In Vitro α-synuclein aggregation assay for studying induced cellular stress, drug targets and compounds

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Abstract
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ANTTILA, SEVERI: In Vitro α-synuclein aggregation assay for studying induced cellular stress, drug targets and compounds
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Parkinson's disease (PD) is most common movement disorder of the central nervous system in the world. Prevalence of PD increases by age, being 3000 per 100 000 in over 70 year olds. Pharmacological treatments of PD are based on relieving symptoms. There is an unmet medical need for disease modulating or preventing pharmacological treatments.

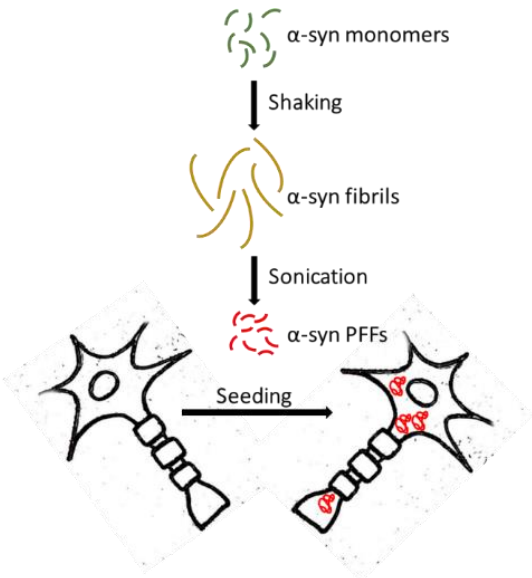
Emerging evidence suggests that formation and accumulation of α -synuclein aggregates in dopaminergic (DA) neurons cause severe axonal and synaptic degeneration. This toxic accumulation leads to death of DA neurons and significant reduction of DA neurons lead to unbalance in nigrostriatal circuitry. By preventing α-synuclein aggregation, disease progression could be modulated. Also, α-synuclein aggregation modulation might have positive effect for other synucleinopathies: Dementia with Lewy Bodies (DLB) and multiple system atrophy (MSA), as well.

This master's thesis describes the development of cell based *in vitro* α-synuclein aggregation assay for target validation and drug screening.

Monomer α-synuclein protein as such does not induce aggregation. Monomers can be transformed to Pre-formed fibrils (pFFs). This requires several days of shaking and sonication step to be transformed to pFFs which can trigger α-synuclein aggregation in cellular systems.

Several different pFFs and their ability induce aggregation were studied and α-synuclein aggregation in HEK-293 and human derived dopaminergic LUHMES cells were achieved.

The pFF induced aggregation will be studied further to optimize the setup for high throughput screening.



Keywords: Parkinson's disease, α-synuclein, aggregation, *in vitro*-model

Tiivistelmä
Turun Yliopisto
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Parkinsonin tauti (PD) on maailman yleisin keskushermostoperäinen liikkumishäiriö. PD:n esiintyvyys kasvaa iän mukaan, ja yli 70-vuotiailla se on 3000 sairastunutta 100000 henkilöä kohden. PD:n farmakologiset hoidot perustuvat oireiden lievittämiseen. Taudin etenemistä hidastavien ja pysäyttävien hoitojen tarve on suuri.

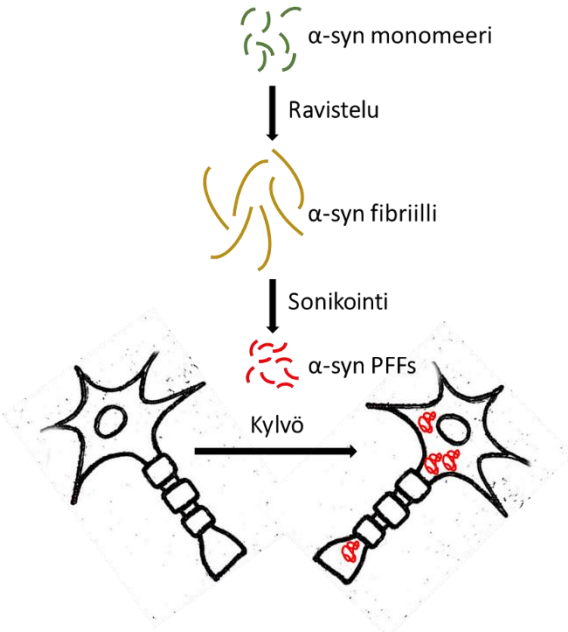
Uudet löydökset viittaavat siihen, että α-synukleiini-aggregaattien muodostuminen ja kertyminen dopaminergisiin (DA) neuroneihin aiheuttaa vaikeaa aksonaalista ja synaptista rappeutumista, joka lopulta johtaa DA-neuronien kuolemiseen. Merkittävä DA-neuronien väheneminen johtaa epätasapainoon aivojen nigrostriataalisessa järjestelmässä. Estämällä α-synukleiinin aggregaatiota, PD:n etenemistä voitaisiin mahdollisesti hidastaa. α-synukleiiniilla on merkittävä rooli myös muissa synukleopatioissa, Lewyn kappale-taudissa (DLB) ja monisysteemiatrofiassa (MSA).

Tässä Pro gradu -työssä esitetään soluihin perustuvan *in vitro* α-synukleiiniaggregaatiomalli lääkekohteiden validointia ja lääkeseulontaa varten.

Yksittäinen α-synukleiiniproteiini ei sellaisenaan aggregoidu. α-synukleiinista pitää muodostaa ravistelemalla ja sonikoimalla valmiiksi muotoutuneita fibrillejä (pFF), jotka pystyvät aiheittamaan aikaan solujen endogeenisen α-synukleiinin aggregoitumisen.

α-synukleiini monomeereistä saatiin muodostettua pFF:ä ja niiden avulla pystyttiin saamaan aikaan α-synukleiinin aggregoituminen HEK-293- ja ihmisestä peräisin olevissa dopaminergisissä LUHMES- soluissa.

Mallin kehittämistä ja optimointia jatketaan, jotta se soveltuisi tulevaisuudessa suren mittakaavan seulontoihin.



Avainsanat: Parkinsonin tauti, α-synukleiini, aggregaatio, *in vitro* -malli

Contents

1. Review of the literature

1.1 Epidemiology

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and the most common movement disorder of the central nervous system in the world. In 2015, it was estimated that up to 10 million individuals worldwide were affected by PD (Elbaz A, Carcaillon L et al. 2015). The incidence of PD is 15 per 100 000 people per year. The prevalence is 200 per 100 000 in unselected population (von Campenhausen, Bornschein et al. 2005). The frequency of PD increases with age as below the age of 50 years it is quite uncommon, but in elderly people above 60 years PD already affects 1% of the population (de Lau, Breteler 2006). Both the prevalence and incidence increase progressively after the age of 60, with a prevalence of over 1000 per 100 000 individuals in people over 70 years of age (Elbaz A, Carcaillon L et al. 2015)

PD is more common in men (1.5 times more) than in women. In Norway, there was an increasing occurrence in neurodegenerative diseases like ALS and PD in 2000 (Seljeseth, Vollset et al. 2000). The number of PD patients is expected to double between 2005 and 2030 in Western Europe (Dorsey, Constantinescu et al. 2007). In Finland, the number of PD patients is growing along with the increasing life expectancy, in the same way as in Europe. Interestingly, risk for PD seem to be slightly lower in the African and Asian countries than in the Western world. Geographical differences cannot, however, be directly compared due to methodological differences and differences in case-finding methods in the published articles and reports. (Elbaz A, Carcaillon L et al. 2015, Tysnes, Storstein 2017)

1.2 Clinical Features

PD commonly manifests as a movement disorder without toxic exposure or other known underlying etiology. The disease starts pre-symptomatically and usually progresses over 10 to 15 years, before producing severe motoric challenges. In PD, motor and non-motor symptoms are common. Bradykinesia, rigidity,

restlessness, and balance problems are most dominant motoric problems in PD. The most common initial symptom observed is the asymmetrical restlessness of the hands or fingers. Typical non-motor symptoms are depression, illusions, bladder dysfunction, sleep disorders, and loss of sense of smell. In addition, medication-related impulsive-compulsive disorders are common when patients are treated with dopamine receptor agonist. (Working group set up by the Finnish Medical Society Duodecim and the Finnish Neurological Society 2019)

1.3 Diagnosis

PD is usually diagnosed when the motor symptoms appear. Before motor symptoms, a prodromal phase can last up to 20 years or more before the diagnosis. Diagnosis of PD is demanding because of lack of specific tests. Diagnose itself is based on medical history, a review of patients' signs and symptoms, and a neurological and physical examination.

Signs that have been associated in increased risk of PD are decreased bowel movement, representing a 2.7-fold increase in risk of PD (Svensson, Henderson et al. 2016). One of the earliest signs of PD is loss of smell, but patients themselves do not usually notice that (Quinn, Rossor et al. 1987). In addition, hyposmia (Haehner, Hummel et al. 2007) and REM-sleep behavior disorder (Janković, Svetel et al. 2015) are associated with the risk of PD. Nevertheless, these findings do not clearly specify that PD causes these symptoms, thus they can not be used as a diagnostic criteria.

There is a great need for early diagnostics tools, but because of a lack of those, medication should be able to restore some of the ability or significantly slow down the disease progression. At the moment, none of the used drugs can cure or restore neuronal function, only symptoms can be relieved for a short period of time. Drugs with disease-modifying properties remain the ultimate unmet medical need.

1.4 Pathophysiology

PD is mainly sporadic but also both autosomal dominant and recessive genetically-linked forms of the disease exist. Heritable forms of Parkinson disease represent 5–10% of all cases (Pringsheim, Jette et al. 2014). Toxins could also induce PD. In 1976, a home chemist synthesized MPPP (Desmethylprodine) opioid class drug with MPTP as a major impurity. MPTP is a prodrug to the neurotoxin MPP+. Three days after self-injection the result, PD like motor symptoms occurred. From autopsy, the Lewy bodies and loss of dopaminergic neurons in the substantia nigra were discovered. (Fahn 1996)

The most important pathological feature is the loss of dopaminergic neurons in the substantia nigra pars compacta. Loss of dopaminergic neurons is first restricted to the ventrolateral substantia nigra, but later spread wider to other midbrain dopaminergic neurons (Fearnley, Lees 1991, Damier, Hirsch et al. 1999). The loss of the nigrostriatal dopaminergic pathway is causing the classic motor symptoms of Parkinson's disease (Kalia, L., Lang, A., 2015).

Another characteristic feature of Parkinson disease is widespread accumulation of intracellular presynaptic α-synuclein protein. The abnormal deposition of monomeric α-synuclein in the cytoplasm leads to oligomerization of the protein and further into aggregated form of α-synuclein. Formation and accumulation of α-synuclein aggregates in DA (dopaminergic) neurons cause severe axonal and synaptic degeneration. Later this accumulation leads to death of DA neurons. Sequentially, significant reduction of DA neurons leads to unbalance in nigrostriatal circuits. (Caligiore, Helmich et al. 2016, Brundin, Dave et al. 2017)

These α-synuclein aggregates have a role in Lewy body formation in the later stages of the disease. Lewy bodies are proposed to be toxic to neurons, causing neuronal loss and the non-motor symptoms of Parkinson's disease. Lewy bodies occur in dopaminergic neurons and in neurons in the olfactory system, causing the loss of smell. Later stage of disease, within 1 year of the onset of motor symptoms, dementia that arises, is referred to Lewy body dementia (LBD). (Kalia, L., Lang, A., 2015)

1.5 α-Synuclein

α‑synuclein has a key role in the pathogenesis of Parkinson's disease. It is prominent in Lewy bodies and in Lewy neurites in all synucleinopathies, Parkinson's disease (PD), Dementia with Lewy Bodies (DLB) and multiple system atrophy (MSA). α-synuclein is a small 140 amino acid protein, encoded by SNCA gene (NP_001139527). Middle part of the protein is hydrophobic region that has a high propensity to aggregate (Giasson, Duda et al. 2000). α-synuclein might exist as an intrinsically disordered protein or unstructured monomer in the cell (Binolfi, Fernández et al. 2012, Burré, Vivona et al. 2013, Fauvet, Mbefo et al. 2012). α-synuclein can be found from neurons, where it normally has a function in synaptic transmission via recycling of synaptic vesicles (Burré 2015, Jacqueline Burré, Manu Sharma et al. 2017). In synaptic transmission, α-synuclein is bound to membranes of synaptic vesicles. It is shown that synucleins have critical function in presynaptic SNARE-complex assembly. SNARE-complex is protein complex which primary role is to mediate vesicle fusion. α-synuclein directly binds to the SNARE-protein and synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) and promotes SNARE-complex assembly (Jacqueline Burré, Manu Sharma et al. 2010). SNARE-complex role is to mediate intracellular vesicle fusion to membrane. In the presynaptic membrane in neurons SNAREs are those that mediate docking of synaptic vesicles and enable release of neurotransmitters to synaptic cleft.

α-synuclein in the cytosol is in soluble and unfolded monomeric state (Burré 2015, Bendor, Logan et al. 2013, Jacqueline Burré, Manu Sharma et al. 2017). Another possible form is stated to be tetramer (Bartels, Choi et al. 2011) but this is debated (Fauvet, Mbefo et al. 2012). In pathological states, for an unknown reason, monomeric α-synuclein forms higher molecular weight insoluble α-synuclein fibrils (Wang, Zhao et al. 2016).

α-synuclein is endogenously expressed in neurons especially in the dopaminergic circuitry. High concentrations of α-synuclein is also present in red blood cells, but the specific function there is not known (Barbour, Kling et al. 2008). In gastrointestinal (GI) tract, gut cells express α-synuclein and α-synuclein aggregates and Lewy bodies are present in gut. Braak have hypothesized that

changes in gut microbiome cause α-synuclein aggregation in the gut and α-synuclein propagates via the vagus nerve to the brain in a prion-like fashion. (Braak, Rüb et al. 2003, Tredici, Braak 2008)

Aggregated α-synuclein and cellular processes that are involved in aggregation are promising drug targets. Lewy pathology inclusions consist mainly of α-synuclein and lipids, and experimental models indicate that α-synuclein aggregation induces neuronal dysfunction and cell death (Iacono, Geraci-Erck et al. 2015, Lewis, Spillane 2019). Mutations and multiplication of SNCA gene can lead to neurodegenerative conditions. One known and widely studied α-synuclein mutation is A53T, which results in a form of α-synuclein more prone to aggregate than the wild type counterparts. On the other hand, overexpression of mutant α-synuclein alone in neuronal and non-neuronal cells as well as primary neurons derived from α-synuclein transgenic mice does not lead to significant α-synuclein inclusion formation (Mazzulli, Mishizen et al. 2006).

In rodents, injections of pre-formed α-synuclein fibrils (pFFs) induce α-synuclein aggregation and wide spreading α-synuclein accumulation in the brain (Mahul-Mellier, Altay et al. 2018). Similar effects can be detected when primary neurons and differentiated dopamine neurons are seeded with pFFs to induce endogenic α-synuclein aggregation (Mahul-Mellier, Altay et al. 2018).

1.5.1 α-synuclein as a drug target

Aggregation and Lewy body formation cascade are not fully understood, and many questions remain open. Aggregation of α-synuclein might be caused by declined cellular functionality, such as declined mitochondrial or lysosomal function. Thus, pathway-specific malfunction would cause α-synuclein aggregation as an outcome. Another major question is that could a normal aging of cells induce dysfunction in upstream of α-synuclein physiological degradation pathway and cause aggregation as an endpoint.

α-synuclein has important physiological functions in neurons but aggregated forms and Lewy body functions are not understood. 10-30% of normally aged individuals exhibit Lewy pathology in the brain without any associated symptoms

(Iacono, Geraci-Erck et al. 2015). It is also unclear whether the α-synuclein aggregates are primarily causing the cell death or is the degeneration due to other forms of α-synuclein. It is not fully understood which form of α-synuclein is the most harmful, but studies suggest that the soluble oligomers of α-synuclein are the toxic species. It has been suggested that α-synuclein aggregates have been actively formed by cell as a protective mechanism, to bind and inactivate the more toxic oligomeric species. Aggregation and formation of Lewy bodies might be intracellular response to reduce acute toxicity. In pathophysiological brain samples, oligomeric forms and fibrils are stated to be in equilibrium. It is not known what specific event or series of events cause changes in this equilibrium. The equilibrium between oligomer and fibrillary forms might be valuable target to modify. When creating medicines against α-synuclein aggregation, it needs to be considered which form is targeted. (Wang, Zhao et al. 2016, Brundin, Dave et al. 2017)

Lewy pathology does not develop simultaneously in every part of the brain. Most vulnerable parts initially are olfactory bulb and dorsal motor nucleus of the vagus. α-synuclein pathology in these parts of the brain does not cause the end phase disease symptoms. The declination in ability to smell is one of the early sign of prodromal PD.

Several studies have shown that α-synuclein pathology can be transmitted in a cell-to-cell fashion especially from gut to brain via vagus nerve (Li, J., Lashley et al. 2008, Luk, Song et al. 2009, Breen, Halliday et al. 2019). Studies and epidemiological findings support the theory that α-synuclein could spread through vagus nerve. When PD patient-derived α-synuclein lysate or monomeric, oligomeric, or fibrillar forms were injected into the intestine of rats, immunoreactivity in the intestinal wall and vagus nerve were detected in a time-dependent manner (Holmqvist, Chutna et al. 2014, Manfredsson, Luk et al. 2018).

In addition, it has been studied that partial resection of vagus nerve (vagotomy) as a treatment on peptic ulcer disease decreases the risk of PD (Liu, Fang et al. 2017). Vagus nerve might offer a way for α-synuclein spreading to the brain, but actually, it is appendix that is stated to be the most abundant part of GI-track for α-synuclein aggregation. Appendix also lacks the blood–tissue barrier and this

might facilitate contact with the enteric nervous system to facilitate even more spreading. Similar to vagotomy, appendectomy has been shown to lower the risk of PD (Breen, Halliday et al. 2019). This supports the possibility that α-synuclein aggregation in the intestine could have an effect on the spreading of α-synuclein and that it may induce central nervous system (CNS) related pathology. α-synuclein aggregation in the GI track might also be an interesting target to modulate spreading of α-synuclein pathology. (Breen, Halliday et al. 2019)

1.5.2 Reducing α-synuclein production

During ageing, people have a decreased α-synuclein mRNA expression in their brain but still high levels of α-synuclein is present. Post-translational stabilization, like phosphorylation, might cause or promote accumulation of monomeric α-synuclein. This accumulation of endogenic α-synuclein is associated with decreased dopamine levels in the striatum but not with the dopaminergic neuronal death. Increased stabilization of α-synuclein during ageing increases protein levels and accumulation, which may also increase the amount of toxic forms of α-synuclein protein in the cytosol. Increased stabilization might also decrease normal protein degradation which in turn increases accumulation (Li, W., Lesuisse et al. 2004). On the other hand, excessive reduction of α-synuclein in substantia nigra of rodent and nonhuman primates leads to nigrostriatal system degeneration. (Collier, Redmond et al. 2016, Gorbatyuk, Gorbatyuk et al. 2010)

It is evident that α-synuclein has a crucial role in physiological function of neurons and thus downregulation of α-synuclein mRNA might not be the optimal target for modulating drug. Currently one antisense therapy is being tested in clinical settings. This antisense therapy targets α-synuclein mRNA to reduce the amount of mRNA and total α-synuclein production (Cole, Paumier et al. 2016).

Another drug on clinical trials for reducing α-synuclein levels is 2-adrenergic agonists that are used for treating asthma. 2-adrenergic agonists reduce α-synuclein mRNA levels probably through an epigenetic mechanism presumably involving Histone 3 lysine 27 acetylation. People that use this kind of asthma medication have reduced prevalence for PD. (Mittal, Bjørnevik et al. 2017)

Reduction of α-synuclein might have limited ability to treat PD. However, controlling the right level of α-synuclein i.e. finding the therapeutic window might be challenging, and thus other means are being investigated for finding a safer and more efficient ways to treat patients.

1.5.3 Inhibiting α-synuclein aggregation

Preventing α-synuclein aggregation is one possible option to prevent loss of dopaminergic neurons. The normal function of α-synuclein in synaptic transmission can be sustained and the toxicity that follows misfolding can be prevented.

Neuropore Therapies in partnership with UCB Pharma have created therapeutic small-molecule lead NPT200–11. It has been reported to prevent the formation of toxic aggregates and improve both behavioral and neuropathological outcome measures in α-synuclein over-expressing transgenic mouse model. NPT200-11 and its analog NPT100-18A interfere the interaction between α-synuclein and lipid membranes. This interaction is a crucial step in the oligomerization and further aggregation of α-synuclein. (Price, Koike et al. 2018, Wrasidlo, Tsigelny et al. 2016)

Pro-clara Bioscience has developed NPT088, a molecule which is a bacteriophage capsid fusion protein designed to simultaneously target multiple misfolded proteins. Pro-clara has reported that NPT088 binds α-synuclein aggregates and has a positive effect on tyrosine hydroxylase levels in an α-synuclein overexpressing transgenic mouse model. (Krishnan, Tsubery et al. 2014)

Modulating an upstream point in the pathogenic α-synuclein misfolding cascade has been considered as a good approach to prevent α-synuclein aggregation. This approach might have the wanted effects on α-synuclein aggregation, but the higher the target is in the cascade the more challenging it is to validate the right pathway and anticipate possible side effects (Sardi, Cedarbaum et al. 2018). Small-molecules targeting specific proteins that enhance autophagy or increase phosphorylation and ubiquitination (which have effects on lysosomal

degradation) may show effects for reducing the amounts of α-synuclein. Phosphorylation and ubiquitination strongly regulates degradation of intracellular protein by affecting lysosomal functions (Nguyen, Kolch et al. 2013). As an example, c-Abl tyrosine kinase activation is a key indicator of oxidative stress (Li, B. 2005) and the activation is strongly associated with PD (Imam, Zhou et al. 2011, Schlatterer, Tremblay et al. 2011). Nilotinib is one of the second-generation c-Abl tyrosine kinase inhibitors, which are more selective and potent compared to first-generation c-Abl inhibitors. It has also moderate brain penetration (Soverini, Martinelli et al. 2012) It has been found to reduce α-synuclein pathology by enhancing autophagy which has critical role in α-synuclein degradation (Karuppagounder, Brahmachari et al. 2014). In safety trials, Nilotinib showed efficacy with acceptable toxic parameters. This proofs that c-Abl has effects on α-synuclein pathology (Pagan, Hebron et al. 2016).

1.6 *In vitro* research of PD

In vitro research of PD has been done from various points of views e.g. neurotoxicity of toxins like 6-OHDA and MPP+ (Xiao-min Zhang, Ming Yin et al. 2014), excitotoxicity, α-synuclein aggregation and its effects on neuron survival, and mitochondrial function (Brundin, Dave et al. 2017). End points of these studies have been T helper cell numbers, α-synuclein aggregates, neurite outgrowth and apoptosis (Brundin, Dave et al. 2017). α-synuclein aggregation is one of promising targets for intervention, and prevention of aggregation and especially degradation of α-synuclein aggregates might relieve patients' symptoms. Here, the focus is on α-synuclein aggregation assays since that is the key question of this master's thesis.

In *in vitro* settings, various study materials have been used to study the aggregation of α-synuclein in PD. Neuronal cells of animal origin e.g. primary mouse or rat cells have been successfully used (Volpicelli-Daley, Luk et al. 2014, Masliah, Rockenstein et al. 2000) but the relevance of these for human disease is vague. Human cells have also been studied, e.g. human embryonic kidney (HEK)-293 cells (Krumova, Meulmeester et al. 2011) as the simplest type. Even

though the disease pathophysiology has been detected in these cells (Guo, Scarlata 2013), the relevance of the HEK cells can be somewhat questioned due to their lack of CNS properties and fast replication. The utilization of immortalized cell lines such as SH-SY5Y (Kovalevich, Langford 2013) might give somewhat elusive results due to the characteristics of an immortalized neuroblastoma cell line.

The cells in other organisms have been promising, but not necessarily relevant for human disease. LUHMES (Lund Human Mesencephalic) cells are human derived cells, which have DA cell properties after differentiation (Scholz, Pöltl et al. 2011a). Previous α-synuclein aggregation studies with LUHMES cells have lasted only for 6 days due to enormous cell death (Höllerhage, Goebel et al. 2014). As formation of α-synuclein aggregates inside the cells takes ~6-8 days. LUHMES neurons survival are not the most suitable for this kind of an assay. For studying ways to protect dopaminergic cells from α-synuclein induced toxicity, a clear need for cells enabling longer culturing periods exist.

Induced pluripotent stem cells (iPSC) provide an excellent and representative model for DA neurons in PD research. With iPSC derived DA neurons α-synuclein aggregation and degradation can be studied. IPSC have indeed been used previously for studying the effects of α-synuclein mutations and small molecule drug development studies (Aflaki, Borger et al. 2016). These cells have been shown to recapitulate some characteristics of PD that cannot be modeled in other *in vitro*-models (Torrent, De Angelis Rigotti et al. 2015). The advantage of iPSC-based models is that they are of human origin and they have not been immortalized. Thus they are quite suitable *in vitro* cell type for PD research (Zhang, Chen et al. 2017).

However, iPSC models for PD are far beyond the proof-of-principle stage, and researchers and industry have started to optimize and standardize the several varieble differentiation protocols (Ryan, Dolatabadi et al. 2013) and efficient cell-sorting methods (Schöndorf, Aureli et al. 2014) for these cells. It is expected that these efforts help towards the discovery of new aspects of disease biology and new targets for therapeutic intervention.

2. Aims, hypotheses and goals

The aim of this Master's thesis was to create a reproducible *in vitro* α-synuclein aggregation assay that can be used to study induced cellular stress, drug targets and compounds. This work consists of two parts, 1) preparation of pre-formed fibrils (pFFs) and testing their capacity to induce aggregation of transfected α-synuclein within HEK-293 cells. In addition, 2) the setup was then tested in more neuronal like cell culture system.

Based on literature, hypothesis was that aggregation could be achieved via introduction of pFFs to cellular systems where α-synuclein is expressed. Expression can be endogenous or induced. When working with non-neuronal cells, external vectors such as plasmid are needed to get high α-synuclein quantity which is likely required for successful aggregation and to meet this requirement. In more neuronal like systems the endogenous expression of α-synuclein could be high enough for the aggregation to occur without extra α-synuclein expression/delivery. It is likely that aggregation influences the cellular stress response pathways and vice versa. Non-neuronal cells were seeded with pFFs, and formation of intracellular α-synuclein aggregation was studied determining changes in protein and RNA levels by immunocytochemistry, western blotting and real-time quantitative PCR. α-synuclein aggregation model has been successfully created for primary hippocampal neurons from C57Bl/6 mice (Volpicelli-Daley, Luk et al. 2014).

3. Results

3.1.1 Cell culturing

HEK-293 (human epithelial kidney) cells were routinely cultured in DMEM containing 10% of FBS and 1% of PenStrep in humified 37°C with 5% CO_2. The cells were split every 3 to 4 days when 90 % confluence was achieved

3.1.2 α-synuclein plasmid vectors

HEK-293 cells express only a small amount of α-synuclein endogenously. Higher α-synuclein expression can be achieved by transfecting HEK-293 cells with an SNCA gene plasmid that encodes α-synuclein production.

Expression plasmids were designed by and ordered from GenScript. Human SNCA transcript variants 1, 2 and 3 were selected for studies. In addition, α-synuclein A53T mutant (a known PD risk mutant), α-synuclein mouse and α-synuclein rat plasmids were ordered for testing as well.

3.1.3 HEK transfection and RNA and protein expression

First transfection experiment with variant 1 was performed by using 1.6 µg of plasmid for 300.000 cells in a well in 6-well plate. Normal HEK-medium was used during transfection. Two hours after initiating the transfection all cells spontaneously detached from the surface. After 24 hours, the cells had re-attached to the surface, but they formed a big cell aggregate in the center of the well (Figure 1). All four plasmids α-synuclein human variant 1, α-synuclein A53T mutant, α-synuclein mouse and α-synuclein rat plasmids showed toxicity to the cells as they detached regardless of the plasmid.

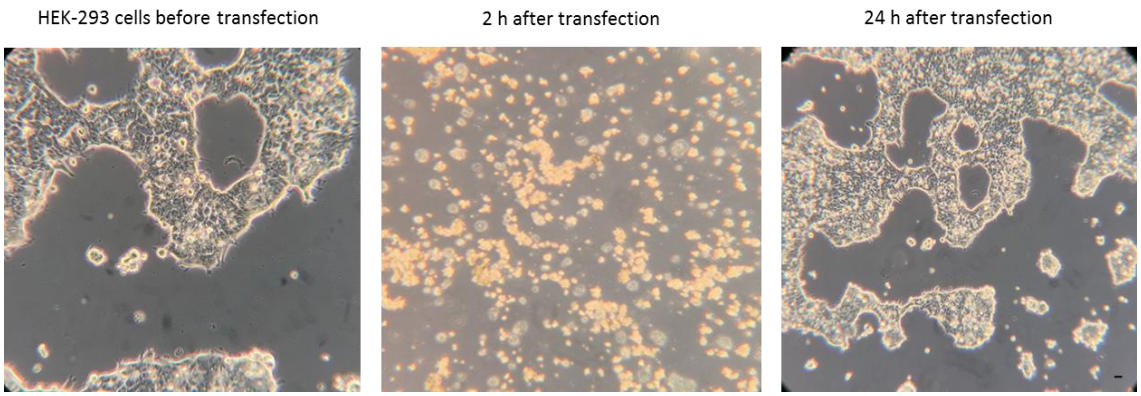


Figure 1. Transfected HEK-293 cells. HEK-239 cells detached during transfection (2 hrs.), but reattached later again (24 hrs.). Scale bar 20 µm

To enhance the cell attachment, next transfection experiment was performed with Poly-D-Lysine coated plastic wares. This time only α-synuclein human variant 1

was used. Cell viability after transfection of 3 µg, 1 µg or 0.3 µg plasmid for T-75 flask was phenotypically better compared to previous transfections. Phenotypic and morphological evaluation indicated that transfection with 10 µg plasmid for T-75 flask caused notable decrease in cell count. Transfection with 3 µg or less was not as toxic for HEK-293 cells than transfection with higher plasmid concentrations (Figure 2). Thus, the next HEK-293 cell transfections were performed on Poly-D-Lysine coated plastic ware.

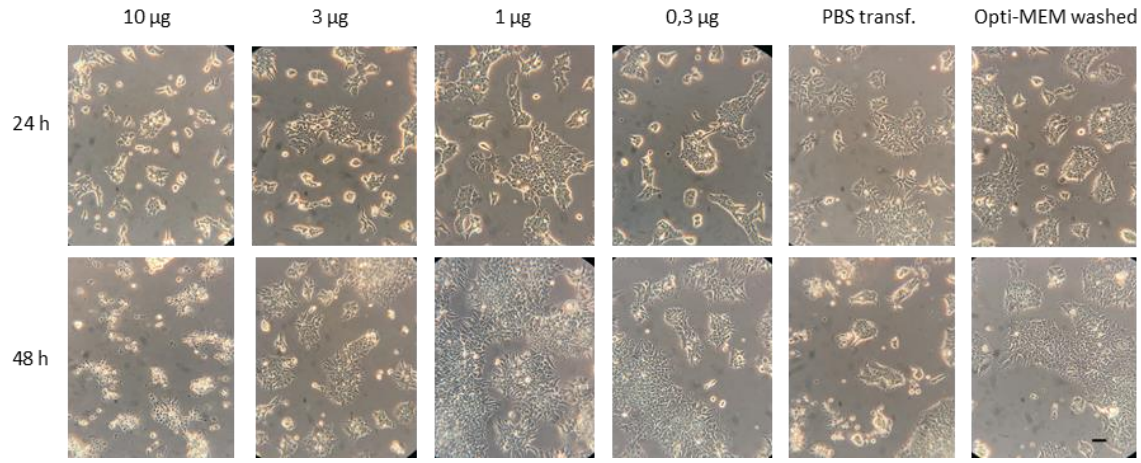


Figure 2. HEK-293 cell survival after transfection. The morphology of HEK-293 cells 24- and 48-hours following transfection with different amounts of plasmid. Scale bar 20 µm.

3.1.4 α-synuclein mRNA levels after transfection

After successful transfection, the mRNA levels of α-synuclein in the cells should transiently increase. Thus, transfection efficiency was measured using quantitative RT-PCR. In order to enable the aggregation of endogenous α-synuclein, this step with HEK-293 cells is crucial and the time of peak value of mRNA needs to be verified. Timeframe between transfection and mRNA peak levels may vary and qRT-PCR analysis can be used to detect this.

qRT-PCR analysis was performed on five different transfections (Fig 3). HEK-293 cells were transfected with 1) 3 µg of α-synuclein human variant 1. 2, 3) 2.5 µg and 5 µg of α-synuclein human variant 2, and 4, 5) 2.5 µg and 5 µg of α-synuclein

human variant 3, in T-75 flask each. Mock transfected HEK-293 cells (transfection without DNA) were used as control group to show level of endogenous α-synuclein.

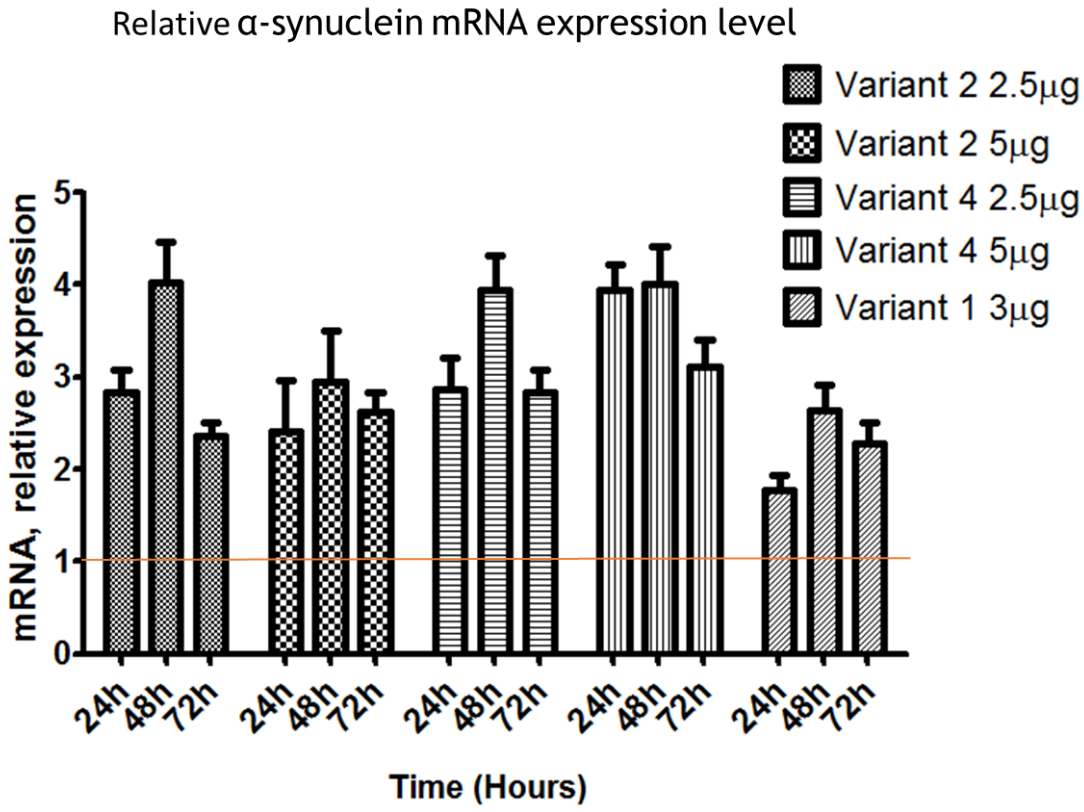


Figure 3. α-synuclein gene expression. Relative mRNA expression level compared to mock transfected HEK-293 cells. Relative expression level to housekeeping gene GAPDH, basal level 1 from MOCK transfected cells is cells endogenous α-synuclein level. All 5 plasmid transfected cell lines α-synuclein mRNA levels increases compared to control cells.

Relative mRNA levels were two to three times higher 24 hours after transfection when compared to the negative control and the expression of α-synuclein was up to 4 times higher than in negative control 48 hours after the transcription (Figure 3). The α-synuclein gene expression started to decline thereafter as seen from expression levels 72 hours post transfection. The results were similar for all of the plasmid variants and concentrations, but with α-synuclein human variant 3, the highest plasmid concentration resulted in highest relative gene expression.

3.1.5 α-synuclein protein levels

Western blot analyses were performed several times to determine the α-synuclein protein levels in the cells collected 48 hours after α-synuclein human variant 1 transfection. Indeed, western blotting clearly showed that transfected HEK-293 cells expressed detectable amounts of α-synuclein protein (Figure 4.).

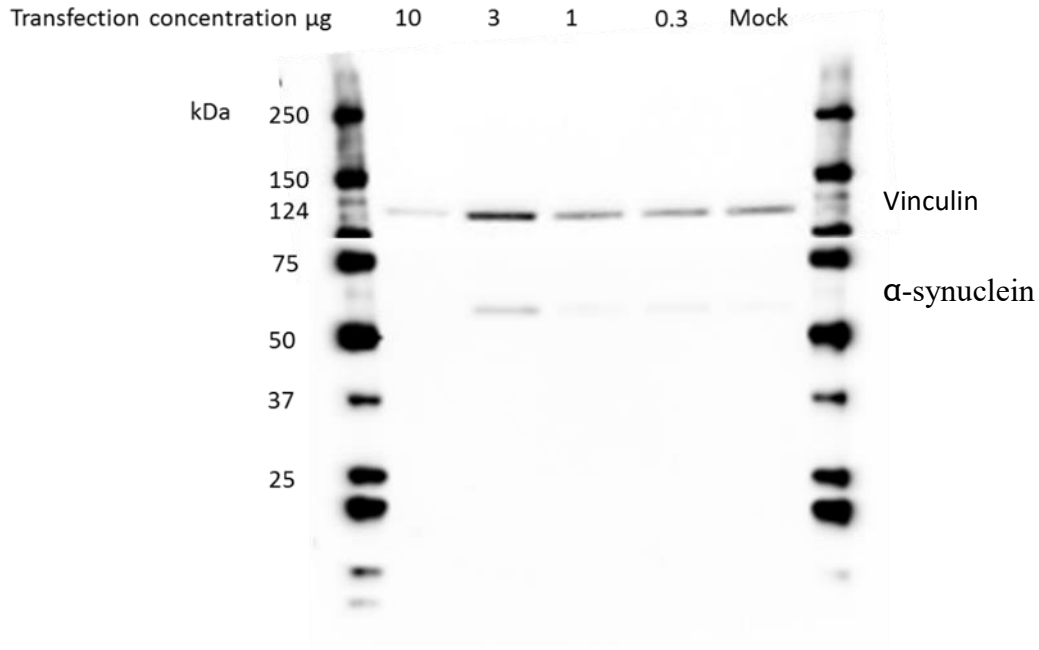


Figure 4. α-synuclein protein expression. The weaker band at 57-60 kDa represents α-synuclein and the stronger band at 124 kDa is the loading control vinculin. 10 µg of protein from α-synuclein human variant 1 transfected cells were loaded to first well. For another well's protein was loaded 26 µg.

3.1.6 Pre-formed fibrils, pFFs

α-synuclein fibrils were made from α-synuclein monomers. Monomers were purchased from Proteos and pFFs were produced by following a protocol published by Michel J. Fox foundation (MJFF) "Protocol for generation of pre-formed fibrils from alpha-synuclein monomer". After 1 week in orbital shaker, monomer solution appeared turbid, which was one of the verification steps of the fibril formation. Aliquots of samples were stored in -80°C and sonicated for pFF

forming prior to use. Fibrils need to be sonicated prior to use to produce pFFs and sonication has effects on seeding capacity and efficiency. Indeed, MJFF and others have stated that the sonication step in this assay is critical for the success of producing suitable pFFs for aggregation studies. Several sonication parameters were tested and the pFFs from those setups were seeded onto HEK-293 cells. (Please see details in materials and methods section). The seeding capacity of the pFFs was assessed by studying the endogenous α-synuclein aggregation.

3.1.7 pFFs seeding experiment

α-synuclein plasmid variant 1 transfected HEK-293 cells were seeded with pFFs. Seeded pFFs should induce endogenous α-synuclein to aggregate. This α-synuclein aggregation phenomenon was studied with immunocytochemical (ICC) stainings and quantified with Operetta imaging system. pFFs which were sonicated the longest induced more efficient α-synuclein aggregation than the pFFs with shorter sonication (Figure 5 and 6). Thus, sonication parameter 1-4 efficiency on pFF seeding quality was compared to sonication parameter 5 (Figure 7 and 8). Cells were fixed 3 days after the pFFs seeding. The primary antibody used in these experiments was α-Synuclein (D37A6) XP®. This primary antibody was tested with WB to detect α-synuclein fibrils (Supplemental figure 1).

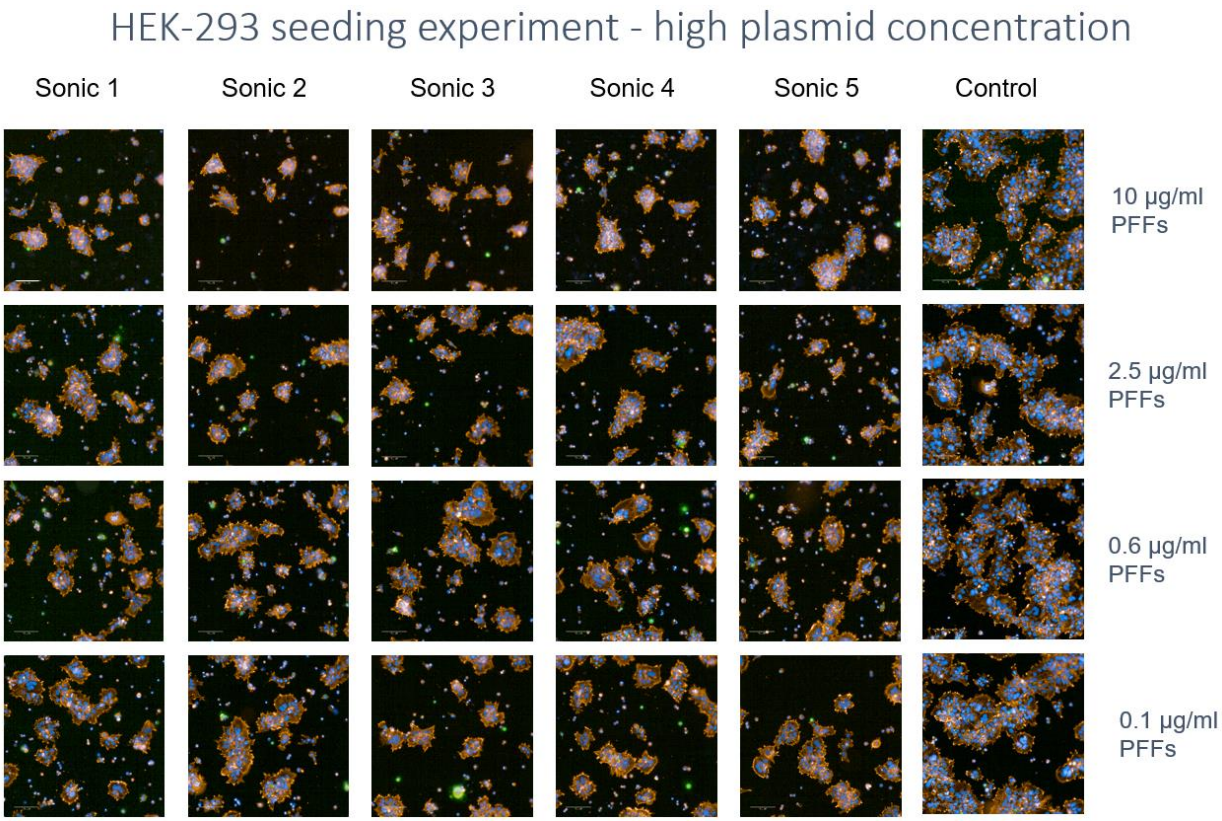


Figure 5. HEK-293 seeding experiment – high plasmid concentration. HEK cells transfected with 6 µg α-synuclein human variant 1 plasmid for T-75 flask. Green; α-synuclein, red; phalloidin, blue; DAPI. Scale bar 100 µm.

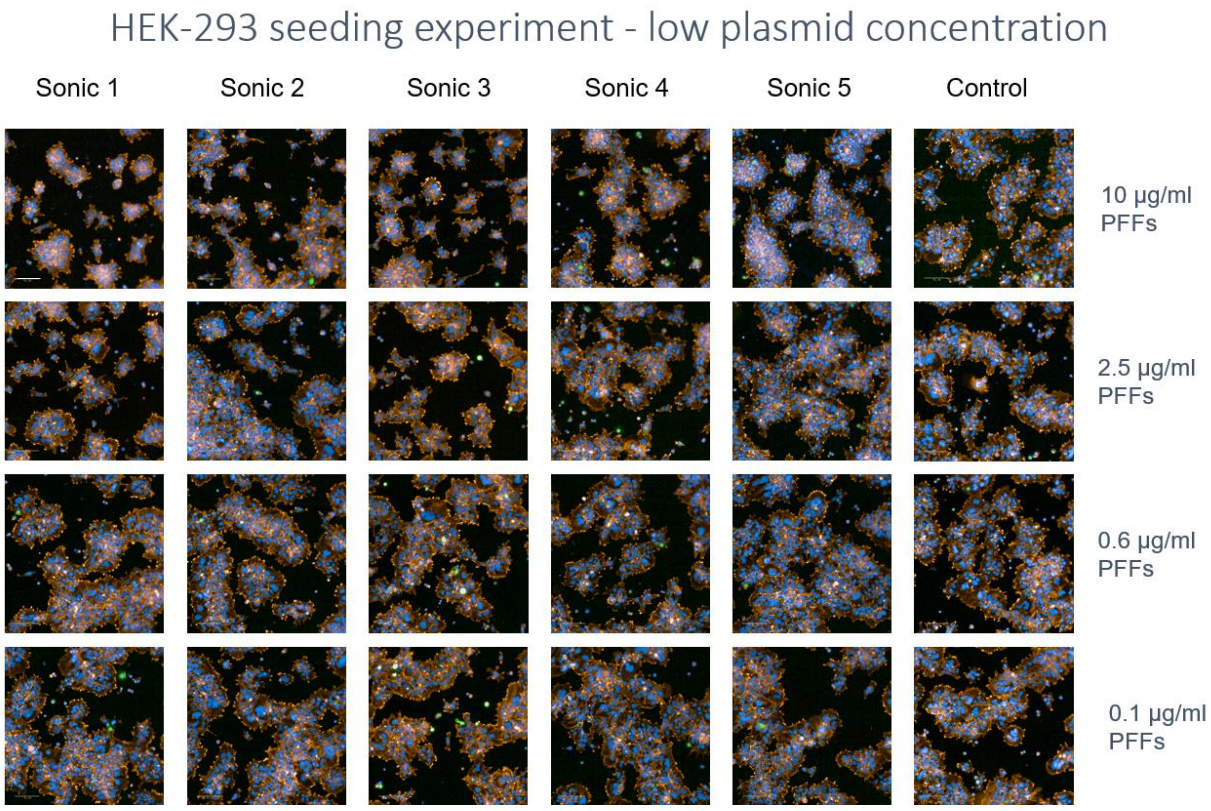


Figure 6. HEK-293 seeding experiment – low plasmid concentration. HEK cells transfected with 3 µg of plasmid for T-75 flask. Cells were fixed 3 days after seeding. Green; α-synuclein, red; phalloidin, blue; DAPI. Scale bar 100 µm.

The tested α-synuclein plasmid concentrations did not have concentration dependent effects on the pFF induced α-synuclein aggregation in HEK-293 cells. High content Operetta images were used for quantifying the differences in seeding efficiency of pFFs sonicated with different parameters (Figure 7 and 8).

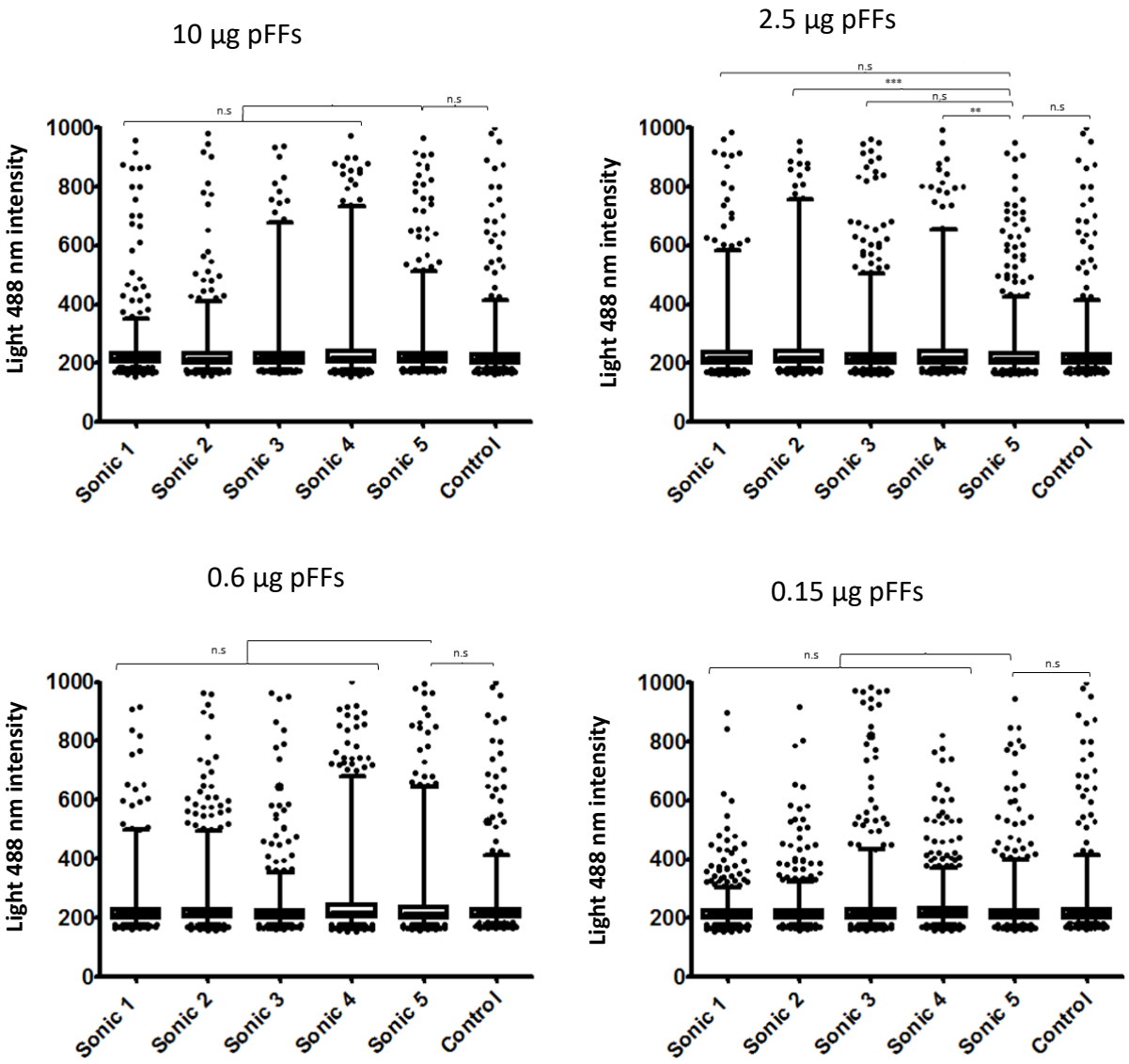


Figure 7. HEK-293 seeding experiment – high plasmid concentration. HEK-293 cells were transfected with 6 µg α-synuclein human variant 1 plasmid. pFF seeding concentration were 10 µg, 2.5 µg, 0.6 µg, 0.15 µg. Control cells were transfected but were not seeded with PFFs. α-Synuclein (D37A6) XP® and Alexa 568 antibody complex light emission were measured and compared between groups (Figure 7 and 8).

When HEK-293 cells were transfected with higher amount of plasmid, differences between groups were not significant. Only differences were found when seeding was done with 2.5 µg of pFFs from sonication parameter 2 and 4 compared to sonication parameter 5.

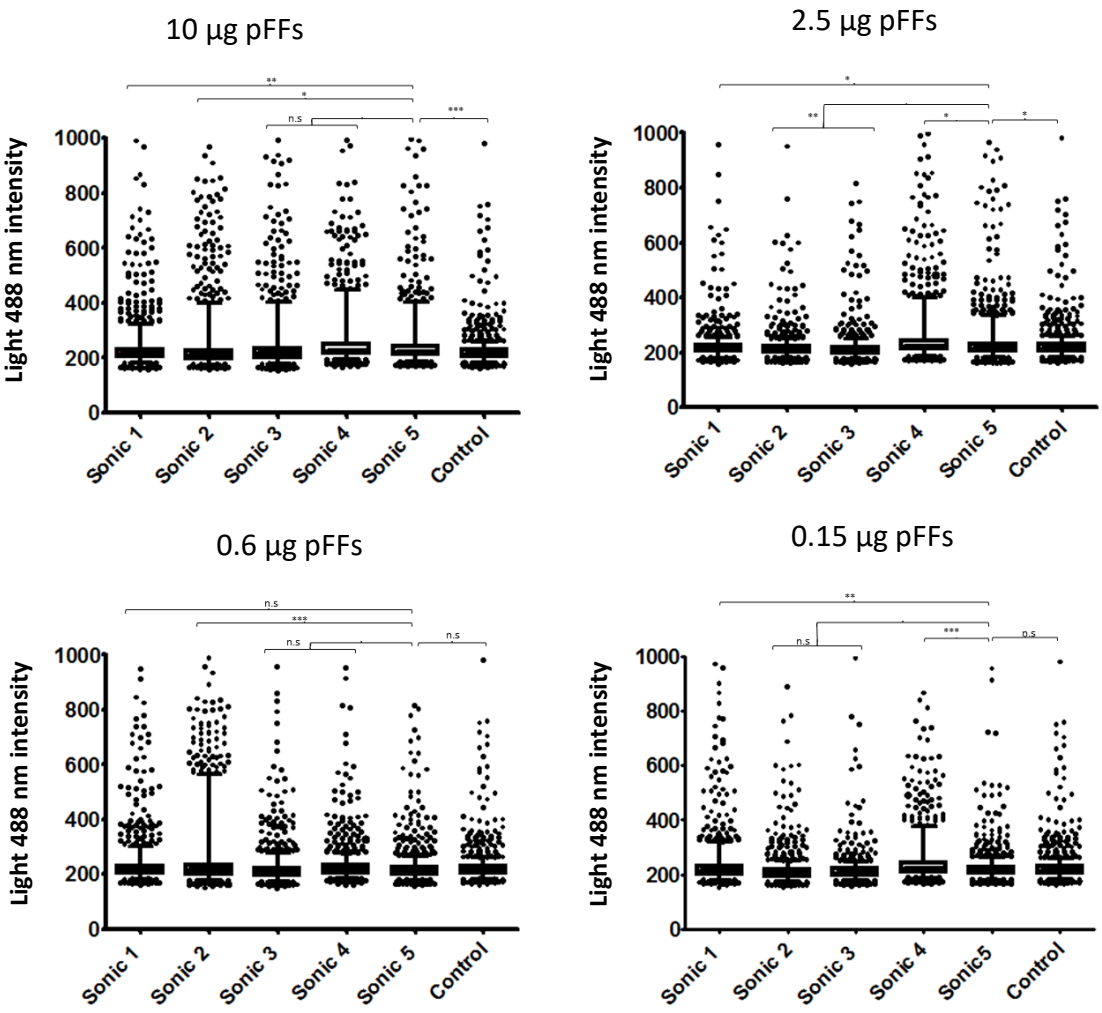


Figure 8. HEK-293 seeding experiment – low plasmid concentration. HEK-293 cells were transfected 3 µg α-synuclein human variant 1 plasmid. Fibril seeding concentration were 10 µg, 2.5 µg, 0.6 µg, 0,15 µg. Control cells were transfected but were not seeded with PFFs. α-Synuclein (D37A6) XP® and Alexa 568 antibody complex light emission were measured and compared between groups (Figure 7 and 8).

3.2 LUHMES *in vitro* experiments

3.2.1 Differentiated LUHMES cell survival

The survival of differentiated LUHMES cells is limited. Previously LUHMES PD models have survived up to 6 days after differentiation (Höllerhage, Goebel et al. 2014). For aggregation studies, longer surviving cell models are needed. Survival

of LUHMES cells under different differentiation protocols (Figure 9) was elucidated.

Next, it was tested whether replating cells already on the day 1 in vitro (DIV) has positive effect for survival and neuronal morphology (Figure 10). Neuronal cells, regardless of the day of replating, survived until DIV 5 (Figure 11). The morphological changes of the cells during differentiation are presented in Figure 10. The somas seemed bigger if the cells were replated on DIV 1 when compared to DIV 2. The cells replated on DIV2 were generally smaller and looked more like neuronal cells (Figure 10). Days in vitro before replating have an effect on maturity of cells. Differentiation to DA neurons continues on 96 well plate after replating, but if the cells are replated on DIV 2, their ability to proliferate after replating is decreased, indicating more neuronal phenotype. This was clearly detected as the DIV 1 replated cultures grew more confluent than the DIV 2 replated cultures.

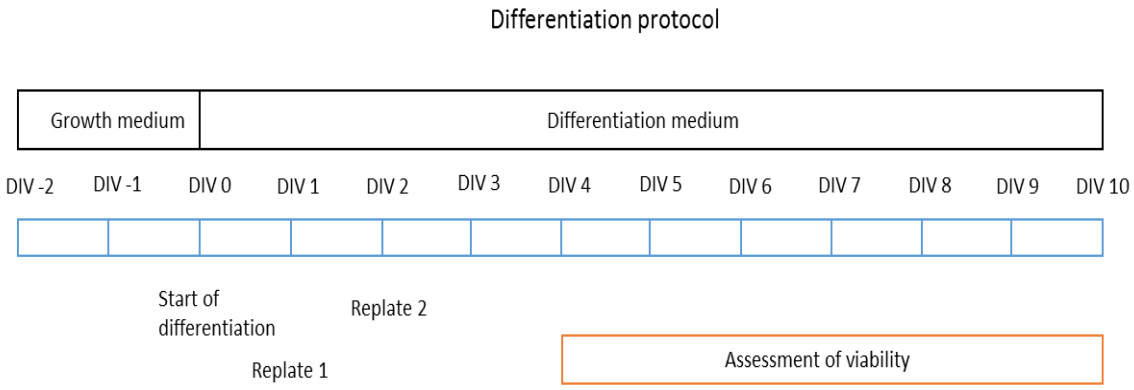


Figure 9. LUHMES cell differentiation protocol.

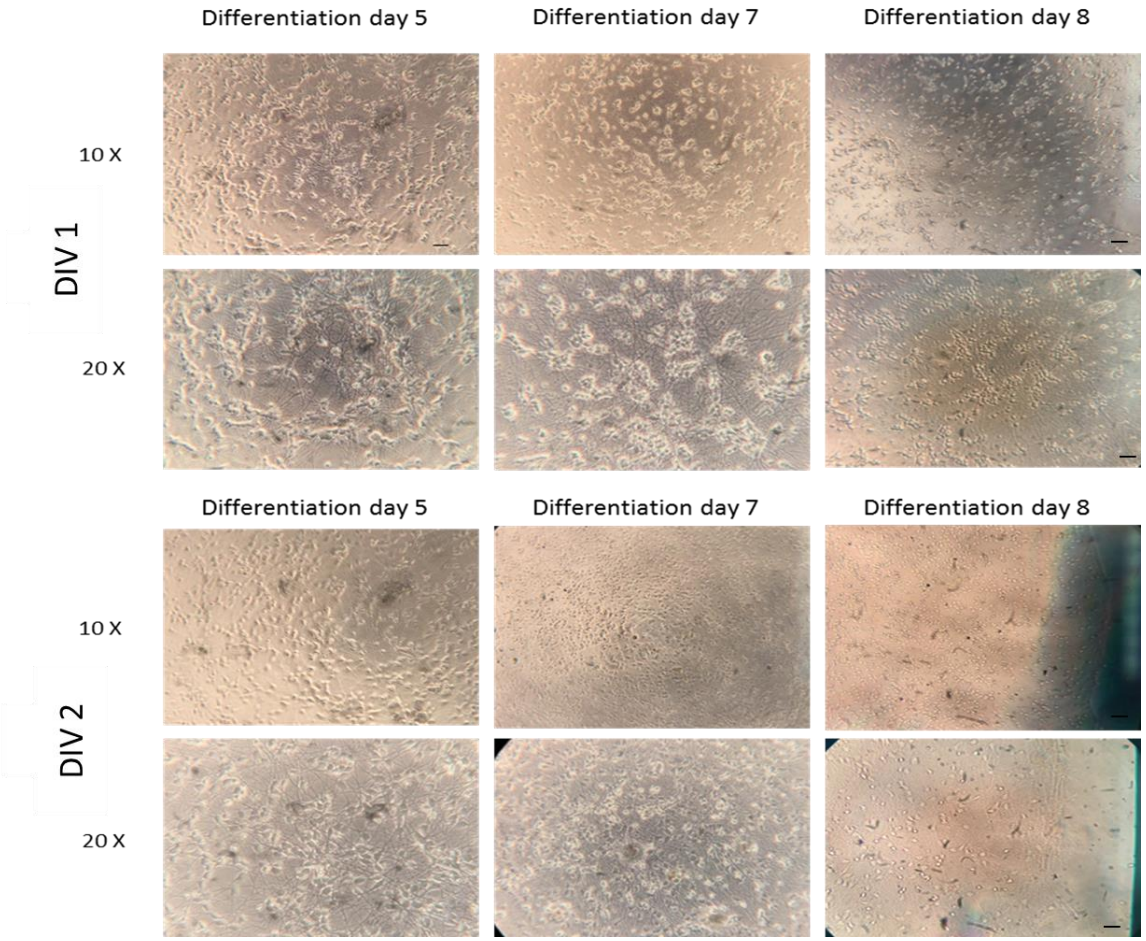


Figure 10. Differentiated LUHMES cells. Cells were replated in 150,000 cells/cm^2 in PDL+ fibronectin coated 96 well plate. The cells replated on DIV 1, upper panel or DIV 2, lower panel. Scale bar 20 µm.

Next, the LUHMES cells' overall survival was assessed in regards to the replating day. After DIV 8, all the DIV 2 replated cells were lost while the survival of DIV 1 replated cells was higher. If the cells were replated on DIV 1, over 90% of the wells contained viable cells on DIV 6. On DIV 7, viability started to decrease in the wells with 150,000 cells/cm2 and 100,000 cells/cm2 cells, but still over 80% of the wells contained viable cells. On DIV 8 decrease in cells' viability could be detected, but the wells with more sparse cultures seemed to survive better (Figure 11). This experiment clearly showed that LUHMES cells survive surely for only 5 days in 96-well plate.

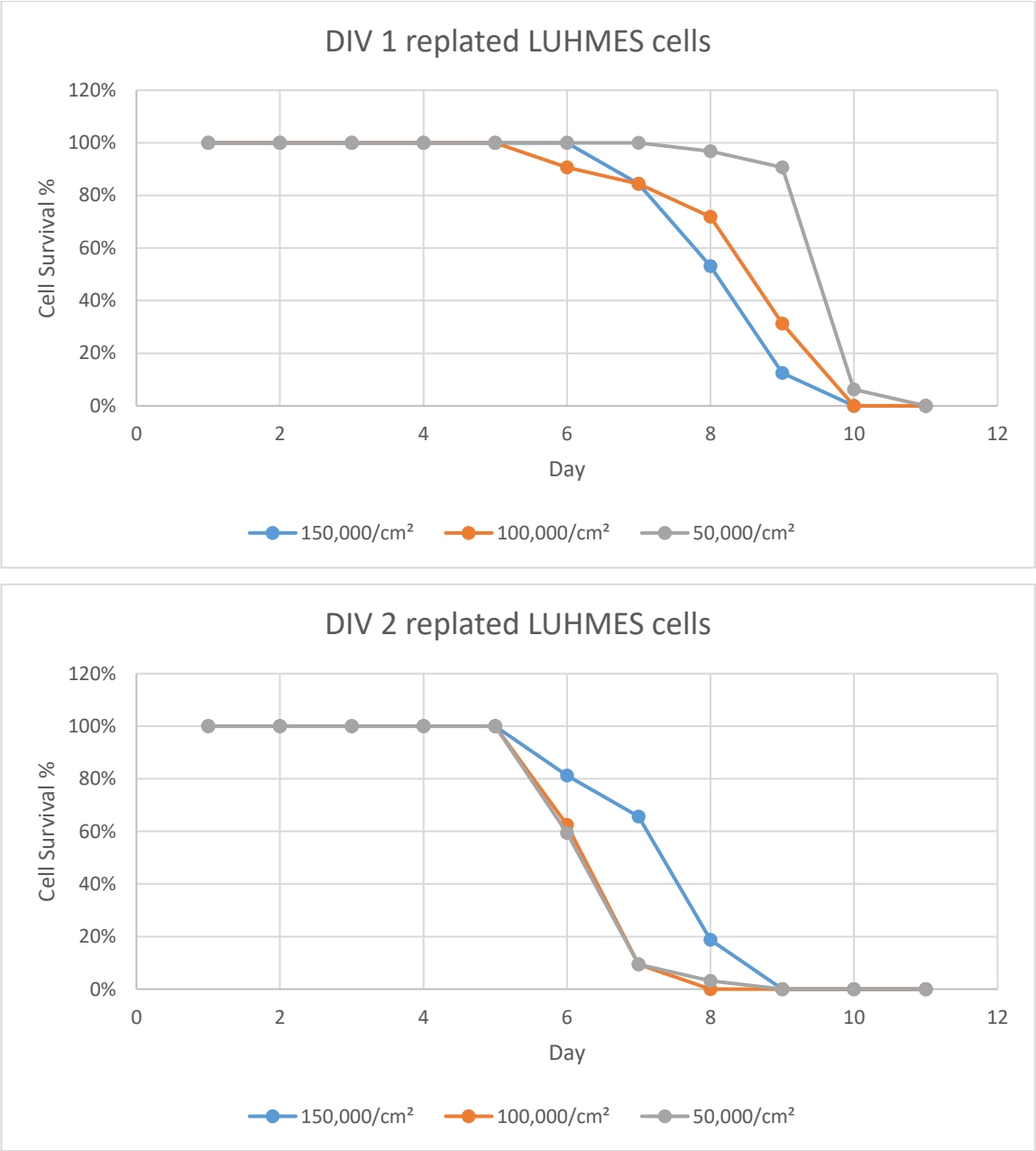


Figure 11. Cell survival after re-plating. Cell survival was studied with visual inspection. Cells were considered dead if neuronal morphology was lost in the well. N=32

3.2.2 GrowDex® effect for LUHMES survival

GrowDex® (GD) is a hydrogel extracted from birch, made by UPM Biomedicals. GD mimics the extracellular matrix (ECM) and it effect for LUHMES survival were studied. GD concentrations 0.015% and 0.03% had no effects on cells survival.

On the contrary, on DIV 6-7 the viability decreased. Control cells without GD or with 0.075% GD survived slightly better. Overall, GD did not show significant effects on LUHMES cells survival at DIV 1 replated cells (Figure 12 A). DIV 2 replated cells with 0.03% GD survived slightly longer than the controls or other concentrations (Figure 12 B).

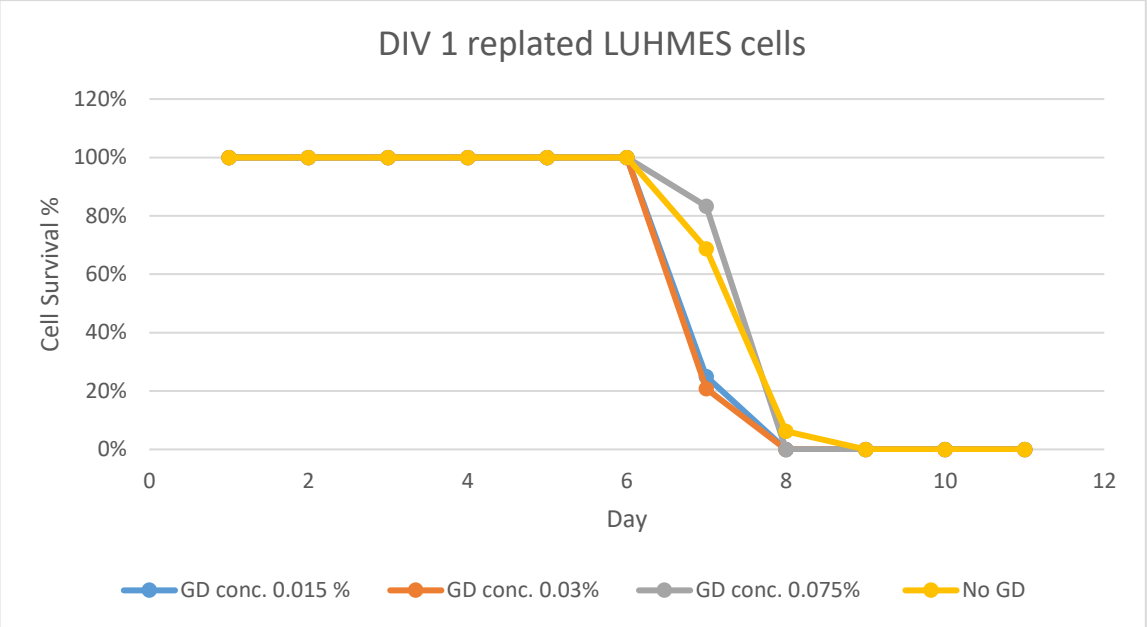


Figure 12 A. Cell survival after re-plating. GD concentration 0.015% n=24, 0.03% n=24, 0.075% n=16 and control cells without GD n=16 effects on LUHMES cells' survival.

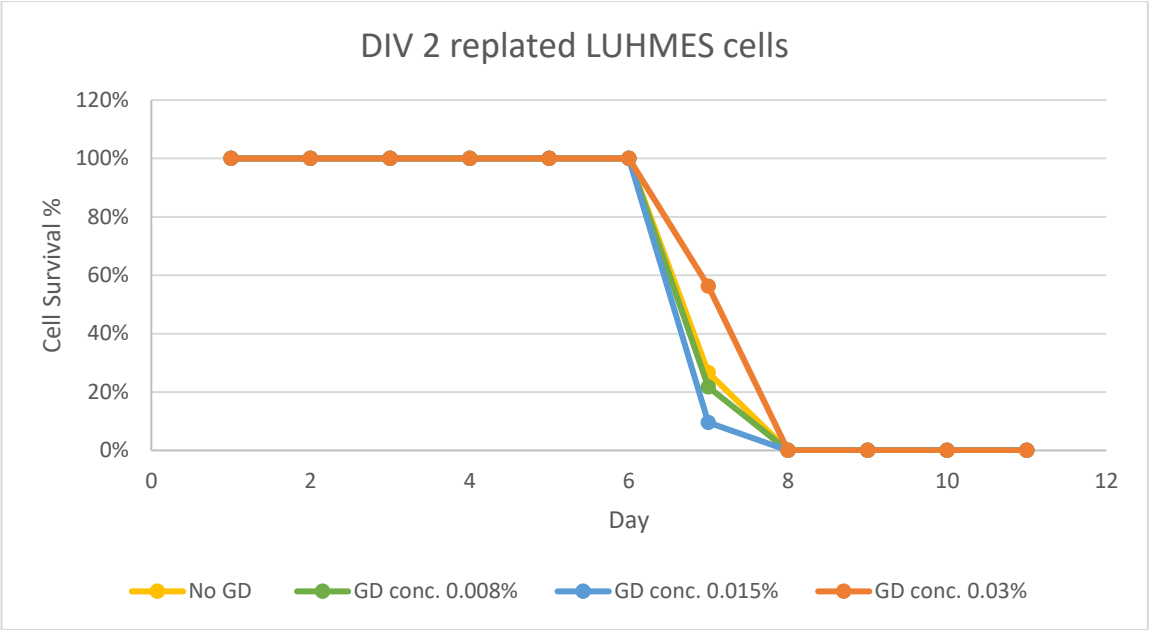


Figure 12 B. Cell survival after re-plating. GD concentration 0.008% n=23, 0.015% n=21, 0.03% n=24 and control cells without GD n=16 effects on LUHMES cells' survival.

3.2.3 LUHMES cells endogenic α-synuclein protein Western Blotting analysis

The protein expression pattern of LUHMES cells changes during differentiation and mature DA neurons have clearly different expression pattern from the precursor state. Endogenous α-synuclein is produced after differentiation initialization and this was confirmed with wester blotting analysis.

Small amount of α-synuclein is expressed in undifferentiated LUHMES cells. After differentiation α-synuclein production increases remarkably and stays quite steady during maturation. Vinculin at 124 kDa was used as a loading control and the used α-synuclein primary antibody detects α-synuclein at 60 kDa. The reactivity of the primary antibody against α-synuclein fibrils (before sonication) was also tested. The used primary antibody did not detect the synthetic α-synuclein (Figure 13).

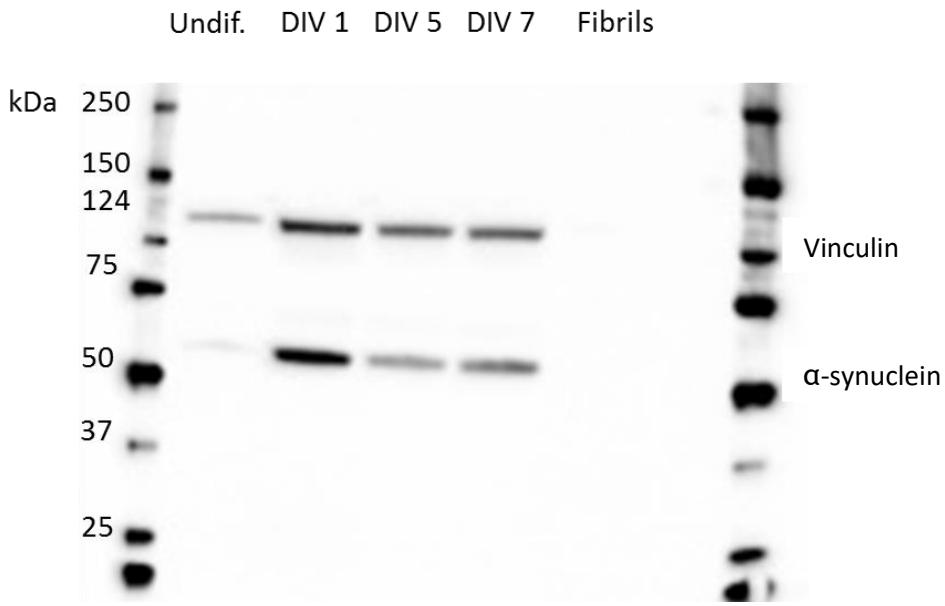


Figure 13. α-synuclein protein expression in LUHMES cells. Loading control was vinculin at 124 kDa and used α-synuclein primary antibody detect α-synuclein at 60 kDa.

3.2.4 LUHMES cells fibril seeding experiments

LUHMES cells were seeded with pFFs similar to HEK-293 cells. Endogenous α-synuclein aggregation was studied with ICC stainings and quantified with Operetta imaging system. Endogenous α-synuclein aggregates were detected in LUHMES cells two days after the seeding. pFFs with longer sonication parameters (4 and 5) induced α-synuclein aggregation better than the other pFFs with shorten sonication (Figure 14 and 15). Highest seeding concentration induce highest fluorescence detection. Control cells were not seeded. The fluorescence in cells seeded with sonication 5 pFFs was statistically significantly higher $p < 0.001$ than in control cells.

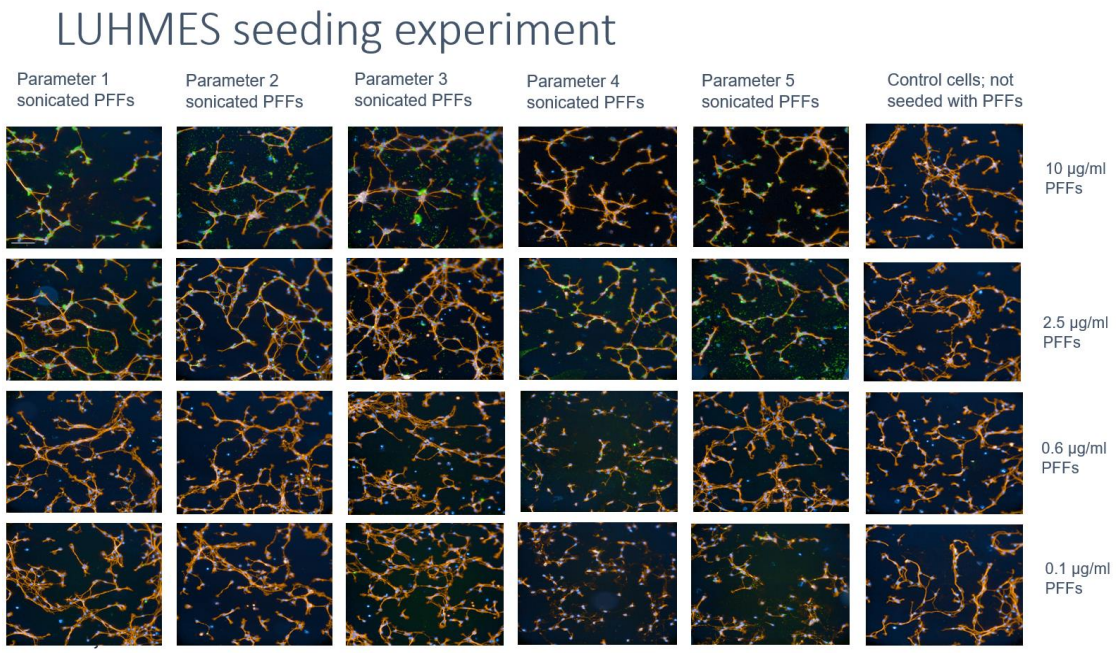


Figure 14. LUHMES seeding experiment. Cells were seeded with pFFs and D37A6 primary antibody was used to detect α-synuclein. Green; α-synuclein, red; TUJ-1, blue; DAPI. Scale bar 100µm

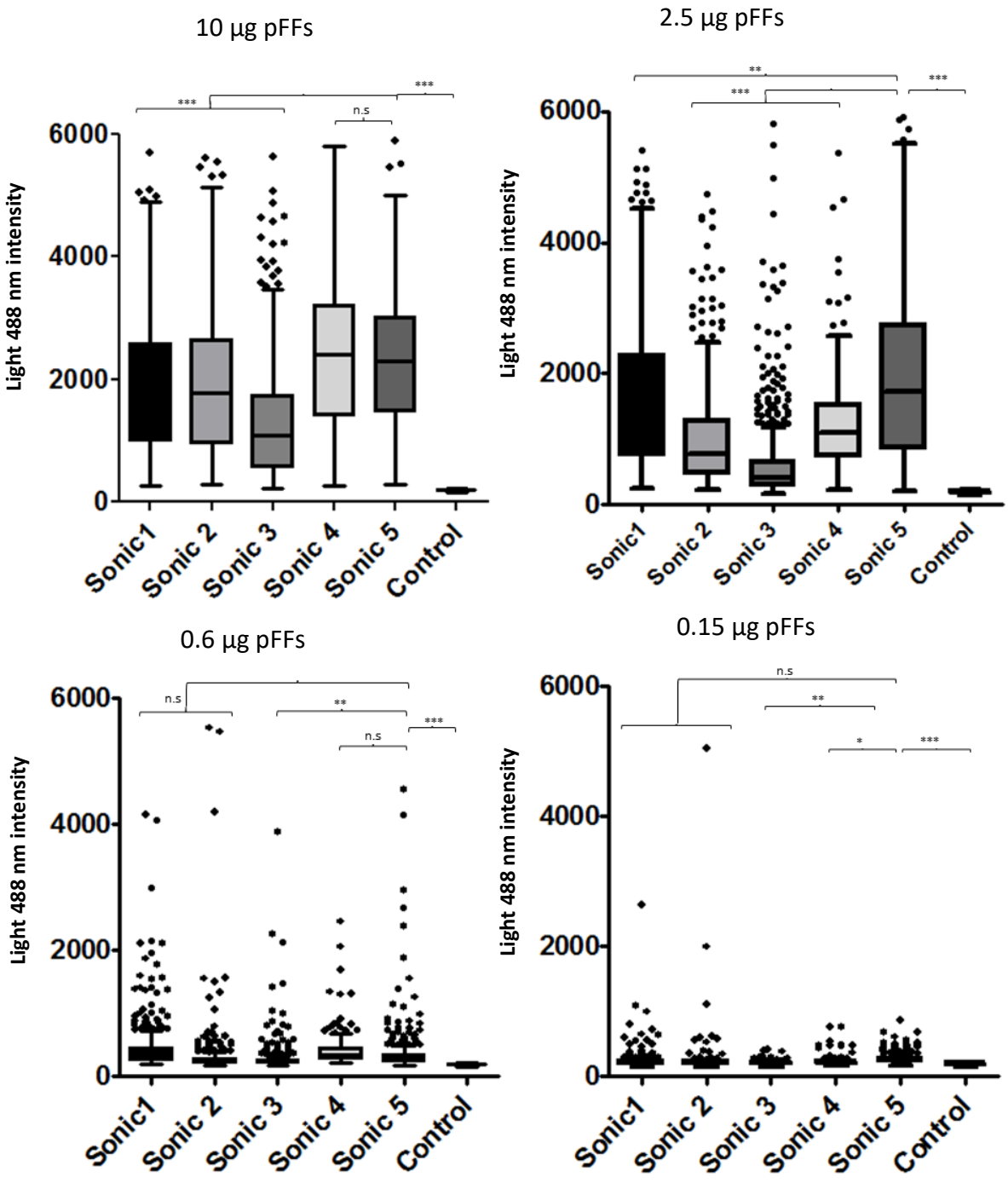


Figure 15. Quantification of α-synuclein from the LUHMES cells. Highest seeding concentration induced highest fluorescence detection. In all four experiments sonication groups were statistically significantly higher than control cells fluorescence detection. Aggregates were detected with D37A6 primary antibody to detect all forms of α-synuclein. Groups equal variances were tested with Bartlett's test. P-Values were derived from Dunnett's multiple comparison

test, sonication parameter 5 was compared to other sonications: n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$

4. Discussion

4.1 Reviewing the methods

α-synuclein has been shown to have key role in PD. In vitro models for studying effects of α-synuclein have been published, but those models have their limitations such as usage of animal cells and their validity for human disease can be questioned. Based on previous studies, focus of this thesis was in developing human neuronal cell based in vitro model for studying induced cellular stress, drug targets and compounds. α-synuclein aggregation has been shown on rodent primary neurons and human derived induced pluripotent stem cells (Volpicelli-Daley, Luk et al. 2014).

Development of the assay was divided into three main parts. 1) Non-neuronal cell model development and instrument suitability testing for detection and quantification of aggregation. 2) pFFs formation and seeding to induce aggregation, and 3) LUHMES cells differentiation, culturing and seeding of pFFs.

Transfection induced cell models have been published previously (Volpicelli-Daley, Luk et al. 2014), but their validity for industrial purposes is vague. Here, HEK-293 cells transfection model development was done to validate the protocol transfer. Non-neuronal HEK-293 cells do not endogenously express enough α-synuclein. Thus, inducing aggregation without plasmid transfection is most likely unsuccessful. High α-synuclein quantity was required and to meet this requirement an external vector plasmid was used to induce α-synuclein expression in HEK-293 cells. After transfection α-synuclein mRNA and protein production were detected and transfection protocol was optimized for future experiments.

pFF production protocol is published by the Michael J. Fox foundation (Michael J. Fox Foundation 2019). Protocol is intended for specially-formulated monomeric α-synuclein to form pFFs. Previously, pFFs generated with this protocol have

been shown to induce aggregation in rodent primary cultures in vitro and in mice in vivo. Our sonication instrument was different from previously reported and needed to be optimized for this purpose.

In this thesis, the used cell lines are suitable to be scaled up for e.g. HTS screening and their relatively low costs and LUHMES cells DA neuronal properties made them good cell lines to be used in these proof of principle studies. LUHMES cells are human derived immortalized mesencephalic cells that are relatively easy to maintain and can be efficiently differentiated into DA neurons. After differentiation, LUHMES cells express α-synuclein endogenously. Human derived α-synuclein endogenously expressing cells have not been previously used in α-synuclein aggregation experiments. The suitability of these cells for aggregation assay was evaluated.

4.2 HEK-293 cells experiments

Transfection of HEK-293 cells was challenging and several plasmid and transfection circumstances were tested to optimize the workflow. Transfections were performed with or without antibiotics in medium, as well as with coated or uncoated plastic ware.

When using 3 µg of variant 1 plasmid in transfection, cells tolerated the stress caused by the handling. Highest amounts, 12 µg and 10 µg, caused a decrease in cell amount and transfection with 6 µg of plasmid had effect on cell morphology. One can conclude that higher plasmid concentrations were more toxic for the cells than lower concentrations. No morphological changes were observed in control cells. Thus, transfection itself was not harmful for the cells, otherwise morphological changes would have been seen in MOCK control cells.

For transfections, plasmids were expanded to yield enough plasmid for multiple transfections. Plasmids were prepared in house in *E. coli* and purified with Machnery-Nagel Endotoxin-free plasmid DNA Purification kit. After purification, the amount of endotoxins should be low. The amount of endotoxins in plasmid stocks, however, were not separately tested, thus possible endotoxin contaminants might have had an effect on transfection efficiency and cell survival.

The proliferation rate of HEK-293 cells is also high and proliferation during and after transfection might influence transfection efficiency. In the future, the proliferation of cells could be inhibited, for example with mitomycin c. This could potentially enhance the transfection efficiency.

4.3 LUHMES cells differentiation

Long-term maintenance of LUHMES cells is more challenging than that of HEK-293 cells. Differentiation protocols have been published for LUHMES cells, but culturing densities and survival of cells have not been properly addressed (Scholz, Pöltl et al. 2011). Alongside the published protocol, some changes to the differentiation protocol were done (Figure 9). Thus, the optimal cell density for differentiation and 96-well plate format was elucidated. After the onset of the differentiation, the cells were replated in desired confluences to the 96-well plates. These replated cells showed neuronal morphology one day after plating and the cultures formed neuronal networks during the following days. At DIV 6 the neuronal network started to detach from the growth surface. This aspect makes the use of LUHMES cells challenging when developing an assay that requires longer times to detect pathological changes.

How one cultures LUHMES cells has crucial effects in this assay development. Once pFFs are seeded on top of cells it might take a few days before effects are taking place. In days, the endogenous monomeric α-synuclein in cells will change into oligomeric, more toxic form which is getting phosphorylated. Indeed, this was detected in neurite tips of iPSC-derived neurons in 7 days (Mahul-mellier 2019). More aggregation along the neurites towards cell soma was detected not earlier than in 14 days. LUHMES cells survival for seven or more days would thus be important and this need to be optimized before conducting long term aggregation experiments. In previous studies, differentiated LUHMES cells have lost viability 7 days after differentiation (Höllerhage, Goebel et al. 2014), thus my findings are in line with previously published work and the challenge with the short survival-time with LUHMES cells still exists.

In the culturing optimization experiments I presented here, the plating confluence and replating day had effect on cells' overall survival. Cell viability was highest if cells were replated at differentiation day 1 at confluence 50.000 / cm2. Same amount of replated cells resulted in higher confluency when comparing D1 and D2. D1 replated cells proliferated more than D2 replated and that implies that the D1 neuronal cells were more immature than D2 cells. D1 replated cells were also slightly bigger in size than D2 replated cells .Even though D2 replated cells were morphologically more neuron-like cells, all cells stained positive with neuronal specific TUJ-1 antibody, which was used to detect neurites. Confluence might also have had an effect on the amount of dendrite junctions after replating. D2 replated cells had a tendency to detach more easily from the plate surface. Time before replating most likely has effects on overall differentiation based on the gained results when comparing cells replated on D1 vs D2.

The viability of differentiated LUHMES cells can be prolonged by 14% with this optimized culturing protocol, but longer survival times would be better. LUHMES culturing and differentiation medium consist from a base medium that contains glutamine that is one of the essential amino acids that the cells need. Glutamine, however, has one notable limiting effect for neuron culturing. It non-enzymatically breaks down into ammonia in 37°C. Ammonia, even at low concentrations is very toxic for neurons. This effect of ammonia could be prevented by changing the cell culture medium regularly, but due to the high risk of LUHMES cell detachment only half volume of the medium was changed at once. Thus, ammonia concentration for sure increased during differentiation because of limited medium renewal. Possible future optimization steps could be to change to medium that contains glutamine that is modified to prevent breakdown. A full 3D culture might address the detachment issue and could be studied further.

LUHMES differentiation medium consists of several supplements such as doxycycline, cAMP and hGDNF. cAMP ja hGDNF are needed to induce expression of DRD2, RET (receptor tyrosine kinase), tyrosine hydroxylase and aromatic amino acid decarboxylase. Addition of doxycycline abolishes v-myc expression, which allows cells to exit the cell cycle and induce differentiation. (Scholz, Pöltl et al. 2011a)

In the future, medium osmolality effects for the viability of LUHMES cells could be studied. Physiologically osmolarity is higher than medium osmolarity. This could have effects on differentiation, aggregation of α-synuclein and viability of differentiated cells. In addition, confluence of differentiated neurons should be reviewed. Currently, confluence is optimized for culturing and long survival of cells, but low confluence might influence cells, especially neurons, physiological properties. More connections between neurites could have positive effects on electrophysiology and that could have an effect for differentiation.

A more in depth characterization of differently differentiated LUHMES needs to perform before choosing optimal differentiation protocol. Differences in protocols may cause variation for physiology of neurons and this need to be characterized further.

4.4 Western blotting

Results from western blotting were somewhat difficult to analyze. The vinculin band detection was as expected with both HEK-293 and LUHMES cell samples, but α-synuclein detection was not. The detected α-synuclein band was four times bigger than expected. α-synuclein proteins is 14 kDa but detected band was about 57 kDa, implying that α-synuclein tetramers were detected here. This helically folded tetramers are published and found to be aggregation resistant (Bartels, Choi et al. 2011). Tetramer α-synuclein is found in transfected HEK cells, but also shorter monomeric forms should be present.

Confirming that the used antibody detects α-synuclein, used pFFs were detected by used α-synuclein (D37A6) XP antibody (Supplemental figure 1). One possible reason of unsuccessful detection of the monomeric α-synuclein could be in primary antibody incubation. Small weight monomeric α-synuclein could be detached from membrane during incubation and washing steps. For preventing this, membrane is to be fixed with formaldehyde to prevent loss of α-synuclein during antibody incubations.

Used commercial M-PER buffer for protein extraction is probably Triton based lysis buffer. Triton is a gently lysing agent that is used when the conformation,

physical properties and function of proteins are to be stored. Proteins from cytosol can be detected with this lysing method. Another possible method could be to use SDS based lysis buffer. With SDS based lysis buffer all cellular proteins can be detected. SDS is a more effective lysing reagent and all membrane bound proteins are detached.

Cell lysing was done with commercial M-PER buffer, but for effective lysing also cell scraper can be used. In these experiments cells were first detached by pipetting PBS strongly towards the cells on well bottom. Suspension was then centrifuged and cell pellets were snap frozen. M-PER buffer was added onto the cells upon further protein isolation procedure, lysing is more gently than using cell scraper. This could be explanation why only tetramerically conformed α-synuclein was detected. Monomeric form is probably bound to membrane and more efficient lysing is needed. In the future, different lysis buffers and detaching methods could be studied. It would also be important to know are tetra- and monomer forms of α-synuclein located freely in cytosol or bound to membranes.

4.5 RT-qPCR

RT-qPCR analysis for mRNA detection was performed to detect if α-synuclein mRNA levels truly increased after plasmid transfection. Results showed α-synuclein peak mRNA levels to be 24-48 h after transfection. qRT-PCR results validated the assumption that mRNA levels of α-synuclein increased after plasmid transfection and that protein levels should be elevated at 48 hours post transfection, assuming that translation machinery works as expected. For HEK-293 cells optimal pFF seeding time point was considered to be 24 or 48 hours post transfection. Peak protein levels might also be later than 48 hours after transfection, and this should also be studied. However, the cell proliferation most likely reduces cellular plasmid derived expression levels/cell. Determination of the highest α-synuclein protein level time is not crucial, because seeded pFFs are kept on cells for few days and during this time pFFs should induce aggregation of expressed α-synuclein.

4.6 Seeding experiment

Seeding experiments were performed for HEK-293 and LUHMES cells. pFFs were kept on cells for two days before fixation. Two-day duration might be too short for endogenous α-synuclein to start aggregating and for α-synuclein post-translational modification like phosphorylation. In LUHMES experiments, detected fibrils were probably mainly the seeded pFFs, not endogenously formed. This might be the reason why aggregates could be detected only with α-syn (D37A6) XP antibody. Anti-Alpha-synuclein (phospho S129) (EP1536Y) antibody detects only phosphorylated form of aggregates and only endogenous α-synuclein is modified post-translationally.

With HEK-293 cells seeding experiment (figure 13 and 14) the phosphorylated α-synuclein was detected with pS129 antibody. This form of α-synuclein has gone through posttranslational modifications and thus most likely it is from plasmid transfected α-synuclein. Detection of phosphorylated α-synuclein validates that plasmid is functional and transfection has been successful at least partly. PCR results show α-synuclein peak mRNA levels to be 24-48 h after transfection. These cells were seeded 48 after transfection and fixed 48 hours after seeding. During this time, protein levels have had time to rise.

Now, when LUHMES cells survival time has been increased up to seven days, longer seeding experiments can be conducted. In addition, the pFF seeding period for HEK-293 cells needs to be optimized and to be tested after transfection optimization.

Sonication of fibrils was tested with five different parameters. It has been stated that pFFs need to be shorter than 50 nm to properly induce aggregation (Volpicelli-Daley et al. 2014). At some point during sonication a plateau phase should be achieved and shorter pFFs not anymore induce aggregation. On the other hand, detected α-synuclein could be only pFFs in cells, because of lack of phosphorylated α-synuclein detection. If this is the case, longer sonication produce better intruding pFFs and higher amount of those are detected in the cells. In the future, emphasis should be put to detect specifically the phosphorylated α-synuclein. That would be a genuine measure for endogenous α-synuclein.

Quality control of fibrils and pFFs should be conducted before assay validation. Quality control steps for sonicated pFFs should be performed before determining the optimal sonication parameters for pFFs production. Electron microscopy imaging could offer proper qualitative data on the size of the used pFFs. Before sonication, fibril formation needs to be verified. In more detail, thioflavin T assay could be suitable and fast verification step before storing samples.

Morphological changes in LUHMES cells after seeding can be caused by pFFs or possible pFFs solution containing endotoxins. pFFs are in orbital shaker for seven days during the generation. During this time, toxin concentration could raise if monomer solution is not sterile. All steps were performed in a sterile manner, but endotoxins could be tested to exclude this possibility.

In the future, the length of the fibrils should be studied and sonication protocol could be optimized accordingly. On the other hand, as over-shortened fibrils should not cause aggregation, it would be crucial to find the appropriate sonication-window for producing aggregation-prone pFFs repeatedly, every time. Here EM imaging would be an optimal method to verify the gained pFF sizes.

This assay could also be used for studying the spreading pathology of PD. Once aggregation is detected, it would be interesting to follow how the oligomeric α-synuclein spreads to neighbouring cells and better yet, how to prevent that from happening. This assay could offer answers on how aggregation develops and spreads and how it could be inhibited. Since PD patients are usually diagnosed only after the pathological processes have had an enormous effects of the dopaminergic system, would be more beneficial to have an in vitro model where disease hallmarks are detected and then rescued with compounds/new modalities. This would be more benefial for the patients today, before specific biomarkers for earlier diagnosis are found.

4.7 Future perspectives

HEK-293 cells and differentiated LUHMES cells express α-synuclein. This was the first time when α-synuclein expression in LUHMES cellswas studied. Next, same tests will be performed on undifferentiated and differentiated SH-SY5Y

cells. Conducted immunostaining experiments and seeding times with HEK-293 and LUHMES cells were quite short. Longer pFF seeding time might be beneficial for phosphorylated α-synuclein detection and will be tested.

More WB tests could be done to perform WB from newly produced samples with M-PER and SDS based lysis buffers to detect differences between lysing methods. Presence of 15 kDa α-synuclein should be detected in gently lysed cells if it is localized in the cytosol. When both forms are detected, the ratio between these two forms could be one outpoint measure for studying mechanism which have effects for this ratio. If the tetrameric form of α-synuclein is poorly aggregating then it would be interesting to study if the ratio between these forms change upon pFF seeding.

In conclusion, this work provides characterization of a reproducible human relevant neuronal model of α-synucleinopathies. This work also shows that LUHMES cell based model could be used as a platform to identify cellular α-synuclein aggregation pathways to screen therapeutic agents based on the modulation of these pathways.

5. Materials and methods

5.1 Cells mediums and growth parameters

HEK-293 cells were routinely growth in DMEM (Gibco 41965) containing 10% of FBS (Gibco 10270) and 1% of PenStrep (Gibco 15140) in 37°C with 5% CO_2.

Lund human mesencephalic (LUHMES) cells purchased from ATCC (ATCC® CRL-2927™). Cells are a subclone of the Doxycycline-controlled, v-myc-over-expressing human mesencephalic-derived cell line MESC2.10 (Lund, Sweden).

LUHMES base medium consists of DMEM (Gibco 11320), 1% of N2 supplement (Gibco 17502-001), 1,5% HEPES Buffer solution (1M) (Gibco 15630-056) and 1% PenStrep (Gibco 15140).

Proliferation/maintenance medium for LUHMES consisted of base medium supplemented by 40 ng/ml human FGF-2 (basic recombinant human fibroblast

growth factor, Miltenyi Biotec, Germany). LUHMES cells were cultured on Poly-L-Ornithine (Sigma-aldrich, Germany, Ref: P4957) and Fibronectin (Sigma-Aldrich, Germany, REF: F0895) coated NUNC EASYFLASK 75cm^2 NUNclon DELTA surface flasks. 96-well plates were, Poly-D-Lysine plate BioCoat Corning, UK REF: 356640. Poly-D-Lysine coated flask BioCoat Corning, UK (356537) were also used.

For LUHMES differentiation, proliferation medium replaced with differentiation medium two days after division. Differentiation medium consisted of LUHMES base medium supplemented by 1 µg/ml doxycycline, 1 mm dibutyryl cyclic AMP (db-cAMP, D0627, Sigma-Aldrich, N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt), and 2 ng/ml glial cell line-derived neurotrophic factor (GDNF, R&D Systems, Minneapolis, MN). Cells were kept for 24 hours in the differentiation medium in T-75 flask, after which the cells were replated to 96-well plate or 6 well plate for an additional two days before experiments. Cells were grown in humidified incubator at 37°C with 5% CO_2.

5.2 Plasmid Production

Plasmids and transient transfections used to induce α-synuclein expression in HEK cells are presented. The following plasmids ordered from GenScript:

Alpha-SYN human, Clone ID: OHu23545D , Vector: pcDNA3.1+/C-(K)-DYK. Alpha-SYN Ala53Thr mutant human, Clone ID: OHu23545DM 1 Ala53Thr, Vector: pcDNA3.1+/C-(K)-DYK. Alpha-SYN Mouse, Clone ID: OMu21967D, Vector: pcDNA3.1+/C-(K)-DYK. Alpha-SYN RAT, Clone ID: Ora12515D, Vector: pcDNA3.1+/C-(K)-DYK.

2 µl of each plasmid concentration and 50 µl Bacterial cells (*Escherichia coli* strain: JM109, WI, USA) were combined. Heat shock was induced by 45 second incubation in + 42 C water bath after which samples were cooled in an ice bath for 2 minutes. Bacterial culture plated onto 50 µg/ml ampicillin containing Lysogeny broth (LB) MILLER agar (Merck, Germany) and incubated overnight at + 37°C. The following day, one colony was picked and transferred into 4 ml LB-medium with 2 µl of ampicillin. The vials then grown for 8 h at + 37°C, 250 rpm.

Next, 1 ml of pre-growth suspension and 200 ml of LB-medium containing ampicillin (50µg/ml) were combined and incubated overnight at + 37°C 250 rpm. Finally, bacteria medium was centrifuged (6000 x g, at +4°C, 15 minutes) and the pellet was collected.

Plasmid-DNAs were purified by Machnery-Nagel Endotoxin-free plasmid DNA Purification kit (Machnery-Nagel GmbH & CO.KG, Düren, Germany Ref: 740426.10 Lot: 1604/014). According to manufacturer's instruction 8April 2017/Rev.07). Purity and yield were determined with Eppendorf BioPhotometer 6131 V1.26.

For transfection, HEK-293 cells were plated day before on a density of $30x10^3$ cells/cm2 in PDL-coated T-75 flasks (BioCoat, UK). For transfections, 500 µl of Opti-MEM 1x + GlutaMAX, (Gibco, USA) and various volumes of the plasmids were combined with the same amount than plasmid PLUS reagent (Thermo Fisher, USA, product of Lithuania) as the same amount as the plasmid. In another tube, 500 µl of Opti-MEM and 50 µl of Lipofectamine LTX (Thermo Fisher, USA, product of Lithuania) combined. Next, these two solutions combined and incubated in room temperature 10 to 20 minutes. After incubation, the solution was diluted to 4 ml of Opti-MEM 1x + GlutaMAX and dropwise added on top of the cells. Samples were collected 24 h, 48 h and 72 h after transfection.

5.3 LUHMES cells culturing experiment with GrowDex

LUHMES cells differentiated in T-75 flasks one to two days before subculturing them to 96-well plate. Tested culturing densities were between $115x10^3$ – $150x10^3$ cells/cm^2. Cells allowed to attach and differentiate one to two days before addition of GrowDex on top of cells. GrowDex® Conc, 1.5% Lot: 119921718g

5.4 Western Blot

Samples for Western blotting were collected 48 hours after transfection, from 6-well plates/T-75 flasks by manually scraping the cells into PBS after one wash

with PBS. Gained cell pellets were snap-frozen and stored in -80°C. Before western blotting analysis, the samples were lysed to M-PER (ThermoFisher) buffer containing cOmplete, EDTA-free protease inhibitor cocktail (ROCHE, Germany) and PhosSTOP, Phosphatase inhibitor cocktail (Roche, Germany). Protein concentrations were measured with pierce BCA assay (Protein Assay Kit, Thermo Fisher Scientific), with standard curve from 2000 µg/ml to zero. Concentration average calculated from duplicates.

Sample preparation for western blotting was done the following way, 1/4 of total sample volume was combined with Laemmle containing 1/10 β-mercaptoethanol. Samples were boiled at 95°C for 5 min before loading them into Criterion TGX Stain-free Precast-gel (BIO-RAD, CA, USA). The running buffer was 1X Tris/Glycine/SDS buffer (BIO-RAD, CA, USA) and samples were run for 95 min with 110 V. Samples were then transferred to nitrocellulose membrane (Transfer-Blot Turbo Transfer pack, BIO-RAD, CA, USA) with Transblot Turbo Bio-Rad Semi-dry transfer method, 1.3A, 25V, 7 minutes.

Before blocking, membrane placed to 0.4% formaldehyde solution to fix protein to membrane. Membrane blocking was done with 5% non-fat dry milk (BIO-RAD, CA, USA) for 1 h at room temperature. After wash, primary antibodies added and incubated at +4°C overnight. Primary antibodies used was 1) α-synuclein Monoclonal Antibody (4B12) (Thermo Fisher) 1:1000 in 5% BSA and 2) Anti-Vinculin (ab129002) (Abcam) rabbit monoclonal antibody 1:10 000 in 5% non-fat dry milk.

Secondary antibody was goat anti-rabbit IgG-HRP 1:3000 (Bio-Rad,CA,USA) and Precision protein streptactin-HRP conjugate 1:10000 (Bio-Rad,CA,USA) was used to visualize molecular weight ladder. After 1 hour incubation at room temperature, the membrane was washed and Clarity Western ECL substrate (Bio-Rad,CA,USA) was added before scanning it with Azure biosystems C400.

5.5 Immunostaining

Cells fixed with 3.7 % Formaldehyde (Sigma-Aldrich, Germany) and washed with PBS (Gibco, UK) before blocking. Blocking solution contained 10% horse serum

(Gibco, New Zealand), 0.1 % TritonX-100, 1% BSA (Sigma-Aldrich, USA) in PBS, and incubated 45 minutes in room temperature. After blocking, cells washed with 1 % serum, 0.1 % TritonX-100, 1% BSA in PBS before adding primary antibodies in the same solution for overnight incubation at +4°C.

Before secondary staining cells were washed two times with 1% BSA in PBS. Secondary antibodies diluted into 1% BSA in PBS and incubated 1 hour in room temperature. 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was diluted (1:1000) in 1% BSA in PBS and added on top of cells for last 5 minutes. Then, cells were washed a couple of times with PBS and stored PB solution: 1.15 ml NaH_2PO_4 stock + 3.85 ml Na_2HPO_4 stock + 95 ml dH_2O. (NaH_2PO_4 stock: 27.6g NaH_2PO_4 / 1000ml dH_2O, Na_2HPO_4 stock: 28.6g / 1000 ml dH_2O) in +4°C. Staining was performed by using primary antibodies Anti-Alpha-synuclein (phospho S129) antibody (EP1536Y) 1:200, α-synuclein (D37A6) XP antibody 1:200, TUJ-1, 1:800 (Anti-Beta-Tubulin III antibody, Clone TUJ1, Stem cell technologies, UK) and Phalloidin 568, (Alexa Fluor, Invitrogen) 1:400. Used secondary antibodies were goat anti mouse (H+L) Alexa 568 1:400 (Life Technologies,CA,USA) and goat anti rabbit (H+L) Alexa 488 1:400 (Life Technologies CA,USA). Imaging performed with EVOS FL and Operetta (Operetta CLS High-Content Analysis System). Quantitative data analysis performed with Columbus and GraphPad Prism softwares. Apple iPhone SE and iDu Optics® LabCam™ Microscope Adapter used to follow morphological changes.

5.6 Production of pre-formed fibrils

Alpha-synuclein monomers (10 mg/ml, 100 ul / vial) were purchased from Proteos (MI, USA). Monomers were diluted to equal volumes of 2xPBS to obtain 5 mg/ml solution. This diluted monomer solution divided into Non-stick surface micro-centrifuge tubes (525-1007 VWR, USA), 100 µl / tube. Tubes placed to orbital shaker (Eppendorf Thermomixer Comfort MTP) at 37°C, 1000 RPM for 7 days. After shaking period the solution was turbid and gray, suggesting fibrillation of α-synuclein monomers. The solutions from each tube were pooled and stored

aliquots à 25µl in Non-stick surface Miro-centrifuge tubes (525-1006, VWR, USA). All aliquots were snap-frozen on dry ice and stored in -80°C.

Fibrils were thawed at room temperature and sonicated immediately before seeding. Fibrils sonicated with Sonicator Q Sonica (Q800R3-110, USA). Sonication total time varied from 30 to 120 seconds. On/Off pulses varied from 1 second on/1 second off to 5 second on/5 second off pulses (Table 1). Sonicator parameter was adjusted to 750 Watts and frequency to 60% of maximum. Water temperature was adjusted to 4°C.

Table 1. Used sonication parameters for Q Sonica water bath sonicator.

Sonication parameter	Pulse on time	Pulse off time	Time
Sonication 1	1	1	30 sec.
Sonication 2	5	5	35 sec.
Sonication 3	1	1	60 sec.
Sonication 4	5	5	65 sec.
Sonication 5	1	1	120 sec.

5.7 Seeding experiment

HEK cells seeded with pFFs two days after α-synuclein transfection and LUHMES cells two days after the onset of differentiation. The concentration of pFFs for seeding was 10 µg/ml with dilution factor 1:4. Control cells without medium change and control cells with seeding medium change were on the same 96-well plate. HEK-293 cells culturing density for seeding experiment was 15x10^3 cells/well and LUHMES cells density was 150x10^3 and 50x10^3 cells/cm^2. Cells kept on seeding media 72 h before fixing cell plates.

Comparisons performed by one-way ANOVA using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA). Data distribution assumed not to be normally distributed, but this was not formally tested. Group's equal variances were tested with Bartlett's test. P-Values were derived from Dunnett's multiple comparison test, sonication parameter 5 were compared to other sonication

parameters: n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$

5.7 RNA isolation

RNA isolation was performed with Machnery-Nagel NucleoSpin RNA kit Ref: 740955.250 Lot: 1604/014 and purity was determined by DeNovix DS-II Fx Spectrophotometer.

5.8 cDNA synthesis

cDNA synthesis made in sterile 0.2 ml 8-strip tubes (PCR strips of 8 caps, BRAND, Germany). Every strip included water control and control that contained all the other components but the enzyme. cDNA synthesis components were 4 µl of 5x TransAmp Buffer (SensiFAST, cDNA Synthesis kit, BIOLINE, UK), 1 µl of Reverse Transcriptase (SensiFAST cDNA Synthesis kit, BIOLINE, UK), up to 1 µg of RNA and total volume up to 20 µl with RT-PCR Grade water (Ambion by life technologies, RT-PCR Grade water, USA). Samples RNA amount were standardized to 1 µg. Run program was 10 min at +25°C, 15 min at +42°C, 5 min at +85°C, and finally at +4°C to cool down samples. Reaction were performed with SImpliAmp™ Termal Cycler (Applied Biosystems, Singapore)

5.9 Real Time-quantitative PCR

RT-qPCR analysis were performed Quantstudio 6 real-time thermal cycler. Reaction mixture contains 19 µl master mix and 1 µl of sample cDNA. Master mix contains 10 µl SYBR mix (2xSensiFAST SYBR mix Lo-ROX kit, BIOLINE, UK), 1 µl qSTAR qPCR SNCA primers pairs (Origene, MD, USA) and total volume up to 20 µl with RT-PCR Grade water (Ambion by life technologies, RT-PCR Grade water, USA). Master Mix and sample were combined to MicroAMP optical 96-well Reaction Plate with barcode (Applied biosystem, China). Plate sealed with Optical adhesive covers, (Applied biosystems, USA)

Every reaction contained three biological replicates and two technical replicates from every biological replicate. Water, master mix and control samples without Reverse Transcriptase in cDNA synthesis were included.

RT-qPCR sample results melting point analysis performed, and Ct-values means calculated to compare results. ΔΔCt analysis were performed to adjust expression to relative expression.

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7. Used abbreviations

DA	Dopaminergic
DLB	Dementia with Lewy bodies
GI	gastrointestinal tract
HEK	Human epithelial kidney
HTS	High-throughput screening
ICC	Immunocytochemistry
PD	Parkinson’s disease
iPSC	Induced pluripotent stem cells
LBD	Lewy body dementia
LUHMES	Lund Human Mesencephalic cell
MPP+	1-methyl-4-phenylpyridinium
MSA	Multiple system atrophy
PBS	Phosphate-buffered saline
pFF	pre-formed α-synuclein fibrils
VAMP2	synaptobrevin-2/vesicle-associated membrane protein 2
6-OHDA	6-hydroxydopamine

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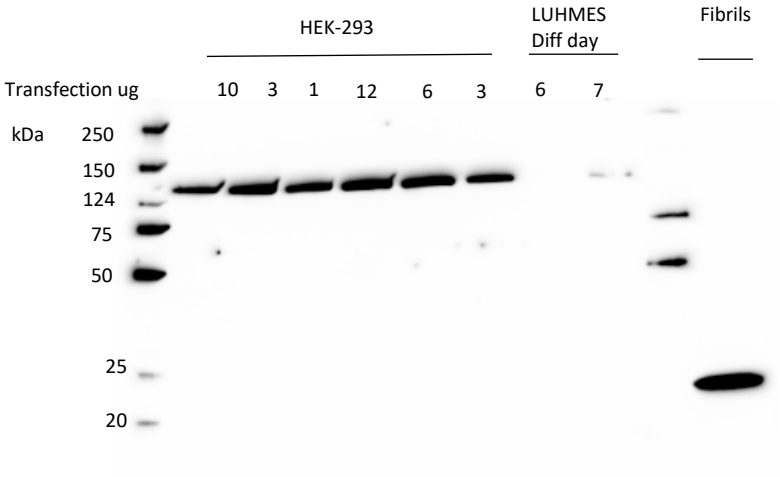
Appendices

Supplemental figure 1



Fibrils before sonication were detected by α-synuclein (D37A6) XP antibody.