SZENT ISTVÁN UNIVERSITY

Animal Husbandry Science PhD School

NEURONAL DISEASE MODELLING WITH RABBIT AND HUMAN STEM CELLS

Thesis for Doctoral degree (PhD)

Anna Dorota Ochałek

Gödöllő

2017

The PhD program

Name: Animal Husbandry Science PhD School

Discipline: Animal Husbandry Science

Leader of the school: Professor Dr. Miklós Mézes, D.V.M., Member of the HAS

Head of Department,

Szent István University, Faculty of Agricultural and Environment Science, Department of Nutrition

Supervisor: Professor Dr. András Dinnyés, D.V.M, D.Sc.

Head of Molecular Animal Biotechnology Laboratory,

Szent István University, Faculty of Agricultural and Environment Science, Institute for Basic Animal Sciences.

Co-supervisor: Dr. Julianna Kobolák, PhD

Scientific Director,

BioTalentum Ltd.

…………………….…………

Approval of the PhD School leader

…………………….…………   .……………………………..…

Approval of the Supervisor   Approval of the Co-supervisor

1. INTRODUCTION AND GOALS

Disease models function as a valuable platform for analysis of the biochemical mechanisms of normal phenotypes and the abnormal, pathological processes during disease progression. Animal models employed in the study of human disorders give new possibilities to investigate disease mechanisms and develop potential therapies. Rodents are the most common type of vertebrates used in disease modelling, and extensive studies have been conducted on mice, rats, rabbits, hamsters and guinea pigs. Rapid progress in animal modelling has resulted in better understanding of basic mechanisms underlying aberrant biological processes of many central nervous system disorders including neurodegenerative origin, motor disabilities in Parkinson's disease, cell death in stroke or optic nerve injury. Modelling of neurological disorders with animals is a source of information about molecular and genetic aspects of the disease and allow for in-depth study of neuropathophysiological mechanisms.

Besides animal models, pluripotent stem cells also offer a valuable *in vitro* system to study events related to development especially complex, multifactorial diseases at the molecular and cellular level. Induced pluripotent stem cells (iPSCs) established by Yamanaka's group in 2008 serve as a model to investigate the mechanisms regulating differentiation of cells of the central nervous system that can be important for further analysis of the diseases with complex etiology including neurodegenerative diseases. The stem cell-based models hold tremendous potential for the study of human neurological diseases bridging the gap between studies using animal models and clinical research.

Most neurological diseases are not yet well examined and there is not efficient treatment available. Thus, animal and *in vitro* cellular models can be utilized in the drug development and testing of possible therapeutic treatments. Within all neurological disorders, neurodegenerative diseases such as Alzheimer's disease (AD) affect the highest percentage of population. The majority of AD cases are sporadic, with unknown etiology, only 5% of all AD patients present familial monogenic form of the disease. Therefore a strong phenotypic model is required to recapitulate the disease pathomechanism.

The aim of the current study was to establish rabbit and human neuron based *in vitro* cellular models to study the pathomechanism of familial and sporadic form of Alzheimer's disease.

Objectives of this study

The key questions behind the study:
- Can rabbit iPSCs differentiate into neuronal cells?
- Can rabbit iPSC-derived neurons be used in human disease modelling?
- Can human iPSCs derived from patients with *PSEN1* mutation (familial AD) and late-onset sporadic AD differentiate into mature cortical neuronal cells which are able to model the disease *in vitro*?
- Which aspects of AD phenotype can be modelled using neurons derived from iPSCs?
- Are iPSCs enable to investigate the pathomechanisms of late-onset sporadic AD?

Specific objectives of the research were:
- Neuronal differentiation of rabbit iPSCs towards neuronal lineage using dual inhibition of SMAD signaling pathway
- Neuronal differentiation of human iPSCs towards neuronal lineage using dual inhibition of SMAD signaling pathway
- Characterization of iPSC-derived neurons by detection of neuronal marker expression at different time points of differentiation and maturation process
- Measurement the amyloid β secretion in control and AD neurons
- Detection of TAU expression and TAU phosphorylation at various epitopes in AD neurons and control lines
- Investigation of GSK3B activation in control and AD neuronal cultures
- Analysis of cellular response to potent oxidative stress inducers in control and AD-derived neuronal cells

2. MATERIALS AND METHODS

The chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and the cell culture reagents and culture plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless specified otherwise.

2.1 Rabbit stem cells and their derivatives

2.1.1 Rabbit iPSC lines

Rabbit embryonic fibroblast (rbEF) cells from Hycole hybrid rabbit foetus were reprogrammed by lentiviral delivery of a self-silencing human OSKM polycistronic vector as previously described by our team (Tancos *et al.*, 2017). The newly established rbiPSCs were maintained in iPSM Medium supplemented with 4 ng/ml human basic fibroblast growth factor (bFGF) and 10^3 units/ml ESGRO LIF. The media was changed daily and the cells were passaged every 3-4 days using 0.05% trypsin- EDTA.

2.1.2 Spontaneous *in vitro* differentiation

The rbiPSCs were treated with 0.05% trypsin-EDTA, resuspended in differentiation medium (Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 20% KSR, 2 mM GlutaMax, 1x NEAA, and 0.1 mM ß-mercaptoethanol), supplemented freshly with 4 ng/ml bFGF, and 50 ng/ml Activin A (R&D systems), and cultured in low cell binding Petri-dishes (Nunc). After 5 days the embryoid bodies (EBs) were plated on 0.1% gelatin covered cover slips in differentiation medium and fixed with 4% PFA on day 14 of differentiation.

2.1.3 Dual SMAD inhibition on rbiPSCs

The dual inhibition of SMAD signaling pathway using LDN193189 and SB431542 was tested on rabbit iPSCs to generate neural progenitor cells (NPCs). Neural induction was initiated upon reaching a desired confluence of rbiPSCs by addition of Neural Induction Medium supplemented with, 5 ng/ml bFGF, 0.1 µM or 0.2 µM or 0.4 µM LDN193189 (Selleckchem) and 5 µM or 10 µM SB431542 (Sigma-Aldrich). The NIM medium was changed every day. At day 15 neural rosettes were picked manually and replated on poly-L-ornithine/laminin (POL/L) (Sigma-Aldrich) coated dishes and expanded in Neural Maintenance Medium (NMM) supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml bFGF.

2.2 Human stem cells and their derivatives

2.2.1 Human iPSC lines

Patients derived iPSC lines used in this study were characterized and published earlier (Chandrasekaran *et al.*, 2016; Nemes *et al.*, 2016; Ochalek *et al.*, 2016; Táncos *et al.*, 2016; Tubsuwan *et al.*, 2016). Non-dement volunteers were used as controls, from whom iPSC lines were established, characterized and maintained under identical conditions as the AD iPSC lines. The hiPSC lines were maintained on Matrigel (BD Matrigel; Stem Cell Technologies) in mTESR1 (Stem Cell Technologies) culture media. The media was changed daily and the cells were passaged every 5-7 days using Gentle Cell Dissociation Reagent, according to the manufacturer's instructions.

2.2.2 Neural induction of human iPSCs

Neural progenitor cells (NPCs) were generated from each of the human iPSCs by dual inhibition of SMAD signaling pathway using LDN193189 and SB431542. Neural induction was initiated upon reaching a desired confluence of iPSCs on Matrigel-coated dishes by addition of NIM medium, which was supplemented with 5 ng/ml bFGF, 0.2 μM LDN193189 (Selleckchem) and 10 μM SB431542 (Sigma-Aldrich). The NIM medium was changed every day. At day 10 neural rosettes were picked manually and replated on poly-L-ornithine/laminin (POL/L) (Sigma-Aldrich) coated dishes and expanded in NMM medium, supplemented with 10 ng/ml EGF and 10 ng/ml bFGF.

2.2.3 Neural differentiation of human NPCs

To generate human neurons, NPCs were plated on the POL/L coated dishes and cultured in Neural Differentiation Medium (NDM) supplemented with 0.2 mM ascorbic acid (AA) (Sigma-Aldrich) and 25 μM β-mercaptoethanol. For terminal differentiation into cortical neurons, the cells were plated on POL/L with NMM medium. The efficiency of terminal differentiation was monitored by immunocytochemical staining and qRT-PCR for TUBB3 and microtubule associated protein (MAP2) expression at week 10. In the current study NPCs from passage 9 up to passage 10 were differentiated up to 10 weeks for ELISA and Western blott experiments, while samples were collected weekly intervals.

2.2.4 Immunocytochemistry (ICC) and AP staining

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min at RT, then permeabilized and blocked for 60 min at RT. The cells were incubated with specific primary antibodies overnight at 4°C. To visualize the signal isotype specific secondary antibodies conjugated with appropriate fluorophores were applied, while nuclei were counterstained with DAPI. For detecting alkaline phosphatase (AP) activity, rbiPSCs were stained using AP live stain kit (Life Technologies) according to the manufacturer's protocol. The cells were observed under fluorescent microscope equipped with 3D imaging module, (Axio Imager system with ApoTome; Carl Zeiss) controlled by AxioVision 4.8.1 microscope software (Carl Zeiss).

2.2.5 Electron microscopy

Electron microscopy was performed in the laboratory of Eötvös Loránt University (ELTE), Anatomy Cell and Developmental Biology Department, by Dr. Kinga Molnár; Dr. Lajos László and Mónika Truszka. The protocol and results presented here by their permission.

To evaluate the neuronal cultures a monolayer of 5 weeks old neurons grown on POL/L treated glass coverslips were fixed with a fixative solution containing 3.2% PFA, 0.2% glutaraldehyde, 1% sucrose, 40 mM $CaCl_2$ in 0.1 M cacodylate buffer for 24 hours on 4°C. Samples were rinsed for 2 days in cacodylate buffer, then postfixed in 1% ferrocyanide-reduced osmium tetroxide for 1 hour (RT). The samples were then treated with aqueous 1% uranyl-acetate for 30 minutes and embedded in Spurr low viscosity epoxy resin medium (Sigma-Aldrich), according to the manufacturer's instructions, and cured for 24 hours at 80°C. Ultrathin sections were stained with Reynolds's lead citrate for 2 minutes and were examined in JEOL JEM 1010 transmission electron microscope operating at 60 kV. Photographs were taken using an Olympus Morada 11 megapixel camera and iTEM software (Olympus).

2.2.6 Flow cytometry

Single cell suspension were fixed with 4% PFA for 20 min at RT, permeabilized and blocked. Cells were stained for 1 hour at RT with Alexa Fluor 647 mouse anti-NESTIN and PE mouse anti-human PAX6 antibodies (BD Pharmingen). Flow cytometry analysis was performed using a Flow Cytometer Cytomics FC 500 (Beckman Coulter). To detect nestin (NES) and PAX6 expression in NPCs an argon laser 488nm and a red solid laser 635nm were used respectively. Flow cytometry data analysis was performed using FlowJo software (version 7.6.5; FlowJo, LLC). (Carl Zeiss).

2.2.7 Immunoblotting

The cell cultures were lysed with RIPA Lysis and Extraction Buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail and Pierce™ Universal Nuclease for Cell Lysis (Thermo Fisher). Lysed samples were sonicated and incubated for 60 min on ice. Total protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher). Cell lysates (2 µg) were separated by electrophoresis on 10% SDS-polyacrylamide gel and transfer to a Immun-Blot® PVDF Membrane (Bio-Rad). The membranes were blocked with TBST containing 5% BSA and then incubated with the respective primary antibody solution overnight at 4°C. After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. Signals were detected with SuperSignal™ West Dura Extended Duration Substrate by KODAK Gel Logic 1500 Imaging System and Kodak MI SE imaging software. Densitometry measurement of protein bands intensity was carried put using Image Studio™ Lite software (LI-COR).

2.2.8 Measurement of Aβ 1-40 and Aβ 1-42 by ELISA

Conditioned media was collected weekly. To prevent protein degradation, 4-(2-aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was added to the media. Extracellular Aβ1-40 and Aβ1-42 levels were measured using human β-Amyloid (1-40) ELISA Kit and human β-Amyloid (1-42) ELISA Kit (Wako), according to the manufacturer's instructions. Data were normalized to total protein content of cell lysate. The signal was detected with Varioskan Flash Multimode Reader (Thermo Fisher). As a control value we used the average value (± SEM) of the four healthy individual derived clones (ctrl-1, ctrl-2, ctrl-3, ctrl-4) in all experiments (LI-COR).

2.2.9 Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from differentiated neurons at different time points using the RNeasy Plus Mini Kit kit (Qiagen) according to the manufacturer's protocol. One µg of RNA was transcribed using the SuperScript™ III VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). The PCR conditions were subjected to 94°C, 3 min, initial denaturation; followed by 40 cycles of 95°C, 5 seconds, denaturation; 60°C 15 seconds, annealing and 72°C 30 seconds, elongation. The amplification reactions were carried out in a total volume of 15 µL using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). Reverse transcription quantitative PCR (RT-qPCR) was run on the Rotor-Gene Q 5plex Platform (QIAGEN) using

oligonucleotide primers. Human *GAPDH* was used as reference gene. The data was analyzed using REST software (2009 V2.0.13).

2.2.10 Cell viability assay after H$_2$O$_2$ and Aβ1-42 treatment

Neurons growing on 96 well plate, at day 28 and 56 of terminal differentiation were treated with 30 μM and 60 μM of hydrogen peroxide (H$_2$O$_2$) (Sigma-Aldrich) or 5 μM Aβ1-42 oligomer solution. For reparation of oligomer solution, iso-amyloid 1-42 peptides were used as detailed in (Bozso *et al.*, 2010) (provided by University of Szeged, Hungary). Viability of the cultures following H$_2$O$_2$ and Aβ treatment was assessed at 24 hours stimulation using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The luminescent signal was detected in Varioskan Flash Multimode Reader (Thermo Fisher Scientific). The neuronal survival was represented as a percentage of control.

2.2.11 Statistical analysis

All results were analyzed using the GraphPad Prism 5 software and Microsoft Office 2010. Analysis of data was presented in the form of mean ± S.E.M. (standard error of the mean). Dunnett's method was performed to compare the individual groups to controls. In all cases, significance was noted at *p<0.05.

3. RESULTS

3.1 Rabbit iPSCs and their neuronal differentiation

3.1.1 Characterization of rabbit iPSCs

Rabbit iPSCs (rbiPSCs) newly generated from embryonic fibroblasts resembled the human iPSCs in that they presented similar morphology and culture requirements: they formed flattened colonies and proliferated indefinitely in the presence of bFGF.

RbiPSCs presented alkaline phosphatase activity and highly expressed OCT4, SOX2 and the membrane surface marker SSEA4. The *in vitro* spontaneous differentiation of rbiPSCs led to formation of three germinal layers ectodermal, mesodermal and endodermal as well as neuroectoderm.

3.1.2 Induced differentiation of rbiPSCs towards neurons

To generate neuronal cells, rbiPSCs were first cultured in 3D environment using hanging drop or suspension culture and then directly differentiated into neurons. Then, EBs were plated on gelatin coated dishes and differentiated towards neuronal lineage. Immunofluorescent analysis revealed very low expression of early neuronal marker (TUBB3), late neuronal marker (MAP2) and neural RNA-binding protein (MUSASHI) in cells obtained from both hanging drop and suspension cultures. Furthermore, rbiPSC-derived neurons presented very short neurites and they were not able to form dense neuronal network. After the failure of long-term culture of EBs, we applied the well-known neuronal human iPSC differentiation protocol, the dual inhibition of SMAD signalling, on rabbit iPSCs. We investigated the effect of different concentration of small molecules in different combinations: in our experiments 0.2 μM LDN193189 and 10 μM SB431542 combination provided the best outcome and provided the highest rosette –like structure formation. However, the efficiency of differentiation forming neurons from rbiPSCs was very low. Furthermore, newly differentiated neurons presented very rapid and dramatic cell death. Thus, we decided to use only human iPSCs for production of neurons and building up the *in vitro* neurodegenerative disease model.

3.2 Human iPSCs and their neuronal differentiation

3.2.1 Characterization of human iPSCs and human neuronal progenitor cells (NPCs)

Early-onset familial (fAD) and late-onset (sporadic) Alzheimer's disease (sAD) patient's samples were reprogrammed and iPSC lines were generated and characterized by our teams

previously. Here, three fAD patients with mutation in *PSEN1*, four patients with late onset sAD and three non-dement individuals as controls' iPSC clones were studied. The iPSC induction process was facilitated by inhibition of BMP4 and TGFB signalling mediated through SMADs using small molecule inhibitors: LDN193189 and SB431542. All AD-iPSCs and control iPSCs were successfully converted into neural progenitor cells and expressed neuroepithelial markers: PAX6 and NES.

3.2.2 Neuronal differentiation capacity of fAD and sAD patient derived iPSCs

The neuronal differentiation (terminal differentiation; TD) of fAD and sAD origin NPCs where investigated and compared with control lines at two different time points (TD28, TD70). Immunofluorescent labeling has shown the increasing expression of neuronal markers: TUBB3, MAP2, NF200 and TAU during the neuronal differentiation, which was comparable among the disease and control cell lines. ICC and RT-qPCR analysis at day 70 of AD-derived and control neuronal cultures revealed the presence of cholinergic, dopaminergic, GABAergic and glutamatergic neurons as well as astrocyte and oligodendrocyte progenitors.

3.2.3 Secretion of Aβ by fAD and sAD iPSC-derived neurons

Upregulation of Aβ production is one of the main pathological hallmark of AD. To determine Aβ production, secreted Aβ1-40 and Aβ1-42 levels were measured by ELISA. Elevated secreted Aβ1-40 and highly increased Aβ1-42 levels were observed in all fAD neural cultures compare to the control lines. The Aβ1-42/Aβ1-40 ratio was also increased up to 2-fold for the fAD-iPSCs-derived neurons. Extracellular level of Aβ1-40 in all sAD neurons was significantly higher compared to control and fAD neurons. The Aβ1-42 secretion in sAD neurons was also elevated compared with the average values of the controls, however the levels did not reach the same levels as in fAD neurons. Interestingly, the measured Aβ1-42/Aβ1-40 ratio in all sAD-iPSCs-derived neurons was similar to those in controls. Furthermore, we detected increased expression of amyloid precursor protein (APP), the direct precursor of Aβ, and its cleavage product APP-CTF in all fAD and sAD neural cultures.

3.2.4 Detection of epitope specific TAU hyperphosphorylation in fAD and sAD lines

One of the proposed mechanisms for the TAU protein pathomechanism in AD are posttranslational modifications through abnormal phosphorylation (hyperphosphorylation). The appearance and accumulation of abnormally phosphorylated TAU lead to mislocalization and aggregation of TAU in the brain. We observed significantly higher phosphorylation of

TAU at five different phosphorylation sites Ser262, Ser396, Ser202/Thr205, Thr181 and Ser400/Thr403/Ser404.

3.2.5 GSK3B activation in AD-derived neurons

Scientific evidences suggest that GSK3B is involved in many pathological hallmarks of AD including hyperphosphorylation of TAU, increased Aβ production, memory impairment and neuronal loss. To verify if AD neurons with elevated TAU phosphorylation have increased GSK3B activity, the percentage of active form of GSK3B was calculated. Our results demonstrated that neurons derived from patients with fAD and sAD exhibited significantly higher amount of active GSK3B than control neurons. Furthermore, higher phosphorylation of TAU at Thr231, which can be catalyzed by GSK3B *in vitro* was observed in all fAD and sAD-derived neurons.

3.2.6 Oxidative stress response in fAD and sAD neuronal cultures

Oxidative stress plays important role in the pathogenesis of neurodegenerative disorders, but its effect on the human iPSC derived neurons from AD patients is still not well studied. In our experiments we examined neuronal response to H_2O_2. In AD and control neurons treatment with increasing doses of H_2O_2 engendered a dose-dependent loss of cell viability, however these two cell groups presented differential susceptibility to the stressor. The fAD and sAD-iPSCs derived neurons were more sensitive to H_2O_2 compared with controls.

A series of evidence about the toxicity of Aβ to neurons has been demonstrated in rodent models and neuronal cell lines. Due to the limited studies on neurons generated from iPSCs we examined the effect of synthetic Aβ1-42 oligomer solution on iPSC derived neurons. Cells treated at day 28 and 56 with Aβ1-42 oligomers showed reduced neuronal survival depending on the neuronal maturation state. Relative to control samples, diseased lines had enhanced sensitivity to cell toxicity induced by Aβ1-42. Furthermore, mature neurons, at TD56, were more susceptible to cell death.

4. NEW SCIENTIFIC RESULTS

1. As a novel finding, I demonstrated that, rabbit iPSCs generated by reprogramming of fibroblast cells, underwent differentiation through dual inhibition of SMAD signalling most efficiently by two small molecules, LDN193189 and SB431542. Newly generated neuronal progenitor cells had a limited proliferation capacity and potential to differentiate into neuronal cells.

2. My experiments showed that an increased extracellular Aβ1-40 and Aβ1-42 secretion was measured in neurons derived from human familiar AD and sporadic AD iPSC lines on a maturation dependent manner. An elevated ratio of Aβ1-42 to Aβ1-40 was detected in PSEN1 mutants, while Aβ1-42/Aβ1-40 ratio in sAD lines remained unchanged and comparable with non-AD controls.

3. For the first time I detected an increased TAU phosphorylation at six different epitopes: Ser262, Ser202/Thr205, Ser396, Ser400/Thr403/Ser404, Thr181 and Thr231 in familiar and sporadic AD iPSC lines-derived neurons.

4. I presented increased level of active GSK3B measured in neurons derived from fAD and sAD iPSCs *in vitro*. Moreover, I showed elevated sensitivity to oxidative stress induced by amyloid oligomers or oxygen peroxide both in familial and sporadic AD-iPSCs differentiated neurons.

5. An *in vitro,* iPSC based cellular model was developed which recapitulated the pathological hallmarks of the target, the Alzheimer's disease. In Hungary this is the first iPSC-based *in vitro* AD study, which analyzes this human neurodegenerative disease in a complex way.

5. DISCUSSION AND SUGGESTIONS

The present study shows that particular key disease phenotypes of the most common age-related neurological disorder, Alzheimer's disease, can be modeled using patient specific iPSC-derived neuronal cells.

We have demonstrated generation of rbiPSCs from rabbit liver somatic cells by the introduction of four human transcription factors: OCT4, SOX2, KLF4 and c-MYC. The newly generated rbiPSCs closely resembled human iPSCs and expressed the endogenous pluripotency markers, and were positive for alkaline phosphatase staining. Moreover in the absence of growth factor, the rbiPSCs spontaneously differentiated into ectoderm, mesoderm and endoderm.

In the next step we investigated if it is possible to generate neurons from rabbit reprogrammed cells. Differentiation of rbiPSCs towards neurons was initiated by formation of three-dimensional multicellular aggregates – EBs in two different systems: liquid suspension culture and culture in hanging drops. It was shown that EBs generated from human stem cells are beneficial in the initiation of differentiation and enhancement of differentiation towards certain lineages such as neural tissues. RbiPSCs maintained in suspension culture formed small uniform-sized spherical EBs, whereas cells growing in hanging drop system aggregated into heterogeneous EBs with a wide variety of sizes. Further culture of these cells in 2D system revealed very poor neuronal differentiation and maturation. In contrast to rbESCs, only few rbiPSCs differentiated into neurons and revealed expression of early neuronal marker. The above results may be the effect of non-optimized conditions for iPSC maintenance, EB formation or EB differentiation in 2D environment. Furthermore, the reprogramming procedure with human OSKM factors might not have been sufficient to produce naive pluripotent stem cells with self-renewing potential and differentiation ability.

Thereafter we investigated if a 2D monolayer based, well defined human iPSC neuronal differentiation protocol, the dual inhibition of SMAD signaling with the combination of two small molecules, could trigger neuronal rosette formation and more efficient neuronal precursor differentiation. Unfortunately, despite of several tested conditions we could not improve the efficiency of the differentiation and produce sufficient neurons from the rabbit iPSCs.

Modelling AD using human iPSCs was initiated with familial cases carrying mutations in *PSEN1*, *PSEN2* and *APP*. Until now there are only few groups that reported generation of

iPSCs-derived neurons from patients with familial AD and still little is known about sporadic AD cases. In our current study, we analyzed samples and generated neurons from patients carrying the pathogenic mutations in *PSEN1* gene and sporadic AD patients with unknown background. Our findings demonstrate that iPSCs derived from fAD and sAD patients can be successfully induced into NPCs with very uniform expression of NESTIN and PAX6, and further differentiated into neurons. Gene expression and immunocytochemistry analysis revealed the presence of various neuronal subtypes including GABAergic, glutamatergic, cholinergic, dopaminergic neurons and progenitor cells of astrocytes and oligodendrocytes in control and AD-derived cells. Therefore, we conclude that, there is no prominent difference in the differentiation and maturation propensity, nor in marker gene expression between control and AD neuronal cells.

One of the most important question in our work was to evaluate if sporadic AD cases can be modelled through iPSCs derived neuronal cultures and represent a suitable model system for neuropathology investigations and drug development studies. Therefore, first of all we analyzed the relevant *in vivo* pathological hallmarks of the disease in an *in vitro* system. In this line the accumulation of Aβ into extracellular aggregates is one of the pathological sign of AD in the human brain. Our study demonstrated an increased extracellular Aβ1-40 and Aβ1-42 levels in neurons derived from all fAD and sAD lines on a maturation dependent manner. Moreover, we observed an elevated ratio of Aβ1-42 to Aβ1-40, one of the AD hallmark, in PSEN1 mutants, while Aβ1-42/Aβ1-40 ratio in sAD lines remained unchanged and comparable with non-AD controls. Additionally, we observed upregulated expression of APP and APP-CTF in all AD-derived cell lines, which is in line with the increased amyloid levels we measured. Based on the above results we can conclude that mutations in PSEN1 gene may change the metabolism of Aβ peptides and drive amyloidosis in fAD patients. Our findings also indicate the possible heterogeneity of familial and sporadic AD. AD-iPSC lines with PSEN1 mutation and sAD do not always recapitulate the same phenotypes.

Herein, we demonstrated that another pathological hallmark characteristic for AD, TAU hyperphosphorylation, is detected in AD-patient specific neurons. Conformational changes and misfolding the protein structure result in aberrant aggregation of TAU into neurofibrillary structures. We analyzed TAU phosphorylation in iPSC-derived neurons from familial and late onset AD patients at six different phosphorylation sites. As a novel finding, we demonstrated an increased TAU phosphorylation at all examined epitopes in all iPSC-derived fAD and sAD neurons. The increasing TAU phosphorylation during the differentiation indicates that an

appearance of the AD-phenotype depends on the maturation state of neuronal culture. Furthermore, we have shown higher levels of active GSK3B in our AD cultures. Our findings revealed that activation of GSK3B might contribute to TAU misregulation and abnormal phosphorylation.

In our study we evaluated cell viability after hydrogen peroxide and extracellular Aβ1-42 exposure in neurons at different maturation stages. Our results demonstrated a significant H_2O_2 dose-related decrease in the survival of fAD and sAD neurons. More mature neurons (TD56) showed a greater sensitivity to H_2O_2 than younger neuronal cultures (TD28). These observations indicate that H_2O_2 may provoke an antioxidant stress response resulting in increased level of ROS and may lead to subsequent cell death. Additionally, we observed that treatment with Aβ1-42 oligomers induced cell death both in fAD and sAD neurons. However, a decreased cell survival was detected in mature (TD56) neurons.

Results of these studies provide an insight into understanding the molecular basis of disease and developing patient cell models that display the AD phenotype. In all fAD and sAD lines we observed higher Aβ1-40 and Aβ1-42 secretion, increased active GSK3B and hyperphosphorylation of TAU at six different epitopes. We showed that iPSC derived neurons from both familial and sporadic cases demonstrated Alzheimer's disease phenotypes. This finding suggests that sAD patients with an unknown disease etiology might have genetic background that resembles neuronal fAD phenotypes. To determine the frequency of such genomes within the sAD population, a larger sample size will be required during further studies.

6. PUBLICATION LIST

International paper publications:

- Zhou S, Szczesna K, **Ochalek A**, Kobolák J, Varga E, Nemes C, Chandrasekaran A, Rasmussen M, Cirera S, Hyttel P, Dinnyés A, Freude K*, Avci HX*. *Neurosphere based differentiation of human iPSC improves astrocyte differentiation* *contributed equally, Stem Cells International 2016; doi: 10.1155/2016/4937689. Impact Factor: 3.687

- Zhou S*, **Ochalek A***, Szczesna K*, Avci HX, Kobolák J, Varga E, Schmid B, Rasmussen M, Cirera S, Hyttel P, Freude K, Dinnyés A. *The positional identity of iPSC-derived neural progenitor cells along the anterior-posterior axis is controlled in a dosage-dependent manner by bFGF and EGF* *contributed equally, Differentiation 2016; doi: 10.1016/j.diff.2016.06.002. Impact Factor: 2.461

- **Ochalek A***, Nemes C*, Varga E, Táncos Z, Kobolák J, Dinnyés A. *Establishment of induced pluripotent stem cell (iPSC) line from a 57-year old patient with sporadic Alzheimer's disease* *contributed equally, Stem Cell Research 2016; doi: 10.1016/j.scr.2016.05.020. Impact Factor: 3.892

- **Ochalek A**, Szczesna K, Petazzi P, Kobolak J, Dinnyes A. *Generation of cholinergic and dopaminergic interneurons from human pluripotent stem cells as a relevant tool for neurological disorders studies,* Stem Cells International. 2016; doi: 10.1155/2016/5838934. Impact Factor: 3.687

Publications submitted for publication:

- Chandrasekaran A, Avci HX, **Ochalek A**, Rösingh LN, Molnár K, László L, Bellák T, Téglási A, Pesti K, Mike A, Phanthong P, Krause K-H, Kobolák J, Dinnyés A. *Comparison of 2D and 3D neural induction methods for the generation of neural progenitor cells from human iPSCs. (under revision)*

- **Ochalek A**, Mihalik B, Avci HX, Chandrasekaran A, Téglási A, Lo Giudice M, Táncos Z, Molnár K, László L, Holst B, Freude K, Hyttel P, Kobolák J, Dinnyés A.*Neurons derived from sporadic Alzheimer's disease iPSCs reveal elevated TAU hyperphosphorylation, increased amyloid levels and GSK3B activation. (under revision)*

International abstract and poster presentations:

- Chandrasekaran A, Roesingh LN, **Ochalek A**, Nemes C, Varga E, Bock I, Zhou S, Szegedi V, Avci H, Raveh-Amit H, Kobolák J, Dinnyes A. *Neuronal differentiation from human induced pluripotent stem cells*. IDPbyNMR Final meeting, Grosseto, Italy 21-26 September 2014

- Chandrasekaran A, Neelchen Roesingh L, **Ochalek A**, Nemes C, Varga E, Bock I, Avci H, Kobolak J, Dinnyes A. *Neuronal differentiation of patient specific induced pluripotent stem cells.* EMBO Conference Stem cells in Cancer and Regenerative Medicine, Heidelberg, Germany 9–12 October 2014

- Szczesna K, **Ochalek A**, Chandrasekaran A, Bellák T, Szegedi V, Varga E, Zhou S, Avci H, Schmidt B, Kobolák J, Dinnyés A.*3D engineered neural tissue from human induced pluripotent stem cells as a tool for modelling the early development stage of central nervous system.* EMBO Workshop: Cortical development in health and disease Rehovot, Israel 26-29 April 2015

- Chandrasekaran A, **Ochalek A**, Bellák T, Szegedi V, Avci H, Varga E, Szczesna K, Schmidt B, Zhou S, Hyttel P, Kobolák J, Dinnyés A. *Modelling Alzheimer's disease in 3D engineered neural tissue from patient-derived induced pluripotent stem cells.* ISSCR Annual Meeting Stockholm, Sweden 24-27 June 2015

- Zhou S, Szczesna K, Avci H, Kobolak J, Varga E, Schmidt B, **Ochalek A**, Rasmussen M, Freude K, Cirera S, Dinnyes A, Hyttel P. *Role of bFGF and EGF in neural rosette formation – modelling human neuroscience in the dish.* ISSCR Annual Meeting Stockholm, Sweden 24-27 June 2015

- **Ochalek A**, Bellák T, Chandrasekaran A, Szegedi V, Bock I, Varga E, Nemes C, Zhou S, Hyttel P, Kobolák J, Dinnyés A, Aci H. *Engineering high and low complex 3D neural tissue from human induced pluripotent stem cells.* Stem Cell Models of Neural Regeneration and Disease Dresden, Germany 1-3 February 2016

- Roman V, Kobolák J, Avci H, Ábrahám Z, Hodoscsek B, Berzsenyi S, Koványi B, Dezső P, Nagy J, Chandrasekaran A, **Ochalek A**, Varga E, Nemes C, Bock I, Pentelényi K, Németh K, Balázs A, Molnár J, Dinnyés A, Lévay G, Lendvai B.*Compromised Neurite Morphology of Induced Pluripotent Stem Cell-Derived Neurons: Similar Patterns from Independent Non-Syndromic Autism Cases.* 2016 International Meeting for Autism Research (IMFAR) Baltimore, MD, USA 11-14 May 2016

- Francz B, Schmidt B, **Ochalek A**, Chandrasekaran A, Kovács E, Mihalik B, Kobolák J, Dinnyés A. *In vitro neurotoxicology models based on human Induced Pluripotent Stem Cells.* 19th International Congress on In Vitro Toxicology ESTIV 2016 Juan-les-Pins, France 17-20 October 2016

Abstracts and posters in Hungary:

- Tancos Zs, **Ochalek A,** Nemes Cs, Varga E, Bock I, Dinnyes A. *Generation of rabbit induced pluripotent stem cells (iPSCs) by human reprogramming factors.* Fiatal Biotechnológusok Országos Konferenciája (FIBOK) 2014 – Young Biotechnologists' National Conference 2014 Szeged, Hungary 7 March 2014

- Chandrasekaran A, Roesingh LN, **Ochalek A**, Nemes C, Varga E, Bock I, Zhou S, Szegedi V, Avci H, Kobolak J, Dinnyes A. *Patient specific induced pluripotent stem cells and their neuronal differentiation*. Annual Meeting of the Hungarian Biochemical Society Debrecen, Hungary August 2014

- Chandrasekaran A, Roesingh LN, **Ochalek A**, Nemes C, Varga E, Bock I, Szczesna K, Avci H, Kobolak J, Dinnyes A. *Comparison of 2D and 3D neuronal differentiation of patient specific induced pluripotent stem cells.* XV. Biannual Conference of the Hungarian Neuroscience Society, Budapest, Hungary 22-23 January 2015

- Bellak T, Chandrasekaran A, **Ochalek A**, Szegedi V, Zhou S, Avci H, Kobolak J, Dinnyes A. *3D engineered neural tissue from human induced pluripotent stem cells.* XV. Biannual Conference of the Hungarian Neuroscience Society, Budapest, Hungary 22-23 January 2015

- Bellak T, Chandrasekaran A, **Ochalek A**, Szegedi V, Varga E, Nemes C, Zhou S, Avci H, Kobolak J, Dinnyes A. *3D engineered neural tissue from human induced pluripotent stem cells.* "Hungarian Molecular Life Sciences 2015″ Conference Eger, Hungary 27-29 March 2015

- Bellak T, **Ochalek A**, Chandrasekaran A, Szegedi V, Avci H, Szczesna K, Varga E, Nemes C, Nógrádi A, Kobolák J, Dinnyés A. *Modelling Alzheimer's Disease with three-dimensional engineered neural tissue.* 19th Congress of the Hungarian Anatomical Society, Szeged, Hungary 11-13 June 2015

- **Ochalek A**, Chandrasekaran A, Bellák T, Avci H, Szegedi V, Varga E, Nemes C, Schmidt B, Bock I, Zhou S, Szczesna K, Kobolák J, Dinnyés A. *3D engineered neural tissue from patient-derived induced pluripotent human induced pluripotent stem cells as a tool for modelling Alzheimer disease.* HuNDoC 2016 Budapest, Hungary 20 January 2016

- Francz B, Schmidt B, **Ochalek A**, Chandrasekaran A, Kovács E, Mihalik B, Kobolák J, Dinnyés A. *Induced Pluripotent Stem cell-based in vitro neurotoxicology models.* HuNDoC 2016 Budapest, Hungary 20 January 2016

- Kovacs E, Tancos Z, **Ochalek A**, Varga E, Schmidt B, Avci H, Kobolák J, Dinnyés A. *Establishment of an in vitro drug screening assay for Alzheimer's disease based on induced pluripotent stem cells.* IBRO Workshop Hungarian Academy of Sciences Budapest, Hungary 21-22 January 2016

- **Ochalek A**, Chandrasekaran A, Bellák T, Avci H, Szegedi V, Varga E, Nemes C, Schmidt B, Bock I, Zhou S, Szczesna K, Kobolák J, Dinnyés A. *3D engineered neural tissue from patient-derived induced pluripotent human induced pluripotent stem cells as a tool for modelling Alzheimer disease.* IBRO Workshop Hungarian Academy of Sciences Budapest, Hungary 21-22 January 2016

- Francz B, Schmidt B, **Ochalek A**, Chandrasekaran A, Kovács E, Mihalik B, Kobolák J, Dinnyés A. *Induced Pluripotent Stem cell-based in vitro neurotoxicology*

models. Fiatal Biotechnológusok Országos Konferenciája (FIBOK) Godollo, Hungary 21-22 March 2016

Oral presentations:

- **Ochalek A,** Tancos Zs, Nemes Cs, Varga E, Bock I, Dinnyes A. *Establishment and characterization of rabbit iPS cells.* RGB-Net (Collaborative European Network on Rabbit Genome Biology) Third RGB-Net meeting, Zagreb, Croatia 6-8 May 2014

- Chandrasekaran A , Roesingh LN, Nemes C, Varga E, Bock I, Zhou S, **Ochalek A**, Szegedi V, Avci H, Dinnyes A, Kobolak J. *Induced pluripotent stem cells for biomedical applications.* Innovations in Medicine, Budapest, Hungary 10 October 2014

- Tancos Zs, **Ochalek A**, Nemes Cs, Varga E, Bock I, Dinnyes A. *Generation of rabbit induced pluripotent stem cells (iPSCs) by human reprogramming factors*. Opening Conference of COST Action BM1308 "Sharing Advances on Large Animal Models – SALAAM" Munich, Germany 15-17 December 2014

- Dinnyes A, Chandrasekaran A, **Ochalek A**, Bellák T, Szegedi V, Avci H, Eszter V, Téglási A, Eszter K, Szczesna K, Schmidt B, Kobolák J.*Betegspecifikus őssejtek differenciójának neurális és kardiális irányokba*. Lecture at Hungarian experimental and clinical pharmacological company's experimental pharmacological IX. symposium, Velence, Hungary 26-28 March 2015

- Kobolák J, Chandrasekaran A, **Ochalek A**, Bellák T, Szegedi V, Varga E, Avci H, Zhou S, Szczesna K, Smidt B, Dinnyés A. *Patient specific induced pluripotent stem cells (iPSCs) and their neuronal differentiation in Alzheimer's disease*. "Hungarian Molecular Life Sciences 2015″ Conference Eger, Hungary 27-29 March 2015

7. ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Professor Andras Dinnyes for giving me the opportunity to carry out my Ph.D. study, for his continuous support, patience, and motivation. I would like to express my sincere gratitude to my co-supervisor Dr Julianna Kobolak for the patient guidance, encouragement and advices she has provided throughout my work. I would have not been able to finish my Ph.D. without their help and support.

I would like to thank my dissertation committee members: Dr Elen Gocza and Dr Laszlo Hiripi for their insightful comments and encouragement, but also for the hard questions which incented me to widen my research from various perspectives.

I gratefully acknowledge the support provided by Dr Balazs Mihalik and Dr Hasan Avci. I appreciate all their contributions of time, ideas, productive discussions and helpful advices.

It is a pleasure to express my thanks to Dr Zsuzsanna Majorne Tancos for providing and characterization of rabbit iPSCs and ESCs, Dr Istvan Bock for performing NGS analysis, and Abinaya Chandresakaran and Annamaria Teglasi for their crucial contribution to the cell culture work.

I greatly appreciate the help I received from Geta Serbana, Katalin Barane and Marianna Toth during my study.

I express my gratitude to all members of Molecular Animal Biotechnology Laboratory and BioTalentum Ltd. who helped me and supported my work.

Last but not the least, I would like to thank with all my heart my family, especially my parents, my sister and my beloved husband for their support, trust and love.