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Oral stem/progenitor cells as a possible cell source for dopaminergic neurons and the role of A disintegrin and metalloprotease 17 in neuronal development and homeostasis

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Table of contents

1 Introduction	1
1.1 Cell models	1
1.1.1 Stem cells	1
1.1.1.1 Induced pluripotent stem cells	1
1.1.1.2 Mesenchymal stem cells	2
1.1.1.3 Human oral stem/progenitor cells	2
1.1.1.4 Gingival mesenchymal stem/progenitor cells	3
1.1.2 SH-SY5Y cell line	3
1.2 Neuronal cell differentiation in SH-SY5Y cells induced by retinoic acid	4
1.3 Midbrain dopaminergic neurons derived from induced pluripotent stem cells	5
1.4 Parkinson's disease	5
1.5 A disintegrin and metalloprotease	6
1.5.1 ADAM17	6
1.5.2 The role of ADAM17 in dopaminergic neuronal cell differentiation	8
16 Aims	9
2 Material and Methods	10
2.1 Material	10
2.1.1 Cell lines	10
2.1.2 Media, Buffers, and Solutions	10
2.1.3 Differentiation supplements	15
2.1.4 PCR: Enzymes and buffers	15
2.1.5 Antibodies	16
2.2 Methods	
2.2.1 Cell biological methods	
2.2.1.1 Cell cultivation	18
2.2.1.2 Cell differentiation	18
2.2.1.2.1 SH-SY5Y cells differentiated into neuronal cells	18
2.2.1.2.2 G-MSCs differentiated into dopaminergic (DA) neurons	18
2.2.2 Molecular biology	20
2.2.2.1 Protein Isolation	20
2.2.2.2 Protein quantification	20
2.2.2.3 SDS-PAGE	20
2.2.2.4 Western blot	
2.2.2.5 KNA isolation	
2.2.2.6 Nucleic acid quantitation (DNA/KNA)	
2.2.2.7 DNA Isolation	
2.2.2.0 Reverse Transcription (CDIVA Synthesis)	23
2.2.2.9 Folymenase-Cham-Reaction (FCK)	24 25
2.2.2.10 Kear-Time Quantitative Forymerase-Cham-Reaction (QKT-FOR)	23
2.2.2.11 Against electrophotesis	20
2.2.2.13 CRISPR/Cas	
2.2.2.14 Single cell dilution	
2.2.2.15 T7-Endonuclease-I-Assay	
2.2.2.16 Cell lysate activity assay	
2.2.3 Microscopic methods	
2.2.3.1 Scanning electron microscopy	
2.2.3.2 Immunostaining	32
2.2.4 Statistical analysis	

3 Results	33
3.1 G-MSC-derived dopaminergic neurons	
3.1.1 Cell morphology	
3.1.2 Biochemical characterization	
3.2 The role of ADAM17 in neuronal development and homeostasis	
3.2.1 Expression levels of ADAM17 during induced neuronal cell differentiation	
3.2.2 ADAMI / knockout affects neuronal cell development	
3.2.4 ADAM17 knockout affects neuronal cell development	
4 Discussion	41
4.1 G-MSC-derived neurons	41
4.2 ADAM17 in neuronal development	41
4.3 Approaches for investigating neuronal diseases and finding treatment strategies	
4.4 ADAM proteases involved in human diseases	
4.5 ADAM17 in cell differentiation and homeostasis	
4.6 Outlook	47
5 Abstract	48
6 Zusammenfassung	49
7 References	50
8 Appendix	56
8.1 Abbreviations	
8.2 Further materials	
8.2.1 Primers and gRNA	59
8.2.2 Kits	60
8.2.3 Chemicals	
8.2.4 Consumables	
8.2.6 Software	
8.3 Publication	
8.4 Acknowledgement	

1 Introduction

1.1 Cell models

1.1.1 Stem cells

Stem cells are unspecialized cells with outstanding characteristics: the ability to differentiate into various cells and potential for continuous self-renewal. They exist in embryos and adult organisms alike and can be categorized into embryonic stem cells (ESCs) and non-ESCs according to their origin [Diomede et al. 2020]. Based on their potential to differentiate into different cell types, stem cells can be classified as totipotent, pluripotent, multipotent and unipotent [Singh et al. 2016]. Totipotent stem cells have the highest differentiation potential and are able to differentiate into any cells of the entire organism, be they embryo or extraembryonic structures. Pluripotent stem cells (PSCs) on the other hand can differentiate into cells of all three germ layers except extra-embryonic structures, while multipotent stem cells (MSCs) have a more limited range of differentiation varieties than PSCs and instead specialize in discrete cells of specific cell lineages [Zakrzewski et al. 2019]. MSCs offer extraordinary potential to understand and develop innovative therapies in regenerative medicine [Diomede et al. 2020], whereas unipotent stem cells only have the capability to differentiate into one cell type. They, however, differ from non-stem cells in their ability to regenerate [Larijani et al. 2012]. Based on regenerative use, stem cells can be categorized as ESCs, tissue specific progenitor stem cells, mesenchymal stem cells, umbilical cord stem cells, bone marrow stem cells and induced pluripotent stem (iPS) cells [Mahla 2016].

1.1.1.1 Induced pluripotent stem cells

In 2012, the discovery that mature cells can be reprogrammed to become pluripotent revolutionized today's research-possibilities tremendously. Induced pluripotent stem (iPS) cells are pluripotent stem cells generated artificially via genetic manipulation of somatic cells [Takahashi and Yamanaka 2006]. More importantly it is possible to generate iPS cells from mature, non-pluripotent cells and convert them into stem cells with comparable pluripotency to that of ESCs [Zunke and Mazzulli 2019]. Therefore, patients can provide their own cell source for iPS cells. Due to the resulting outstanding biocompatibility the iPS cell technology is of great interest in understanding neurodevelopmental defects [Shafique 2022] and drug screening [Ebert et al. 2012].

Nevertheless, stem cells with less differentiation capabilities such as mesenchymal stem cells are also of great importance for research purposes.

1.1.1.2 Mesenchymal stem cells

Human mesenchymal stem cells (MSCs) are adult multipotent stem cells [Diomede et al. 2018]. They were initially identified as a subpopulation of bone marrow cells with osteogenic potential [Keating 2012], but have come to be characterized by their distinguished ability to differentiate into osteogenic, chondrogenic adipogenic, myogenic and neurogenic-like cell lineages [Phinney et al. 2017]. Moreover, they are acknowledged as primary growth factor secretors and immunomodulatory promoters. MSCs feature remarkable potential in animal and human models and enable great possibilities for developing novel therapies in regenerative medicine [Diomede et al. 2020]. Further, the human oral cavity also provides a variety of stem/progenitor cells.

1.1.1.3 Human oral stem/progenitor cells

Teeth and their surrounding tissue have the great advantage of being the most natural and noninvasive source of stem cells. An example of the common use of stem cells in dentistry is tissue engineering for treating periodontal diseases [Zakrzewski et al. 2019]. There are six orally derived human MSCs successfully isolated and characterized from a variety of oral tissues, including dental pulp, apical papilla, exfoliated deciduous teeth, dental follicle, periodontal ligament, and gingiva [Silvestro et al. 2020] shown in Figure 1.



Figure 1: **Overview localization of human oral stem cells**. Dental MSCs can be isolated either from the tooth itself or its associated tissues. Therefore, they can be derived from the inner tooth pulp of adult molars (light blue, DPSCs), from the pulp of deciduous exfoliated teeth (purple, SHEDs) and from the apical papilla (blue, SCAPs). The supporting tissues provide dental MSCs from the dental follicle (green, DFPCs), the periodontal ligament (turquoise, PDLSCs) and the gingiva (pink, GMSCs). Illustration adapted from: [Diomede et al. 2020].

The oral cavity has been identified as an easily accessible reservoir of human MSCs [Silvestro et al. 2020]. Oral stem cells (OSCs) are different from other MSCs in many aspects. They proliferate rapidly in culture with an average population doubling time of ≤ 20 h [Huang et al. 2009]. Dental tissues are specialized tissues that do not undergo continuous remodeling as shown in bone tissue. Therefore, dental-tissue-derived stem cells may be more committed or restricted in their differentiation potency in comparison to bone marrow stem cells [Huang et al. 2009].

OSCs originate from migrating cranial neural crest cells [Chai et al. 2000]. They are thought to have a greater neurogenic potential than other MSCs [Karaöz et al. 2011]. Further, dental pulp stem cells and gingival stem/progenitor cells (G-MSCs) appear to be more neurogenic compared to other OSCs and non-OSCs including bone marrow stem cells [Li et al. 2019]. The characteristics and benefits of G-MSCs are outlined in the following section.

1.1.1.4 Gingival mesenchymal stem/progenitor cells

The gingiva is a unique oral tissue attached to the alveolar bone and part of the periodontium. It contains multiple layers that serve as a biological mucosal barrier and is important for oral mucosal immunity [Zhang et al. 2009]. Its remarkable wound healing properties are described as fetal-like, healing occurs rapidly without inflammation. Further, it shows rapid re-epithelialization characteristics and little, if any, evidence of scarring [Irwin et al. 1994]. It is the most easily accessible tissue compared to all other sources for stem/progenitor cells from the oral cavity and harbors both stem-cell-like and immunomodulatory properties [Zhang et al. 2009].

Previous studies have discovered that excised gingival tissue contains gingiva-derived mesenchymal stem cells (G-MSCs) [Li et al. 2019]. For this study, G-MSCs were isolated from the free gingival margin. This requires a sample of human de-epithelized gingiva [Venkatesh et al. 2017]. Alternatively, a well-established cell model for neuronal research proposition is described in the next section. In this thesis, it was used for basic research of molecular mechanisms.

1.1.2 SH-SY5Y cell line

It is necessary to have appropriate cell models for in vitro studies in order to understand the pathophysiological mechanisms of specific disorders, as Parkinson's disease (PD) for example. The neuroblastoma SH-SY5Y cell line is a suitable in vitro model widely used in PD research [Xicoy et al. 2017]. SH-SY5Y neuroblast-like cells are a subclone of the parental neuroblastoma cell line SK-N-SH generated from a bone marrow biopsy. These cells contain

two morphologically distinct phenotypes: neuroblast-like and epithelial-like cells [Kovalevich and Langford 2013]. SH-SY5Y cells are adherent, genetically stable [Shipley et al. 2016] and possess the capability to proliferate in culture for a long time [Xie et al. 2010]. They can be differentiated into mature human neurons through several methods, including the use of all-trans retinoic acid. Alternative methods are treatments with phorbol esters or specific neurotrophins such as brain-derived neurotrophic factor (BDNF) [Shipley et al. 2016].

1.2 Neuronal cell differentiation in SH-SY5Y cells induced by retinoic acid

All-trans retinoic acid (RA) is a metabolite of vitamin A, which has the ability to induce postmitotic neural phenotypes in many cell lines [Janesick et al. 2015]. The effect of RA on SH-SY5Y cells is well characterized. RA induces neuronal differentiation through regulation of the transcription of neurotrophin receptor genes, the Wnt signaling pathway, and pathways involving type II protein kinase A [Xie et al. 2010]. RA binds to the retinoic acid receptors (RARs), which then heterodimerizes with the retinoic X receptors (RXRs). Both receptor classes, RARs and RXRs, are non-steroid receptors that function as ligand-regulated transcription factors. The heterodimer RAR/RXR binds to the RA response element (RARE), which modifies the transcriptional activity of specific genes. It downregulates the differentiation-inhibiting genes ID1, ID2 and ID3, while mRNA levels of the differentiationpromoting factors NEUROD6 and NEUROD1 increase [López-Carballo et al. 2002]. The treatment with RA results in neurite outgrowth and partially inhibited cell growth. Neuronal markers, such as neuron-specific enolase (NSE), neuronal nuclei protein (NeuN), β3-Tubulin, Synapsin, Synaptophysin and dopaminergic markers such as tyrosine hydroxylase (TH) and dopamine transporter (DAT) increase [Shipley et al. 2016]. Treatment with RA augments survival and attenuates basal apoptotic pathways in SH-SY5Y cells, thereby increasing cell viability [Waetzig et al. 2019].

1.3 Midbrain dopaminergic neurons derived from induced pluripotent stem cells

In 2011, a protocol was established to successfully differentiate ESC into midbrain dopaminergic (DA) neurons [Kriks et al. 2011]. The protocol is a floorplate-based method inducing co-expression of the floorplate marker FOXA2 and the roof-plate marker LMX1A. Therefore, the bone morphogenetic protein (BMP) pathway and transforming growth factor (TGF) β -signaling, involved in embryogenesis, proliferation, differentiation and SMAD signaling [Miyazawa and Miyazono 2017], first becomes inhibited, followed by the activation of sonic-hedgehog (Shh) signaling. Shh is expressed by the floorplate and regulates neural tube formation, developmental processes, and differentiation of midbrain dopaminergic neuron [Patel et al. 2017]. The inhibition of BMP during early development mediates the differentiation of the neuroectoderm from the ectoderm [Bond et al. 2012]. Wnt-signaling, which is involved in neuronal patterning during embryonic development and cell differentiation and proliferation [Routledge and Scholpp 2019], is then induced by CHIR99021 inhibiting glycogen synthase kinase (GSK) -3β , which in turn is involved in energy metabolism, neuronal cell development, and body pattern formation. In order to ensure the survival of neurons outside the brain, the brain-derived and glia cell-derived neurotrophic factors BDNF and GDNF are added. These help during the process of neurons adapting to external influences [Popova et al. 2017]. BDNF also triggers the activation of ERK signaling and downstream MAPK-interacting kinases [Lee et al. 2016]. At this point, TGFβ-signaling is also induced. Lastly, the gamma secretase inhibitor DAPT is added to block Notch signaling and promote further neuronal differentiation and proliferation [Cheng et al. 2021].

1.4 Parkinson's disease

Parkinson's disease (PD) is a long-term neurodegenerative disorder that mainly effects the motor system. This disease affects about one percent of the world's population over the age of 60 years. PD is therefore the second most common neuronal disease worldwide after Alzheimer's disease. It is caused by the cell death of dopaminergic neurons in the substantia nigra pars compacta, a region of the midbrain, and primarily leads to movement disorder [Ding et al. 2015]. Some of the motor symptoms provoked include resting tremor, bradykinesia, postural instability, and rigidity of skeletal muscle and freezing of gait [Salari and Bagheri 2019]. The exact mechanisms causing dopaminergic cell death are still unknown [Opara et al. 2017].

Besides the characteristic motor symptoms, PD patients can exhibit non-motor symptoms such as apathy, pain, bowel incontinence, and sleep disorders. All in all, the symptoms, and especially the non-motor symptoms, can be embarrassing for patients and are sometimes not clearly linked to PD [Chaudhuri and Schapira 2009]. Further, cognitive impairment with prevalence of dementia is also a frequent comorbidity in PD [Wu et al. 2018]. PD is mostly recorded in patients after the age of 50 years [Opara et al. 2017]. Several causative monogenetic mutations have been discovered. These however only explain less than 10% of all PD cases [Lau and Breteler 2006]. According to currently available information, alpha-synuclein pathology (presence of Lewy bodies), mitochondrial dysfunction, and oxidative stress are related to PD pathogenesis [Surmeier 2018]. Therefore, it is a matter of urgency to further understand the pathogenesis of non-genetically caused PD and to develop effective therapeutic strategies.

1.5 A disintegrin and metalloprotease

A disintegrin and metalloproteinases (ADAMs) are a family of membrane-anchored multidomain glycoproteins. ADAMs are involved in cell adhesion, migration, cellular signaling and proteolysis [Jones et al. 2016]. As functional metalloproteinases they are implicated in ectodomain shedding of several transmembrane proteins such as growth factors, cytokines and chemokines, whose receptors and ligands are involved in apoptosis and cell migration [Khatoon et al. 2018]. ADAMs thereby play a crucial role in fertilization, angiogenesis, neurogenesis, heart development, but are also key players in diseases like cancer [Horiuchi et al. 2005]. One of the biologically most studied members of the ADAM family are ADAM10 and

ADAM17. Their function in the immune system and central nervous system has been a matter of interest in several previous studies and seems to be very similar to one another [Palau et al. 2019].

1.5.1 ADAM17

ADAM17 is involved in the regulation of many body functions and developmental processes. Also known as tumor necrosis factor- α (TNF- α) -converting enzyme (TACE), ADAM17 is of great importance for signaling pathways associated with tumor progression [Mochizuki and Okada 2007]. ADAM17 knockout mice are not viable and die prenatally (Peschon et al. 1998). This demonstrates the important role of ADAM17 in vivo. However, it is known that ADAM17 has more than 80 substrates, including cytokines, growth factors and receptors to many cell adhesion molecules [Scheller et al. 2011].

In regeneration, ADAM17 can regulate epidermal-growth-factor-receptor (EGFR) signaling by cleaving EGFR-ligands such as transforming growth factor (TGF)α, heparin-bound (Hb)-EGF and Epiregulin (EREG) from their membrane-anchored precursor (Blobel 2005). The EGF

family of receptor tyrosine kinases (ErbBs) has a crucial role in regulating cell proliferation, survival, division, differentiation and migration [Sabbah et al. 2020]. Due to the various impact of ADAM17, it does not come as a surprise that the structure is as complex as its function. ADAM17 is a transmembrane protein and consists of five extracellular domains and a small cytoplasmic tail as shown in Figure 2 [Zunke and Rose-John 2017].



Figure 2: Structure of ADAM17. The transmembrane protein consists of six domains, five extracellular domains and a cytoplasmic tail. ADAM17 occurs in two forms. The pro-domain (red) blocks the catalytic center (blue), which inactivates the metalloprotease. Furin activates ADAM17 by cleaving the pro-domain. Illustration adapted from: [Zunke and Rose-John 2017].

As type I transmembrane protein, ADAM17 contains an N-terminal signal sequence that directs newly synthesized proteins into the endoplasmic reticulum (ER) followed by maturation in the Golgi apparatus [Jones et al. 2016].

The metalloproteinase-domain or catalytic domain contains the protein's catalytic center that mediates shedding. The N-terminal pro-domain is a chaperone and inhibits the catalytic activity of the protease. It is cleaved by the pro-protein convertase Furin in the Golgi apparatus [Wong et al. 2015]. The other domains are responsible for interaction with integrins and substrate recognition [Zunke and Rose-John 2017]. Therefore, ADAM17 occurs in two forms: as a full-length inactive protein, and as a mature form without the pro-domain, but catalytically active [Palau et al. 2019].

1.5.2 The role of ADAM17 in dopaminergic neuronal cell differentiation

Due to the cleavage and secretion release of epidermal growth factor receptor (EGFR) ligands, ADAM17 can activate the EGFR and is therefore involved in various downstream signaling pathways [Wei et al. 2015]. Previous studies implicate a regulation of the dopamine D2 receptor (D2R) by ADAM10 or 17, inducing the shedding of EGF shown in Figure 3. Further, it has been discovered that the absence of D2R leads to alteration in dopaminergic neuronal development (Kim et al. 2006). EGFR transactivation leads to extracellular signal-regulated kinase (ERK) activation, which plays an important role in the development of dopaminergic mesencephalic neurons [Yoon and Baik 2013]. Regarding the regulation of the development of dopaminergic neurons by the Wnt5a protein, it has further been shown that Wnt5a interacts with D2R, and that this regulation is dependent on ERK signaling [Yoon et al. 2011]. Consequently, ERK activation is not only regulated by D2R, but also by EGFR signaling, which in turn is related to the shedding activity of ADAM10 and/or ADAM17.



Figure 3: Dopamine D2 receptor mediated ERK signaling. ADAM17 is regulated by the dopamine D2 receptor, shedding the EGFR-ligand, and activating ERK signaling pathway leading to dopaminergic neuronal cell differentiation. Illustration adapted from: [Yoon and Baik 2013].

1.6 Aims

The aim of this thesis is firstly to explore the potential of a new human cell source for developing dopaminergic neurons in vitro. The human oral cavity provides a variety of stem/progenitor cells with easy access. Although these cells have already been a matter of high interest for dental research, the neuronal research area has only been giving little attention to. Currently, stem cells provide a variety of research possibilities with great potential in medical therapeutics. So far, studies have indicated that the iPS-cell technology offers enormous potential for autologous cell transplantation in numerous diseases. Because the iPS cell system is expensive and elaborative, this study investigates the potential of gingival-mesenchymal-stem/progenitor cells (G-MSCs) as a possible cellular source for dopaminergic neurons in vitro. The advantage of these cells is an easy access via biopsy and fetal-like scarless wound healing. For the differentiation of G-MSCs into dopaminergic neurons, the well-established protocol for iPS cell-derived dopaminergic neurons serves as a template.

The second aim was to investigate the relevance of the metalloprotease ADAM17 during neuronal cell differentiation and homeostasis. To better understand the mechanisms of neuronal diseases and for the purpose of enabling new therapeutic strategies it is necessary to further explore the molecular mechanisms during neuronal cell differentiation. Therefore, this study analysis the role of ADAM17 in dopamine D2 receptor (D2R) mediated ERK signaling via an ADAM17 knockout in human neuroblastoma SH-SY5Y cells, performed with CRISPR/Cas9 technology.

2 Material and Methods

Each method used for this thesis is described in this chapter in detail. Further equipment required is listed in the appendix (6.2 Further materials).

2.1 Material

2.1.1 Cell lines

Table 1: Name and source of the cell lines used for this thesis.

Name	Source
Human neuroblastoma SH-SY5Y	ECACC #94030304
Human Gingival Mesenchymal Stem	kindly provided by the working group of Prof. Dr.
Cells (G-MSCs)	Christof Dörfer and PD Dr. Karim M. Fawzy El-
	Sayed from UKSH Kiel

2.1.2 Media, Buffers, and Solutions

Table 2: List of all manufactured media, buffers, and solutions.

Name	Company	Reference number
Advanced DMEM F12 (<i>Dulbecco's</i> <i>Modified Eagle's medium/Ham's F-</i> <i>12</i>)	Sigma-Aldrich Chemistry GmbH	12634010
DMEM F12 no glutamine	Sigma-Aldrich Chemistry GmbH	21331046
MEM (Minimum Essential Medium Eagle)	Sigma-Aldrich Chemistry GmbH	M4526
Neurobasal Media	invitrogen	21103049
Neurocult SM1 (B27 supplement) 50x	StemCellTech	05711
L-Glutamine solution	Sigma-Aldrich Chemistry GmbH	G7513
Amphotericin B solution	Sigma-Aldrich Chemistry GmbH	A2942
Penicillin/Streptomycin (100x)	PAA Laboratories GmbH	P11-010

Trypsin- EDTA Solution	Sigma-Aldrich Chemistry GmbH	T4299
Laminin (from mouse EHS sarcoma)	Roche LifeScience	11243217001
Gelatin from cold water fish skin	Sigma-Aldrich Chemistry GmbH	G7765
PageRuler [™] Prestained Protein	Thormo Fisher Scientifie	26616
Ladder	Thermorisher Scientific	20010
SureCast [™] Stacking Buffer	ThermoFisher Scientific	HC2112
SureCast [™] Resolving Buffer	ThermoFisher Scientific	HC2212
Novex WedgeWell 8-16% Tris-	ThormoFisher Scientifie	XP08162BOX
Glycine Gel	Thermorisher Scientific	

Table 3: List of media used for cell culture.

Name	Composition	
Advanced DMEM F12	89% Advanced DMEM F12	
	10 % FBS	
	1 % Penicillin/Streptomycin	
DMEM/F12 no glutamine	89% DMEM F12 no glutamine	
	10% FBS	
	1% Penicillin/Streptomycin	
MEM	70% MEM	
	15% FBS	
	1% Penicillin/Streptomycin	
	1% L-Glutamine	
	1% Amphotericin B	
SM1 (filtered)	Neurobasal Media	
	2% SM1 50x	
	Penicillin/Streptomycin	
	Glutamine	

Name	Composition
SH-SY5Y and G-MSC wild type	1% Triton X100
	50 mM Tris-HCl
	150 mM NaCl
	2 mM EDTA
	1% NP ₄ O
	2.5 pills per 50 mL cOmplete Protease Inhibitor
	Cocktail
G-MSC after differentiation	1% Triton X100
(Base-Buffer)	10% Glycerol
	150 mM NaCl
	25 mM HEPES pH 7.4
	1 mM EDTA
	1.5 mM MgCl ₂
	50 mM NaF
	$2 \text{ mM Na}_3 V_4$
	1 mM PMSF
	2.5 pills per 50mL cOmplete Protease Inhibitor
	Cocktail
For Activity Assay	50 mM Tris-HCl pH 7.4
	150 mM NaCl
	1% Triton X-100
	4% Glycerol
	10 mM 1.10-Phenanthroline
	1 pill per 50mL cOmplete Protease Inhibitor
	Cocktail EDTA free

Table 4: List of buffers regarding protein isolation.

Name	Composition
Stacking gel	1.7mL ddH ₂ O
	500µL Polyacrylamidsolution (30%)
	760µL Tris-HCl (0.5M) pH6.8
	10µL SDS (10%)
	30µL APS (10%)
	3μL TEMED
Resolving gel (10%)	4mL ddH ₂ O
	3.3mL Polyacrylamidsolution (30%)
	2.5mL Tris-HCl (1.5M) pH 8.8
	100µL SDS (10%)
	100µL APS (10%)
	4µL TEMED
5 x loading dye (Laemmli)	50% Glycerol
	0.3 M Tris-HCl pH 6.8
	10% SDS
	5% Bromophenol blue
	20% β-Mercaptoethanol
1 x SDS-running Buffer	192 mM Glycin
	25 mM Tris-HCl pH 8.3
	0.1% SDS
Blotting Buffer	20% Methanol
	192 mM Glycin
	25 mM Tris-HCl pH 8.3
1 x TBS	10 mM Tris-HCl pH 8.0
	150 mM NaCl
TBS-T	1 x TBS
	0.05% Tween 20
Fish-Protein Blocking Buffer	2% Gelatin from cold water fish skin
	1 x TBS-T

Table 5: List of buffers and solutions regarding SDS-PAGE and Western blots

Name	Composition
TBE (0.5x)	44.5 mM Boric acid
	10 mM EDTA pH 8.0
	44.5 mM Tris-HCl
Agarose gel	0.5x TBE
	1% Agarose
	0.002% Ethidium bromide
6 x loading dye	30% Glycerol
	0.25% Bromophenol blue
	0.25% Xylene cyanol FF

Table 6: List of buffers and solutions regarding agarose-gel electrophoresis.

Table 7: List of buffers regarding immunofluorescence staining.

Name	Composition
Fixing Buffer	1 x PBS
	4% Paraformaldehyde (10%)
Permeabilization Buffer	1 x PBS
	0.3% Triton X-100
IF Blocking Buffer	2% BSA
	5% FBS
	0.3% Triton X-100
	1 x PBS
Embedding media	1 mL Moviol
	100 μL Dabco
	1 μL DAPI

Table 8: Buffer used for scanning electron microscopy.

Name	Composition
Crosslinking solution	3% Glutaraldehyde
	97% Phosphate buffer (0.1%) pH7.4

Article	Company	Reference number
All Trans Deting is Asia	Sigma-Aldrich Chemistry	D2625 500MC
All-Irans Relinoic Acid	GmbH	K2023-300MG
Ascorbic acid (AA)	Sigma-Aldrich Chemistry	A 5060 25C
	GmbH	A3900-23G
CHIR 99021	StemCellTech	72054
DAPT, 5mg	StemCellTech	72082
dibutyryl cAMP	Enzo Life Sciences	BML-CN125-0100
Dimethyl sulfoxide (DMSO)	Carl-Roth GmbH Co. KG	A994.1
GI 254023X	Tocris Bioscience	3995
GW 280264X	Iris Biotech	-
LDN-193189	StemCellTech	72147
Purmorphamine, 5mg	StemCellTech	72204
RecHu BDNF, ACF 10ug	StemCellTech	78133
RecHu GDNF	R&D Systems	212-GD-010
ReHuSONIC Hedgehog (Shh)	R&D Systems	1845-SH-025
RecHuTGF-beta 3	R&D Systems	243-B3-002
SB 431542, 1 mg	R&D Systems	1614/1

Table 9: List of supplements used for differentiation purpose.

2.1.4 PCR: Enzymes and buffers

Table 10: List of enzymes and buffers used for PCR.

Name	Company	Reference number
Q5 High-Fidelity DNA	New England BioLabs	M04010
Polymerase	GmbH	M0491S
	New England BioLabs	D0020 A
Q5 High GC Enhancer	GmbH	B9028A
Q5 Reaction Buffer 5x	New England BioLabs	B9027S
	GmbH	
NE Buffer2 10x	New England BioLabs	B7002S
	GmbH	
Dream Taq DNA Polymerase	ThermoFisher Scientific	EP0702

Dream Taq Buffer 10x	Thermo Fisher Scientific	B65	
dNTP Solution Mix	New England BioLabs	N0447S	
	GmbH		
T7 Endonuclease I	New England BioLabs	M0302L	
	GmbH	11105021	
RevertAid Reverse Transcriptase	Thermo Fisher Scientific	EP0441	
5x Reaction Buffer for RT	Thermo Fisher Scientific	Supplied with Revert	
		Aid	
RiboLock RNase Inhibitor	Thermo Fisher Scientific	EO0381	
Oligo(dt) ₁₈ Primer	Thermo Fisher Scientific	SO131	
LightCycler 480 Probes Master	Roche Life Science	04707494001	

2.1.5 Antibodies

Table 11: List of primary antibodies used for Western blot.

Name	Target (-sequenz)	Species	Company	Reference number	LOT
Anti	Synansin	rabbit	ThermoFisher	A-6442	2186047
Synapsin	Synapsin	nuoon	Scientific	11 0112	2100017
Anti β3-	63-Tubulin	mouse	Biol evend	657401	B264428
Tubulin		mouse	DioLogona	007101	D 201120
Anti NSE	NSE	mouse	BioLegend	804501	B294828
	ADAM17			F777 1 . 1	
A300D	(disintegrin-like	mouse	In-house	[1rad et al. 2011]	
	domain)			. 1	
Anti HNF-	FOXA2	mouse	Santa Cruz	sc-101060	J3017
3b RY-7				50 101000	
Anti				DSHB-	
GAPDH	GAPDH	mouse	DSHB	hGAPDH-	CCSE0418021
				2G7-s	
Anti B-			Sigma-Aldrich		
Actin	β-Actin	mouse	Chemistry	A5441	127M4866V
			GmbH		

Name	Target	Species	Company	Reference number	LOT
Anti β3-Tubulin TUJ1 (PRB-435P)	β3-Tubulin	rabbit	BioLegend	802001	B304527
Anti β3-Tubulin TUJ1 (MMS-435P)	β3-Tubulin	mouse	BioLegend	801202	
Anti-Tyrosine Hydroxylase (TH)	TH	rabbit	Merck Milipore	AB5986	2783118
Anti-FOXA2 HNF- 3b RY-7	FOXA2	mouse	SantaCruz	sc-101060	J3017
Anti-NeuN, clone A60	NeuN	mouse	Merck Milipore	MAB377	

Table 12: List of primary antibodies used for immunofluorescence staining.

Table 13: List of secondary antibodies used for Western blot.

Name	Target	Species	Company	Reference number
IRDye® 680RD Goat anti-				
mouse IgG Secondary	mouse-IgG	goat	LI-COR Biosciences	926-68070
Antibody				
IRDye® 800CW Donkey				
anti-rabbit IgG Secondary	rabbit-IgG	donkey	LI-COR Biosciences	926-32213
Antibody				
Alexa Fluor Plus 648 anti-	and InC	1 1	ThermoFisher	# 1 2 2 8 4 0
goat (Cy5)	goat-1gG	donkey	Scientific, Waltham	#A32849

Table 14: List of secondary antibodies used for immunofluorescence staining.

Name	Target	Species	Company	Reference number	
Alexa Fluor 488 anti-		1 1	ThermoFisher	A 11000	
rabbit	rabbit-lgG	donkey	Scientific, Waltham	A-11008	
Alexa Fluor 594 anti-	mana IaC	~~~*	ThermoFisher	A 21202	
mouse	mouse-1gG	goat	Scientific, Waltham	A-21203	

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Cell cultivation

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified eagle's medium with nutrient mixture F12 (DMEM/F12) no glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100x). Human Gingival Mesenchymal Stem Cells (G-MSCs) were grown in Minimum essential medium eagle (MEM) supplemented with 15% FBS, 1% penicillin/streptomycin (100x), 1% Glutamine and 1% Amphotericin. Cells were maintained under standard conditions (37°C, 95% humidity, 5% CO₂), and cultured until subconfluent (70%) to harvest for cell tests and experiments.

Cells stock were stored at -145°C dissolved in 90% FBS and 10% Dimethyl sulfoxide (DMSO).

2.2.1.2 Cell differentiation

2.2.1.2.1 SH-SY5Y cells differentiated into neuronal cells

For cell differentiation, cells were seeded at a density of 1×10^4 cells cm⁻². After 24 h, neuronal differentiation was induced by treatment with retinoic acid (30 μ M) dissolved in DMSO and hydroxamate inhibitors (3 μ M) in Advanced DMEM/F12 for 10 days. The temporal sequence is shown in Figure 4.



Figure 4: Timeline of neuronal differentiation of SH-SY5Y cells with retinoic acid. Cells were treated for 10 days and the media consisting of $30\mu M$ retinoic acid was changed every 2 days.

2.2.1.2.2 G-MSCs differentiated into dopaminergic (DA) neurons

For cell differentiation, cells were seeded at a density of 6.4×10^3 cells cm⁻² (3×10^4 cells per 6well). After 48 h neuronal differentiation was induced by treatment with supplemented medium (see Table 15) for 40 days. For 6-wells and 24-wells a medium volume of 1 mL and 500µL per well were used.

Day	Factors supplemented	Medium
0	LDN (100 nM), SB (10 µM)	MEM
1	LDN, SB, Shh (100 mg/mL), Purmorphamine (2 µM), FGF8a (200 ng/mL)	MEM
2	LDN, SB, Shh, Purmorphamine, FGF8a	MEM
3	LDN, SB, Shh, Purmorphamine, FGF8a, CHIR (3 µM)	MEM
4	LDN, SB, Shh, Purmorphamine, FGF8a, CHIR	MEM
5	LDN, Shh, Purmorphamine, FGF8a, CHIR	MEM 3 : 1 SM1
6	LDN, Shh, Purmorphamine, FGF8a, CHIR	MEM 3 : 1 SM1
7	LDN, CHIR	MEM 1 : 1 SM1
8	LDN, CHIR	MEM 1 : 1 SM1
9	LDN, CHIR	MEM 1 : 3 SM1
10	LDN, CHIR	MEM 1 : 3 SM1
11	CHIR, BAGTC*, DAPT (10 µM)	SM1
12	CHIR, BAGTC, DAPT	SM1
13	BAGTC, DAPT	SM1
14	BAGTC, DAPT	SM1
15	Keep same media composition and feed every 3 days	SM1

Table 15: Supplemented factors for differentiating G-MSCs into DA neurons.

* Table 16: Components of BAGTC

Component	concentration
BDNF	20 pg/µL
Ascorbic Acid (AA)	200 µM
GDNF	5 pg/µL
TGF-beta 3	1 pg/µL
cAMP	100 µM

2.2.2 Molecular biology

2.2.2.1 Protein Isolation

Cells from a 10 cm culture dish were washed with ice-cold PBS before being mechanically detached. After pelleting by centrifugation (1,000 x g for 5 min at 4°C), SH-SY5Y cells were lysed by resuspension in lysis buffer thoroughly by pipetting up and down, followed by rolling incubation at 4°C for 1 h. G-MSCs were lysed in the same buffer following the freeze-thaw method (3 repetitions of freezing the sample for 5 min at -80°C followed by thawing for 1 min at 37°C). G-MSCs differentiated into DA neurons were lysed in base-buffer and homogenized at a rotation speed of 6,800 min⁻¹ for 30 sec (Precellys). The cells were then incubated on ice on a shaking plate for 1 h. Cells used for activity determination were lysed in an EDTA-free lysis buffer, followed by incubation at 4°C on a tube roller for 1 h. The composition of all lysis buffer is listed in section 2.1.2, Table 4. After lysis, the lysates were centrifuged at 14,000 x g for 15 min at 4°C.

2.2.2.2 Protein quantification

The amount of total protein was determined by BCA-Assay (BCA-kit, Pierce TM, Thermo Fisher Scientific) according to the manufacturer's manual. The method is based on the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium. The resulting Cu^{+} forms a purple color complex with bicinchoninic acid (BCA), which exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. This was measured in a plate reader (Infinite 200 PRO, TECAN) together with a standardized protein curve.

2.2.2.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their molecular weight despite their electric charge. The gel consists of two phases, the stacking and running gel, with the latter being responsible for the separation. Small proteins run faster through the running gel and proteins with higher molecular weight need more time. Therefore, low- percentage of polyacrylamides are recommended for analysis of larger proteins and high-percentage gels for small proteins. Sodium dodecyl sulfate (SDS) is an anionic surfactant and has amphiphilic properties that allow it to act as a detergent. It is supplied to disrupt non-covalent bonds in the proteins, denaturing them and thereby providing all proteins with a similar net negative charge.

The samples were supplemented with 5x Laemmli sample buffer (see section 2.1.2, Table 5) and heat inactivated at 95°C for 5 min. Equal amounts (20-40 µg total protein) were loaded on a 10% polyacrylamide gel and separated by SDS-PAGE.

2.2.2.4 Western blot

After separation by SDS-PAGE, the proteins were transferred to a PVDF membrane (Merck Millipore) to enable the detection of proteins with antibodies. The membrane must be activated in methanol before use. The blotting-construct ("sandwich") consists of a cathode (-), an anode (+), two blotting pads, filter-paper (Whatman), the SDS-Gel, and a membrane. The sandwich is assembled as shown in Figure 5. Blotting was performed at 4°C with 120 V for 90 min. After transferring, the membrane was blocked for 1 h at room temperature in 2% fish-blottefield (11BS). Primary antibodies (see section 2.1.5, Antibodies) were incubated overnight (4°C). Before adding the secondary antibody, the membrane was washed three times for 20 min in PBS. This was repeated directly before detection. Secondary antibodies were incubated for 1 h at room temperature and detected by phosphor imaging (Amersham Typhoon IP).



Figure 5: Set-up of blotting-construct. The blotting-sandwich consists of an anode, followed by a blotting pad, three layers Whatman paper, the PVDF membrane, the SDS-Gel, another three layers of whatman paper, another blotting pad, and is completed by the cathode.

2.2.2.5 RNA Isolation

The cells were washed with ice-cold PBS before being mechanically detached from the culture dish and pelleted by centrifugation (1,000 rpm for 5 min at RT). RNA was isolated (by use of RNeasy Mini Kit from Thermo Scientific) as follows:

- 1. Add appropriate volume of Buffer RLT to cell pellet
 - $< 5 \text{ x} 10^6$ cells (< 6cm dish) $\rightarrow 350 \mu$ L
 - $< 1 \text{ x } 10^7 \text{ cells (6-10 cm dish)} \rightarrow 600 \text{ }\mu\text{L}$
- 2. Add 1:100 volume β -Mercap (3.5 μ L / 6 μ L)

From this point the following steps were done one sample after the other

- 3. Add 1:1 volume 70% ethanol
- 4. Mix well by pipetting
- 5. Transfer up to 700 μ L to column
- 6. Centrifuge for 15 sec at > 8,000 x g
- 7. Discard the flow-through
- 8. Add 700 µL Buffer RW1

Steps 3-8 were then repeated with the remaining samples

- 9. Centrifuge for 15 sec at > 8,000 x g
- 10. Discard the flow-through
- 11. Add 500 μL Buffer RPE
- 12. Centrifuge 15sec at > 8,000 x g and discard the flow-through
- 13. Add 500 μL Buffer RPE
- 14. Centrifuge for 2 min at > 8,000 x g
- 15. replace collection tube with new one
- 16. centrifuge for 1 min at maximum speed to dry the membrane
- 17. place the column in 1.5 mL collection tube (supplied)
- 18. elute: add 30 µL RNase-free water (place it directly in the center of the membrane)
- 19. 5 min incubation at RT
- 20. centrifuge for 1 min at > 8,000 x g

2.2.2.6 Nucleic acid quantitation (DNA/RNA)

The DNA and RNA concentration was measured in ng/ μ L by utilizing a NanoDrop® spectrophotometer (Peqlab Biotechnology GmbH). The spectrophotometer was first washed twice, calibrated, and blanked with 1 μ L nuclease free water. Then 2 μ L of the sample was applied to measure the DNA concentration. Wavelengths between 230 and 280 nm were used for the spectrophotometric reading of optical density (OD) because nucleic acids exhibit a specific absorption spectrum in that range. An OD of 1 at 260 nm reveals a concentration of 50 μ g/mL. Moreover, the quotient of the absorption at 260 nm and 280 nm gives information about the purity of the DNA/RNA sample. Contaminations with proteins or solvents would show here and a value between 1.8 and 2.0 is generally accepted as pure.

2.2.2.7 DNA Isolation

The cells were washed with ice-cold PBS before being mechanically detached from the 10 cm culture dish and pelleted by centrifugation (1,000 rpm for 5 min by 4°C). DNA was isolated according to the instructions of the kit manual (DNeasy Blood&Tissue Kit), although DNA was

eluted by adding 200 μ L nuclease free water instead of Buffer AE as it was described in the manual.

2.2.2.8 Reverse Transcription (cDNA Synthesis)

The reverse transcription is a process that generates complementary DNA (cDNA) from a single-stranded RNA template. This happens naturally in retroviruses for example. However, in this case, cDNA was generated from mRNA so that gene expression in single cells could be analysed via real time PCR. Therefore, RNA was isolated, and the concentration was measured as described before (see 2.2.2.5 RNA Isolation and 2.2.2.6 Nucleic acid quantification). The reaction mixture (assembled as shown in Table 17) was incubated at 42°C for 1 h, heated to 70°C for 10 min, and could then be stored at 4°C.

Component	21µl reaction
RNA	1 µg
Oligo(dT)- Primer	1 µl
Nuclease free water	to 14.7 µl
5x Reaction Buffer	4 µl
RiboLock RNase Inhibitor	0.5 µl
dNTP Mix25mm	0.8 µl
RevertAid Reverse Transcriptase	1 µl

Table 17: Ingredients of cDNA Synthesis

To confirm the success of the cDNA synthesis a test-PCR was done. To this end, DreamTaq DNA polymerase was used to amplify the control gene β -actin. The reaction was assembled as shown in Table 18. The PCR cycle is shown in Table 19.

Table 18: Ingredients DreamTaq-PCR

Component	23µl reaction
10x Buffer DreamTaq	2.5 µl
MgCl ₂	1 µl
dNTPs	1 µl
Primer β -actin up (1:10)	1 µl
Primer β -actin down (1:10)	1 µl
DreamTaq	0.1 µl
Nuclease free water	16.4 µl
cDNA (1:4)	2 µl

Step	Temperature	Time
Initial denaturation	95°C	5 min
35 cycles	Denaturation 95°C	30 sec
	Annealing 52°C	30 sec
	Elongation 72°C	1 min
Final extension	72°C	10 min
Hold	4°C	∞

Table 19: DreamTaq-PCR cycle β-actin

2.2.2.9 Polymerase-Chain-Reaction (PCR)

The PCR is a method used to amplify specific DNA regions exponentially. Because DNA polymerase (Q5) can only add nucleotides onto a preexisting 3'OH group of single-stranded DNA, an oligonucleotide (primer) is needed. Therefore, primers determine the region of template sequence that should be amplified. Further, every enzyme of each reaction step has an optimum temperature at which it works most efficiently (see Table 20). PCRs consist of three main reaction steps (Denaturation, Annealing, Elongation) that are repeated cyclically, thereby amplifying DNA exponentially. The reaction was assembled as shown in following Table 21.

Step	Temperature	Time
Initial denaturation	98°C	30 sec
35 cycles	Denaturation 98°C	5-10 sec
	Annealing 50-72°C	10-30 sec
	Elongation 72°C	20-30 sec/kb
Final extension	72°C	5 min
Hold	4°C	∞

Table 20: Standard-PCR cycle (Q5)

Table 21: Ingredients of Standard-PCR assemble (Q5)

Component	50µl reaction
5x Q5 Reaction Buffer	10 µL
dNTPs	1 µL
Primer fwd (1:10)	2.5 μL
Primer rev (1:10)	2.5 μL
5x Q5 Enhancer	10 µL
Q5 polymerase	0.5 μL
Template DNA	< 1µg
Nuclease free water	to 50 µL

2.2.2.10 Real-Time Quantitative Polymerase-Chain-Reaction (qRT-PCR)

The real-time quantitative PCR (qRT-PCR) is a method used to quantify nucleic acids indirectly. Apart from a ribonuclease protection assay, nucleic acids are not quantified at mRNA level, but indirectly with the help of cDNA. In this case, qRT-PCR was used to analyse the transcriptional levels of specific proteins.

The detection technique is based on fluorescence resonance energy transfer (FRET) via hybridization probes as shown in Figure 6. Each probe consists of two oligonucleotides that are blocked through a dideoxynucleotide or phosphate group so they cannot function as a primer, but as a fluorescent reporter. Further, one of the oligonucleotides has an acceptor at the 3'-end, and the other a donor at the 5'-end. Both bind at the same DNA-strand close to each other (maximum distance 5 nucleotides). The mechanism of FRET is to excite fluorochromes with a specific wavelength, which leads to the emission of their excessed energy in the form of light. In this scenario, the first fluorochrome (acceptor) can be excited and then passes its energy onto the second (donor), which converts it into light. Consequently, emission can only be detected when both oligonucleotides are bound to the DNA-strand [Mülhardt 2013].



Figure 6: Mechanism of FRET via hybridization probes. After the double strand is opened through denaturation, the acceptor- and donor- primer bind. If the acceptor then gets excited, it passes on the energy to the donor, which converts it into light. The emission can then be measured. Illustration adapted from: [Mülhardt 2013].

The quantification is based on the kinetic of PCR-reactions. PCR products increase exponentially at the beginning, which turns into a linear growth over time. Therefore, the crossing point (C_P) at which the amplification curve crosses the vertical threshold is used for analysis. The C_p is determined by the 2nd derivative maximum of each curve, and the measured crossing point is the cycle at which PCR amplification begins its exponential phase. This point is considered being most reliably proportional to the initial concentration.

The 20 x Assay was assembled as shown in Table 22.

Component	20µl reaction
Primer up	1.2 μL
Primer down	1.2 µL
Probe #	4 µL
Nuclease free water	13.6 µL

Table 22: Ingredients of 20 x Assay for qRT-PCR

The reaction was assembled as shown in Table 23.

Table 23: Ingredients of the reaction for qRT-PCR

Component	Triplicates
20 x Assay	1.75 μL
2 x MasterMix (applied: Probes Master, Roche Life Science)	17.5 μL
Nuclease free water (applied)	8.75 μL
cDNA (1:4)	7 μL

2.2.2.11 Agarose electrophoresis

Agarose-gel electrophoresis is a molecular biological method used to separate DNA molecules according to their molecular weight. To this end, 1% agarose gels in TBE buffer were used to separate and analyse PCR products. Agarose in TBE was carefully melted in a microwave. To visualize DNA, ethidium bromide was added to the gel (1%), which intercalates into the DNA and shows under UV light. The gels were prepared by dissolving agarose in TBE buffer through heating in the microwave and cooling them down in an electrophoresis chamber at RT for 30 min. The samples were supplemented with 6x loading buffer and loaded onto the gel. Additionally, a size reference of 100 bp ladders was applied. The electric field to separate the negative charged DNA molecules was applied for 30-40 min at 100 mV.

2.2.2.12 DNA extraction from gel using centrifuge

The GeneJET Gel Extraction Kit (Thermo Scientific) was used to extract DNA from the

Agarose-gel.

- 1. pre-weigh tube
- 2. excise gel slice, cut as close to the DNA as possible
- 3. record the weight of the gel slice
- 4. add 1:1 volume of Binding Buffer
- 5. incubate at 60°C until gel is *completely* dissolved
- 6. vortex briefly
- optional (only if DNA fragment is < 500 bp or > 10 kb): add 100% Isopropanol (1:1 gel volume)
- 8. transfer up to $800 \ \mu L$ of the solubilized gel solution to the column
- 9. centrifuge at >12,000 x g (10,000 14,000 rpm) for 1 min
- 10. discard the flow-through
- 11. if you want to sequence later: add 100 µL of Binding Buffer
- 12. centrifuge at >12,000 x g (10,000 14,000 rpm) for 1 min
- 13. discard the flow-through
- 14. add 700µL of Wash Buffer
- 15. centrifuge at >12,000 x g (10,000 14,000 rpm) for 1 min
- 16. discard the flow-through
- 17. centrifuge the empty column at >12,000 x g for 1 min to remove residual wash buffer
- 18. transfer column into a clean 1.5 mL microcentrifuge tube
- 19. elute by adding 20-50 μL nuclease free water to the center of the membrane
- 20. 15 min incubation at RT
- 21. centrifuge at >12,000 x g (10,000 14,000 rpm) for 1 min
- 22. store purified DNA at -20°C

2.2.2.13 CRISPR/Cas

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) /Cas method revolutionized genetic engineering. It is based on using the ability of Cas9 to bind guide RNA (gRNA) followed by a DNA double strand break. The induced double strand break adjusts DNA repair mechanisms to rebuild the missing piece. In the absence of a DNA template the reconstruction can result in insertion or deletion of nucleotides or frameshift (shown in Figure 7) [Merenda et al. 2017].

The ADAM17 knockout was performed by using the Gene Knockout Kit v2 (Synthego) and Neon Electroporation (Thermo Scientific). The cells were seeded two days before electroporation in an appropriately sized vessel for them to be 70-80% confluent on the day of

transfection. The RNP complex was prepared as shown in Table 24 below (9:1 sgRNA to Cas9 ratio).

Component	Molarity	Volume
sgRNA	30 µM	3 µL
Cas 9	20 µM	0.5 µL
Resuspension buffer		3.5 µL

Table 24: Assemble RNP Complexes (9:1 sgRNA to Cas9 ratio)

Total Volume 7 µL/transfection

The media, <u>without antibiotics</u>, was pre-warmed and the destination plate (12-well) was prepared for after the transfection. The cells were washed twice with 1x PBS, removed with trypsin (5 min incubation), and resuspended in antibiotic free media. In addition, the cells were counted and $5x10^6$ cells were transferred to a sterile microfuge tube containing 1mL media. One tube is sufficient for ~10 transfections. The cells were centrifuged for 5 min at 500 x g, then the medium was aspirated, and the cells were washed once with PBS and centrifugation was repeated. After aspirating the PBS, the pellet was resuspended in 50 µL (5 µL/transfection) resuspension buffer R (provided with Neon Transfection System Kit). 5 µL cell suspension was then added to each 7 µL RNP solution, resulting in a 12 µL cell-RNP solution per reaction. For transfection, the Neon tube was prepared with 3 mL Electrolytic buffer E. The Neon Pipette

tip was loaded with 10 μ L of cell-RNP solution and plugged into position in the Neon transfection device. The selected protocol was 1,200 V, 20 ms, 3 pulse, tip type 10 μ L (depending on cell line). Immediately after transfection the pipette was unplugged, and the cells were transferred to a pre-warmed 12-well plate. They were then maintained for two to three days in an incubator under standard conditions, before analysing.



Figure 7: Mechanism of CRISPR/Cas9 with multiple guide RNAs. Three guide RNAs (gRNA) that formed a complex with Cas9 are inserted through electroporation. At each gRNA that binds the DNA, Cas9 can provoke a double strand break, which can either lead to an insertion or deletion of nucleotides, a frameshift, or no change in the sequence.

2.2.2.14 Single cell dilution

Single cell cloning separates transfected cells from non-transfected ones. To do this, cells of the mixed clone were counted and seeded with 0.5 cells/well on a 96-well plate in 100 μ L media without antibiotics. An additional 100 μ L Medium containing antibiotics (P/S) was added three days later. Thereafter, the medium was changed twice a week. After two and a half weeks, 20 wells presumed to have one cell per well were transferred to 12-well dishes. The cells were cultured until being 70% confluent and were then transferred from 12-well to 6-well dishes, with the clones being split 1:2. After the cells were cultured until they were again 70% confluent, they were transferred onto 10 cm dishes and were then ready for analysis.

2.2.2.15 T7-Endonuclease-I-Assay

The T7-Endonuclease-I-Assay (T7E1) is a suitable method to check the success of a previously performed knockout.

T7 endonuclease recognizes mismatches in DNA heteroduplexes and cleaves them as shown in Figure 8. The process starts by isolating DNA from wild type and mutant cells, followed by a PCR (as described before, see Table 20). Afterwards the amplifications of the wild type (WT) and mutant gens get mixed and heated to 96°C again. While slowly cooling to room temperature, the single strands reattach and new assemblies arise [Vouillot et al. 2015].



Figure 8: Mechanism of T7E1 assay. After denaturation, the DNA double-strand breaks and the single-strands rematch. Thereby, strands with and without mutations mix and reanneal so that wildtype, homodimer, or heterodimer DNA double-strands can be formed. If there is a mutation in one strand (heterodimer) the endonuclease T7 cleaves at the mismatch. Consequently, DNA fragments, which are resolved by gel electrophoresis, occur.

Table 25: Ingredients for T7 Assay

Component	19µl annealing reaction
DNA	200 ng
10X NEBuffer 2	2 µL
Nuclease-free water	Το 19 μL

The PCR products were annealed using the conditions detailed in Table 26.

Table 26: Annealing-protocol of T7 Assay

Step	Temperature	Ramp rate	Time
Initial Denaturation	95°C		5 min
Annoaling	95°C-85°C	-2°C/ sec	
Anneaning	85°C-25°C	-0.1°C/ sec	
Hold	4°C		00

After the PCR 1 μ L T7 Endonuclease I was added to each reaction and incubated for 1 h at 37°C. The reaction was stopped by adding 1.5 μ L of 0.25 M EDTA. The cleavage products were supplemented with 6x loading dye and then applied on a 1.5% agarose gel.

2.2.2.16 Cell lysate activity assay

Cells were lysed as described before (2.2.2.1 Protein Isolation). The protein concentration was determined using BCA assay (Thermo Fisher Scientific). Protein amounts of 150 μ g were transferred to a black 96 well plate (Merck, Sigma-Aldrich GmbH) and a 20 μ M substrate (TACE-Substrate IV, 5 mg/mL) in 100 μ L PBS was added. After 30 min incubation at room temperature, ADAM17 activity was measured for 120 min at emission 405 nm and extinction 320 nm with a plate reader (Infinite 200 PRO, TECAN).

2.2.3 Microscopic methods

2.2.3.1 Scanning electron microscopy

For scanning electron microscopy, SH-SY5Y cells were seeded on glass slides before differentiation, while G-MSCs were differentiated as usual in 6-well culture dishes as already described in section 2.2.1.2. At the point in time of interest, cells were washed with PBS and fixed twice in 3% glutaraldehyde for 30 min. In the case of the G-MSCs, samples were provided by carefully milling out a smaller piece from the culture dish, keeping the cells attached to the surface of the culture dish. These pictures were taken by PD Dr. Philipp Arnold (Institute of Anatomy, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany).

2.2.3.2 Immunostaining

The cells were washed in PBS and fixed for 15 min in 4% PFA in PBS at room temperature, and the washed three times for 5 min in PBS. Before blocking in IF-Block (2% BSA) for 60 min at room temperature, the cells were permeabilized for 30 min in 0,3% Triton X-100 in PBS. After blocking, the cells were incubated with the primary antibody mix (see section 2.1.5 Antibodies) overnight at 4°C. After being washed in PBS the next day, the samples were incubated with Alexa488 and Alexa594-conjugated secondary antibody (Thermo Scientific) in IF-Block (see section 2.1.2 Media, Buffers, and Solutions) for 1 h at room temperature in darkness, before being washed again in PBS three times. The samples were mounted using embedding media (including DAPI) to stain the nuclei/DNA.

2.2.4 Statistical analysis

All values are expressed as mean \pm standard error of the mean (SEM), whereas *n* equals the number of independently repeated experiments. Data analysis was performed in Excel and GraphPad Prism version 7. Further, Image J was used for image analysis and Image Studio Ver3.1 for Western blot analysis. In all analyses, the null hypothesis was rejected at *p* < 0.05. To determine if the means of two sets of data are significantly different from each other, a t-test was used. One-way ANOVA was used for three or more sets, and two-way ANOVA for grouped analysis.

3 Results

3.1 G-MSC-derived dopaminergic neurons

3.1.1 Cell morphology

To simplify the development of dopaminergic neurons in vitro, this study aimed to look for a new possible cell source with an easier access. Therefore, the suitability of the established protocol for iPS cells [Kriks et al. 2011] was examined and adjusted to the needs of G-MSCs. The protocol is based on activating sonic hedgehog and WNT signaling. The morphology of the cells changed due to the treatment and attained a more neuronal character as shown in Figures 10A (cell clustering after 28 and 40 days) and 10B (development of neurite outgrowth).



Figure 10: Morphology of G-MSCs during and after differentiation. A, Transmitted light microscopy pictures. Cells before differentiation and after 15, 28 and 40 days of differentiation. Scale bar, 200 μ m. B, Scanning electron microscope pictures, two representative examples after 15 and 40 days of differentiation. Scale bar, 20 μ m.

3.1.2 Biochemical characterization

Besides morphological changes, the cells were characterized biochemically. The expression of the neuronal markers β 3-tubulin and neuron-specific enolase (NSE) increased after 15 days of differentiation in comparison to non-differentiated cells. Further, the dopaminergic marker FOXA2 was also increased at this early point of differentiation (Fig.11A, B).



Figure 11: Protein expression of G-MSCs during and after differentiation. A Representative Western blot. **B**, Relative intensity analysis from Western blots of neuronal marker proteins from WT and differentiated cells normed to loading control. Mean values SEM (error bars) are shown, n=3. *, p < 0.04; unpaired t-test.

Immunofluorescence staining also showed the increase of neuronal and dopaminergic character as described before. The cells were stained positive for the neuronal markers β 3-tubulin and NeuN after 15 days of differentiation (Fig. 12A) and for the dopaminergic marker tyrosine hydroxylase after 40 days (Fig. 12B).



Figure 12: Immunofluorescence of G-MSCs during and after differentiation. A, Immunofluorescence showing positive staining for neuronal markers β 3-tubulin and NeuN after 15 days of differentiation. **B**, positive staining for the dopaminergic marker TH and defined neuronal outgrowth with non-positive cells in the background (see DAPI staining) after 40 days of differentiation. Scale bar, 50 μ m.

To provide additional evidence for the neuronal and dopaminergic character of these cells, the transcriptional levels of neuronal and dopaminergic markers were also analysed (Fig. 13). The mRNA expression levels of β 3-tubulin and synaptophysin increased significantly after 15 days, while NeuN and FOXA2 exhibited augmented expression after 40 days of differentiation.



Figure 13: **mRNA expression levels of G-MSCs during and after differentiation** measured by qRT-PCR. β 3-tubulin and synaptophysin increased after 15 days, and NeuN and FOXA after 40 days. Mean values SEM (error bars) are shown, n=3 (for every approach). *, p < 0.02; **, p < 0.004; ***, $p \le 0.0003$ for control versus differentiated cells; Ordinary one-way ANOVA.

3.2 The role of ADAM17 in neuronal development and homeostasis

3.2.1 Expression levels of ADAM17 during induced neuronal cell differentiation

Previous studies indicated that ADAM10 and/or ADAM17 regulate the dopamine D2 receptormediated ERK signaling via EGFR, which is associated with the regulation of dopaminergic neuron development [Yoon and Baik 2013]. In the following, the role of ADAM17 in this signaling cascade was analysed more specific. First, when differentiating SH-SY5Y cells (a well-established cell model for neuronal in vitro studies), an increase of the total protein amount of ADAM17 during the process could be detected via Western blot (Fig. 14A, B). Further, the mRNA expression levels of ADAM17 increased after induction of neuronal differentiation, as shown by an increase of NSE and synapsin levels (Fig. 14C).



Figure 14: ADAM17 expression level in neuronal differentiation. SH-SY5Y cells from WT were treated with RA ($30\mu M$) for 10days. A, Representative Western blot and B, Quantitative analysis of ADAM17 signal intensity in Western blot from WT and differentiated cells normalized to loading control and relative to day0. Mean values SEM (error bars) are shown, n=5. **, p < 0.0006 for control versus differentiated cells after 10 days; Ordinary one-way-ANOVA. C, mRNA expression levels of neuronal markers and ADAM17 amplified by qRT-PCR. Mean values SEM (error bars) are shown, n=4. *, p=0.03; **, p=0.002; ***, p=0.0003 for control versus differentiated cells after 10 days; Ordinary one-way-

3.2.2 ADAM17 knockout affects neuronal cell development

To exclude a role of ADAM17 in neuronal development, a CRISPR Cas9 knockout SH-SY5Y cell line was generated using a multiple gRNA system. The gRNA design is shown in Fig.15.



Figure 15: Schematic of gRNAs targeting region, in Exon 5 of the ADMA17 gene region.

Before single cell dilution was performed, the mixed clone already showed that a loss of ADAM17 led to less neuronal character after induced neuronal differentiation in the cells as indicated by the total protein amount of ADAM17 (Fig. 16A). Western blot analysis showed an increase of the neuronal markers synapsin, β 3-tubulin and NSE in WT cells between 5 and 10 days of differentiation, whereas the increase in the ADAM17 KO mixed clone was far less (Fig. 16B). The analyses compared the total protein amount of neuronal marker proteins in WT and KO cells after 5 and 10 days of differentiation.



Figure 16: ADAM17 expression level after neuronal differentiation in ADAM17 knockout mixed clone. A, Western blot and B, Quantitative analysis of synapsin, β 3-tubulin and NSE signal intensity in Western blot analyses off WT and ADAM17 KO cells (mixed clone) normalized to GAPDH loading control, n=1.

3.2.3 CRISPR/Cas9-mediated ADAM17 knockout in SH-SY5Y cells

Varies different tests have shown that the single cell dilution revealed clonal selection. Based on following analysis clone 2 and clone 15 were chosen for further experiments.

The sanger-sequence alignment of clone 2 with WT PCR products showed the deletion of 91 bp in clone 2 after the transfection in detail (Fig. 17). Due to technical difficulties, there are no sanger-sequencing results for clone 15, which leads to the hypothesis that clone 15 might be a heterozygous clone, while clone 2 seems to be homozygous.



Figure 17: **Sanger-sequencing results** of ADAM17 coding sequence from subcloning the PCR product. In between the two gRNA cleavage sites, there is a 91 bp fragment that was deleted in the examined clone compared to the WT sequencing result.

Western blot analysis showed almost fully depleted levels of endogenous ADAM17 (Fig.18A, B). As a test for genomic modification T7-Endonuclease-I leads to cleavage of DNA if there are mismatches present. DNA fragments in agarose-gel electrophoresis are the result. The cleavage products of clone 2 are shown in Fig.18C. Further, the shorter PCR product compared to WT demonstrated the partly deleted sequence in clone 15 (Fig. 18C). Lastly, the activity of ADAM17 in the whole lysate of both cell clones appeared to be reduced after transfection indicated by the activity levels not rising above the background activity of SH-SY5Y cells (Fig. 18D, E).



Figure 18: ADAM17 knockout in SH-SY5Y cells through CRISPR/Cas9. Two cell clones are characterized after utilizing CRISPR/Cas9 method for ADAM17 knockout in SH-SY5Y cell line. A, Representative Western blot and B, Quantitative analysis of ADAM17 signal intensity in Western blot from WT and ADAM17 KO cells normalized to loading control and relative to WT. C, T7 endonuclease 1 assay (T7E1 assay) of WT and transfected cells. Representatives of the PCR product after cleavage by T7E1 assay (+) and negative control (-). D, ADAM17 activity assay in whole cell lysate of WT and ADAM17 KO clones 2 and 15, blank equals the background signal of non-treated cells. E, Area under curve analysis from ADAM17 activity assay. Mean values SEM are shown for WT and ADAM17 KO cells, n=3 (B). For all statistics ****, p < 0.0001 for WT versus transfected cells; Ordinary one-way-ANOVA.

3.2.4 ADAM17 knockout affects neuronal cell development

After successfully reducing ADAM17 expression in SH-SY5Y single-clones, the neuronal differentiation treatment was repeated as before with RA for 10 days. When the transfected cells were analysed afterwards, a decrease of neuronal morphology compared to WT cells after differentiation was observed. The length of the neurite outgrowth was significantly lower in ADAM17 KO cells than in WT cells (Fig. 19A, B). On closer inspection, ADAM17 KO cells looked more cubic after induced neuronal differentiation than before, and quite different to WT cells (Fig. 19C). ADAM17 deficient cells lifted off plate after having been maintaining in culture for two to three months.



Figure 19: Effect of ADMA17 on neurite outgrowth during neuronal differentiation in SH-SY5Y cells (WT and clone 15). A, Transmitted light microscopy pictures. WT and ADAM17 KO cells after neuronal differentiation. Scale bar, $60\mu m$. B, Analysis of neurite outgrowth in WT and ADAM17 KO cells after neuronal differentiation. C, Scanning electron microscopy pictures of WT and ADAM17 KO cells before and after differentiation. Scale bar, $100\mu m$. Mean values SEM (error bars), n=7 (7 independent experiments with 25 measurements each). ****, p < 0.0001, two-way ANOVA.

Lastly, the structure of ADAM17 knockout cells after neuronal differentiation also showed less morphologic traits for neuronal cells compared to the WT cells as exhibited by not only a far less increase but also decrease of neuronal marker proteins in ADAM17 knockout cells compared to WT cells during and after neuronal differentiation (Fig. 20A). Western blot

analysis showed that the total protein amount of the neuronal markers synapsin, β 3-tubulin and NSE decreased in the transfected cells after neuronal differentiation compared to WT cells differentiated for 10 days (Fig. 20B). Similar results were obtained for the mRNA levels when analysing the neuronal markers NSE and synaptophysin after 10 days in ADAM17 KO and WT cells. While the expression level of NSE was significantly lower in ADMA17 KO cells as in WT cells after 10 days compared to before the differentiation the analysis of synaptophysin shows even a significant decrease in ADAM17 KO cells (Fig. 20C).



Figure 20: ADAM17 expression level after neuronal differentiation in ADAM17 knockout cells. A, Representative Western blot and B, Quantitative analysis of synapsin, β 3-tubulin and NSE signal intensity in Western blot from WT and ADAM17 KO cells after neuronal differentiation for 10 days normalized to loading control and relative to day 0. C, mRNA expression levels of neuronal markers amplified by qRT-PCR. Mean values SEM (error bars) are shown, n=3. *, p < 0.05 (B) 0.03 (C); ***, p < 0.0003 for differentiation rate of WT versus ADAM17 knockout cells after 10 days; unpaired t-test.

In summary, the data illustrate G-MSCs having potential to differentiate into neurons in vitro and ADAM17 playing an important role in this process.

4 Discussion

4.1 G-MSC-derived neurons

The neuronal morphology throughout the differentiation-treatment is captured in Figure 10. Further, immunofluorescence staining shows positive cells for neuronal and dopaminergic markers with negative controls in the background. This underlines the assumption of a successfully induced differentiation process. Although the analysis of neuronal and dopaminergic expression levels so far confirms the development of dopaminergic neurons, it might be of interest to determine the endogenous protein levels of further dopaminergic marker proteins via Western blot analysis. The neuronal marker β 3-Tubulin seems to show an increase at first, but then decreases after 40 days of differentiation. This effect might be due to the fact that non-differating (stem) cells continue to divide and proliferate, leading to a mixed population in the sample. Therefore, if the amount of β 3-Tubulin does not increase more than WT cells, the rate will drift and analysis results will show an alleged decrease over time. Despite this observation, an extension of the differentiation period could make the obtained data clearer. Moreover, investigating the functionality of the provided cells would support the assumption that mature neurons have been developed, for example by patch clamp analysis measuring ionic currents in individual isolated living cells, thereby proving neuronal activity.

4.2 ADAM17 in neuronal development

In this study, neuronal differentiation in human neuroblastoma SH-SY5Y cells was induced by all-trans retinoic acid (RA), a metabolite of retinol (vitamin A). The latter functions as a ligand for nuclear RA receptors, which leads to regulation of transcriptional mechanisms to activate or repress genes. Further studies are needed to identify the key genes regulated by RA signaling [Moutier et al. 2012]. Although, it cannot be entirely ruled out whether RA plays a role in the expression levels of single proteins, a wildtype control was always running simultaneously during every differentiation approach to verify the sufficiency of neuronal differentiation.

To exclude the involvement of ADAM17 in signaling pathways during neuronal celldifferentiation, CRISPR/Cas9 technology was used to knockout ADAM17 expression in SH-SY5Y cells. The success of the knockout was verified by multiple analysis including sanger sequencing. Two single clones, clone 2 and 15, were observed to be successful. Clone 15 proved extremely difficult to obtain sequencing results. As the 5' region is very thymin-rich, it is possible that there might be a secondary structure of the DNA that aggravates sanger sequencing analysis. Moreover, an effect was observed in Western blot analysis, where a minimal revival of ADAM17 expression after culturing the cells of a longer time was shown. This might also reinforce the hypothesis of clone 15 being a heterozygous clone. However, the expected expression levels were hardly detectable. Even if ADAM17 expressing cells might have overgrown the KO cells due to contaminations of non-transfected cells in the population, it can be expected that the levels would be so low that it would not have a significant impact to disprove the results this study shows, because the ADAM17 signal intensity was analysed for control purposes during the study. Also, the activity assay did not show more signal in the ADAM17 KO clones than created by the background untreated SH-SY5Y cells. Lastly, when using CRISPR/Cas9 technology, off-target effects must be taken under consideration. But because the results of clone2 and clone 15 were so similar and both showed the same effect during neuronal differentiation, this thesis does not provide further analysis regarding off-target effects. Results implicating that ADAM17 has an impact on neuronal development and a new cell source generating possibly dopaminergic neurons in vitro more easily, helps further understanding neuronal diseases and the development of new treatment strategies.

4.3 Approaches for investigating neuronal diseases and finding treatment strategies

Previous studies have shown that G-MSCs are a very promising cell source for future biologically based therapeutic techniques and could be useful and superior to conventional clinical treatment modalities [Fawzy El-Sayed and Dörfer 2016]. Since Parkinson's disease (PD) is the second most common neuronal disease worldwide and the neurodegenerative process is still not fully understood, it is of interest to find ways to further investigate molecular pathways and develop treatment strategies safely in vitro. Up until now neither early diagnosis, nor efficient therapies that can stop neurodegeneration are available.

Over the years, animal models have come to the center of attention in cell-transplantation research. Ongoing development has provided a variety of different cells for treatment strategies including fetal dopaminergic neurons [Thompson and Parish 2013] as well as embryonic, neuronal, mesenchymal and iPS cells [Freed et al. 2003; Hedlund and Perlmann 2009; Xu et al. 2016]. Clinical trials using intrastriatal grafts of human fetal mesenchymalic dopaminergic neurons provided proof of sustained survival of the transplanted cells postmortem [Deierborg et al. 2008].

Although clinical trials have yet to report the hoped-for success, some studies in animal models do confirm the potential of cell replacement for treatment of PD [Tom Robert Barrow 2015]. Due to these highly variable results, it is strongly argued that some of the key requirements to

make this therapeutic approach work are still unknown. Nonetheless, even if future studies optimize the procedure, there are still ethical and logistical problems with fetal tissue, such as sufficient and steady availability of material, especially considering the large number of patients.

Therefore, recent discoveries on iPS cell-derived dopaminergic neurons are a promising solution in providing a reliable source and obviating ethical issues compared to fetal tissue [Björklund and Lindvall 2017]. Despite the enormous potential that the iPS cell technology offers, it is still limited by issues relating to clinical safety and technical shortcomings. Although it enables the generation of cells genetically identical to the graft recipient, the required reprogramming-process makes it costly and more difficult to provide enough material [Allan et al. 2010]. Fortunately, recent studies have optimized the reprogramming method and made it more efficient. Therefore, it is now possible to generate iPS cell lines that are free from genomic integration and oncogenes, thereby improving clinical safety [Heng et al. 2010]. Furthermore, the therapeutic potential depends on efficient differentiation of functional iPS cell-derived midbrain dopaminergic neurons. In this regard, the release of a reliable protocol in 2011 [Kriks et al. 2011] was an important break-though. The protocol constitutes the basis for a feasible treatment-strategy in the future.

Still, the iPS cell-system remains costly and elaborative, which is why it is of interest to look for an alternative cell source with similar benefits. The cells whose potential this study is aiming to show provide both patient individuality and steady availability. Whereas all oral soft tissues are descended from a common neural crest ectomesenchymal origin, the lamina propria of the free gingival margin is thought to be formed from cells stemming from the outer layer of the dental follicle. Due to the developmental contribution of the inner layer of the dental follicle and everything related to it the free gingival margin is assumed to be unlike other oral soft tissues [El-Sayed et al. 2015]. While previous studies revealed the osteogenic, adipogenic and chrondrogenic differentiation potential of oral stem/progenitor cells [Sharpe 2016], the aim now is to investigate their capabilities in neuronal research. The Huang group was the first to examine the capability of G-MSCs to differentiate toward neuronal lineages in 2018. Their research has shown, that G-MSCs and dental pulp stem cells appear to be more neurogenic than other oral stem cells [Li et al. 2019]. Furthermore, because G-MSCs already feature stem cell like properties, exogenous reprogramming (as it is the case with iPS cells) is not necessary, hence potential issues considering clinical safety can be avoided. Additionally, the tissue harboring the proposed alternative cells is widely available for use and is routinely removed during dental grown lengthening and periodontal surgical procedures. The tissue is also reported to have a remarkable periodontal regenerative capacity. While the use of G-MSCbased regenerative therapy for a variety of disorders (skin disorders, autoimmune and inflammatory diseases, nerve regeneration, and oral disorders) has already been noted [Kim et al. 2021], G-MSCs have the potential to become a more established personalized cell model in vitro that enables the investigation of molecular pathways and can be of great use to clinical trials.

4.4 ADAM proteases involved in human diseases

Previous studies have reported ADAM proteases in cancer, neurological and cardiovascular diseases, asthma, infection and inflammation [Mochizuki and Okada 2007]. ADAMs may also play a role in the promotion of tumor invasion and metastasis via cleavage of extracellular matrix proteins. Many ADAMs influence cell-cell communication via adhesive interactions. Because of their increasing relations with human diseases and involvement in inflammatory processes, ADAMs are attractive targets for novel therapies [Edwards et al. 2008].

Several members of the ADAM family have been thoroughly studied and are associated with different types of cancer. ADAM8, 9, 10, 12, 15 and 17 are associated with breast and/or prostate cancer [Valkovskaya et al. 2007; McCulloch et al. 2000; Sjöblom et al. 2006; Kuefer et al. 2006; Schütz et al. 2005; McGowan et al. 2007], while ADAM19 and 22 are associated with gliomas [Wildeboer et al. 2006; D'Abaco et al. 2006]. Further, ADAM8 and ADAM17 are also associated with Rheumatoid arthritis [Gómez-Gaviro et al. 2007; Charbonneau et al. 2007]. However, the relation between ADAMs and neurodegenerative diseases has only been published mainly regarding ADAM10 [Bernstein et al. 2003]. There is only little information regarding ADAM17, although it is well known that the targets of ADAM10 and ADAM17 are very similar.

4.5 ADAM17 in cell differentiation and homeostasis

In 2013, the Yoon and colleagues group inferred that ADAM10 and ADAM17 may play a crucial role in the regulation of the dopamine D2 receptor (D2R) mediated ERK signaling pathway, leading to neuronal differentiation [Yoon and Baik 2013]. They however did not distinguish between ADAM10 and ADAM17. This study looks more specifically into the role of ADAM17 in neuronal development and homeostasis. The increase of endogenous ADAM17 expression in SH-SY5Y cells due to induced neuronal differentiation with retinoic acid for 10 days confirmed the hypothesis and gives rise to the call for further investigation.

A CRISPR/Cas9 knockout approach was used as a crosscheck to observe what happens when ADAM17 is not involved in the process. The preliminary data with the mixed knockout clone directly after transfection already demonstrated a decrease of neuronal character after induction of neuronal differentiation. Further research with ADAM17 KO single clones has confirmed this observation, whereas neuronal marker proteins did not increase after differentiation for 10 days compared to WT cells. Regarding the specific signaling pathways described in previous studies [Yoon and Baik 2013], that the dopamine D2 receptor regulates ADAM17 leading to ERK signaling, additional investigation would be necessary to fully uncover the coherence between ADAM17, D2R and ERK signaling. Therefore, the expression levels and activity of all actors involved in the signaling cascade would have to be examined via Western blot and qRT-PCR while repeating the differentiation protocol. Especially the EGFR, being a substrate of ADAM17, would need to be analysed. A decrease of the EGFR-ligand would strengthen the hypothesis that ADAM17 regulates D2R mediated ERK signaling, by indicating a direct correlation between ADAM17 and EGFR shedding.

However, the immense importance of ADAM17 is impressively shown in mouse models. Due to its wide range of functions and its almost omnipresence in all tissues throughout the organism, it is not surprising that ADAM17 knockout mice are not viable and die prenatally [Peschon et al. 1998]. In 2007 the Horiuchi and colleagues group generated a conditional ADAM17-deficient mouse model [Horiuchi et al. 2007]. These mutant mice survive up to 9-10 months, although they exhibited severe growth retardation, skin defects and infertility [Horiuchi et al. 2009]. Therefore, in 2010 a cre-transgenic mouse model with tissue-specific deletion of the ADAM17 gene was developed. The so called ADAM17^{ex/ex} mice show compromised shedding of ADAM17 substrates and are viable. The phenotype is characterized by blindness, skin defects and abnormal heart and lung development. It appears that no abnormalities can be detected histologically in the brain (for instance in basal ganglia, internal capsule and hippocampus) of ADAM17 ^{ex/ex} mice compared to controls [Chalaris et al. 2010]. Further, motor symptoms have not been sufficiently investigated while a change of movement patterns could be a result of their blindness. Additionally, up to now it has not been possible to conduct a survey with respondents to examine symptoms of dementia or otherwise.

Finally, a few words about the diverse possibilities targeting ADAM17 in therapeutic strategies illustrating the enormous importance of understanding the molecular mechanisms of ADAM17 in general. TGF- α , being a key substrate of ADAM17 is associated with liver, gastrointestinal, breast, skin, lung, brain, and ovarian cancers, as it seems to serve as a marker for human cancers [Yeh and Yeh 1989]. Also, TGF- α binds the EGFR, the same receptor that plays a part in the

previously described ADAM17 induced ERK signaling, leading to neuronal development. Further, as ADAM17 is for example also highly associated with the development of lung adenocarcinoma, an understanding of cancer related ADAM17 actions could also help to explain neuronal diseases. In 2019, promising data indicating the inhibition of ADAM17 as a potential therapeutic strategy to treat the deadly malignancy of lung adenocarcinoma was published [Saad et al. 2019]. Previous studies have illustrated that the selective inhibitor INCB7839 (targeting ADAM10 and 17) could decrease tumor growth in a non-small cell lung cancer model. The results of this study show that the inhibition affects multiple EGFR pathways and has great potential for pharmacological intervention, either alone or in combination with other drugs [Zhou et al. 2006]. Up until now, three patients have been reported to suffer from ADAM17 deficiency, two of them died from complications of the deficiency. While the first patient was not older than two when he died [Tsukerman et al. 2015], the second died at the age of 12 from heart muscle inflammation. While patients mainly suffer under increased susceptibility to infections the third affected person reports to live a normal life apart of recurring inflammatory skin lesion [Blaydon et al. 2011]. The absence of ADAM17 shows to not only have serious consequences on the health in mouse models. Therefor the understanding of ADAM17 regulated pathways is relevant in order to better evaluate the possible side effects of an ADAM17 inhibition in therapeutic context.

In conclusion, ADAM17 was proven to be an essential element in the regulation of several cellular and body functions and developmental processes. To fully understand its complex regulatory capacity, ADAM17 must be examined at all levels. This study contributes to this aim by showing the crucial role of ADAM17 in neuronal development and homeostasis. The fact that ADAM17 deficient mice are not able to survive demonstrates the importance of proper ADAM17 function. As indicated in previous studies, ADAM17 seems to be a promising target in therapeutic strategies and must thereby be further explored and understood.

4.6 Outlook

Even though this study provides preliminary data, nevertheless it already illustrates a promising lead of discovering a new possible cell source and shows that G-MSCs are worthwhile further investigating in terms of being a personalized cell model for exploring molecular pathways and testing treatment strategies in vitro (Fig. 21).

Moreover, this research contributes towards the aim to further enlighten cellular pathways during neuronal differentiation. The ADAM17 knockout approach also helps ongoing examination and enables better understanding of the role ADMA17 plays in general.



Figure 21: Simplified procedure providing personalized cell-based therapy for PD. After the tissue is collected there is no exogenous reprogramming needed to gain stem/progenitor cells. After dopaminergic neurons are generated in vitro, they can be used to test treatment strategies and develop personalized medicine.

5 Abstract

This thesis examines neuronal cell development in vitro. The focus is on the role a disintegrin and metalloprotease ADAM17 plays in this process. Further, this project reports initial trials of developing neurons from a human oral stem cell source. The potential of a unique subpopulation of human mesenchymal stromal/stem cells isolated from gingival tissue (G-MSCs) as a cell source to derive functional neurons has not received much attention until now though G-MSCs would be of benefit to stem cells research because of the easy access and stem-cell like characteristics were no reprogramming is needed. In this study, an established differentiation method that has already been successfully used on induced pluripotent stem cells, was used to guide G-MSCs towards neuronal lineages. This thesis provides initial insight and preliminary data in developing dopaminergic neurons from G-MSCs. Under treatment with sonic-hedgehog and Wnt-signaling activators, the cells developed dendritic outgrowth, a characteristic morphological feature of neurons. Further, expression of the neuronal markers β 3-tubulin, NSE and synaptophysin increased significantly, during the first 15 days of differentiation. After another 25 days, increase in the neuronal and dopaminergic marker proteins NeuN, tyrosine hydroxylase and FOXA2 could be observed. In conclusion, this study shows that G-MSCs have the potential to differentiate into neuron-like cells and demonstrates the possibility of a new cell model for neuronal in vitro studies.

Moreover, previous research has associated ADAM10 and ADAM17 with dopamine D2 receptor (D2R) mediated ERK signaling that induces neuronal cell development, by showing that the absence of D2R leads to alteration in dopaminergic neuronal development. Therefore, this thesis investigates uniquely the role of ADAM17 in neuronal development and homeostasis. For this purpose, CRISPR/Cas9 technology was used to provide an ADAM17 knockout in human neuroblastoma SH-SY5Y cells. ADAM17 deficient cells expressed decreased neuronal markers, Synapsin, β 3-tubulin, NSE and Synaptophysin, significantly compared to wildtype cells after neuronal cell differentiation. Further, the morphology of the ADAM17 deficient cells demonstrated less neuronal character. Also, the cells became more cubic during the differentiation process until they died off. This data supports the hypothesis that ADAM17 plays a noteworthy role in neuronal cell development and homeostasis. Also, it provides an ADAM17 knockout approach to investigate the role of ADAM17 in further molecular pathways.

6 Zusammenfassung

Diese Arbeit befasst sich mit neuronaler Zellentwicklung in vitro. Der Fokus liegt dabei auf der Ergründung, welche Rolle speziell die Disintegrin und Metalloprotease (ADAM) 17 während dieses Prozesses spielt. Außerdem präsentiert diese Arbeit erste Ergebnisse zur Erkundung einer neuen human-oralen Zellressource für dopaminerge Neurone. Das Potential einer einzigartigen Subpopulation von humanen mesenchymalen Stroma/Stamm-Zellen, isoliert aus gingivalem Gewebe (G-MSCs) als Zellressource für dopaminerge Neuronen hat bisher kaum Hier wird ein etabliertes Differenzierungsmodell verwendet, um Beachtung gefunden. G-MSCs in neuronale Zellen zu verändern. Dieses wird bereits bei induzierten pluripotenten Stammzellen mit Erfolg angewandt. Unter Verwendung dieser Methode zeigten die G-MSCs morphologisch, durch die Formation von dendritischen Ausläufern, einen neuronalen Charakter. Außerdem exprimierten die Zellen bereits nach 15 Tagen Differenzierung die neuronalen Marker β3-tubulin, NSE und Synaptophysin signifikant mehr als undifferenzierte Zellen. Nach weiteren 25 Tagen stieg die Expression des neuronalen Markers NeuN und der dopaminergen Marker Tyrosinhydroxylase und FOXA2 an. Zusammenfassend zeigt diese Arbeit das Potential von G-MSCs in neuronale Zellen zu differenzieren und die damit verbundene Möglichkeit, G-MSCs als neues Zellmodell für neuronale in vitro Studien zu verwenden.

Des Weiteren haben vorherige Studien ADAM10 sowie ADAM17 mit dem Dopamin D2 Rezeptor vermittelten ERK Signalweg, welcher zu neuronaler Zellentwicklung führt, assoziiert, indem gezeigt wurde, dass sich die dopaminerge neuronale Entwicklung verändert, wenn der D2 Rezeptor abwesend ist. Deshalb wird in dieser Arbeit speziell die Rolle von ADAM17 in neuronaler Zellentwicklung und Homöostase untersucht. Zu diesem Zweck wurde mittels der CRISPR/Cas9 Technologie ein ADAM17 "knockout" in humanen Neuroblastom SH-SY5Y Zellen induziert. Die ADAM17 defizienten Zellen exprimierten die neuronalen Marker Synapsin, β3-tubulin, NSE und Synaptophysin nach der Differenzierung signifikant weniger als Zellen des Wildtyps. Außerdem demonstrierte die Morphologie der ADAM17 defizienten Zellen nicht nur einen weniger neuronalen Charakter und eine deutlich kubischere Form, darüber hinaus starben sie nach einiger Zeit sukzessive ab. Folglich unterstützen die Daten dieser Arbeit die Hypothese, dass ADAM17 eine wichtige Rolle in der neuronalen Zellentwicklung spielt und stellt eine ADAM17 defiziente Zelllinie zur Verfügung, welche ermöglicht, den Einfluss von ADAM17 auf weitere Zellprozesse zu untersuchen.

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8 Appendix

8.1 Abbreviations

<i>Tuble 27. List of ubbreviation</i>	Table	27:	List	of	abbr	evia	tions
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%	Percentage
°C	Degree Celsius
±	Confidence interval / standard deviation
∞	Infinity
\leq	Less or equal
ADAM	A disintegrin and metalloprotease
ANOVA	Analysis of Variance
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BMP	Bone Morphogenic Proteins
bp	Base pairs
cAMP	Cyclic adenosinmonophosphate
Cas9	CRISPR associated endonuclease and ribonucleoprotein
CBGTC	Cortico-basal ganglia-thalamo-cortical loop
cDNA	Complementary DNA
cm ²	Square centimeters
CRISPR	Clustered regularly interspaced short palindromic repeats
ctrl	Control
d	Day
D2R	Dopamine D2 receptor
DA	Dopamine
DAPI	4',6-Diamidino-2-phenylindoldihydrochlorid
DAT	Dopamine transporter
ddH ₂ O	Double-destilled water
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTPs	Desoxyribonucleotidtriphosphate
EDTA	Ethylendiamintetraacetat

EGF	Epidermal-growth-factor
EGFR	Epidermal-growth-factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
ESC	Embyonic stem cells
ex/ex	Tissue specific deletion/knockout
FBS	Fetal bovine serum
Fig.	Figure
FRET	Fluorescence resonance energy transfer
fwd	Forward
g	Gravitational force equivalent
G-MSC	Gingival mesenchymal stem cells
GAPDH	Glycerinaldehyd-3-phosphate-dehydrogenase
GDNF	Glia-derived neurotrophic factor
gRNA	Guide RNA
GSK-3β	Glycogen synthase kinase
h	Hour
Hb	Heparin-bound
ID	Differentiation-inhibiting genes
iPS	Induced pluripotent stem cells
iRHOM	Inactive-rhomboide Intramembraneserinprotease
kb	Kilobase
L-DOPA	3,4-Dihydroxy-Phenylalanin
MAPK	Mitogen activated protein kinase
MEM	Minimum essential medium eagle
mg	Milligram
MgCl ₂	Magnesium chloride
min	Minute
mL	Milliliter
mM	Millimole
mRNA	Messenger RNA
ms	Millisecond
MSC	Multipotent stem cells
mV	Millivolt

n	Size of statistical sample
NeuN	Neuronal nuclei: neuronal marker protein
ng	Nanogram
nm	Nanometer
NSE	Neuron-specific enolase: neuronal marker protein
OD	Optical density
OSC	Oral stem cells
р	p-value (in the context of null hypothesis, statistical significance)
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's diesease
PFA	Perfluoroalkoxy alkane
pН	Potential hydrogen
PSC	Pluripotent stem cells
Q5	High-Fidelity DNA-Polymerase
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RA	Retinoic Acid
RAR	Retinoic acid receptor
RARE	Retinoic response element
rev	Reverse
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RXR	Retinoic X receptor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error
sgRNA	Single guide RNA
Shh	Sonic hedgehog
T7E1	T7-Endonuclease-1
TACE	Tumor necrose factor converting enzyme
TBE	Tris/Borate/EDTA

TGF	Transforming growth factor
TGFβ	Transforming Growth Factor
TH	Tyrosine hydroxylase
TNF	Tumor necrose factor
Tris	Tris-(hydroxymethyl)-aminomethan
UV	Ultraviolet
V	Volt
WT	Wildtype
μL	Microliter
μΜ	Micromolar
μm	Micrometer

8.2 Further materials

8.2.1 Primers and gRNA

Table 28: List of companies the primers and gRNA were obtained from

Name	Company
UPL's	Roche LifeScience
Primer	Sigma-Aldrich Chemistry GmbH
Gene Knockout Kit v2	CRISPRevelotion by Synthego

Table 29: List of sequences of gRNA used for CRISPR/Cas9

gRNA	Sequence 5' to 3'
ADAM17 Guide 1	RNA UGUCUACUAACCCUUUU
	DNA TGTCTACTAACCCTTTT
ADAM17 Guide 2	RNA CAGUCUCCAAAAGUGUG
	DNA CAGTCTCCAAAAGTGTG
ADAM17 Guide 3	RNA UUAACAAAUCUCCAAAG
	DNA TTAACAAATCTCCAAAG

Table 30: List of primer sequence used for PCR

Gen	Annealing Temp		Sequence 5' to 3'
ADAM17 (ICE-2)	54°C	fwd	TTATCTAAGAACAAGCAAAG
ADAWI / (ICE-2) 5	54 C	rev	TCCCTAATCTTTTTTTAAAGTC
ADAM17 (ICE 2)	DAM17 (ICE-3) 62°C	fwd	TGCATCCTCTGAAAGATTTCAC
ADAMIT (ICE-3)		rev	CACTCCATTTCCCTTTTCCC

TRAC 68°C	(800	fwd	TCAGGTTTCCTTGAGTGGCAGG
	rev	TAAGGCCGAGACCACCAATCAG	
β-Actin 52°C	5200	fwd	GATGGTGGGAATGGGTCA
	52 C	rev	CACGCACGACGATTTCCCTCT

Table 31: List of primer sequence used for qRT-PCR

Gen	Probe #		Sequence 5' to 3'
B-Actin	#11	fwd	ATTGGCAATGAGCGGTTC
p-Aetin	π 11	rev	CGTGGATGCCACAGGACT
β3-Tubulin	#78	fwd	GCAACTACGTGGGCGACT
,		rev	CGAGGCACGTACTTGTGAGA
ADAM17	#78	fwd	CCTTTCTGCGAGAGGGAAC
	1110	rev	CACCTTGCAGGAGTTGTCAGT
Dopamine	#/1	fwd	GTGCTGGTCATCGTGCTG
Transporter	# 4 1	rev	GGCTGTGATCCATACCACCT
FOYA2	#68	fwd	CGTTCCGGGTCTGAACTG
TOAA2 #00	#08	rev	TCGTGCCCTTCCATCTTC
GAPDH	#60	fwd	AGCCACATCGCTCAGACAC
OAI DII	#00	rev	GCCCAATACGACCAAATCC
NSF	#80	fwd	ACCAATGTGGGGGGATGAAG
INSE #80		rev	GCTTCCTTAACCAGCTCCAA
Synantonhygin	#21	fwd	CGCCTTGTAAGAAGAAAGCAGTA
Synaptophysin	#21	rev	CATTTCCTCCCCAGAGTATCAT
Tyrosine	#42	fwd	TCAGTGACGCCAAGGACA
Hydroxylase	# -1 2	rev	GTACGGGTCGAACTTCACG

8.2.2 Kits

Table 32: List of used kits for this thesis

Name	Company	Reference number
DNeasy Blood&Tissue Kit	Qiagen GmbH	69504
GeneJET Gel Extraction Kit	Thermo Fisher Scientific Inc.	K0692
Pierce [™] BCA Protein Assay Kit	Thermo Fisher Scientific Inc.	23225
RNeasy Mini Kit (50)	Qiagen GmbH	74104

8.2.3 Chemicals

Table 33: List of chemicals used for this thesis

Article	Company
1.10-Phenantrolin	Sigma-Aldrich Chemistry GmbH
Acrylamid (Rotiphorese® Gel 30)	Carl Roth GmbH Co. KG
APS	Carl Roth GmbH Co. KG
Bis(trimethylsilyl)acetamide (BSA)	Carl Roth GmbH Co. KG
β-Mercaptoethanol	Carl Roth GmbH Co. KG
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH Co. KG
EDTA	Carl Roth GmbH Co. KG
Ethanol (pure)	Carl Roth GmbH Co. KG
Ethanol (denatured)	Carl Roth GmbH Co. KG
Fetal bovine serum (FBS)	PAA Laboratories GmbH
Glutaraldehyde 25% Aqueous Solution	Merck Chemicals GmbH
Glycerin	Carl Roth GmbH Co. KG
Glycine	Carl Roth GmbH Co. KG
Hydrochloric acid	Carl Roth GmbH Co. KG
Isopropanol (pure)	Carl Roth GmbH Co. KG
Isopropanol (technical)	Carl Roth GmbH Co. KG
Methanol	Walter CMP
Paraformaldehyde (10%)	Polysciences, Inc.
Potassium chloride	Carl Roth GmbH Co. KG
Potassium dihydrogen phosphate	Carl Roth GmbH Co. KG
SDS	Carl Roth GmbH Co. KG
Sodium chlorite	Carl Roth GmbH Co. KG
Sodium hydroxide	Carl Roth GmbH Co. KG
Sulphuric acid	Carl Roth GmbH Co. KG
TEMED	Carl Roth GmbH Co. KG
Tris	Carl Roth GmbH Co. KG
TRITON X-100™	Carl Roth GmbH Co. KG
TWEEN® 20	Carl Roth GmbH Co. KG

8.2.4 Consumables

Table 34: List of gloves and disinfection

Material	Company	Reference number
bümedes® nonwoven tissues, 100%	Büttner KG	-
PET		
ASEPTOMAN® Hand disinfection	DESOMED - Dr. Trippen	DT-403-010
	GmbH	
Disposable gloves Nitril 35black,	Bingold GmbH+Co. KG	635101
latex-free, powder-free		
pursept® A Xpress 1L spray bottle	Schülke & Mayr GmbH	230131

Table 35: List of reaction tubes

Reaction tube	Company	Reference number
qPCR assay plate 384-well, white	Biozym Scientific GmbH	710886
Microtestplate 96 Well, F	Sarstedt AG & Co	82.1581
Corning 96 well black polystyrene	Merck, Sigma-Aldrich GmbH	CLS3603-48EA
Reaction tube 1,5mL	Sarstedt AG & Co	72.690.001
Reaction tube 2,0mL	Sarstedt AG & Co	72.691
pipe 15ml, 120x17mm, PP	Sarstedt AG & Co	62.554.502
pipe 50ml, 114x28mm, PP	Sarstedt AG & Co	62.547.254
pipe 50ml, 114x28mm, PP braun	Sarstedt AG & Co	62.547.354

Table 36: List of pipette tips

Name	Company	Reference number
Pipette tips 10µL	Sarstedt AG & Co	70.1130
Pipette tips 200µL	Sarstedt AG & Co	70.760.002
Pipette tips 1000µL	Sarstedt AG & Co	70.762
Filter Tip 10µL	Sarstedt AG & Co	70.1115.410
Filter Tip 200µL	Sarstedt AG & Co	70.760.441
Filter Tip 1000µL	Sarstedt AG & Co	70.762.411
Serological pipette 5mL	Sarstedt AG & Co	86.1253.001
Serological pipette 10m L	Sarstedt AG & Co	86.1254.001
Serological pipette 25mL	Sarstedt AG & Co	86.1685.001
Pasteurpipetts, without cotton stuffing	Carl Roth GmbH Co. KG	4518

Name	Company	Reference number
TC-dish 60, Standard	Sarstedt AG & Co	83.3901
TC-dish 100, Standard	Sarstedt AG & Co	83.3902
TC-bottle T75, Standard	Sarstedt AG&Co	83.3911
TC-bottle T175, Standard	Sarstedt AG&Co	83.3912
Cellometer® Cell Counting	Nexcelom Bioscience Ltd	CHT4-SD100-002
Chambers		
6 Well Cell Culture Plate, clear,	Greiner Bio One International	657 160
CELLSTAR [®] , lid with	GmbH	LOT: E19123D5
condensation rings, sterile, single		
packed		

Table 37: List of requirements needed for cell culture

8.2.5 Devices

Table 38: List of devices used for incubation, centrifugation, lysis, and electroporation

Machine	Company	Reference number
Drying and heating cabins	Binder	9010-0153
FP053-230V	Dilidoi	5010 0125
Thermomixer Compact	Eppendorf	2050-110-04
Shaking water bath Typ 1083	GFL mbH	-
Roll Mixer Analog	Cole Parmer scientific experts	SRT09
Tilt and roll mixer RS-TR05	Carl Roth GmbH Co. KG	XK30.1
Heraeus Megafuge 2.0R	Heraeus Holding GmbH	75003080
Homogenisator, Precellys 24	PEQLAB-Life Science VWR	432-3750
Neon Transfection System 10µL-Kit	Thermo Fisher Scientific	MPK 1025
Precellys 24	PEQLAB Life Science	

Table 39: List of devices used for recording and measuring

Machine	Company	Reference number
Amersham Typhoon	GE Life Sciences	29187194
Axiovert 20PM fluorescence microscope	Zeiss	-
Cellometer® Auto T4 Plus cell counter	Nexcelom Bioscience Ltd	Z692441
CKX41 optical microscope	Olympus	-
FV-1000 confocal fluorescence	Olympus	-
microscope		

CLSM (FACILITY line)	Abberior Instruments	
NanoDrop1000 Spectrophotometer	Peqlab Biotechnology	-
(ND1000)	GmbH	
SEM	Philips XL20	
Spectra Rainbow Microplate Reader	SLT Labinstruments	F039046
Infinite 200 PRO	TECAN Trading AG	
Roche Light Cycler 480 II	Roche Life Science	26300

Table 40: List of electrophoresis devices and transfer membrane

Machine	Company	Reference number
Immobilon-FL PVDF, 0,45 μm	Merck Chemicals GmbH	IPFL00010
Invitrogen Mini Gel Tank	ThermoFisher Scientific	A25977
Invitrogen SureCast Gel Handcast	ThermoFisher Scientific	HC1000S
System		
Mini-PROTEAN [®] 3 Cell	Bio-Rad Laboratories, Inc.	165-3301
PowerEase 300W Power Supply	ThermoFisher Scientific	PS0301
PowerPac 200 TM Basic power supply	Bio-Rad Laboratories, Inc.	#1645050

Table 41: List of devices used for pipetting

Machine	Company	Reference number
Eppendorf [®] Research [®] pipette 0,5-	Eppendorf	7368083
10 µL	Lppendon	2500005
PIPETMAN® P20	Gilson	F123600
PIPETMAN® P200	Gilson	F123601
PIPETMAN® P1000	Gilson, Inc.	F123602
Eppendorf® Easypet®	Eppendorf	4421 000.013
Transferpette® S -8	Brand	-
Eppendorf® Multipipette®	Eppendorf	-

8.2.6 Software

Table 42: List of all softwares used for analysis, statistics and illustrations

Name	Company
Adobe®Illustrator® CS6 Ver 16.0.3	Adobe Systems Incorporated
Cellometer Auto Counter	Nexeclom Bioscience
EndNote X7	Thomson Reuters
Excel 2016	Microsoft
Fiji	Fiji contributors
FW10-ASW (Fluoview 10) Ver 4.2b	Olympus
GraphPad PRISM®6 Ver 6.01	GraphPad Software, Inc.
Image Studio TM Lite	Li-Cor Biosciences
Magellan	Tecan
Powerpoint 2016	Microsoft
Word 2016	Microsoft

8.3 Publication

SCHLOTHAUER, T., ARNOLD, P., DÖRFER, C.E., ROSE-JOHN, S., EL-SAYED, K.F., ZUNKE, F. 2021. Oral stem cells as a possible cell source for dopaminergic neurons. *Poster, SY-STEM-* 3rd *Symposium on Stem Cell Research, Vienna.*

SCHLOTHAUER, T., DROBNY, A., ARNOLD, P., ROSE-JOHN, S., ZUNKE, F. 2021. Neuronal differentiation in different cell lines and oral stem cells as a cell source for dopaminergic neurons. *Young Investigator Oral at the 14th Göttingen Meeting of the German Neuroscience Society.*

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