Functional and Transcriptional Studies of Human Dopaminergic Neurons

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Functional and Transcriptional Studies of Human Dopaminergic Neurons

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2020

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Abstract

Parkinson's Disease (PD) is the most common movement disorder and second most common neurodegenerative disease. The principal hallmark of the pathology is represented by a loss of mesencephalic Dopaminergic neurons (mesDA) that reside in the Substantia Nigra pars compacta (SNpc). Another feature of the disease is represented by formation of abnormal protein aggregates, known as Lewy Bodies (LBs), mainly composed by the α-synuclein protein. The etiology of mesDA death is still unknown, however LBs formation could represent one of the factor contributing to neuronal mesDA death and PD progression.

Cell Replacement Therapy for PD aims at restoring the function of the dopaminergic neurons through the transplantation of the lost cells in the brain. Recently, cell sources derived from stem cells such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSC) have been investigated and implicated in clinical trials for PD. Another route for generating neurons is represented by the direct reprogramming of terminally differentiated cells. With the overexpression of specific transcription factors (TFs) and/or micro RNA (miRNA) is possible to target somatic cells *in vitro* or resident brain cells *in vivo* for reprogramming into mesDA neurons.

The overall aim of my thesis has been to study functional and transcriptional profile of newly generated mesDA neurons *in vitro* and *in vivo* for cell-based therapies of PD. Indeed the transplantation outcome depends on the ability to generate mesDA neurons that are as similar as possible to the endogenous DA neurons. However, our knowledge of human DA neurons is limited by the inaccessibility of developing and adult brain tissues. In the first part of my thesis I focused on studying the properties of directly reprogrammed cells to determine their phenotypic and functional profile. In the second part of this thesis, I performed an extensive molecular, transcriptional and functional analysis of human fetal mesDA neurons to increase our understanding of DA neurons. Lastly, I focused on establishing a stem cell derived organoid system that allowed for the generation of authentic human DA neurons.

Key words: Dopaminergic neurons, cell reprogramming, cell therapy, induced neurons, *in vitro* reprogramming, *in vivo* reprogramming

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Representation of a boat transporting human fetal dopaminergic neurons in a sea of recordings.

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To my family

"The mind is not a vessel to be filled
but a fire to be kindled."
Plutarch

ἀλήθεια

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ABSTRACT

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The overall aim of my thesis has been to study functional and transcriptional profile of newly generated mesDA neurons *in vitro* and *in vivo* for cell-based therapies of PD. Indeed the transplantation outcome depends on the ability to generate mesDA neurons that are as similar as possible to the endogenous DA neurons. However, our knowledge of human DA neurons is limited by the inaccessibility of developing and adult brain tissues. In the first part of my thesis I focused on studying the properties of directly reprogrammed cells to determine their phenotypic and functional profile. In the second part of this thesis, I performed an extensive molecular, transcriptional and functional analysis of human fetal mesDA neurons to increase our understanding of DA neurons. Lastly, I focused on establishing a stem cell derived organoid system that allowed for the generation of authentic human DA neurons.

LAY SUMMARY

Parkinson's Disease (PD) is the most common movement disorder and second most common neurodegenerative disorder after Alzheimer Disease. The symptoms experienced by patients are mainly related to motor impairment however some patients experience neuropsychiatric disturbances, autonomic and sensory dysfunctions and sleep problems. The principal hallmark of the pathology is represented by a loss of neurons in the brain that in healthy conditions modulate motor output and control by releasing the neurotransmitter Dopamine (DA).

Cell Replacement Therapy aims at restoring the function of these neurons through the transplantation of new cells in the brain of PD patients. Notably, stem cells have the capability of generating neurons when specific protocols are applied for their differentiation in the laboratory. Clinical trials for PD are nowadays taking place using these cells, however, stem cells have the main feature of being highly proliferative in an undifferentiated state giving rise to concerns for tumor formation in their application for cell transplantation approaches. Other venues for generating neurons are currently under investigation such as the direct conversion of skin fibroblasts so called induced neurons (iNs) or resident brain cells into the desired neurons. This allows for the generation of neurons without passing through a proliferative step, potentially decreasing the risk of tumor formation.

An important aspect to consider when generating neurons for transplantations, is how closely these new cells resemble the authentic DA neurons residing in the human brain. However, our knowledge of human dopaminergic neurons is limited by the inaccessibility of the brain tissue during and after development.

In the first part of my thesis I focused on determining properties of gene and protein expression together with functional aspects of directly reprogrammed cells starting from human skin cells or resident mouse brain cells. In the second part of the thesis, human fetal brain tissue was studied in order to increase our current knowledge of authentic DA neurons. I therefore performed an extensive molecular, transcriptional and functional analysis of human fetal DA neurons. Lastly, I used stem cells for replicating physiological DA development and maturation using the organoid technology. Differently from standard monolayer cell cultures, the organoid system allows cells self-structural organization in three dimensional cultures, closely resembling the process taking place during development.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Parkinsons sjukdom är den vanligaste rörelsehindrande, och den näst vanligaste neurodegenerativa sjukdomen efter Alzheimers sjukdom. De symptom som patienter ofta upplever är normalt relaterade till nedsatt motorfunktion, men en del patienter upplever neuropsykiatriska störningar, autonom och sensorisk dysfunktion, och sömnproblem. Det klassiska kännetecknet för Parkinsons sjukdom är förlusten av nervceller i hjärnan som under normala förhållanden kontrollerar motorsignaler genom att frisätta signalsubstansen dopamin.

Cellersättningsterapi har som mål att återställa funktionen och balansen i hjärnan som har gått förlorad hos patienter med Parkinsons sjukdom. Stamceller har förmågan att generera nervceller genom att applicera specifika protokoll i laboratoriet. Kliniska prövningar för Parkinsons sjukdom sker för närvarande med dessa celler, men stamceller har huvudsakligen kännetecknet av deras stora förmåga att dela sig i ett tidigt stadium vilket ger upphov till oro för tumörbildning. För närvarande undersöks även andra metoder som kan användas för att generera nervceller, såsom direkt omprogrammering av hudceller till så kallade inducerade nervceller. Det pågår även forskning där man riktar in sig på hjärnceller i hjärnan för direkt omprogrammering till önskade nervceller. Metoden direkt omprogrammering möjliggör generering av nervceller utan att genomgå ett proliferativt stadium, vilket minskar risken för tumörbildning.

En viktig aspekt att tänka på när man genererar nervceller för transplantationer är hur lika dessa nya celler är de äkta dopaminerga nervceller som är bosatta i människans hjärna. Vår kunskap om humana dopaminerga nervceller begränsas av hjärnvävnadens otillgänglighet under och efter utvecklingen. I min avhandling behandlade jag dessa olika aspekter av celltransplantation, och delade upp mitt arbete i två delar.

I den första delen av min avhandling fokuserade jag på att klarlägga egenskaperna för gen- och proteinuttryck tillsammans med funktionella aspekter av direkt omprogrammerade celler från mänskliga hudceller eller mushjärnceller i den levande mushjärnan. I den andra delen av avhandlingen studerades människans fosterhjärnvävnad för att öka vår nuvarande kunskap om äkta dopaminerga hjärnceller. Jag utförde därför en omfattande molekylär, transkriptionell och funktionell analys av mänskliga dopaminerga hjärnceller från foster. Denna kunskap tillämpades sedan för att möjliggöra fysiologisk dopaminerg nervcellsutveckling och mognad med hjälp av organoidtekniken. Till skillnad från vanliga cellkulturer tillåter organoidsystemet cellerna att strukturera och organisera sig i tredimensionella kulturer som efterliknar den process som äger rum under utveckling.

RIASSUNTO IN ITALIANO

La malattia di Parkinson é la più comune tra i disordini del movimento e la seconda malattia neurodegenerativa dopo il morbo di Alzheimer. Solo il 10% dei casi riportati é correlato a mutazioni genetiche ed il restante 90% dei casi non ha una causa conosciuta. I sintomi sono generalmente collegati a difficoltá motorie, anche se sono stati riscontrati altri sintomi come disturbi neuropsichiatrici, disfunzioni del sistema nervoso autonomo e disturbi del sonno. La caratteristica principale della malattia é rappresentata dalla morte dei neuroni dopaminergici localizzati nel cervello che in condizioni fisiologiche permettono la modulazione ed il controllo dell´attivitá motoria tramite il rilascio del neurotrasmettitore Dopamina.

La terapia con cellule staminali é un approccio che si basa sul trapianto intracerebrale di nuovi neuroni generati in laboratorio per permettere la ricostituzione delle funzioni dei neuroni dopaminergici. Le cellule embrioniche staminali e pluripotenti sono studiate per la loro abilitá nel generare neuroni dopaminergici e sono attualmente implicate in studi clinici. Un alternativa all´uso delle cellule staminali é la riprogrammazione di cellule somatiche come le cellule della pelle. Questa tecnica prevede l´espressione forzata di specifici fattori di trascrizione (TFs) o RNA messaggeri (miRNAs) che permettono il passaggio da un fenotipo cellulare ad un altro senza passare attraverso uno stato di proliferazione. Questo aspetto é considerato un vantaggio rispetto all´uso delle cellule staminali in quanto limita la possibilitá di formazione di tumori. Inoltre, la riprogrammazione cellulare puó essere effettuata direttamente nel cervello, somministrando TFs e miRNAs attraverso particelle virali. Quest´ultima applicazione prevede la riprogrammazioni di cellule che risiedono nel cervello ma che hanno funzione di supporto neuronale, permettendo di mantenere inattti i circuiti neuronali preesistenti.

Un´ importante aspetto da considerare é quanto le cellule generate in laboratorio siano simili ai neuroni dopaminergici che si trovano nel cervello umano. Nonostante anni di ricerca, molti aspetti rigurado ai neuroni dopaminergici ancora non sono ancora chiari. Nella prima parte della tesi ho studiato neuroni generati da tecniche di riprogrammazione usando cellule e modelli animali. Nella seconda parte ho poi eseguito un´analisi dettagliata di neuroni dopaminergici ottenuti da embrioni umani. Questo ha permesso di accrescere l´ attuale conoscenza dello sviluppo del cervello umano per quanto riguarda i neuroni dopaminergici. Infine ho generato neuroni tramite il differenziamento di cellule staminali utilizzando organoidi, un sistema che mima lo sviluppo e la maturazione di neuroni in modo piú accurato rispetto ai sistemi tradizioni di colture cellulari.

ABBREVIATIONS

AAV	Adeno-Associated Vector
APs	Action Potentials
COMT	Catechol-O-methyltransferase
DA	Dopamine
DBS	Deep Brain Stimulation
DIV	Days *In Vitro*
FP	Floor Plate
GS3Ki	Glycogen Synthase 3 inhibitor
hESCs	Human Embryonic Stem Cells
hiPSC	Human Induced Pluripotent Stem Cells
HLA	Human Leukocyte Antigen
iDANs	Induced Dopaminergic Neurons
iNs	Induced Neurons
LBs	Lewy Bodies
IsO	Isthmic Organizer
IZ	Intermediate Zone
LV	Lentivirus
MAOB	Monoamine Oxidase Type B Inhibitor
mesDA	Mesencephalic Dopaminergic neurons
miRNA	micro RNA
MOI	Multiplicity of Infection
MZ	Mantle Zone
ORF	Open Reading Frame
Patch-Seq	Patch-sequencing
PD	Parkinson's Disease
PGK	Phosphoglycerate Kinase
PV	Parvalbumin
PSC	Pluripotent Stem Cells
REST	RE1-Silencing Transcription factor
RG	Radial Glia
RMP	Resting Membrane Potential
scRNA-seq	Single Cell RNA Sequencing
shRNA	Short Hairpin RNA
SNpc	Substantia Nigra pars compacta
TH	Tyrosine Hydroxylase
TFs	Transcription Factors
VM	Ventral Midbrain
VTA	Ventral Tegmental Area
VZ	Ventricular Zone
wpc	Weeks Post Conception
w.p.i.	Weeks Post Injection

INTRODUCTION

Parkinson´s Disease

Parkinson's Disease (PD) is the most common movement disorder and second most common neurodegenerative disorder after Alzheimer Disease, affecting around 1% of the population over 60 years of age (de Lau and Breteler, 2006). The incidence of PD reflects a correlation with age as 90% of the cases are among 50 years or older individuals. However, an early onset of the disease is also reported, with 10% of the PD patients at the age of 21-49 years old (Mehanna et al., 2014).

PD is mostly seen in sporadic cases accounting for 90% of the overall cases (Ascherio and Schwarzschild, 2016), nonetheless few causative monogenic mutations have been discovered (Greenamyre and Hastings, 2004). The symptoms experienced by patients were first described by James Parkinson in "An assay on the Shaking Palsy", 1817, and they are nowadays well known to involve motor dysfunctions, such as tremor, rigidity, bradykinesia, hypokinesia, akinesia and freezing. Other symptoms such as neuropsychiatric disturbances, autonomic dysfunctions, sleep problems and sensory symptoms have been linked to PD (Kalia and Lang, 2015).

One disease feature is represented by formation of abnormal protein aggregates, known as Lewy Bodies (LBs), firstly discovered in PD patients´ brains by Spillantini et al., 1997. LBs are mainly composed by the α-synuclein protein that in physiological conditions retains functional roles in different neuronal subcellular compartments (Bendor et al., 2013). However, in pathological conditions it has been shown to spread in a prion-like manner between cells and brain regions (Braak et al., 2003) recruiting functional proteins and favoring the process of LBs formation. This leads to the disruption of normal cellular functions related to mitochondrial, lysosomal and synaptic activity.

Another hallmark of the pathology is represented by a loss of mesencephalic Dopaminergic neurons (mesDA) that reside in the Substantia Nigra pars compacta (SNpc) and connects to the caudate-putamen in the basal ganglia circuit where they modulate motor output and control by releasing the neurotransmitter Dopamine (DA) (Björklund and Dunnett, 2007). The etiology of mesDA death is still unknown, however LBs formation could represent one of the factor contributing to neuronal death and PD progression (Stefanis, 2012).

Treatments and Therapies for PD

Current treatments are mainly characterized by drug administration including Levodopa, DA agonists, Monoamine oxidase type B inhibitor (MAO-B) and catechol-O-methyltransferase (COMT). These treatments can restore dopaminergic activity in the striatum and alleviate the impaired motor

deficits of PD patients. However, they do not treat many of the non-motor features and they are associated with several side effects (Kalia and Lang, 2015).

Another approach to treat PD is represented by deep brain stimulation (DBS), where electrodes are surgically implanted in the brain to deliver stimulating electrical signals for DA release. The difficult surgical procedure as well as a short action range of the electrodes limit the application of DBS for PD (Lozano et al., 2019).

Cell Replacement Therapy is an alternative approach to restore the function of the dopaminergic neurons through the transplantation of the lost cells. This field was initiated in the 1980´s when human fetal ventral midbrain tissue (VM) was transplanted intracerebrally into patients (Lindvall et al., 1989). This treatment resulted in the restoration of DA release and long-term clinical improvements in some patients (Lindvall et al., 1990, 1994; Wenning et al., 1997; Brundin et al., 2000; Barker et al., 2015). Despite the positive results, the outcome has been very variable and graft-induced dyskinesia have been reported and hypothesized to be due to serotoninergic contaminant neurons in the graft. These complications, together with the restricted tissue availability, limit the use of fetal VM in cell replacement therapy for PD. New renewable sources of cells derived from stem cells such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSC) have now been investigated and implicated in clinical trials for PD (Barker et al., 2017; Barker et al., 2018).

A possible future source of mesDA neurons is also represented by neurons directly converted from skin fibroblasts, so called induced neurons (iNs) (Caiazzo et al., 2011; Pfisterer et al., 2011). This process, known as direct reprogramming, is achieved via virus-dependent delivery of specific transcription factors (TFs), micro RNAs (miRNA) and/or small molecules. It allows for short and cost effective protocols, favoring the possibility of establishing personalized medicine. It also limits concerns regarding tumorigenicity which are present when using hESCs and hiPSCs. However, upon transplantation, low cell survival and integration have been reported (Kim et al., 2011; Caiazzo et al., 2011; Dell'Anno et al., 2014). On the other hand, iNs have been shown to retain the aging signature of the donor cells (Mertens, et al., 2015; Huh et al., 2016) and their potential application for cell disease modeling is been investigated (Drouin-Ouellet et al., 2017).

Another future venue for restoring neuronal functions in the brain is depicted by *in vivo* reprogramming of resident cells. Indeed, non neuronal resident brain cells can be targeted through systemic virus delivery and directly reprogrammed into the desired neuronal subtype (Buffo et al., 2005; Torper et al., 2013; Grande et al., 2013; Niu et al., 2013; Magnusson et al., 2014; Heinrich et al., 2014; Guo et al., 2014; Niu et al., 2015; O Torper et al., 2015; Liu et al., 2015; Brulet et al., 2017; Rivetti Di Val Cervo et al., 2017; Weinberg et al., 2017; Mattugini et al., 2019; Qian et al., 2020; Zhou et al., 2020). However, this promising approach has major challenges to be circumvent for clinical application, such as neuronal survival and innervation in injured or diseased brain and efficient reprogramming into human mesDA neurons.

DA neurons

Generating DA Neurons trough *in vitro* technologies or via *in vivo* reprogramming requires molecular, transcriptional and functional understanding of human mesDA neurons. Here a summary of

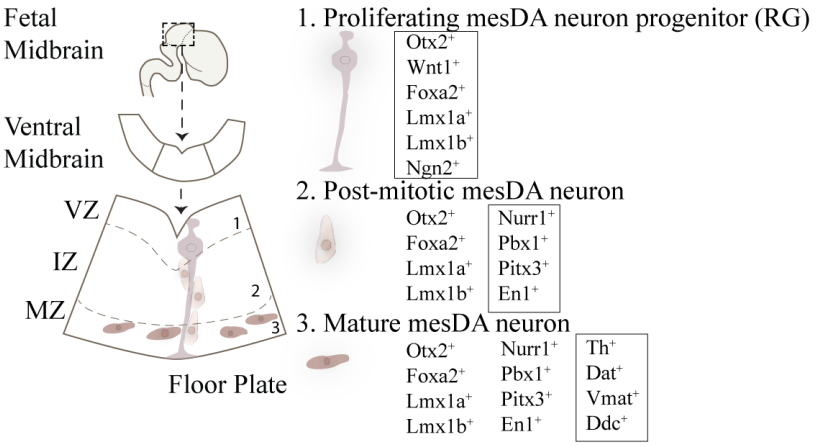


Figure 1 *Schematic representation of midbrain dopaminergic neurons development and their gene expression at different stages*.

Abbreviations: VZ, ventricular zone; IZ, intermediate zone; MZ, mantel zone; mesDA, mesencephalic dopaminergic neurons; RG, radial glia.

the current knowledge in these fields and highlights of subjects that need to be investigated for moving forward in finding treatments for PD.

Origin of Dopaminergic Neurons

The mesDA neurons arise from the most ventral part of the mesencephalon and they are derived from proliferating Radial Glia (RG) cells located in the ventricular zone (VZ) of the medial floor plate (FP) (Figure 1)(Ono et al., 2007; Hebsgaard et al., 2009; Nelander et al., 2009). At the boundary between the midbrain-hindbrain a signaling center, the isthmic organizer (IsO), is responsible for the expression of the TF *Otx2* (Millet et al., 1996; Broccoli et al., 1999) and for the secretion of the morphogen Wnt1 in the midbrain (Nordström et al., 2002). These signals are essential in establishing the midbrain progenitor domain and the following mesDA neurogenesis (Ásgrímsdóttir and Arenas, 2020). Upon specification, DA progenitors begin to express transcription factors required for mesDA neuron development, *Foxa2, Lmx1a, Lmx1b* (Andersson et al., 2006; Ferri et al., 2007; Nelander et al., 2009; Marklund et al., 2014). These progenitors expand and subsequently undergo neurogenesis, a process regulated by the proneural genes *Neurog2* and *Mash1* (Kele et al., 2006) that results the generation of post-mitotic mesDA neuroblasts that maintain the expression of *Otx2, Foxa2, Lmx1a/b* and

start to express new genes such as the TF *Nr4a2 (Nurr1)*(Zetterström et al., 1996; Ásgrímsdóttir and Arenas, 2020). Post-mitotic cells migrate from the VZ to the intermediate zone (IZ) and finally reach the mantle zone (MZ) where the mesDA post-mitotic cells mature into functional neurons secreting DA into their target. During this migration process, the cells acquire the expression of other TFs required for mesDA neuron development, *Pbx1* (Villaescusa et al., 2016), *PITX3* and *Engrailed1* (Smidt et al., 2004; Maxwell et al., 2005; Veenvliet et al., 2013). Maintaining the expression of these key genes, cells subsequently are found to be enriched for genes related to DA function, such as the enzyme for DA production, *tyrosine hydroxylase* (*TH*), dopamine and monoamine transporters, *Slc6A3/DAT* and *Slc18a2/Vmat2* (Molinoff and Axelrod, 1971; Miller et al., 1999; Nelander et al., 2009).

Transcriptional profile of DA neurons

Adult midbrain DA neurons are traditionally classified based on their location and projection in two main subtypes. The A9 subclass, that populates the SN and is mainly involved in motor control and the A10 populations that populate the ventral tegmental area (VTA) and generates connections through the mesolimbic and mesostriatal pathways (Björklund and Dunnett, 2007). However recent studies have examined the molecular diversity of mesDA neurons through transcriptional analysis at the single cell level, single cell RNA-sequencing (scRNA-seq) determining cell heterogeneity and developmental trajectories (Poulin et al., 2014; La Manno et al., 2016; Hook et al., 2018; Tiklová et al., 2020). These studies have almost exclusively been performed in mice where up to 7 different DA populations were found (Table 1)(reviewed in (Poulin et al., 2020). Only one study (La Manno et al., 2016) has compared mouse and human development using scRNA-seq and profiled VM suggests the emergence of 3 different DA subtypes during early development. However in order to elucidate the exact molecular profile of mesDA neurons, more studies should confirm these human mesDA developmental groups and correlate these populations with mature DA subtypes.

Functional profile of mesDA neurons

mesDA neurons in the SN make connections with the striatum through the nigrostriatal pathway. Here they modulate the activity of medium spiny projection neurons releasing the neurotransmitter DA (Freund et al., 1984; Voorn et al., 1988). This modulation depends on the ability of DA to activate D1-receptor expressing spiny neurons and inactivate D2- neurons. Activation of dopaminergic neurons is regulated through the presence of D2 autoreceptors as well as NMDA receptors on mesDA dendrites and axons (GluR1 and AMPA) (Christoffersen et al., 1995; Albers et al., 1999).

Studies from rat midbrain DA neurons suggest at least two distinct firing patterns among A9 and A10 neurons (Grenhoff et al., 1988) with less regular discharge and more burst firing in the A10 group (Ungless and Grace, 2012). Other characteristics such a resting membrane potential around -60 mV, threshold of -41/-36 mV, long duration of action potentials (APs) (>2 ms), input resistance around 700-800 MΩ (Grace and Onn, 1989; Shepard and Bunney, 1991; Kang and Kitai, 1993; Pacheco-Cano et al., 1996) were characterized in DA neurons from rat slice preparations.

Furthermore, DA neurons are characterized by a unique pacemaking like firing: a slow membrane depolarizing conductance depolarizes the neuron from its resting membrane potential threshold for spike generation, typical of DA neurons. The action potential (AP) is followed by a calcium-dependent afterhyperpolarization followed by initiation of a slow depolarization. However, detailed knowl-

Table 1 *Classification of 7 dopaminergic clusters based on gene expression and localization*.
Table adapted from Poulin et al., 2020. Abbreviations: SNpc, substantia nigra pars compacta; VTA, ventral tegmental area.

	Aldh1a1$^+$ Sox6$^+$	Aldh1a1$^-$ Sox6$^+$	Vglut$^+$	Vglut$^+$	Vgat$^+$	Aldh1a1$^+$ Otx2$^+$	Vip$^+$
Genes	*TH* *Aldh1a1* *Sox6* *Aldh1a7* *Ndnf* *Igf1* *Sncg* *Vcan* *Anxa1* *Grin2c*	*TH* *Sox6* *Ndnf* *Igf1* *Sncg* *Calb1* *Lypd1* *Tacr3* *Cyp26b1*	*TH* *Vglut2* *Calb1*	*TH* *Vglut2* *Calb1*	*TH* *Vgat* *Calb1* *Crhbp* *Gad2* *Wnt7b* *Vglut2-* *(Slc17a6)* *Dat*	*TH* *Aldh1a1* *Otx2* *Lpl* *Neurod6* *Gpr83* *Grp* *Cbln4* *Vglut2*	*TH* *Vip* *Gipr* *Calb1* *Vglut2*
Location	Ventral SNpc	SNpc, Parabrachial pigmented region of VTA, Retrorubral area	Dorsolateral SNpc	VTA	VTA	Ventromedial VTA	Periaqueductal gray, Dorsal raphe

edge of how human mesDA neurons function *in vitro* or *in vivo* and in relation to their transcriptional profile needs to be elucidated.

Generating mesDA neurons *in vitro*

mesDA neurons from hESCs

hESCs were first successfully isolated from the inner cell mass of the blastocyst (Figure 2) by Thomson et al 1988, a major breakthrough for developmental studies and cell replacement therapies. The main characteristics of the derived cells are their infinite potential of expansion in culture and their possible ability to differentiate into any of the three germ layers cells upon cell-specific signals activation. Thereafter scientists succeeded in generating neurons through hESCs differentiation and embryoid body (EB) formation (Itskovitz-Eldor et al., 2000; Reubinoff et al., 2001; Zhang et al., 2001).

An important improvement of neuronal differentiation took place when a protocol for an optimized neuralization was obtained (Chambers et al., 2009). This protocol is based on the addition of Nogging and SB431542 to inhibit bone morphogen proteins (BMPs) and blocking pathways of Lefty, Activin and Transforming growths factor beta (TGFb). Along with the discovery of the FP origin of the DA neurons, another group subsequently showed the possibility of FP induction through the use of Sonic Hedgehog (SHH) (Fasano et al., 2010). In this protocol, forebrain neurons were obtained. Only with the application of patterning factors such as WNT through the use of a chemical inhibitor of glycogen synthase kinase 3 (GSK3) brought to the generation of bona fide mesDA neurons (Kriks

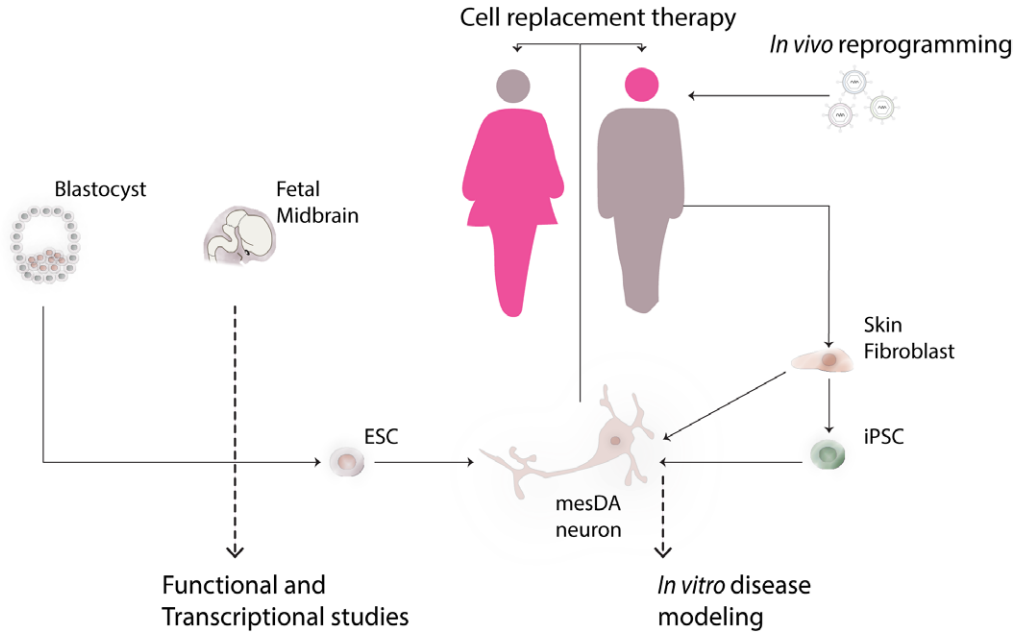


Figure 2 *Schematic overview of different cell sources used for therapies and studies of Parkinson´s Disease*.
Abbreviations: mesDA, mesencephalic dopaminergic; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

et al., 2011; Kirkeby et al., 2012; Nolbrant et al., 2017). These cells express the FP and midbrain markers such as *OTX2, LMX1A, FOXA2* and *TH*, and they shows functional properties of DA neurons and they integrate upon transplantation (Kriks et al., 2011; Doi et al., 2014; Grealish et al., 2015; Cardoso et al., 2018; Adler et al., 2019).

mesDA neurons from iPSC

In 2006 another groundbreaking discovery in the field of pluripotent stem cell and development took place. Yamanaka and colleagues showed how mouse and human somatic cells can be reprogrammed into iPSCs using virus mediated delivery of four pluripotency factor (Takahashi et al., 2007). This allows to revert any somatic cell into a pluripotent state and subsequent differentiation of this into any specific cell type.

Of particular importance, this discovery lead to the possibility of generating patient specific lines or match human leukocyte antigen (HLA) donors for cell-based therapies. Such applications of these cells are currently ongoing and results will answer key questions on functionality and integration of these cells (Barker et al., 2017; Parmar and Björklund, 2020).

With this technique, fibroblasts or peripheral blood mononuclear cells (PBMCs) obtained from PD patients can be reverted back to pluripotency, and then differentiated into mesDA neurons allowing to study cellular mechanisms related to the pathology, vulnerability, and degeneration of these neurons (Figure 2).

mesDa neurons from skin fibroblasts

iNs are reprogrammed somatic cells that are forced to change their fate without passing through a pluripotent state (Figure 2) thanks to the viral delivery of genes related to neuronal induction and maturation (Vierbuchen et al., 2010).

The main advantages of direct reprogramming are represented by a fast protocol needed for the generation of the neurons of interest, a low risk of genetic mutations insertion or tumor formation due to the absence of a pluripotent step, a homogeneity of the target population produced with low line to line variability and the possibility to resemble the patient-aged cellular phenotype (Tanabe et al., 2015).

Several works have been carried out in order to generate dopaminergic (iDANs) neurons from somatic cells in vitro (Addis et al., 2011; J Kim et al., 2011; Caiazzo et al., 2011b; Pfisterer et al., 2011; Liu et al., 2012; Dell'Anno et al., 2014; Torper et al., 2015) indicating that different factor combinations can successfully generate iDANs. The functionality of these cells have been analysed *in vitro* and upon transplantation in animal models (Kim et al., 2011; Caiazzo et al., 2011b; Dell'Anno et al., 2014) showing mature DA neuronal profile for iDANs generated from mouse skin fibroblasts or human fetal skin fibroblast. Nevertheless studies applying direct reprogramming on human adult fibroblasts to neurons are very few (Table 2) and physiological activity of direct reprogrammed DA neurons from human adult fibroblasts has been so far reported only in one study (Caiazzo et al., 2011) and transcriptional studies to highlight differences and similarities with human mesDA neurons are still missing.

Generating DA neurons *in vivo*

In vivo reprogramming is based on the idea of converting resident brain cells into a specific cell of interest that are impaired or lost in a diseased brain (Figure 2). A particular suitable target cell for this approach is represented by glia cells, proliferative and widely distributed cells in the brain parenchyma (Dimou and Götz, 2014). *In vivo* reprogramming eliminates the introduction of external cells into the brain, avoiding the risk of transplant rejection. Many studies have successfully generated neurons that acquire a diverse neuronal subtype, such as GABAergic, glutamatergic and DA phenotype (Grande et al., 2013; Niu et al., 2013; Torper et al., 2015; Rivetti Di Val Cervo et al., 2017). Recently published works showed improvements in the generation of mesDA neurons by targeting the RNA-binding protein PTB in a chemically induced mouse model of PD (Qian et al., 2020; Zhou et al., 2020) that resulted in high reprogramming efficiency and motor skills recovery. Whether these approaches will show similar results in different animal models of PD will have to be addressed in the future, however these results provide further proofs supporting the use of in vivo reprogramming as a restorative approach in PD.

Table 2 *Reports of direct neuronal reprogramming of human adult skin cells into neurons (iNs) in vitro*.

	Factor Combination	Neuronal Subtype	Efficiency	Functional assessment	RNA- sequencing
Caiazzo et al. 2011	*Ascl1* *Nurr1* *Lmx1a*	Dopaminergic	Tuj1$^+$ 5% ±1 TH$^+$3% ±1	Whole-Cell Patch-Clamp (details not specified)	NA
Ladewig et al. 2012	*Ascl1* *Ngn2* Small Molecules	Gabaergic Glutamatergic	bIII-tub$^+$ 13.2% ±1.4	NA	NA
Hu et al. 2015	Small Molecules	Glutamatergic	Tuj1$^+$ and Map2$^+$ Min 3.9 ±1.2 Max 12.6 ± 1.1	Whole-Cell Patch-Clamp + Calcium Imaging (co-culture active at 14 DIV)	NA
Ouellet et al. 2017	*Ascl1* *Brn2* shREST Small Molecules	NA	MAP2$^+$ 40% ca TAU$^+$ 50% ca	Whole-Cell Patch-Clamp (co-culture active at 90-100 DIV)	Bulk RNA-Seq
Yang et al. 2019	Small Molecules	Glutamatergic	TUJ1$^+$ 40.3% 36 ± 2.6 MAP2$^+$ 35.1 ± 2.4, TAU$^+$ 32.7% ± 3.1	NA	NA

Abbreviations: NA, not assessed; DIV, days *in vitro*.

Caiazzo, M. et al. (2011) 'Direct generation of functional dopaminergic neurons from mouse and human fibroblasts', Nature, 476(7359), pp. 224–227. doi: 10.1038/nature10284.

Drouin-Ouellet, J. et al. (2017) 'REST suppression mediates neural conversion of adult human fibroblasts via microRNA-dependent and -independent pathways', EMBO Molecular Medicine, 9(8), pp. 1117–1131. doi: 10.15252/emmm.201607471.

Hu, W. et al. (2015) 'Direct Conversion of Normal and Alzheimer's Disease Human Fibroblasts into Neuronal Cells by Small Molecules', Cell Stem Cell. Cell Press, 17(2), pp. 204–212. doi: 10.1016/j.stem.2015.07.006.

Ladewig, J. et al. (2012) 'Small molecules enable highly efficient neuronal conversion of human fibroblasts', Nature Methods. Nat Methods, 9(6), pp. 575–578. doi: 10.1038/nmeth.1972.

Yang, Y. et al. (2019) 'Rapid and Efficient Conversion of Human Fibroblasts into Functional Neurons by Small Molecules', Stem Cell Reports. Cell Press, 13(5), pp. 862–876. doi: 10.1016/j.stemcr.2019.09.007.

Bridging the gap between *in vitro* and *in vivo* studies: 3D systems and organoids with midbrain profile

In 2013 the work from Lancaster et al., launched a new era in the research of the human brain with the generation of "cortical organoids".

These 3D structures are made of self-organizing human PSCs that differentiate without patterning factors (Kadoshima et al., 2013) giving rise to different brain regions including hindbrain, midbrain, forebrain and retinal cells in a single organoid. Remarkably, patterning events taking place in the organoids closely resembled the ones occurring in the brain (Renner et al., 2017). Variability in brain regions formation across organoids was however a limitation of this protocol, resulting in new protocols involving patterning factors for generating selected brain structures such as the cortex (Sloan et al., 2018). More recently human midbrain organoids have been generated from regionally patterned neural stem cells (NSC) (Tieng et al., 2014; Jo et al., 2016; Qian et al., 2016; Monzel et al., 2017; Kim et al., 2019; Smits et al., 2019). Cells committed to the FP identity of the mesencephalon, have been subjected under 3D condition to specific spatio-temporal signaling following previously established protocols in 2D cultures (Kriks et al., 2011; Kirkeby et al., 2012; Reinhardt et al., 2013). The generated organoids showed the expression of mature DA markers such as *TH* and *DAT* together with signs of mature neuronal cells, as myelin formation (Faivre-Sarrailh and Devaux, 2013). Electrophysiological properties measured by Multi-Electrode Array (MEA) (Tieng et al., 2014) or with whole-cell patch-clamp recordings (Jo et al., 2016; Qian et al., 2016; Monzel et al., 2017; Kim et al., 2019) indicated presence of mature network of DA neurons. Ultimately functionality of mesDA organoids was detected in form of DA release (Smits et al., 2019) and presence of Neuromelanin deposits (Jo et al., 2016).

Overall, the use of these systems supply a unique way for researchers to address transcriptional and functional questions in a human context, bridging the gap between *in vitro* cultures and animal models.

AIMS OF THE THESIS

The overall aim of my thesis has been to assess the functionality and transcriptional profile of neurons derived from stem cells or via direct reprogramming *in vitro* and *in vivo*. A major focus has been to relate functional and transcriptional profile of newly generated mesDA neurons *in vitro* and *in vivo* with the final aim to contribute to new cell-based therapies of PD.

The specific aims of my thesis were to:

1. Optimize the generation of functional neurons from directly reprogrammed human adult skin fibroblasts *in vitro* (**Paper I**)

2. Generate DA neurons from healthy and PD human adult skin fibroblasts and assess their electrophysiological properties (**Paper II**)

3. Evaluate the profile of newly reprogrammed neurons generated via AAV delivery of DA fate determinants in animal models of PD (**Paper III**)

4. Develop a 3D culture system for characterizing transcriptional and functional properties of human fetal VM DA neurons (**Paper IV**)

5. Analyse the ability of a stem cell derived brain organoid system to retain molecular, functional and transcriptional characteristics of the VM (**Paper V**)

ADDITIONAL PAPERS AND REVIEW ARTICLES NOT INCLUDED IN THE THESIS

In addition to the papers included in this thesis, additional studies performed during my PhD studies have resulted in the following publications:

Direct reprogramming intro interneurons: potential for brain repair.

Pereira M, **Birtele** M, Rylander Ottosson D.

Cellular and Molecular Life Science. 2019 Oct;76(20):3953-3967

In Vivo Direct Reprogramming of Residual Glial Cells into Interneurons by Intracerebral Injections of Viral Vectors.

Pereira M, **Birtele M**, Rylander Ottosson D.

Journal of Visualized Experiments. 2019 Jun 117;(148)

Single Cell Gene Expression Analysis Reveals Human Stem Cell-Derived Graft Composition in a Cell Therapy Model of Parkinson´s Disease.

Tiklová K, Nolbrant S, Fiorenzano A, Björklund Å K, Sharma Y, Heuer A, Gillberg L, Hoban D B, Cardoso T, Adler A F, **Birtele M**, Lundén-Miguel H, Volakakis N, Kirkeby A, Perlmann T, Parmar M.

Nature Communication. 2020 11:2434

3D- Printed Soft Lithography for Complex Compartmentalized Microfluidic Neural Devices.

Kajtez J, Buchman S, Vasudevan S, **Birtele M**, Rocchetti S, Pless CJ, Heiskanen A, Barker A R, Martinez-Serrano A, Parmar M, Ulrik Lind J U, Emnéus J.

Advanced Science. 2020, 202001150

SUMMARY OF RESULTS AND DISCUSSION

Cell based therapies for PD rely on the capability of differentiation and reprogramming protocols to successfully generate authentic mesDA neurons. To investigate this, I have been focusing on understanding functional and transcriptional properties of newly generated cells as well as human fetal mesDA neurons. In **paper I** I show how human skin fibroblasts from adult donors can be directly reprogrammed into functional neurons *in vitro*. However, their subtype-specific identity resemble an heterogenous neuronal population. In the subsequent study, **paper II**, I focused on generating DA neurons *in vitro* via direct reprogramming of human adult fibroblasts from healthy and PD donors. In **paper III** I applied the direct reprogramming technique *in vivo* and evaluated the profile of newly generated neurons. However, reprogrammed neurons did not show the desired DA profile. Results from this study highlighted the gap between *in vitro* and *in vivo* experiments, leading to the need of expanding our knowledge in the DA neuronal development. We therefore established a relevant system where to study functional and transcriptional profile of authentic VM neurons, **paper IV**. Lastly, in **paper V**, I set up a brain organoid model of VM from hPSC to reproduce the generation of authentic DA neurons.

Improving functional maturation of directly reprogrammed neurons from human adult fibroblasts in long term *in vitro* cultures (Paper I)

Neuronal conversion of human adult cells into functional neurons is of value for both disease modelling and patient-specific cell therapy treatments. However, reports have shown how human cells are harder to reprogram compared to rodent cells (Caiazzo et al., 2011; Xue et al., 2013, 2016) and how adult donors have lower reprogramming efficiencies compared to fetal cells (Pfisterer et al., 2011; Liu et al., 2013). To address this challenge, our group previously published a study showing how the suppression of the RE1-Silencing Transcription factor (REST) complex using a short hairpin RNA (shREST) is a key factor for generating neurons at high efficiency from human adult cells (Drouin-Ouellet et al., 2017a). In Ouellet et al., a single vector expressing shREST and the genes *ASCL1* and *BRN2* (AB-shREST), was developed. The neural conversion via AB-shREST was found to be in part, but not fully, mediated via microRNAs upregulation. This led us to investigate whether the AB-shREST cocktail together with miRNAs could improve the functional profile of the new iNs.

In this study, we decided to use an upregulation of mir9 and mir124, already known to improve neuronal reprogramming (Yoo et al., 2011; Drouin-Ouellet et al., 2017), to see if they were influencing the maturation of cells when delivered together with our new "single vector" conversion protocol (Drouin-Ouellet et al., 2017a; Shrigley et al., 2018).

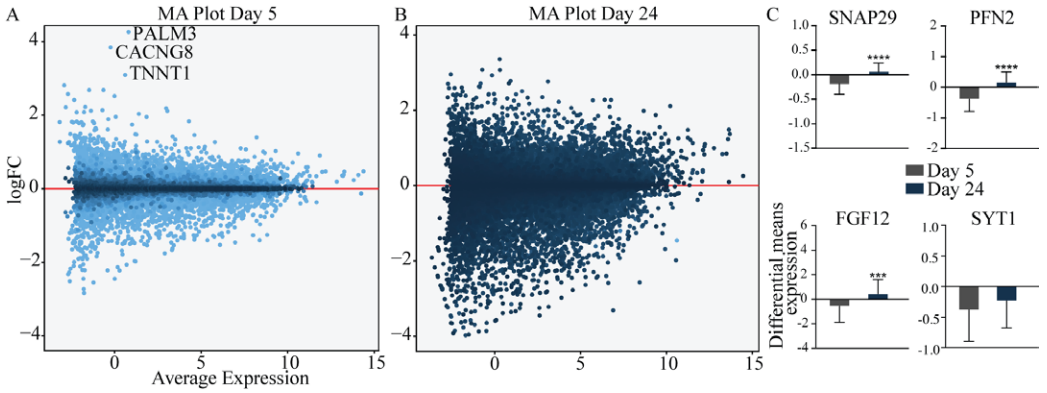


Figure 3 *Differential gene expression in iNs converted with AB-shREST-miR9/124 vs AB-shREST.*

(A) MA plot at day 5 indicating average gene expression of the samples AB-shREST-miR9/124 (n = 3) compared to AB-shREST (n = 3). At this time point, genes related to ion and calcium regulation such as *PALM3*, *CACNG8*, and *TNNT1* were found to be upregulated in the AB-shREST-miR9/124 condition. (B) MA plot at day 24 indicating average gene expression of the samples AB-shREST-miR9/124 (n = 3) compared to AB-shREST (n = 3). At this time point, the general high divergence of gene expression can be found in between the conditions. (C) Plots of differential expression for genes related to neuronal maturation and function such as *SNAP29, FGF12, SYT1*, and PFN2 in AB-shREST-miR9/124 samples at day 5 and day 24. Plots show an increase in these genes at day 24 in AB-shREST-miR9/124-converted cells.

miRNAs added to the reprogramming factors increase the expression of genes associated with neural development and cell communication at early stages of the conversion

To investigate the effect of miR9 and miR124 in the direct reprogramming of human adult fibroblasts I performed global gene expression analysis of fibroblasts converted with AB-shREST and cells converted using miR9 and miR124 in addition to *Ascl1, Brn2,* and shREST (AB-shREST-miR9/124) at 5 days post-conversion. Results showed that a few genes associated with calcium signaling (*CACNG8, TNNT1, PALM3*) were more highly expressed in the ABshREST-miR9/124 group (Figure 3A). Next, I performed similar analysis at 24 days after conversion and found that there was more divergent gene expression between the microRNA- and non-microRNA-reprogrammed cells (Figure 3B). Interestingly, comparing the gene expression between days 5 and 24, I found that synaptic or ion channel related genes *SNAP29, PFN2*, and *FGF12* were increased significantly over time (Figure 3C). However, there were no signs of physiological neuronal maturation at this time point with either conversion methods (Figure 4). This suggests that despite some differences in the expression of genes related to neuronal maturation and synaptic function between the two conditions, none of the conditions were functionally mature at this relatively early time point.

In long term cultures, the expression of miRNA9/124 together with shREST leads to iNs maturation and neuronal subtype specification

Next, I analyzed the effect of miR9/miR124 and shREST on functional maturation at a later stage of the reprogramming process (Figure 5A). After 80 – 85 days *in vitro* the majority of iNs converted

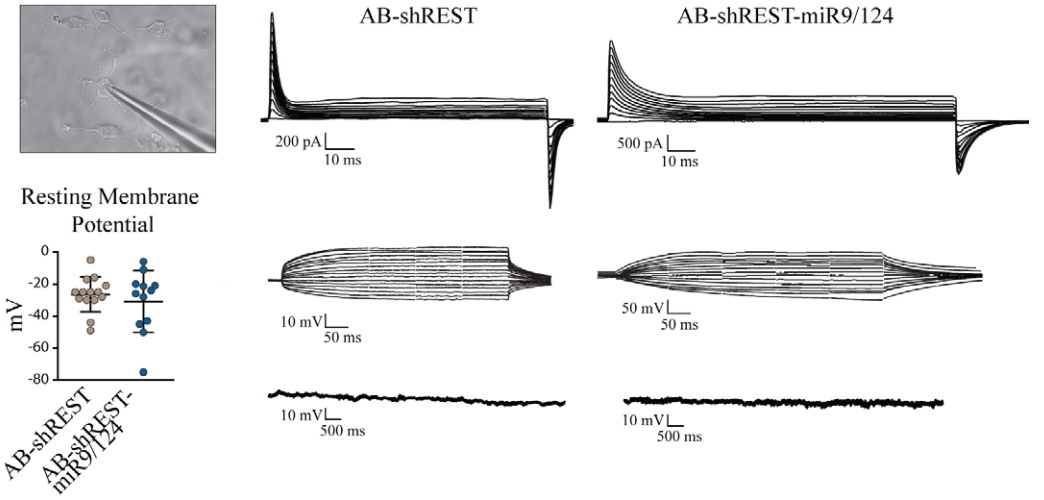


Figure 4 *Whole-cell patch-clamp recordings of AB-shREST and AB-shREST-miR9/124 conditions at day 24.*

Bight field image representing patch pipette targeting a single neuron. Plot of RMP values from AB-shREST condition and AB-shREST-miR9/124 condition. Values show immature RMP for both conditions. Plots indicating the mean of RMP and relative SEM calculated from Student's t-test analysis. (Upper figure) Examples of inward sodium/outward potassium currents from whole-cell patch-clamp recordings for AB-shREST condition (left panel) and AB-shREST-miR9/124 condition (right panel). All recordings showed an absence of currents. (Middle figure) Examples of induced APs from whole-cell patch-clamp recordings for AB-shREST condition (left panel) and AB-shREST-miR9/124 condition (right panel). All recordings showed an absence of induced APs. (Lower figure) Examples of spontaneous firing from whole-cell patch-clamp recordings for AB-shREST condition (left panel) and AB-shREST-miR9/124 condition (right panel). All recordings showed an absence of activity.

with AB-shRESTmiRNA124/9 showed presence of inward sodium currents (Figure 5B) and a higher proportion of cells were capable of firing current-induced APs (Figure 5C). Furthermore, the APs generated were of higher amplitude in this group, indicating a greater maturation level in comparison with cells reprogrammed with miR9/124 or shREST only, in which only immature APs could be detected. In these iNs, the presence of spontaneous firing was detected in current clamp mode, indicating that the maturation level in this group was higher compared to the reprogramming conditions with miR9/124 or shREST only, where spontaneous firing was absent.

When looking for specific neurotransmitter phenotypes, cells showed a similar expression of somatostatin-, GABAergic-, glutamatergic-, acetylcholinergic-, and dopaminergic-related genes *(SSTR1, GABRA1, GRIA2, CHRMA43, and DRD1)* (Figure 5D).

Overall these data support the finding that mir9 and mir124 are involved in the neuronal maturation, particularly it seems to improve functionality over long periods of time when reprogramming human adult skin cells. The established approach results in functional neurons, however it does not seem to generate a single neuronal cell type but rather an heterogenous neuronal population.

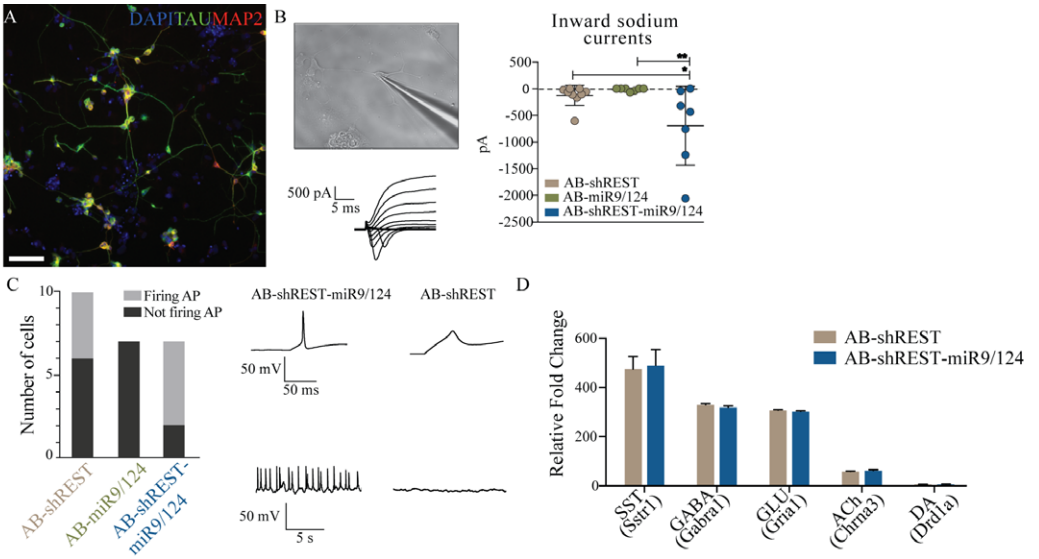


Figure 5 *Whole-cell patch-clamp recordings of iNs converted with different vectors at 80 DIV.*

(A) Representative immunofluorescence image showing TAU+ and MAP2+ cells on PFL coating at 60 DIV. (B) Representative image of a patched iN and representative inward sodium/outward potassium electrophysiological recording of iNs reprogrammed using AB-shREST1-miR9/124. Quantification of Inward sodium current (AB-shREST, n = 9; AB-miR9/124, n = 7; AB-shREST-miR9/124, n = 7) revealed higher sodium currents in AB-shREST-miR9/124 condition. (C) Quantification of the number of cells firing APs in each group
(AB-shREST, n = 9; AB-miR9/124, n = 7; AB-shREST-miR9/124, n = 7). (Upper figure) Example of mature induced AP in an iN cell reprogrammed using AB-shREST1-miR9/124 and immature induced AP present in an iN cell reprogrammed using AB-shREST. (Lower figure) Example of spontaneous firing present in the group ABshREST-miR9/124 and absence of spontaneous firing in the group AB-miR9/124. (D) Relative fold change expression for specific genes such as *SSTR1, GABRA1*, *GRIA1*, *CHRNA3*, and *DRD1* indicating similar expression of neuronal subtype generated in both AB-shREST1 (n = 3) and AB-shRESTmiR9/124 (n = 3) conditions.

Generating functional neurons with DA specific phenotype *in vitro* via direct reprogramming of human adult skin fibroblasts from healthy and PD donors (Paper II)

Next, we investigated the possibility to generate dopaminergic neurons (iDANs) through different TFs combinations in addition to *Ascl1, Brn2*, and shREST. Our group previously showed the ability to directly reprogram human fibroblasts for generating DA neurons from different cell sources such as fetal skin, fetal lung and newborn foreskin (Pfisterer et al., 2011). In the same year, another group (Caiazzo et al., 2011) demonstrated the generation of iDANs from mouse embryonic and human adult fibroblasts through the forced expression of *Mash1, Nurr1* and *Lmx1a*. However the extent of the maturation levels of these cells was not determined in iDANs derived from healthy and PD donors.

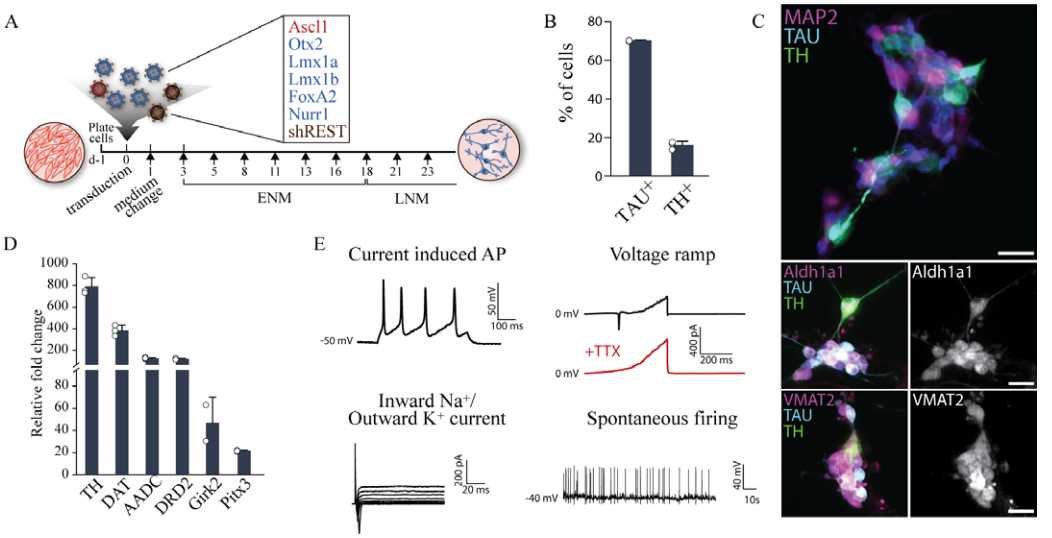


Figure 6 *Successful generation of iDANs from human adult fibroblasts*.

(A) Overview of the protocol used for generating iDANs. (B) Quantification of TAU+ and TH+ cells (mean average of 13,527 TAU+ and 2,826 TH+ cells assessed per well from 3 biological replicates). (C) Double TAU+ and TH+ iDANs expressing Aldh1a1 and VMAT2. Cells are counterstained with DAPI (in blue). (D) Gene expression quantification of DA genes relative to parental fibroblast levels (from 3 biological replicates). (E) Patch clamp recordings of iDANs (at day 65) showing presence of current induced APs, Inward NA+ - Outward K+ currents, selectively blocked signals with tetrodotoxin (ttx) and spontaneous firing.

In this study my focus was to investigate how to efficiently generate iDANs with mature DA phenotype and functionally characterize iDANs generated from healthy and PD donors.

A combination of known DA genes generates functional iDANs from human adult skin fibroblasts

We screened different reprogramming factors that were selected based on: their role during normal DA neurogenesis, their expression in human fetal ventral midbrain, and their role on midbrain-specific chromatin modeling. All factors were expressed in combination with the knockdown of REST. The best TH+ cell yield was obtained with the combination that includes shREST, *Ascl1, Lmx1a/b, Foxa2, Otx2, Nurr1*(Figure 6A). This combination gave rise to 70.33 %± 0.31 % of cells expressing the neuronal marker TAU+ of which 16.1 % ± 2.01 expressed TH (Figure 6B).

Further characterization of the iDANs showed that in addition to TH, these cells also expressed ALDH1A1, which is found in a subset of A9 DA neurons that are more vulnerable to toxins associated with the development of PD, and VMAT2 a key DA marker (Figure 6C). Gene expression profiling confirmed an up-regulation of key genes related to the DA patterning and identity *(FOXA1, OTX1, SHH, PITX3)*, as well as DA synaptic function including the receptors *DRD1* to *DRD5*, the DA transporter *DAT*, the enzymes *DDC, MAOA, ALDH1A1* and the A9-enriched DA marker

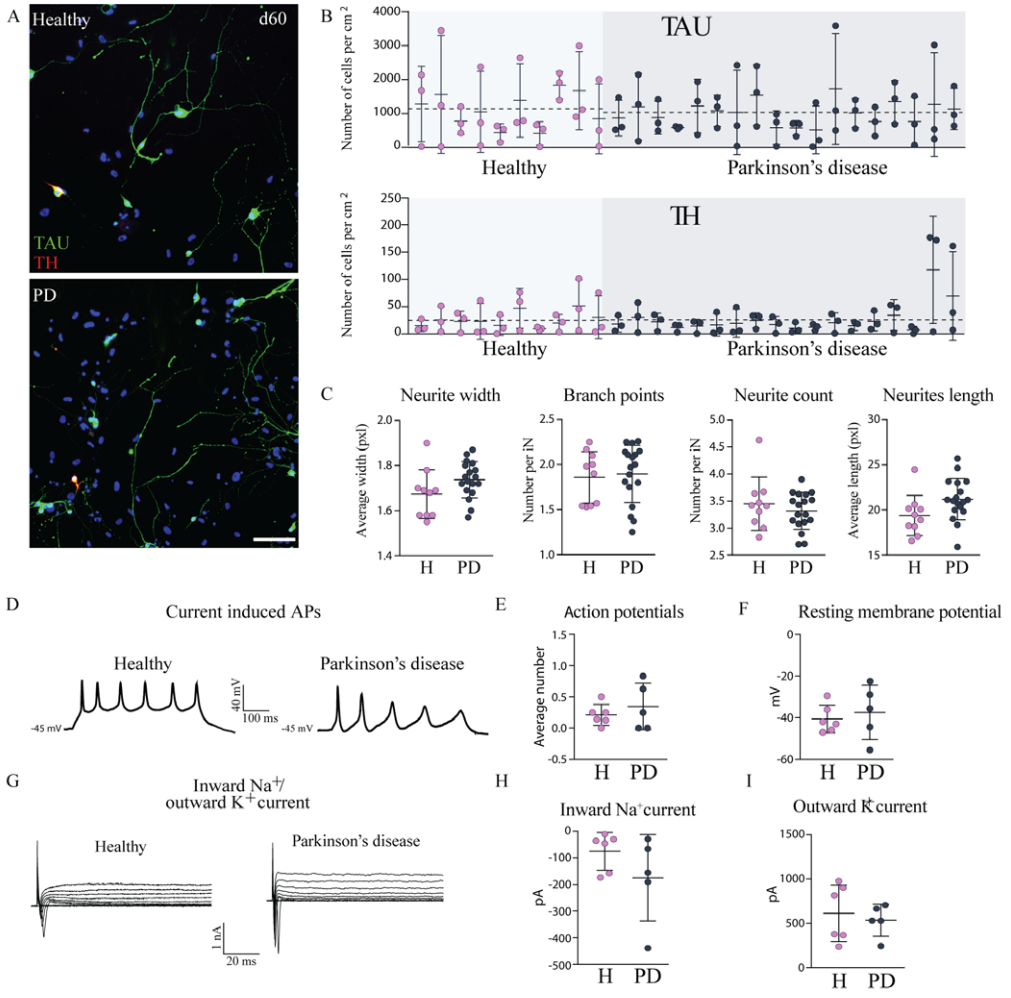


Figure 7 *iDANs from PD and heathy donor lines*.

(A) Quantification of TAU+ and TH+ cells (experiment has been repeated independently 3 times). Dashed lines represent the mean. (B) Double TAU+ and TH+ H-iDANs and PD-iDANs at day 60. Scale bar = 100µm. (C) Quantification of neurite profile in TAU+ H-iNs and PD-iNs. (D) Current-clamp recordings of evoked action potentials. (E) Quantification of current-clamp recordings of evoked action potentials (n = 8-10 neurons per lines, n = 5-6 lines per group). (F) Resting membrane potential of H-iNs and PD-iNs. (n = 4-9 neurons per lines, n = 5-6 lines per group). (G) Representative traces of Inward NA+- Outward K+ currents following voltage depolarization steps in H-iNs and PD-iNs. (H) Quantification of inward NA+ current (n = 4-9 neurons per lines, n = 5-6 lines per group). (I) Quantification of outward K+ current (n = 4-9 neurons per lines, n = 5-6 lines per group).

GIRK2 (Figure 6D). All of these were present 25 days after initiation of conversion. Moreover, the iNs showed mature electrophysiological properties 65 days post transduction. They displayed the ability to fire repetitive APs upon injection of current as well as exhibited inward sodium (Na+) - outward potassium (K+) currents with depolarizing steps (Figure 6E). When a continuous depolarizing voltage ramp was applied, the currents in the cells were specifically blocked by the neurotoxin tetrodotoxin (TTX), indicating an involvement of voltage-gated sodium channels in the currents. Furthermore, cells displayed spontaneous firing at resting membrane potential, indicating a mature profile.

Neuronal Reprogramming is successfully achieved from healthy and sporadic PD donors

Next, we investigated whether iDANs could be successfully generated from healthy and sporadic PD donors. We reprogrammed 10 healthy cell lines and 19 sporadic PD lines and we found that fibroblasts obtained from PD patients reprogrammed at a similar efficiency to those obtained from age- and sex-matched healthy donors and displayed a similar neuronal morphological profile (Figure 7 A-C). Moreover, when measuring their functional properties with patch-clamp electrophysiological recording, we confirmed that iNs derived from healthy donors (H-iNs) and from PD patients (PD-iNs) displayed similar functionality in terms of the number of current induced APs (Figure 7 D-E), resting membrane potential (Figure 7 F) and the inward Na+- outward K+ current (Figure 7 G-I).

In this study, we generated subtype specific iNs directly converted from human fibroblasts using a new combination of transcription factors that resulted in DA neurons. We found that fibroblasts from both healthy controls and PD patients converted into functional neurons at similar degree.

Application of direct reprogramming *in vivo*: turning resident glia into neurons (Paper III)

In order to investigate the potential of direct reprogramming *in vivo*, we sought to convert resident NG2 glia cells into functional and subtype specific neurons by delivery of reprogrammed factors in the brain. To this end we made use of factors that have been previously used for dopaminergic conversions, *Ascl1, Lmx1a* and *Nurr1* (ALN)(Caiazzo et al., 2011). At 12 weeks post injection (w.p.i.) we analyzed molecular, functional and gene expression of the reprogrammed neurons in order to characterize their profile.

***In vivo* conversion using ALN combination give rise to mature neurons with interneuron phenotype**

We performed the delivery of CRE-dependent ALN conversion vectors into NG2-Cre mice with a GFP reporter that labels reprogrammed neurons.

At 12 w.p.i. we estimated the neuronal conversion efficacy as being 66.81% ± 38.38%. Immunohistochemical analysis revealed the presence of markers common to interneurons (IntNs) such as Parvalbumin (PV), choline acetyltransferase (ChAT), Neuropeptide Y (NPY), or the striatal projection neuron marker DARPP32 (Figure 8A). Quantifications showed that the majority (41.27% ±2.99%)

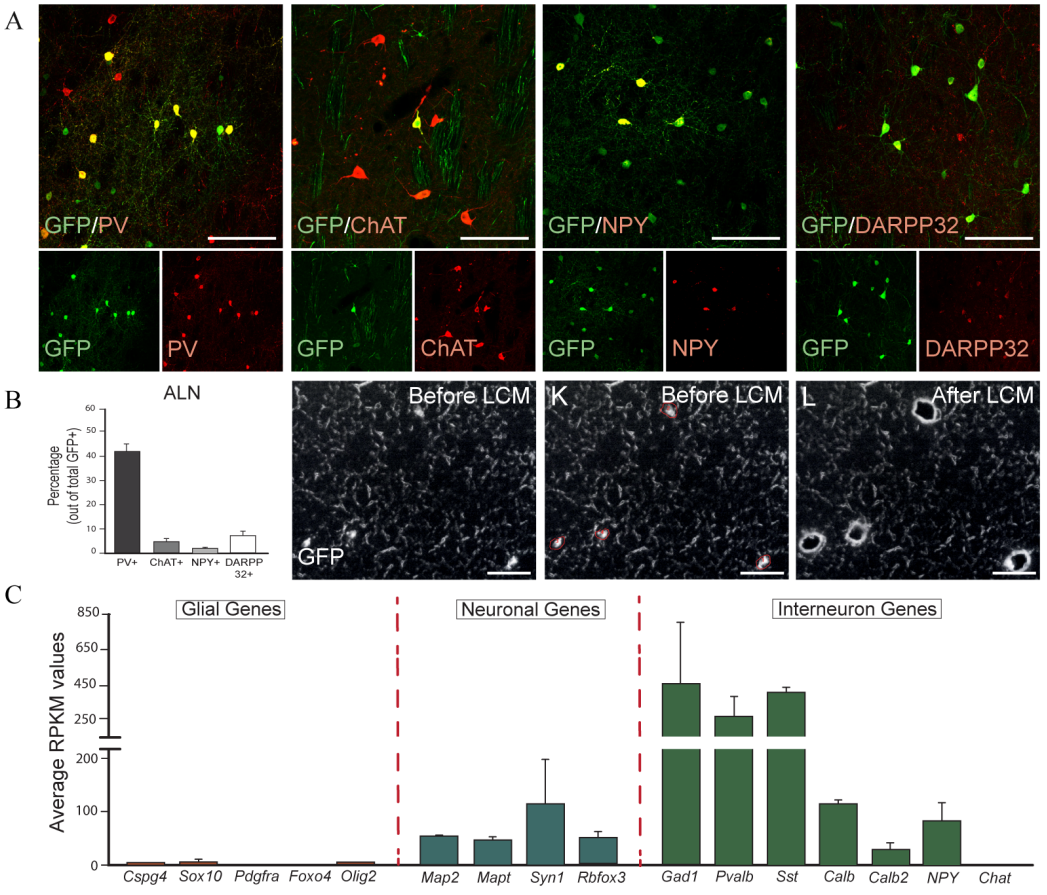


Figure 8 *Phenotypic Identities of In Vivo Reprogrammed Neurons*.

(A) Confocal images showing co-localization of GFP and the interneuron markers PV, ChAT, NPY , and projection neuron marker DARPP32.

(B) Percentage of neurons expressing the markers from (A) shows that the majority of ALN-converted neurons are PV+ (n = 9 brains).

(C) RNA-seq results, presented as average RPKM (reads per kilobase per million) values, show the downregulation of glial genes and upregulation of pan-neuronal genes and interneuronal-linked genes (n = 12–65 cells from n = 2–3 brains).

co-expressed PV, whereas less than 10% of the GFP+ cells were co-labeled with any of the other markers (Figure 8B). These data were confirmed by laser capture microscopy (LCM) (Figure 8C) and functional assessment. Interestingly, similar results were found in animal models of PD where DA denervation in the SNpc was obtained through 6-OHDA toxin injection.

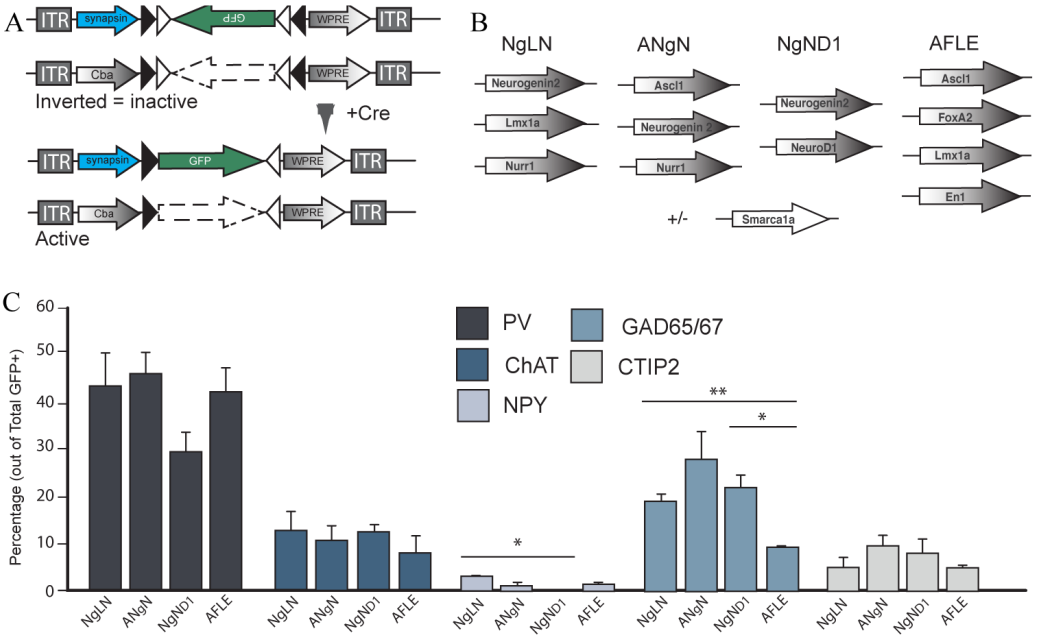


Figure 9 *Different Gene Combinations Expressed in Striatal NG2 Glia Lead to Minor Differences in Neuronal Phenotype*. (A) Schematics of AAV5 constructs used for in vivo reprogramming.(B) Genes were grouped into four different combinations: NgLN, ANgN, NgND1, and AFLE. These groups of factors were used alone or in combination with Smarca1a. (C) Quantification of neurons reprogrammed with different factor combinations that express the markers PV, ChAT, NPY, GAD65/67, and CTIP2 shows that the majority of neurons obtained are PV-positive in all conditions (n = 3 brains/combination).

Delivery of different factor combinations results in similar interneronal phenotype

Next, we investigated the reprogramming output using additional factors combinations with proneural *(Ascl1, Ngn2, NeuroD1)* and DA-*(Lmx1a, Nurr1, FoxA2, En1)* genes (Figure 9A). Four different combinations were used, NgLN *(Neurogenin2, Lmx1a,* and *Nurr1)*, ANgN *(Ascl1, Neurogenin2, and Nurr1)*, NgND1 *(Neurogenin2* and *NeuroD1)*, and AFLE *(Ascl1, FoxA2, Lmx1a,* and *En1)* (Figure 9B). These were injected either alone or together with the midbrain-specific chromatin remodeler Smarca1 (Metzakopian et al., 2015) into the striatum of intact NG2-CRE mice. Similar to ALN, the largest proportion expressed the interneuron marker PV, ChAT+ and NPY+ neurons were found in lower percentages and CTIP2 was found in less than 10% of the reprogrammed neurons (Figure 9C).

Here we showed that we can generate functional neurons through *in vivo* direct reprogramming, supporting its application for brain repair. However, when delivering different factors combinations previously used for generating TH neurons from fibroblasts and astrocytes *in vitro*, no TH-expressing neurons were generated via *in vivo* reprogramming. This raises the question of how cell fate is influenced during *in vivo* conversion and poses the issue of establishing appropriate *in vitro* systems to better investigate fate determinants for reprogramming studies.

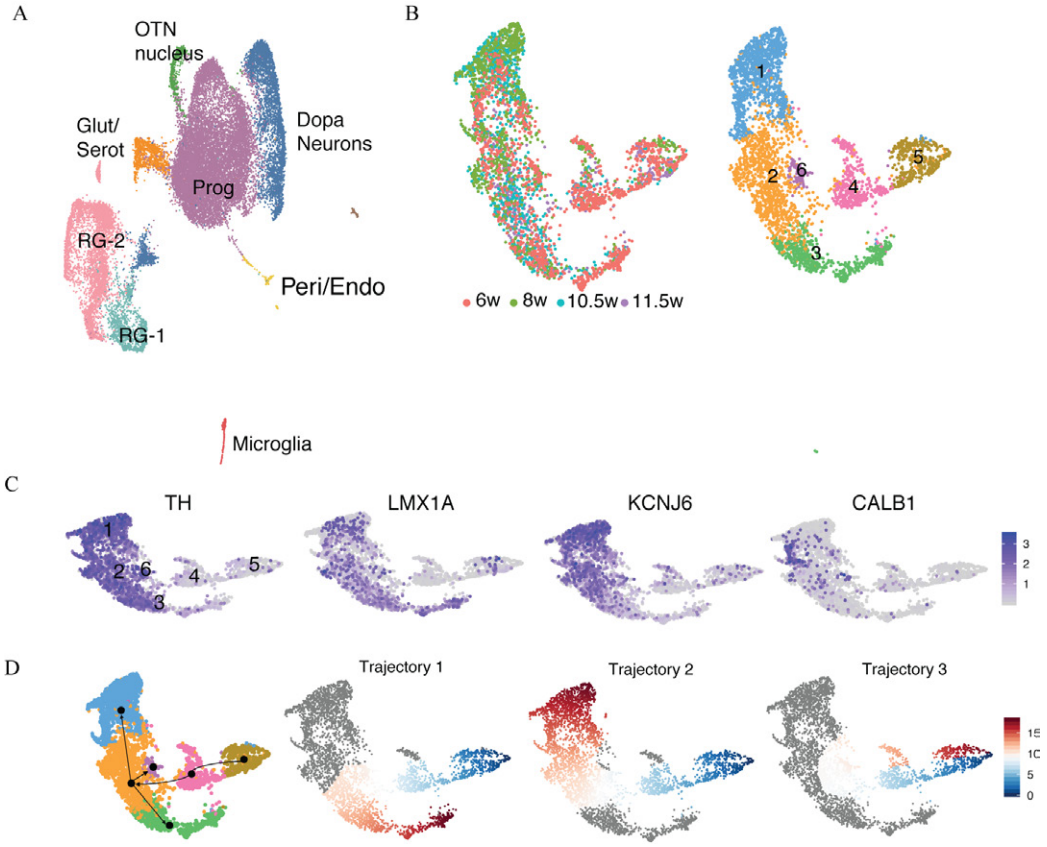


Figure 10 *Three different Human Dopaminergic Trajectories are revealed during fetal development*.

(A) UMAP embedding of integrated data set from four embryos shows presence of 9 different clusters: Dopamine Neurons (Dopa Neurons), Glutamatergic/Serotoninergic Neurons (Glut/Serot), Microglia, Radial Glia 1 and 2 (RG-1, RG-2), Oculomotor/Trochlear Nucleus (OTN nucleus), Pericytes/Endothelial Cells (Peri/Endo), Progenitors (Prog) and Red Blood Cells (RBC). (B) UMAP embedding of dopamine neurons from all time points (n=3838) colored by sample identity. Results identify integration and overlapping expression among embryos at 6, 8, 10.5 and 11.5 weeks PC. UMAP embedding of dopamine neurons from all time points (n=3838) colored by cluster. Six putative dopaminergic subtypes are found to be expressed in embryos at 6, 8, 10.5 and 11.5 weeks PC. (C) Featureplot of normalized expression for selected dopamine neuron marker genes, *TH, LMX1A, KCNJ6, CALB1*. Purple indicates high expression. Dopaminergic Markers are found to be highly expressed in cluster 1, 2, 3, 6. (D)Three lineage trajectories were identified by Slingshot Analysis, with cluster 5,4 and 2 as common path and 3,1 and 6 as leaf clusters. UMAP plot showing the arrangement of cells in pseudotime according to the three trajectories.

Developing a 3D culture system to study human fetal dopaminergic neurons (Paper IV)

Efforts to develop more refined and precise reprogramming and differentiation protocols to generate sub-type specific DA neurons both *in vitro* and *in vivo* are continuously ongoing. In this process, a better understanding of human DA neuron specification and maturation is vital.

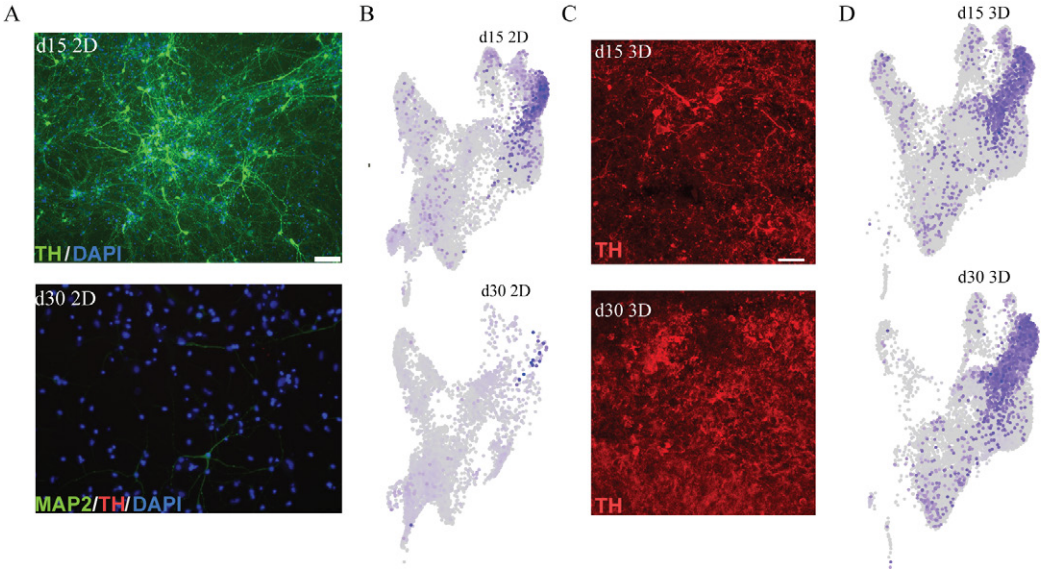


Figure 11 *Dopaminergic development is not sustained in 2D culture systems*

(A)Immunostaining of hVM 2D cultures at d15 for TH, post-mitotic DA neuronal marker, showing presence of DA neurons in culture, at d30 TH marker is not expressed and low levels of MAP2 are found. (B) Feature plots from scRNA-seq analysis of 2D cultures at d15 and d30 for TH showing a decrease in the expression over time. (C) Immunostaining of hVM 3D cultures at d15 for TH, showing presence of DA neurons in culture, at d30 TH marker is expressed at high levels. (D) Feature plots from scRNA-seq analysis of 3D cultures at d15 and d30 for TH showing a stable expression over time.

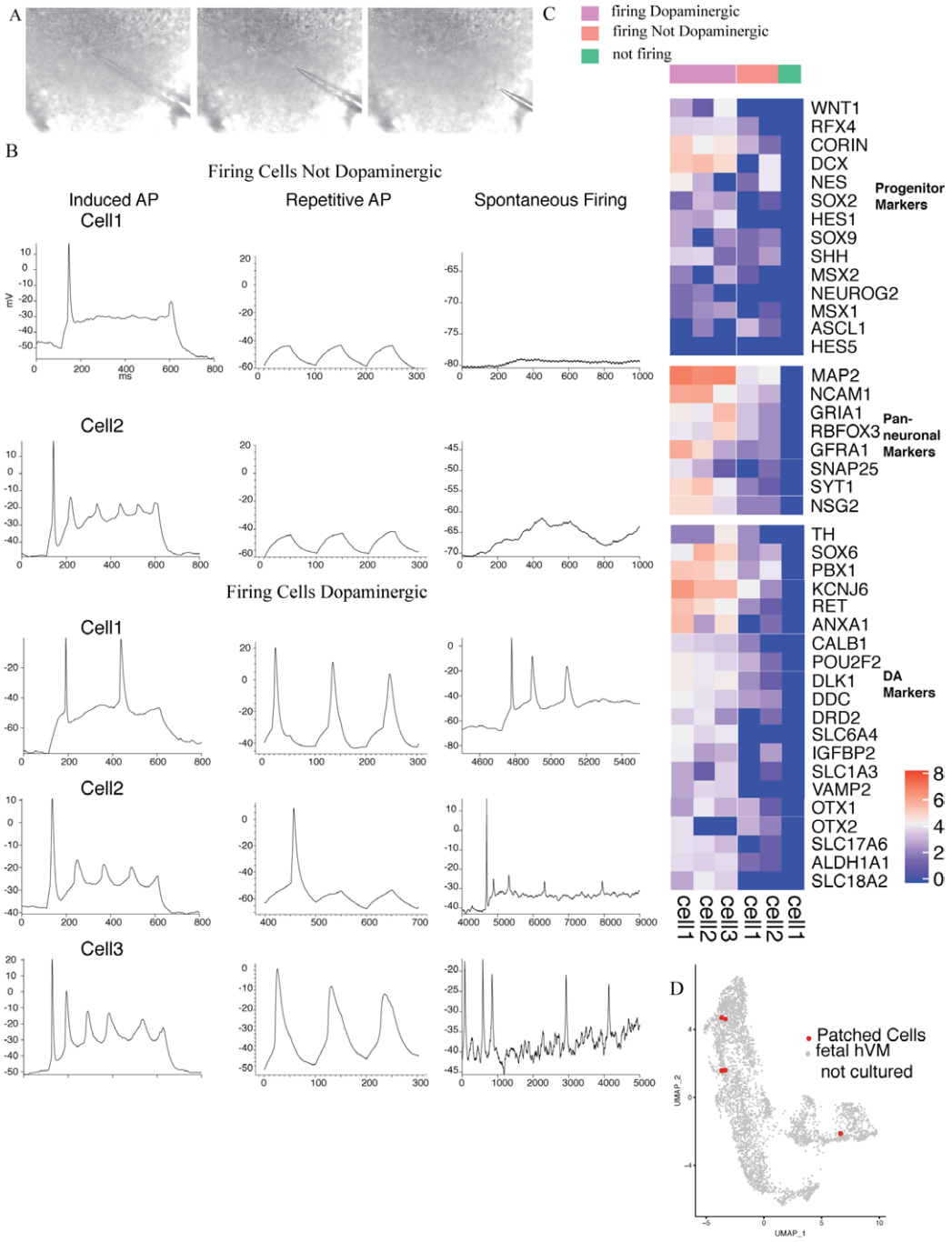
Human fetal tissue is of great value for understanding the human brain development. Previously this tissue has been characterized in 2D culture conditions (Hebsgaard et al., 2009; Nelander, et al., 2009) for its gene and protein profile.

In this study we wanted to explore if it is possible to culture hVM cells in 3D organoid-like cultures, with the aim of maintaining fetal DA neurons in long term cultures. Ultimately, we wanted to characterize fetal human VM-derived DA neurons at the level of gene expression, phenotypic identity and functional properties.

Distinct dopaminergic trajectories are found in the developing human brain

To determine the cellular composition of the developing VM at the molecular level, we subdissected VM from human embryos of gestational ages 6 to 11.5 weeks post conception (wpc). We performed droplet based scRNA-seq on the dissected VM tissue from 4 separate fetuses (6, 8, 10 and 11.5 wpc) (Figure 10A). Among different embryos we were able to find 6 different DA clusters (Figure 10B-C), suggesting the presence of transcriptionally distinct dopaminergic subtypes at these early timepoints.

When applying Slingshot analysis (Figure 10D), three different trajectories were found to link the DA clusters, pointing at different developmental pathways in the DA generation.


A
B
C
D
Firing Cells Not Dopaminergic
Induced AP
Repetitive AP
Spontaneous Firing
Cell1
Cell2
Firing Cells Dopaminergic
Cell1
Cell2
Cell3
mV
ms
firing Dopaminergic
firing Not Dopaminergic
not firing
WNT1
RFX4
CORIN
DCX
NES
SOX2
HES1
SOX9
SHH
MSX2
NEUROG2
MSX1
ASCL1
HES5
Progenitor Markers
MAP2
NCAM1
GRIA1
RBFOX3
GFRA1
SNAP25
SYT1
NSG2
Pan-neuronal Markers
TH
SOX6
PBX1
KCNJ6
RET
ANXA1
CALB1
POU2F2
DLK1
DDC
DRD2
SLC6A4
IGFBP2
SLC1A3
VAMP2
OTX1
OTX2
SLC17A6
ALDH1A1
SLC18A2
DA Markers
8
6
4
2
0
cell1
cell2
cell3
cell1
cell2
cell1
Patched Cells
fetal hVM
not cultured
UMAP_1
UMAP_2

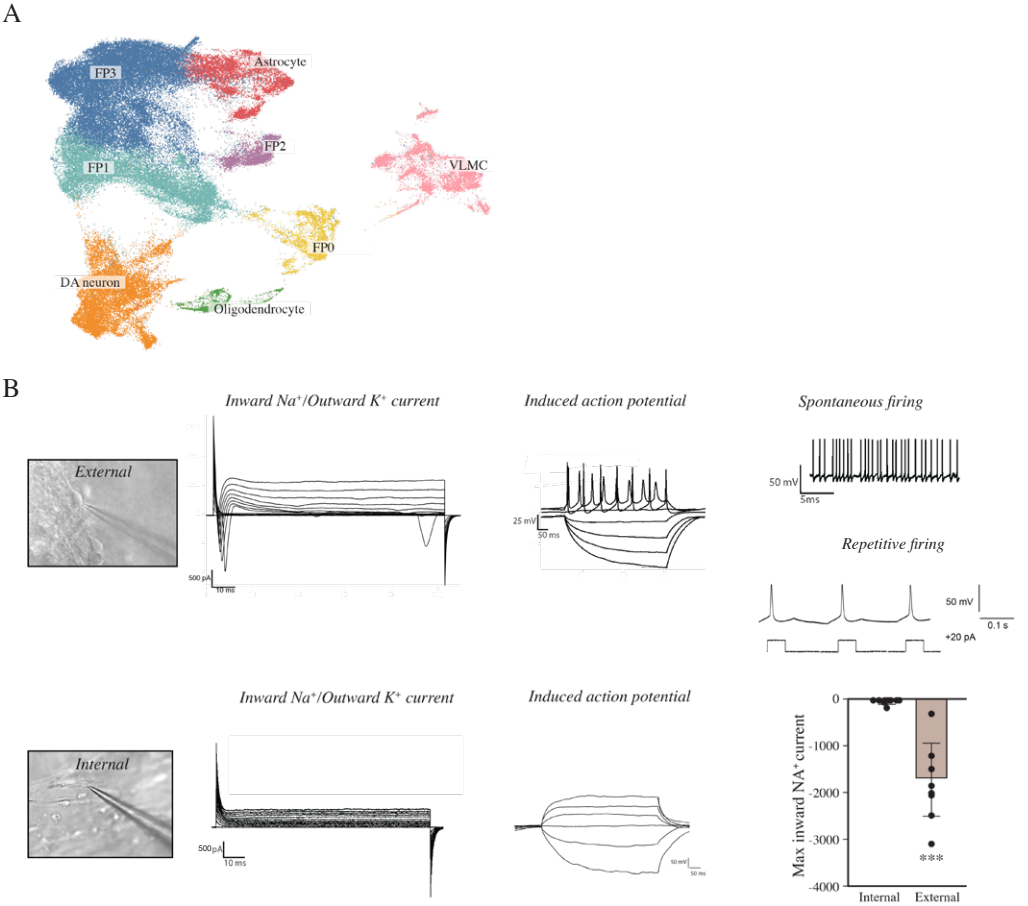


Figure 13 *Single cell transcriptomics and whole-cell patch clamp recordings of standard VM organoid.*

(A) UMAP plot showing clustering of 91,034 analysed cells from VM organoids at day 15, 30, 60, 90, and 120. Clusters identified: neural progenitors, floor plate progenitors, DA neurons, astrocytes, oligodendrocytes and vascular leptomeningeal cells. (B) Electrophysiological properties of externally and internally located neurons within standard VM organoids demonstrating inner core immaturity by whole-cell patch-clamp recordings.

<- Figure 12 *3D system allows transcriptional and functional studies oh human DA neurons in long term cultures.*

(A) Representative images showing patch pipette targeting a single cells during whole-cell patch-clamp recording, followed by cell aspiration from the 3D hVM culture for scRNA-seq preparation. (B) Traces from whole-cell patch-clamp recordings showing induced APs, repetitive firing upon small current injection and spontaneous firing. Cells presenting induced APs, repetitive firing and spontaneous firing were classified as Firing Cells Dopaminergic, cells that presented only induced APs were classified as Firing Cells Not Dopaminergic. In the analysis was included also one cell, here not presented, that did not show any induced APs and classified as Not Firing. (C) Heatmap showing single cell expression for selected genes (progenitors, pan-neuronal and dopaminergic) in each recorded cell. Different transcriptional profile is visible among Dopaminergic, Not Dopaminergic Firing cells and Not Firing cell. (D) UMAP plot showing DA subclusters from hVM not cultured resulted in the 10x dataset merged with Patch-Seq Smartseq processed cells. Majority of the patched cells with a mature profile cluster within the late DA populations observed in 10x data.

Over long period of time DA neurons are better preserved in 3D cultures than standard 2D system

When culturing the human fetal VM, we found that over time, DA neurons were lost in the cultures, as shown by the loss of TH-positive cells from scRNA-seq and immunocytochemistry analysis (Figure 11A-B). We therefore optimized a protocol for self-aggregation using low attachment plates to obtain cell-cell self-interaction. In stark contrast to the in 2D cultures, in the 3D condition the TH neurons were shown to be maintained at day 30 (Figure 11C-D).

3D culture system enable to capture different molecular subtypes of functionally mature human DA neurons

In an attempt to relate molecular subtype to functional properties, we performed Patch-Sequencing (Patch-Seq) on fetal DA neurons from 3D cultures at day 30 (n=6) (Figure 12A). We found that cells presenting dopaminergic firing (Figure 12B) displayed higher expression levels for genes related to dopamine pathway such as *TH, SLC32A1, CALBINDIN* and their transcriptome was clearly different from non-DA neurons. Specific ion channels and receptors were also found to be upregulated in the dopaminergic firing cells (Figure 12C). The cell that did not show the ability to fire evoked APs, did not express any of the dopamine markers expressed by the firing cells .

When merging the dataset from Patch-seq and 10x of fetal VM, the majority of patched cells (in red) are overlapping with the late DA neurons confirming that the late DA neuron cluster indeed is representing a functionally mature DA population (Figure 12D).

In this work we found that fetal DA neurons cluster in 6 different groups that appeared to follow distinct development trajectories. Next we established a culture condition that allowed us to maintain and analyse human mature DA neurons in culture. This enabled us to study the physiology and transcriptional profile of mature DA neurons that resembled the characteristics of a late developmental stage of DA neurons.

Establishing VM organoids from PSCs as a source of authentic DA neurons (Paper V)

In this study, we wanted to generate mesDA neurons from hESC in an organoid system to understand the physiological characteristics of the midbrain organoids, as well as their affinity with fetal 3D VM organoids. Here we adapted a commonly used protocol for forebrain organoid generation with the addition of dual-SMAD inhibition (Nolbrant et al., 2017), combined with exposure to the neural tube ventralizing secreted factor SHH and GSK3i. GSK3i activates Wnt signaling, thereby promoting neurogenic conversion of VM FP progenitors toward DA neuron fate in standard 2D differentiations (Nordström et al., 2002).

Human DA neurons are successfully generated in VM organoids

scRNAseq analysis of generated organoids revealed the presence of a neuronal cluster defined by

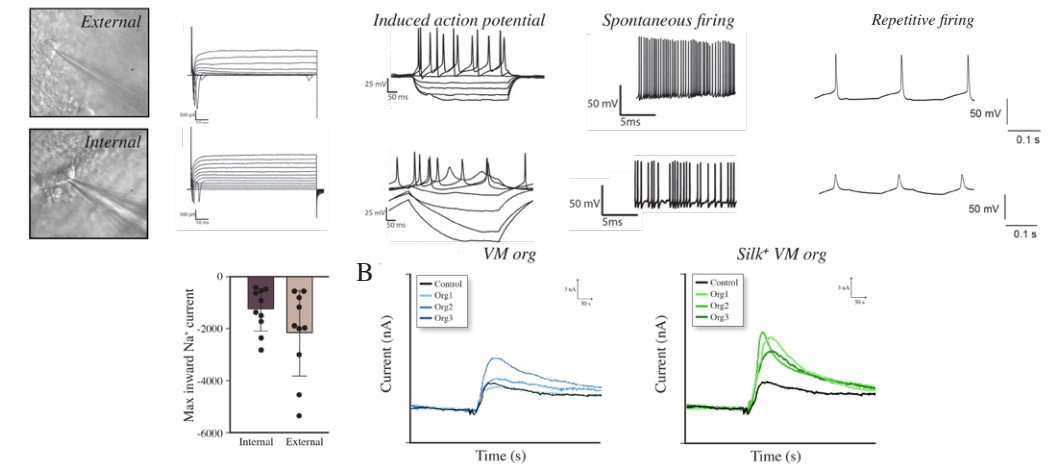


Figure 14 *Silk-VM organoids are functionally homogenous*.

(A)Whole-cell patch-clamp recordings of external and internal silk+Lam111 VM showing presence of mature neurons both in the internal and external region of the organoids. (B) Representative analysis of real-time DA release chrono-amperometric measurements in conventional and silk+Lam111 organoids showing less variability in DA release in Silk-VM organoids.

expression of *DCX, SYT1, STMN2* and primarily expressed genes associated with DA fate identity *(PBX, NR4A2, EN1, TH, DDC)*. VM organoids also contained astrocytes *(GFAP, AQP4, EDNRB)* and a small group of oligodendrocyte progenitors *(OLIG1/2, PDGFRa, SOX10)*, as well as a newly discovered class of perivascular-like cells termed vascular leptomeningeal cells (VLMCs) expressing *PDGFRa, COL1A1, COL1A2,* and *LUM* (Marques et al., 2016; Vanlandewijck et al., 2018) (Figure 13A). However, when analyzing the functional activity of VM organoid, we found that the system used did not support a homogenous differentiation. Indeed, as revealed by absence of Inward Sodium/Outward Delayed Rectifier Potassium currents, and lack of ability to fire induced APs (Figure 13B), the inner core of the organoid was found to be differentiated to a less extent compared to the outer layers.

Silk-bioengineered VM organoids allows for homogeneous and reproducible pattering

To address the limitation of immature inner core and variability in terms of TH yields among organoids, we used a biomaterial made of recombinant spider silk protein (Widhe et al., 2010; Åstrand et al., 2020) which functions as a biocompatible and bioengineered cell scaffold. Silk fibers were used either alone or functionalized with Lam-111 (termed silk(-) and silk(+)), shown to promote DA patterning and support DA differentiation in 2D cultures. scRNAseq and immunohistochemical analysis of silk hVM organoids revealed a similar developmental progression and similar cell populations to that observed in conventional organoids. To support this data, functional analysis was performed using whole-cell patch-clamp recordings and revealed mature DA neurons in both inner and outer layer of the organoids (Figure 14). DA release was analysed from standard and silk hVM organoids and confirmed the high maturation and functionality of DA neurons. In conventionally generated orga-

noids, only four out of eight VM organoids recorded showed a release of DA while seven out of eight silk+Lam111 organoids released DA (Figure 14B). Although the quality of DA neurons generated in 3D organoids is comparable between conventional and silk organoids, the silk-based tissue engineering technology is more robust and results in less variation within and between organoids.

This work shows how hVM brain organoids optimized with Silk scaffold were efficiently patterned into a VM identity, leading to the formation of DA progenitors and their subsequent differentiation into mature DA neurons with electrophysiological properties of DA neurons and the ability to release the neurotransmitter DA.

CONCLUSIONS AND FUTURE PERSPECTIVES

DA neurons play an essential role in PD as they are found to be selectively lost in the SNpc and their main released neurotransmitter, DA, is found to be present at lower levels in patients when compared to healthy individuals (Dauer and Przedborski, 2003). Indeed studies that allow restoration of these cells in the brain and aim at understanding the causes of this selective loss are essential for progressing towards finding a cure for PD.

To allow such studies, a generation of *bona fide* DA neurons is vital and a detailed characterization of the newly generated neurons is essential for their application in pre- and clinical context.

In **paper I**, direct neuronal reprogramming was applied to human adult fibroblasts with the aim of achieving cells capable of neuronal maturation and function for future applications. Indeed, only few studies previously provided successful neuronal maturation from *in vitro* reprogramming of human adult skin fibroblasts (Caiazzo et al., 2011; Drouin-Ouellet et al., 2017b). Global gene expression analysis at 5 and 24 DIV showed that using miR9 and miR124 in addition to *Ascl1, Brn2*, and shREST as reprogramming factors correlates with an upregulation of genes related to calcium and functional neuronal properties. However, at these time points cells were not functionally active. Only between 80-90 DIV cells displayed neuronal activity, with a more pronounced mature neuronal population in miR9/miR124, *Ascl1, Brn2*, shREST reprogrammed neurons. These results indicate that reprogramming of human adult neurons requires a long culturing period to reach maturation when applying *Ascl1, Brn2*, and shREST. The presence of mir9 and mir124, factors previously used for reprogramming studies (Yoo et al., 2011), here helped the maturation process leading to a high degree of functionality that was reported for the first time in the context of human adult skin cells reprogramming. Furthermore, gene analysis of late time points showed a heterogeneous neuronal profile present in the cultures, indicating that the expressed genes activate an intracellular cascade related to a pan-neuronal profile. In the future, a superior characterization of the iNs cultured together with other cell types, such as astrocytes, will help in determining if they retain the ability to form circuits and complex cell to cell interactions, aspects that are essential for both cell transplantation and disease modelling.

In **Paper II** direct reprogramming was used for generating DA neurons from human adult skin cells. Similar to the work from Caiazzo et al. 2011, a successful conversion into DA neurons was achieved, however, in this work a new factor combination was found to reprogram both healthy and diseased skin adult samples with high neuronal yield. The newly generated cells showed the expression of pan-neuronal as well as DA specific proteins. Functional analysis showed that reprogrammed cells from healthy and PD derived fibroblasts retain a similar neuronal maturation. These results confirm the successful establishment of a protocol for iDANs generation. Subsequent analysis of the reprogrammed neurons showed impairment of different autophagic pathways in iDANs generated from PD fibroblast lines, supporting previous findings that show maintenance of epigenetic and

phenotypic signatures during direct reprogramming (Mertens et al., 2015). Finally, these results show how this protocol can have applications for disease modeling studies, however it provides indications that cell transplantations for PD patients may require HLA matched donors for the generation of healthy iDANs.

In **Paper III** we applied direct reprogramming for generating DA neurons *in vivo*. Mouse resident NG2 glia was targeted and successfully converted into neurons when delivering *Ascl1, Lmx1a* and *Nurr1* as reprogramming factors. Interestingly, the factor combination that *in vitro* generates DA neurons, here gave rise to interneurons, mostly with properties of PV+ cells. To explore the possibility that environmental cues could affect the reprogramming output, injections of ALN factors in a 6-OHDA model of PD were performed. However, no TH+ cells were detected, but rather cells converted to an interneuronal phenotype were assessed in the reprogrammed cells. In addition, when delivering ALN but also other factor combinations, PV+ interneurons were found to be the main cell type generated through reprogramming. These results mark the gap between *in vitro* and *in vivo* outcomes in reprogramming studies. This study is therefore pointing at a need for new models where to study and generate DA neurons in a human physiologically relevant context.

Generation of mesDA neurons from stem cells reprogramming is often based on current knowledge regarding hVM formation and maturation, however, the restricted access to human material during brain development limits a deep understanding of the development of this brain region.

In **paper III**, I explored the possibility to study human fetal VM to expand our knowledge of mesDA neurons. With this work, I provide insights into dopaminergic diversity among human embryos collected at different developmental stages. A similar approach has been adopted in the study by La Manno et al., nonetheless here the yield of cells collected for scRNA-seq was improved, generating a robust dataset composed by more than 20´000 hVM fetal derived cells. Furthermore, I cultured human fetal VM for allowing cell specification and maturation. Previous works (Hebsgaard et al., 2009; Nelander et al., 2009; Ribeiro et al., 2013) showed the possibility to culture human fetal VM tissue in 2D for a short period of time. Here, 3D culturing resulted in a successful approach for maintaining DA neurons alive outside the human embryo up to 4 months. Lastly, DA neurons were characterized by Patch-Seq technique resulting in gene and functional analysis of mature DA neurons.

These results provide a valuable tool that can be used as a reference dataset when generating or studying human mesDA neurons.

In **Paper V**, the generation of neurons that resemble authentic mesDA development was assessed in VM organoid. Organoid system represent a technique that has been recently applied in the field of Neuroscience (Lancaster et al., 2013). Generation of organoids with characteristics of specific brain regions has been achieved through the use of specific patterning protocols (Renner et al., 2017; Sloan et al., 2018). Similarly to other works (Tieng et al., 2014; Jo et al., 2016; Qian et al., 2016; Monzel et al., 2017; Kim et al., 2019; Smits et al., 2019) VM organoids were here successfully established. Furthermore in this study an inner undifferentiated core was found, limitation that is commonly known in the field (Qian et al., 2019). Therefore, a new culturing method that employs the use of silk microfibers was implemented and resulted in VM organoids that homogenously resemble gene, protein and functional profile of authentic DA neurons.

Overall, the studies in this thesis show how transcriptional and functional analysis are applied for studying DA neurons and for establishing the authenticity of DA neurons generated through reprogramming and differentiation studies. Indeed transcription and function are two essential aspects to be determined when generating neurons for therapeutic applications. These features are used in a comparison between newly generated and authentic mesDA. However, most of our current knowledge on the DA populations is based on rodent studies and/or performed with underpowered technologies. To allow a better understanding of human DA neurons, I established a 3D system to study single-cell transcriptome and physiological profile of human fetal VM. Finally, PSCs VM organoid were estabished for generating autentic mesDA neurons. This provide evidence that VM organoids can be used for future applications aiming at bridging the gap between *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

In this chapter, I will describe key methods used in the studies that are included in my thesis. For additional details about these procedures and others not referred here, I kindly refer the reader to the method section of the respective papers (see appendix).

In vitro direct reprogramming

Culturing of human fibroblasts

Adult dermal fibroblasts were obtained from the Parkinson's Disease Research clinic at the John van Geest Centre for Brain Repair (Cambridge, UK) and used under local ethical approval (REC 09/H0311/88). For biopsy sampling information see Drouin-Ouellet et al., 2017. Fibroblasts were expanded in T75 flasks with standard fibroblast medium (DMEM, 10% FBS, 1% penicillin-streptomycin) at 37°C in 5% CO2. After thawing, cells were kept a minimum of 2 days in culture before starting experiments. When confluent, the cells were dissociated with 0.05% trypsin and plated at a lower density for expansion. To freeze the fibroblasts from a confluent T75 flask, the cells were detached after 5 minutes incubation in 0.05% trypsin 37°C, spun for 5 minutes at 400g and frozen in a 50/50 mixture of DMEM and FBS with 10% DMSO. For details of cell lines used in Paper I and II see Table 3.

Lentiviral Vectors

The lentiviruses (LV) used are third generation vectors containing a non-regulated ubiquitous phosphoglycerate kinase (PGK) promoter. In Paper I the DNA plasmids used are expressing the open reading frames (ORFs) for *Ascl1, Brn2* with short hairpin RNA (shRNA) targeting REST or ORFs for *Ascl1, Brn2* with miRNA loops for miR-9/9* and miR-124 in combination with shRNA targeting REST. The single vector containing *Ascl1, Brn2*, shREST was used at multiplicity of infection (MOI) of 20. The vector containing *Ascl1, Brn2*, miR9/9* and miR124 was used in combination with shRNA targeting REST at MOI 20. All viruses used in this study titered between 3 $x10^8$ and 6 $x10^9$ pfu/mL. In Paper II DNA plasmids expressing ORFs for *Ascl1, Lmx1a, Lmx1b, FoxA2, Otx2, Nurr1, Smarca1, CNPY, En1 or Pax8,* as well as two shRNAs targeting REST containing a non-regulated U6 promoter. The single vector containing *Ascl1, Brn2*, shREST was used to reprogram iNs for RNAseq. Transduction was performed at a MOI of 5 for each vector (all viruses used in this study titered between 1 x 10^8 and 9 x 10^9) or MOI of 20 in the case of the single vector containing *Ascl1, Brn2,* shREST.

Table 3 *Details of human fibroblasts used in these studies*.

Group	Sex	Age at biopsy	Age of onset	Study
Healthy	M	69		Paper II
Healthy	F	67		Paper I and II
Healthy	M	80		Paper II
Healthy	F	75		Paper II
Healthy	M	70		Paper II
Healthy	F	70		Paper I and II
Healthy	M	71		Paper II
Healthy	F	61		Paper II
Healthy	F	66		Paper II
Healthy	F	58		Paper II
PD	M	56	34	Paper II
PD	M	60	48	Paper II
PD	F	77	65	Paper II
PD	F	67	56	Paper II
PD	F	59	45	Paper II
PD	F	80	69	Paper II
PD	M	80	49	Paper II
PD	F	87	72	Paper II
PD	F	77	56	Paper II
PD	M	75	63	Paper II
PD	M	77	66	Paper II
PD	F	71	62	Paper II
PD	M	72	70	Paper II
PD	M	81	76	Paper II
PD	F	44	40	Paper II
PD	F	79	NA	Paper II
PD	M	61	60	Paper II
PD	F	68	55	Paper II
PD	M	57	50	Paper II

Neuronal Reprogramming

Fibroblasts were plated at a density of 27 800 cells per cm^2 in 24-well plates (Nunc). Cells used for electrophysiological recordings were directly plated on glass coverslips coated with Polyornithine (15 μg/mL), Fibronectin (0.5 ng/μL) and Laminin (5 μg/mL) (PFL). One day after plating, cells were transduced with Lentiviral Vectors with fibroblast medium. Three days after the viral transduction, the medium was replaced with neural differentiation medium (NDiff227; Takara-Clontech) supplemented with growth factors at the following concentrations: LM-22A4 (2 μM, R&D Systems), GDNF (2 ng/mL, R&D Systems), NT3 (10 ng/μL, R&D Systems) and db-cAMP (0.5 mM, Sigma) and the small molecules CHIR99021 (2 μM, Axon), SB-431542 (10 μM, Axon), noggin (0.5 μg/ml, R&D Systems), LDN-193189 (0.5 μM, Axon), valproic acid sodium salt (VPA; 1mM, Merck Millipore). Half medium changes were performed every 2 days for the first 30 days of conversion, whereas in the later stages of conversion the medium changes were done every 3 days. At 18 days post-transduction, the small molecules were withheld, and the neuronal medium was supplemented only with LM-22A4, GDNF, NT3 and db-cAMP until the end of the experiment.

In vivo direct reprogramming

Transgenic animals

GFAP-Cre (Jackson) transgenic mice were backcrossed onto a C57BL/6J background, and NG2-Cre (Jackson) transgenic mice were backcrossed onto a B6129PF2/J background, bred at the in-house BMC animal facility. Heterozygotes were identified by PCR.

Viral Vectors

Cre-inducible AAV5 vectors were created by inserting the cDNA for the genes of interest in a reverse orientation flanked by two pair of heterotypical, antiparallel LoxP (FLEX) sequences. Constructs were mixed in equal ratios, each at a 5% dilution from stock (for detailed protocol see Pereira et al., 2019).

Immunohistochemistry

Brain sections were rinsed three times in potassium phosphate buffered saline (KPBS) and pre-incubated in blocking solution (5% serum and 0.25% TritonX-100 in KPBS) (TKPBS) for 1 hour at room temperature. Primary antibodies were then diluted as described in Table 4 in the same pre-incubation solution, added to sections and left over-night at 4°C. The primary antibody was removed and sections were rinsed twice with TKPBS followed by pre-incubation in 5% serum-TKPBS solution for 30 minutes at room temperature. Details on the antibody used can be found in Table 4. Sections were then incubated in secondary antibodies (Jackson Laboratories, Life Technology) conjugated with cy2/488, cy3/555 and cy5/647 for 2 hours at room temperature. Sections were then rinsed 3 times in KPBS, mounted on glass slides and coverslipped using glycerol or xylen based media.

Table 4 *Details of antibodies used in Paper III*.

Antibody	Host	Dilution	Provider
MAP2	Mouse	1:500	Sigma
NeuN	Mouse	1:100	Millipore
hGFAP	Rabbit	1:1000	DAKO
hNCAM	Mouse	1:1000	SanatCRuz
HuNu	Mouse	1:200	Millipore
GFP	Chicken	1:1000	Abcam
GFP	Rabbit	1:5000	Millipore
TH	Rabbit	1:1000	Millipore
Parvalbumin	Mouse	1:2000	Sigma
ChAT	Goat	1:200	Chemicon
NPY	Rabbit	1:5000	Immunostar
DARPP32	Rabbit	1:1000	Abcam
GAD65/67	Rabbit	1:1000	Abcam
vGlut1	Rabbit	1:1000	Synapitc Systems
Tbr1	Rabbit	1:300	Abcam
CTIP2	Rat	1:1000	Abcam
Calretinin	Rabbit	1:200	Abcam

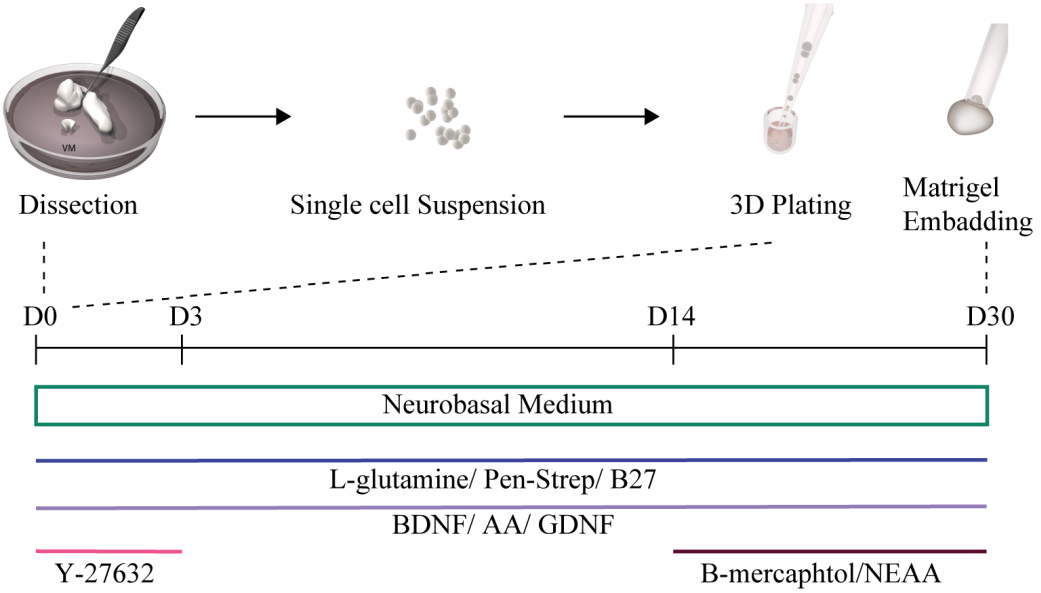


Figure 15 *Overview of fetal hVM 3D cultures procedures*.

3D structures and Organoids

Human Fetal Dissection and 3D culture

Human fetal tissues were collected from WPC 6–11.5 old legally terminated embryos at Malmö Hospital (Malmö, Sweden) and Addenbrooke's Hospital (Cambridge, U.K.). Ethical approval for the use of postmortem human fetal tissue was provided by the the Swedish national board of health and welfare in accordance with existing guidelines including informed consent from women seeking abortions and by the National Research Ethics Service Committee East of England - Cambridge Central (Local Research Ethics Committee, reference no. 96/085). Samples from U.K. were shipped overnight on ice in HIBERNATE media (Thermo Fisher Scientific) to Sweden. Tissue from both Sweden and U.K. was dissected in HIBERNATE media. Narrow subdissection of human VM were performed and tissue was later washed in phosphate buffered saline (PBS solution). After 3 washes, the tissue was treated with accutase (PAA Laboratories) for 20 min at 37°C degrees. After incubation, single cell suspensions were generated by mechanical dissociation and the cells plated at a density of 70,000 cells/well (36,842 cells/cm2) in culture media (Figure 15). Culture media used was formulated as follow: Neurobasal Medium, 2 nM L-Glutamine, 100 µg/mL pen/strep, 20 ng/ml BDNF, 10 ng/ml GDNF, 0.2 mM AA, 1/3 B27 (Figure 15). Only on plating day after dissociation, the culture media was supplemented with Y-27632 (10 µM) for improving neuronal survival. 1% minimum essential medium-non essential amino acids (MEM-NEAA) and 0.1% 2-mercaptoethanol was added to the culture media from day 14. Media changes were performed every 2 days. 2D cultures were performed

in standard plates coated with a combination of Polyornithine (15 μg/mL), Fibronectin (0.5 ng/μL) and Laminin (5 μg/mL). 3D cultures were performed using U-bottom shaped ultra-low attachment 96-well plates (Corning). Droplets of Matrigel were applied as embadding at day 30 to sustain long term cultures. At the time of embedding, 3D hVM cultures were transferred into ultra-low attachment 24-well plates (Corning).

3D cultured organoids used for calcium imaging were left attaching on glass coverslips coated with Polyornithine, Fibronectin and Laminin at day 90.

VM Organoids Culture

To start 3D VM organoid differentiation, RC17, H9, HS999, HS1001, and TH-Cre hPSC cells were detached from the culture dish with 0.5 mM accutase (Thermo Fisher Scientific, #A1110501) to yield a single cell suspension. Differentiation was initiated by plating 2,500 single cells in each well of a 96-well U-bottom plate (Corning, #CLS7007) in iPS Brew with 10 μM Y-27632 dihydrochloride (Miltenyi, #130-106-538), as previously described (Quadrato et al., 2017; Renner et al., 2017). After three days in culture, embryoid bodies were transferred to differentiation medium consisting of 1:1 DMEM/F12:Neurobasal medium (Thermo Fisher Scientific, #21331020 and #A1371201), 1:100 N2 supplement (Thermo Fisher Scientific, #A1370701), 10 μM SB431542 (Miltenyi, #130-106-543), 150 ng/ml rhNoggin (Miltenyi, #130-103-456), 400 ng/ml SHH-C24II (Miltenyi, #130-095-727), and 1.5 μM CHIR99021 (Miltenyi, #130-106-539), with 200 mM L-glutamine (Thermo Fisher Scientific, #25030081) and 10,000 U/mL penicillin-streptomycin (Thermo Fisher Scientific, #15140122). During the whole differentiation period 1% minimum essential medium-non essential amino acids (MEM-NEAA; Sigma-Aldrich, #M7145) and 0.1% 2-mercaptoethanol (Merck, #8057400005) were maintained. On day 11, developing VM organoids were transferred to a 24-well plate containing 1:50 Neurobasal medium, B27 supplement without vitamin A (Thermo Fisher Scientific, #12587010), and 100 ng/mL FGF-8b (Miltenyi, #130-095-740). On day 14, 20 ng/mL BDNF (Miltenyi, #130-096-286) and 200 mM L-Ascorbic acid (Sigma-Aldrich, #A4403-100MG) were added. At this point, VM organoids were embedded in 30 μL droplets of Matrigel (BD Biosciences), as previously described (Lancaster et al., 2014). From day 16 onwards, 0.5 mM db-cAMP (Sigma-Aldrich, #D0627-1G) and 1 μM DAPT (R&D Systems, #2634) were added to the culture medium for terminal maturation for up to four months.

scRNA-seq

For 10x Genomics single cell RNA sequencing, single cell suspensions were loaded onto 10x Genomics Single Cell 3' Chips along with the mastermix as per the manufacturer's protocol (https://support.10xgenomics.com/single-cell-gene-expression/index/doc/technical-note-chromium-single-cell-3-v3-reagent-workflow-and-software-updates) for the Chromium Single Cell 3' Library to generate single cell gel beads in emulsion (GEMs, version 3 chemistry). Resulting libraries were sequenced on either a NextSeq500 or a NovaSeq 6000 with the following specifications Read1 28 cycles, Read2 98 cycles, Index1 8 cycles using a 200 cycle kit. Raw base calls were demultiplexed and converted fastq files using cellranger mkfastq program (bcl2fastq 2.19/cellranger 3.0). Sequencing data was first

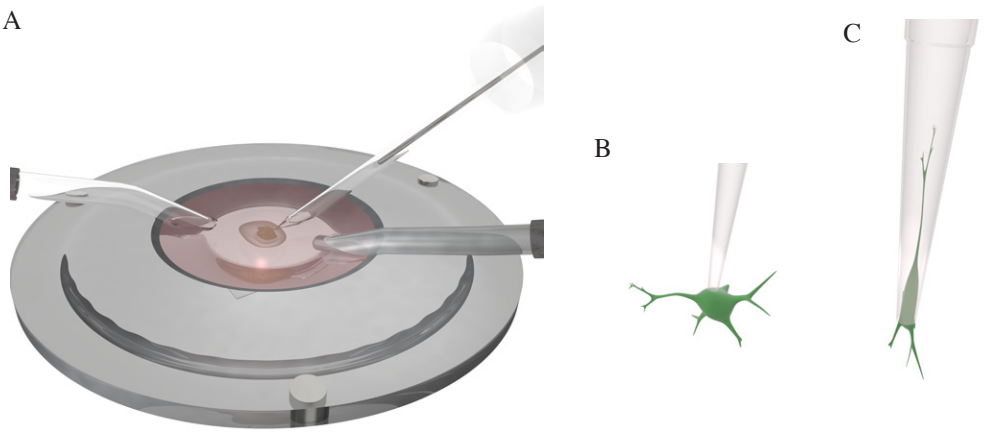


Figure 16 *Representative image of Patch-Seq procedure*.

The cell of interest is targeted by a patch recording pipette and aspirated from the culture. Collected cell is further processed for scRNA-seq.

pre-processed through the Cell Ranger pipeline (10x Genomics, Cellranger count v2) with default parameters (expect-cells set to number of cells added to 10x system), aligned to GrCH38 (v 3.1.0) and resulting matrix files were used for subsequent bioinformatic analysis.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp electrophysiological recordings were performed on glass coverslips for Paper I-II, on whole 3D cultures of fetal VM in Paper IV and on sliced VM organoids for Paper V. Cell on glass coverslips were with constant flow of Krebs solution gassed with 95% O2 - 5% CO2 at room temperature in the recording chamber. 3D structures and sliced VM organoids were transferred to a recording chamber with Krebs solution gassed with 95% O2 - 5% CO2 at room temperature without constant flow rather the Krebs solution was exchanged manually at the end of the recording of each cell. The composition of the Krebs solution was (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 25 Glucose and 26 NaHCO3. For recordings Multiclamp 700B amplifier (Molecular Devices) was used together with borosilicate glass pipettes (5–7 MΩ) filled with the following intracellular solution (in mM): 122.5 potassium gluconate, 12.5 KCl, 0.2 EGTA, 10 Hepes, 2 MgATP, 0.3 Na3GTP and 8 NaCl adjusted to pH 7.3 with KOH. Data acquisition was performed with pClamp 10.2 (Molecular Devices); current was filtered at 0.1 kHz and digitized at 2kHz. Cells with neuronal morphology and round cell body were selected for recordings. Resting membrane potentials were monitored immediately after breaking-in in current-clamp mode. Thereafter, cells were kept at a membrane potential of -45 mV to -70mV. For detailed Voltage and Current protocol, please refer to methods sections of each papers (see appendix).

Patch Sequencing

Patch-Seq procedure was established similarly to (Bardy et al., 2016). Following electrophysiological recording, slight additional negative pressure was applied (Figure 16). The neuron was then transferred in a volume of ~2 µl of internal patch solution into a PCR tube by slowly retracting the patch pipette from the chamber bath and breaking the tip of the electrode along the inside wall of the tube. Cells were immediately frozen placing the tube on dry ice. For scRNA-seq procedure, cells of interest were thawed at 4°C. Sample buffer was added to the cell and internal solution to reach a total volume of 10 ml following instructions supplied by Clontech with the SMARTer Ultra Low RNA Kit . Successful removal of the cell from the 3D fetal culture was always confirmed by DIC optics. Collected single cells were processed for SMARTer cDNA synthesis following manufacturer's instructions (Clontech, Mountain View, CA, USA). Briefly, first-strand cDNA was synthesized from poly(A)+ RNA by incubation with 1 µl of 3' SMART CDS Primer II A (24 µM) for 3 min at 72 °C, followed by reverse transcription in a 20-µl final reaction volume using 200 units of SMARTScribe Reverse Transcriptase for 90 min at 42 °C and inactivation for 10 min at 70 °C. First-strand cDNA was then purified using Agencourt AMPure XP SPRI Beads (Beckman Coulter Genomics, Danvers, MA, USA) and amplified by long-distance PCR using the Advantage 2 PCR Kit (Clontech) with the following PCR thermocycler program: 95 °C for 1 min, 18 cycles of 95 °C for 15 s, 65 °C for 30 s, 68 °C for 6 min, and 72 °C for 10 min. PCR-amplified double-stranded (ds) cDNA was immobilized onto SPRI beads, purified by two washes in 80% ethanol, and eluted in 12 µl of purification buffer (Clontech). The quality (Agilent 2100 Bioanalyzer High Sensitivity DNA Kit; Agilent Technologies, Santa Clara, CA, USA) and quantity (Qubit dsDNA High Sensitivity Assay Kit; Invitrogen/Thermo Fisher Scientific) of each ds cDNA sample were assessed before library preparation. Construction of single-cell mRNA-seq libraries was performed using the Nextera XT DNA sample prep kit (Illumina) with 0.25 ng of input cDNA.

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