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STUDYING THE PROPERTIES OF GABAERGIC ENRICHED NEURONS DERIVED FROM DRAVET SYNDROME PATIENTS'

Bachelor's thesis
Faculty of Medicine and Health Technology
October 2022

ABSTRACT

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The nervous system is the most important system of communication, control, and regulation of the entire human body. It is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). CNS includes the brain and spinal cord, while PNS covers all parts outside the CNS. The basic unit of the nervous system is the neuron, which consists of the cell body, a single axon, and one or more dendrites. They are responsible for communication between neurons, so they form the neuronal network for information transmission throughout the body. Mutations or dysfunctions in neurons may cause various diseases with severe symptoms, and their treatment is especially important because of high morbidity and mortality. For this reason, research on neurological diseases is critical.

Dravet syndrome (DS) is a rare neurodevelopmental disease of the nervous system characterized by severe epilepsy with various co-morbidities. In the majority of patients, DS is caused by a mutation in the SCN1A gene, which causes a dysfunction in the sodium channel. A dysfunctional sodium channel promotes brain agitation and seizure formation, as it is responsible for sending action potentials between neurons. GABA is an inhibitory neurotransmitter that regulates the activity of excitatory neurons. Impaired inhibition causes an imbalance in neurotransmission, which is the main pathogenic mechanism underlying the seizure formation of DS. However, the study of neurons differentiated from induced pluripotent stem cells (iPSC) derived from DS patients provides new information on underlying disease mechanics and potentially helps to find novel treatments for the patients.

This thesis focuses on *in vitro* models of human-specific stem cells derived neuronal cells, which can be used to study DS with a focus on GABAergic function. In DS, impaired neuronal excitation has previously been shown to act via GABAergic interneurons rather than excitatory neurons. Thus, this study aims to differentiate neurons isolated from the DS patient cell line and the control hESC (human embryonic stem cell) line into GABAergic enriched interneurons. The success of differentiation is characterized at the protein level with immunocytochemistry and gene level with a quantitative polymerase chain reaction. The study aims to optimize the yield of GABAergic neurons during differentiation by testing different passaging methods to increase the yield and survival of neurons for long term in cultures.

In this study, the control hESC line was successfully differentiated into GABAergic interneurons. The yield of the patient cell line was unexpectedly low. The information obtained from this study can be used as a basis for future *in vitro* studies for modelling DS with various GABAergic neurons differentiated from stem cells. This study together with others creates the basis for epilepsy research both in the field of drug treatment and disease modelling and helps refining animal-based research.

Keywords: Dravet syndrome, Gamma-aminobutyric acid, Immunocytochemistry, Neuron, Quantitative polymerase chain reaction

TIIVISTELMÄ

Eveliina Taimela: Dravet'n oireyhtymää sairastavista potilaista eristettyjen hermosolujen ominaisuuksien tutkiminen
Kandidaatin tutkielma
Tampereen yliopisto
Bioteknologian ja biolääketieteen tekniikan tutkinto-ohjelma
Lokakuu 2022

Hermosto on koko ihmiskehon tärkein viestintä-, ohjaus- ja säätelyjärjestelmä, joka voidaan jakaa anatomisesti keskus- ja ääreishermostoon. Keskushermostoon kuuluu aivot ja selkäydin, kun taas ääreishermosto kattaa kaikki keskushermoston ulkopuoliset osat. Hermoston perusyksikkönä toimii hermosolu, joka koostuu soomasta, aksoneista sekä dentriiteistä, ja sen tehtävänä on vastata kommunikaatiosta hermosolujen välillä. Hermoston normaali toiminta on välttämätöntä, koska hermosolujen muodostamat aktiopotentiaalit ovat vastuussa tiedon välittämisestä koko kehossa. Mutaatiot ja toimintahäiriöt hermosolujen välisessä viestinnässä voivat aiheuttaa erilaisia sairauksia, joihin liittyy vakavia oireita. Niiden hoito on erityisen tärkeää korkean sairastuvuuden ja kuolleisuuden vuoksi. Tästä syystä neurologisten sairauksien tutkimus on kriittistä.

Dravet'n oireyhtymä on harvinainen hermoston kehityssairaus, jonka näkyvin tunnusmerkki on monimuotoinen epilepsia erilaisten samanaikaissairauksien kanssa. Suurimmalla osalla potilaista sairauden aiheuttaa mutaatio SCN1A geenissä, joka aiheuttaa toimintahäiriön natriumkanavassa. Vioittunut natriumkanava lisää aivojen aktivaatiota ja kohtausten muodostumista, sillä se on vastuussa aktiopotentiaalien lähettämisestä neuroneissa. GABA on keskushermoston hermosolujen toimintaa inhiboiva eli estävä välittäjäaine, joka säätelee kiihdyttävien eli eksitatoristen neuronien toimintaa. Heikentynyt aivokuoren inhibitio aiheuttaa epätasapainoa neurotransmissiossa, joka on pääasiallinen mekanismi Dravet'n oireyhtymän kohtausten muodostumisen takana. Potilaista johdetuista indusoiduista pluripotenteista kantasoluista erilaistettujen neuronien tutkimus luo uutta tietoa hermostosairauksista.

Tässä työssä keskitytään ihmisspesifeistä kantasoluista erilaistettujen hermosolujen *in vitro* -malleihin, joiden avulla Dravet'n oireyhtymää ja varsinkin GABA:n toimintaa hermosoluissa voidaan tutkia. Aiemmin on osoitettu, että hermosolujen aktivaation heikkeneminen vaikuttaa inhiboiviin GABAergisiin interneuroneihin eikä eksitatorisiin hermosoluihin. Täten tässä tutkimuksessa pyritään erilaistamaan Dravet'n oireyhtymää sairastavasta potilaasta eristettyjä kantasoluja ja kontrolli hESC-linjan soluja GABA rikastetuiksi interneuroneiksi. Erilaistumisen onnistumista karakterisoidaan sekä proteiini- että geenitasolla käyttäen immunosytokemiallisia värjäyksiä sekä kvantitatiivista polymeraasi ketjureaktiota. Tutkielman tavoitteena on optimoida hermosolujen tuotanto erilaistuksen aikana testaamalla erilaisia siirrostusmenetelmiä, jotta hermosolujen tuotto kasvaisi sekä niiden elinikä pidentyisi soluviljelmässä.

Tässä tutkimuksessa hESC-linja onnistuttiin erilaistamaan inhiboiviksi interneuroneiksi, kun taas potilas linjan tuotto jäi tehottomaksi. Tästä tutkimuksesta saatua informaatiota voidaan hyödyntää perustana tuleville *in vitro* tutkimuksille, jotka mallintavat Dravet'n oireyhtymää ja joita suoritetaan erilaisille kantasoluista erilaistetuille GABA rikastetuille hermosoluille. Nämä tutkimukset luovat perustaa epilepsia tutkimukselle niin lääkehoidon kuin tautimallien saralla, ja edesauttavat vähentämään eläimillä tehtävää tutkimusta.

Avainsanat: Dravet'n syndrooma, Gamma-aminovoihappo, Hermosolu, Immunosytokemia, Kvantitatiivinen polymeraasiketjureaktio

PREFACE

This thesis is a part of my Bachelor of Science studies at the Faculty of Medicine and Health Technology (MET) at Tampere University.

I would like to express my gratitude to Adj. Prof. Susanna Narkilahti for giving me an opportunity to join the Neuro Group to do this thesis. Special thanks to my supervisor Ph.D. student Ropafadzo Mzezewa for great guidance and help. I would like to thank all the people I have worked with in the Neuro Group and the MET faculty. In addition, special thanks to my family and friends for their support.

Tampere, 20 October 2022

Eveliina Taimela

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

AA	ascorbic acid
BSA	bovine serum albumin
Camp	cyclic adenosine monophosphate
CNS	central nervous system
DS	Dravet syndrome
GABA	γ-aminobutyric acid
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
hPSC	human pluripotent stem cell
ICC	immunocytochemistry
KSR	knockout serum replacement
NDS	normal donkey serum
NIM	neural induction media
NPC	neural progenitor cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
PLO	poly-L-ornithine
PNS	peripheral nervous system
Pur	purmorphamine
qPCR	quotative polymerase chain reaction
RNA	ribonucleic acid
SHH	sonic hedgehog

INTRODUCTION

The nervous system is the most important system of communication, control, and regulation of the entire human body. It is divided anatomically into two parts, the central nervous system (CNS), and the peripheral nervous system (PNS). The CNS includes the brain and spinal cord, while the PNS covers all parts outside the CNS. The nervous system can also be functionally divided into somatic and autonomic nervous systems. (1) The function of the nervous system is essential for homeostasis because action potentials have the responsibility for information transmission throughout the body. Many nervous system diseases have severe symptoms, and their treatment is especially important because of high morbidity and mortality. For this reason, research on neurological diseases is critical.

The basic unit of the nervous system is the neural cell, of which there are two types: neurons and glial cells. Neurons in the neuronal network are responsible for communication between neurons and they consist of the cell body, single axon, and one or more dendrites. (1) Cortical neurons are divided into two subclasses: inhibitory neurons using mainly the neurotransmitter GABA (γ-aminobutyric acid) and excitatory neurons using mainly the neurotransmitter glutamate (2). The other cell type is the glial cells, that are astrocytes and oligodendrocytes. They provide support for the neurons. (1)

When a neuron conducts nerve impulses to another neuron, impulses travel along the axon until they reach the synapse, where the electrical signals are converted into a chemical signal. In this case, the axon releases neurotransmitters into the synaptic cleft, which causes the receiving neuron's dendrites to convert the signal to chemical again. These signals called action potentials allow neurons to pass information through the body and thus maintaining homeostasis of the human body. Mutations or dysfunctions in neurons may cause various neurological diseases. (1)

This thesis deals with the neurological disease which mainly manifest itself as epilepsy. More specifically on Dravet syndrome (DS) with a focus of GABAergic neurons. In chapter one, the main background behind this thesis and the aims of the study, are discussed. Materials and methods are presented in chapter two. In chapter three, the research results are presented, and discussion section based on the results is in chapter four.

1. BACKGROUND

In this chapter, the background information related to the research methods, materials, and results, are presented at a level that is necessary to understand the rest of the text. The chapter is divided into four separate sections: 1.1 Dravet syndrome, 1.2 In vitro model of Dravet syndrome, 1.3 Characterization of neurons, and 1.4 Aims of the study.

1.1 Dravet syndrome

Epilepsy is a neurological disorder characterized by repeated seizures that can vary widely between individuals. Epileptic seizures are caused by abnormal electrical signals produced by dysfunctional neuronal cells. Abnormal brain functions in epilepsy can be caused by various congenital or acquired factors. (3) Epilepsy can be diagnosed by electroencephalograph (EEG), neuroimaging (CT, MRI), metabolic evaluation or genetic testing. Drugs that decrease electrical activity of the brain or prevent the recurrence of seizures can be used to treat epilepsy. There are numerous antiseizure medications on markets that act on different mechanisms, and the medication is chosen individually. Despite medication, the symptoms and seizures do not disappear in about a third of patients. Epilepsy is estimated to affect 60 million people. (4)

Dravet syndrome (DS) is a rare neurodevelopmental disease that manifests itself as severe and multi-dimensional drug-resistant epilepsy. It is characterized by refractory seizures, which are often triggered by fever. (5) In addition to seizures, other morbidities like cognitive and behavioural impairments are often associated with DS. The syndrome often occurs in early childhood (within the first 12 months of life) and is associated with high mortality although the symptoms progress more endurable with age. (6) In adults, the syndrome is underdiagnosed. (7)

In majority of DS patients (70 % − 80 %) syndrome is induced by a heterozygous loss-of-function mutation in the *SCN1A* gene, which causes a dysfunction in the α-subunit of the neuronal sodium channel Na_V 1.1, which *SCN1A* encodes (5). Na_V channels control action potential by opening in response to depolarization and that's why they are essential for neuronal excitability. A dysfunctional sodium channel promotes brain excitation and seizure formation, as the loss of sodium currents and action potentials reduces excitation of GABAergic neurons. (8)

GABA is the main inhibitory neurotransmitter in the CNS that regulates the activity of excitatory neurons. Its action is associated with cellular chloride balance regulated by two chloride transporters NKCC1 and KCC2. (5) Impaired GABAergic inhibition causes an imbalance of excitatory to inhibitory neurotransmission, which is the main pathogenic mechanism underlying the seizure formation of DS (7). Studies in SCN1A heterozygous mouse models have showed loss of sodium current and dysregulated action potential firing in GABAergic inhibitory interneurons (6). However, the role of factors affecting the severity of the seizure in Dravet's syndrome, is still unknown. This is at least partially due to the lack of human tissue-based research. For example, many drug development trials have failed when moving from preclinical animal studies to clinical trials. Modelling epilepsy by using stem cells and *in vitro* models may be a solution for that. (9)

1.2 In vitro models of Dravet syndrome

The in vivo and in vitro mouse models of DS have helped to understand the main neurobiology behind the DS. Although they have been used to model for example the seizure formation, it's clear that mouse models do not behave the same way as human-based models. Many drug development projects have failed at stage of clinical trials because of species-specific differences. That's why human induced pluripotent stem cells (hiPSCs), or human embryonic stem cells (hESC) can be used in research alongside the mouse models in the future to conform the results. In recent years various neurological disease models with patient hiPSCs derived neuronal cells have been developed to discover neurobiological abnormalities and different types of epilepsy. (7,10) Characterizing the cellular effects of epilepsy and also DS mutations in a human genetic context is important and now, when human pluripotent stem cell (hPSC) technology is available, it is possible to generate neurons from patients with DS mutations. (11,12)

Mouse in vivo and in vitro hPSC studies that have modelled DS shows that the impairment of neuronal excitation affects inhibitory GABAergic interneurons and not excitatory neurons (6). Reduced GABAergic inhibition and hyperexcitability of neuronal circuits have been demonstrated by *in vitro* functional analysis. It relates to majority of both Na_V 1.1 and GABA receptor mutation, but it's still unclear how mutations of Na_V 1.2 and sodium channel β1 occurs. (8) These studies showed that heterozygous SCN1A variants decreases sodium currents (6).

Most electrophysiological studies performed on DS-hiPSC derived cells are performed at the single cell level, with very rare studies performed at network level. Also, only few

studies have analysed hiPSC derivates enriched cortical GABAergic neurons and its effect of SCN1A variants. More research is needed because the molecular mechanisms and the biological processes behind the pathological characteristic of DS are not entirely clear. (6) With human-specific in vitro models, there could be promising results in disease modelling of epilepsy and drug screening, and the research with use of animal models could be refined (10,12).

Table 1 summarizes some previous publications related to DS modeling by using GABAergic interneurons. Maroof et al. publication focuses on cortical neurons differentiation and maturation protocol which other studies have adapted (13). These studies on Table 1 serve as a basis for this work as well. The table contains information about the differentiation protocols used in those studies and how they characterized neurons. The GABA differentiation of all studies resembles the differentiation protocol performed in this study. The protocols of Higurashi et al. and Woo et al. mainly differ in terms of the small molecules, medium, and differentiation schedule. Schuster's protocol has been analyzed more in the following sections, but overall, their differentiation protocol was efficient. The results of these studies look promising that patient derived iPSCs may provide the opportunity for a reliable in vitro system that can be used to model DS (7,14). Research is still needed to optimize the in vitro system reliable.

Table 1. *Summary of previous studies of neurons derived from DS patient. Definitions of all abbreviations in the list of symbols and abbreviations above.*

Article	Aim	Cell source	Media	Small molecules	Gene Expression	Protein Expression
Directed Differentiation and Functional Maturation of Cortical Interneurons from Human Embryonic Stem Cells. Maroof et al. (13)	Efficient induction of human cortical interneurons by Small-molecule-based strategy	hESC (NKX2.1::GFP) and iPSC (C72, SeV6)	Neural induction: KSR+N2, neuronal differentiation: neurobasal + B27 + N2	AA, BDNF, Camp, DKK1, FGF2, Noggin, SHH, Pur, XLS	DLX2, EMX2, LHX6, NKX2.1, OLIG, SIX6, etc.	ASCL1, FoxA2, FoxG1, GABA, GFAP, GFP, MAP2, NKX2, Pax6, VGAT
A human Dravet syndrome model from patient induced pluripotent stem cells. Higurashi et al. (7)	GABAergic neurons differentiation for DS modelling	DS patient iPSC with a c.4933C>T substitution in SCN1A (D1-1, D1-6) and control iPSC (201B7)	Neural induction: DMEM/F12 + knockout serum, neuronal differentiation: serum-free neurosphere medium + B27	AA, BDNF, BMP4, GDNF, Pur, SHH	NKX2.1, SCN1A, SCN2A, SCN3A, SCN8A, Sox2, Oct4	Btub3, GABA, GAD67, GFAB, GFP, Nanog, Nav1.1, Oct 4, SSEA4, Tra-1, VGlut
Transcriptomes of Dravet syndrome iPSC derived GABAergic cells reveal dysregulated pathways for chromatin remodelling and neurodevelopment. Schuster et al. (6)	Differentiate GABAergic neurons from Maroof et al. strategy	DS patient iPSC with different SCN1A variants (DS1, DS4, DS5) and control iPSC (Ctl8)	Neural induction: HFF medium+ supplements/ E8, neuronal differentiation: NIM, NBN + B27 + N2 and KSR	AA, BDNF, Camp, Rock inhibitor, SHH, Pur, XLS	DLX2, EMX2, GAD67, LHX6, NKX2.1, OLIG, SIX6, SCN1A etc.	Btub3, GABA, Nav1.1, SSEA4, NANOG, TRA-1-60
Differential effects on sodium current impairments by distinct SCN1A mutations in GABAergic neurons derived from Dravet syndrome patients. Woo et al. (14)	Role of SCN1A mutation in forebrain GABAergic neurons	DS patient iPSC with different mutation in SCN1A (DS1 missense mutation, DS2 nonsense mutation) and control iPSC	Neural induction: DMEM/F12 + N2, neuronal differentiation: DMEM/F12 + N2	AA, BDNF, Camp, GDNF, IGF-1, Pur	GAD67, NKX2.1	DCX, GABA, NANOG, OCT4, SOX2, SSEA4, TRA-1-60, TRA-1-81, TUJ1

1.3 Characterization of neurons

In this thesis, characterization is needed for analysing neurons. Successful neuronal differentiation is essential for obtaining functional neuronal networks (10). For analysing the differentiation efficacity, different characterization methods are used: quantitative polymerase chain reaction (qPCR) and immunocytochemistry (ICC). In this chapter, different methods are presented briefly.

qPCR, the real-time amplification, and measurement of targeted DNA, is a technology which is used for measuring the gene expression levels. It amplifies DNA and measures the amount of amplified product with fluorescent dye, or the signal generated by probes in real time. It will tell how much of a specific mRNA there is in sample. qPCR has three different temperature stages, and it requires fluorescent reporter DNA molecule that binds to PCR-product and reports on the results. After each cycle, the intensity of the fluorescence emitted by the probe is measured, and the device draws an amplification curve from the signal. A qPCR curve has an exponential phase, where the PCR cycle (Ct) measure needs to be taken. The Ct is the value where the PCR curve crosses the threshold. (15) Because our samples are RNA, first we must reverse transcribe that into cDNA. In this thesis, this technique is used to study the expression of key neural markers relevant in the GABAergic differentiation as well as sodium channels.

ICC is semi-quantitative analysing tool which will assess the presence key protein markers essential for the GABAergic patterning by means of microscope analysis. Furthermore, the generation of neurons or neuronal morphology, conformation and localization can be characterized by ICC. The outcome of this characterization will enable us to compare efficiency of differentiation with the DS cells to the control cells. A basic ICC protocol includes these steps: fixation, permeabilization, blocking, immunolabeling, counterstaining, mounting and microscope analysing. Fixation is important step which prevents the cells from decay and retain cells morphology. Permeabilization is performed with using detergent like Triton X-100. The blocking phase prevents the emergence of unwanted interactions. In this study, the indirect detection method in immunolabelling phase is used, which means that first fixed cells are stained with unlabelled primary antibody. Then the cells are washed, and labelled fluorochrome-conjugated secondary antibody is added. After counterstaining with DAPI and after mounting, the result is seen under fluorescence microscope. (16)

1.4 Aims of the study

The aim of this study was to differentiate control hESC line and DS patient derived hiPSC line into GABAergic (inhibitory) enriched interneurons by optimizing the yield-product of GABAergic neurons during differentiation by testing different passaging methods to increase yield and survival of neurons for long term cultures. Characterization of key markers of the differentiation was analysed at the gene and protein level at specific time points.

2. MATERIALS AND METHODS

Here the experimental part in the laboratory is presented. This chapter is divided into four sections: 2.1 Cells, 2.2 Cell culture platforms and differentiation, 2.3 qPCR characterization, and 2.4 ICC characterization.

2.1 Cells

In this study, in-house derived hESC line (08/017) and DS patient derived hiPSC line (DD1C) which contained a de novo frame shift variant of *SCN1A* gene (6), are used. DS cell line were provided to us by our collaborators at Uppsala University, Sweden. Before neuronal differentiation, hESCs were transferred and expanded in feeder-free culture on recombinant human laminin-521 (LN521, Biolamina) and E8 medium (Thermo Fisher Scientific). Both cell lines were derived at the Faculty of Medicine and Health Technology (MET), Tampere University, Finland. The hPSCs used in this study were acquired from voluntary subjects who had given written and informed consent. The project has supportive statement from Pirkanmaa Hospital District to use the named hPC lines in neuronal research (R20159).

2.2 Cell culture platforms and differentiation

Cell culture platform were coated by using human recombinant laminin-521 (LN521-05, Biolamina) substrate and poly-L-ornithine (PLO, Sigma). Cells were plated to 24- and 48-well plates with density of 100 000 cells/cm2. Cells were cultured at +37 ℃ in a 5% CO_2 humidified incubator in neural induction media (NIM) for the first 10 days and then NBN media. NIM was NBN KSR combination media, where KSR media contained DMEM knock out (Invitrogen), 15% KnockOut Serum Replacement (Invitrogen), 1x GlutaMax (Invitrogen), Non-essential amino acid (Invitrogen), and 1% Penicillin/Streptomycin (Invitrogen). In passaging day 2, NIM also contained ROCK Inhibitor (Y-27632, Sigma). NBN media during neural progenitor cell (NPC) maturation contains Neurobasal medium (Invitrogen), N2 (Invitrogen), B27 without vitamin A (Invitrogen), and 1% Penicillin/Streptomysin (Invitrogen).

The differentiation of the GABAergic interneurons was performed according to a previously published method (6,13). Here briefly: The differentiation of GABA interneurons starts with neural induction (days 0-10) when NIM (consisted of KSR media that was gradually changed to NBN until day 10) was supplemented with the small molecules XAV939 (2 μM, Sigma), LDN193189 (100 nM, Miltenyi biotech), and SB431542 (2 μM, Millipore). In the neural ventral forebrain stage (days 10-17), NBN media was supplemented with Sonic Hedgehog (SHH, 5 nM, R&D Systems), Purmorphamine (1 μM, Miltenyi biotech), BDNF (10 ng/ml, Thermofisher Scientific), cyclic adenosine monophosphate (cAMP, 100 μM, Sigma), and Ascorbic Acid (200 μM, Sigma).

At day 17 (final plating day), the cells were replated at density of 100 000 cells/cm^2 in 24- and at density of 50 000 cells/cm^2 in 48-well plates which were newly coated with laminin-521 and PLO in NBN media containing ROCK Inhibitor. At this time point, two passaging methods were tested to optimize the yield of neurons and neurons survival in long term cultures. Briefly: in Method 1, cells are washed gently with sterile PBS, prior to incubating with TrypLE Select (Thermo Fisher Scientific) for 2 mins to detach the cells. TrypLE Select is then replaced with resuspension media in preparation of counting and replating cells. Method 2 is adapted from our collaborators at Uppsala. Briefly cells are firstly incubated for 4 mins with TrypLE Express (Thermo Fisher Scientific) to detach the cells. TrypLE Express is carefully removed and cells are washed with sterile DPBS. This is followed by resuspension of cells in resuspension media, in preparation for counting and replacing as in Method 1. Following the last stage (day 17->) NPCs-GABAergic interneurons were cultured in NBN media including essential growth factors BDNF (10 ng/ml, Thermofisher Scientific), cAMP (100 μM, Sigma), and Ascorbic Acid (200 μM, Sigma). The supplements are added to the warm media. Figure 1 below illustrates the differentiation protocol.

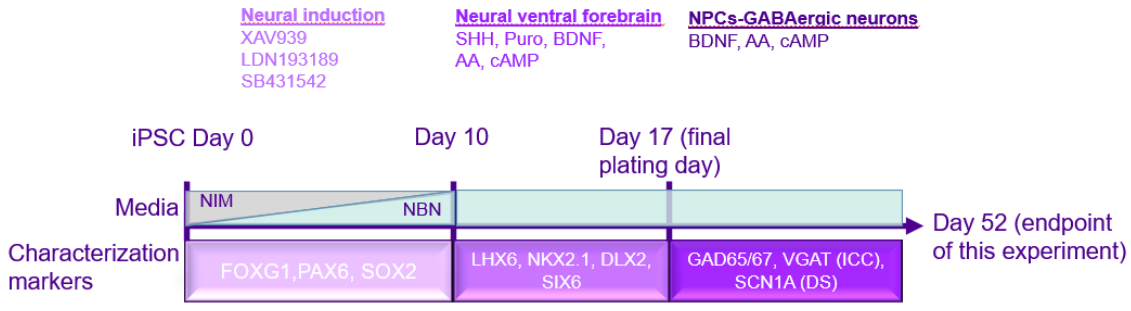


Figure 1. *Protocol outline of GABAergic interneuron differentiation. (Image modified from R.Mzezewa)*

Media was changed 3 times a week and laminin-521 (LN521-05, Biolamina) was added once a week (1:1000). Phase contrast images were taken at each time when media was changed. Images can be seen in Figure 11.

2.3 qPCR characterization

RNA for the qPCR was collected from four wells of 24-well plate at specific time points 1, 12, 19, 34, and 52 and from single cell pellet on day 17 with DPBS Dulbecco's Phosphate Buffered Saline without calcium or magnesium (RA1, Lonza 17-512F) and 2-mercaptoethanol (Sigma M3148). Then collected RNA was isolated with a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany). The purity and concentration of RNA were quantified with a NanoDrop 1000 (Thermo Fisher Scientific). After RNA isolation, 400 ng of total RNA was converted to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression levels were analysed with TaqMan assays using a real-time PCR system ABI QuantStudio 12K Flex System with 5 ng of the cDNA. TaqMan assays are listed in Table 1. Each 15 µl reaction contained 15 ng cDNA, 0.75µl 20X TaqMan Gene Expression Assays, and 7.5 µl 2X TaqMan Gene Expression MasterMix. All the samples were run in three technical replicates.

Table 2. *Primer details for qPCR.*

Gene Symbol	**Gene Name**	**Assay ID**
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Hs99999905_m1
NKX2.1	NK2 Homeobox 1	Hs00968940_m1
DLX2.1	Distal-Less Homeobox 2	Hs00269993_m1
LHX6	LIM Homeobox 6	Hs01030943_m1
SIX6	SIX Homeobox 6	Hs00201310_m1
GAD67/GAD1	Glutamate Decarboxylase 1	Rn00690300_m1
SCN1A	Sodium Voltage-Gated Channel Alpha Subunit 1	Hs00374696_m1

Between days 10 and 19 *LHX6, NKX2.1, DLX2*, and *SIX6* are main characterization markers. After day 19 main characterization markers were *GAD67* and *SCN1A* (6). The data were analysed with relative quantification (RQ) values from delta-delta Ct method by using *GAPDH* as endogenous control housekeeping gene (15). The data is analysed by GraphPad Prism software.

2.4 ICC characterization

Immunocytochemical staining was performed as previously described (17). Briefly: cells were fixed with 4% paraformaldehyde (PFA) 15 min. Cell blocking started after washing by using blocking serum: 10% normal donkey serum (NDS), 0.1% Triton X-100, and 1% bovine serum albumin in PBS (BSA) at room temperature for 45 min. The samples were incubated overnight on shaker in +4°C with primary antibodies in mixture of primers: 1% normal donkey serum (NDS), 0.1% Triton X-100, and 1% BSA in PBS. Primary antibodies consisted of βIII-tubulin (βIII-tub, chicken, 1:400), βIII-tubulin (mouse, 1:1000), FOXG1 (rabbit, 1:500), GAD67 (mouse, 1:100), MAP2 (chicken, 1:4000), Oct4 (goat, 1:200), Pax6 (rabbit, 1:1000), Sox2 (mouse, 1:200), and VGAT (rabbit, 1:500) (all from Thermo Fisher Scientific).

Samples were incubated in room temperature 1 hour with secondary antibodies in mixture of secondaries: 1% BSA in PBS. Fluorescent labelled secondary antibodies consisted of Alexa fluor 488 (donkey anti-goat, 1:400), Alexa fluor 488 (A21206, donkey anti-rabbit, 1:400), Alexa fluor 568 (A10037, donkey anti-mouse, 1:400), Alexa fluor 568 (A10042, donkey anti-rabbit, 1:400), Alexa fluor 647 (A21449, goat anti-chicken, 1:200), and Alexa fluor 647 (A21449, goat anti-chicken, 1:200) (all from Thermo Fisher Scientific). After washing, the well plates are dried and mounted with Prolong gold and cover slips. For fluorescence imaging, an Olympus IX51 inverted fluorescence microscope (Japan) was used.

3. RESULTS

The results of this experiments are presented in this chapter. Experiment is divided into seven specific time points. RNA was collected at 1, 12, 17, 19, 34, and 52 timepoints to measure gene expression of specific markers, whilst protein expression was analysed at day 12, 19, 34, and 46. This chapter is divided based on the characterization methods.

3.1 Gene level characterization

Reverse transcription quantitative PCR (RT-qPCR) reaction was performed to quantify the amount of nucleic acid and to compare the expression of target genes. The data is based on relative quantification (RQ) values, which measures the fold changes compared to the calibrator, which means the sample that all the others are compared to (15). The calibrators were specific target at day 1 for each cell line separately. On day 1, the RQ value was 1 for both cell lines. *DLX2.1, GAD67, LHX6, NKX2.1, SCN1A*, and *SIX6* genes were analysed between the cell lines at the specified time points. Statistical analysis was performed with multiple unpaired t-test to compare differences between the groups at each time point. P-value under 0.05 was considered significant. The # -mark reports if there is no data at that time point so the test could not be performed. In Figure 2, all the RQ values are presented.

In Figure 2a, the expression of *NKX2.1* between cell lines at different time points can be seen. *NKX2.1* is a medial ganglionic eminence (MGE) marker, which should be highly expressed between day 10 to 19 (6). The gene expression of *NKX2.1* was increased from day 1 to day 12 in both cell lines, though in the diseased line DD1C much lower than in the control line. The RQ value of control cell line was the highest on day 12 and was approximately 350, which means that the gene is upregulated from day 1. In the patient cell line, the expression of *NKX2.1* was much lower, approximately 15. In the control line, the expression remained relatively constant at day 17, whilst it decreased in the patient line, DD1C. Statistically significant (p-value < 0.05) difference in the expression of *NKX2.1* between cell lines was found at day 12 and day 17 meaning that control line expressed more the *NKX2.1* than patient line during the same period.

The expression of *SIX6*, which is ventral forebrain marker (6), is presented in Figure 2b. The highest expression of both cell lines was on day 12. The difference between cell lines is between day 17 and day 19, where expression of control line first decreased and

then increased but with patient cell line, it decreased steadily. The variation within the control group was also much greater than in the patient line, as the fold change varied from 35 to 330 between those days. The RQ-value of the patient line, on the other hand, was between 10 and 30, and during days 12 and 17 it was almost the same. Statistically significant (p-value < 0.05) difference in the expression of *SIX6* between cell lines was found only on day 19.

Figure 2c shows the expression of interneuron marker *DLX2.1* (6), which should be highly expressed at neural ventralization stage and after that. The expression of control line was around 200-300 between day 17 and day 34, and on day 34, the RQ-value was highest (over 300). The expression of patient line increased steadily until day 19, when the RQ-value was highest. After that it decreased from 100 to approximately 0,5 on day 34. On day 52, the expression of control line was still around 25 whilst with patient line, it was almost 0. Statistically significant (p-value < 0.05) difference in the expression of DLX2.1 between cell lines was found from day 12 to day 34.

The expression of migratory interneuron marker *LHX6* (6) is presented in Figure 2d. The highest expression of control cell line was on day 34, when the RQ value was 40. Before that it was around 10 on day 17 and on day 19. With patient line, the Ct values were undetermined after day 19, so the highest point cannot be stated. At day 17, the RQ value was approximately 4, so it was much lower than the RQ-value of control cell line. Statistically significant (p-value < 0.05) difference in the expression of *LHX6* between cell lines was found at day 17 and 19. On the other time points, there were not statistically significance changes observed.

The expression of *GAD67* can be seen in Figure 2e. It shows expression of GABAergic interneurons, which should normally show after day 19. The expression of control group was extremely high compared to other genes RQ-values. On day 34 it was over 1000, so *GAD67* is 1000 times more expressed in control line sample than in the calibrator sample. The DD1C groups highest RQ value was approximately 7 on day 19 and after that it decreased to 3. On day 52, the RQ-value of DD1C line was 0.1 so in that timepoint, *GAD67* was downregulated from day 1. Statistically significant (p-value < 0.05) difference in the expression of *GAD67* between cell lines was found from day 17 to day 52.

In Figure 2f, the expression of *SCN1A* is presented. *SCN1A* is a marker for the alpha-subunit of the voltage gated sodium channel (6). As with other genes, the control line expressed much more than the patient line. The highest expression of both lines was on day 34, where RQ value of control line was 33 and with patient line it was around 1. Before that both cell lines were less expressed than at say 1 timepoint, because RQ-

values were under 1. Statistically significant (p-value < 0.05) difference in the expression of *SCN1A* between cell lines was found at day 17 and 19.

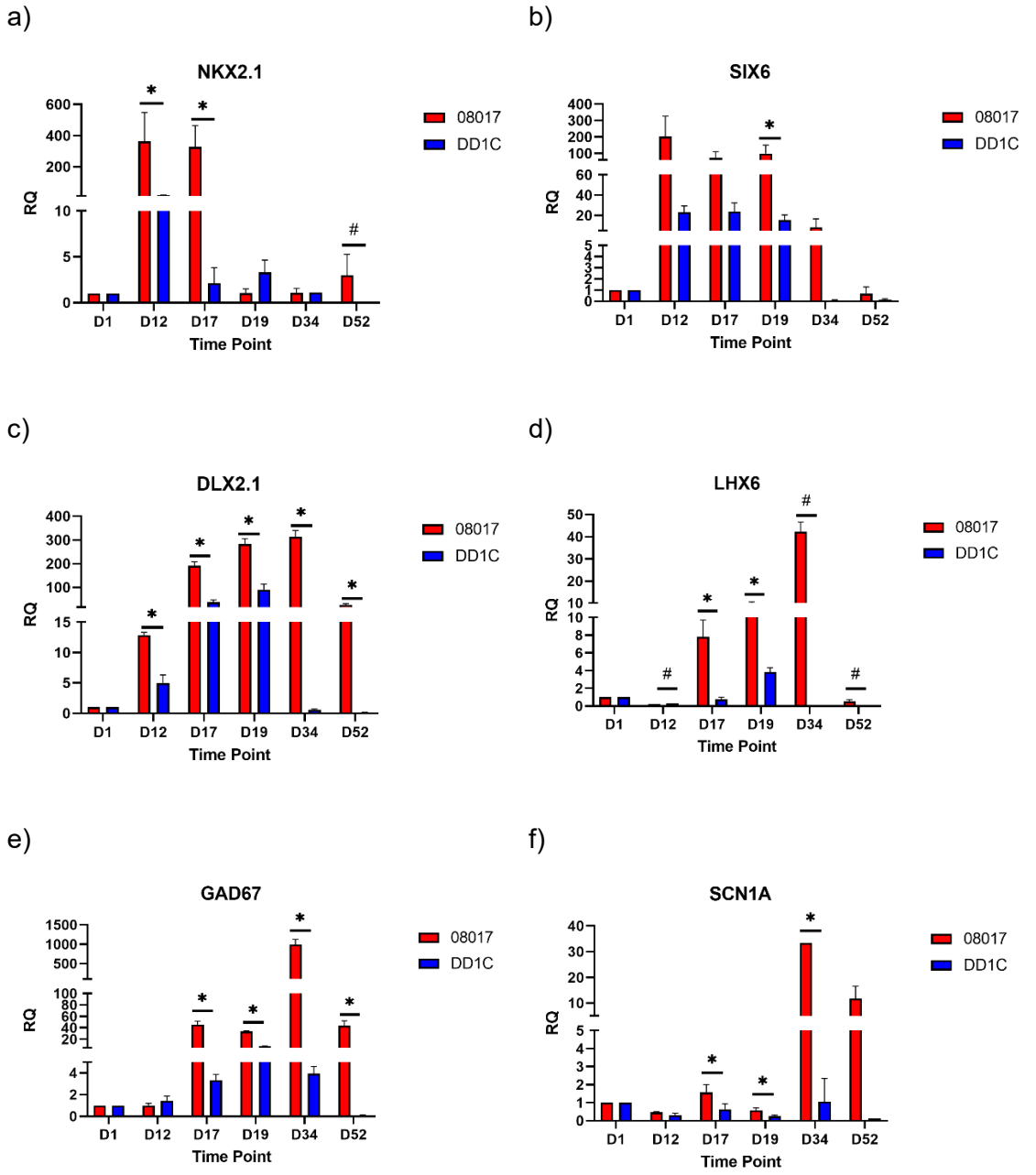


Figure 2. *Graph is presenting expression of genes from day 1 to day 52. Control cell line 08017 is presented in red and patient line DD1C in blue. Multiple unpaired t-test was performed to compare differences between the groups at each time point. Significance is marked as *p<0.05. Data error is marked with #. (a) The expression of NKX2.1, (b) The expression of SIX6, (c) The expression of DLX2.1, (d) The expression of LHX6, (e) The expression of GAD67, and (f) The expression of SCN1A.*

3.2 Protein level characterization

The success of the differentiation can be confirmed by means of immunostainings for both control hESC line (08/017) and patient line (DD1C) at each time point. After 10-day neural induction stage, on day 12, the cells were characterized with FOXG1, Pax6, and Sox2 antibodies which are early neuroectodermal markers and with Oct4, which is marker for undifferentiated hPSCs (Figure 3). Both cell lines showed expression of every marker, which indicate efficient neural conversion and promising to become neuroectodermal cells. The biggest difference between control and patient cell lines were in expression of Pax6. According to visual evaluation, the expression of Pax6 in the patient cell line was lower than in control line (Figure 3).

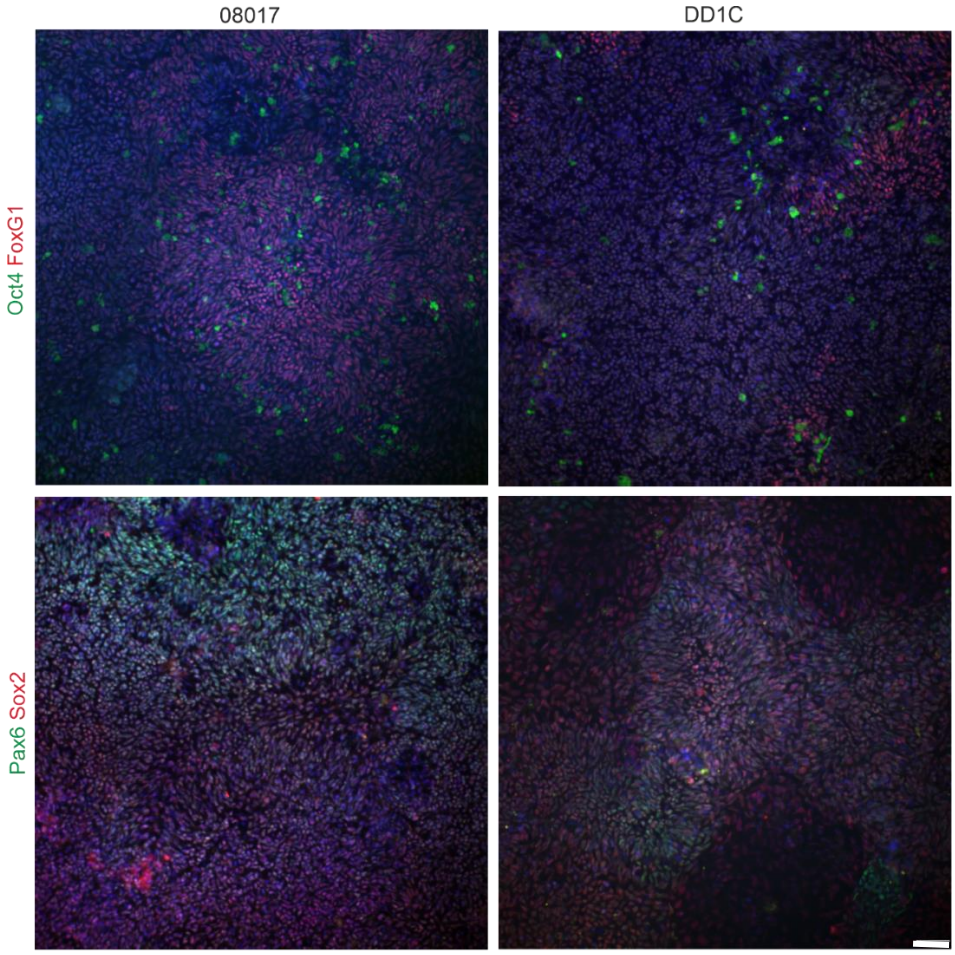


Figure 3. *Control and patient cell lines on day 12 characterized by Oct4, FoxG1, Pax6 and Sox2 for their efficiency to become neuroectodermal cells. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

Two days after final plating day, on day 19, cells were characterized with FoxG1, βIII-tub, GAD67, MAP2, and VGAT (Figure 4). The same characterization markers and GABA marker were also used in the remaining immunostainings. In Figure 4, in method

1 plate, the cell expression of neuroectodermal markers FoxG1 and GAD67 wasn't so high, but a positive expression of axonal and dendritic markers, βIII-tub and MAP2, were visible in both cell lines. The results of immunostainings looked promising on day 19, although expression of VGAT marker in patient line wasn't that high compared to control line. Immunostainings showed evident of cell morphology like cell bodies, dendrites, and axons. Results showed that the neurons could possibly progress into GABAergic enriched interneurons because of started neuronal network formation.

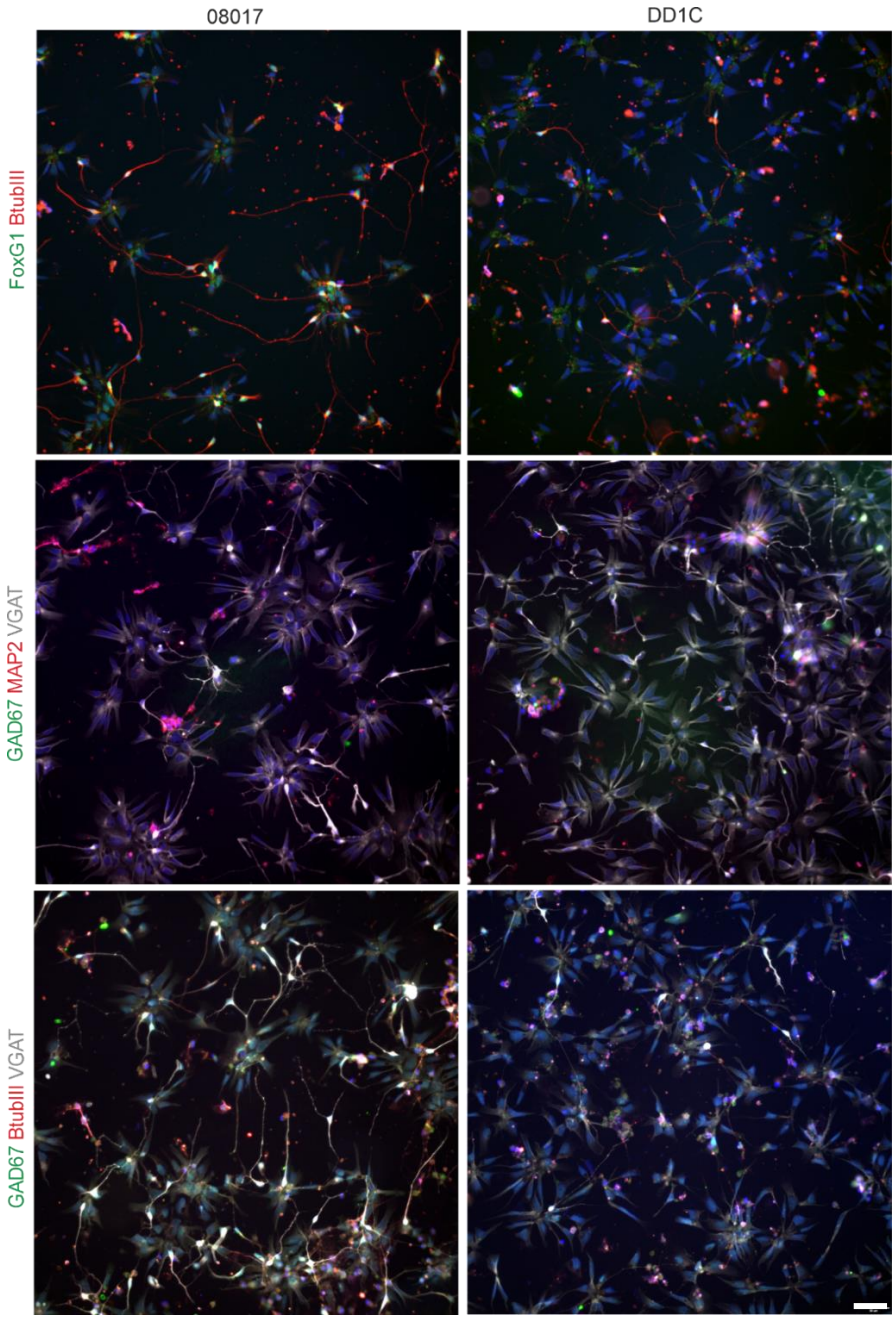


Figure 4. *Control and patient cell line fluorescence images on day 19 with method 1. The cells were characterized with FoxG1, GAD67, βIII-tub, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

On day 34 and day 46, in addition to the normal plate named method 1, there was additional plates named coverslip plate and method 2 plate. In coverslip plate, method 1 was tested in different substrates. In method 2, different plating and passaging method from our collaborators in Uppsala University (6) was tested. Two methods were tested on the final plating day and onwards, to optimize the yield-production and survival of neurons. The difference between the two methods were the procedure of passage the cells. Method 1 is the main method used in Neuro group, so method 2 had fewer plates and that's why they were kept longer in culture to compare the maturity and there are no images from day 19.

On day 34 in every plate, neuronal markers were expressed higher in the control cell line than patient line differentiated samples and neuronal network formed more robustly in the control. The cell amount was pretty much same regardless of the cell line, but there were much more dead cells in the patient cultures based on visual evaluation. There were also more neurons with axonal projections and paths in control group than patient line. Based on the images of immunostaining (Figure 5, 6, 7), the DD1C line differentiated more poorly than the control line in all different methods on day 34.

With both methods on day 34, the expression of GAD67, βIII-tub, MAP2, and especially GABA and vesicular GABA transporter VGAT was high. According to the morphology of cells that can be seen in Figure 5, 6, 7, neuronal network formation between neurons has started indicating maturation of interneurons. Especially coverslip plate expressed main markers highly and thus looked the most promising plate in this stage. However, no major differences between the methods were noticeable to day 34.

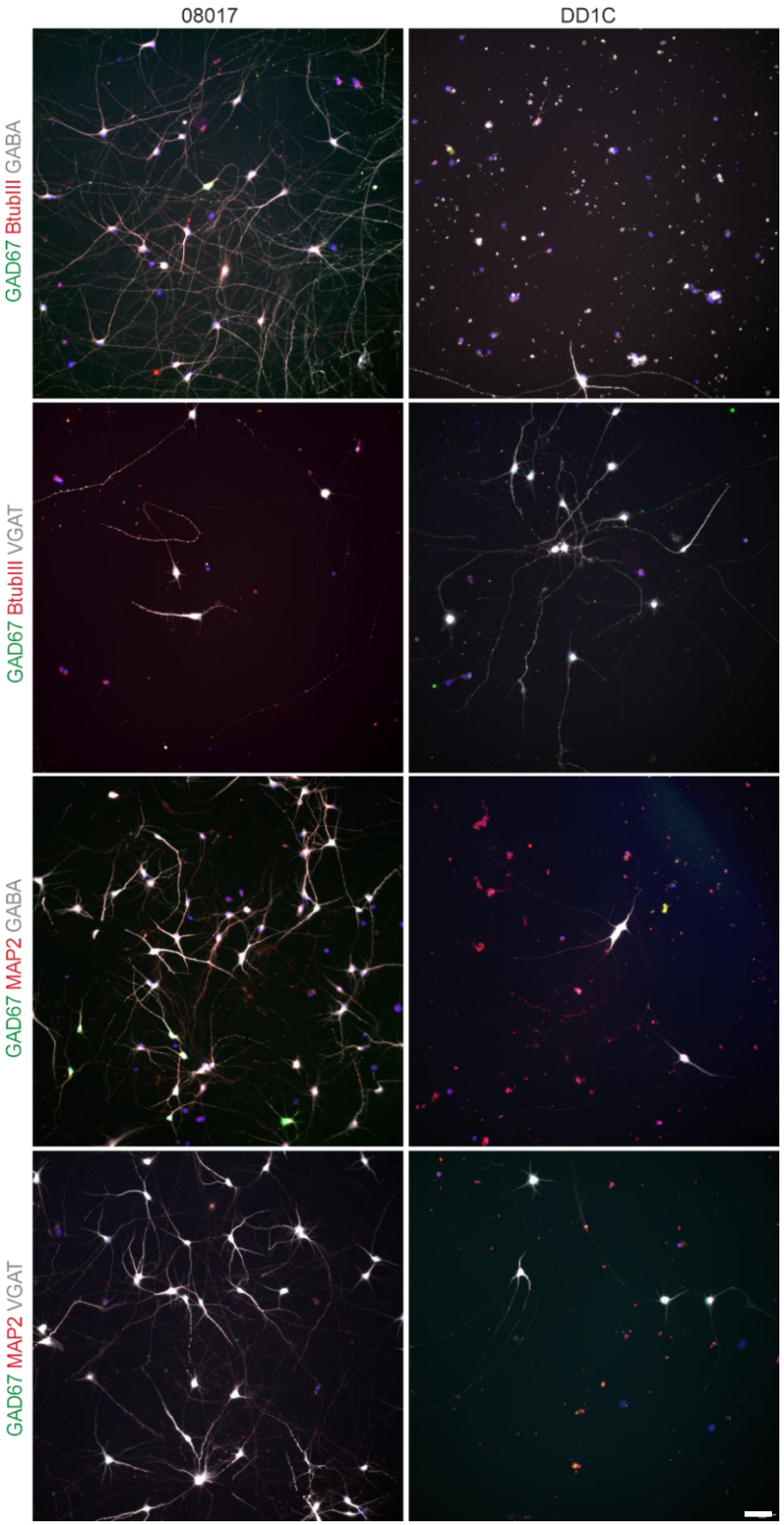


Figure 5. *Control and patient cell line fluorescence images of maturing neurons on day 34 in method 1 plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

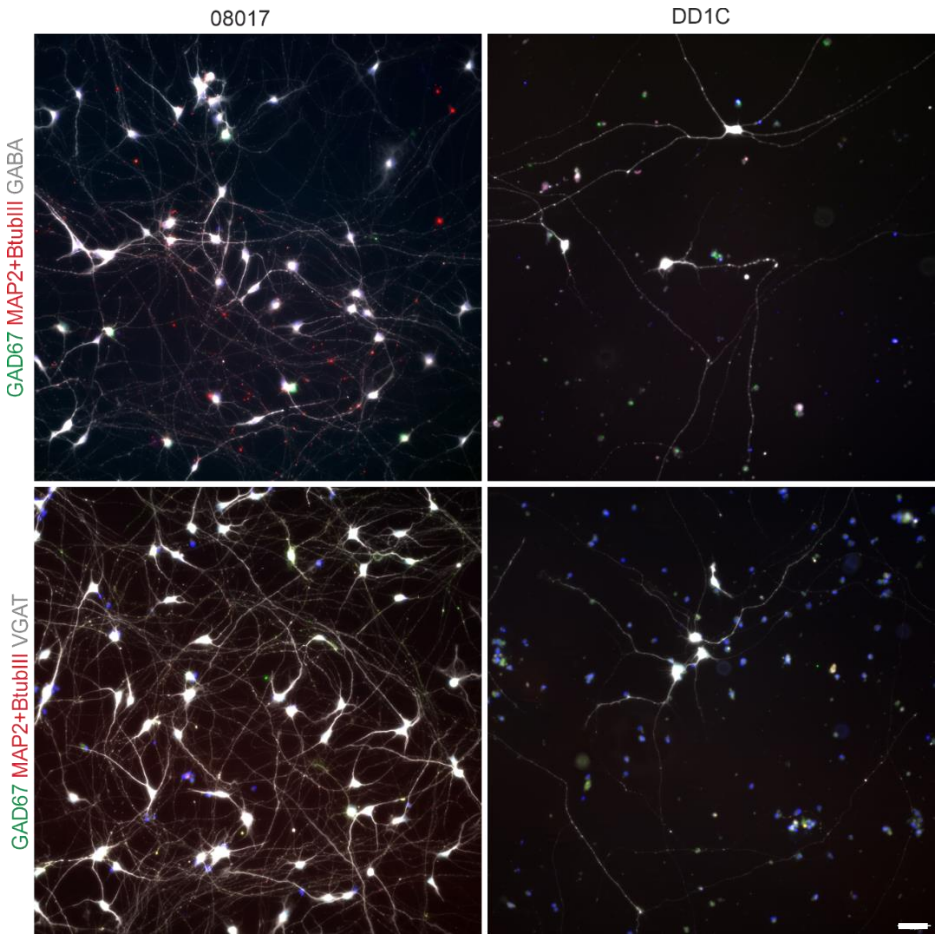


Figure 6. *Control and patient cell line fluorescence images of maturing neurons on day 34 in coverslip plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

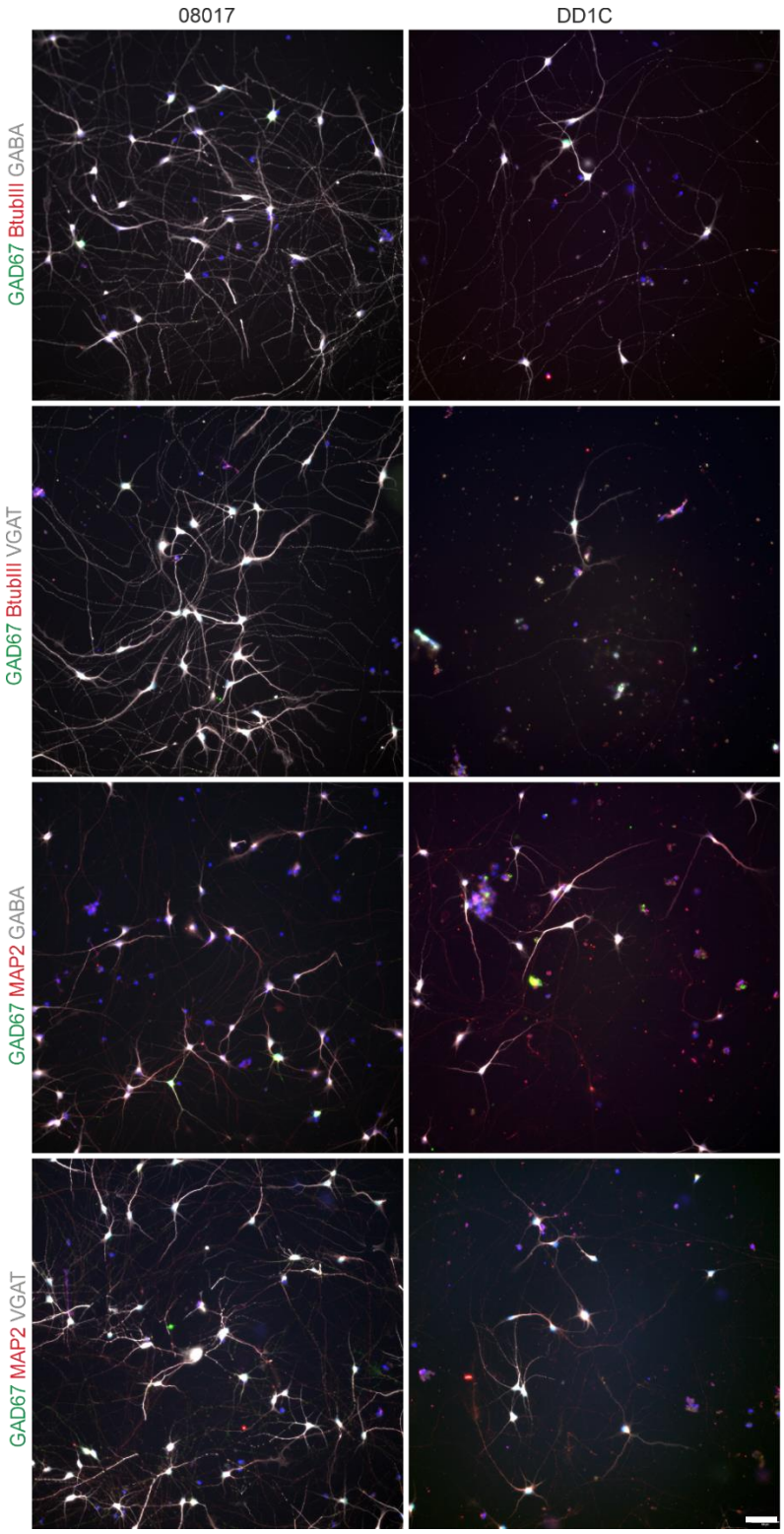


Figure 7. *Control and patient cell line fluorescence images of maturing neurons on day 34 in method 2 plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

Between day 34 and day 46 with method 1, there was development of neuronal network in the control cell line mostly indicating healthy culture. Although the networks had developed from day 34, on day 46 there were also more dead cells. In the patient line, there was network connection only on day 34, and on day 46 all the cells had suffered. However, the patient's line did not look promising day 34 and the outcome was expected. (Figure 5, 8)

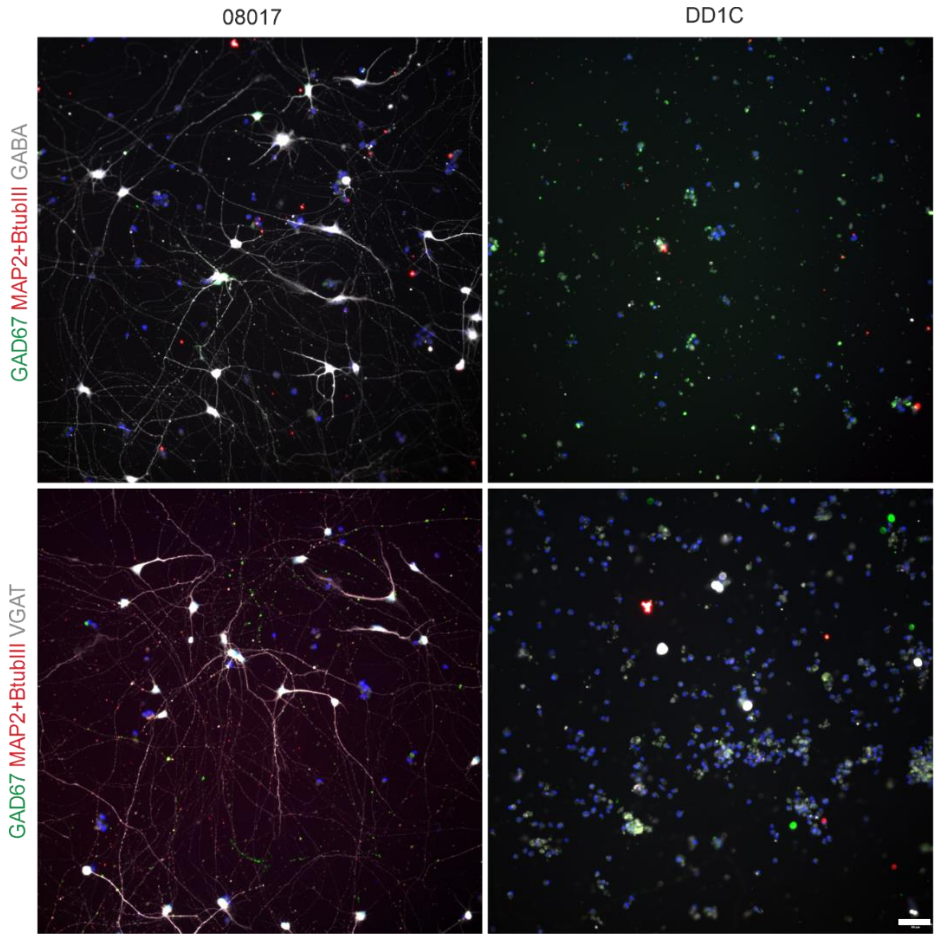


Figure 8. *Control and patient cell line fluorescence images of neurons on day 46 in method 1 plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

Coverslip plate looked most promising on day 34, but the yield of interneurons was not favourable at day 46, even though there were a lot of cells and networks on the coverslip plates at day 34. The entire network has suffered, both on the control and patient lines. (Figure 6, 9)

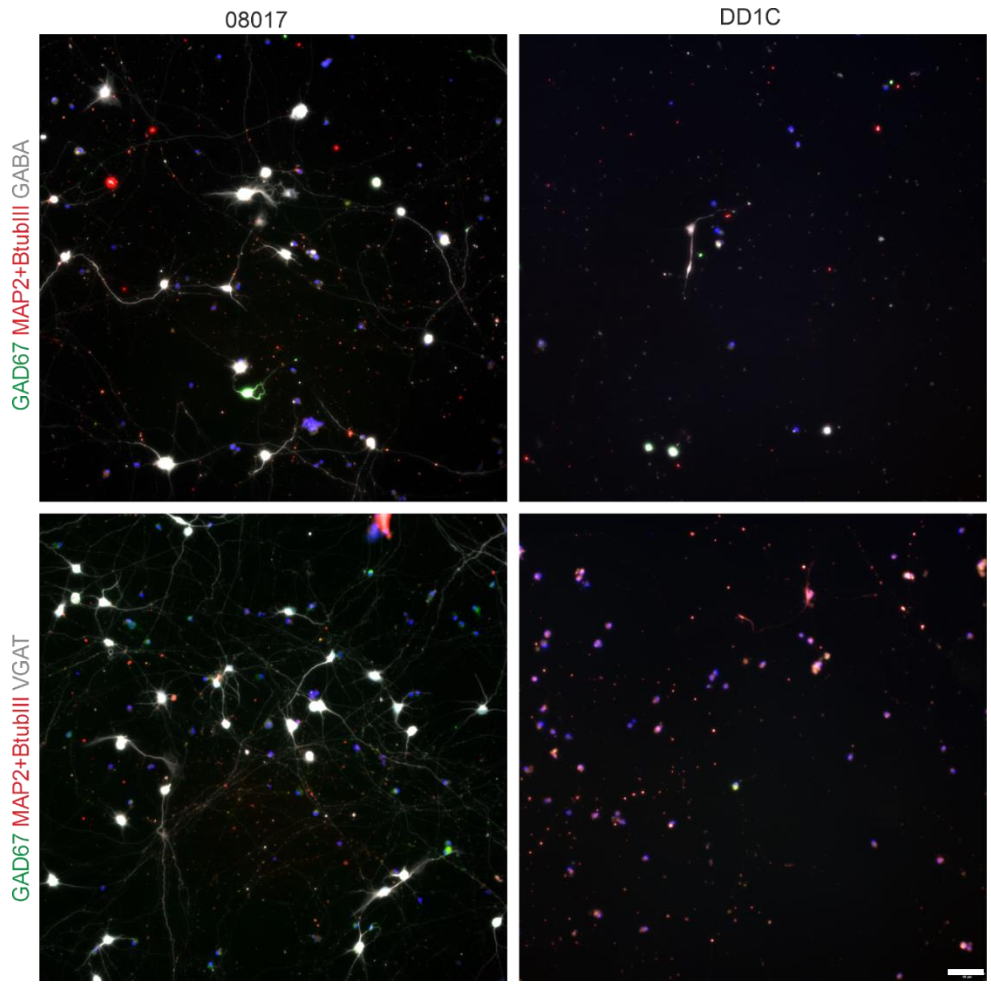


Figure 9. *Control and patient cell line fluorescence images of neurons on day 46 in coverslip plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

In method 2, the expression of βIII-tub, GABA, MAP2 and VGAT was high at both timepoints (34 & 46) in control cell line. The patients line expression of those genes was low, but it expressed VGAT little, so a few living cells with axons were there in method 2 culture. (Figure 7, 10)

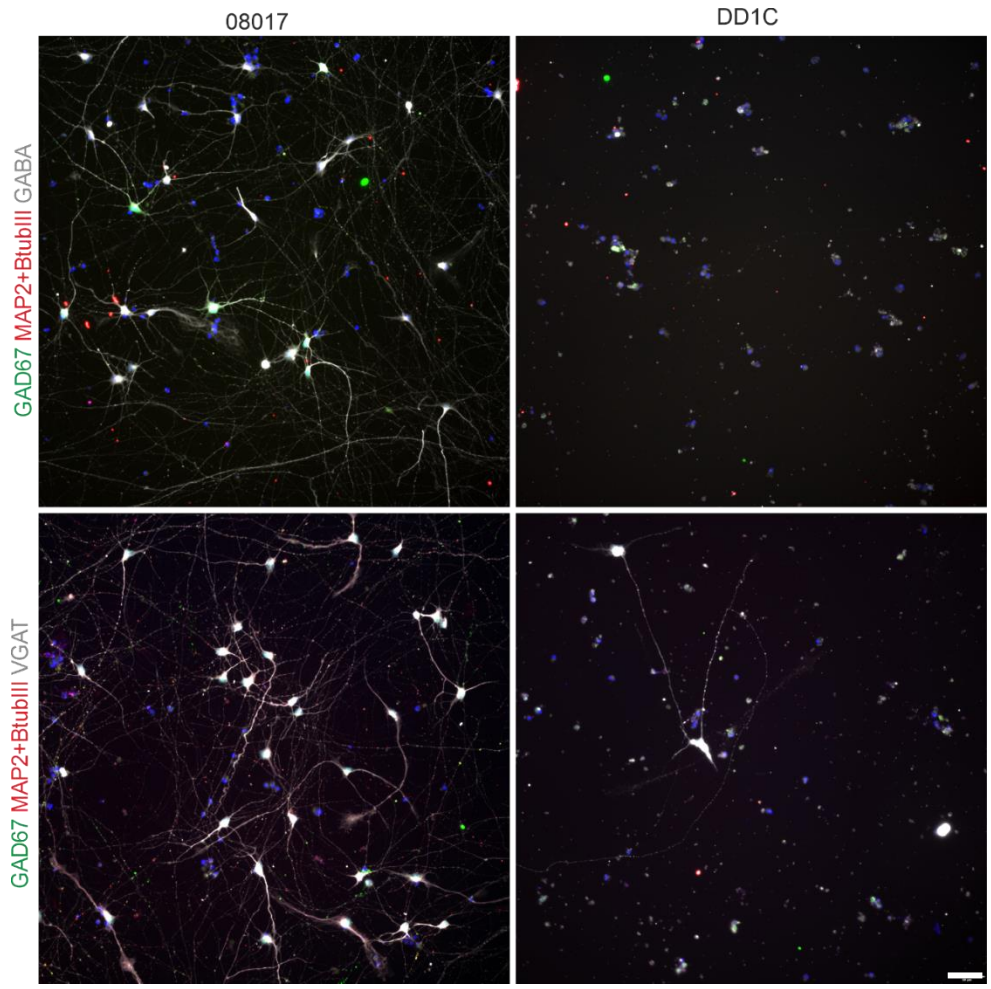


Figure 10. *Control and patient cell line fluorescence images of neurons on day 46 in method 2 plate. The cells were characterized with βIII-tub, GABA, GAD67, MAP2, and VGAT. Dapi nuclear staining is shown in blue. Scale bar is 50 µm.*

Overall, the expression of *GAD67* was very low at each time point. The expression of both *VGAT* and *GABA* were high and especially the morphology of control cells were good. Expression of all genes were higher on day 34 than day 46. However, the differentiation of control cell line was success and yield of patient interneurons was week. In Appendix 2, phase contrast images show how cells were looking in culture from the final plating day 17, until cells were fixed at day 46.

4. DISCUSSION

In this chapter, the discussion regarding the achieved results, their significance, error sources, deviations from the expected results, and the reliability of research, are presented. Aim of this study was to differentiate DS patient cell line DD1C and the control hESC line 08017 into GABAergic enriched interneurons. The purpose was to optimize the yield and survival of interneurons in long term culture by using different passaging methods on the final plating day. The neurons generated were characterized at the gene and protein level to study the success of the differentiation. The differentiation protocol was conducted according to Maroof et al. 2013, which was modified by Schuster et al. 2019.

The results showed that the neuronal differentiation into GABAergic interneurons was overall successful in both cell lines, even though the patient cell line differentiated poorly in comparison to the control line. The expression of *NKX2.1* and *SIX6* genes increased during neuronal differentiation in both studied cell lines indicating ventral patterning for the cortical progenitors (NPCs), although the expression levels was significantly higher in control line. As previous publications showed (6), the expression of MGE marker *NKX2.1* should increase until day 19 or even day 35, but here it was not observed, because expression levels of patient line started to decrease after day 12. However, the expression of *SIX6* should be high between day 12 to day 35 (6), which it was with both cell lines, to indicate starting of differentiation. It can be concluded that the ventralization was successful. The same result is also seen at the protein level in ICC analysis on day 12, where the expression of early neuroectodermal markers was high. Morphology of cells in immunostainings on day 19 were evident in cell bodies, dendrites, and axons, which were characterized with FoxG1, βIII-tub, GAD67, MAP2, and VGAT markers. Based on these results, the differentiation of GABAergic interneurons started successfully in this experiment especially with control line.

The analysis supports the fact that the patient cell line DD1C differentiated into GABAergic interneuron poorly in this study. Based on the findings of similar studies, there should be no big differences in expression levels between patient line and control line (14). Actually, DS line has a higher expression for many genes than the control line in the study by Schuster et al. 2019. The expression of *LHX6* and *NKX2.1* markers was lower with the patient line compared to the control line in this study, contrary to the study by Schuster et al. 2019. However, the expression curves of other genes like *SIX6, DLX2.1* and

GAD67 were mostly similar in this study compared to Schuster et al. 2019, even though the data of *DLX2.1* may not be reliable due to the lack of data.

The expression results of the *SCN1A* gene were poor, because the expression should be highest at day 19 as reported earlier (6), but in our study, the *SCN1A* expressed less than the calibrator in DD1C group at every timepoint. Higurashi et al. 2013 has done a similar study, where they found that the *SCN1A* tended to be expressed higher in the patient neurons than in control neurons, which was not observed in our study. Expression levels of *SCN1A* were much lower than was expected in patient line, but there was an error in our results, so the statement is not entirely reliable. The error was because there were no data available from the patient line from day 17 to day 34. In their study (7), they generated iPSCs from a DS patient with a point mutation in *SCN1A*. Because the mutation is not the same as in this study, we can't directly compare these results.

In this study, two different passaging and plating methods were compared for optimizing the neuronal differentiation. Method 2 had fewer plates and the difference between methods could be characterized only in protein level on day 34 and 46. Method 1 and method 2 had different plating and passaging strategy. On coverslips plate, method 1 was used. ICC characterization shows that especially the DD1C line did not survive on method 1 plate as well as in method 2 or coverslip plate. The coverslip plate wasn't the optimal plate for the control line due to the poor condition of the cells on day 46. Then the expression of VGAT and GABA in the immunostaining were not high. Both cell lines seem to survive most successful in method 2 culture. However, there was no big difference between methods, so it's difficult to say which one of the methods would be the most optimal to produce neurons in the long term in this experiment. Cells started to suffer earlier than expected and phase contrast images in Appendix 2 also support this statement. Our collaborators Schuster et al. 2019 recommended to use modified method 2, because it has worked well there. Method 1 has been tested at Neuro group twice before, and it has also proven to be promising.

Higurashi et al. 2013 used mostly similar differentiation protocol to differentiate GABAergic neurons for modelling Dravet syndrome. In the study by Higurashi et al. 2013, the immunocytochemical characterizations looked similar with our study in control group. They found that if neuronal morphology includes large and complex cell body with growth of more than four dendrites, the neurons are mature (7). From this it can be concluded that the 08017 neurons in this work differentiated into mature interneurons. Expression of GAD67 and VGAT indicates that the neurons were GABAergic interneurons which they should be at least after day 34 (6,7). DD1C group didn't grow complex cell body and the expression of GAD67 and VGAT wasn't that high, so they differentiated poorly to

GABAergic interneurons. Also, the expression of βIII-tub and GABA markers on day 65 in Schuster et al. 2019 were high, but in this study, the DD1C cell line suffered already on day 46 (Figure 10) indicating the same result.

It can be concluded that something unexpected happened in differentiation process because Schuster et al. 2019 succeeded in differentiation up to day 65 with all cell lines they used. This can be seen in the expression of key markers βIII-tub, GABA, GAD67, MAP2, and VGAT at maturation stage. Differentiation protocols were similar between cell lines and in both methods the DD1C line differentiated more poorly, so the reason cannot be in passaging methods. There was cell passaging and plating day on day 17 in this experiment, which may have affected cell growth and differentiation, because after that time point, expression of neural maturation markers decreased with patient line compared to control cell line. At the gene level, the expression of all genes varies between time points, so there is no clear sign of any error. It can also be stated with qPCR characterization, that the RQ-values of the patient line were much lower than the control line in every gene. However, nothing abnormal was reported, so the low expression levels can be due to the chosen amounts of nucleic acids to cDNA calculations, or the reasons could be inducted to the poor differentiation or too low amount of DD1C cells.

Our collaborators from Uppsala succeed in differentiating both cell lines until day 65 with using modified method 2 (6). Their method is modified from Maroof et al. 2013 study, where they noticed the importance of small-molecule-based strategy in efficient induction stage. We used same small molecules in this experiment, but they had different passaging days compared to the experiment in this study, which may affect the poor yield and survival of DD1C interneurons. They passaged cells on day 10, as final plating day, as in this study the final plating day was day 17. Neural induction, media changes and addition of ventralization and maturation growth factors were same. However, one experiment does not allow conclusions to be drawn as to which method would be more profitable to use. Also in our study, the cell amounts of GABA positive cells were not calculated, so it's harder to compare our results to others studies (7), because they have calculated GABA positive cell amounts. Because of this, the result should be examined critically. More research is needed.

The information obtained from this study will help to better understand the protocols behind the differentiation and further development of methods 1 and 2, in order to optimize the yield and survival. Future studies need to be done and it could be good to try other passaging and plating methods for differentiation, because the cells have not been able to be kept alive as long as would have been desired. The culture conditions in our study did not permit fully differentiation into GABAergic neurons, and more research is needed

to establish to enhance understanding of these DS models. However, this study illustrates markable potential of use of interneurons derived from patient for applications in disease models and regenerative medicine.

5. CONCLUSION

This chapter summarizes results of this thesis. The differentiation and characterization of the control hESC line 08017 and DS patient cell line DD1C was overall successful even though the patient line differentiated into GABAergic interneurons more poorly in comparison to the control line. There weren't any big differences in successful of differentiation between method 1 and method 2, though the survival of interneurons seems to be a little higher with method 2 in both cell lines. This was the third time using this differentiation protocol, so more experiments and studies need to be performed to optimize the yield and survival of differentiated interneurons in long term cultures. However, this study created valuable information of this protocol and these different methods and is the basis of the following experiments in Neuro group.

REFERENCES

1. Stiles J, Jernigan TL. The Basics of Brain Development. Neuropsychol Rev. 2010 Dec;20(4):327–48.

2. Fitzgerald M, Sotuyo N, Tischfield DJ, Anderson SA. Generation of cerebral cortical GABAergic interneurons from pluripotent stem cells. Stem Cells. 2020 Nov 1;38(11):1375–86.

3. Miller JW, Goodkin HP, editors. Epilepsy. Chichester, West Sussex: Wiley-Blackwell; 2014. 1 p.

4. Stafstrom CE, Carmant L. Seizures and Epilepsy: An Overview for Neuroscientists. Cold Spring Harb Perspect Med. 2015 Jun 1;5(6):a022426–a022426.

5. Ruffolo G, Cifelli P, Roseti C, Thom M, van Vliet EA, Limatola C, et al. A novel GABAergic dysfunction in human Dravet syndrome. Epilepsia. 2018 Nov;59(11):2106–17.

6. Schuster J, Laan L, Klar J, Jin Z, Huss M, Korol S, et al. Transcriptomes of Dravet syndrome iPSC derived GABAergic cells reveal dysregulated pathways for chromatin remodeling and neurodevelopment. Neurobiol Dis. 2019 Dec;132:104583.

7. Higurashi N, Uchida T, Lossin C, Misumi Y, Okada Y, Akamatsu W, et al. A human Dravet syndrome model from patient induced pluripotent stem cells. Mol Brain. 2013 Dec;6(1):19.

8. Mantegazza M. Dravet syndrome: Insights from in vitro experimental models: In Vitro Studies of Dravet Syndrome Mutations. Epilepsia. 2011 Apr;52:62–9.

9. Griffin A, Hamling KR, Hong S, Anvar M, Lee LP, Baraban SC. Preclinical Animal Models for Dravet Syndrome: Seizure Phenotypes, Comorbidities and Drug Screening. Front Pharmacol. 2018 Jun 4;9:573.

10. Hyvärinen T, Hyysalo A, Kapucu E, Aarnos L, Vinogradov A, Eglen SJ, et al. Functional characterization of human pluripotent stem cell-derived cortical networks differentiated on laminin-521msubstrate: comparison to rat cortical cultures. Sci Rep. 2019 Aug 29;16.

11. Sun Y, Paşca SP, Portmann T, Goold C, Worringer KA, Guan W, et al. A deleterious Nav1.1 mutation selectively impairs telencephalic inhibitory neurons derived from Dravet Syndrome patients. eLife. 2016 Jul 26;5:e13073.

12. Mzezewa R, Lotila J, Kiiski H, Vinogradov A, Kapucu FE, Peltola J, et al. A kainic acid-induced seizure model in human pluripotent stem cell-derived cortical neurons for studying the role of IL-6 in the functional activity. Stem Cell Res. 2022 Apr;60:102665.

13. Maroof AM, Keros S, Tyson JA, Ying SW, Ganat YM, Merkle FT, et al. Directed Differentiation and Functional Maturation of Cortical Interneurons from Human Embryonic Stem Cells. Cell Stem Cell. 2013 May;12(5):559–72.

14. Kim HW, Quan Z, Kim YB, Cheong E, Kim HD, Cho M, et al. Differential effects on sodium current impairments by distinct SCN1A mutations in GABAergic neurons derived from Dravet syndrome patients. Brain Dev. 2018 Apr;40(4):287–98.

15. Aarskog N, Vedeler C. Real-time quantitative polymerase chain reaction. Hum Genet. 2000 Dec 1;107(5):494–8.

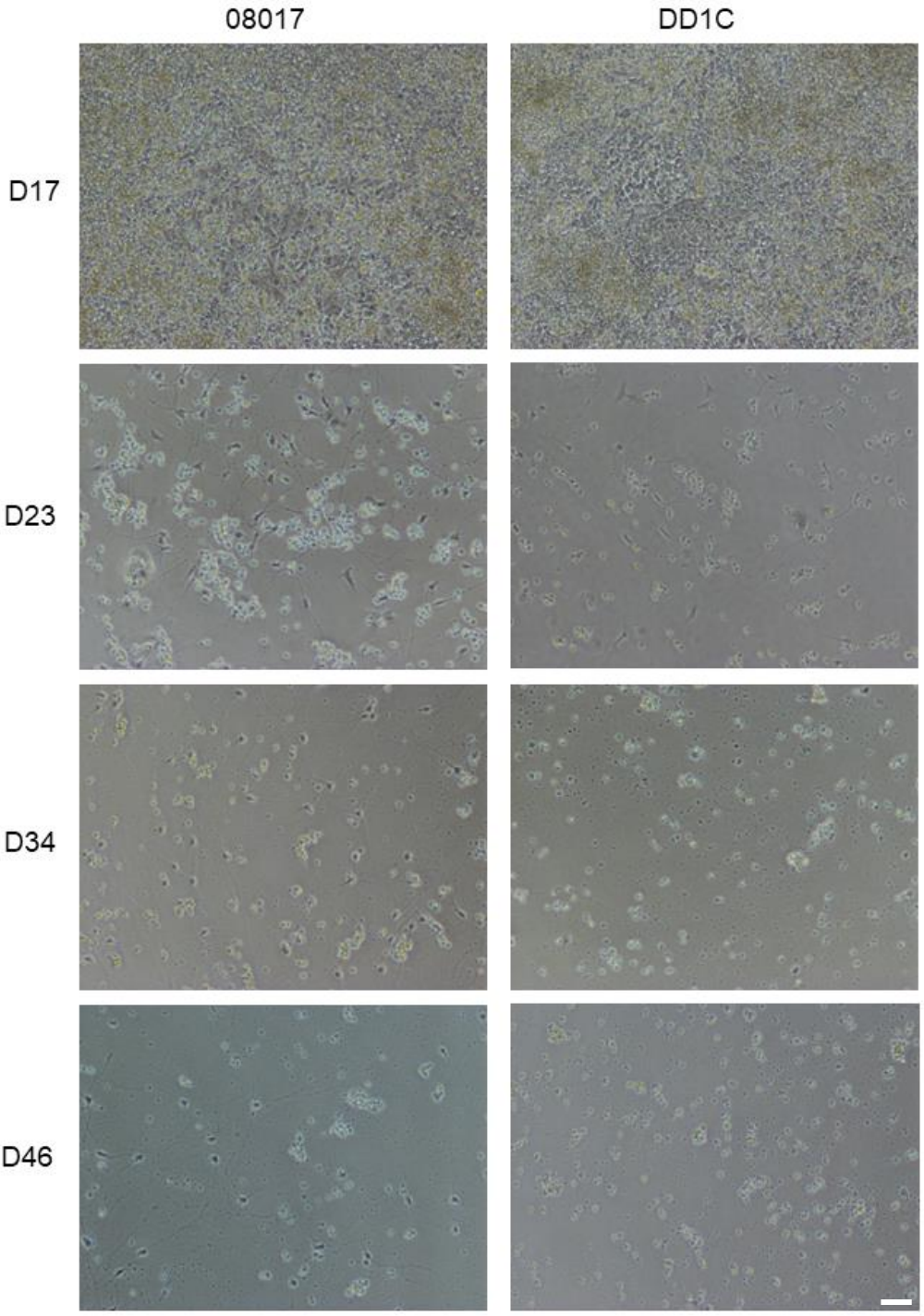
16. Burry RW. Immunocytochemistry [Internet]. New York, NY: Springer New York; 2010 [cited 2022 Jun 18]. Available from: http://link.springer.com/10.1007/978-1-4419-1304-3

17. Pelkonen A, Mzezewa R, Sukki L, Ryynänen T, Kreutzer J, Hyvärinen T, et al. A modular brain-on-a-chip for modelling epileptic seizures with functionally connected human neuronal networks. Biosens Bioelectron. 2020 Nov;168:112553.

APPENDIX 1: LIST OF PRIMARY AND SECONDARY ANTIBODIES

Time Point	Primary antibodies	Secondary antibodies
D12	*Staining 1:* Oct4 (goat, 1:200), FoxG1 (rabbit, 1:500) *Staining 2:* Pax6 (rabbit, 1:1000), Sox2 (mouse, 1:200)	*Staining 1:* donkey anti-goat 488, donkey anti-rabbit 568 (A10042) *Staining 2:* donkey anti-rabbit 488 (A21206), donkey anti-mouse 568 (A10037) All diluted 1:400, from Thermo Fisher Scientific
D19	*Staining 1:* FoxG1 (rabbit, 1:500), Btub3 (mouse, 1:1000) *Staining 2:* GAD67 (mouse, 1:100), MAP2 (chicken, 1:4000), VGAT (rabbit, 1:500) *Staining 3:* GAD67 (mouse, 1:100), Btub3 (chicken, 1:400), VGAT (rabbit, 1:500)	*Staining 1:* donkey anti-rabbit 488 (A21206), donkey anti-mouse 568 (A10037) *Staining 2-3:* donkey anti-rabbit 488 (A21206), donkey anti-mouse 568 (A10037), goat anti-chicken 647 (A21449, 1:200) All diluted 1:400 (except one marked), from Thermo Fisher Scientific
D34	*Staining 1:* GAD67 (mouse, 1:100), MAP2 (chicken, 1:4000), VGAT (rabbit, 1:500) *Staining 2*: GAD67 (mouse, 1:100), Btub3 (chicken, 1:400), VGAT (rabbit, 1:500) *Staining 3:* GAD67 (mouse, 1:100), MAP2 (chicken, 1:4000), GABA (rabbit, 1:1000) *Staining 4:* GAD67 (mouse, 1:100), Btub3 (chicken, 1:400), GABA (rabbit, 1:1000)	*Staining 1-4:* donkey anti-rabbit 488 (A21206, 1:400), donkey anti-mouse 568 (A10037, 1:400), goat anti-chicken 647 (A21449, 1:200) All from Thermo Fisher Scientific
D34 + D46 co-verslip + D46	*Staining 1:* GAD67 (mouse, 1:100), MAP2+Btub3 (chicken, 1:4000/1:400), VGAT (rabbit, 1:500) *Staining 2:* GAD67 (mouse, 1:100), MAP2+Btub3 (chicken, 1:4000/1:400), GABA (rabbit, 1:1000)	*Staining 1-2:* donkey anti-rabbit 488 (A21206, 1:400), donkey anti-mouse 568 (A10037, 1:400), goat anti-chicken 647 (A21449, 1:200) All from Thermo Fisher Scientific

APPENDIX 2: PHASE CONTRAST IMAGES



Method 1 control and patient cell line phase contrast images from day 17 to day 46. Scale bar is 50 µm.