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Academic dissertation

Directing and Dissecting the Fate of Dopaminergic Neurons

- Multiple Avenues towards Cell Replacement Therapy in Parkinson's Disease

Sara Nolbrant

2020

With approval of the Faculty of Medicine of Lund University,
this thesis will be defended
at 09:00 on January 31st, 2020 in Segerfalksalen,
Wallenberg Neuroscience Center, Lund, Sweden

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Abstract

The brain is a complex organ with a limited inherent capacity to regenerate. Restoration of the damaged or diseased central nervous system therefore relies on therapeutic interventions. Cell replacement therapy offers an appealing strategy to treat many neurodegenerative diseases, amongst which Parkinson's disease (PD) is considered a particularly promising therapeutic target due to the focal degeneration of dopaminergic (DA) neurons in midbrain. Building on the success of early clinical trials using human fetal tissue as a cell source for brain repair in PD, protocols for developing DA neurons from human embryonic stem cells (hESCs) have been developed and are currently undergoing clinical translation. In the future, a potential alternative strategy for replacing lost DA neurons could be through direct conversion, where somatic cells are directly reprogrammed to induced neurons without passing through an intermediate pluripotent stage.

The objectives of this thesis have been to direct and dissect the fate of DA neurons to generate cells that can be further developed for cell replacement therapy in PD. These efforts have resulted in a refined and clinically adapted hESC-based cell differentiation protocol and the identification of DA progenitor markers in vitro that can successfully predict the outcome of grafts in vivo, thereby predicting the safety and efficacy of the grafted cell product. In addition, we have increased our understanding of the cellular diversity of DA grafts by employing single cell sequencing for an unbiased assessment of the composition of functionally mature fetal- and hESC-derived grafts. Finally, we developed a hESC-based system for direct conversion of human glial progenitors into induced DA neurons and demonstrated their promise as a source of new neurons in the brain. Collectively, this work has advanced our development of a hESC-based DA differentiation protocol for clinical translation and has further advanced the use of direct neuronal reprogramming as a future avenue for brain repair.

Key words: Dopaminergic neurons, cell replacement therapy, Parkinson's disease, human pluripotent stem cells, cell differentiation, direct neuronal reprogramming, glial progenitors, midbrain development

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- Multiple Avenues towards Cell Replacement Therapy in Parkinson's Disease

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2020

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To my family

"And all the lives we ever lived
And all the lives to be,
Are full of trees and changing leaves"
– *Virginia Wolf*

"I have one power. I never give up."
– *Batman*

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ORIGINAL PAPERS AND MANUSCRIPTS INCLUDED IN THE THESIS

Paper I

Predictive markers guide differentiation to improve graft outcome in clinical translation of hESC-based therapy for Parkinson's disease.

Kirkeby A, **Nolbrant S**, Tiklová K, Heuer A, Kee N, Cardoso T, Rylander Ottosson D, Lelos M, Rifes P, Dunnett S, Grealish S, Perlmann T, Parmar M.

Cell Stem Cell 2017 Jan 5; 20(1):135-148

Paper II

Single-cell analysis reveals a close relationship between differentiating dopamine and subthalamic nucleus neuronal lineages.

Kee N, Volakakis N, Kirkeby A, Dahl L, Storvall H, **Nolbrant S**, Lahti L, Björklund ÅK, Gillberg L, Joodmardi E, Sandberg R, Parmar M, Perlmann T.

Cell Stem Cell 2017 Jan 5; 20(1):29-40.

Paper III

Generation of high-purity ventral midbrain dopaminergic progenitors for *in vitro* maturation and intracerebral transplantation.

Nolbrant S, Heuer A, Parmar M, Kirkeby A.

Nature Protocols 2017 Sep; 12(9):1962-1979.

Paper IV

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 ÅK, Sharma Y, Heuer A, Gillberg L, Hoban DB, Cardoso T, Adler AF, Birtele M, Lundén-Miguel H, Volakakis N, Kirkeby A, Parmar M and Perlmann T. (*Equal contribution)

Manuscript under revision, available at bioRxiv 2019.

Paper V

Direct reprogramming of hESC-derived glial progenitors into induced midbrain dopaminergic neurons

Nolbrant S, Hoban DB, Giacomoni J, Bruzelius A, Birtele M, Pereira M, Ottosson DR, Goldman SA, Parmar M.

Manuscript 2019

ADDITIONAL FINDINGS AND APPLICATIONS STEMMING FROM THE WORK PRESENTED IN THIS THESIS

1) A better understanding of cell integration and innervation after grafting and human cell targeting by viral transduction

-Target-specific forebrain projections and appropriate synaptic inputs of hESC-derived dopamine neurons grafted to the midbrain of parkinsonian rats

Cardoso T, Adler AF, Mattsson B, Hoban DB, **Nolbrant S**, Wahlestedt JN, Kirkeby A, Grealish S, Björklund A, Parmar M.

Journal of Comparative Neurology 2018 Sep 1; 526(13): 2133-2146

-hESC-derived dopaminergic transplants integrate into basal ganglia circuitry in a preclinical model of Parkinson's disease

Adler AF, Cardoso T, **Nolbrant S**, Mattsson B, Hoban DB, Jarl U, Wahlestedt JN, Grealish S, Björklund A, Parmar M.

Cell reports 2019 Sep 24; 28(13):3462-3473

-A systematic capsid evolution approach performed *in vivo* for the design of AAV vectors with tailored properties and tropism

Davidsson M, Wang G, Aldrin-Kirk P, Cardoso T, **Nolbrant S**, Hartnor M, Mudannayake J, Parmar M, Björklund T.

Accepted in PNAS

2) A better understanding of midbrain development

-Single-cell RNA sequencing reveals midbrain dopamine neuron diversity emerging during mouse brain development

Tiklová K, Björklund ÅK, Lahti L, Fiorenzano A, **Nolbrant S**, Gillberg L, Volakakis N, Yokota C, Hilscher MM, Hauling T, Holmström F, Joodmardi E, Nilsson M, Parmar M, Perlmann T.

Nature communications 2019 Feb 4; 10(1):581

3) GMP cell manufacturing to enable clinical use in patients.

- 300 patient doses of cells have now been manufactured and are undergoing safety and efficacy studies prior to the initiation of the clinical trial (STEM-PD). The STEM-PD trial is planned as a multicenter transplantation study at Skåne University Hospital (SUS) and Addenbrooke's Hospital in Cambridge as outlined by (Barker and consortium, 2019; Kirkeby et al., 2017).

ABSTRACT

The brain is a complex organ with a limited inherent capacity to regenerate. Restoration of the damaged or diseased central nervous system therefore relies on therapeutic interventions. Cell replacement therapy offers an appealing strategy to treat many neurodegenerative diseases, amongst which Parkinson's disease (PD) is considered a particularly promising therapeutic target due to the focal degeneration of dopaminergic (DA) neurons in midbrain. Building on the success of early clinical trials using human fetal tissue as a cell source for brain repair in PD, protocols for developing DA neurons from human embryonic stem cells (hESCs) have been developed and are currently undergoing clinical translation. In the future, a potential alternative strategy for replacing lost DA neurons could be through direct conversion, where somatic cells are directly reprogrammed to induced neurons without passing through an intermediate pluripotent stage.

The objectives of this thesis have been to direct and dissect the fate of DA neurons to generate cells that can be further developed for cell replacement therapy in PD. These efforts have resulted in a refined and clinically adapted hESC-based cell differentiation protocol and the identification of DA progenitor markers *in vitro* that can successfully predict the outcome of grafts *in vivo,* thereby predicting the safety and efficacy of the grafted cell product. In addition, we have increased our understanding of the cellular diversity of DA grafts by employing single cell sequencing for an unbiased assessment of the composition of functionally mature fetal- and hESC-derived grafts. Finally, we developed a hESC-based system for direct conversion of human glial progenitors into induced DA neurons and demonstrated their promise as a source of new neurons in the brain. Collectively, this work has advanced our development of a hESC-based DA differentiation protocol for clinical translation and has further advanced the use of direct neuronal reprogramming as a future avenue for brain repair.

LAY SUMMARY

Parkinson's disease (PD) is a common neurodegenerative disorder. The disease becomes increasingly prevalent with age and affects around 1% of the population over 60 years, but 1 patient out of 10 is diagnosed already at an age between 20-50 years. PD is primarily characterized by a set of motor symptoms which include tremor, rigidity and bradykinesia (difficulties to initiate movements). These symptoms arise as a consequence of the progressive loss of the dopaminergic (DA) neurons in the midbrain. In the healthy brain, DA neurons secrete the signaling substance dopamine which is important for controlling and fine tuning our movements. The motor symptoms that are associated with PD are therefore commonly treated with drugs to restore dopamine levels in the brain. However, this treatment approach does not modify or halt the disease progress, and the effect of these drugs decline over time and many patients experience severe and debilitating side effects.

Cell replacement therapy offers an alternative restorative treatment strategy for PD. The idea is to replace the DA neurons that are lost with new and healthy dopamine producing cells that take over the function of cells lost in the disease. The potential efficacy of this therapeutic approach was shown in clinical trials 30 years ago, through grafting of cells isolated from human fetal tissue into the brains of patients with PD. The use of fetal tissue for cell replacement purposes is however ethically complicated and is further limited by a low tissue availability. More applicable sources of DA neurons have therefore been investigated and stem cells have been identified as a promising candidate source. Pluripotent stem cells hold the potential to become any mature cell type within the human body if given the right instructive signals. The work in this thesis has been important for discerning the correct combination of factors and signals for making stem cells into DA neurons. Stem cell-derived DA neurons have shown very promising results when grafted into preclinical animal models of PD and function the same way as the fetal derived DA neurons that we know can provide beneficial effects for patients.

In this thesis, I describe how we identified markers that can be used to predict the outcome of transplantations prior to grafting the DA cells, which is important for ensuring the safety and functional benefit for each and every patient. The new predictive markers were then used in the development of a clinically compatible protocol for producing DA neurons from stem cells, bringing this treatment strategy one step closer to the clinic. In a subsequent study, the cellular composition of the DA grafts was investigated and the cell types present in the graft in addition to the DA neurons were identified. This study revealed a previously unappreciated cellular diversity of the grafts.

In the last part of this thesis, I also investigated direct reprogramming as an alternative strategy to repair the damaged brain. Through virus-mediated delivery of certain cell fate specific genes, mature specialized cells can be directly reprogrammed into other specialized cell types, including DA neurons. This approach could potentially be applied directly in the brain by targeting resident glial cells in the

brain. In this study, the potential for converting such glial cells *in vitro*, in a cell culture dish, was assessed and the results showed that these cells can be efficiently reprogrammed into functional DA neurons, thus opening up for new possibilities for brain repair.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Parkinsons sjukdom är en vanligt förekommande neurodegenerativ sjukdom. Antalet fall ökar med en stigande ålder och sjukdomen drabbar omkring 1% av befolkningen över 60 år, men 1 patient av 10 diagnostiseras redan vi en ålder av 20 till 50 år. Parkinsons sjukdom karaktäriseras av motoriska symptom så som skakningar, muskelstelhet och svårigheter att påbörja rörelser. Symptomen är ett result av förlusten av de dopaminproducerande nervcellerna i en specifik region i hjärnan som kallas mitthjärnan. Dessa så kallade dopaminerga (DA) nervceller utsöndrar normalt signalsubstansen dopamin, som är viktig för att kontollera och finjustera våra rörelser. Sjukdomens motoriska symptom behandlas därför vanligtvis med läkemedel som höjer dopaminnivån i hjärnan. Denna behandlingsstrategi kan varken modifiera eller sakta ner sjukdomsförloppet och effekten av läkemedlet minskar efterhand och många patienter utvecklar svåra bieffekter.

En alternativ metod för att behandla patienter med Parkinsons sjukdom skulle kunna vara med hjälp av cellterapi. Tanken är att ersätta de förlorade DA cellerna i hjärnan med nya friska dopaminproducerande celler. Klininska prövningar som utfördes med hjälp av transplantation av celler som isolerades från fostervävnad visade redan för 30 år sedan att denna behandlingsstartegi kan ge en positiv effekt för patienter med Parkinsons sjukdom.

Användet av celler från aborterade foster för cellterapi är dock komplicerad och försvåras ytterligare av en begränsad tillgång av vävnad. Mer applicerbara källor av DA nervceller har därför efterforskats och ett lovande alternativ är stamceller. Pluripotenta stamceller karaktäriseras av att de har förmågan att bli vilken celltyp som helst i kroppen om de får de rätta signalerna och genom många års forskning så vet vi nu hur man får dem att bli till DA nervceller. De DA nervceller som produceras från stamceller har gett lovande resulat efter transplantation i djurmodeller av Parkinsons sjukdom och de fungerar likvärdigt med de fosterceller som vi sedan tidigare vet kan ge en positiv effekt efter transplantation i patienter.

I denna avhandling har jag beskrivit hur vi identifierade markörer som kan förutsäga resultatet av transplantationen redan innan cellerna transplanteras. Detta är en viktig förutsättning för att kunna säkerställa att alla patienter får en behandling som på ett säkert sätt kan förbättra deras rörelseproblem. De nya markörerna användes också för att utveckla en kliniskt relevant metod för att producera DA nervceller från stamceller, som en del i processen att föra denna behandling närmare kliniska prövningar. En påföljande studie fokuserade på att noggrant granska cellkompositionen av transplantaten för att undersöka vilka olika celltyper dessa innehåller förutom DA nervceller. Denna studie avslöjade en tidigare underskattat diversitet av celler i transplantatet och försåg oss därmed med viktig ny kunskap.

I den sista delen av avhandlingen har jag undersökt direkt omprogrammering som en alternativ strategi för att reparera den skadade hjärnan. Direkt omprogrammering innebär att mogna special-

iserade celler programmeras om till andra mogna celltyper, däribland DA celler, med hjälp av virus som bär på celltypsspecifika gener. Denna strategi skulle potentiellt kunna appliceras direkt i hjärnan, genom omprogrammering av hjärnans gliaceller. I denna studie undersöktes möjligheten att omprogrammera dessa gliaceller *in vitro*, det vill säga i en cellodlingsplatta. Resultaten visade att dessa celler effektivt kan programmeras om till funktionella DA nervceller, vilket öppnar upp för nya framtida möjligheter för reparation av hjärnan.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ABM	Ascl1, Brn2 and Myt1l
ALN	Ascl1, Lmx1a and Nurr1
CNS	Central nervous system
DA	Dopamine/dopaminergic
DBS	Deep brain stimulation
Dox	Doxycycline
EB	Embryoid body
EN1	Engrailed 1
FP	Floorplate
FACS	Fluorescence-activated cell sorting
FGF8	Fibroblast growth factor 8
GFAP	Glial fibrillary acidic protein
GMP	Good manufacturing practice
GFP	Green fluorescent protein
hESCs	Human embryonic stem cells
hGPCs	Human glial progenitor cells
hfVM	Human fetal ventral midbrain
hPSCs	Human pluripotent stem cells
OPCs	Oligodendrocyte progenitor cells
iDANs	Induced dopaminergic neurons
iNs	Induced neurons
iPSCs	Induced pluripotent stem cells
IsO	Isthmus organizer
PCA	Principal component analysis
PD	Parkinson's disease
PDGFRα	Platelet-derived growth factor receptor A
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RA	Retinoic acid
REST	RE1-silencing transcription factor
scRNA-seq	Single cell RNA sequencing
SHH	Sonic hedgehog
SNpc	Substantia nigra pars compacta
STN	Subthalamic nucleus
TF	Transcription factor
TH	Tyrosine Hydroxylase
VLMCs	Vascular and leptomeningeal cells
VM	Ventral Midbrain
VTA	Ventral tegmental area

INTRODUCTION

Parkinson's disease

Over 200 years ago, James Parkinson published his essay on the "shaking palsy", in which he described what he called *Paralysis agitans* (Parkinson, 2002). This debilitating disease later became known as Parkinson's disease (PD) and is today the second most common neurodegenerative disorder, affecting around 1% of the population over 60 years of age (de Lau and Breteler, 2006). The disease becomes increasingly prevalent later in life, and though it is strongly associated with increased age, approximately 10% of the cases occur between the ages of 21-49 and are classified as "young onset" (Mehanna et al., 2014; Schrag and Schott, 2006). The etiology of the PD remains elusive, and while a number of genetic mutations are known to have a causative link to the disease, the vast majority of cases are considered sporadic (idiopathic) (Ascherio and Schwarzschild, 2016; de Lau and Breteler, 2006). The cardinal motor symptoms of PD comprise tremor, rigidity, bradykinesia and postural instability and result from the progressive depletion of the midbrain dopaminergic (DA) neurons and a subsequent DA signaling deficiency in the forebrain. The midbrain DA neurons reside in the substantia nigra pars compacta (SNpc) from where they project to the caudate-putamen and release DA to regulate basal ganglia circuits, which are involved in motor output and control (Björklund and Dunnett, 2007).

The prevailing therapeutic strategy to manage the motor symptoms of PD is by pharmacological elevation of DA levels in the brain through the administration of drugs such as levodopa, DA agonists and inhibitors of DA degrading enzymes (Connolly and Lang, 2014). This approach generally provides an efficient symptomatic relief for most patients at an early stage of the disease. However, over time the pharmacological increase of DA becomes less effective for symptom management, and patients commonly develop side effects including motor fluctuations and dyskinesias which result from non-physiological DA levels and off-target effects associated with the systemic delivery of DA (Jankovic, 2005; Thanvi and Lo, 2004). To address the motor fluctuations and dyskinesias associated with advanced PD, intestinal gels for continuous administration of levodopa/carbidopa are sometimes employed. These gels are delivered though percutaneous gastronomy via a jejunal tube and can be effective in reducing the "off" time during which the patient's motor symptoms cannot be efficiently managed, but the benefits are limited by safety issues related to the intestinal infusion system (Wirdefeldt et al., 2016). Deep brain stimulation (DBS) represents a non-pharmacological advanced stage treatment approach and involves implantation of electrodes into the brain to stimulate inhibitory nuclei of the basal ganglia (Groiss et al., 2009). DBS can result in an improvement of the motor fluctuations and a reduction of tremors, but is complicated by the invasiveness of the strategy and has been associated with certain behavioral and cognitive issues (Rossi et al., 2018).

In addition to the motor symptoms, patients with PD often suffer from a variety of non-motor symptoms including depression, cognitive deficiencies, sleep disturbances and autonomic dysfunc-

tions (Duncan et al., 2014). These symptoms likely arise from a more widespread distribution of pathological Lewy bodies and neurites and from the degeneration of non-dopaminergic brain nuclei and are therefore treated in a patient-specific manner (Braak et al., 2004; Connolly and Lang, 2014; Giguere et al., 2018).

While effective therapeutic options for managing the motor symptoms of PD exist, the treatment alternatives that are currently in practice are neither disease modifying, nor restorative (AlDakheel et al., 2014). The ability to halt or even prevent DA degeneration would greatly impact the disease progression and would have important implications for the life quality of the patients. Another appealing prospect would be to have the ability to regenerate the cells in the brain. This applies in particular for the individuals who develop the disease at an early age and who therefore are expected to live with the disease for a significant part of their lives. Cell replacement therapy offers such a potential restorative treatment, with the idea of replacing the lost neurons with new and healthy DA-producing cells.

Cell replacement therapy for Parkinson's disease – the past

The rationale

Cell replacement therapy is being developed as a treatment alternative for a number of neurodegenerative disorders and is currently being translated into multiple clinical trials (Steinbeck and Studer, 2015; Tabar and Studer, 2014). PD is considered to be particularly well-suited for this therapeutic strategy due to the relatively local degeneration of a small number of DA neurons, making it a relatively easy target compared to other neurodegenerative disorders. Additionally, DA enhancing medicines provide efficient relief of the motor symptoms of the disease, but the unregulated release of DA across the whole brain leads to complications (Connolly and Lang, 2014). Through cell replacement therapy, DA could be secreted locally in a more physiological way, thus circumventing the development of side effects associated with current pharmacological approaches.

The idea to replace DA neurons lost to PD was introduced many years ago. Starting in the 1980s, multiple different cell types and cell sources have been assessed in clinical trials, including porcine fetal ventral midbrain tissue, retinal pigmental epithelial cells, adrenal medullary cells and carotid body cells (Barker et al., 2015a). However, the trials in which these cell sources were used all built on limited and unconvincing preclinical data and consequently provided very modest or only temporary clinical improvements when transplanted into patients. The one cell source from these early trials that did inspire hope was cells isolated from the human fetal ventral midbrain (hfVM) (Lindvall et al., 1990). This cell source had previously provided positive preclinical results including DA neuron survival, extensive innervation, DA release and behavioral recovery in DA-depleted rats (Brundin et al., 1986; Brundin et al., 1988; Strömberg et al., 1986). Collectively, these early grafting trials showed that only authentic human midbrain DA neurons represent an appropriate cell source for cell replacement therapy in PD, and that when entering clinical trials, sufficient supporting preclinical data is critical for predicting therapeutic success (Figure 1).

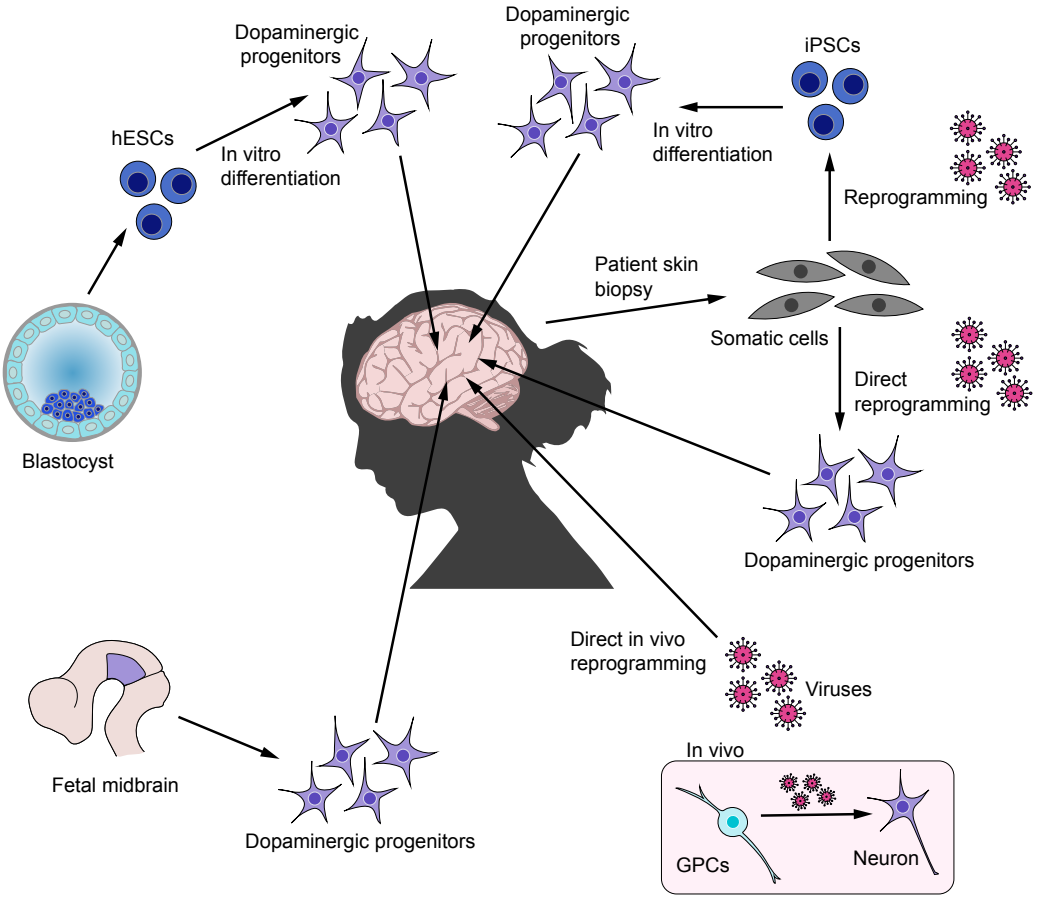

Figure 1: *Alternative sources of DA neurons for cell replacement therapy in PD*

Transplantable midbrain DA neurons can be isolated from the developing fetal midbrain, differentiated from pluripotent stem cells including hESCs and iPSCs, or generated via direct reprogramming of somatic cells to iNs. iPSC technology and direct reprogramming can be applied to cells that have been isolated by a skin biopsy and allows for patient specific treatments. Direct reprogramming could also be done directly in the brain, by the conversion of brain resident glial cells.

The history – fetal tissue trials

The first hfVM transplantations into patients with PD took place in Lund in 1987 and the encouraging results from these early grafts led to the initiation of a larger open-label study, with a subsequent 13 patients being transplanted over the 1990s (Brundin et al., 2000; Hagell et al., 1999; Lindvall et al., 1990; Lindvall et al., 1994; Wenning et al., 1997; Widner et al., 1992). In this transplantation series, tissue from three to six fetuses of gestational age 5–9 weeks was transplanted unilaterally or bilaterally into the putamen or caudate-putamen and the patients were on an immunosuppressive regime for at least one year. The outcome of this trial provided additional proof-of-principle for the potential

efficacy of this treatment strategy with marked clinical recovery in a subset of the patients, accompanied by restored DA release in the striatum, functional graft integration, and in the most successful cases, a complete discontinuation of DA replacement medication (Piccini et al., 1999; Piccini et al., 2000). Additionally, long term follow-up studies of patients grafted in this trial have shown functionally maintained for over 15 years and histological analysis of postmortem tissue from a transplanted patient has provided evidence for the survival and maintained phenotypic identity of hfVM derived DA neurons for 24 years (Kefalopoulou et al., 2014; Li et al., 2016).

At the same time as the open-label study was taking place in Lund, additional hfVM clinical studies for PD were initiated globally. In the US, encouraging clinical results paved the way for two NIH funded studies were the efficacy of the hfVM grafts were assessed in the form of double-blind placebo-controlled trials (Freed et al., 1992; Kordower et al., 1995). However, when the results from these trials were published in the early 2000s, the reports were both negative, with none of the trials being able to reach their primary endpoints (Freed et al., 2001; Olanow et al., 2003). Even more concerningly, a subset of patients developed side effects in the form of graft-induced dyskinesias. This was a major setback for the field, and many decided to completely abandon the idea of pursuing cell replacement therapy for PD. However, multiple aspects of these NIH funded clinical trials made them fundamentally different from the open label trials (Barker et al., 2013). This included factors such as patient selection, tissue preparation and the number of fetuses used for transplantation, as well as the immunosuppressive regime employed post-grafting. Additionally, the design of the trial was suboptimal, so that any statistically significant beneficial effects of the grafts were difficult to detect even though a subset of patients with less-advanced disease did indeed show recovery over time (Ma et al., 2010).

As a consequence of these inconsistencies, a working group in Europe set out to define factors that were associated with a positive grafting outcome from all previously conducted grafting studies. On the basis of the findings of this extensive investigation, the European multicenter open label trial TRANSEURO was initiated in 2009 (Barker et al., 2015a). Over a 3-year period, 11 patients received bilateral grafts in either Lund or Cambridge (Barker and consortium, 2019). The last patient was grafted in Lund in 2018 and the outcome of the study will be evaluated in 2021. However, a major shortcoming associated with the use of fetal tissue that had already previously been recognized was further emphasized during the TRANSEURO trial – the low availability of hfVM tissue resulted in few grafting opportunities and a total of 87 surgeries were cancelled due to inadequate tissue supply. There is a restricted ability to store and culture cells from hfVM tissue and together with the low availability, this results in limited opportunities for quality control and standardization, which leads to increased variability between patients. An unlimited and standardized cell source for obtaining midbrain DA neurons was therefore a prerequisite for making this treatment strategy accessible for larger patient groups. During the planning and execution of the TRANSEURO trial, an increased understanding of midbrain DA neuron development, coupled with the emergence of more sophisticated protocols for generating authentic midbrain DA neurons from human pluripotent stem cells (hPSCs) made these objectives feasible to realize.

hPSC-based cell replacement therapy for PD – the present

The development of midbrain DA neurons

The early regionalization of the neural tube is initiated through the emergence of signaling centers that instruct the cells about their position along the rostral-causal and dorsal-ventral axes (Kiecker and Lumsden, 2012). In the midbrain, concentration gradients of the signaling molecules Wnt1, Fgf8 and Shh provide positional information and the formation of the isthmus organizer (IsO) and the floor plate (FP), from which these factors are secreted, marks the initiation of regionalization and patterning of the midbrain (Kiecker and Lumsden, 2012; Liu and Joyner, 2001). The homeodomain transcription factors Otx2 and Gbx2 are crucial for the positioning of the IsO, which specifies the sharp boundary between the midbrain and the hindbrain (Martinez-Barbera et al., 2001). In addition to the role of Otx2 in regulating the identity of the progenitor domain of the VM, this factor has also been identified to confer neurogenic potential to the FP (Ono et al., 2007; Puelles et al., 2004). FP cells were long considered to only play their roles as organizers, through Shh mediated ventralization of the neural tube, therefore the discovery of radial glia-like cells of the FP as the origin of midbrain DA progenitors came as a surprise (Bonilla et al., 2008; Hebsgaard et al., 2009; Ono et al., 2007).

Years of extensive studies of the transcriptional profile of midbrain DA neurons have provided a thorough understanding of the development of these cells (Figure 2). The transcription factor (TF) Lmx1a and its downstream effector Msx1 are among the earliest DA fate determinants and play crucial roles in the specification of a DA identity (Andersson et al., 2006b). The FP marker FoxA2 is another early determinant of the midbrain DA fate that is expressed together with FoxA1 in the ventral midbrain and regulate multiple stages of DA development and maturation (Ferri et al., 2007; Lin et al., 2009; Stott et al., 2013). These early fate determinants together promote the expression of the pro-neuronal TF Ngn2 that is required for DA neuron differentiation (Andersson et al., 2006a; Kele et al., 2006). Additional important TFs for acquiring a DA identity include Engrailed 1/2 (En1/2) and Lmx1b (Simon et al., 2001; Smidt et al., 2000). The expression of these genes is initiated already in early progenitors and is sustained in both immature and mature neurons and required for the generation, survival and maintenance of mature midbrain DA neurons. At the stage when the midbrain DA progenitors become post-mitotic, the expression of the TF Nurr1 is initiated (Zetterstrom et al., 1996). Nurr1 is important for the long-term survival of DA neurons in the midbrain and functions as a master regulator in the adoption of a DA neurotransmitter identity by inducing and maintaining the expression of Tyrosine hydroxylase (*Th*), dopamine transporter (*Dat*), vesicular monoamine transporter 2 (*Vmat2*) and L-aromatic amino acid decarboxylase (*Ddc*) (Saucedo-Cardenas et al., 1998; Smits et al., 2003). The expression of the TF Pitx3 is also limited to postmitotic DA progeny and appears to work in concert with Nurr1 to promote the survival and terminal differentiation of midbrain DA neurons (Chakrabarty et al., 2012; Jacobs et al., 2009; Nunes et al., 2003).

The diversity of midbrain DA neurons

The developmental program of midbrain DA neurons is highly complex and the profiling of the cells is further complicated by the fact that they give rise to different subpopulations that display phenotypically and functionally diverse properties after grafting (Mendez et al., 2005; Thompson et

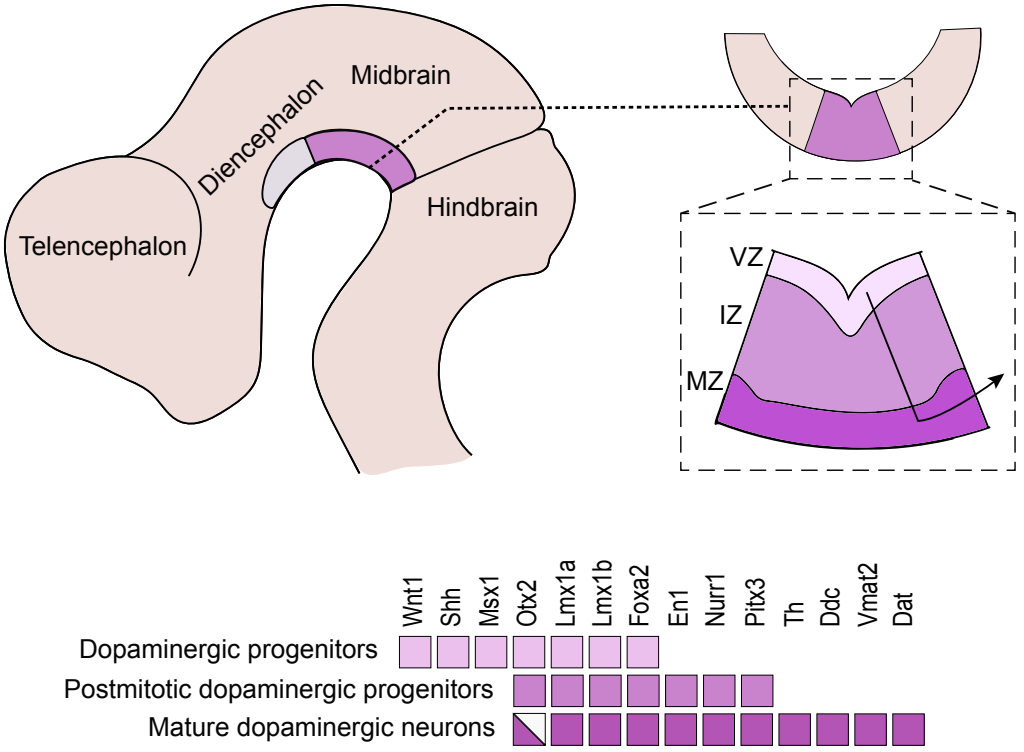

Figure 2: *Development of midbrain DA neurons*

Midbrain DA neurons develop in the ventral part of the midbrain. The acquisition of a DA identity is initiated in the ventricular zone (VZ) and postmitotic DA progenitors in the intermediate zone (IZ) migrate first radially to the mantel zone (MZ) and then tangentially as they mature into DA neurons. The arrow indicates the axes of migration. The genes expressed at the different stages of DA neuron development are indicated as colored boxes. Otx2 is predominantly expressed by mature DA neurons in the ventral tegmental area (VTA).

al., 2005). Initial subcategorization of the DA neurons in the midbrain based on morphology, location and projections divided the cells into three distinct groups termed A8 (located in the retrorubral field, RRF), A9 (located in the SNpc), A10 (located in the ventral tegmental area, VTA) (Dahlström and Fuxe, 1964). Out of these groups, the A9 DA neurons of the SNpc are selectively vulnerable to degeneration in PD (Damier et al., 1999). The A8-A10 subgrouping of the midbrain DA cells offers a simplified description of DA diversity and includes limited descriptions of molecular differences or any developmental aspects of the different lineages. With the development of single cell RNA sequencing (scRNA-seq) as a more refined tool for understanding cell diversity, an increased understanding of cell development and lineage diversification is emerging. Through the comprehensive molecular characterization provided by these studies, an even more complex picture of DA neuron development and diversity is being unveiled (La Manno et al., 2016; Poulin et al., 2014; Tiklova et al., 2019). The functional consequences of this molecular diversity are however still far from being fully

understood and we have only just started to comprehend what factors dictate the projection patterns and properties of different subtypes of DA neurons (Chabrat et al., 2017; Grealish et al., 2014; Panman et al., 2014).

Generation of midbrain DA progenitors from hPSCs

The first account of successful derivation of human embryonic stem cells (hESCs) was reported by Thomson et al. in 1998 and represented a major breakthrough for the possibility to study human development and disease, as well as for the development of cell-based therapies (Thomson et al., 1998). This study showed that pluripotent hESCs that are isolated from the inner cell mass of early blastocysts can be maintained and expanded over a long period *in vitro* and hold the potential to differentiate into any cell of the three germ layers if given the correct instructive signals. Only a few years after the derivation of hESCs, the first reports of hESC-based generation of neuronal cells trough spontaneous differentiation and embryoid body (EB) formation were published (Itskovitz-Eldor et al., 2000; Reubinoff et al., 2001; Zhang et al., 2001). However, these protocols generated few if any TH^+ DA neurons, which was considered of high interest for multiple fields due to the involvement of DA neurons in not only movement, but also emotions and reward, implicating these cells for the study and treatment of PD, schizophrenia and addiction (Klein et al., 2019).

In the original protocols for differentiating hESCs into DA neurons, patterning was achieved through sub-culturing on murine stromal cells with or without the addition of signaling factors such as SHH and FGF8 to the medium (Park et al., 2005; Perrier et al., 2004; Zeng et al., 2004). These protocols gave rise to a high number of TH^+ neurons *in vitro*, but few if any TH^+ cells survived when grafted into the rodent brain and in some cases the grafting resulted in tumor formation due to an inefficient and non-synchronized differentiation of the cells (Roy et al., 2006; Sonntag et al., 2007). Additionally, the DA neurons generated from these protocols did in fact not co-express the midbrain DA markers LMX1A and FOXA2, thus questioning the true midbrain DA identity of these cells.

The unexpected discovery of the FP origin of midbrain DA neurons combined with the development of better protocols for a more efficient neuralization inspired new hope of resolving some of these issues. In 2009, Lorenz Studer's group at Sloan-Kettering institute published a Dual-SMAD inhibition protocol for achieving a rapid and synchronized neuronal induction through the addition of Noggin and SB431542 to inhibit bone morphogen proteins (BMPs) and to block the pathways of Lefty, Activin and Transforming growth factor β (TGFβ) (Chambers et al., 2009). One year later, the same group published a protocol for generating FP cells from hPSCs by adding a highly potent form of the ventralizing agent SHH to the medium (Fasano et al., 2010). Without supplementation with any additional patterning factors, this protocol generates cells of a forebrain identity. However, by a dose-dependent activation of WNT singling through the addition of a chemical inhibitor of glycogen synthase kinase 3 (GSK3), the cells can adopt more posterior identities, including midbrain DA fates (Kirkeby et al., 2012; Kriks et al., 2011). In these two initial reports of the generation of FP derived midbrain DA neurons from hPSCs, the obtained progenitors co-expressed midbrain and FP markers such as OTX2, LMX1A and FOXA2 and they expressed TH after terminal differentiation *in vitro* or after transplantation and maturation *in vivo*. Additionally, when grafted into DA lesioned rodents, the cells released DA and could restore the motor impairments of the animals. Importantly, the complete and synchronized neuralization that is achieved through Dual-SMAD inhibition also resulted in a

complete absence of tumor formation. These two papers both showed that it is possible to generate authentic midbrain DA neurons from hPSCs, thereby opening the possibility to use these cells for cell replacement therapy in PD.

With the discovery that human somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) through virus mediated delivery of four pluripotency factors, an accessible source of autologous hPSCs emerged that is considered less ethically problematic than hESCs, since it does not require the disruption of human embryos and provides possibilities for patient specific cells or human leukocyte antigen (HLA) matching (Takahashi et al., 2007). The finding by Shinya Yamanaka truly revolutionized the field as it allowed for any mature human cell type to be reprogrammed back into a pluripotent stage, thus opening up the doors for better ways to model disease and to screen for drugs, and also allowing for the possibility of patient specific treatments (Robinton and Daley, 2012). Another group in Japan, led by Jun Takahashi, developed an iPSC-based protocol for generating midbrain DA neurons, building on the same principle of a FP intermediate progenitor (Doi et al., 2014).

Developing a hPSC-based cell replacement therapy for PD

The publication of protocols for generating authentic midbrain DA neurons from hPSCs sparked a revived interest in cell replacement therapy for PD (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011). A number of follow up studies were designed to look at the functionality of the grafts, and to compare these grafts to hfVM grafts that previously had shown efficacy in clinical trials (Chen et al., 2016; Grealish et al., 2014; Grealish et al., 2015; Hallett et al., 2015; Kikuchi et al., 2017; Niclis et al., 2017; Steinbeck et al., 2015). The report that hESC-derived DA neurons showed similar preclinical efficacy and potency compared to graft derived from hfVM tissue when grafted into 6-OHDA lesioned rats therefore became an important landmark study for the field (Grealish et al., 2014). This paper also showed that hESC-derived DA neurons restore the DA neurotransmission in the striatum as assessed by positron emission tomography (PET) imaging, that fetal- and hESC-derived DA neuron show similar subtype specific marker expression after maturation *in vivo* and that both cell sources provide correct target-specific innervation. Through the use of optogenetic and chemogenetic approaches, the activity of hPSC-derived DA neurons that had been engineered to express either light sensitive ion channels or designer receptors exclusively activated by designer drugs (DREADDs) could be modulated in real time when grafted into DA lesioned mice (Chen et al., 2016; Steinbeck et al., 2015). These experiments showed that the activity of the grafts could be fine-tuned and provided evidence for the dependence of graft function on neuronal activity and DA release, and also showed that the grafts were able to modulate host glutamatergic synaptic transmission onto striatal medium spiny neurons in a similar way as endogenous midbrain DA neurons.

Through the use of a modified rabies virus, the afferent and efferent connectivity of DA grafts has been assessed monosynaptically in a series of studies conducted by our group in Lund. These studies show that graft connectivity is observed already after 6 weeks post-grafting, that it remains stable for at least 6 months, and that it is comparable to endogenous midbrain connectivity when the cells are grafted in the midbrain (Cardoso et al., 2018; Grealish et al., 2015). For clinical purposes, the DA progenitors will be transplanted directly into the caudate and putamen, to the immediate site of action for DA secretion in order to reduce the distance that the axons need to grow to reach their

targets. Importantly, additional rabies tracing experiments were able to show that ectopically placed DA grafts in the striatum of DA lesioned rats receive monosynaptic inputs from the same appropriate host structures as homotopically placed grafts in the midbrain (Adler et al., 2019). By combining rabies tracing with an additional retrograde tracer, this study further showed that the same individual host neurons in fact collateralize and contact neurons in the striatum and midbrain simultaneously.

The functionally of hPSC-derived DA neurons has in addition to rodent models also been successfully assessed using parkinsonian primates (Hallett et al., 2015; Kikuchi et al., 2017). Grafted human iPSC-derived midbrain DA progenitors survive long term in the MPTP-lesioned primate brain, mature into subtype specific midbrain DA neurons, secrete DA, provide extensive innervation and amelioration of the lesion induced motor symptoms. These effects were seen regardless if the iPSCs were derived from heathy donors or individuals with PD, thereby arguing for the possibility to use patient specific treatment (Kikuchi et al., 2017). One beneficial aspect of using patient specific cells would be the eliminated need of immunosuppression post-grafting. However, the long-term aspects of using cells that are predisposed to the disease still need to be determined, especially since an increased accumulation of pathogenic alpha-synuclein containing Lewy bodies has been detected in fetal derived grafts over time in some patient grafts (Kordower et al., 2008; Li et al., 2008; Li et al., 2016). An alternative approach until these questions have been resolved could be to graft major histocompatibility complex (MHC)-matched allogeneic cells, which have been shown to reduce the immunogenic response to iPSC-derived DA graft (Morizane et al., 2017). However, a recent study in which autologous, MHC-matched and mis-matched iPSC-derived neurons were grafted in a primate model of Huntington's disease challenged this concept since the MHC-matching failed to prevent long-term graft rejection (Aron Badin et al., 2019). The applicability of better immunologically matched cells for preventing graft rejection in the absence of immunosuppression therefore remains uncertain.

Moving to the clinic

The success of the pre-clinical studies of hPSC-based cell replacement therapy for PD has led to the initiation of several translational programs with the aim to bring these cells to a clinical setting (Barker et al., 2017). In 2014, the global initiative G-FORCE PD was formed, with the purpose to take a collaborative approach to the development of a stem cell-based therapy for PD (http://www.gforce-pd.com). The consortium consists of translational teams from Europe, Asia and the US who meet annually to discuss the progress and potential pitfalls associated with the clinical translation (Barker et al., 2015b). A whole new set of challenges arise when moving from an academic- to a clinical setting. This includes cryopreservation of the cells, good manufacturing process (GMP) adaptation of the cell differentiation protocol, as well as the production process and fulfilling all standards set forth by regulatory authorities (Kirkeby et al., 2017; Studer, 2017; Takahashi, 2017). Additionally, the design of the trial needs to be carefully planned to avoid issues with the readout of the trial and to be able to make well supported conclusions (Barker and consortium, 2019).

In October 2018, the first patient was transplanted in Japan using iPSC-derived DA progenitors in a study designed primarily to assess the safety and tolerability of the grafted cells (http://www.cira.kyoto-u.ac.jp/e/pressrelease/news/180730-170000.html). Additional trials using hESC-derived cells are planned in both Sweden and in the US and will take place during the next couple of years. In

Lund, my work on the development of GMP adapted protocols for generating and cryopreserving the hESC-derived DA progenitors marked an important milestone for the clinical translation of this therapy (Nolbrant et al., 2017). These efforts have now led to the successful production of a GMP cell bank of 300 patient doses that are currently undergoing safety and efficacy studies before use in the clinical STEM-PD trial.

Direct reprogramming for brain repair – the future

The concept of direct reprogramming

For many years, cell differentiation was considered to be an exclusively unidirectional process wherein a pluripotent cell becomes progressively specialized, without the ability to de-differentiate to a less mature state or to transdifferentiate across lineages (Waddington, 1957). This concept was challenged by Sir John Gurdon in the 1960s through his nuclear transfer experiments in frogs (Gurdon, 1962). However, at the time it was still undetermined if mammalian cells could exhibit the same level of plasticity and it took decades until additional evidence for reprogramming was obtained through forced expression of transcription factors to drive the conversion of cells within the same germ layer (Davis et al., 1987; Tapscott et al., 1988). Twenty years later, the discovery of the possibility to reprogram both mouse and human mature somatic cells back into pluripotency (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) motivated scientists to explore the question whether other combinations of transcription factors could be used to reprogram mature somatic cells directly to other specialized cell types.

Direct neuronal reprogramming *in vitro*

In 2010, a group led by Marius Wernig at Stanford University was able to show viral mediated direct reprogramming *in vitro* of mouse embryonic and postnatal fibroblasts into induced neurons (iNs) by overexpression of the three TFs Ascl1, Brn2 and Myt1l (ABM) (Vierbuchen et al., 2010). One year later, additional studies reported successful conversion of human embryonic and postnatal fibroblasts as well as mature hepatocytes into functional iNs using the ABM transcription factor combination with or without the addition of the TF NeuroD1, thereby providing evidence not only for the possibility of direct neuronal conversion of human cells but also reprogramming across the germ layers (Marro et al., 2011; Pang et al., 2011; Pfisterer et al., 2011).

Mechanistic studies of the reprogramming process have identified Ascl1 as a pioneering factor that can open up closed chromatin and recruit other transcription factors to its targets (Wapinski et al., 2013). Multiple studies have shown that the reprograming process is not dependent on cell division to occur (Fishman et al., 2015; Karow et al., 2012; Marro et al., 2011). For neuronal conversion of adult human cells, the RE1-silencing transcription factor (REST) complex function as a potent barrier of reprogramming, and down regulation of REST consequently leads a significant improvement of reprogramming efficiency (Drouin-Ouellet et al., 2017; Masserdotti et al., 2015). By using scRNA-seq technology, recent studies have been able to dissect the reprogramming process in more detail (Biddy et al., 2018; Karow et al., 2018; Treutlein et al., 2016). When adult human pericytes were transduced with Ascl1 and Sox2, the reprogramming cells pass through a neural stem cell intermediate before adopting either a glutamatergic or GABAergic fate, and the cellular context of the starting cell dictates the ability of the cell to reprogram (Karow et al., 2018). Sequencing technology has also been used to identify factors associated with different lineage trajectories that lead to either successful or failed reprograming – information that could be used to improve the reprogramming process further (Biddy et al., 2018).

The addition of small molecules during the reprogramming process to activate signaling pathways and modify chromatin structures markedly enhance the efficacy of reprogramming (Karow et al., 2018; Rivetti di Val Cervo et al., 2017), and can in some systems completely replace delivery of TFs. In a recent study, timeline RNA-seq data across the course of conversion allowed for a methodical identification of signaling pathways that could be modulated to enhance the reprogramming process further (Herdy et al., 2019). Several studies also report the reprogramming of human fibroblasts and astrocytes to functional iNs *in vitro*, through a sophisticated scheme of small molecules and growth factors applied in sequence without the addition of any viral vectors (Hu et al., 2015; Yang et al., 2019; Yin et al., 2019; Zhang et al., 2015).

In the initial reprogramming studies, the conversion of human cells using the ABM cocktail resulted mainly in iNs exhibiting either a glutamatergic or GABAergic fate. However, by combining a pioneering proneuronal factor such Ascl1 or Ngn2 with lineage specific transcription factors, many different kinds of subtype-specific neurons have been generated, including sensory neurons, noradrenergic neurons, motor neurons, striatal neurons and neurons of a DA fate (Blanchard et al., 2015; Caiazzo et al., 2011; Li et al., 2019; Pfisterer et al., 2011; Son et al., 2011; Victor et al., 2014).

Direct *in vitro* reprogramming to a DA fate

The detailed knowledge of midbrain DA development provided an important basis for the direct conversion of somatic cells to induced DA neurons (iDANs) (Arenas et al., 2015). The first report of successful reprogramming of human somatic cells into a DA fate using the factors ABM in combination with Lmx1a and Foxa2 was published in 2011 (Pfisterer et al., 2011). Within the same year, three additional studies described conversion of human and mouse fibroblasts and mouse astrocytes into iDANs using slightly modified combinations of TFs: Ascl1, Lmx1a and Nurr1 (ALN); Ascl1, Lmx1b and Nurr1; or Ascl1 and Pitx3 (Addis et al., 2011; Caiazzo et al., 2011; Kim et al., 2011). The obtained iDANs showed correct DA gene expression profiles, subtype-specific markers, electrophysiological properties comparable to endogenous DA neurons, as well as DA release. In a more recent study, an increased efficiency and improved functionality of iDANs reprogrammed from human astrocytes *in vitro* was achieved with the transgene combination NeuroD1, Ascl1, Lmx1a and mir218 (NeAL218) in combination with a more refined administration scheme of small molecules to promote chromatin remodeling and to induce TGFβ, SHH and WNT signaling (Rivetti di Val Cervo et al., 2017).

One of the early studies on DA conversion showed successful transplantation of iDANs generated from mouse fibroblasts after conversion *in vitro* (Caiazzo et al., 2011). A few years later, the same group also showed functional integration of stably converted mouse iDANs *in vivo* and a capability of these cells to provide a substantial amelioration of the motor symptoms in a rat model of PD (Dell'Anno et al., 2014). Furthermore, chemogenetic DREADD technology was used to modulate the activity of the grafted iDANs, which markedly improved the beneficial effect of the graft. Human fibroblasts transduced with doxycycline (dox) inducible viral vectors carrying the TFs ABM, Lmx1a/b, Foxa2 and Otx2 have been transplanted into rats after transgene activation *in vitro*, or transplanted and converted *in vivo* upon administration of dox to the drinking water of the animals (Pereira et al., 2014; Torper et al., 2013). However, although some TH^+ cells were detected *in vivo*, the efficiency was much lower compared to what had been observed previously *in vitro*.

In vivo reprogramming in the brain

For future therapeutic applications, direct conversion of resident brain cells *in situ* by delivery of the viral vectors directly to the brain is an appealing alternative approach that circumvents the need for cell transplantation and immunosuppression (Li and Chen, 2016; Vignoles et al., 2019). The first account on *in vivo* reprogramming was reported in 2005 and described retrovirus mediated conversion of reactive glia though overexpression of Pax6 or a negative-dominant form of Olig2 in the cortex following a stab wound injury (Buffo et al., 2005). Subequent studies have been able to show *in vivo* conversion in both injured and intact brain regions, using different starting cells, factor combinations and vector systems (Gascon et al., 2017).

In vivo conversion into a DA fate has proven to be a more elusive task than what could be anticipated, as factors combinations that effciently convert cells *in vitro* failed to reprogram both mouse astrocytes and glial progentitor cells *in vivo*, independent of brain region and the local environment (Pereira et al., 2017; Torper et al., 2015). In one study, TH^+ neurons were observed *in vivo* in the mouse following conversion of astrocytes using the NeAL218 factor combination (Rivetti di Val Cervo et al., 2017). However, the origin of these cells was not determined since no reporter system was used and since TH^+ cell bodies can arise in the striatum as a consequence of DA depletion (Björklund and Dunnett, 2007; Pereira et al., 2017, Tepper and Koós, 2010). Interstingly, in a recent study, the *in vivo* transduction of the factor combination Sox2, Foxa2, Lmx1a and Nurr1 together with the administration of valproic acid (VPA) failed to convert mouse glia into DA neruons, but resulted in an induction of DA fates in striatal neurons, which was coupled with emergence of electrophysiological properties of DA neurons rater than those of striatal neurons (Niu et al., 2018). This informs us that reprogramming into neurons with DA properties may be achieved *in vivo,* but that there may be additional roadblocks associated with the reprogramming of glial cells to DA neurons *in vivo* that yet need to be identified and resolved. In addition, validation of the origin of the reprogrammed neurons will require the employment of good reporter systems and the characteristics of the cells need to be carefully assessed using appropriate tests to ensure the subtype specific authenticity of the neurons.

Identifying appropriate target cells for *in vivo* reprogramming

To be able to consider *in vivo* reprogramming as a feasible treatment strategy for brain repair, appropriate target cells need to be identified. Numerous studies describe the conversion of human astrocytes *in vitro* and mice astrocytes into subtype specific neurons *in vitro* and *in vivo* (Guo et al., 2014; Mattugini et al., 2019; Niu et al., 2015; Rivetti di Val Cervo et al., 2017). The conversion of reactive astrocytes could provide an additional beneficial aspect under certain pathological circumstances, by removing potentially harmful cells and at the same time providing a source of new neurons (Torper and Götz, 2017). However, astrocytes play complex and multiple roles related to brain physiology and cell signaling as they regulate processes such as ion homeostasis, neurotransmitter clearance, and synapse maintenance (Khakh and Sofroniew, 2015). Therefore, when targeting these cells, measures need to be taken in order to not deplete cells that confer important physiological functions, but to only target the deleterious cells.

Pericytes are associated with the brain vasculature where they contribute to the maintenance of the blood-brain-barrier and the regulation of capillary blood flow, but are capable to proliferate and

migrate into the parenchyma upon injury (Armulik et al., 2011; Goritz et al., 2011). Human pericytes have been successfully reprogrammed into neurons *in vitro,* but the potential for targeting these cells *in vivo* is yet to be resolved (Karow et al., 2018; Karow et al., 2012). The first report on *in vivo* neuronal conversion of microglial was published recently (Matsuda et al., 2019). Homeostatically self-renewing microglia are spread throughout the brain and could represent an interesting candidate cell source since they are activated upon injury and migrate to the damaged brain area (Li and Barres, 2018).

One study reports neuronal conversion of oligodendrocytes in the adult brain (Weinberg et al., 2017). However, just like mature astrocytes, myelinating oligodendrocytes provide important trophic support to neurons and they enable a fast transmission of neuronal signaling though saltatory conduction of action potentials along axons, making them crucial components for a maintained physiological brain function (Nave, 2010). Oligodendrocyte progentitor cells (OPCs, also called glial progenitor cells or NG2 glia) are uniquely intersting candidate target cells for *in vivo* conversion. OPCs migrate continuously throughout brain, they remain proliferative though adulthood and they maintain a homeostatic control of cell density, meaning that the risk of depleating these cells is reduced (Hughes et al., 2013; Robins et al., 2013; Simon et al., 2011). Additionally, OPCs are able to recive synaptic input from neurons in the brain, and therfore already have part of the machinery to become electrophysiologically active when reprogrammed into neurons (De Biase et al., 2010; Lin and Bergles, 2004). The ability to convert OPCs to subtype specific neurons in mouse models *in vivo* has been shown in multiple studies (Guo et al., 2014; Heinrich et al., 2014; Pereira et al., 2017; Torper et al., 2015). However, the study of human OPC conversion has been challenged by the fact that these cells are not born until late in the second trimester, thus limiting their accessibility from human embryos for experimental studies and *in vitro* cultures (Jakovcevski and Zecevic, 2005; Sim et al., 2011).

The development of glial progenitor cells

The glial cells of the central nervous system (CNS) are generated late, as gliogenesis follows after neurogenesis in the developing brain (Rowitch and Kriegstein, 2010). Following the neuron–glial-cell switch, progenitor regions that previously produced neurons will start to produce cells of the astrocyte- and oligodendrocyte lineages. In the spinal cord, the pMN region that initially produced motor neurons will start to generate OPCs. This switch in lineage production is associated with a downregulation of pro-neuronal factors such as Ngn2 and a concurrent upregulation of the pro-glial genes Sox9 and NFI and is dependent on a number of prerequisites including a maintained but perhaps modified activity of Shh and Olig2 and an upregulation of notch signaling (Deneen et al., 2006; Esain et al., 2010; Li et al., 2011; Park and Appel, 2003).

Oligodendrogenesis has been extensively studied in the mouse spinal cord where it starts around day E12.5 in the ventral pMN region. Shh signaling is both required and sufficient for this first wave of OPC production and drives the induction of Nkx6.1/2 controlled Olig1 and Olig2 expression (Orentas et al., 1999; Vallstedt et al., 2005). A second wave of OPC production that is independent of Shh signaling starts around day E15.5 from the dorsal neural tube (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005), and a third wave occurs after birth (Rowitch and Kriegstein, 2010). The same concept of three waves of OPC production takes place in the forebrain, with ventrally derived

OPCs developing first (Kessaris et al., 2006). The functional relevance of these subsequent waves is not fully understood as OPCs from the different region are transcriptionally and functionally similar and some of the later dorsally derived OPC will replace cells generated from the first wave (Clarke et al., 2012; Marques et al., 2018; Tripathi et al., 2011).

The expression of PDGFRα represents an important stage for the developing OPCs, as this receptor specifically marks myelinogenic and migration competent cells, while inhibiting their premature differentiation (Noble et al., 1988; Sim et al., 2011). The developing cells migrate throughout the white and gray matter until they reach their positions. A subset of OPCs will exit the cell cycle and differentiate along the oligodendrocyte lineage, but many persist as OPCs, making up around 3-5% of the cells in the adult human and mouse brain (Roy et al., 1999; Scolding et al., 1999). OPCs remain in the brain parenchyma into adulthood and while they start as a homogenous population, they become increasingly heterogenous in both function and potential with age which correlates with the electrophysiological properties of the cells (Spitzer et al., 2019).

Protocols for generating glial progenitors from hESCs

The detailed knowledge of OPC development and differentiation has been translated into protocols for generation these cells from hPSCs, with the idea of reproducing the temporal order of signaling events that generates first neuroepithelial cells and then glial restricted progenitors (Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005). To achieve this, fibroblast growth factors (FGFs) and Retinoic acid (RA) are used to induce neuralization and caudalization of the cells, while SHH is applied as a ventralizing factor, as well as a mitogen. The growth factors PDGF, NT3 and IGF-1 all work as potent mitogens for OPCs, and PDGF also promotes the motility of OPCs while inhibiting their premature differentiation (Barres et al., 1994b; Bibollet-Bahena and Almazan, 2009; McMorris et al., 1986; Noble et al., 1988). Apart for the mitogenic properties of IGF-1, this growth factor also potentiates the maturation of OPCs together with thyroid hormone (T3) (Barres et al., 1994a; Bibollet-Bahena and Almazan, 2009)

Building on the early protocols for OPC generation, more refined protocols have been developed. In 2013, Steven Goldman's group described a protocol for generating bipotent glial progenitors from multiple different hPSC-lines (Wang et al., 2013). When these cells were transplanted into neonatal myelin deficient shiver mice, the cells dispersed though the brains of the recipient mice and differentiated into myelinating oligodendrocytes, which in a subset of the animals provided a rescue of the otherwise lethal phenotype. While some of the cells differentiated into mature oligodendrocytes and astrocytes, the main proportion of cells remained as newly incorporated glial progenitor cells (GPCs). Interestingly, human GPCs appear to have a competitive advantage over murine GPCs and actively replace the resident cells through a mechanism that is yet to be determined (Windrem et al., 2014). Also, the fate of transplanted human GPCs has been shown to be context dependent as the cells will only produce new myelin in a hypomyelinated environment. By using patient derived iPSC-lines, this human glial chimeric model has been harnessed to understand the glial contribution to human diseases such as schizophrenia and Huntington's disease in an *in vivo* environment (Benraiss et al., 2016; Liu et al., 2019b; Windrem et al., 2017).

While this protocol generates a high proportion of highly expandable human GPCs, the timeline for doing so is very long. Several protocols have since then been published with the aim to simplify and shorten the process. One approach to achieve this has been through culturing the cells in low oxygen tension to mimic the hypoxic environment in the developing brain (Stacpoole et al., 2013). Another strategy has been to include Dual-SMAD inhibition to accelerate and potentiate the neuroepithelial differentiation (Douvaras et al., 2014). When Dual-SMAD inhibition and RA was applied at the start of the protocol, 30% $O4^+$ OPCs could be obtained in only 55 days, and the purity could be further increased through fluorescence-activated cell sorting (FACS) (Douvaras and Fossati, 2015). Most of the developed protocols incorporate RA for posteriorizing the cell, but a few describe alternative RA-independent routes of OPC production, favoring a forebrain identify of the cells (Piao et al., 2015; Stacpoole et al., 2013). These cells have been shown to efficiently provide remyelination and rescue following radiation induced demyelination (Piao et al., 2015).

To further resolve the issue of the prolonged culture time required for obtaining OPCs, direct reprogramming has been assessed as an alternative approach for producing these cells. In 2013, two independent studies were able to show successful conversion of mouse and rat embryonic fibroblast or mouse embryonic and lung fibroblasts into induced OPCs (iOPCs) through forced expression of the TFs Sox10 and Olig2 in combination with either Nkx6.2 or Zfp536 (Najm et al., 2013; Yang et al., 2013). The expression profiles of these iOPCs matched those of authentic OPCs and the cells were able to ensheath host axons and provide compact myelin structures when transplanted into hypomyelinated mice. Subsequent studies have reported *in vivo* and *in vitro* conversion of mouse astrocytes into iOPCs using only Sox10, and chemically induced conversion of mouse embryonic fibroblasts or human astrocytes using small molecules and epigenetic modifiers (Liu et al., 2019a; Mokhtarzadeh Khanghahi et al., 2018; Zare et al., 2018). However, the number of reports describing direct reprogramming for obtaining iOPCs is still limited and the potential to generate human iOPCs from more distantly related cell lineages remains to be demonstrated.

Current approaches for hPSCs-derived generation of OPCs describe glial induction in 3D cultures for rapid and large-scale production of functional and transplantable cells (Rodrigues et al., 2017). With the rise of the organoid field, successful attempts have also been made to induce OPC generation in human cortical spheroids, and to generate human oligodendrocyte spheroids as appropriate models to study different aspects of oligodendrocyte development, myelination and cell interaction in health and disease *in vitro* (Madhavan et al., 2018; Marton et al., 2019).

The recent advances for obtaining more accessible sources of human glia and the development of more applicable models for studying the role and function of these cells in the healthy and diseased brain will enable us to answer questions that were previously difficult to address. This includes the potential to use these cells as targets for direct neuronal conversion and the subsequent assessment of the therapeutic potentials of this strategy for treating disorders of the CNS, including PD. Compared to stem cells-based treatments, direct neuronal reprogramming still remains far away from realizing practical clinical applications. However, if the reprogramming field continues to evolve along the same promising trajectory, this could very well develop into an alternative strategy for cell replacement therapy in the future (Figure 3).

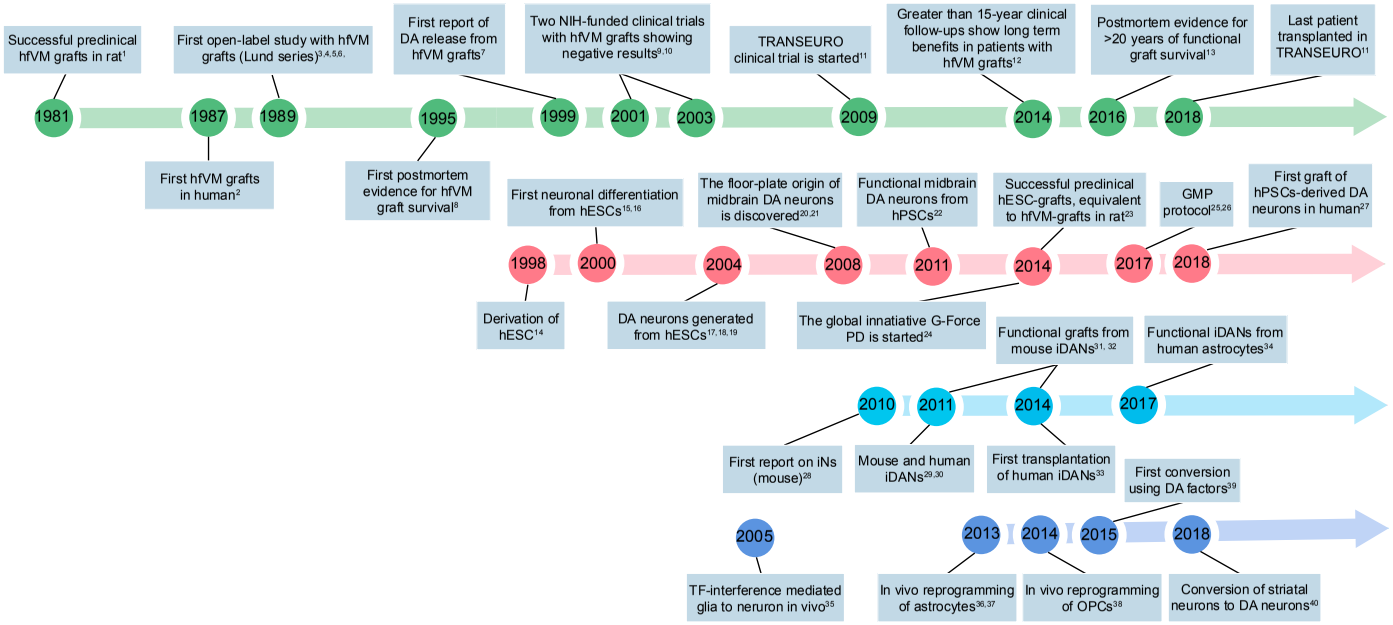

Figure 3: *Timelines of different strategies for cell replacement therapy for PD*

Important milestones for the development of cell replacement therapy through the use of hfVM tissue (green, top), hPSCs (pink, middle), *in vitro* direct reprogramming (light blue, middle) and *in vivo* direct reprogramming (violet, bottom). References related to the timeline are presented on the following of pages.

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AIMS OF THE THESIS

The overarching aim of my thesis has been to develop authentic DA neurons for cell replacement therapy in PD. Building on previous work using human pluripotent stem cells as a cell source for cell therapy I have worked to refine and better define hESC-derived DA neurons, as we are approaching clinical trials using these cells. For future applications, I have also explored direct neuronal programming of somatic cells as a route for brain repair in PD.

The specific aims of my thesis were to:

1. Identify predictive markers of a successful hESC-derived DA graft outcome **(Paper I and II)**

2. Develop a GMP-compatible DA progenitor cell differentiation protocol for clinical translation **(Paper I and III)**

3. Dissect DA graft composition through scRNA-seq of transplanted cells, and to validate these findings using histological methods **(Paper IV)**

4. Identify factors that can successfully reprogram human primary- and stem cells derived glial progenitor cells into DA neurons **(Paper V)**

SUMMARY OF RESULTS AND DISCUSSION

Cell replacement therapy using fetal cells have been successfully explored to treat PD, and the first clinical trials using hPSC-derived DA progenitors for brain repair are on the horizon (Barker et al., 2017). At the same time, neuronal reprogramming is being investigated as a future opportunity to replace the lost DA neurons *in situ* in the brain, offering a more direct way of cell replacement and thus circumventing the need for cell transplantation (Li and Chen, 2016; Vignoles et al., 2019). In **paper I and II**, novel markers that are able to predict the grafting outcome using hESC-derived DA progenitors in a rat model of PD were successfully identified. These markers were then used in the development of a GMP adapted cell differentiation protocol for highly efficient and reproducible production of DA progenitors from hPSCs that can be used for transplantations in patients (**paper III**). In **paper IV**, single cell sequencing and immunohistochemical analyses were used to determine the graft composition of hESC-derived grafts, and to compare this to the composition of hfVM grafts. Finally, in **paper V** the possibility to generate midbrain DA neurons from human glial progenitors via direct reprogramming *in vitro* was explored, with the ambition of setting up an *in vivo* reprogramming model of neuronal conversion of human cells. Throughout these studies, I have addressed questions related to early cell fate specification in the developing human brain and in human stem cell models, with the aim to generate, molecularly characterize and compare endogenous- and hESC-derived human DA neurons.

Common midbrain DA markers correlate poorly with a successful grafting outcome (Paper I)

Cell therapy offers an interesting opportunity to replace the DA neurons that are lost in PD. Limitations inherent with the use of fetal tissue as a source of transplantable DA neurons have resulted in a lack of standardization and limited possibilities of quality controls, which has led to variabilities in the outcomes of the transplantations in patients (Barker et al., 2015a). The use of hPSCs as a source for generating midbrain DA neurons has the potential to overcome these issues since hPSCs offers an unlimited cell source which can be used to generate large batches of DA progenitors that can undergo extensive quality controls, including safety and efficacy assessments, prior to use in humans (Kirkeby et al., 2017). In order to bring hPSC-based cell therapy to a clinical setting it is crucial to have a cell differentiation protocol which produces a cell product that generates predictable and reproducible results, with good survival and functional maturation after transplantation. Between the years of 2010 to 2014 our group had transplanted over five hundred rats with cells from over thirty different hESC-derived cell batches. From these transplantation experiments we had seen highly reproducible graft survival, neuralization and absence of tumor formation, but not always a high DA content *in*

vivo. In order to get a better understanding of the variability in transplantation outcome we decided to systematically reanalyze all the brains of animals that had been grafted in the lab.

Considerable inter-experimental variation in transplants derived from cell-batches with high yields of LMX1A$^+$/FOXA2$^+$ cells

In all these transplantation experiments, the cells had been assessed for a high co-expression of FOXA2, LMX1A and OTX2 (≥80 %) prior to grafting in a 6-OHDA rat model. To determine the inter-experimental variation of transplantation outcomes from these experiments, we did a retrospective analysis of the grafts by quantifying the DA yield (number of TH$^+$ cells per 100,000 cells grafted), graft volume (mm^3) and DA neuron density (TH$^+$ cells/mm^3). This reanalysis of transplantation outcomes revealed a considerable inter-experimental variation in grafts from cell batches with high yields of FOXA2/LMX1A/OTX2 (Figure 4A-D). In addition, we determined that the gene expression levels of commonly known DA progenitor markers at the time point of transplantation (day 16) did not correlate with the DA yield *in vivo* (Figure 4E-F). At this point, the cells are still progenitors, since grafting of mature DA neurons results in a decreased survival and less TH rich grafts (de Luzy et al., 2019; Ganat et al., 2012; Jönsson et al., 2009). We therefore maintained the DA progenitors in culture for an additional 23-29 days to let them mature into TH-expressing DA neurons and confirmed that also the expression levels of more mature DA markers *in vitro* were unable to predict the outcome of the grafting in terms of DA yield (Figure 4E, G). From these results we concluded that the expression of TH *in vitro* and *in vivo* correlates poorly, and that there was a need for additional markers that could better predict functional maturation *in vivo*.

RNA-seq analysis reveals positive correlations between DA yield and markers of the caudal VM

Over the years of transplantation studies, RNA from each grafted cell batch had been isolated and kept for long term storage in the freezer. In order to identify truly predictive makers of grafting outcome, RNA samples from the transplanted cell batches were sequenced with the aim comparing and correlating the sequencing data with the *in vivo* outcomes of the grafting. For this purpose, we took an unbiased approach where we divided the graft experiments into DA^{high} and DA^{low} groups, based on the total number of DA cells in the grafts (Figure 5A). When these groups were analyzed by a principal component analysis (PCA) we could see a clustering of the two groups on the PC1 axis, which confirmed that the samples from the DA^{high} and DA^{low} groups had distinct expression profiles (Figure 5B). The DA^{high} samples clustered on the positive PC1 axis containing genes such as *FGF8, EN2, PAX5* and *CNPY1* which are expressed in the midbrain-hindbrain boundary (MHB) region and the caudal part of the VM (Asano and Gruss, 1992; Hirate and Okamoto, 2006; Ye et al., 2001). To identify all differentially expressed genes between the two groups, we next employed a DEseq2 analysis. Interestingly, this analysis confirmed the results from the PCA as we again found a high representation of genes that are expressed in the caudal VM and MHB region to be positively associated with high DA yields of the grafts (Figure 5C-D).

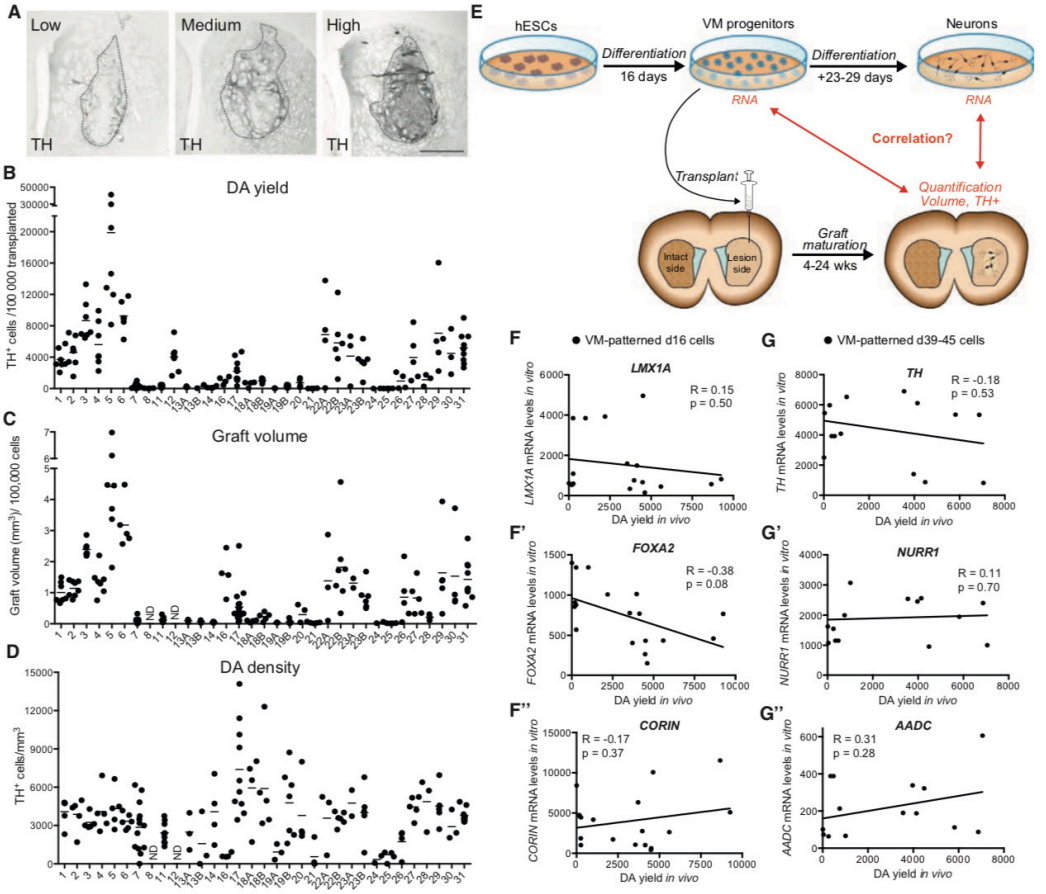


Figure 4. *VM-patterned hESC-derived progenitors result in variable graft outcomes after transplantation and maturation in vivo*

(A) Examples of hESC-derived DA grafts with a low, medium and high DA content as shown by immunohistochemical labeling of TH.

(B-D) Quantification of DA yield (B), graft volume (C) and DA density (D) in hESC-derived DA grafts. The numbers on the x-axis correspond to different grafting experiments and each dot represent an individual animal.

(E) Schematic overview of the strategy for assessing the correlation between known DA progenitor markers on day 16 and mature DA markers after terminal differentiation *in vitro* with the DA yield of mature grafts *in vivo*.

(F-G) Commonly used DA progenitor markers (F), as well as mature DA makers (G) did not correlate with the DA yield *in vivo*. To assess the correlations, the mean DA yield in each graft experiment was plotted against the mRNA levels of the indicated genes.

Spearman correlation analysis was used to assess correlations and the results are presented as R and p values. Tendencies of correlation are shown by linear regression lines. In (B–D), the bars represent means. Scale bar represent 500 μm.

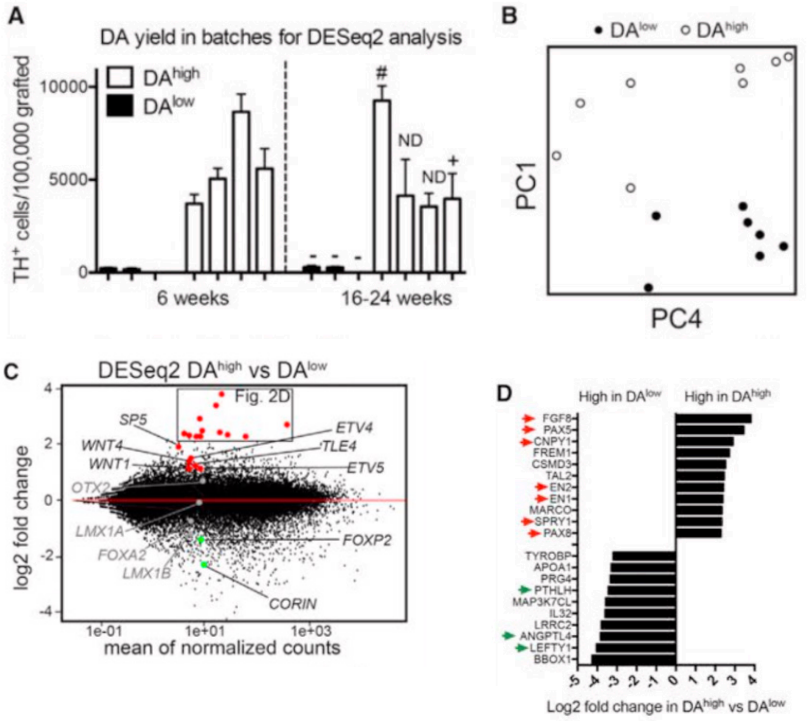


Figure 5. *Unbiased identification of novel markers associated with successful grafting outcomes*

(A) Graft experiments were divided into DA^{high} and DA^{low} groups based on the total number of DA neurons in the grafts (normalized to the number of transplanted cells). For long term experiments "-" marks grafting experiments that did not result in a functional motor recovery in the grafted animals, while experiment with functional recovery of amphetamine induced rotation is marked with "+" and experiments with functional PET imaging are marked with "#". Experiments without functional assessment are marked "ND".

(B) Principal component analysis shows a segregation of samples belonging to the DA^{high} and DA^{low} groups on the PC1 axis.

(C) DESeq2 analysis was employed to look at all differentially expressed genes between the DA^{high} and DA^{low} groups. For indicated genes, red corresponds to an upregulation in DA^{high} samples, green corresponds to an upregulation in DA^{low} samples, and gray indicate genes that were not differentially expressed.

(D) Among the top genes that were associated with a high DA yield, many were related to the caudal midbrain and the MHB region (red arrows). On the contrary, several genes associated with a low DA yield were related to the diencephalic domain (green arrows).

Common midbrain DA markers are shared with subthalamic nucleus (STN) progenitors (Paper I and II)

In parallel to our work in Lund, Thomas Perlmann's group at The Karolinska Institute in Stockholm was conducting a study of the development of Lmx1a-expressing cells in the mouse fetal brain **(Paper II)**. In this study they took advantage of a $Lmx1a^{EGFP}$ transgenic mouse line and isolated

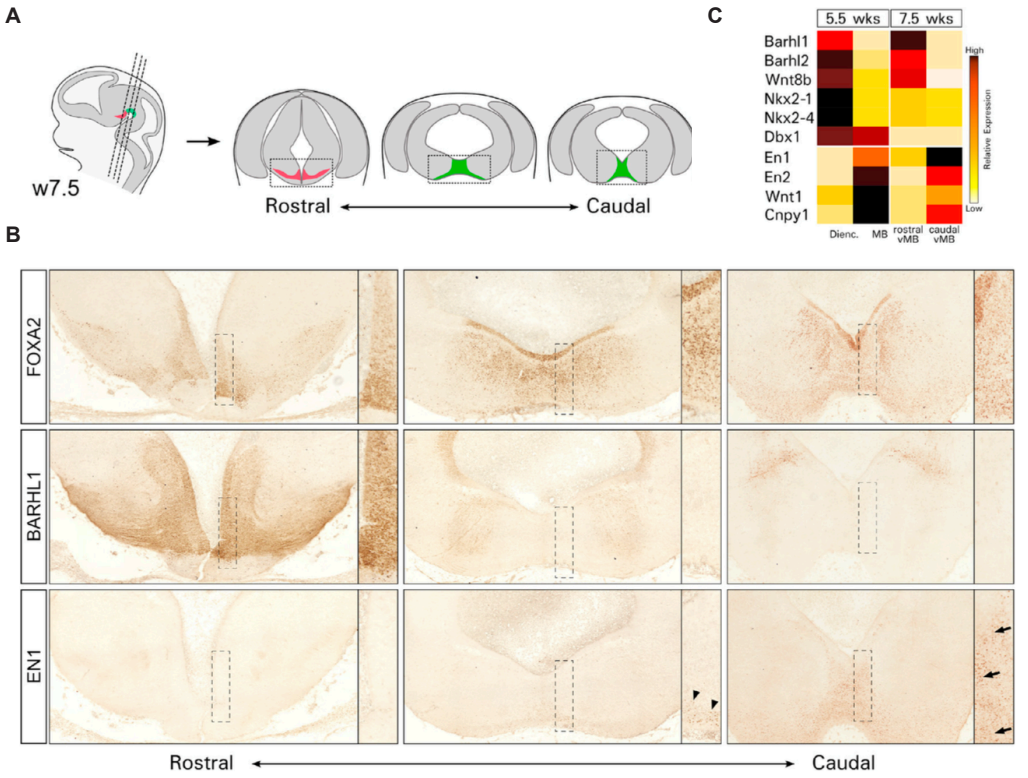


Figure 6. *Distinct populations of FOXA2⁺ progenitors along the rostro-caudal axis during human midbrain development*

(A) Schematic overview of the 7.5-week old human embryonic brain with indications of the rostro-caudal origin of the pictures in (B).

(B) Immunohistochemistry of FOXA2, BARHL1 and EN1 in the developing diencephalon and mesencephalon showing distinct progenitor domains along the rostro-caudal axis.

(C) qPCR analysis of the sub-dissected diencephalon and midbrain in 5.5- and 7.5-week old embryos show a segregation of DA and STN related genes.

EGFP-positive and -negative midbrain cells between day E10.5 and E13.5, which represents the developmental time span during which the majority of DA neurons are born (Bayer et al., 1995). The expression profiles of these cells were then analyzed at a single cell level to study midbrain development. In this study, two different axes of Lmx1a-expressing cells were discovered and many known DA markers, including *Foxa2* and *Lmx1a*, were expressed in both. However, an additional set of DA markers, including *En1, Cnpy1, Th* and *Pitx3*, were exclusively expressed in only one of the axes. Cells in the second axis lacked the expression of these DA related genes but instead expressed another set of genes including *Barhl1/2, Dbx1, Epha3* and *Pitx2*, which are expressed by glutamatergic progenitors of the subthalamic nucleus (STN). This discovery revealed the existence of two closely related cell lineages in the developing mouse midbrain, with progenitor cells that share the expression of many DA progenitor associated genes and which can only be separated by a small set of genes.

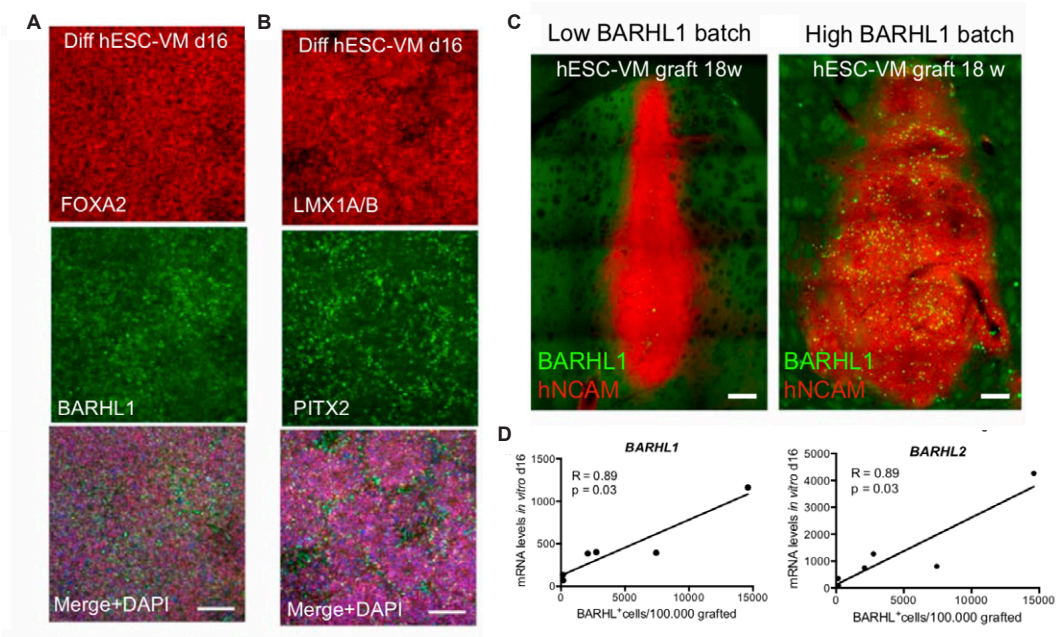


Figure 7. *VM-patterned cell batches contain cells of a STN identity in vitro and after grafting and maturation in vivo*

(A-B) Immunocytochemistry of VM-patterned hESC-derived progenitors on day 16 show cells that are positive for both BARHL1/FOXA2 and PITX2/LMX1.

(C) Variable numbers of BARHL1⁺ cells were also found in mature grafts after transplantation and maturation *in vivo*.

(D) The expression level of *BARHL1* and *BARHL2* in the transplanted cell batches at day 16 correlate with the number of BARHL1⁺ *in vivo*.

Spearman correlation analysis was used to assess correlations and the results are presented as R and p values. Tendencies of correlation are shown by linear regression lines. Scale bars represent 100 μm (A-B) and 200 μm (C).

Close relationship between DA- and STN progenitors during human brain development

To investigate the existence of these two progenitor cell populations during human brain development we performed immunohistochemistry on the developing midbrain of a 7.5-week old human embryo (Figure 6A). This analysis revealed that there is indeed a rostral population of cells that are positive for FOXA2, BARHL1, but not EN1 and a population of cells in the caudal midbrain that are positive for FOXA2, EN1 but not BARHL1 (Figure 6B). Sub-dissection of the developing human midbrain at 5.5-weeks and 7.5-weeks, followed by qRT-PCR gene expression analysis of the rostral and caudal midbrain regions, further strengthen this theory since there was a subdivision of midbrain DA progenitor markers and STN progenitor markers between these two regions (Figure 6C).

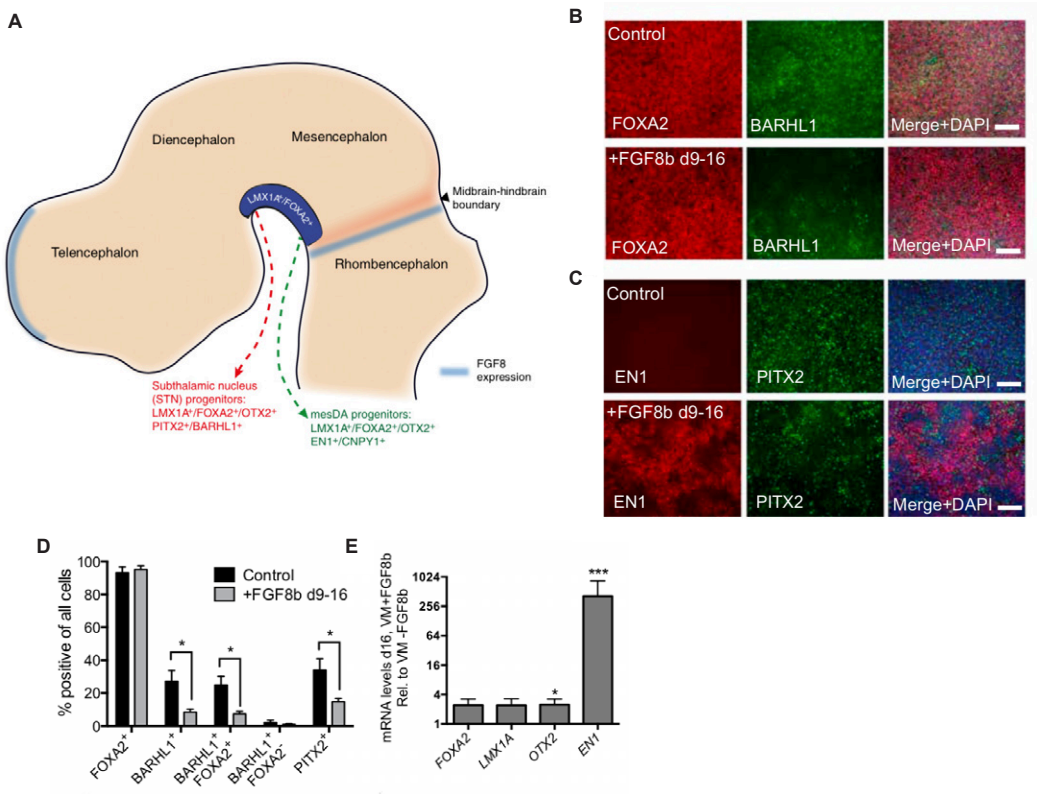


Figure 8. *Timed addition of FGF8b to hESC-derived VM cultures results in a more precise patterning to a caudal VM identity*

(A) Schematic picture indicating the sources of FGF8 signaling from the MHB and from the anterior neural ridge (ANR) in the developing brain.
(B-C) Timed addition of FGF8b results in a decreased number of BARHL1$^+$ and PITX2$^+$ cells and an increased number of EN1$^+$ cells as shown by immunocytochemistry.
(D-E) Quantifications of the number of BARHL1$^+$, PITX2$^+$ and FOXA2$^+$ cells from the immunocytochemistry (D) and the relative expression of EN1 in VM cultures with or without the addition of FGF8b (E). Scale bars represent 100 μm.

VM-patterned hESC-cultures contain cells of a diencephalic STN identity

The refined understanding on midbrain development prompted us to consider the implications this close lineage relationship could have on our protocol for generating hESC-derived DA progenitors, especially since the majority of DA markers that we were using at the time were shared between DA- and STN progenitors. We speculated that some of the observed variation in transplantation outcome perhaps could be attributed to an unappreciated presence of these STN progenitors within our transplanted cell batches. Indeed, we did discover that in some cell batches with a high proportion of cells positive for LMX1A/FOXA2/OTX2 on day 16, there were a considerable number of progenitors co-labeled for both FOXA2/BARHL1 and LMX1A/PITX2, indicative of a STN

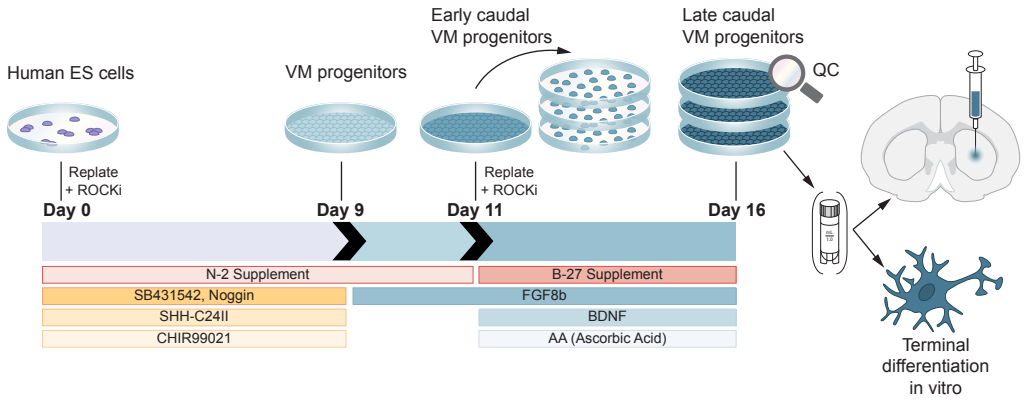


Figure 9. *Schematic overview of the GMP adapted cell differentiation protocol for generation of transplantable DA progenitors*

The GMP adapted cell differentially is fully defined and relies on timed addition of FGF8b for precise patterning to a caudal VM identity. DA progenitors are generated in 16 days and can be cryopreserved or used directly for transplantation studies or for terminal maturation *in vitro*.

progenitor identity (Figure 7A-B). Looking back into previous transplantation experiments we also found a variable presence of BARHL1$^+$ cells after transplantation and maturation *in vivo* and we could confirm that the levels of BARHL in the cell batches prior to grafting correlated with the amount of BARHL1$^+$ cells in the mature grafts (Figure 7C-D).

Development of a GMP-compatible cell differentiation protocol (Paper I and III)

Given the observed variation in transplantation outcome we next wanted to investigate if we could fine tune the patterning of our differentiation protocol towards the caudal part of the VM, since markers of this region correlated with a high DA yield after transplantation *in vivo* and since genes expressed in the caudal VM could discriminate DA progenitors from STN progenitors. As we were aiming to generate a cell product that could be used in a clinical setting, we also wanted to adopt our cell differentiation protocol to make it compliant with GMP standards. This means that the protocol must be fully defined and free from any animal derived products, which will in turn increase the reproducibility of the protocol and facilitate technical transfer of the protocol to different sites of production.

Timed delivery of FGF8b causes a fate switch from diencephalic to caudal VM progenitors

One of the genes that showed up in both the PCA and DEseq2 analysis in association with DA rich grafts was *FGF8*. FGF8 is a signaling molecule that is secreted from the MHB and is important for the patterning of the caudal midbrain (Figure 8A) (Joyner et al., 2000). We therefore assessed if the addition of a high affinity form of FGF8 to the cultures would result in a more precise patterning of the progenitors to a caudal VM fate. Indeed, when FGF8 was added to the VM cultures between day 9 to 16 a decreased number of PITX2$^+$ and BARHL1$^+$ cells were observed in the cultures, while the expression of EN1 was increased (Figure 8B-E). From this we concluded that a timed delivery of FGF8b to the VM cultures could cause a fate switch from diencephalic to caudal VM progenitors.

Our GMP-compatible protocol can be adapted to different pluripotent stem cell lines and allows for cryopreservation of the generated DA progenitors

For the purpose of clinical translation, it is crucial that the protocol can be implemented with clinical grade stem cell lines and transferred to different sites of production. The final cell product should also be cryopreservable for long term storage, to enable extensive quality controls including safety and efficacy assessments before use, as well as to enable on-demand transplantation (Figure 9). By a slight adjustment of the timing and concentration of the patterning factors, we could successfully adopt the protocol to a range of different GMP-derived stem cell lines *in vitro* (Figure 10). Additionally, we developed a protocol for cryopreservation of the cells and showed that cryopreserved DA progenitors were able to generate a similar extent of innervation, behavioral improvements and correct marker expression *in vivo* as their non-cryopreserved counterparts when transplanted into 6-OHDA lesioned rats (Figure 11A-B, D-E, G-H and J-K). Similar successful *in vivo* results were also obtained after transplanting cells that had been generated from one of the additional hESC-lines, thus providing further proof for the general applicability of our cell differentiation protocol (Figure 11C, F and I-K).

Single cell gene expression analysis of grafted hfVM tissue and hESC-derived DA progenitors after 6 months of maturation *in vivo* (Paper IV)

From many years of extensive efforts of preclinical validation, it has been established that the hPSC-derived DA neurons are authentic, functional and equivalent to fetal DA neurons when transplanted in animal models (Chen et al., 2016; Doi et al., 2014; Grealish et al., 2014; Grealish et al., 2015; Kirkeby et al., 2012; Kriks et al., 2011; Steinbeck et al., 2015). Both cell types show good survival and maturation, functionality in pre-clinical animal models, and equipotency – meaning that they have a similar potency and therefore work at a similar dose. Both sources of cell also show similar innervation patterns and, importantly, an outgrowth capacity that is compatible to a scale up in a human-size brain. However, one aspect that remained largely unknown was the complete cellular composition of these grafts, since we had been limited mainly to immunohistochemistry to address this question. Therefore, in this study we decided to take an unbiased approach to characterize the composition of both hfVM- and hESC-derived DA grafts through scRNA-seq.

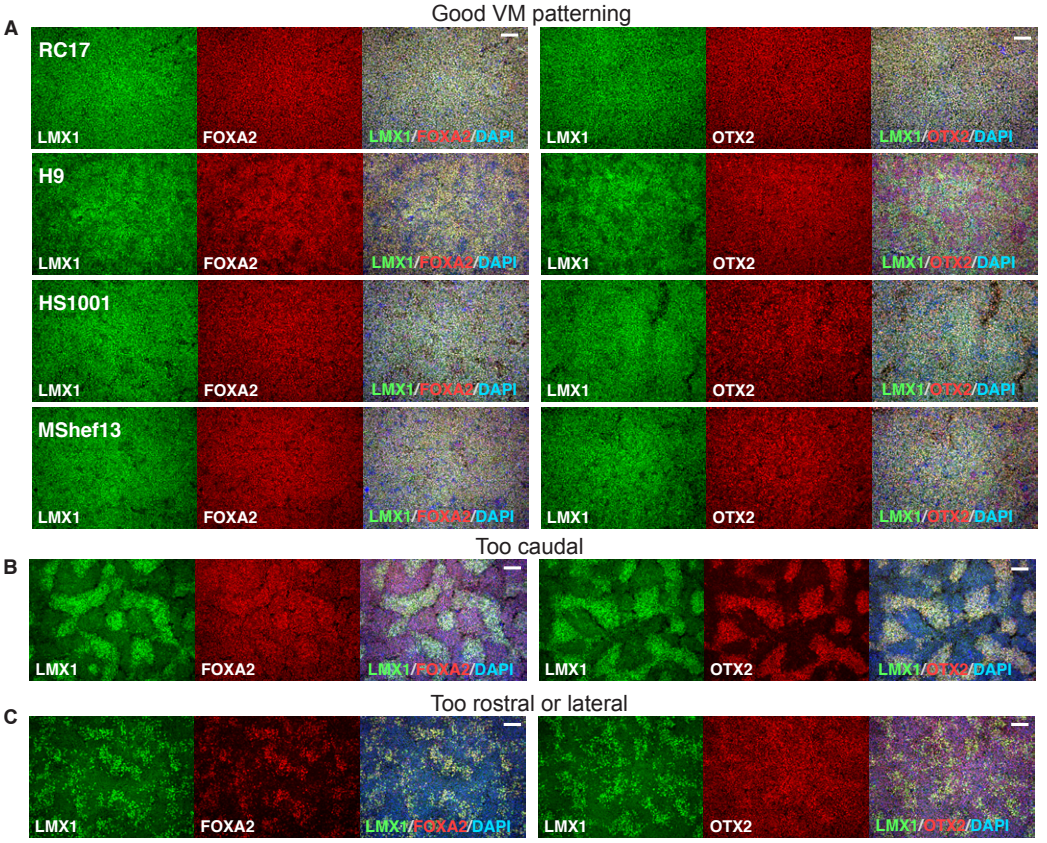
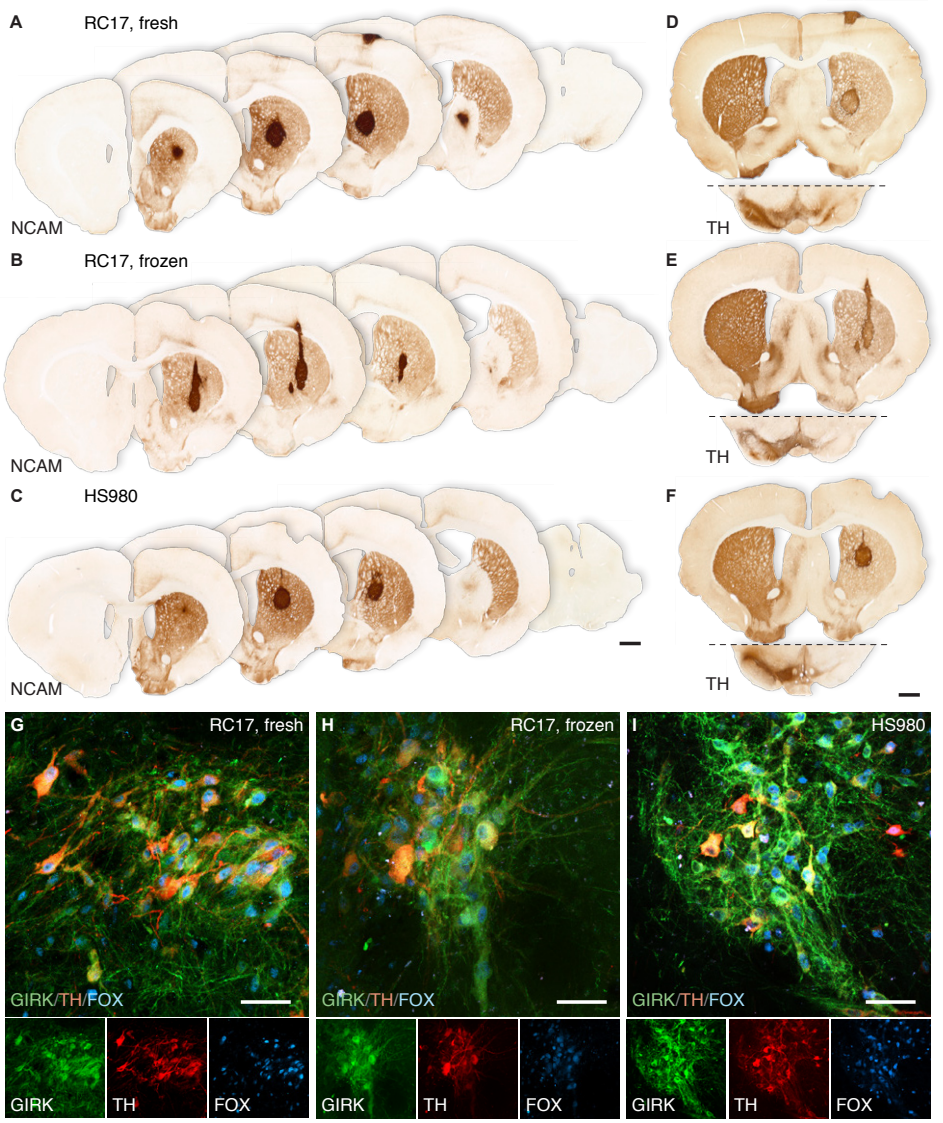


Figure 10. *Successful patterning of multiple hESC-lines to midbrain DA progenitors*

(A) Immunocytochemistry of LMX1/FOXA2 and LMX1/OTX2 show successful VM patterning at day 16 using different hESC-lines including RC17, H9, HS1001 and MShef13.
(B) Example of a differentiation with too caudal patterning, as determined by the presence of an $OTX2^-$ population.
(C) Example of a differentiation with a too rostral or lateral patterning, as determined by the presence of an $LMX^-/FOXA2^-$ population, but with a high proportion of $OTX2^+$ progenitors (qPCR can be used to further determine the identity of the cells). Scale bars represent 100 μm.

Figure 11. *Subtype specific maturation of functional DA grafts in vivo using the GMP adapted cell differentiation protocol*

(A-C) The neural content and innervation of the DA grafts can be assessed after long term maturation *in vivo* for 6 months by immunohistochemistry of the human specific neuronal marker hNCAM. A comparable extent of innervation is achieved using two different hESC-lines; RC17 (A) and HS980 (C), as well as using cryopreserved progenitors (B).
(D-E) Corresponding TH immunohistochemistry of the grafts represented in (A-C) show that all the grafts are DA rich.
(G-I) Fresh RC17 (G), frozen RC17 (H) and HS980 (I) all give rise to subtype specific A9 DA neurons that co-label GIRK2/TH/FOXA2 after maturation *in vivo*.
(J) Quantifications of the mean DA yield, graft volume and DA density form fresh and frozen RC17 and HS980. The cryo-preservation does not affect the DA density (nonsignificant in Student's t test between fresh and frozen RC17).
(K) Behavioral motor recovery in 6-OHDA lesioned rats is assessed by amphetamine induced rotations and is obtained using fresh RC17 (n=13), frozen RC17 (n=13) and HS980 (n=10) after 6 months. Scale bars represent 1 mm (C, F) and 50 μm (G-I).



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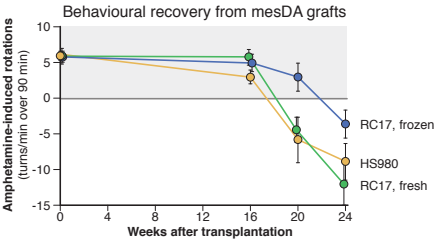
Cell source	n	DA yield TH⁺ cells/10⁵ grafted cells	Graft volume mm³/10⁵ grafted cells	DA density TH⁺ cells/mm³
RC17, fresh	11	2,700	0.29	8,500
RC17, frozen	10	1,700	0.28	6,900
HS980	9	3,700	0.39	8,200



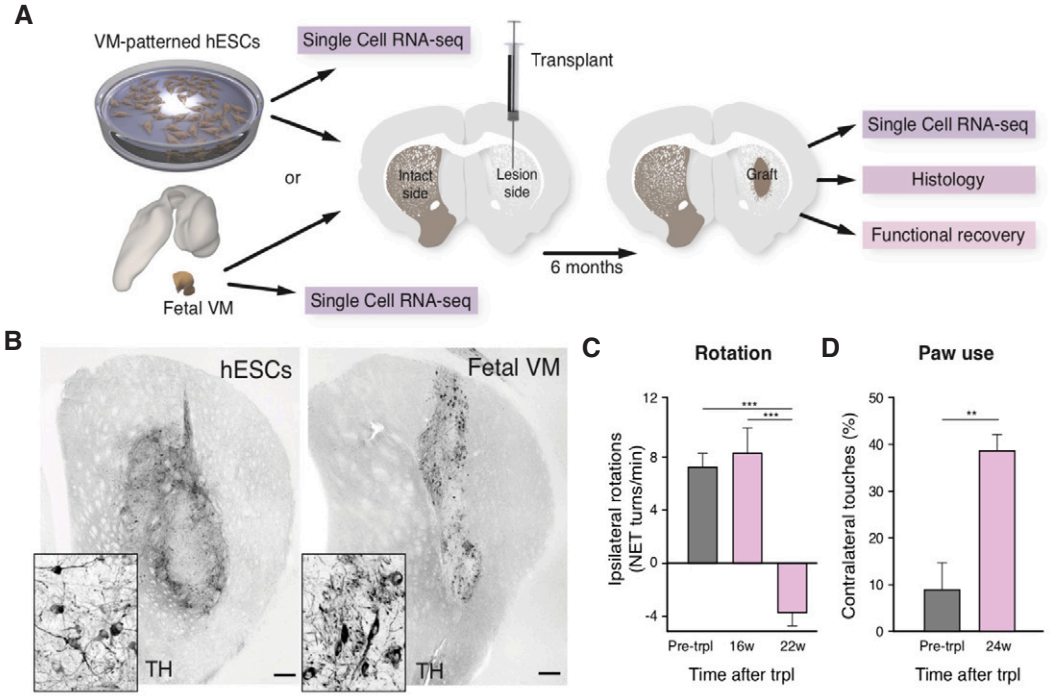


Figure 12. *scRNA-seq of functional fetal- and hESC-derived DA grafts*

(A) Schematic overview of the experimental design for sequencing of fetal- and hESC-derived VM progenitors and cells isolated from functional DA grafts.

(B) Both fetal- and hESC-derived grafts where rich in DA neurons as determined by TH immunohistochemistry. Insets show high power magnifications of the DA neuron morphology.

(C-D) The functionality of the hESC-derived DA grafts was assessed using amphetamine induced rotations (C) and spontaneous paw use in the cylinder test (D) (n=7). Data are presented as means ± SEM; the motor functions of the rats post-lesion and after grafting were compared suing a two-tailed paired t-test, $p < 0.05$. Scale bars represent 250 μm.

scRNA-seq of grafted VM progenitors reveals four major clusters of cells

For the purpose of conducting a comprehensive assessment of cellular graft compositions, both hfVM tissue and hESC-derived DA progenitors were transplanted into the DA depleted rat striatum (Figure 12A). Six months after grafting, the functionality and DA subtype specificity of the grafts was confirmed through behavioral assessment of rats that had received grafts and immunohistochemistry for TH (Figure 12B-D). The mature grafts were isolated from the rat brains through dissection, dissociated to single cells, purified with FACS, and then analyzed through scRNA-seq. To investigate the cellular diversity of the sequenced cells, graph-based clustering was used to visualize transcriptional similarities among the analyzed cells. The clustering of the cells revealed four major clusters of graft-derived cells corresponding to neurons (*GAP43* and *RBFOX3*), astrocytes (*AQP4* and *GFAP*) and oligodendrocytes (*OLIG1* and *OLIG2*) (Figure 13A-D). In cells within the neuron cluster, DA markers like *DDC, TH* and *VMAT2 (SLC18A2)* were expressed in addition to pan-neuronal marker indicating a high DA proportion among neurons (Figure 13E).

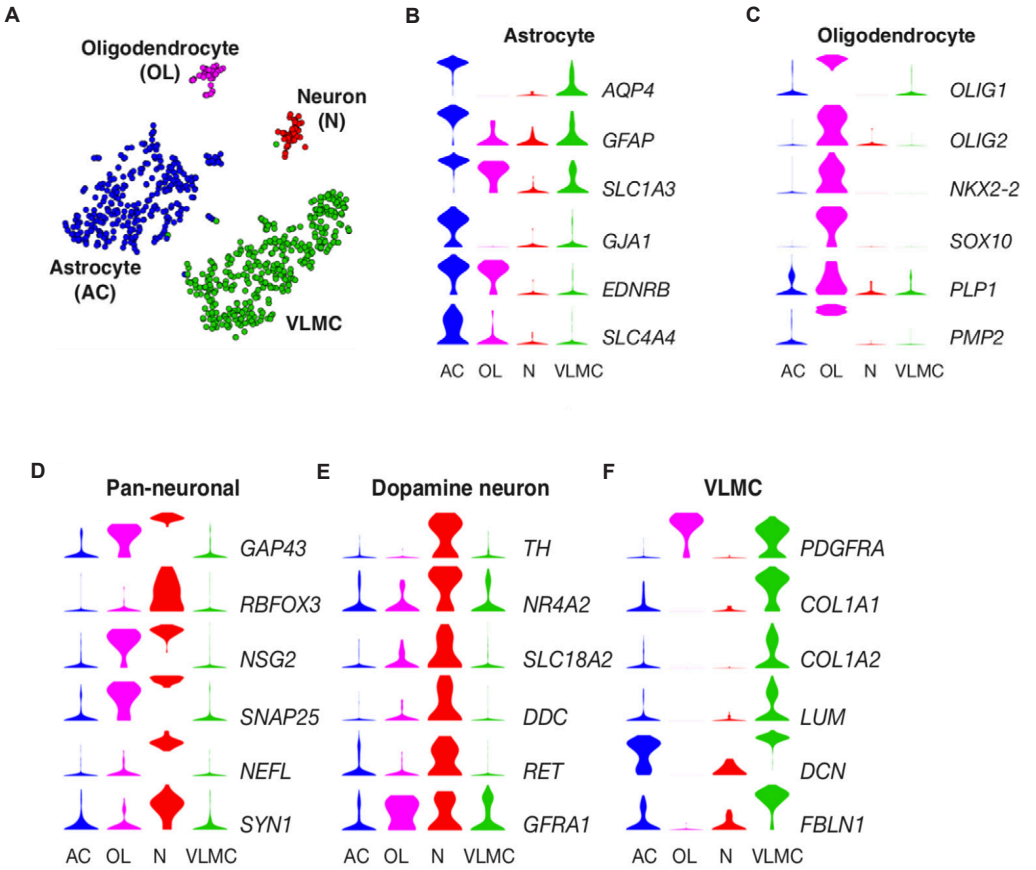


Figure 13. *Mature fetal- and hESC-derived grafts consists of four major cell types*

(A) Clustering of cells isolated from mature grafts using t-SNE revealed four major clusters of cells.
(B-F) The expression levels of indicated genes per cluster reveal clusters of astrocyte identity (B), oligodendrocyte identity (C), DA neuron identity (D-E) and VLMC identity (F). All indicated genes are significantly enriched for the respective clusters and are established markers for the corresponding cell types.

Vascular leptomeningeal cells (VLMCs) are identified as a new component of the grafts

The identity of the fourth cluster of cells that was identified as a component of mature grafts was more difficult to assign. Top genes in this cluster were associated with the extracellular matrix and collagens (*COL1A1* and *COL1A2*) and *PDGFRA*, which is expressed by oligodendrocyte progenitors (Figure 13F). To pinpoint the identity of the cells of the fourth cluster, we compared the expression profile of these cells to a large data set of scRNA-seq from the mouse brain containing over 400,000 cells in 250 clusters representing diverse cell types including neuronal subtypes and various glia, but also immune cells and brain vascular cell types (Zeisel et al., 2018). In this data set, enteric glia (specifically enteric mesothelial fibroblasts) and vascular leptomeningeal cells (VLMCs) were identified as

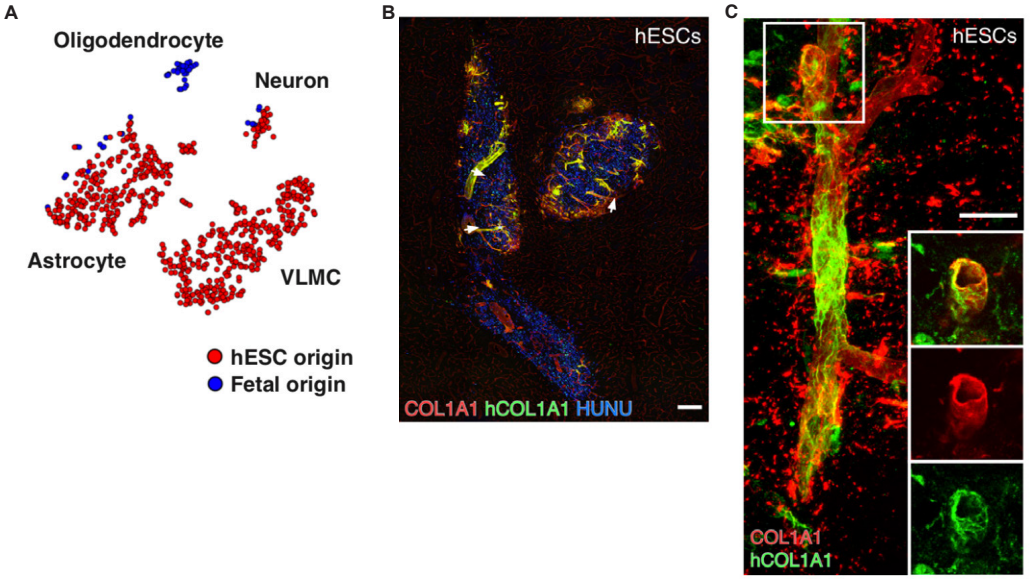


Figure 14. *VLMCs are unique components of hESC-derived grafts*

(A) Clustering of cells isolated from mature grafts using t-SNE and labeling of the cells according to their origin from either fetal-derived (blue) or hESC-derived (red) grafts.
(B) Immunohistochemistry of a hESC-derived graft from a rat that was transplanted in parallel with the same cell batch as was used for the scRNA-seq experiment. Human COL1A1$^+$ cells are present in the graft and are largely associated with the graft vasculature.
(C) Confocal high magnification image from a section of the graft in (B) showing intermingling of host derived COL1A1$^+$ cells and human COL1A1$^+$ cells in close association with the graft vasculature. Scale bars represent 200 μm (B) and 20 μm (C).

cell types with high transcriptional similarity to the cells of the fourth cluster. These VLMCs (also called brain perivascular fibroblast-like cells) have recently been described by two additional scRNA-seq studies (Marques et al., 2016; Vanlandewijck et al., 2018) and represent a type of barrier forming fibroblast, localized on blood vessels and meninges, with an unknown developmental origin. When comparing the cells of the fourth clusters with these recent scRNA-seq data sets, the gene expression profiles appeared to be remarkably similar as they shared the expression of genes such as *PDGFRA*, *COL1A1, LUM* and *DCN*, and we therefore refer to the cells of the fourth cluster as VLMCs.

Distinct cell populations in hfVM- and hESC-derived grafts

The dissection of cellular graft diversity at a single cell level enabled us to detect both similarities and differences between fetal- and stem cell-derived grafts (Figure 14A). While both graft types contained astrocytes and neurons, cells of the oligodendrocyte lineage were exclusive components of hfVM-derived grafts and VLMCs only appeared in hESC-derived grafts. These findings were further validated through immunohistochemistry of fetal- and hESC-derived grafts generated from the same cells as the grafts that were used for the sequencing experiment. In the hESC-derived grafts, human cells positive for COL1A1 were found in close proximity to blood vessels penetrating the grafts, which is in line with their normal role in association with brain vasculature (Figure 14B-C).

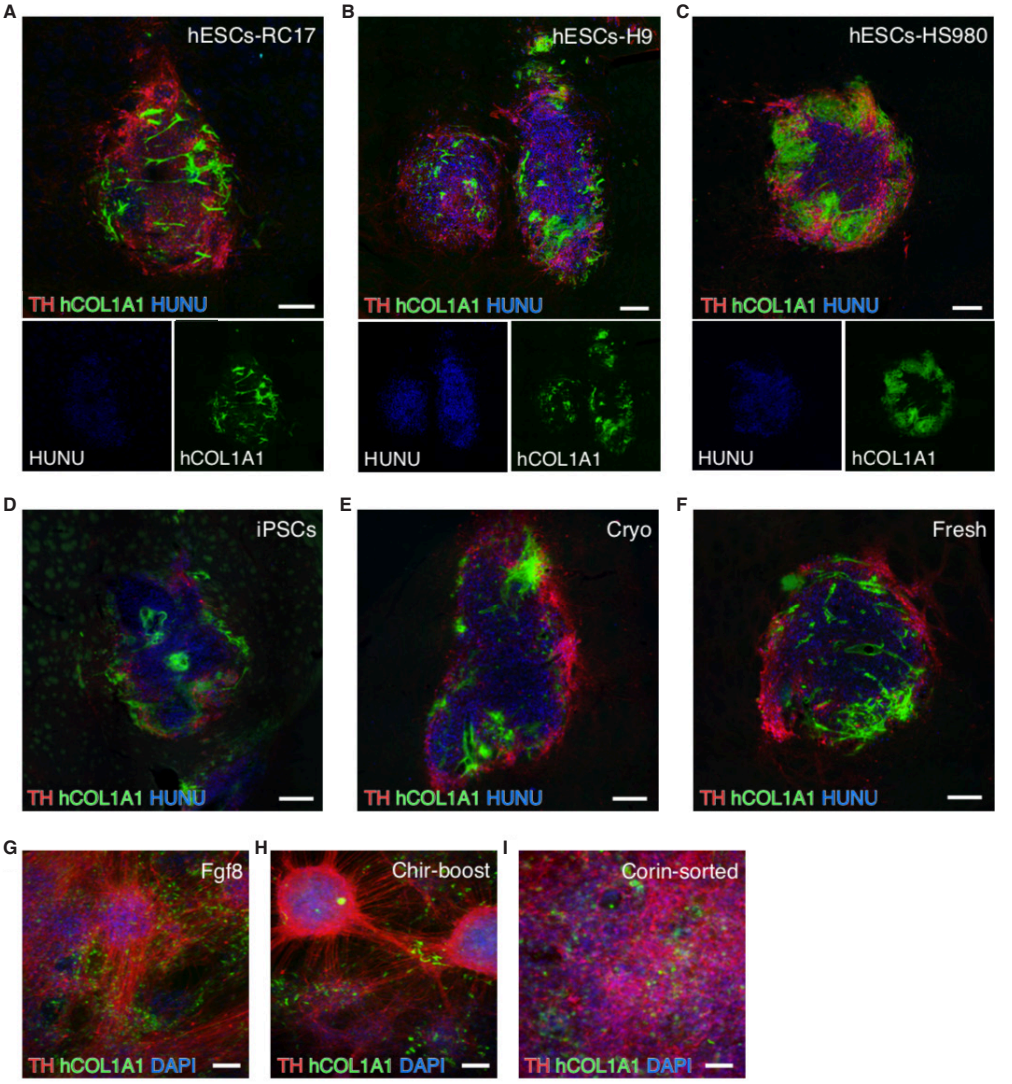


Figure 15. *VLMCs are present in all functional hPSC-derived grafts and after terminal maturation in vitro*

(A-C) Immunohistochemistry of TH/hCOL1A1/HUNU reveals the presence of human COL1A1$^+$ cells in grafts derived from three different cells lines; RC17 (A), H9 (B) and HS980 (C).

(D) Human COL1A1$^+$ are also present in grafts derived from an iPSCs-line with cell sorting for the cell surface marker IAP prior to grafting.

(E-F) Human COL1A1$^+$ are present in grafts from both cryopreserved (E) and fresh cells (F).

(G-I) Human COL1A1$^+$ cells also arise after terminal differentiation *in vitro* using three different clinically relevant protocols for differentiating VM progenitors. The presence of Human COL1A1$^+$ cells is observed *in vitro* using the protocol employed in this study (G), the protocol developed by the Studer lab (H) and the protocol developed by the Takahashi lab (I). Scale bars represent 200 μm (A-F) and 100 μm (G-I).

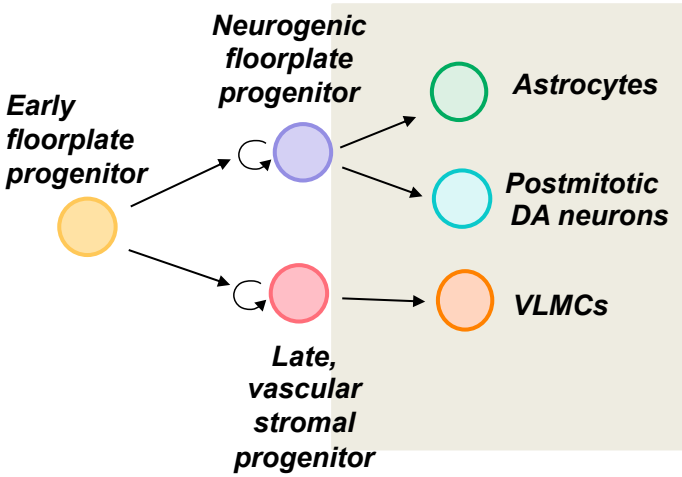


Figure 16. *Model of a common progenitor that generates both DA neurons, astrocytes and VLMCs*

Schematic model of an early bipotent FP progenitor that can generate neurogenic progenitors as well as vascular stromal progenitors that subsequently give rise to the different cell lineages within the hESC-derived grafts.

VLMCs are present in all functional hPSC-derived DA grafts and in hESC-derived DA neuron cultures *in vitro*

To investigate if VLMCs were uniquely present in the grafts of this specific transplantation experiment, or if they were a common component of all functional hPSC-derived DA grafts we stained additional grafts from previous experiments for human COL1A1. This confirmed that VLMCs appeared in grafts generated from different hPSC-lines, using either our research grade or GMP grade protocol, with or without cell sorting before transplantation, as well as after cryopreservation of the transplanted cells (Lehnen et al., 2017; Nolbrant et al., 2017) (Figure 15A-F). Furthermore, the appearance of these cells was not related to the transplantation procedure per se, since they also emerged after terminal differentiation of the DA progenitor cultures *in vitro*, which was confirmed using both our cell differentiation protocol, as well as two additional protocols that are either currently being used in clinical trials or are intended for clinical use (Doi et al., 2014; Kikuchi et al., 2017, see also https://patents.justia.com/patent/20180094242) (Figure 15G-I).

Origin of the hESC-derived VLMCs

The identification of VLMCs as a cellular component of hESC-derived grafts encouraged us to investigate the origin of these cells. Through scRNA-seq analysis of the hESC-derived progenitors prior to transplantation, we could confirm our previous notion that the transplanted progenitors con-

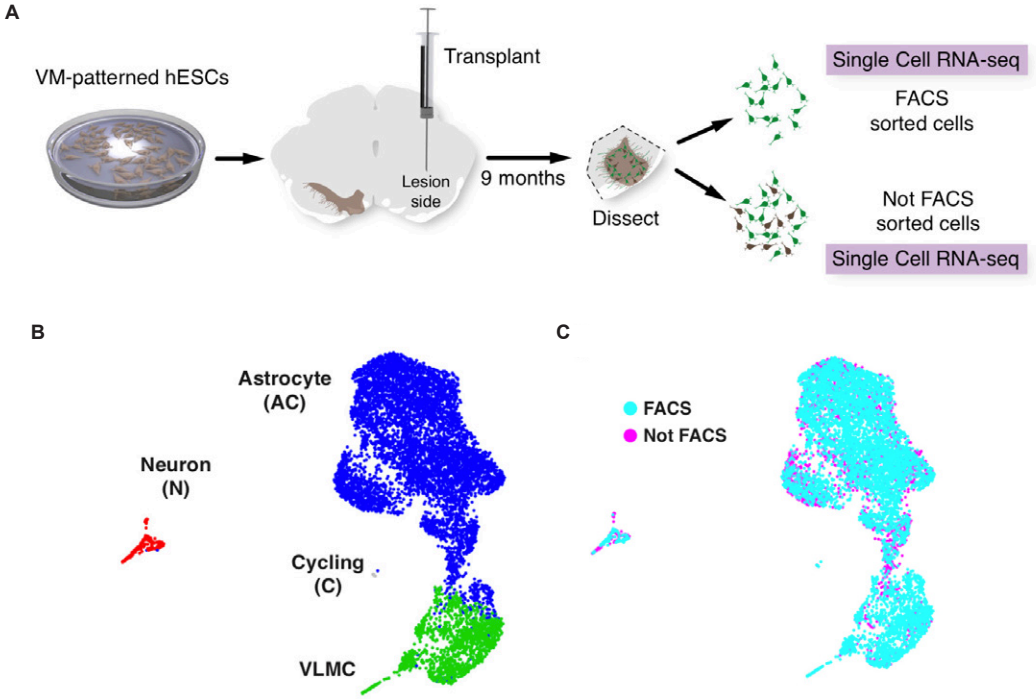


Figure 17. *Assessment of graft composition of intranigral transplants using droplet-based sequencing*

(A) Schematic overview of the experimental design for sequencing of cells from hESC-derived DA grafts in the nigra. (B-C) UMAP based clustering of the grafted cells revealed four major clusters of cells (B). Cells isolated by FACS (blue) or without FACS (magenta) contributed to all of the four clusters (C).

sist of a homogenous group of cells. Interestingly, the hESC-derived progenitor cells co-expressed neural progenitor genes, DA neurogenesis related genes as well several genes associated with a VLMC fate, suggesting the potential of a common hESC-derived progenitor cells to generate neurons, astrocytes and VLMCs (Figure 16).

The scRNA-seq results can be confirmed using a different grafting paradigm and a different cell isolation and sequencing method

In order to validate the findings from the scRNA-seq of graft derived cells, we transplanted hESC-derived DA progenitors into the rat midbrain and re-isolated the grafted cells after 9 months of maturation *in vivo* (Figure 17A). These cells were then sequenced with the 10x Genomics Platform, with or without being subjected to FACS to assess if the GFP-based selection or cell isolation method would bias the result. The higher throughput of the 10x Genomics Platform allowed for 7875 cells to be analyzed and once again the hESC-derived cells were divided into three clusters corresponding

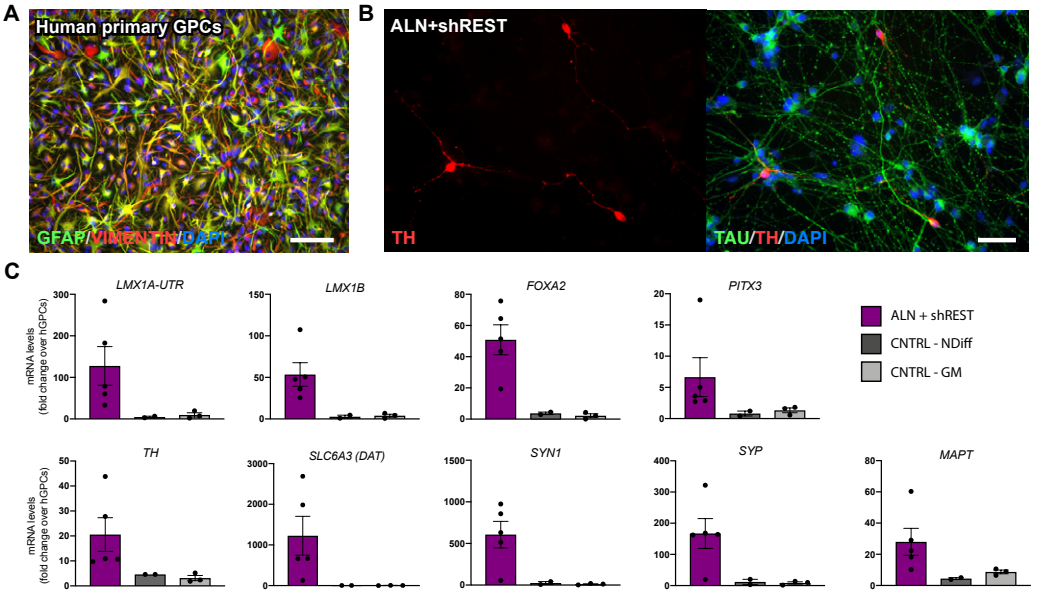


Figure 18. *Human fetal primary GPCs can be converted to iDANs*

(A) Fetal primary hGPCs visualized by GFAP and Vimentin immunocytochemistry.
(B) Reprogrammed TH^+/TAU^+ neurons 3 weeks after transgene activation.
(C) Gene expression analysis of reprogrammed cells 3 weeks after transgene activation shows an upregulation of pan-neuronal and DA related genes. Data are presented as mean ± SEM and all data points have been visualized in the graphs. Scale bars represent 100 μm (A) and 50 μm (B).

to neurons, astrocytes and VLMCs, regardless if they had been isolated and sequenced directly or isolated by FACS based on GPF-expression (Figure 17B-C). Importantly, this reveals that the hESC-derived cells contribute to three major clusters of cells corresponding to neurons, astrocytes and VLMCs independently of the grafting site and the method of isolation.

Human glial progenitors can be reprogrammed into DA neurons *in vitro* (Paper V)

Direct neuronal reprogramming offers an alternative route for brain repair in PD. This strategy allows for the possibility of neuronal replacement *in situ* in the brain by targeting resident brain cells (Li and Chen, 2016). One good candidate cell type to target could be GPCs (also known as oligodendrocyte progenitors or NG2 glia), since these cells are spread throughout the adult brain parenchyma and since their proliferation is homeostatically controlled, thus limiting the risk of depletion (Hughes

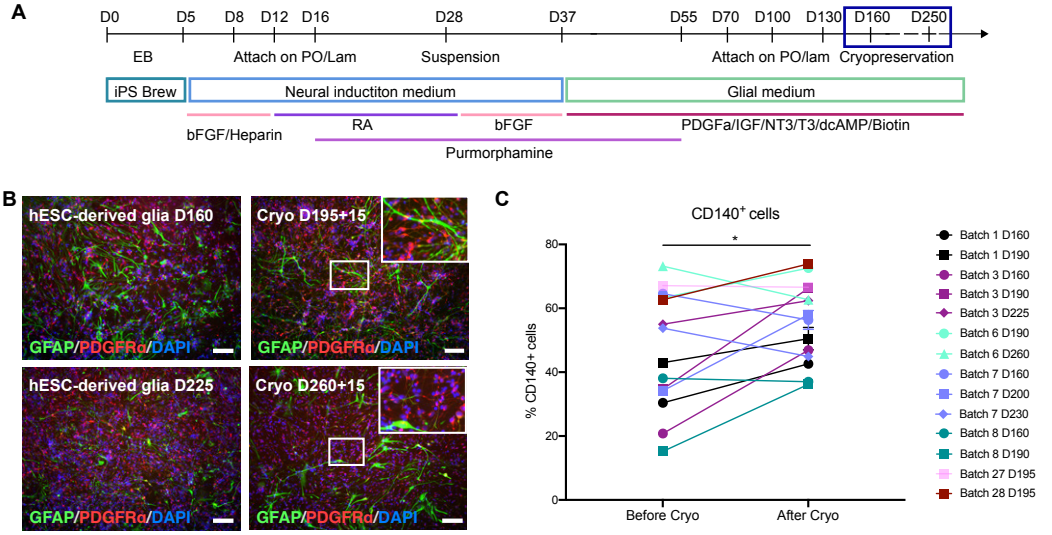


Figure 19. *Generation of an expandable and standardized source of hGPCs from hESCs*

(A) Schematic overview of the protocol for generating hESC-derived hGPCs, with an indication of the time frame used for cryopreservation.

(B) Immunocytochemistry of GFAP/PDGFRα in the hESC-derived hGPC cultures at different stages and before and after cryopreservation. Insets show high magnification images of the morphology of cells in the boxed areas.

(C) Percentage of CD140⁺ cells within the cultures before and after cryopreservation as determined by flow cytometry. The proportions of CD140⁺ cells were compared using a paired two-tailed t-test; *P<0.05 (p=0.0327). Scale bars represent 100 μm.

et al., 2013; Robins et al., 2013; Simon et al., 2011). Proof-of-principle for the potential to convert GPCs into neurons have been obtained through studies *in vivo* in mice (Heinrich et al., 2014; Pereira et al., 2017; Torper et al., 2015). However, there is still a lack of studies showing neuronal conversion of human GPCs. In this study we therefore wanted to assess the possibility to directly reprogram hGPCs, into functional DA neurons *in vitro*, in order to evaluate hGPCs as a target cells for neuronal conversion.

Reprogramming of human fetal primary GPCs

In order to assess the possibility to directly reprogram hGPCs into DA neurons, fetal primary hGPCs isolated from the brain of a 20-week old fetus (Figure 18A) (Benraiss et al., 2016) were transduced with doxycycline (dox) regulated vectors carrying the transgenes *Ascl1, Lmx1a* and *Nurr1* (ALN), and short hairpin RNA targeting the RE1-silencing transcription factor (REST) complex. Three weeks after transgene activation, TH⁺ neurons were observed in the transduced culture and the expression of pan-neuronal genes, as well as DA related genes were upregulated, thus confirming the possibility to convert hGPCs into induced DA neurons (iDANs) (Figure 18B-C).

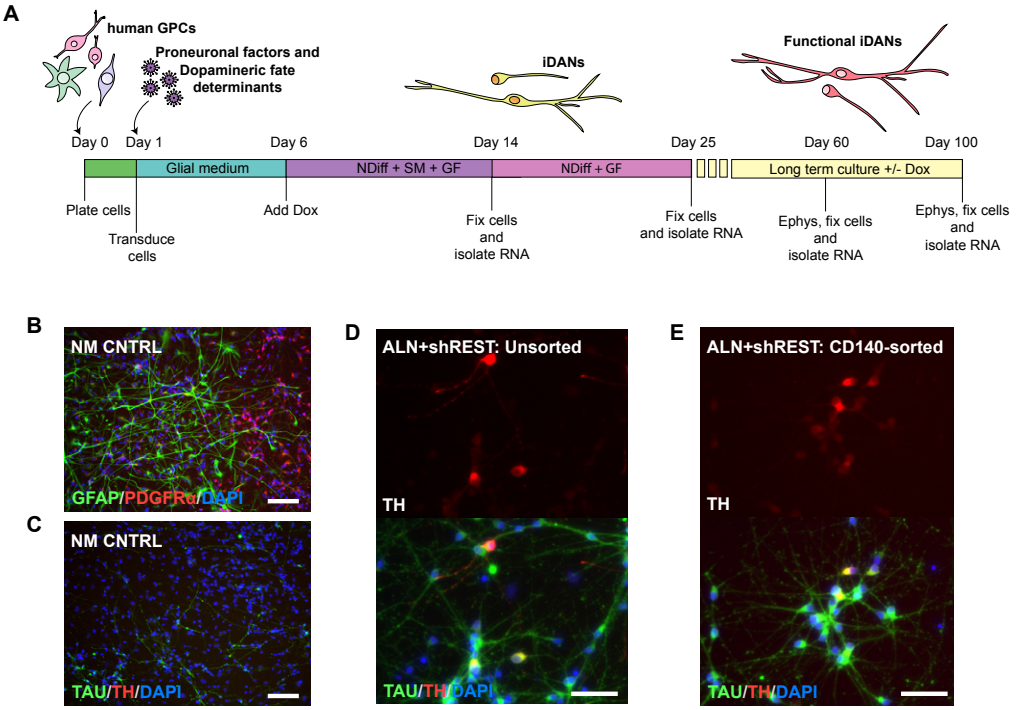


Figure 20. *hESC-derived hGPCs can be converted to iDANs*

(A) Schematic overview of the neuronal reprogramming protocol with indications of the different time points of analysis. (B-C) GFAP$^+$ and PDGFRα$^+$ cells are maintained when the hESC-derived hGPCs are kept in neuronal conversion medium for 3 weeks (B), and no TH$^+$/TAU$^+$ neurons are observed in these cultures (C).
(D-E) Unsorted (D) and CD140-sorted (E) hESC-derived hGPCs give rise to a comparable number of TH$^+$ neurons 3 weeks after transgene activation. Scale bars represent 100 μm (B-C) and 50 μm (D-E).

Establishing a renewable and reproducible stem cell based hGPC *in vitro* model of direct neural conversion

Human GPCs are not born until late in the second trimester of fetal development and these cells are therefore hard to obtain for experimental studies (Jakovcevski and Zecevic, 2005; Sim et al., 2011). To generate a more accessible source of hGPCs we adopted a previously published protocol for generating hESC-derived hGPCs (Wang et al., 2013) and introduced a cryopreservation step to the protocol in order to improve the logistics and reproducibility of the protocol (Figure 19A). Using this protocol, we obtained cultures with a high proportion of highly proliferative PDGFRα$^+$ (CD140$^+$) hGPCs and we could confirm that the phenotype of these cells was maintained after cryopreservation (Figure 19B-C).

We next reprogrammed these cryopreserved hESC-derived hGPCs using the same protocol and reprogramming factors as we had used for converting primary fetal hGPCs (Figure 20A). While untransduced control cultures that were kept in parallel in neuronal conversion medium maintained

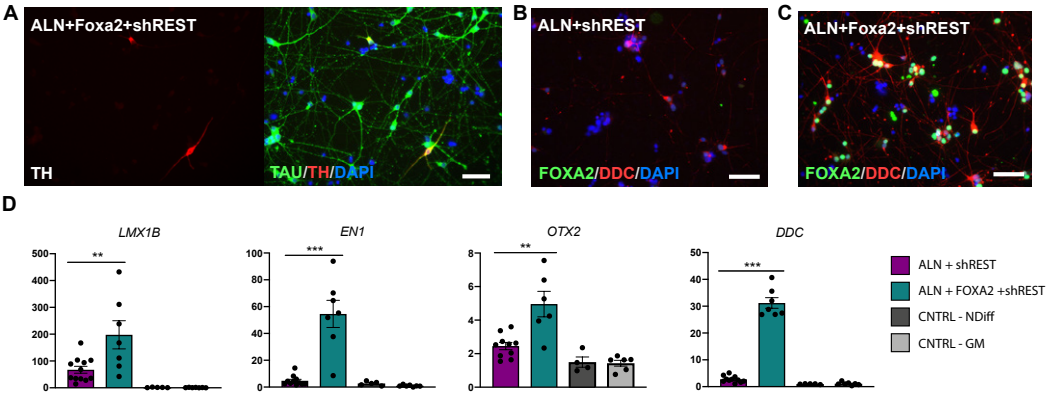


Figure 21. *Addition of FOXA2 to the reprogramming cocktail results in an increased induction of a subset of midbrain DA genes after 3 weeks*

(A) hESC-derived hGPCs can be reprogrammed into TH⁺/TAU⁺ neurons with ALN+FOXA2+shREST 3 weeks after transgene activation.
(B-C) Addition of Foxa2 to the reprogramming cocktail results in more DDC⁺ cells after 3 weeks of reprogramming as determined by immunocytochemistry.
(D) Conversion using the reprogramming factors ALN+Foxa2+shREST results in an upregulation of certain midbrain DA markers compared to ALN+shREST, as determined by qRT-PCR. Data are presented as mean ± SEM and all data points have been visualized in the graphs. The gene expression levels for the conditions ALN+shREST and ALN+FOXA2+shREST were compared using a Mann-Whitney test; **p<0.01 (p=0.0098 for *LMX1B*, p=0.0075 for *OTX2*); ***p<0.001 (p=0.0001 for *EN1*, p<0.0001 for *DDC*). Scale bars represent 50 μm.

their glial identity, TH$^+$ neurons were observed in the transduced cultures 3 weeks post transgene activation (FIG 20B-D). To directly demonstrate that pure populations of CD140$^+$ hGPCs can be reprogrammed to iDANs, we next sorted CD140$^+$ hGPCs by FACS to be able to specifically target these cells. Importantly, by converting CD140-sorted and unsorted cells from the same batch side by side we could confirm that the reprogramming efficiency appeared to be equivalent and that CD140$^+$ hGPCs can be converted into iDANs *in vitro* (Figure 20D-E).

Assessing optimal factor combinations to reprogram hGPCs into iDANs

Using this stem cell-derived model system of hGPCs neuronal conversion, we next went on to screen for additional DA fate determinants. Through these efforts, we identified Foxa2 as a factor that can further increase the endogenous expression of several DA related genes after 3 weeks of transgene activation (Figure 21A-D). Addition of Foxa2 to the initial reprogramming cocktail resulted in a higher expression of the midbrain DA markers *OTX2, LMX1B* and *EN1*, as well as *DDC*, which is involved in the synthesis of DA (Figure 21D).

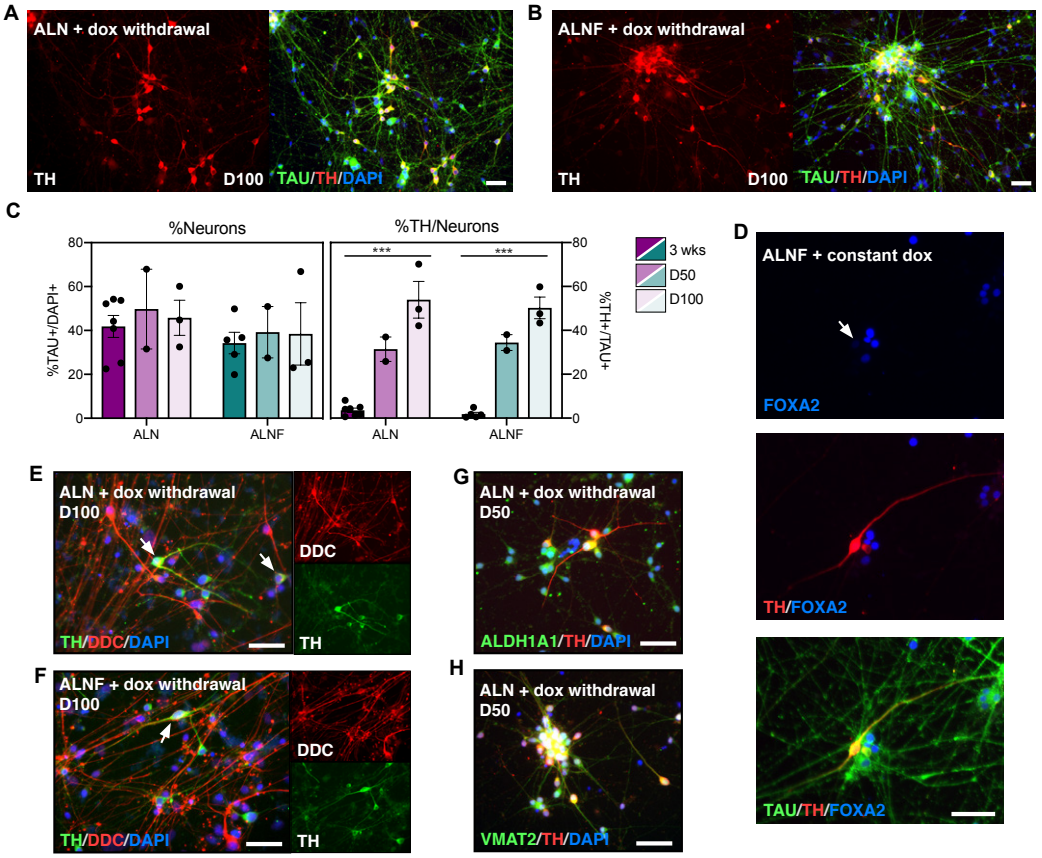


Figure 22. *Progressive subtype specific maturation of iDANs over time*

(A-B) Immunocytochemistry of TH/TAU after 100 days in culture (75 days after dox withdrawal) in hESC-derived hGPCs cultures reprogrammed using either ALN+shREST (A) or ALN+FOXA2+shREST (B).

(C) The proportion on TH^+ iDANs increase over time in culture, while the proportion of TAU^+ neurons remains comparable across the time points.

(D) A unphysiological high and/or prolonged transgene expression of Foxa2 seems to directly interfere with the expression of TH, since cells that show a high expression of FOXA2 do not co-label TH when dox is maintained it the medium. White arrow indicates a TH^+ cell with weak nuclear labeling of FOXA2.

(E-F) TH^+/DDC^+ double-positive cells are observed in long term cultures of both ALN+shREST (E) and ALN+Foxa2+shREST (F) reprogrammed cells. White arrows indicate double-labeled cells.

(G-H) The generated iDANs are positive for ALDH1A1 (G) and VMAT2 (H). The proportion of TAU^+ and TAU^+/TH^+ cells at the 3-week time point was compared to the late timepoints Day 50 and Day 100 combined using an unpaired, two-tailed, t-test. ***$p<0.001$ ($p<0.0001$ for TH^+ cells in ALN+shREST, $p<0.0001$ for TH^+ cells in ALNF+shREST); n.s. ($p=0.5307$ for TAU^+ cells in ALN+shREST, $p=0.6637$ for TAU^+ cells in ALNF+shREST). Scale bars represent 50 μm.

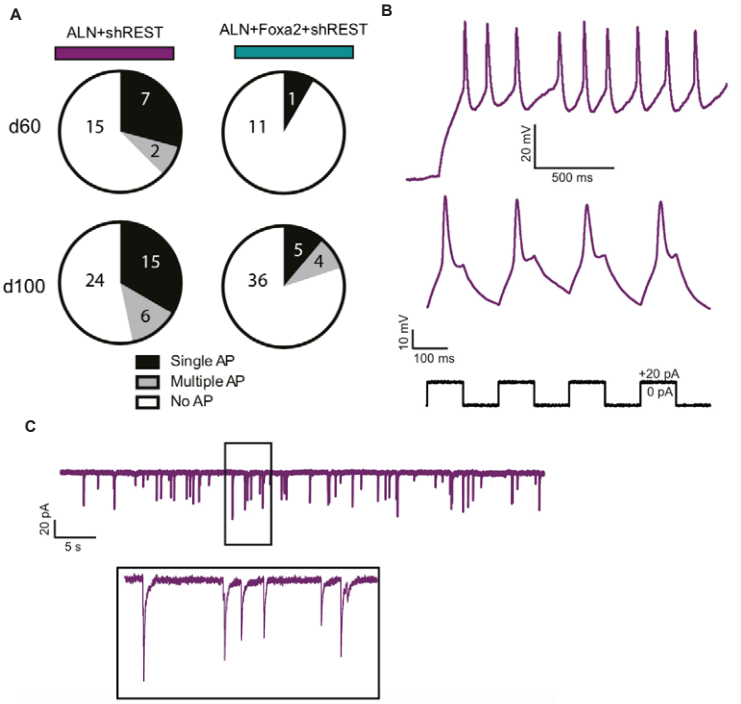


Figure 23. *The iDANs adopt mature and functional electrophysiological properties*

(A) Pie chart showing the number of cells exhibiting multiple, single or no current induced action potentials (AP) after 60 and 100 days of reprogramming with ALN+shREST (left) and ALN+Foxa2+shREST (right).
(B) Spontaneous firing at resting membrane potential in an ALN+shREST reprogrammed neuron (top) and repetitive induced AP at small current injections (bottom).
(C) Spontaneous postsynaptic currents in an ALN+shREST reprogrammed iDAN.

Progressive sub-type specific maturation of iDANs

At this early time point 3 weeks after transgene activation, the neuronal proportions of the reprogrammed cultures were high, but the TH^+ neuronal proportion remained low, indicating an efficient neuronal reprogramming but a limited sub-type specific maturation. To assess if the proportion of TH^+ iDANs increased over time, we used the ALN+shREST and ALN+Foxa2+shREST reprogrammed cells for long term culture, with either maintained dox administration to the medium or with dox withdrawal after 3 weeks (Figure 20A). Quantifications of the neuronal content and the TH^+ neuronal proportion over time showed a maintained neuronal proportion even 75 days after dox withdrawal, while the proportions of TH^+ iDANs were significantly and progressively increased (Figure 22A-C).

Interestingly, while a maintained transgene expression of ALN+shREST did not interfere with the adoption of a TH^+ neuronal phenotype, high numbers of TH^+ cells were only observed after discontinued exogenous expression of *Foxa2*, and a non-physiologically high and/or prolonged expression of *Foxa2* seemed to directly interfere with the adoption of a TH^+ phenotype (Figure 22D).

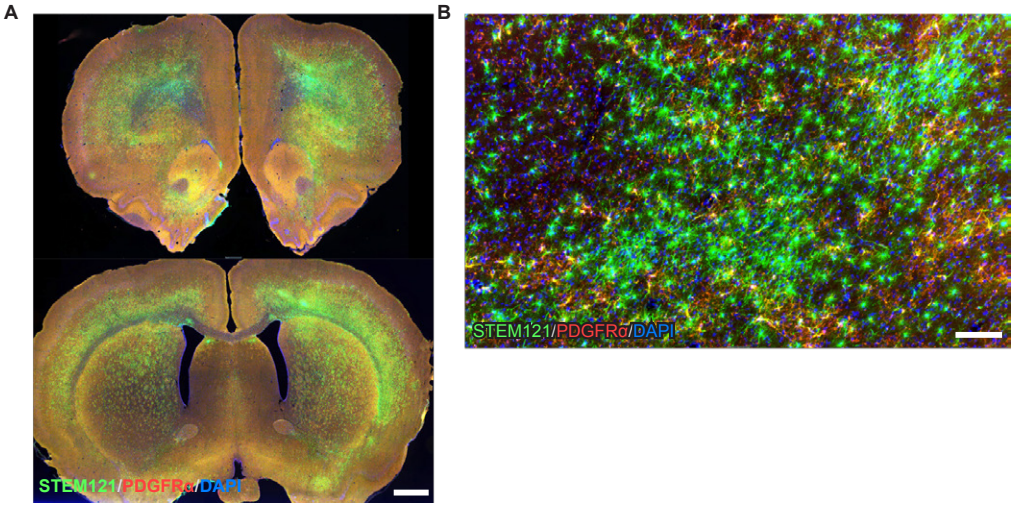


Figure 24. *Grafting of cryopreserved hESC-derived hGPCs into the neonate rat brain results in a widespread distribution of hGPC after 7 months*

(A) Immunohistochemistry of human PDGFRα and a human cytoplasmic marker (STEM121) show widespread distribution of human cells 7 months after grafting into the neonate rat brain.
(B) High magnification image showing the morphology of hGPCs in the rat brain. Scale bars represent 1 mm (A) and 100 μm (B).

The expression of mature neuronal- and DA neuron markers SYN1, VMAT2 and ALDH1A1 was confirmed in long-term cultures of ALN$^+$shREST reprogrammed iDANs by immunocytochemistry and at this late time points DDC$^+$ cells were present at equal quantities in ALN+shREST and ALN+Foxa2+shREST reprogrammed cultures in contrary to what was observed 3 weeks after transgene activation (Figure 21B-C, 22E-H).

Reprogrammed iDANs become functionally mature

In order to assess the functionality of the induced neurons that had been reprogrammed from hESC-derived hGPCs using ALN+shREST and ALN+Foxa2+shREST, whole-cell patch clamp recordings were performed after 60 and 100 days of reprogramming. These recordings showed that the ALN+shREST reprogrammed cells exhibited mature functional characteristics by day 60, but that in comparison, few cells in ALN+Foxa2+shREST condition adopted mature functional characteristics even after 100 days in culture (Figure 23A). Both conditions also showed spontaneous postsynaptic activity and spontaneous firing at resting membrane potential and repetitive firing with small current injections, features that are indicative of a DA neuron phenotype (Figure 23B-C). However, the proportion of cells that showed these features at day 100 was higher in the ALN+shREST condition. These results suggest that hESC-derived hGPCs convert into functional iDANs when reprogrammed with ALN+shREST and achieve more mature profiles compared to ALN+Foxa2+shREST reprogrammed cells even after long term culture. These observations also emphasized the importance of

assessing the reprogrammed neurons after long term culture in terms of phenotypic development and functional maturity.

Cryopreserved hESC-derived GPCs can be grafted into neonatal and adult rats (Ongoing study)

Our ultimate goal is to set up a human glial chimeric rat model (Wang et al., 2013), in order to assess the ability to directly reprogram human GPCs in an *in vivo* environment. Preliminary results show that when the hESC-derived hGPCs are transplanted to the adult rat striatum the cells survival, they maintain their glial identity and there is an increased spread of the cells away from the graft core over time. The migratory potential is one of the defining features of hGPCs, and when cryopreserved hGPCs were transplanted into the neonate rat brain bilaterally into corpus callosum and striatum, human PDGFRα^+ cells could be observed throughout the entire brain after 7 months (Figure 24). This chimeric rat model will provide us with an excellent testbed to assess the potential to reprogram hGPCs *in vivo* into subtype specific neurons, and to further assess their therapeutic potential in a PD model.

CONCLUSIONS AND FUTURE PERSPECTIVES

Cell replacement therapy for PD has been pioneered through clinical trials using fetal derived VM tissue for grafting in patients. However, one of the shortcomings with this cell source has been a lack of standardization which has resulted in variable outcomes in transplanted patients (Barker et al., 2015a). Stem cells have the potential to overcome this issue since large batches of cells can be generated and extensively quality controlled prior to grafting. However, to be able to ensure that all patients will receive a safe and efficacious treatment, we first need to understand what factors dictate graft outcomes. Predicting outcomes *in vivo* is complicated by the fact that the grafted cells are still progenitors, since mature neurons survive transplantation poorly (de Luzy et al., 2019; Ganat et al., 2012; Jönsson et al., 2009). The final cell cycle exit, subtype specific maturation and development of functional properties thus occurs within the host brain, and the possibility to predict the future development of the progenitors at the time of transplantation and to monitor this process has been limited.

In **paper I** and **II**, we identified DA progenitor markers that could better predict the outcome of grafting and at the same time discovered an unexpected close lineage relationship between developing DA neurons in the midbrain and glutamatergic STN neurons in the rostral midbrain/caudal diencephalon. These insights increased our knowledge of DA neuron development and provided us with important tools for improving the predictability and robustness of our transplantable cell product. Interestingly, the predictive marker *Dlk1* was subsequently identified to be associated with excellent outcomes in terms of number of surviving TH^+ cells, innervation and DA uptake also when grafting iPSC-derived DA progenitors in a primate model of PD, thus validating the broader applicability of our findings (Kikuchi et al., 2017).

In **paper III**, we used our refined understanding of midbrain development and our newly identified predictive markers in the development of a GMP adapted cell differentiation protocol for clinical translation. We showed that this protocol can be initiated from different hPSC-lines that are approved for clinical applications and generates DA progenitors that can be cryopreserved, enabling long term storage, extensive quality controls and off-the-shelf grafting on demand. Since the development of this protocol, we have been able to successfully transfer the procedure to multiple sites of production across Europe. Earlier this year, the cell bank that will be used in our first clinical trial was produced at Royal Free hospital in London. Before taking these cells into humans, the safety, dosing and functional benefits will be extensively assessed in rodent models which is enabled by the great number of cryopreserved vials containing cells from an identical production run.

One aspect of both fetal- and hESC-derived DA grafts that has been difficult to study due to methodological limitations is the complete cellular composition of the grafts. With the development of scRNA-seq technology, we were able to for the first time to address this question in **paper IV.** This study revealed that while both fetal- and hESC-derived grafts shared important similarities, they were also fundamentally different in certain aspects. In particular, we found that oligodendrocytes

are only present in fetal grafts, and we identified a new type of cells, VLMCs, as a previously unrecognized graft component that is exclusively present in hPSC-derived grafts. From the sequencing of cells prior to grafting it seems like VLMCs and DA neurons might arise from a shared progenitor cell, but future lineage tracing studies are needed to elucidate the origin of these cells and the dynamics of their development.

This study took the first important steps for providing a detailed assessment of the cellular graft composition as it introduced a new methodology for unbiased evaluation of the different cell types existing in the graft. Importantly, this method could be further employed to study the components of various types of grafts, thus extending the relevance of this study to other fields of research. However, we still lack knowledge about the functional aspects of the different components in the grafts and the consequences of the differences observed between fetal- and hESC-derived grafts. It still remains unaddressed whether the VLMCs are beneficial for the graft function, or if the therapy would be improved further if these cells were abolished. To be able to answer this question, we first need to identify were these cells come from as well as when they arise, since the transplanted progenitor cells express both DA markers and markers associated with a VLMC-fate, which hampers cell sorting based elimination.

For future applications, direct *in vivo* reprogramming could present a fast track to replacing the lost DA neurons directly in the brain. The benefits of this therapeutic strategy are related to the facts that it circumvents the need of cell transplantation and the associated immunosuppressive demands and that it involves a reduced risk of tumor formation since the cells do not go through a pluripotency stage. In **paper V**, we were able to successfully reprogram hGPCs into functional iDANs, encouraging the feasibility of targeting these cells for *in vivo* conversion. Attempts to convert mouse GPCs into iDANs *in vivo*, using factor combinations that have been used to convert mouse and human astrocytes and fibroblast *in vitro*, have failed previously, as the converted neurons instead adopted interneuron fates (Pereira et al., 2017; Torper et al., 2015). The chromatin state of cells has been shown to strongly influence the capacity of the cells to reprogram and the epigenetic environment needs to be permissive for TFs to act (Wapinski et al., 2013). A recent study investigating the chromatin environment around interneurons genes in OPCs identified the presence of a unique histone code (Boshans et al., 2019). In OPCs, key interneuron genes appeared to be transcriptionally "poised", perhaps reflecting the close developmental origin of these two cell lineages arising from the ventral forebrain. One can speculate that this might result in a favored ability of OPCs to adopt interneuron fates compared to other neuronal fates and that additional strategies might be required in order to switch the interneurons program to a DA program during reprogramming.

Whether hGPCs can be reprogrammed into iDANs *in vivo* is yet to be determined. One barrier for reprogramming of human adult cells has previously been identified in REST, which is part of the repressor complex that suppress neuronal fates. Down regulation of REST consequently results in the possibility to reprogram human adult cells which otherwise are highly refractory to reprogramming (Drouin-Ouellet et al., 2017; Masserdotti et al., 2015). It is possible that additional roadblocks exist when reprogramming cells *in vivo,* and that the success of this approach therefore relies on identifying and eradicating these.

Taken together, the studies that are included in this thesis have been crucial for the development of a stem cell-based treatment for PD, which is currently being translated into a clinical trial in hu-

mans. However, at the same time as many questions and issues have been resolved, these studies have opened up for additional questions and future studies related to the function of the different graft components and the developmental potential of FP-derived progenitors. Work included in this thesis has also recognized hGPCs as a possible target cell for generating new neurons though direct reprogramming. Moving to *in vivo* human chimeric conversion models, the prospects of this therapeutic strategy will be further investigated.

These are exciting times for cells replacement therapy for PD, with several stem cell-based clinical trials being either recently commenced or on the verge of being initiated (Kirkeby et al., 2017; Studer, 2017; Takahashi, 2017). Decades of research has brought us to where we are today, and future efforts will be focused on developing second generation stem cell-based products for improved safety, efficacy and control starting from non-immunogenic hPSC-lines that could potentially be used without immunosuppression (Greco et al., 2015; He et al., 2017; Zheng et al., 2016). Additionally, parallel lines of research will continue to investigate direct reprogramming as potential new strategy for replacing the lost neurons directly in the brain (Li and Chen, 2016; Parmar et al., 2019).

MATERIAL AND METHODS

In this section I will describe some selected methods that have been important in the studies that are included in my thesis. For more details about additional methods employed in this work, including cell transplantations, assessments of the cells *in vivo* and bioinformatic analyses, I kindly refer the reader to the method section of the respective papers (see appendix).

Culturing of hESCs

The majority of work included in my thesis has been conducted using the hESC-line RC17 (Roslin Cells, cat. no. hPSCreg RCe021-A). However, several additional hESC-lines have been employed in order to assess the robustness and reproducibility of the DA progenitor differentiation protocol, including; H9 (WiCell, cat. no. hPSCreg WAe009-A); MShef7 and Mshef13 (both from the University of Sheffield); and HS980, HS983a, HS999 and HS1001 (all from Karolinska Institute).

For maintenance of pluripotent hESCs, the cells were cultured on tissue culture plates coated with recombinant human (rh) laminin 521 (LN521, 0.5 μg/cm^2, BioLamina) in iPS-Brew XF medium (StemMACS; Milteny) (Yap et al., 2019). This cell culture system offers the benefits of being fully defined and free from any animal-derived components, resulting in highly consistent cultures and minimal spontaneous differentiation of the pluripotent cells. Furthermore, this culture medium is available in a GMP version, which is currently being used for clinical cell manufacturing. The cells were passaged weekly with EDTA (0.5 mM) and seeded at a density of 2,500 cells per cm^2. To enhance the survival of cells seeded at low-density, ROCK inhibitor (10 μM Y-27632) was added to the medium for the first 24 hours after passaging (Watanabe et al., 2007).

Differentiation of hESCs into midbrain DA progenitors

To initiate the differentiation of RC17 hESCs into midbrain DA progenitors, the pluripotent cells were seeded onto Laminin 111 (LN111, 1 μg/cm^2, BioLamina) coated tissue culture plates at a density of 10,000 cells per cm^2 in N2 medium (see table 1) + Y-27632 (10 μM). In order to achieve a neuralization of the cultures, dual SMAD inhibition was employed by supplementing the medium with SB431542 (10 μM) + rhNoggin (100 ng/ml), thus inhibiting both the TGFβ/Activin/Nodal pathway, as well as bone morphogenetic proteins (BMP) signaling (Chambers et al., 2009). For floor-plate induction, high-potency SHH (rhSHH-C24II, 300 ng/ml) was added to the medium to ventralize the cultures and the GSK3 inhibitor CHIR99021 (0.6–1.0 μM) was added to activate WNT-signaling in order to adjust the rostral-caudal identity of the cells to adopt a midbrain fate (Fasano et al., 2010; Kirkeby et al., 2012). Up until day 9 of the differentiation, the cells were kept in N2 medium supplemented with SB431542, rhNoggin, rhSHH-C24II and CHIR99021, with fresh medium being ap-

Table 1: *N2 medium*

Component	Dilution	Final Conc.	Stock Conc.	Vendor	Catalog #
DMEM/F12	1:2	0.5X	1X	Thermo Fisher	21331020
Neurobasal CTS	1:2	0.5X	1X	Thermo Fisher	A1371201
N2 suppl. CTS	1:100	1X	100X	Thermo Fisher	A1370701
L-Glutamine	1:100	2 mM	200 mM	Thermo Fisher	25030081
Pen/Strep	1:500	0.2%	10,000 U/ml	Thermo Fisher	15140122

Note: Used in midbrain DA progenitor induction protocol between day 0-11.

plied every 2-3 days. On day 9, the medium was changed to N2 medium supplemented with FGF8b (100 ng/ml) to fine-tune the patterning of the cells towards the caudal part of the midbrain. Two days later, the expanding progenitors were dissociated to a single cell suspension with Accutase and replated on LN111 coated plates at a density of 800,000 cells per cm^2 in B27 medium (see table 2) supplemented with Y-27632, BDNF (20 ng/ml), ascorbic acid (AA, 0.2 mM) and FGF8b (100 ng/ml). Fresh B27 medium containing BDNF, AA and FGF8b was applied to the cells on day 14, and on day 16 the patterning of the midbrain DA progenitors was complete and the cells were frozen for cryopreservation, transplanted directly or replated for terminal differentiation.

To adopt the differentiation protocol to other hESC-lines than RC17, a higher seeding density was sometimes necessary on day 0. The appropriate concentration of CHIR99021 to achieve a correct caudalization varies between cell lines and was adapted accordingly. Certain cell lines also require an enhanced ventralization, in which case the concentration of SHH-C24II was increased or alternatively Purmorphamine, the Hedgehog receptor Smoothened-agonist, was added.

Terminal maturation of midbrain DA progenitors *in vitro*

The 16-day differentiation protocol generates midbrain DA progenitors and continued culturing is required to achieve mature neuronal cultures. For terminal differentiation of the progenitors, the cells were passaged between day 16 and 18 to prevent the cultures from becoming over-confluent. The cells were dissociated with Accutase and seeded onto LN111 coated plates (2 μg/cm^2) at a density of 155,000 cells/cm^2 in B27 medium supplemented with BDNF, AA (0.2 mM), GDNF (10 ng/ml), db-cAMP (500 μM), DAPT (1 μM) and Y-27632 (10 μM). Every 2-3 days, the medium was then replaced and fresh B27 medium containing BDNF, AA, GDNF, db-cAMP and DAPT was added. From day 25 and on, only two-thirds of the medium was replaced in order to minimize the disturbance of the cells to prevent neuronal cell detachment. Mature cultures of DA neurons were analyzed between day 35 and 55.

Table 2: *B27 medium*

Component	Dilution	Final Conc.	Stock Conc.	Vendor	Catalog #
Neurobasal CTS	-	1X	1X	Thermo Fisher	11140050
B27 suppl.	1:50	1X	50X	Thermo Fisher	12587010
L-Glutamine	1:100	2 mM	200 mM	Thermo Fisher	25030081
Pen/Strep	1:500	0.2%	10,000 U/ml	Thermo Fisher	15140122

Note: Used in midbrain DA progenitor induction protocol from day 11 and onwards.

Differentiation of hESCs into glial progenitor cells

For differentiation of hESCs into hGPCs, the protocol developed in the Goldman lab (Wang et al., 2013) was employed with some minor modifications. The protocol contains several different stages: 1) an initial free-floating embryoid body (EB) stage (day 0-8), 2) an adherent neuroepithelial stage during which the cells are patterned to a ventral spinal cord identity (day 9-28), 3) a free-floating and later adherent pre-OPC stage where the glial induction is initiated (28-130 days), and 4) an adherent OPC stage, where the hGPCs are expanded and can be cryopreserved (130-270 days).

To initiate the differentiation, the hESCs were detached from the plate using Accutase and dissociated to single cells and small cell clusters and kept in iPS-Brew XF medium in ultra-low adhesion flasks to allow for EB formation. On day 0 –1, the iPS-Brew XF medium was supplemented with 10 μM Y-27632 to improve the survival of single cells. On day 5, the medium was switched to neural induction medium (NIM, see table 3) supplemented with bFGF (20 ng/ml) and Heparin (2 μg/ml). On day 9, the EBs were attached on Poly-Ornithine/Laminin (PO/Lam) coated tissue culture plates and two days later the medium was changed to NIM supplemented with Retinoic acid (RA, 0.1 μM) and on day 16 the medium was changed to NIM/B27 medium (see table 4) + RA + Purmorphamine (1 μM). Daily medium changes were performed between day 0-16 and thereafter every second day.

Around day 26-29, the cells were detached from the plates and were seeded as free-floating clusters in ultra-low attachment plates. At this stage, the neuroepithelial identity of the cells was assessed by immunocytochemistry (ICC) of PAX6, SOX1 and OLIG2 and by Flowcytometry (FC) of CD133 and SSEA4. Following the detachment, the free-floating cell clusters were kept in NIM/B27 medium + Purmorphamine + bFGF (10 ng/ml) up until day 37.

On day 37, the glial induction was initiated and the medium was switched to glial medium (GM, see table 5) supplemented with T3 (60 ng/ml), db-cAMP (1 μM), Biotin (100 ng/ml), rhPDGF-AA (10 ng/ml), rhIGF-I (10 ng/ml) and rhNT-3 Protein (10 ng/ml). In addition, Purmorphamine was added to the medium up until day 55 and medium changes were performed every 2-3 days.

Around day 70, the free-floating clusters were manually cut into smaller pieces using disposable sterile scalpels under a dissection microscope and seeded onto PO/Lam coated tissue culture plates. Thereafter, the cells were passaged and expanded every 30 days, when the clusters were mechanically

Table 3: *Neural induction medium (NIM)*

Component	Dilution	Final Conc.	Stock Conc.	Vendor	Catalog #
DMEM/F12	-	1X	1X	Thermo Fisher	11330032
NEAA	1:100	1X	100X	Thermo Fisher	11140050
N2 suppl.	1:100	1X	100X	Thermo Fisher	17502048
Ab/Am	1:200	0.5X	100X	Thermo Fisher	15240062

Note: Used in hGPCs induction protocol between day 6-16.

detached from the plates using a cell scraper, cut at the dissection microscope and re-seeded again at lower density (around 1:2). At every passage, the cells were analyzed by FC for CD44 and CD140 and by ICC for various glial markers, and for culture that had reached a threshold level of 50% $CD140^+$ (PDGFRα) part of the cells were cryopreserved between D160-250 and part of the cells were re-plated for further expansion.

Direct conversion of human glial progenitor cells to induced neurons

For direct neuronal conversion, primary fetal hGPCs or hESC-derived hGPCs were seeded in their normal culturing medium on plates that had been serially coated with PO, Lam and Fibronectin (FN), at a density of 50,000 cells/cm^2. For electrophysiological analysis, the cells were seeded onto PO/Lam/FN coated coverslips that had been pre-treated according to (Richner et al., 2015). One day after seeding, the cells were transduced with doxycycline-regulated vectors (with transgene expression under the control of the TetOn promotor) together with the trans-activator FuW.rtTA-SM2. Viral vectors were delivered at a multiplicity of infection (MOI) of 0.5-1 per vector (primary hGPCs) or MOI 1-2 per vector (hESC-derived hGPCs) and the medium was changed the next day to remove the viral vectors from the cells. Doxycycline (2 μg/ml) was added to the culture medium 5 days after transduction to activate the transgenes and 2 days later the medium was switched to neural differentiation medium (NDiff227; Takara-Clontech) containing small molecules (SMs): CHIR99021 (2 μM), SB-431542 (10 μM), noggin (0.5 μg/ml), LDN-193189 (0.5 μM), and valproic acid sodium salt (VPA; 1 mM); and growth factors (GFs): LM-22A4 (2 μM), GDNF (2 ng/ml), NT3 (10 ng/ml) and db-cAMP (0.5 mM). Two-thirds of the medium was changed every 2-3 days. Two weeks post-transduction, the SMs were withdrawn from the neuronal differentiation medium and the medium was supplemented with only the GFs (LM-22A4, GDNF, NT3, and db-cAMP) until the end of the experiment. For long term assessment of the maturation of the reprogrammed cells, doxycycline was either kept in the medium until the end or withdrawn 3 weeks post transgene activation.

Table 4: *NIM/B27 medium*

Component	Dilution	Final Conc.	Stock Conc.	Vendor	Catalog #
DMEM/F12	-	1X	1X	Thermo Fisher	11330032
NEAA	1:100	1X	100X	Thermo Fisher	11140050
N2 suppl.	1:100	1X	100X	Thermo Fisher	17502048
B27 suppl.	1:50	1X	50X	Thermo Fisher	12587010
Ab/Am	1:200	0.5X	100X	Thermo Fisher	15240062

Note: Used in hGPCs induction protocol between day 16-37.

Table 5: *Glial medium (GM)*

Component	Dilution	Final Conc.	Stock Conc.	Vendor	Catalog #
DMEM/F12	-	1X	1X	Thermo Fisher	11330032
B27 suppl.	1:50	1X	50X	Thermo Fisher	12587010
N1 suppl.	1:100	1X	100X	Sigma Aldrich	N6530
NEAA	1:100	1X	100X	Thermo Fisher	11140050
Ab/Am	1:200	0.5X	100X	Thermo Fisher	15240062

Note: Used in hGPCs induction protocol from day 37 and onwards.

Cell analysis methods

Immunocytochemistry

For immunocytochemistry, the cells were fixed with 4% paraformaldehyde (PFA) solution for 15 min in room temperature. To avoid unspecific binding of secondary antibodies, the cells were pre-incubated in blocking solution containing 0.1 M phosphate buffered saline with potassium (KPBS) + 0.1% Triton + 5 % serum (of secondary antibody host species) for 1-3 hours in room temperature. The cells were then incubated in the blocking solution containing primary antibodies (see table 6) over night at 4°C and the following day washed three times with KBPS. Secondary fluorophore-conjugated antibodies (1:200, Jackson ImmunoResearch Laboratories) and DAPI (1:500) was added to the cells in the blocking solution for 2 hours in room temperature and the cells where then finally washed with KPBS.

Table 6: *Primary antibodies*

Antigen	Species	Company (catalog #)	Dilution	Application
ALDH1A1	Rabbit	Abcam (ab24343)	1:200	1
BARHL1	Rabbit	Novus Biologicals (NBP1-86513)	1:500	2, 5, 6
CALBINDIN	Rabbit	SWant (CB38)	1:1,000	6
COLLAGEN I α1 (hu)	Sheep	R&D systems (AF6220)	1:200	2, 6
COLLAGEN I	Rabbit	Abcam (ab34710)	1:100	2, 6
CRE	Mouse	Abcam (ab24607)	1:1,000	2, 3
CRE	Rabbit	Biolegend (908001)	1:500	2, 3
DDC	Rabbit	Merck Millipore (AB1569)	1:500	1
DAT	Rat	Merck Millipore (MAB369)	1:800	5
EN1	Mouse	DSHB (4GII)	1:20	2, 5, 6
FOXA2	Goat	R&D systems (AF2400)	1:1,000	2
FOXA2	Goat	Santa Cruz (sc-6554)	1:500	1, 5, 6
GIRK2	Rabbit	Alamone Labs (APC006)	1:500	2
GIRK2	Goat	Merck Millipore (AB65096)	1:200	6
GFAP	Mouse	Biolegend (SMI 21)	1:500	2, 3, 4, 6, 7
HNCAM	Mouse	Santa Cruz (SC106)	1:1,000	6, 7, 8
HUNU	Mouse	Merck Millipore (MAB1281)	1:200	6, 7, 8
LMX1	Rabbit	Merck Millipore (AB10533)	1:1,000	2, 5, 6
MAP2	Mouse	Sigma Aldrich (M1406)	1:500	1, 2
MAP2	Rabbit	Millipore (AB5622)	1:500	1, 2
NEUN	Mouse	Millipore (MAB377)	1:500	1, 6
NEUN	Rabbit	Millipore (ABN78)	1:500	1, 6
NFIA	Rabbit	Sigma Aldrich (HPA006111)	1:200	3, 6
OLIG2	Rabbit	Neuromics (RA25081)	1:500	3, 6, 8
O4	Mouse	Merck Millipore (MAB345)	1:100	3
OTX2	Goat	R&D Systems (AF1979)	1:2,000	2
PAX6	Rabbit	Biolegend (901301)	1:1,000	3
PDGFRα	Rabbit	Cell Signaling Technology (5241S)	1:300	2, 3, 4, 6, 7, 8
SOX1	Goat	R&D systems (AF3369)	1:200	3
SOX10	Goat	R&D systems (AF2864)	1:25	3
STEM121	Mouse	Takara Bio (Y40410)	1:500	6, 7
SYNAPSIN 1	Rabbit	Merck Millipore (AB1543)	1:200	1
TAU (HT7)	Mouse	Thermo Fisher (MN1000)	1:500	1
TH	Rabbit	Merck Millipore (AB152)	1:1,000	1, 5, 6, 8
TH	Sheep	Merck Millipore (AB1542)	1:1,000	1, 2, 6
TH	Mouse	Immunostar (22941)	1:5,000	2,6
VIMENTIN	Chicken	Merck Millipore (AB5733)	1:2,000	3, 4
VMAT2	Rabbit	Sigma Aldrich (AB1598P)	1:200	1

1) iNs (*in vitro*)
2) hESC-derived DA cultures (*in vitro*)
3) hESC-derived hGPCs cultures (*in vitro*)
4) Primary fetal hGPCs cultures *(in vitro)*
5) hfVM tissue
6) hESC-derived DA grafts
7) Grafted hGPCs
8) hfVM grafts

qRT-PCR

To analyze the gene expression of the cells, RNA was isolated using the RNeasy Microkit (Qiagen). To obtain cDNA, reverse transcription was performed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific), with 0.2-1 μg of extracted RNA. The cDNA (1 μL) was then mixed with the relevant primers (4 μL, see table 7; Integrated DNA Technologies) and LightCycler 480 SYBR Green I Master (5 μL, Roche) using a Bravo pipetting robot instrument (Agilent) and analyzed by quantitative RT-PCR on a LightCycler 480 II instrument (Roche) using a 40× cycle two-step protocol with a 60°C, 1 min annealing/elongation step and a 95°C, 30 second denaturation step. The average CT values were calculated from three technical replicates and were used to determine the relative gene expression using the ΔΔCT method. The average fold change was based on two different housekeeping genes (ACTB and GAPDH) and the relative gene expression is described in relation to appropriate controls for each experiment.

Flow cytometry

For flow cytometry analyses of hGPCs between day D130-270 of differentiation, or following cryopreservation, the cultured cells were mechanically detached from the cell culture plates and dissociated to single cell suspensions using Accutase. The cells were then resuspended in Miltenyi wash buffer (PBS + 0.5% BSA Fraction V + 2 μM EDTA + 0.05‰ Phenol Red to a concentration of 1 million cells/ml. For each sample, 100,000 cells were incubated with fluorophore-conjugated antibodies in Miltenyi wash buffer for 15 min at 4°C: PE anti-human CD140a (BD Biosciences, cat. no. 556002, 1:10); APC anti-CD44 (Miltenyi, cat. no. 130-095-177, 1:500); APC anti-human CD133/1, (Miltenyi, cat. no. 130-113-668, 1:50); FITC anti-human SSEA-4, Biolegends, cat. no. 330410, 1:20). The cells were then washed with Miltenyi wash buffer and transferred to 5 ml polystyrene tubes through cell-strainer caps at a final density of around 400,000 cells/ml in DMEM/F12 + DNAse. To exclude dead cells, Propidium iodide (PI, Miltenyi, cat. no. 130-095-177, 1:500) was added to the samples. For each sample, 10,000 cells were analyzed on a FACSAria III sorter (BD Biosciences). Gates were set based on Fluorescence Minus One (FMO) controls and compensation was performed using single-stained cells.

Histological assessment of dopaminergic grafts

Immunohistochemistry

For histological validation of grafted cells, rats were terminally anaesthetized with sodium pentobarbital and sacrificed by transcardial perfusion with a 4% PFA solution. The Brains were post-fixed in 4% PFA, cryopreserved in a 30% sucrose solution and sectioned coronally at 35μm in series of 1:8 or 1:12. Immunohistochemistry was performed on free-floating sections and all washing steps were done three times in KPBS. All incubation steps were performed in room temperature, unless otherwise stated. Sections were incubated in Tris-EDTA pH8 for 30 min at 80°C for antigen retrieval, washed and incubated with blocking solution KPBS + 0.5% Triton + 5 % serum (of secondary antibody host species) for 1 hour. The sections were then incubated in the blocking solution containing

Table 7: *qRT-PCR primers*

Gene	Full gene name	Primer sequence (fwd/rev)
ACTB	Beta-actin	CCTTGCACATGCCGGAG GCACAGAGCCTCGCCTT
BARHL1	BarH like homeobox 1	GTACCAGAACCGCAGGACTAAA AGAAATAAGGCGACGGGAACAT
BARHL2	BarH like homeobox 2	GGAGATTACGAGTAGCCGTGAG AAGCTACGCTCCAGTTGATTGA
CNPY1	Canopy FGF signaling regulator 1	TTGGCCTCTCAAACACCATTCT GAGCGAAACAAAACGCAATCAC
CORIN	Corin, serine peptidase	CATATCTCCATCGCCTCAGTTG GGCAGGAGTCCATGACTGT
DDC	DOPA decarboxylase	GGGGACCACAACATGCTGCTCC AATGCACTGCCTGCGTAGGCTG
EN1	Engrailed 1	CGTGGCTTACTCCCCATTTA TCTCGCTGTCTCTCCCTCTC
ETV5	ETS variant 5	TCATCCTACATGAGAGGGGGTT GACTTTGCCTTCCAGTCTCTCA
FEZF1	FEZ family zinc finger 1	GGTACATTCCACATTCGTGAGC TCACGTGCAATAATCAAAACCA
FGF8	Fibroblast growth factor 8	ACAGCGCTGCAGAATGCCAAGT GAAGTGGACCTCACGCTGGTGC
FOXA2	Forkhead box A2	CCGTTCTCCATCAACAACCT GGGGTAGTGCATCACCTGTT
FOXG1	Forkhead box G1	TGGCCCATGTCGCCCTTCCT GCCGACGTGGTGCCGTTGTA
FOXP2	Forkhead box P2	ATGAGCACTCTAAGCAGCCAAT GTTGCAGATGCAGCAGTTCTAC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TTGAGGTCAATGAAGGGGTC GAAGGTGAAGGTCGGAGTCA
GFAP	Glial fibrillary acidic protein	TCATCGCTCAGGAGGTCCTT CTGTTGCCAGAGATGGAGGTT
HOXA2	Homeobox A2	CGTCGCTCGCTGAGTGCCTG TGTCGAGTGTGAAAGCGTCGAGG
LMX1A (UTR)	LIM homeobox transcription factor a	CGCATCGTTTCTTCTCCTCT CAGACAGACTTGGGGCTCAC
LMX1B	LIM homeobox transcription factor b	CTTAACCAGCCTCAGCGACT TCAGGAGGCGAAGTAGGAAC
MAPT (TAU)	Microtuble-associated protein tau	CTCCAAAATCAGGGGATCGC TTTTTATTTCCTCCGCCAG
NCAM1	Neural cell adhesion molecule 1	GTCAGAGGCCACCGTCAACGTG CTTCCCCCTCCCGGAACTCCTG
NEUROG2	Neurogenin 2	ATCCGAGCAGCACTAACACG GCACAGGCCAAAGTCACAG
NKX2-1	NK2 homeobox 1	AGGGCGGGGCACAGATTGGA GCTGGCAGAGTGTGCCCAGA
NKX2-4	NK2 homeobox 4	AACTGCGATTCAAAACGAACCG GCCTCGTGGCATAATGTTACAC
NR4A2 (NURR1)	Nuclear receptor subfamily 4, group A, member 2	CAGGCGTTTTCGAGGAAAT GAGACGCGGAGAACTCCTAA
OTX2	Orthodenticle homeobox 2	ACAAGTGGCCAATTCACTCC GAGGTGGACAAGGGATCTGA
OTX1	Orthodenticle homeobox 1	TATAAGGACCAAGCCTCATGGC TTCTCCTCTTTCATTCCTGGGC
PAX6	Paired box 6	TGGTATTCTCTCCCCCTCCT TAAGGATGTTGAACGGGCAG
PAX8	Paired box 8	ATAGCTGCCGACTAAGCATTGA ATCCGTGCGAAGGTGCTTT

PDGFRA	Platelet derived growth factor receptor alpha	CCTTGGTGGCACCCCTTAC TCCGGTACCCACTCTTGATCTT
PITX2	Paired-like homeodomain 2	AACTCTATGAACGTCAACCCCC CGACATGCTCATGGACGAGATA
PITX3	Paired-like homeodomain 3	GGAGGTGTACCCCGGCTACTCG GAAGCCAGAGGCCCCACGTTGA
SHH	Sonic hedgehog	CCAATTACAACCCCGACATC AGTTTCACTCCTGGCCACTG
SIM1	Single-minded family bHLH transcription factor 1	AAAGGGGGCCAAATCCCGGC TCCGCCCCACTGGCTGTCAT
SLC6A3 (DAT)	Solute carrier family 6 member 3	CACTGCAACAACTCCTGGAA AAGTACTCGGCAGCAGGTGT
SOX1	SRY-box 1	GGGAAAACGGGCAAAATAAT TTTTGCGTTCACATCGGTTA
SPRY1	Sprouty RTK signaling antagonist 1	GCCCTGGATAAGGAACAGCTAC GCCGAAATGCCTAATGCAAAGA
SYP	Synaptophysin	ACCTCGGGACTCAACACCTCGG GAACCACAGGTTGCCGACCCAG
SYN1	Synapsin 1	CCCGTGGTTGTGAAGATGGGGC TGCCACGACACTTGCGATGTCC
TH	Tyrosine hydroxylase	CGGGCTTCTCGGACCAGGTGTA CTCCTCGGCGGTGTACTCCACA
WNT1	Wnt family member 1	GAGCCACGAGTTTGGATGTT TGCAGGGAGAAAGGAGAGAA
WNT8B	Wnt family member 8B	CTAGTGGGGCAATGACTTTCCT TTCCTAGACCTTCGGGGTATGT

primary antibodies (see table 6) overnight. The following day, the sections were washed and incubated in blocking solution for 30-45 min.

For detection of primary-secondary antibody complexes by fluorescent microscopy, the sections were then incubated with fluorophore-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories) for 2 hours, washed and mounted on gelatin coated slides and cover slipped with PVA-DABCO containing DAPI (1:1,000).

For detection of antibody complexes by peroxidase driven precipitation of di-amino-benzidine (DAB), the sections were incubated with secondary biotinylated antibodies (1:200, Vector Laboratories) for 1 hour, washed and then incubated with avidin-biotin complex (ABC) for 1 hour for amplification. For developing the DAB staining, the sections were incubated in 0.05 % DAB for 1-2 minutes before 0.01% H_2O_2 was added for 1-2 minutes. The sections were then washed and mounted on gelatin coated slides and then dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped with DPX mountant.

Dopaminergic neuron count

All DAB-stained TH^+ graft neurons were counted directly at the brightfield microscope at 20x magnification. One series of sections from each animal was analyzed and TH^+ neurons were quantified in each section containing areas of the graft. An estimation of the total number of TH^+ cells per animal was achieved by multiplying counted TH^+ neurons in one series for the total number of series generated from that animal (8x or 12x) and the number of TH^+ neurons per animal was normalized to 100,000 transplanted cells to enable comparison between experiments.

Estimation of graft volume

To estimate the volumes of the grafts, brain sections with DAB-staining of HUNU (labeling human nuclei) were scanned using a DUOSCAN f40 AGFA and analyzed with Image J software. The graft area was extrapolated in every section of 1:8 or 1:12 series that showed $HUNU^+$ staining, and the volumes of the grafts were calculated using Cavalieri's principle (Cavalieri, 1966). The graft volume was normalized to 100,000 transplanted cells to enable comparison between experiments.

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