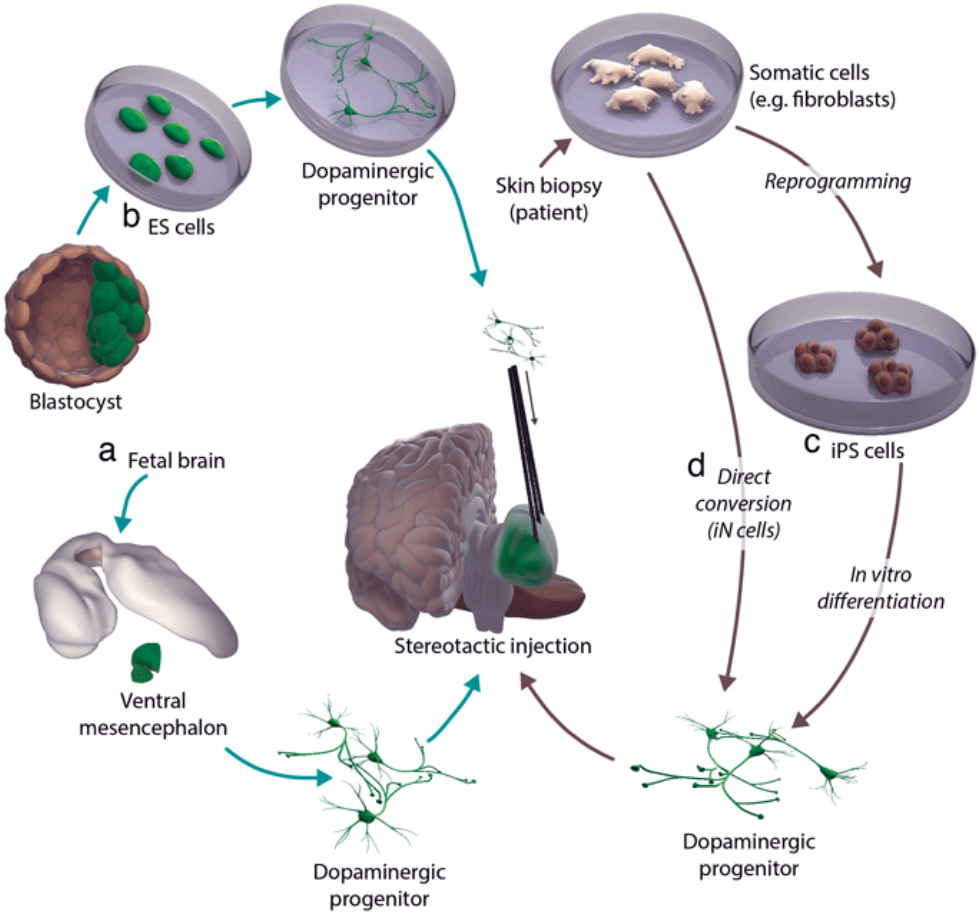


Figure 3. Different cell sources currently being developed for clinical use.
(http://www.acnr.co.uk/2014/09/cell-therapies-for-parkinsons-disease/)

The doctor Martinez- Serrano laboratory dedicated it's work to this topic and produced a generation of a stable cell line of human neural stem cells derived from ventral mesencephalon (hVM1) based on v-myc immortalization. The avian retroviral v-myc oncogene is a viral homolog of c-myc transduced by several acute transforming retroviruses, many of which encode this gene as a Gag-Myc fusion protein (Lee and Reddy, 1999). The hVM1 cells express neural stem cell and radial glia markers like nestin, vimentin and 3CB2 under proliferation conditions. After withdrawal of growth factors, proliferation and expression of v-myc were dramatically reduced and the cells differentiated into astrocytes, oligodendrocytes and neurons. hVM1 cells yield a high number of dopaminergic neurons (about 11% of total cells are TH+) after differentiation, which also produce dopamine (Martinez-Serrano et al., 2016). In addition to proneural genes (NGN2, MASH1), differentiated cells show expression of several genuine mesencephalic dopaminergic markers such as: LMX1A, LMX1B, GIRK2, ADH2, NURR1, PITX3, VMAT2 and DAT, indicating that they retain their regional identity (Tønnesen, 2010). Also the cells are functional in terms of calcium handling, electrophysiology, and DA synthesis and release (Martinez- Serrano et al., 2011).

Also, the ReNeuron Group plc, a biotech company that specializes in using human somatic stem cells for therapeutics developed a similar cell line, and named it ReNcell VM. ReNcell VM is an immortalized human neural progenitor cell line with the ability to readily differentiate into neurons and glial cells. ReNcell VM was derived from the ventral mesencephalon region of human fetal brain. Immortalized by retroviral transduction with the v-myc oncogene, this cell line grows rapidly as a monolayer on laminin with a doubling time of 20-30 hours (Donato et al., 2007). Karyotype analyses indicate that the ReNcell VM retains a normal diploid karyotype in culture even after prolonged passage (>45 passages). In experiments performed by the ReNeuron Group plc, ReNcell VM can be differentiated in vitro to a high level of human dopaminergic neurons. Neurons differentiated from ReNcell VM have furthermore been shown to be electophysiologically active. ReNcell VM may be used for a variety of research applications such as studies of neurotoxicity, neurogenesis, electrophysiology, neurotransmitter and receptor functions (Marchetto et al., 2009).

As already noted, hNSCs of VM origin generally show a poor capacity to generate DAn, and consistently loose this potential with passaging. This phenomenon also affects the immortalized VM NSCs (including hVM1 and ReNcell cell lines) as well as the primary ones. There have been various attempts to increase the neurogenic potential o NSCs, mostly through the expression of developmental transcription factors (Martinez- Serrano et al., 2011). The Bcl-XL gene was found to be the most successful with that objective. Bcl-XL (basal cell lymphoma-extra large) belongs to the Bcl-2 (B-cell lymphoma 2) protein family, playing an important antiapoptotic role in mammals (Boise et al., 1993), particularly during central nervous system development (González-García et al., 1995), but also modulating neuronal differentiation (Shim et al., 2004; Ko et al., 2009). The results of a research demonstrate that Bcl-XL enhances the maintenance of the neuronal and dopaminergic competence in long term expanded cultures and protects the cells from apoptotic cell death during differentiation. Bcl-XL modulates fate decisions, increasing neuronal and dopaminergic differentiation by a dose-dependent mechanism, in parallel with a decrease in glial cell generation (Liste et al., 2007). Also, the hVM1-derived cell lines survive transplantation in a rat model of PD, differentiating into neurons and glia and generating mature DAn, and that Bcl-XL enhances functional recovery of Parkinsonian rats. In spite of its relevant contribution to the phenotypic stability of the cells, the overexpression of Bcl-XL represents a problem for the translation to the clinic being a proto-oncogene. In addition to classical anti-apoptotic actions, it is known that Bcl-XL acts directly on neuronal differentiation through non-canonical pathways (Garcia-Garcia et al., 2012). For this reason it is important to determine the mediators of the neurogenic effect of Bcl-XL, with the ultimate goal of finding a substitute gene capable of promoting neuronal differentiation, but without being associated with any component that would endanger the biosecurity of the therapy. With studies of microarray analysis (Seiz, 2010) some potential Bcl-XL effectors were identified (namely; INSM1, NHLH1, GADD45G, GFRA1, ELAVL3, ST18, CDKN1C) that were previously not linked to dopaminergic neurogenesis. Their expression in hVM1 differentiated cells decreases with time in culture, but when Bcl-XL is overexpressed, this decrease is prevented (Seiza et al., 2012). Thus, the forced overexpression of Bcl-XL prevents the decay of the expression of a certain set of genes and at the same time blocks the decline of dopaminergic neurogenesis. Therefore those genes may be implicated in

neurogenesis and for that reason were included in a selection of genes of interest (GOIs) that were determined as potential effectors of Bcl-XL. Special attention was given to the following genes:

GADD45G (Growth arrest and DNA-damage-inducible protein gamma; Beadling et al., 1993; _Zhang et al., 1999). It can interact with different proteins in the processes of control of the cell cycle, apoptosis, senescence in response to cell stress, in tumor suppression and recently it was observed that it also has an effect on the development of the nervous system (Kaufmann et al., 2011; McLean et al., 2011; Sultan and Sweatt, 2013).

INSM1 (Insulinoma-associated 1), zinc-finger transcriptional factor, able to bind the DNA. It plays a key role in neurogenesis and in the differentiation of neuroendocrine cells during the embryonic and fetal development (Lan and Breslin, 2009). Its overexpression together with NHLH1 was identified as new target in the Hedgehog signaling pathway of medulloblastoma (De Smaele et al., 2008).

CDKN1C (Cyclin-Dependent Kinase Inhibitor 1C (P57, Kip2)) is a tumor suppressor human gene on chromosome 11 (11p15) and belongs to the cip/kip gene family. The encoded protein is a tight-binding, strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation. Thus p57KIP2 causes arrest of the cell cycle in G1 phase. Here we show that p57Kip2 is expressed in postmitotic differentiating midbrain dopamine cells. Induction of p57Kip2 expression depends on Nurr1, an orphan nuclear receptor that is essential for dopamine neuron development. Moreover, analyses of p57Kip2 gene-targeted mice revealed that p57Kip2 is required for the maturation of midbrain dopamine neuronal cells (Bertrand et al., 2003).

The **GFRA1** gene encodes the GDNF family receptor alpha-1 (GFRα1), also known as the GDNF receptor. Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are two structurally related, potent neurotrophic factors that play key roles in the control of neuron survival and differentiation. This receptor is a glycosylphosphatidylinositol (GPI)-linked cell surface receptor for both GDNF and NTN, and mediates activation of the RET tyrosine kinase receptor (Gorodinsky et al. 1998; Angrist et al. 1998).

The **NHLH1** gene encodes a helix-loop-helix protein 1 in humans. The helix-loop-helix (HLH) proteins are a family of putative transcription factors, some of which have been shown to play an important role in growth and development of a wide variety of tissues and species (Begley et al. 1992). Basic-helix-loop-helix transcription factors regulate neurogenesis and neuronal differentiation by as yet unknown mechanisms. It is also known that embryonic neuronal-specific basic-helix-loop-helix protein, NHLH, interacts with 'LIM only' proteins (Bao et al. 2000), controlling the transition from proliferation to differentiation of progenitor cells (De Smaele et al., 2008).

2. Aim of the study

The basic idea is that the increase in the neurogenic potential doesn't occur only because of Bcl-XL, but also thanks to the action of series of genes which lay downstream, whose expression is enhanced by Bcl-XL. By managing to detect these downstream genes, it is possible to work on them avoiding the use of Bcl-XL. For these reasons, the Dr. Martinez Serrano research group has carried a number of experiments through massive analysis of the gene expression by using microarray of DNA and comparing hVM1 and Bcl-XL-hVM1 low and high passage cell lines (Courtois, E. T. et al. 2010) which were later validated through qPCR. At the end, according to the results of the research, a small group of genes was selected which are linked to dopaminergic neurogenesis.

The purpose of the present work was to characterize and analyze the capacity of hVM1 and ReNcell cells to promote a generation of dopaminergic neurons in different media settings, and furthermore to examine and measure the ability of hVM1 cells transfected with different genes of interest to elevate dopaminergic neuron production.

The objectives of the study to examine the above hypothesis are:

•Optimization of the cell culture conditions for NSC regarding media supplements B-27, N-2, SCM005 and evaluation of their influence

• Study of the effect of each of the candidate genes, CDKN1C, GADD45G, GFRA1, INSM1 and NHLH1, on the ability to generate NSC dopaminergic neurons.

3. Materials and Methods

3.1. Media supplements

B-27 Supplement is an optimized serum-free supplement used to support the low or high density growth and short or long-term viability of hippocampal and other CNS neurons (Chen et al., 2008).

N-2 Supplement is a chemically defined, serum-free media supplement based on Bottenstein's N-1 formulation formulated to provide optimal growth conditions for neural stem cell expansion. N-2 Supplement is recommended for growth and survival of neuroblastomas as well as post-mitotic neurons in primary cultures from both the peripheral nervous system (PNS) and the central nervous system (CNS). N-2 is composed of Bovine Insulin, Human Transferrin, Putrescine, Selenite, and Progesterone (https://www.thermofisher.com).

SCM005 media or ReNcell NSC Maintenance Medium is a defined serum-free, growth factor-free medium that has been optimized for the growth and in vitro differentiation of ReNcell immortalized human neural progenitor cells. When used in conjunction with FGF and EGF, the maintenance medium will allow for the proliferation of ReNcell immortalized VM and CX neural stem cells (https://www.merckmillipore.com).

3.2. Cell culture

This study focuses on the polyclonal hVM1 cell line. Its isolation and immortalization were discussed previously in (Villa et al., 2009, Courtois et al., 2010). Briefly, cells were isolated from the ventral mesencephalic region of a 10- week old aborted human fetus at the Lund University Hospital (Sweden), in accordance with the principles set in the Declaration of Helsinki and with the ethical guidelines of the European Network of Transplantation (NECTAR). Procedures were approved by the Lund University Hospital Ethical Committee, and was in conform by Spanish law 35/1988 on Assisted Reproduction. After the acquisitions of the cells, they were

immortalized by the infection with a retroviral vector coding for v-myc (LTR-vmyc-SV40p-Neo-LTR; as reported in Villa et al., 2000).

Cultures of this line are maintained at 37 ° C, with 95% humidity in an atmosphere of low oxygen pressure (5% O2) and 5% CO2. This cell line is maintained in proliferation in HSC medium (Human Stem Cells), characterized by (Villa et al., 2000). It is a serum free defined medium consisting of DMEM: F12 (1: 1) (Gibco) with Glutamax I (Gibco), 20% Albumax (Gibco), 5nM Hepes (Gibco), 30% Glucose (Sigma), 1x supplement N2 (Gibco), B27 (Gibco) or SCM005 (Gibco), 1x non-essential amino acids, a mixture of antibiotics penicillin / streptomycin (100µg / mL), supplemented with hr-EGF (*human recombinant Epidermal Growth Factor*, R&D Systems #236-EG) and hr-bFGF (*human recombinant basic Fibroblast Growth Factor*, R & D Systems # 233-FB) (20ng / ml each). These adherent cells are expanded by seeding them at a density of 4x10 4 cells / cm 2 in plates pretreated with 10µg / mL poly-L-lysine (Sigma # 1274). Proliferating cells are passaged by trypsinization every 3-4 days, when the number of cells has been multiplied by about 4 (reaching a density of about 1.6 × 10 4 cells / cm 2).

Cell differentiation is induced by removing the growth factors (hr-EGF and hr-bFGF) from the culture medium and adding 2 ng / mL hr-GDNF (human recombinant Glial Derived Neurotrophic Factor, Peprotech # 450-10) and 1mM dibutyryl-cAMP (Sigma # 1274) (Lotharius et al., 2002). To differentiate them, 2x104 cells / cm2 are seeded on pre-treated plates with 10µg / mL poly-L-lysine or on glass coverslips coated with 30µg / mL poly-L-lysine. They are maintained 24h in proliferation medium before making a complete change to differentiation medium (day 0). 24h after (day1) another complete change of medium is made, after which 2/5 of medium is replaced every 72h.

3.3. Plasmid DNA Transfection

To transfect the cells with the genes of interest we used the Lipofectamine® 2000 which increases the transfection efficiency of RNA (including mRNA and siRNA) or plasmid DNA into in vitro cell

cultures by lipofection for protein expression, gene silencing, and functional assays. Lipofectamine reagent contains lipid molecules that can form liposomes in an aqueous environment, which entrap the transfection content, i.e. DNA plasmids (Invitrogen, 2012).

To start off, 24.5µl of OptiMEM was mixed with 0.5µg of DNA in an eppendorf to a total of 25µl. Also, 24µl of OptiMEM and 1µl of Lipofectamine 2000 were combined in another eppendorf and the mixture was left to incubate for 5 minutes at room temperature. Afterwards, both eppendorfs were mixed, making a mixture of Lipofectamine2000+OptiMEM: DNA at a ratio of 1µg: 2µL / 1µg: 3µL, and were left to incubate for 20 minutes following the manufacturer's instructions. During the 20 minutes the medium from the cells was removed, and the cells were washed twice with PBS and OptiMEM (6,4 ml) was added. After 20 minutes, the mixture coming from OptiMEM+DNA and OptiMEM+Lipofectamine, was introduced drop by drop onto the cells. The composite was left to incubate for 4 hours. Finally, the medium was removed and fresh growing medium was added. At 48 h they are analyzed by microscopy and flow cytometry.

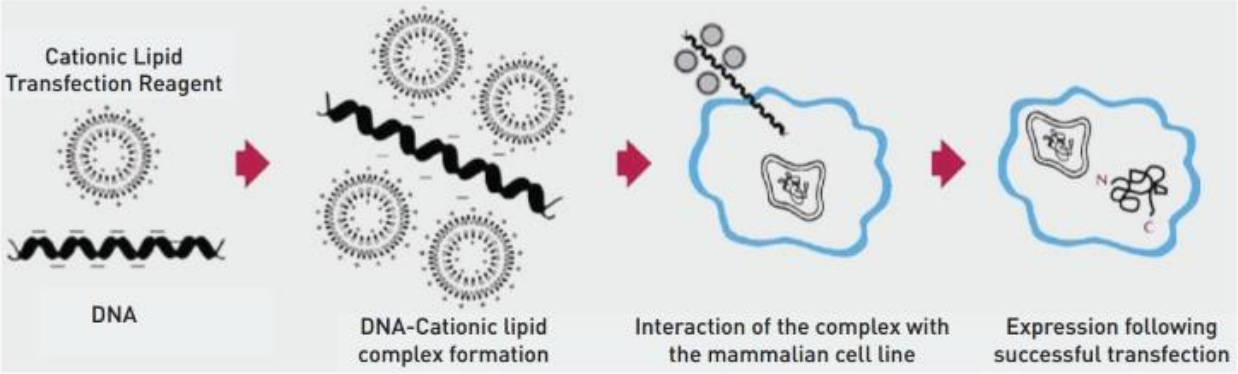


Figure 4. Mechanism of cationic lipid-mediated delivery (https://www.thermofisher.com)

3.4. Immunocytochemistry (ICC)

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody that binds to it. The antibody allows visualization of the protein under a microscope. Immunocytochemistry is a valuable tool to study the presence and sub-cellular localization of proteins.

Cultured cells were fixed on 12mm diameter glass coverslips with parafomaldehyde (PFA) 4% for 20 minutes at room temperature and stored at 4°C in PBS until use. The coverslips with cultured cells were washed three (3) times with PBS; 5 minutes each time. While removing solutions from the cells, we were careful that suction does not dry out the cells. Also, we were always sure to have the next solution on hand to add to cells before removing the solution that coats them so cells are not left to dry. The cells must be treated with a blocking agent to prevent non-specific binding of the antibody. Either goat (GS) or horse serum (HS) was used as a blocking agent depending on the host in which the secondary antibody was raised. If the antigen of interest is inside the cell, the cell membrane must be made permeable to allow entry of the antibody. To permeabilize cells, we used 0.3 % Triton X-100 solution in TBS1X. The blocking solution is made by dissolving 3% goat or horse serum into 0.3 % Triton X-100 solution in TBS1X. It should be pipetted slowly because Triton is viscous, and it should be made sure that the detergent and antibodies are completely dissolved in PBS before adding to cells. The cells were blocked in 3% GS or HS/TBS-tx0.3% solution for 1 hour at room temperature. Afterwards, the primary antibody (which is specific for the protein of interest) was diluted to a pre-determined concentration in 1% GS or HS/TBS-Tx 0.3%, and the coverslip with fixed cells were picked up, such that the cells face up, and were laid on a glass plate filled with filter papers soaked in distilled water covered with parafilm (humidity box). Finally, diluted primary antibody (50 µl per coverslip) was placed over the coverslip so that the cells are in contact with the antibody, and were incubated at 4°C overnight.

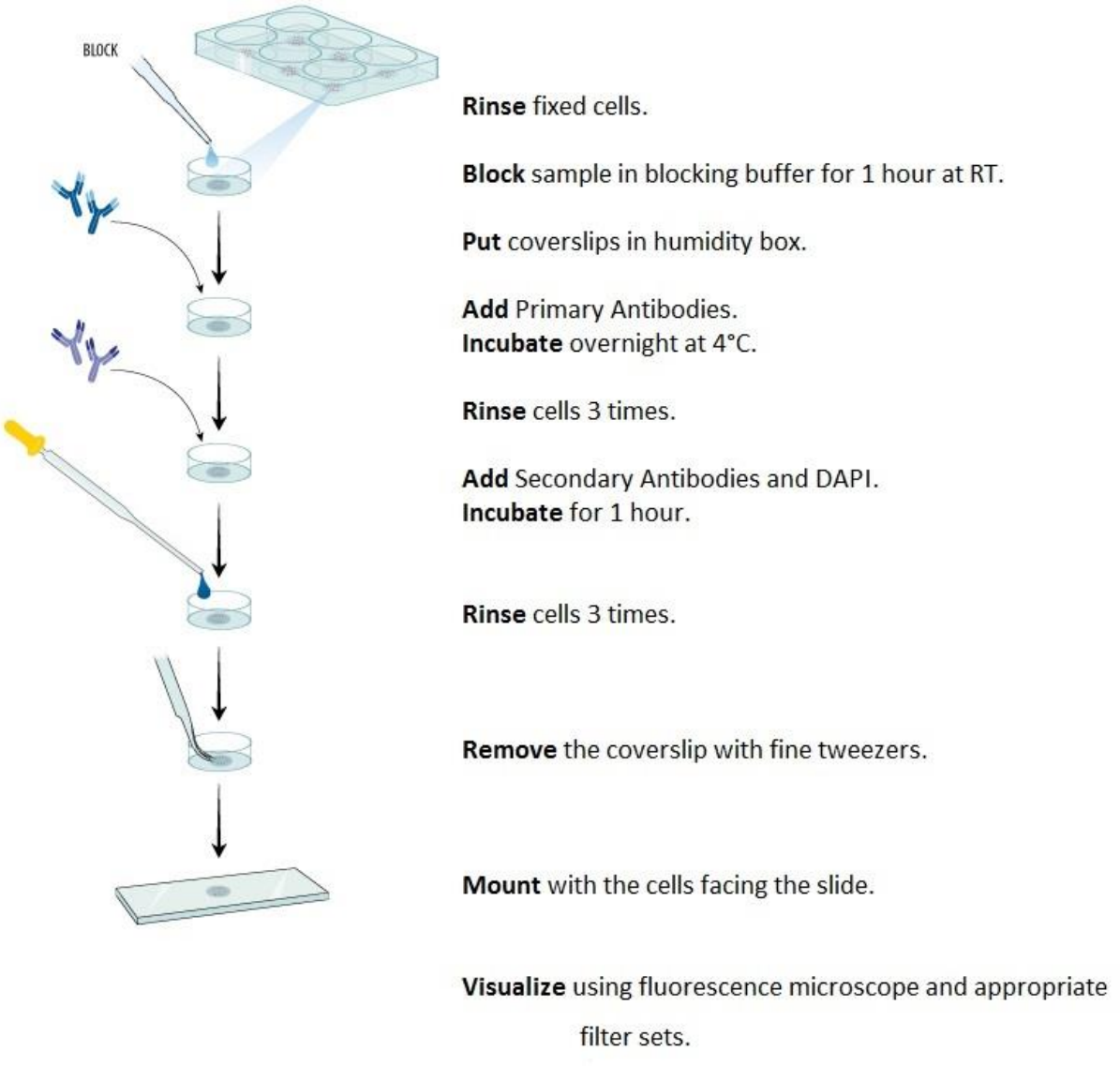


Figure 5. Protocol for the fluorescent ICC staining of cultured cells on coverslips.

The second day we began by carefully collecting the primary antibodies off the coverslip and washing the cells 3 times with TBS1X, 5 minutes each time. We choose a secondary antibody that will detect the primary antibody, for example, if the primary antibody was made in a rabbit, the secondary antibody should recognize rabbit IgG. If using multiple primary antibodies, we made sure they are from different species, and plan to detect with different

fluorochromes attached to the secondary antibodies. For example, an anti-rabbit secondary coupled to a red fluorophore with an anti-mouse secondary coupled to a green fluorophore was used. We incubated cells in the secondary antibody solution diluted to an appropriate concentration in 1% GS or HS/TBS-Tx 0.3% for 2 hours in the dark at room temperature, using the same technique as for primary antibody. Because we were using fluorescent molecules to visualize the secondary antibody, the sample needs to be protected from light once the secondary antibody has been added, so we incubated it in the dark in a foil covered plate. Next, cells were washed 2 times with TBS-TX 0.3%, 5 minutes each time, followed by a 5 minute wash in TBS1X. The coverslips were mounted on a slide with mounting medium for visualization on a microscope. In cases in which a fluorescent molecule is used for visualization of the secondary antibody, the mounting medium should contain agents to minimize photobleaching. Mowiol, a solution of polyvinyl alcohol, was used as mounting medium for fluorescence. An appropriate size drop of Mowiol was placed on a microscope slide. We carefully picked up the coverslip, dabbed off excess water on a paper towel, and laid on the drop of Mowiol with cells facing down. Finally, we placed the coverslip on at an angle and allow to descend slowly to avoid trapping air bubbles. The slide was labeled with the date and any sample information. Slides mounted with coverslips were placed in the dark, and were allowed to dry.

Visualization of ICC was done with an inverted fluorescence microscope (Leica DM-IRB) combined with a CCD camera (Chare Coupled Device). Images were taken at the highest resolution possible and then analyzed with Image J, using plugins for cell counting and merge.

Table 1. Antibodies used for Immunocytochemistry analysis

Antibody		Concentration	Time	Host	Company
1˚ Antibodies	α-GFP	1 /1000	2h	Rabbit	Life Technologies A-6455
	α-ß-Tubulin III	1 /1000	ON	Mouse	Sigma T-220
	α-MAP-2	1 /200	ON	Mouse	Sigma M-4403
	α-TH	1 /250	ON	Mouse	Sigma T-1299
	α-GFAP	1/1000	ON	Rabbit	DAKO #Z0334
2˚ Antibodies	α-rabbit Alexa 488	1/1000	2h	Goat	Molecular Probes A-11034
	α-mouse Alexa 488	1/1000	2h	Goat	Molecular Probes A-11034
	α-mouse Alexa 546	1/1000	2h	Goat	Molecular Probes A-11034
	α-rabbit Alexa 546	1/1000	2h	Goat	Molecular Probes A-11035
	α-mouse	1/200	2h	Horse	Vector BA 2001
Counterstaining	Streptavidine CY3	1/400	30 min		Jackson Inmuno Research 016-160-084
	DAPI	1/1000	2h		AnaSpec (53942)

3.5. ICC data quantification

Using the data of immunocytochemistry, useful information about cell density and percentage were obtained. I calculated the cell density (cells/mm^2) by counting the cells stained with DAPI (4',6-diamidino-2-phenylindole) a nuclear and chromosome counterstain which emits blue fluorescence upon binding to A-T regions of DNA. The total number of cells was determined with photos of representative areas of the coverslip using ImageJ. Then the average is calculated using the ratio of total cells per area and the cell density of each transfection.

3.6. Western blot

3.6.1. Sample preparation

To prepare samples for running on gel, cells and tissues need to be lysed to release the proteins of interest. This solubilizes the proteins so they can migrate individually through a separating gel. RIPA buffer (RadioImmunoPrecipitation Assay buffer) is useful for whole cell extracts and membrane-bound proteins, as well as for extracting nuclear proteins. It will disrupt protein-protein interactions and may therefore be problematic for immunoprecipitations/pull down assays.

RIPA Buffer was added to the cell pellet, using 1mL of RIPA buffer for 40mg of wet cell pellet. Then the mixture was pipetted up and down to suspend the pellet. To increase yields, we sonicated the pellet in pulse mode at low frequency, 4 pulses of 1 second. Afterwards, we centrifuged the mixture at ~14,000 × g for 15 minutes in the cold room (4˚C) to pellet the cell debris. Finally, the supernatant was transferred to a new tube for further analysis. The cells were kept on ice during the whole process.

3.6.2. Bradford assay

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex. A set of standards is created from a stock of protein whose concentration is known, in our case we used bovine serum albumin (BSA- Albumin Standard, Pierce #23209). The Bradford values obtained for the standard are then used to construct a standard curve to which the unknown values obtained can be compared to determine their concentration. The linear concentration range we set is 800-0 µg/ml of protein, using BSA as the standard protein.

Using a P96 well plate, the standard curve 0-800 µg/ml was set up using 2 mg/ml BSA in the following order:

S1: 40 µl standard + 60 µl water (800 µg/ml)

S2: 30 µl standard + 70 µl water (600 µg/ml)

S3: 20 µl standard + 80 µl water (400 µg/ml)

S4: 50 µl of S3 + 50 µl water (200 µg/ml)

S5: 50 µl of S4 + 50 µl water (100 µg/ml)

S6: 50 µl of S5 + 50 µl water (50 µg/ml)

S7: 50 µl of S6 + 50 µl water (25 µg/ml)

S8: 100 µl water (0 µg/ml)

The Bradford reagent (Bio-Rad Protein Assay reagent concentrated, BioRad #500-0006) was diluted using 4 ml water and 1 ml Bradford reagent. Next, we diluted the samples in H2O Milli-Q, using only 5 µl of sample. Then, 10 µl of standards and 10 µl of samples (duplicates) were pipetted into new wells. Afterwards, 200 µl of Bradford reagent was added to each well and the plate was incubated for 5 minutes on a shaker. To quantify the plate we used a plate reader spectrophotometer at 595 nm, making sure all protein absorbance measurements were within the standard curve. To prepare the samples for loading into the gels, based on the results from the spectrophotometer the volume of protein, H2O Milli-Q and 5X SDS-PAGE sample loading buffer (Nzytech #MB11701) were calculated to a final concentration of 0,1 mg/ml. Finally, we mixed all the reagents and place them on thermoblock at 95ºC for 5 minutes.

3.6.3. Electrophoresis

Western blotting is a technique that identifies specific proteins in a given sample or extract after their separation using polyacrylamide gel electrophoresis. The polyacrylamide gel is placed adjacent to a membrane, which is typically nitrocellulose or PVDF (polyvinylidene fluoride), and the application of an electrical current induces the proteins to migrate from the gel to the membrane on which they become immobilized. The membrane is then a replica of the gel protein and can subsequently be stained with an antibody.

Firstly, we cleaned the gel apparatus and glass plates thoroughly with soap and water, rinsed with 100% ethanol and let air dry. Afterwards, we made 4% polyacrylamide stacking and 10% separating gels, the gel percentage required is dependent on the size of protein of interest. Then, the 10% separating gel was poured with a p1000 pipette into the gel rack. Isopropanol was added on top to get rid of bubbles. We waited for gel to set (checking the remaining gel in the tube), so we can decant the isopropanol onto a kimwipe and wash the gel three times with H2O Mili-Q. The 4% stacking gel was poured on top of mold and a comb was inserted at an angle to ensure no bubbles. After casting, the gel was transferred to the tank apparatus with shorter glass facing inside, and filled the tank and wells with 1X running buffer. Equal amounts of protein were loaded into the wells of the SDS-PAGE gel, along with the NZY Colour molecular weight marker II (15011). The loaded sample was 10 µg of total protein from cell lysate. We ran the gel for 1-2 hours at 120V, until the dye was all the way at end of gel.

3.6.4. Protein Transfer to Nitrocellulose membranes

Fresh transfer buffer was prepared, every time we did the transfer, and let to cool down at 4ºC. We soaked the Whatman papers, the Amersham nitrocellulose membrane and the gel 10 minutes prior to transfer. We soaked the gel because it removes any contaminating electrophoresis buffer salts, it allows the gel to shrink to its final size before transfer and it prevents the gel and membrane from drying out if you can’t transfer right away. The semi-dry

transfer was performed for 30 minutes in the Turbo-Blot-BioRad machine by setting the voltage to a constant 25V while the amperes were set to 0.8 A maximum.

3.6.5. Membrane incubation

The membrane incubation is initiated by blocking it in 5% non-fat milk in TBST (Tween20, 0,1%) at room temperature for 1 hour, followed by a few quick washes with TBS 1X. Afterwards, we put the primary antibody diluted to a proper concentration in 0.05% TBST on the membrane and left it to incubate overnight at 4ºC. The next day we started off with three washes, 5 minutes each, with 0.05% TBST. Finally, the membrane was incubated with the secondary antibody, diluted in 0.05% TBST, at room temperature for 1.5 hour. Before developing the membrane it was washed with 0.05% TBST 3 times for 5 minutes. After incubating the membrane in the Amersham ECL Western Blotting Detection Reagent (RPN2106) for 1 minute, we developed the membrane in the ImageQuant™ LAS 4000 mini biomolecular imager.

ECL normal

- 1 ml Reagent 1: 1 ml Reagent 2

Normal transfer buffer

- 100 ml 10X Tris Glycine in H2O
- 700 ml ddH2O
- 2 ml 20% SDS
- 200 ml methanol (add last)

Running buffer

- 1 L 10X Tris Glycine in H2O
- 50 ml 20% SDS
- Mix and dilute to 1X with ddH2O

Table 2. Antibodies used for Western Blot

Antibody		Concentration	Company	Host	Weight
1° Antibodies	MAP-2	1/500	Sigma M 4403	Mouse	70 kDa
	Synaptofisin	1/500	Millipore MAB332	Mouse	38 kDa
	TH	1/1000	Pel-Freez P40101-0	Rabbit	68 kDa
	ß-Tubulin III	1/1000	Sigma T2200	Rabbit	55 kDa
	b-actin	1/2000	Sigma A5441	Mouse	45 Kda
	GAPDH	1/2000	Santa Cruz 32233	Mouse	37 kDa
	Nestin	1/500	Millipore AB5922	Rabbit	200 kDa
	Neuro D	1/500	Santa Cruz sc-1084	Goat	50 Kda
	Musashi	1/500	Millipore MABE268	Mouse	39 kDa
	Pitx3	1/500	Chemicon AB5722	Rabbit	42 kDa
2° Antibodies Peroxidases	a- Mouse	1/2000	Vector	Horse	
	a- Goat	1/2000	Sigma	Rabbit	
	a- Rabbit	1/2000	Nordic Inmuno	Goat	

4. Results

4.1. Western Blot analysis of hVM1 and ReNcell cells cultivated in different media

Western blot analysis of differentiated hVM1 and ReNcell cells day 7 grown in different media, reveal the expression of various neural markers. The markers used to detect dopaminergic neurons in the Western blot analysis were the Tyrosine hydroxylase (TH), an enzyme that converts L-tyrosine to L-3,4-dihydroxyphelylalanine (L-DOPA) which is a rate limiting step in the synthesis of dopamine, as well as Pitx3, a transcription factor expressed selectively in the midbrain and that regulates the differentiation and survival of dopaminergic neurons. Beta III tubulin antibody was used to detect immature neurons (it detects class of tubulin whose expression is limited to neurons) and the marker for NeuroD1, a transcription factor that promotes neuronal development, along with the Musashi-1 marker, a neural RNA-binding protein putatively expressed in CNS stem cells and neural progenitor cells. Mature neurons I marked with the MAP-2 antibody which is a neuron-specific protein that promotes assembly and stability of the microtubule network, including the Synaptophysin marker, a synaptic vesicle protein that regulates vesicle endocytosis in neurons. Neuroepithelial (NE) cells were labeled for Nestin, an intermediate filament protein expressed in NE cells. Its expression persists in radial glia until astrocyte development. And finally, GFAP was used to detect astrocytes. It has been reported that cells with astrocytic property can serve as an origination of new neurons during adult neurogenesis. This proves that cell cultures grown with different media supplements are able to differentiate into the desired neuronal type.

To accurately determine protein expression and interpret Western blot results, it is important to use loading controls. A loading control antibody helps determine if samples have been loaded equally across all wells and confirms effective protein transfer during the western blot protocol. Beta-Actin is commonly chosen as a loading control due to its general expression across all eukaryotic cell types. The expression levels of this protein do not vary drastically due to cellular treatment, which is another reason the protein makes a suitable control. Results in the loading control Beta-Actin indicates little protein in hVM1 samples (Figure 6.). GAPDH is integral for

glycolysis and plays many roles in nuclear function; such as transcription regulation and apoptosis. The stable and ubiquitous expression of GAPDH also make it a suitable loading control for many experiments. When using GAPDH as a loading control, it is important to keep in mind that its expression level does vary between tissues.

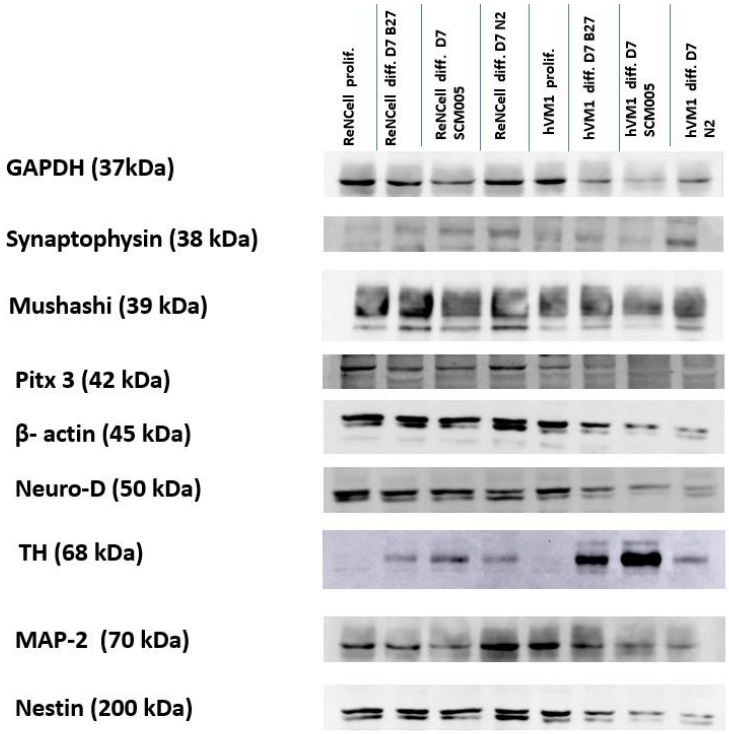


Figure 6. Western blot analysis shows that differentiated hVM1 and ReNcell cell cultures grown with different media supplements are able to differentiate into the desired neuronal type.

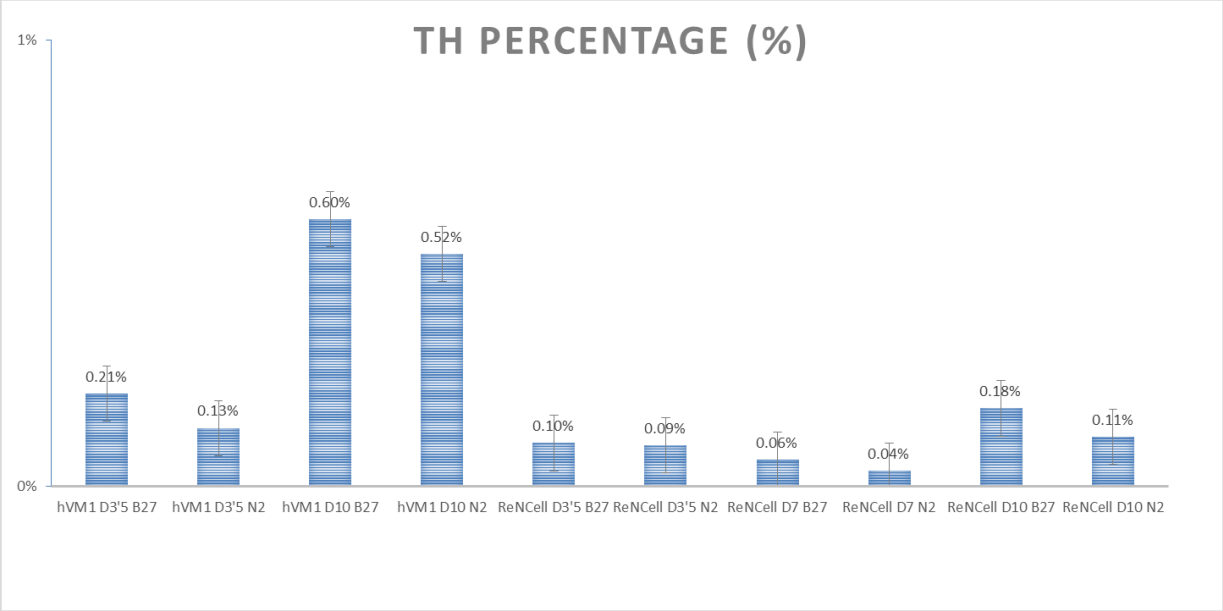
4.2. Study of the generation of dopaminergic neurons (TH +) of hVM1 and ReNcell cells cultivated in different media

To verify the correct neuronal differentiation after transfection, we analyze the presence of markers of different stages of neural differentiation by ICC, and in the same way examine the influence of different media supplements. The percentage of the population expressing different

markers is examined and determined for cells transfected with each media supplement and for each GOI. We hypothesized that GOIs are downstream effectors of Bcl-XL and we studied if any of them is able to induce the expression of dopaminergic neurons the same way Bcl-XL is, as well as how different culturing media effects their growth.

Due to extremely low, or no signal at all on the ICC samples, the SCM005 media supplement could not be quantified. The highest number of dopaminergic neurons (TH+) between cells cultivated in different media is detected in the case of media supplemented with B27 (Figure 7. A), being slightly increased with respect to cells cultivated with other media supplements. The same phenomenon was observed for MAP-2 staining at different days of differentiation where the B27 media supplement generated a slight, but not a statistically significant, increase in the amount of MAP-2 positive cells in the hVM1, while the ReNcell cultures showed no significant positive or negative effect in either media supplement (Figure 7. B). MAP-2 expression is weak in neuronal precursors but it increases during neuron development process. In general, its expression is confined to neurons; specifically in the perikarya and dendrites of these cells. Antibodies to MAP2 are therefore excellent markers on neuronal cells, their perikarya and neuronal dendrites.

A



B

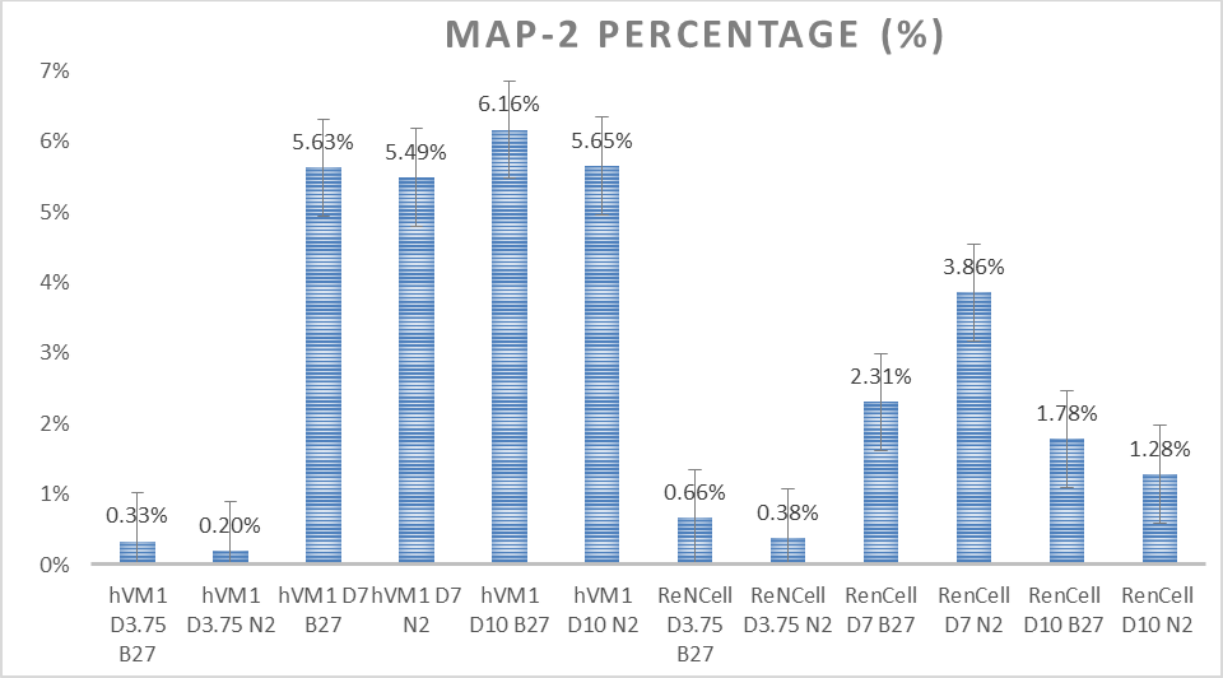


Figure 7. ICC study of differentiated hVM1 and ReNcell cells at days 3.5, 7 and 10 cultivated with different media supplements. A) Percentage of the generation of dopaminergic neurons by different culturing media (TH +) and total of cells per coverslip (DAPI+)- day 7. B) Percentage of MAP-2 positive cells from the total cell counts (DAPI+)

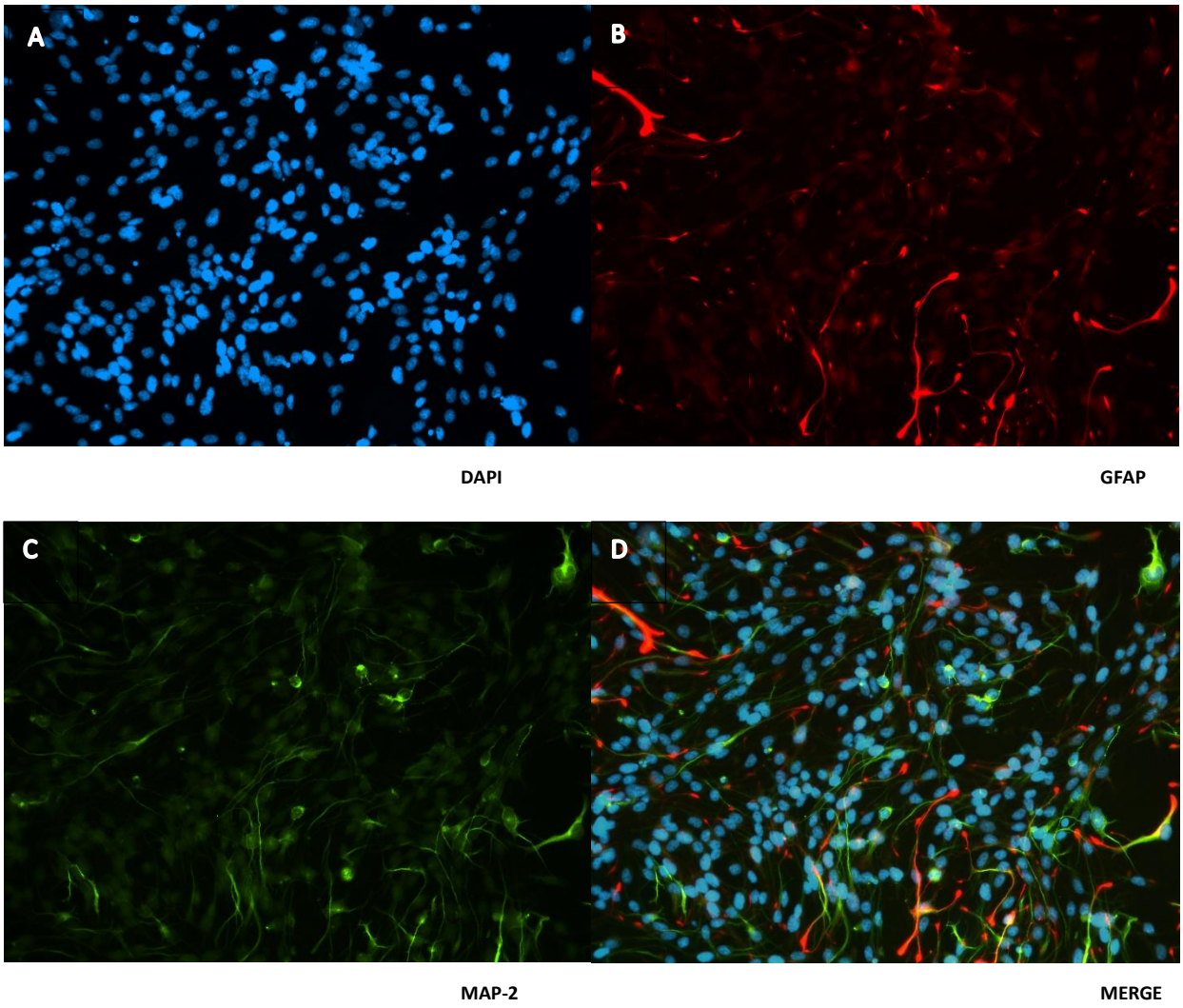


Figure 8. Detail of hVM1 cells differentiated at day 7, cultured with B27 media supplement; Scale bar = 15µm. A.) DAPI - nucleic acid stain, preferentially stains dsDNA; B.) GFAP- astrocytic marker; C.) MAP-2- marker for a cytoskeletal protein, its expression is confined to perikarya and dendrites of cells; D.) Merged picture of a DAPI / GFAP + / MAP-2 + positive cell

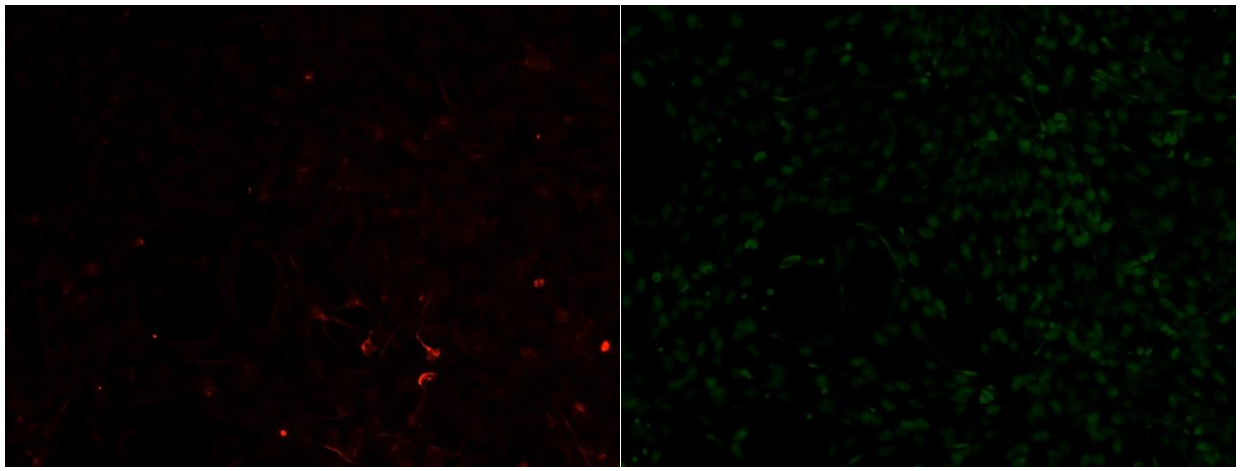
4.3. Study of the generation of dopaminergic neurons (TH +) of transfected and differentiated hVM1 cells

Since neuronal differentiation was better in hVM1, we chose to go for transfections in hVM1. Through a more detailed analysis of the cell density of the culture transfected with different vectors and differentiated at day 7, there were statistically significant differences in the production of dopaminergic neurons for pCAGGS Bcl-XL, GADD45G, INSM1 and GFRA1, relative to control with the empty vector.

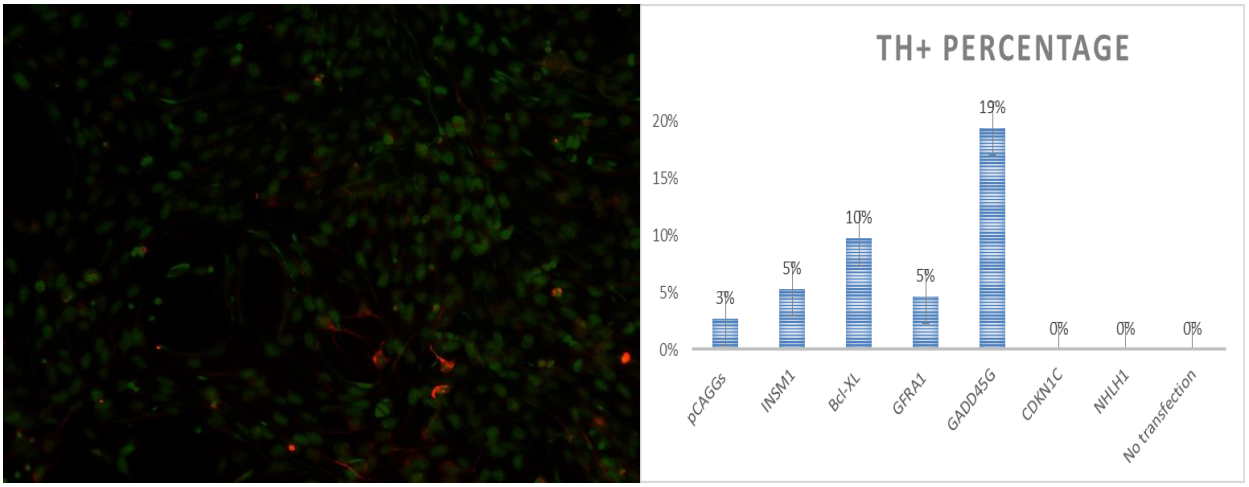
The main objective of this work is the study of the generation of dopaminergic neurons (TH + cells) by hVM1 cells transfected with the different vectors of gene expression and differentiated until day 7. The ICC for TH and GFP performed allows the detection of double positive cells (TH + GFP) and single positive cells (GFP + or TH +). In this way, the transfected cells that have (GFP + TH +) and TH + cells that were generated from the population of non-transfected cells (TH + GFP-) (Figure 9. A).

The highest number of dopaminergic neurons between transfected cells (TH + GFP +) is detected in the case of pCAGGS Bcl-XL, GADD45G, INSM1 and GFRA1, being statistically significant with respect to control cells with the empty vector (Figure 9. B). When TH + cells are obtained from non-transfected cells (TH + GFP-), no significant differences are found (not shown); although there is a greater tendency for increased expression of TH in the cultures transfected with pCAGGS GADD45G, INSM1 and GFRA1. The lowest rates of dopaminergic neurons were obtained for cells transfected with CDKN1 and NHLH1 genes. In those samples, the quantity of TH + GFP+ cells was very low (Figure 9. B).

A



TH+ GFP+



MERGE (GFP+TH+) B

Figure 9. A) Detail of cells viewed with different filters (upper photographs). Detail of a positive double cell TH + / GFP +, Scale bar = 15µm (lower photograph). B) Study of the generation of dopaminergic neurons by transfected cells (TH + GFP +)

5. Discussion

Neuronal cultures are widely used to study neuronal development including neurite and synapse formation, neurotransmitter release, subcellular distribution and trafficking of neuronal proteins such as neurotransmitter receptors, and homeostasis of electrical signaling. Initially those cultures depended on the use of sera for factors that are critical for cell survival and growth. Media supplements such as B27, SCM005 and N2 were developed with defined components that eliminate the need for supplementation with serum (Chen et al., 2008). Such supplements were widely welcomed. In theory the use of defined supplements reduces the variability of the culture conditions. It thereby limits the potential for detrimental effects of components that could affect the health of cultures. However, a number of laboratories have experienced large differences in their neuronal cultures over the last 4-5 years when using commercially available supplements (Schluter et al., 2006; Tsui and Malenka, 2006). Commercial supplements available earlier supported neuronal cultures of excellent quality including neurons derived from hippocampus, retinal ganglia (RGCs), and dorsal root ganglia (DRG) cells.

The ability to manipulate human neural stem cells (hNSCs) in vitro provides a means to investigate their utility as cell transplants for therapeutic purposes as well as to explore many fundamental processes of human neural development and pathology. Using traditional passaging techniques and culture mediums the rate of growth was extremely slow, and only a 12-fold expansion in total cell number can be achieved (Hall et al., 2008). That's why optimization of culturing conditions is required. To measure the influence of different media supplements on the hNSC growth, we established culture conditions using B27 and N2 - supplemented medium. We found that B27 added to the medium at the time of plating resulted in an increase in the number of dopaminergic (TH+) and mature (MAP-2+) neural cells compared with N2 over 3.5, 7 or 10 days in both hVM1 and ReNcell cultures. B27 includes a range of hormones, anti-oxidants and retinal acetate in addition to the Bottenstien and Sato (1985) basic formulation of transferrin, insulin, putrescine, progesterone and sodium selenate (Brewer et al. 1993). Attempts were made to isolate which factors in B27 may be responsible

for the increased survival of precursor cells, by the addition of retinal acetate, tocopherol, catalase and super oxide dismutase to cultures grown in EGF supplemented N2 medium, although the exact concentration of each factor within B27 is proprietary information of Gibco. None of these factors alone or together appeared to have the dramatic effect of B27 itself, suggesting that it may be the combination of all these factors that underlies the enhanced survival of precursors found when using this supplement. It is possible that B27 may increase the division rate of precursor cells rather than enhance their survival (Svendsen et al, 1995). N-2 is composed of Bovine Insulin, Human Transferrin, Putrescine, Selenite, and Progesterone. This supplement is recommended for the growth and expression of neuroblastomas and for the survival and expression of post-mitotic neurons in primary cultures from both the peripheral nervous system (PNS) and the central nervous system (CNS) (Bottenstein et al., 1985).

Addition of B27, a medium supplement that increases neuronal survival in primary CNS cultures, resulted in an increase of the number of dopaminergic and mature cells in vitro after 3.5, 7 or 10 days of differentiation. We conclude from this that the potential of NSC to differentiate into dopaminergic and mature neurons is insignificantly higher with the B27 medium than as revealed by N2 medium, and therefore both B27 and N2 represent an appropriate choice of medium supplement for assays of human NSC behavior and dopaminergic neuron differentiation. B27 supplement has been shown to increase the differentiation in culture of a range of CNS cell types, whereas N2 supplement is sufficient to maintain them.

Several research groups are currently focused on the search for a universal strategy to obtain a source of neural stem cells (NSC) suitable for the development of cell therapies against Parkinson's disease. This source must provide a sufficient number of clinically safe cells to restore homeostasis or slow the progression of this neurodegenerative disease. The immortalization of NSC with v-myc (hVM1 line) allows these cells to expand in culture and to increase their number, however, it does not prevent the loss of neurogenic capacity with the passages in culture. This limitation has been solved by the forced expression of Bcl-XL (in the line hVM1 high Bcl-XL). So far it has been demonstrated, that by overexpressing Bcl-XL it is possible to enhance the generation of mature dopaminergic neurons from their precursors, the VM neuronal stem cells (Courtois et al. 2010). However, the antiapoptotic activity of the Bcl-XL would endanger the

biosecurity of its therapeutic use, so it has been necessary to undertake a search for alternatives from a series of genes that would act as its effectors. The ultimate goal is to find a substitute gene for Bcl-XL whose overexpression would be feasible for the development of a therapy in the clinic. Other authors have previously studied the effect of overexpression of transcription factors, such as ASCL1 (Kim et al., 2009) or Lmx1a and Msx1 (Roybon et al., 2008), in neural progenitors derived from human ventral midbrain, without much success in terms of generation of dopaminergic neurons. Interestingly, and repeatedly in studies with hVM1 cells, the expression of the transcription factors FOXA2 and LMX1A was not detected, although they were described as necessary requirements in dopaminergic differentiation (Stott et al. 2013.). In spite of this, the cells are perfectly capable of differentiating themselves and generating DAns, which might suggest that the expression of such genes is not a necessary condition for dopaminergic neurogenesis from hNSCs. In the present research the effect of several candidate genes on the neurogenic potential of the hVM1 cells has been evaluated, as well as the effect of different culturing media on hVM1 and ReNcell development. Previous laboratory studies gave rise to a selection of genes whose expression was lost in hVM1 cells that lost neurogenic capacity with the passages in culture. However this did not happen when the cells overexpressed Bcl-XL, considering as possible effectors of this. Candidate genes were proven to promote dopaminergic commitment and DAn generation while the neurogenic potential is preserved.

Cells derived from VM lose ability of dopaminergic differentiation in its expansion in culture (Kim et al. 2007). In the case of hVM1 cells this happens in a drastic manner when reaching the passage 20, seriously affecting the survival of cell cultures. However in previous passages the dopaminergic potential is still preserved and the growing conditions are more favorable, making it possible to study the effect of GOI's. Therefore, at 3.5 day of differentiation the candidate genes are already capable of promoting the dopaminergic compromise of neural precursors, inducing the expression of genes responsible for the dopaminergic specification in embryonic development. Although at day 3.5 of differentiation is when the first wave of expression of pro-dopaminergic genes takes place, it takes at least 7 days to be able to define the cells as DAns. At this moment they acquire characteristic of the cellular type as the TH expression. These results

on early differentiation are consistent with those described in the past for Bcl-XL (Curtois et al. 2010).

To determine if any of the genes of interest promotes the generation of dopaminergic neurons in the hVM1 line, as Bcl-XL did, we analyzed the percentage of GFP and TH positive cells generated in the transfected and differentiated cultures. Studies conducted by Dr. Martinez- Serrano research group proved that the pCAGGS expression vector is functional for the transfection of the genes of interest to the hVM1 cell line, as evidenced by detection of the GFP reporter gene, hence it was used for the transfection. With the first results, we found significant data differences for pCAGGS Bcl-XL, GFRA1, INSM1 and GADD45G, regarding the empty vector. The positive effect of Bcl-XL was already known within the hVM1 high Bcl-XL line. Therefore, these data would be suggesting that GFRA1, INSM1 and GADD45G also have a positive effect on the generation of dopaminergic neurons of the hVM1 line (Figure 8). We do not rule out that in addition to acting on the transfected cells themselves the genes could be exerting a paracrine effect on cells neighboring the culture and could thus also increase the number of total dopaminergic neurons. This paracrine effect could be due to multiple factors, one of them being the mere fact of increasing the population of dopaminergic neurons in the culture. It is also possible that the transfected cells may be secreting into the medium some protein and / or signaling molecule that could have a positive action. This would require a detailed proteomic study of mixed and pure cultures.

Further study of the effect of these genes on the neurogenic ability of the hVM1 line should be further investigated to verify these preliminary results. To do this, the number of biological and technical replicates of the experiments must be increased to achieve sufficient statistical power. In addition, GFP + and GFP-cell populations of the transfected cultures have been isolated by flow cytometry under proliferation conditions and after 7 days of differentiation. In this way, gene expression (q-RT-PCR) and protein (western-blot, ICC) analyzes can be performed in the populations of interest. Thus, one could definitively conclude whether candidate genes promote the expression of genes involved in the commitment and differentiation of NSCs to dopaminergic neurons.

To conclude, the lack of an effective alternative to regain homeostasis or prevent the progression of Parkinson's disease makes efforts to focus on options away from traditional pharmacological treatments. Experimental therapy with fetal tissue has shown encouraging results, however, there are many problems related to the use of fetal tissue, both ethical and technical. Through the use of gene therapy, we propose a shift in the approach of cell therapy to Parkinson's disease, minimizing ethical - reducing the number of donors needed when working with an immortalized line - and technical issues - less variability and greater efficiency in the generation of mature phenotypes of interest.

6. Conclusions

The studied media supplements showed no differences in promoting the dopaminergic commitment of hVM1 and ReNcell cells during their early differentiation. Both, B27 and N2 media supplements were shown to be equally good media for NSC culturing.

Similarly to Bcl-XL, candidate genes INSM1, GFRA1 and GADD45G increased cell density in culture with respect to the control.

The INSM1, GFRA1 and GADD45G genes promote greater differentiation of NSCs into dopaminergic neurons in terms of positive tyrosine hydroxylase cells and could be good candidates for replacing Bcl-XL in the present strategy.

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8. Curriculum vitae

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female, born 30.4.1989.

EDUCATION

Department of Biology, Faculty of Science, Zagreb **univ.bacc.biol.**	**2009-2012**
Department of Experimental Biology, Faculty of Science **M.A. in Experimental Biology**	**2012-2016**
Universidad Politecnica de Madrid (UPM), Madrid **Student exchange**	**Jan 2014- Sep 2014**
Department of Molecular Biology, Faculty of Science **M.A. of Molecular Biology**	**2014-2017**

RELATED EXPERIENCE

Laboratory Internships, Clinical hospital center Zagreb (KBC Rebro) Did my training in the Clinical Unit of Molecular Diagnostics gaining skills in the laboratory use of PCR, FISH technique and flow cytometry.	**February 2012– June 2012**
Research Internship, The Croatian Institute for Brain Research (CIBR)- The Laboratory for Neurooncology "The role of the Wnt signaling pathway in epithelial to mesenchymal transition". Studying changes in Dishevelled, TCF/LEF, APC, beta-catenin and E-cadherin and Axin genes in order to understand molecular and genetic mechanisms that govern the formation and progression of different types of brain tumors and in the process of epithelial to mesenchymal transition.	**January 2015 – January 2016**

Research Internship, Centro de Biología Molecular Severo Ochoa (CBMSO)- Potential for cell and gene therapy in neurodegeneration (Alberto Martínez-Serrano) **March 2016 – February 2017**

Knowledge, skills (intellectual and practical) and competences acquired:
I was trained in Molecular Biology techniques to be able to handle samples of nucleic acids and protein to study expression on different markers. Also, I was trained in Microscopy, Immunocytochemistry and Gene Expression Analysis. Furthermore, I was taught to use the samples collected from laboratory animal experimentation studies to determine protein expression by Western Blot or Immunohistochemistry.

Speaker, Night of Biology 2017. (Noć Biologije 2017.) **April 2017.**
Held a presentation about Genetic engineering; mentored by Dr. sc. Malenica

LANGUAGES

Croatian- native language
English- highly proficient in spoken and written (C1)
Spanish- very good command (B2)
German- very good command (B2)

SPECIFIC EXPERIMENTAL SKILLS

Molecular and Cell Biology and Biochemistry

- DNA, RNA and protein isolation	- Bradford protein assay
- PCR	- Lipofectamine® 2000 Transfection
- immunocytochemistry (ICC)	-immunohistochemistry (IHC)
- RT-PCR	- in situ (ISH)
- Westerns	- cytological and histological imaging analyses
- Tissue staining and fixation	- FISH

Microscopy

- fluorescence microscopy	- light/phase microscopy

COMPUTER SKILLS

Proficient with Image J, BioEdit, Microsoft Word, Excel, Access, and Power Point. I'm very comfortable using these programs and have a lot of experience doing so. I'm interested in learning how to use any other programs as well. I'm very comfortable using computers and am confident in my ability to learn any new programs quickly.

ADDITIONAL INFORMATION

Licenses

Class B driver’s license - active driver.

Work experience

Stylist assistant for Adria media- selecting the clothing for published editorial features, print or television advertising campaigns, music videos, concert performances, and any public appearances made by celebrities, models or other public figures.
Two year experience working as a promotor for Dekra d.o.o., Alert d.o.o. and Ex-alto d.o.o. where I have proven ability to initiate and maintain excellent relations on a face to face level with a high-end clientele demographic. Worked closely with customers face to face to identify needs, tactfully answer questions, sell products and services, and solve problems.

Hobbies and Interests

Was five times national champion of Croatia in show jumping and went to various jumping courses all over Europe to improve my riding skills which shows my persistence, focus and good ability to adapt to multicultural environments as well as my team spirit.